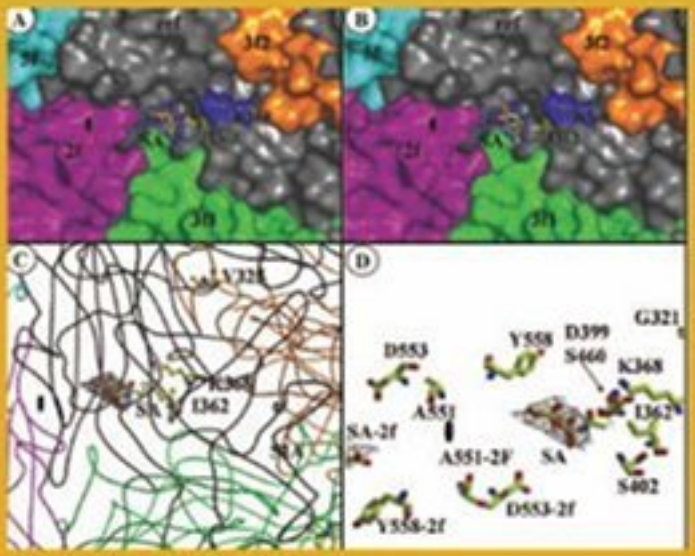
Advances in VIRUS RESEARCH



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KARL MARAMOROSCH

CHAPTER 1

Viruses, Vectors, and Vegetation: An Autobiography

Karl Maramorosch

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I. FROM CHILDHOOD IN EUROPE TO AMERICA

When the suggestion was made to write my biographical chapter for *Advances in Virus Research*, I did not realize how difficult a task this would be—where to start, what to say, and what to omit? I decided to start with my childhood and describe events in my life that inspired me to become a virologist and that were responsible for my scientific career.

In the summer of 1914, shortly after World War I started and the Tsarist army approached the family farm located in the village of Soroki in the eastern part of Austria, my parents escaped to Vienna, the capital of

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the Austro-Hungarian Monarchy. There I was born in 1915. The farm did not move, but the borders moved many times. The family estate found itself under no less than seven different regimes: Austria, Poland, Petlura's Ukraine, Romania, again Poland, USSR, Nazi Germany, USSR, and currently Ukrainian Republic.

My father, a graduate of the Vienna Agricultural University, started Ph.D. studies in Halle/Saal, Germany in 1898 but after 1 year returned home to manage a 4000 acres estate, Kamionki Wielkie near Kolomyja, owned by my grandfather. Around 1900, the estate was sold and the smaller farm, Soroki, was purchased. My father considered himself a Pole of Jewish creed. My mother, born in Zagreb, Croatia, was an accomplished pianist and a linguist, fluent in German, English, French, Italian, and Serbo-Croatian. My siblings, Alfred, 6 years older, and Karla Bronia, 5 years older, spoke only Polish with my father and only German with my mother. I grew up into this system, not realizing that it was not usual for everyone to speak only Polish to one's father and only German to one's mother. I grew up bilingual and only realized this clearly when I left home and started writing letters to my parents—my thoughts were in Polish when I addressed my father, and German toward my mother, and I had to write not one, but two letters during my studies in Warsaw. I was often asked how my parents spoke to each other. They spoke German because, despite the great language skills of my mother, she could not speak Polish without an accent, and it was, unfortunately, customary in Poland to make fun of everybody who mispronounced Polish words. My mother used Polish only when she went shopping or when she spoke with people who helped at home, but never with friends or visitors.

My third language was Ukrainian, which was spoken by all peasants in the village where our farm was located. In high school I had 4 years of Ukrainian and learned the Cyrillic script and some Ukrainian poetry by Taras Shevchenko and Ivan Franko.

When I was 14 years old, my brother came home for his winter vacation from Lwow (Lviv), where he was studying medicine. He told me how his biology professor, Rudolf Weigl, invented a vaccine during World War I that protected against exanthematous typhus. I was completely fascinated, hearing how Professor Weigl was giving enemas to individual body lice. Weigl infected the lice with *Rickettsia prowazekii*, inserting glass micropipettes into their anal openings. Afterward he maintained the inoculated lice on human volunteers for several days. Subsequently, he removed the intestines from batches of 140 inoculated lice, crushed the intestines in a tiny glass mortar with a few drops of formalin, and obtained a single doze of his vaccine. Later I found out that this was in Europe the only available vaccine against trench fever until the end of World War II (Szybalski, 1999). The information about the

currently used vaccine, developed by Harold Cox around 1940/1941, did not reach Europe during the war because of Pearl Harbor.

My brother's description of Weigl's work was spell binding and I decided then to become a medical researcher and try experiments similar to those carried out by the developer of the typhus vaccine.

I received my baccalaureate degree at the top of my class and applied to the same Medical School in Lwow, where my brother had just graduated. I was not accepted because of ''*numerus clausus*,'' as only 10 Jewish students were accepted every year—and I was not among the lucky ones in 1933. I lost 1 year and remained at home, finishing my piano studies. I was practicing every day, an average of 8 h, during my 12th year of piano study. By the end of the year, I graduated from the Music Conservatory but realized that I would not become a famous concert pianist to compete with Arthur Rubinstein, but, at best, a good piano teacher. This did not appeal to me and, to the joy of my father, I decided to follow in his footsteps and study agriculture. In 1934, I applied to the Warsaw Agriculture University (SGGW) and I was accepted without difficulty. After 4 years, I received the degree of Agricultural Engineer (an MS equivalent).

Quoting Harold S. Ginsberg (1999) (*Advances in Virus Research* 54, p. 1), ''I had the extremely good fortune to be in the right place at the right time,'' not just once, but several times during the following years. On May 24, 1935, I joined a group of Jewish students of agriculture to visit the oldest Polish agricultural experiment station, located in Pulawy near Lublin. The very inexpensive trip to Pulawy was on the deck of an old boat on the Vistula River. We arrived in early morning and the whole group walked through the ancient park of the Czartoryski estate toward the station building. Across came a very nicely dressed girl, with a book in her hand. She paid no attention to the 20 students but when she passed me at the very end of the group, she glanced for a fraction of a second at me. Her shiny black eyes struck me and a colleague noticed the shock that I experienced. He told me that he saw the same young lady in Warsaw in the company of the chemistry student who joined our group, and he offered to help me meet her that afternoon. During the following 3 years I was ''going steady'' with Irene Ludwinowska and after I graduated in 1938, we got married.

We returned to the family farm where I worked till September 1939. On September 1, World War II started and by the middle of September the western part of Poland was already occupied by the rapidly advancing Nazi army. On September 17, the Soviet army entered from the east. Our farm was 14 miles from the Romanian border and less than 200 miles from the Soviet border. My wife and I decided to escape across the nearby border to Romania. The nearest route was already occupied by Soviet tanks and we proceeded to the town Kuty on the Czeremosz River to cross the bridge linking Poland and Romania. However, the Polish authorities

prohibited civilians from crossing the bridge, permitting only uniformed armed forces to flee. We were again lucky. A Polish major, Karol Krzyzanowski, stopped his car and asked my pretty wife for directions to the bridge. We offered to guide him and asked to be taken along in his car, driven by a sergeant. Major Krzyzanowski agreed, and just before the bridge ordered the sergeant to take the major's overcoat from the trunk. After I hastily put it on, the major removed his cap and placed it on my head. I looked like a rather young Polish major—I was 24 years old. At the bridge a Polish officer saluted, looked carefully into the car, and dictated to his companion: ''Two majors, one sergeant.'' Then he asked: ''And who is she?'' Major Krzyzanowski replied: ''She is my wife. OK, proceed.'' A moment later we were on the Romanian side in the town of Vishnitza.

Night fell and the endless column of cars moved very slowly through Romanian villages. Rumors were spreading that all officers and soldiers will be placed in refugee camps. Shortly before midnight, we noticed lights in a palatial home on top of a hill. I decided to separate from the military convoy and tried my luck again. We thanked the major for helping us and walked up the hill to the lighted home. The daughter of the owner opened the door and very friendly took us upstairs to a bedroom, then apologized that practically no food remained in the house because more than 200 Polish refugees, now sleeping in the barn, consumed everything during the evening. In the morning, we found out that the lady mistook us for relatives of her husband, whom she expected to arrive from Poland. The owner of the estate, Mr. Orenski, a gentlemen farmer, was a known conductor and composer. The huge living room, with two grand pianos and chandeliers, made an impression of a Hollywood setup. Then we met the charming son, Dr. Stefan Orenski, a microbiologist, who later became our close friend. He became one of my associates after he was able to escape from Romania 20 years later.

Our ''freedom'' lasted only 2 weeks. Polish civilian refugees were soon confined to camps, located in several localities far from the border. The first year we were in the town of Braila on the Danube, followed by Craiova, where we survived till August 24, 1944, when Romania was liberated by Marshall Malinowsky's Third Army and the country came under Soviet domination. We were able to move to Bucharest and I enlisted at the university to obtain a Ph.D. degree. In 1946, a few weeks before my final exam, the US Agricultural Attache helped us to escape from Romania to Sweden. There I got a first preference immigration visa to the United States, as ''skilled agriculturist.''

We were lucky, having survived the holocaust in refugee camps in Romania, but my parents and my brother perished, as did my wife's parents, her sister, and 127 closest relatives living in the Polish cities of Warsaw, Pulawy, and Czestochowa, in Vienna, Austria, and in Zagreb, Croatia.

In Stockholm, waiting to depart on the Swedish America line's old Drotningholm, I worked as volunteer at the Plant Protection Institute. The Swedish plant virologist Dr. Daniel Lihnell helped me to improve my rudimentary knowledge of English and one day he gave me the popular book by Kenneth M. Smith, ''Beyond the Microscope'' (Penguin Edition). Reading the story of the discovery of viruses and the current state of knowledge of their nature was so interesting and stimulating as was, many years earlier, Paul de Kruif's ''Microbe Hunters.'' I decided then that I would become a virologist in the United States.

A few days after we arrived in New York, my wife was hired by the New York Public Library, even though her knowledge of English was very limited and she was unable to complete the form handed her at the library's admission office. She started at the world's largest public library as a page, but soon advanced, becoming eventually the Head of the Searching Section in the Preparation Division. Her knowledge of seven languages, her love of books, and her inherent ability to read extremely fast were certainly among the assets that were helpful in her career. Twelve years earlier, at the time we first met, she used to read two books every day. At first I could not believe that she was actually reading so fast, comprehending the contents, and remembering all the details. I tried to examine her, only to find that she actually knew the contents and remembered all described details of the novels. In those days, speed reading was not taught in Poland and it became popular in the United States only after President Kennedy took speed reading instructions in the White House.

II. BROOKLYN BOTANIC GARDEN

My luck continued when I was hired as technician at the Brooklyn Botanic Garden. My boss and first mentor was Dr. Lindsay M. Black (Fig. 1), who had moved from the Rockefeller Institute Branch in Princeton to the Botanic Garden a year earlier. He hired me to assist in his studies of plant viruses transmitted by leafhopper vectors. I learned how to maintain leafhopper colonies and how to transfer individual leafhoppers to test plants. Catching the tiny insects and placing them on caged plants took many hours every day. I figured out how to construct tiny cages and move them rapidly from plant to plant, omitting the use of an insect-catching device. The individual ''leaf cages'' saved 5–6 h of work every day and Dr. Black suggested that I describe the method and publish it as sole author. He corrected my manuscript and polished my Polish–English text before I submitted it to the *Journal of Economic Entomology*. There Dr. Poos, Editor in Chief of the journal, promptly rejected my paper and wrote a personal letter, stating that entomologists are not interested in keeping

FIGURE 1 Lindsay MacLeod Black. Photo by the author, 1949.

leafhoppers alive but are interested in destroying the pests. I was shocked but my mentor consoled me and suggested to resubmit my paper to the *Journal of the New York Entomological Society*. It was accepted and published in 1951 (Maramorosch, 1951a). Twenty years later, I became Editor in Chief of the *Journal of the New York Entomological Society* and remained in that capacity for a dozen years.

After a few weeks, Dr. Black suggested that I should continue my doctoral studies at Columbia University, and he gave me time off to take courses and laboratory sessions. One day he suggested that I should apply to the American Cancer Society for a predoctoral fellowship that would pay $200 per month plus tuition at the university. When I read the application form, I noticed immediately that it specified that the applicant must be a US citizen. I was less than 1 year in the United States and thus was at least 4 years from applying for US citizenship. Therefore, I put the form aside and did nothing about it. A few days later Dr. Black asked me whether I have filled out the form and when I replied that I could not do this, he said, with a poker face: ''Karl, I thought that you wanted to become a scientist, but now I am disappointed.'' I explained that I could not fill out the form because it specified that the applicant must be a US citizen. I was quite surprised when Dr. Black said: ''If you want to be a scientist, you have to be accurate and logical. Filling out the form is one thing, while being a US citizen is another. I can help you in filling out the form. Simply add a first page, calling attention to the fact that you are not yet a US citizen because you arrived recently, but you expect to become one in four years.'' While I did not believe that my application would be

FIGURE 2 Wendell Meredith Stanley. Photo by the author, 1951.

considered, I followed my mentor's advice and mailed the application. On April 15, 1948, Dr. Black called me to his office and informed me that he had received a phone call from Dr. Wendell M. Stanley (Fig. 2) and that my application had been approved. Dr. Stanley was at that time a reviewer of predoctoral applications at the American Cancer Society. He stated that the formal notification would arrive in a few weeks. I remember the date because it was again one of the very important, lucky moments in my life. We postponed having a child while we were in refugee camps in Romania and, after arriving in the United States, our financial situation was not conducive to starting a family. But Stanley's phone call changed our prospects drastically and precisely 9 months later, our daughter, Lydia Ann, was born. Stanley not only crystallized TMV but also indirectly was responsible for timing our very personal decision.

The predoctoral fellowship from the American Cancer Society and my wife helped me financially to complete my studies at Columbia University in less than 2 years. My Ph.D. diploma was signed by the President of Columbia University, Dwight Eisenhower, before he became Harry Truman's successor in the White House.

My childhood dream to follow Weigl's lice experiments soon became a reality, although not with lice nor with enemas of tiny insects. In Black's laboratory, I learned that in the 1930s Dr. H. H. Storey, FRS, in East Africa successfully transferred a virus to corn leafhoppers using needle inoculation and extracts from diseased corn or from leafhopper vectors (Storey, 1933). Dr. Black carried out similar transmissions using the aster yellows pathogen, considered at that time to be a virus, and later recognized as a phytoplasma (Black, 1953). Now I decided to try whether the *Wound tumor virus*, studied by Black, could also be transmitted to leafhopper vectors by needle inoculation. I learned how to draw very thin glass needles and I connected them to metal needles using various types of glue (Maramorosch, 1951b). Virus transmission was successful and my

FIGURE 3 Louis Otto Kunkel. Photo by the author, 1950.

first paper was published in *Science* (Maramorosch *et al.*, 1949). The mechanical virus transmission permitted the first titration of the *Wound tumor virus* in subsequent experiments (Brakke *et al.*, 1953). When I presented the experimental procedure at a seminar at Columbia University, Dr. Black invited my future mentor, Dr. L. O. Kunkel (Fig. 3) from Rockefeller University, to listen to my presentation. It was again a lucky strike.

I constructed a temperature control box from World War II supplies purchased in New York and studied the influence of temperature on the intrinsic incubation period of the *Wound tumor virus* in leafhopper vectors (Maramorosch, 1950). After finishing my Ph.D. studies at Columbia, I applied to Dr. Kunkel and was accepted, becoming his last assistant in his Department of Plant Pathology at Rockefeller University. Dr. Kunkel headed the department where earlier, at the Princeton Branch of the Rockefeller Institute, Drs. Wendell M. Stanley, Max A. Lauffer, W. C. Price, Philip R. White, Lindsay M. Black, Francis O. Holmes, and a score of other famous virologists had worked (Corner, 1964).

My title of assistant turned out to be just a formal title. Dr. Kunkel never published jointly with others and when I asked him what he wanted me to do, he replied that I can do whatever I wish, since I have my own ideas. If I would come to him and ask for advice, he would do his best to help, but I would have no boss and would be completely independent. This wonderful situation of being completely independent continued throughout my career, but I did not feel that, like Dr. Kunkel, I would publish always as sole author. I tried to find postdoctoral associates who would know techniques, that I did not know, in electron microscopy, tissue culture, and other areas, and these associates permitted me to advance more rapidly and obtain outside support from various sources.

I started at Rockefeller University on July 1, 1949. Dr. Kunkel told me that he will be away, taking a vacation for the first time in his life. He suggested that I also should start by taking a vacation because work during the summer, in hot greenhouses and laboratories that were not air-conditioned, would be very difficult. I thought that Dr. Kunkel was testing me and that he did not expect me to postpone my start for 2 months, till Labor Day. I eagerly began my work the following day, despite the heat and high humidity that prevailed throughout the summer. I had no technician and did everything myself, including the construction of cellulose insect cages. At the Brooklyn Botanic Garden, I learned how to make them from cellulose nitrate sheets, but I was told by the head of the Purchasing Department at Rockefeller that cellulose nitrate cannot be brought to New York City because it was too flammable. Instead, I was told to order cellulose acetate sheets. A shipment soon arrived and I made numerous insect cages and started a large experiment. A few days later, I noticed that my plants, covered by the new cages, looked unhealthy. Shortly thereafter all plants died and the caged insects, devoid of food, also died. I repeated the large test several times, but each time the same happened and all caged plants died a few days after the tests started. I struggled for 2 months, suffering in the hot greenhouses and losing all plants and insects. At lunch time I mentioned my misfortune to one of the chemists who offered to test the cellulose material in his laboratory. It turned out that the plasticizer, diethyl phthalate, used to make cellulose acetate sheets, was the culprit. Repeated washing in running water did not remove the toxicity. When I inserted a tiny piece of the cellulose material into a jar with water and placed a goldfish in the jar, the fish died within a few minutes. I described the toxic effect in *Science* (Maramorosch, 1952b) and this early short paper became better known than any of my later papers on viruses. Scores of reprint requests were received and I had to order additional reprints for interested food scientists and manufacturers.

Eventually I found out that Rockefeller University had a special permit to bring cellulose nitrate to the buildings. This permit was obtained earlier, when the Director, Nobel laureate Dr. Herbert Gasser decided to purchase inexpensive, large quantities of cellulose nitrate photographic film for his experiments in neurophysiology. When I substituted the cellulose acetate with cellulose nitrate sheets to make new cages, I sustained no further losses of plants. However, by the end of the year, I had no publishable results. Again, help came from my former mentor, Dr. Black. He and Dr. Myron Brakke published a paper and since they used my technique of leafhopper injection, they added me as an author to their report (Brakke *et al.*, 1953).

More important was the delay by Dr. Black in publishing his results of a serial passage of the *Wound tumor virus* in leafhopper vectors, after I told

him that I was carrying out a serial passage of the ''aster yellows virus'' in insect vectors (Maramorosch, 1952a). I was just finishing my last, 10th passage and Dr. Black decided that he would wait with his publication until mine would come out. Can you imagine that occurring today?

In 1952, I described the multiplication of the aster yellows pathogen (Maramorosch, 1952a) (considered at that time to be a virus, and in 1968 recognized as a phytoplasma) and I entered the detailed description of the serial passage of the aster yellows ''virus'' for the Cressy Morrison Prize competition of the New York Academy of Sciences. My winning of this prize started my intensive activities at the New York Academy, where I became chair of the Microbiology Section, and later Recording Secretary and Vice President. Work as a member of the committee responsible for the organization of academy conferences gave me the experience in organizing later comparative virology and other national and international conferences.

In 1952, I attended a New York Academy conference on virus taxonomy. Among the invited participants were Dr. Kenneth M. Smith from Cambridge and Sir Frederick C. Bawden (Fig. 4) Director of the Rothamsted Experimental Station in Harpenden, Hearts, United Kingdom. The two plant virologists were recognized as the world's leading authorities on plant viruses. I met both for the first time and

FIGURE 4 Frederick Charles Bawden. Photo by the author, 1952.

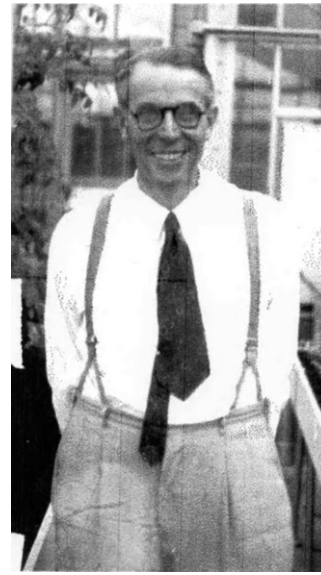

FIGURE 5 Kenneth Manley Smith. Photo by the author, 1953.

I was very lucky when Sir Frederick agreed to visit me at Rockefeller University the following day. Until then Sir Frederick was very skeptical about the work of Professor Teikichi Fukushi in Japan, who was the first to provide evidence for the multiplication of a plant pathogenic virus in leafhopper vectors (Fukushi, 1935). My detailed presentation of 10 serial passages of the aster yellows ''virus'' in leafhopper vectors convinced Sir Frederick that certain plant viruses were actually able to multiply in invertebrate animals. He was working on the second edition of his seminal textbook. Following his visit, he modified the part where he severely criticized Fukushi. He inserted a paragraph describing my work and since his textbook was very widely accepted it promoted my work worldwide. In 1953, Dr. Kenneth M. Smith (Fig. 5) invited me to write a chapter for Vol. 3 of ''Advances in Virus Research'' on the multiplication of plant viruses in insect vectors (Maramorosch, 1955).

In December 1955, I presented a paper, dealing with my first attempt to maintain the aster yellows ''virus'' in tissue cultures of insect vectors (Maramorosch, 1956) at the American Association for Advancement of Science (AAAS) Annual Meeting in Atlanta, GA. My paper won one of the two AAAS Prizes awarded that year. The other prize winner was my former statistics professor from Warsaw's Agriculture University, Dr. Jerzy Splawa Neuman, the head of the Statistics Department at the University of California in Berkeley. When I read in the Atlanta newspapers about it, I wrote to Professor Neuman, jokingly asking what the statistical

probability was of a former professor and a former student of the Warsaw Agriculture University to win the only two AAAS Prizes. Professor Neuman promptly replied that while it would seem highly unlikely to occur, the fact that it happened indicated that the Warsaw ''SGGW'' University was not a bad school.

In 1957, I flew to Hamburg, Germany, to participate in the Plant Protection Congress. A week before my departure I hired a new technician and I tried to explain to her how to take care of the colonies of leafhoppers and how to handle virus-free and viruliferous insects. When I returned, I was horrified to find that my technician did not follow my instructions and that she placed corn leafhoppers, *Dalbulus maidis*, on aster yellows-infected China aster plants. I knew that corn leafhoppers could only survive on corn and teosinte and I thought that the corn leafhoppers were misplaced in the morning when I returned. However, the labels on the cages indicated that the insects were on the improper plants for several days. Was the labeling also erroneous? I confronted Miss Lynn Foster and found out that the labels properly indicated the misplaced insects. To my great surprise, the corn leafhoppers had not died and flourished on aster yellows-diseased plants. When the ''misplacement'' was repeated, I confirmed that exposure to aster yellows-infected China asters, *Callistephus chinensis*, altered the survival abilities of corn leafhoppers. This finding could have implications in the emergence of new plant diseases, but whether it was limited to phytoplasma-caused diseases or also applied to plant virus diseases has not yet been established. I lost track of my technician, Miss Foster, who was responsible for this important discovery. She eloped soon afterward, to get married to her boyfriend, drafted into the Air Force.

I described the beneficial effect of the diseased plants on nonvector insects (Maramorosch, 1958), but before my paper came out, the finding became widely known thanks to an article published by Dr. Earl Ubell, science editor of the *Herald Tribune*. Dr. Ubell read the title of the seminar that I was to present at Rockefeller University. A day before the seminar he visited me in my office and asked for the details. Although he made no notes during our conversation, his description, published the following day, was completely accurate and better written than my own scientific article. *Newsweek* followed with a brief description, based on Dr. Ubell's article.

When Merck discontinued the production of gibberellic acid, I received from them a leftover spray can with the compound. At the suggestion of Dr. D. W. Woolley, who called my attention to the rediscovery of the action of gibberellic acid on plants, I sprayed a number of aster yellows, corn stunt, and wound tumor-diseased plants. The treatment resulted in growth stimulation of the stunted plants, but it had no curative effect. The results were published in *Science* (Maramorosch, 1957) and at the Crop Protection Congress in Hamburg (Maramorosch, 1959). This was the first

report of gibberellic acid treatment of ''virus-diseased plants.'' A few years later it became apparent that, while wound tumor was a virus disease, the two other diseases were not viral, but phyto- and spiroplasma diseases.

III. VIRUS NOMENCLATURE AND CLASSIFICATION

For several years I was actively involved in virus nomenclature and classification (Maramorosch, 1974). My interest stemmed from the finding that several leafhopper-borne viruses that were inducing plant diseases were multiplying not only in plants but also in their invertebrate animal vectors. The finding that little or no harm was observed in the virus-carrying insects could suggest that these viruses originated as insect viruses and over long periods of evolution became harmless to their animal hosts, while their newer plant hosts were severely affected and often killed. Should these viruses be considered as plant, or as insect, viruses? Plant pathogenic viruses may exert a beneficial, or a harmful, effect on their specific insect vectors (Maramorosch, 1968, 1969, 1970; Maramorosch and Jensen, 1963). My popular article in *Scientific American* (Maramorosch, 1953) also focused on these aspects. Incidentally, the honorarium received for this article provided funds for my first movie camera and my new hobby, that later changed to video photography.

I thought that the affinity of vector-borne viruses to certain plant or animal hosts should not be used as a classifying criterion. The naming of viruses was for a long time highly controversial, particularly the naming of plant viruses. European colleagues opposed the use of Latin names for many years and plant virologists had little, if any, contact with animal virologists who created their separate system of virus nomenclature and classification. Already in 1947, shortly before I came to the United States, at the International Microbiological Congress in Copenhagen, it had been approved that the bacterial code in its Latin form applies to viruses and bacteria. This was also stated at the 1953 International Microbiology Congress in Rome, which I attended. Yet, even in 1966, papers were still being published in which disease organisms were described as belonging ''in between viruses and bacteria,'' which Dr. Andre Lwoff pointedly called complete nonsense, since an organism defined as a virus is entirely different from one that is a bacteria. The International Committee of Microbiological Societies appointed a provisional committee to deal with the nomenclature of viruses and since then the nomenclature was in the hand of a powerless committee, which could make recommendations but these were not binding to anyone concerned. In 1963, Sir Christopher Andrews as Chairman of the provisional committee dissolved it and the International Committee for Virus Nomenclature (ICVN) was created for the first time. This ICVN consisted of members nominated by all

the National Microbiological Societies that belong to the International Association of Microbiological Societies. For each of the 10 member countries, 5 delegates were nominated. The United States representatives were Drs. Harold S. Ginsberg, Chair, Jordi Casals, Karl Maramorosch, Joseph L. Melnick, and William R. Romig. I was happy when Dr. Riley D. Housewright, President of the American Society for Microbiology, informed me of my election.

In 1966, a symposium was held in Moscow and two papers dealing with plant viruses, Dr. B. D. Harrison's and mine, met with a very lively discussion. There were 600 virologists seated in the auditorium and another 200 were listening by shortwave receivers outside the hall. When the Executive Committee was elected, only one plant virologist, Dr. A. J. Gibbs, was included. He was in violent opposition to all proposals that were not in conformity with his own postulates. Following the symposium, I decided to devote my time to my laboratory research and field work and I lost interest in the controversial fights between plant and animal virologists.

My luck continued at Rockefeller University when one day at lunch Dr. Rene Dubos asked whether I knew of a virus that would be beneficial. I replied that during the past centuries the smallpox virus was probably beneficial, by marking afflicted women and making them homely and less likely to fall pray to invading and raping enemy solders. This was not what Dr. Dubos was interested in at that time. He told me that he was working on an article describing how once variegated tulips became the vogue in the Netherlands and how the ''tulipomania'' rewarded a few families that knew how to transmit the variegation virus to healthy tulip bulbs. A few days later, also in the Rockefeller lunchroom, Dr. Dubos told me that he was requested to give a popular course on viruses at the New School in Manhattan, but that he was too busy and suggested me instead of him as a lecturer. A day later I was called by phone and offered the teaching position. It involved 14 weekly 2-h lectures and the remuneration was of considerable help. The students in the New School had a variety of backgrounds. One was a microbiology professor at a medical school, another was a nurse in a local hospital, but at the other end there was a cashier at an A&P store and a barber who had no high school education. The course was my first teaching experience and I learned how to avoid technical terms when explaining viruses to a lay audience.

IV. COLD SPRING HARBOR

In 1951 Dr. Keith Porter, a leading cytologist, suggested that I apply to Dr. Milislav Demerec, Director of the Cold Spring Harbor Laboratories, for accommodations during the summer. Dr. George Palade, also a distinguished cell biologist at Rockefeller and later a Nobel laureate, was

driving with Dr. Porter to take part in the June symposium and they took me along, to see the beautiful location and to apply to Dr. Demerec personally. With no written application and no formalities, the permission was granted and this stroke of good luck had a profound influence on my career.

During the 1950s, I spent eight summers at the Cold Spring Harbor Laboratories on Long Island, New York. Dr. Barbara McClintock permitted me to use her greenhouses while she was working outdoors with corn (*Zea mays*). Each year at the end of August, Dr. Alfred Hershey organized a bacteriophage symposium for invited bacterial virologists. Although I did not work on bacteriophages, I was permitted to attend these meetings, where as yet unpublished findings were presented by the virologists. Throughout the summers, Drs. Max Delbruck, Salvator Luria, and Ernst Mayr were working in Cold Spring Harbor, lecturing, and socializing with the small group of scientists. Dr. Luria was working on his textbook on viruses and I was greatly impressed watching him dictate into a tape recorder each morning, and then mailing the tape to his secretary in Urbana for typing. When the typed version came back to Cold Spring Harbor, Dr. Luria made small corrections and each chapter was ready for publication. Few times he asked me for comments and when the book was published, he donated a copy to me and I found that he acknowledged my reviewing of a couple of chapters in his book.

One day Dr. Luria suggested that I should invite Japanese postdoctoral scientists to my laboratory and he added: ''Get a good Japanese postdoc, but never more than one. You will rapidly advance with your work, but if you get more than one Japanese associate, you will no longer have any time with your daughter and your wife, because you will try to keep up with your Japanese postdocs and spend 7 days a week in the lab.'' I remembered the first part of Luria's suggestion and followed it when I left Rockefeller University in 1961 and moved to the Boyce Thompson Institute. But I did not follow the advice concerning the limitation of invited Japanese postdocs. I soon found out how correct Luria was when he told me never to get more than one Japanese coworker at a time. When I got three Japanese associates at the same time, my own working habit changed as I felt compelled to keep up with my Japanese coworkers.

Thanks to Dr. McClintock's generosity in Cold Spring Harbor where she permitted me to use her greenhouses during the summer, I could carry out an experiment in which I injected antibiotics into abdomens of leafhoppers, exposed to presumptive viruses of aster yellows and corn stunt. I used penicillin, streptomycin, and tetracycline, convinced that the causative agents of the two plant diseases were viruses. Penicillin and streptomycin injections did not prevent transmissions, but tetracycline-injected leafhoppers failed to infect the exposed seedlings. Convinced that

tetracycline has no effect on viruses, I did not believe the results of the tests, and assumed that the failed transmission was due to the heat in the greenhouses. I did not repeat the experiment after I returned to the Rockefeller greenhouses and I published the results and my wrong conclusion in the *Transactions of the New York Academy* (Maramorosch, 1954). Had I repeated the tests, I would have found that not the summer heat but tetracycline interfered with the presumptive viruses. Ten years later, my Japanese colleagues in Tokyo discovered the phytoplasma nature of the aster yellows disease, but I missed the boat.

V. THE CADANG-CADANG DISEASE

A Food and Agriculture Organization assignment in the Philippines in 1960 was an eye opener to a very important, different, and most interesting world. I was expected to find the vector of the presumptive virus that had already killed 30 million coconut palms on Luzon and nearby islands (Maramorosch, 1961). While trying to find an insect vector, I learned also first hand about people in the Philippines. I made the decision to combine future laboratory basic research with applied field work to increase food and fiber production in developing countries.

In the Philippines, I was not able to find a vector of the palm disease but I became well acquainted with numerous owners of larger and smaller coconut plantations. One observation which I made, but did not dare to publish in my final FAO report, had to wait 14 years before it made some sense. I found that palm owners, who spoke the local Bicolano language, were losing their palms to the cadang-cadang disease, while owners who spoke Tagalog, the official Philippine language, had healthy palms. The spread of the disease seemed halted at the provinces where Tagalog was the predominant language, sparing completely areas close to Manila and Los Banos. Of course, I did not dare to mention that the virus, or its vector, seemed to distinguish whether the palm owners used one or another language, but the consistent correlation was striking and puzzling.

Fourteen years passed before the viroid cause of the cadang-cadang disease was established by the Australian virologist Dr. J. W. Randles (1975). Almost all viroids require humans to spread from plant to plant and only Avocado blotch is transmitted by pollen. The transmission of cadang-cadang viroids seemed linked to the Bicolano-speaking plantation workers and the contamination of their tools used to make incisions in the palms and the flowers of the trees. Bicolano plantation owners were hiring Bicolano-speaking workers, while plantation owners who spoke Tagalog employed ''their own'' Tagalong workers. Apparently, one group carried viroid-contaminated ''bolos'' (machetes), while the other

did not. It would seem easy to stop the spread of the cadang-cadang viroid by dipping the cutting tools of plantation workers in a calcium chloride solution (Maramorosch, 1985, 1993). As far as I know, this has not been implemented and more than 50 million palms have been destroyed by cadang-cadang in the Philippines (Maramorosch, 2004). Losses are partially alleviated by replanting with susceptible, but earlier maturing, coconut palms.

VI. DARK CLOUDS ON THE HORIZON

Not everything was rosy during my last year at Rockefeller University. When I asked President Detlev Bronk about my future at the University, he first congratulated me to my AAAS Prize but then said that the study of virology was declining all over the world and that it reached its peak in 1935, when Stanley crystallized TMV. ''It no longer is an important science,'' he said. Therefore in the US National Academy, where Dr. Bronk was the President, botany, zoology, chemistry, physics, history, mathematics, and so on were represented, but there was no virology. Although my work was interesting and important, it did not fit into his university and he, as university president, decided to remove both plant and animal virology from Rockefeller University. Dr. Igor Tamm was heading animal virology at that time and Dr. Bronk mentioned him as well as the group in which I and Dr. F. O. Holmes were working with plant viruses. When I got up to leave the President's office, I could barely walk. In the corridor, the newly appointed vice president, Dr. Douglas M. Whittaker, met me and noticed that I looked sick. He put his arm around my shoulder, took me to his office, and asked whether I was ill. I repeated, almost verbatim, what I was told by Dr. Bronk. Dr. Whittaker assured me that my position was secure and he tried to console me. Just that week I received an invitation to go to the Philippines for 6 months, to work on the devastating cadang-cadang coconut palm disease that was believed to be caused by a virus. Dr. Whittaker told me that there was a recent precedent of a leave of absence request and that he, and not Dr. Bronk, could therefore give me permission to take a paid leave of absence for the UNDP consultancy in the Orient. The precedent was a leave granted by President Bronk to Professor Paul Weiss, to teach for 4 months at Harvard University.

VII. INSECT VIRUSES AND CELL CULTURE

When I realized that my tenure at Rockefeller University could be ended by President Bronk, who considered virology an unfit subject for university studies, I approached Dr. Richard Shope to assist me in searching for

a different position elsewhere. Dr. Shope left the Rockefeller branch at Princeton in 1945, when the decision to close the Princeton Laboratory was announced. He went to Merck Laboratories in Rahway, New Jersey, but returned to the Rockefeller University a few years later. When I told him about my predicament, he called Merck's President, Dr. Max Tishler, and the latter contacted Dr. Maurice R. Hilleman, Director of Merck's Virus & Cell Biology Research Institute for Therapeutic Research at West Point, PA. Dr. Hilleman invited me to West Point and offered me a research position at a salary that was 50% higher than my Rockefeller salary. My European prejudice against industrial research prevented me from accepting the very tempting offer. When I discussed this with my wife, she reminded me that I have never been unhappy with any kind of work and that, when I was making a living at the refugee camps in Romania as a cobbler, or as a piano teacher of a young singer, I seemed quite happy. She did not think that I would miss the glamour of Rockefeller University if I would accept Dr. Hilleman's job offer at West Point. Yet, I was unable to decide and mentioned this to Dr. Shope. A few days later he told me that another, temporary position would be offered by Merck. The pharmaceutical company decided to investigate the feasibility of producing insect viruses for biological control of pests. I was hired as consultant for 6 months to organize a conference on insect viruses. For the following half year, I was reading the available literature on a subject that was completely new to me—baculoviruses. I gave the names of all prominent insect pathologists to Merck in Bradenton, where their animal farm was located. A 3-day conference was arranged and I met Professor Edward Steinhaus and a score of prominent US and Canadian insect virologists. After the conference I prepared a report and my final recommendation was that it was too early to start commercial production of baculoviruses because the subject was still in its early stage of university investigation. The 6 months during which I prepared the baculovirus conference got me very interested in insect viruses. I thought that their growth in tissue culture, rather than in living insects, could eventually lead to large-scale commercial production. This did not yet materialize because *in vitro* production of viral pesticides remained more costly than production *in vivo* (Maramorosch, 1979a,b, 1991).

During the following years, at the Boyce Thompson Institute and later at the Waksman Institute of Microbiology, I worked with invertebrate cell culture and baculoviruses. I was joined by excellent postdoctoral associates from Argentina, Australia, Canada, Chile, China, France, Germany, India, Israel, Japan, Korea, the Netherlands, Philippines, Poland, Romania, Slovakia, Turkey, the United States, and Yugoslavia. I shall mention but a few. Among the first was Dr. Robert R. Granados, an insect virologist, who came to my laboratory from Madison, Wisconsin. In subsequent years, he became Program Director for biological control. His research

interests focused on insect pathology, the ultrastructure of insect virus replication and insect cell culture methods for virus studies, mechanisms of infection, and pathogenesis of insect viruses. Dr. Granados provided the first evidence for the accumulation of the *Wound tumor virus* in various organs of an inefficient vector (Granados *et al*., 1967) and for insect viremia, caused by the virus invasion of vector hemocytes (Granados *et al*., 1968). From Japan I was joined by Professor Jun Mitsuhashi who spent 2 years with me at Boyce Thompson Institute. He developed the widely used M&M insect cell culture medium and aseptically grew plant virus vectors (Mitsuhashi and Maramorosch, 1963). Using these vectors, he inoculated plant tissue cultures with the aster yellows ''virus'' (Mitsuhashi and Maramorosch, 1964). After returning to Japan, Professor Mitsuhashi became known for his work on mosquito cell lines and the development of new invertebrate cell culture media for virus propagation. We also published several books jointly. Among my Japanese associates Dr. Hiroyuki Hirumi worked for 10 years with me. He distinguished himself studying the aster yellows ''virus'' in various organs of an insect vector (Hirumi and Maramorosch, 1963). His extensive work with Hemiptera cell culture (Hirumi and Maramorosch, 1971) included the *in vitro* cultivation of embryonic leafhopper tissues (Hirumi and Maramorosch, 1964) and the localization of the *Wound tumor virus* in embryonic nonvector cells (Hirumi and Maramorosch, 1968). Dr. Hirumi also studied the Friend murine leukemia virus in mosquitoes (Hirumi *et al*., 1971), Marek's herpes virus, and Type C virus (Hirumi *et al*., 1974), and the growth of mouse trophoblastic cells stimulated in culture by polyoma virus (Koren *et al*., 1971). After I moved from Boyce Thompson Institute to Rutgers University, Dr. Hirumi joined the International Laboratory for Animal Diseases (ILRAD) in Nairobi, Kenya. During the following years, he made significant contributions to the study of the tsetse fly borne Nagana disease of cattle.

Attempts were made to maintain aphid cells *in vitro*. My daughter, Lydia, assisted during her summer high school vacation and learned how to remove unborn aphids from adult insects by cesarean section. Dr. Takashi Tokumitsu was able to maintain surviving aphid cells for limited period *in vitro* (Tokumitsu and Maramorosch, 1966). He also studied cytoplasmic protrusions that formed in cultured leafhopper cells during mitosis *in vitro* (Tokumitsu and Maramorosch, 1967). From Tubingen, Germany came Dr. Gert Streissle, who worked with me for 7 years. He was the first who compared immunologically animal reo viruses with the plant pathogenic *Wound tumor virus*. Subsequently, the latter was classified as a plant reo virus. I was alerted to the striking morphological resemblance of the plant and the animal reo viruses by Drs. Albert Sabin and Andre Lwoff. Both suggested that we try to compare them immunologically. In our article in *Science* (Streissle and Maramorosch, 1963),

we acknowledged their suggestions. Dr. Streissle returned to Germany to head the antiviral research at Bayer in Wuppertal. My international connections became a most gratifying experience. I would like also to brag about my former graduate students and I shall mention but two. Dr. Dennis M. Schmatz from Merck's Research Laboratories is currently a Vice President, heading research at the Merck Research Building in Tsukuba, Japan. Professor Kenneth E. Sherman, Ph.D., M.D., leads a large group of virologists working with hepatitis virus in Cincinnati, OH.

The etiology of whitefly-borne pathogens remained an enigma for many years. Repeated attempts made in my and in other laboratories to find viruses in extracts of diseased plants or by electron microscopy in thin sections of plant tissues were fruitless. Finally, in 1975, Professor Robert M. Goodman, at the Plant Pathology Department, Illinois University in Urbana, IL, made the brilliant discovery of the whitefly-borne Gemini viruses and of their single-stranded DNA genome (Goodman, 1977). Professor Goodman left Urbana for the University of Wisconsin in Madison and others continued his seminal work on Gemini viruses. I was very happy when he came to Rutgers University 2 years ago, becoming my Executive Dean. In 1975, he generously provided some of his excellent electron micrographs of Gemini viruses for our book on tropical diseases of legumes (Bird and Maramorosch, 1975; Maramorosch, 1975).

After I joined the faculty at Rutgers' Waksman Institute, I was joined by Dr. Arthur H. McIntosh, who for 7 years worked with me on baculoviruses. At Rutgers University, Dr. McIntosh studied the retention of insect virus infectivity in mammalian cell cultures (McIntosh and Maramorosch, 1973) and the localization of a baculovirus in a vertebrate cell line (McIntosh *et al*., 1979). He continued his baculovirus studies after joining the Biological Control Laboratory of the US Department of Agriculture in Columbia, MO. Among my later postdoctoral Japanese associates was Dr. Ken-ichi Yamada, who carried out studies on *Heliothis zea* nuclear polyhedrosis virus (Yamada and Maramorosch, 1980, 1981; Yamada *et al*., 1981). He continued his research in Japan at Tokyo's National Institutes of Health.

Over the years, I organized several national and international conferences dealing with this subject. I edited a number of books, published by Academic Press and by others (Section IX).

In later years, I improved and modified the leafhopper injection technique. In 1958, I saw in Warsaw the last remaining insect holder used by Professor Weigl, years earlier. A similar holder for 20 leafhoppers was constructed for me by the head of the Rockefeller University Instrument Shop, Mr. Niels Jernberg (Maramorosch and Jernberg, 1970). Using carbon dioxide and this modified device, it was easy to inject 20 leafhoppers in 1 min and perform statistically significant tests with several plant viruses, phytoplasmas, and spiroplasmas.

During the past three decades, invertebrate cell culture became widely used in biotechnology and basic research in virology. Use of baculoviruses in insect cell cultures is gaining popularity for the production of recombinant proteins, viral insecticides, and the production of vaccines. *In vitro* techniques are indispensable for studies of insect virus expression systems. Application of invertebrate cell culture and molecular biology is leading to significant progress in the understanding of cellular and molecular interactions between insect cells and viruses. Often unexpected results are obtained as was the case with our M&M medium, developed for leafhopper cell culture, and later found best suitable for mosquito cell cultivation and the study of arboviruses in mosquito cells (Maramorosch, 1979b).

VIII. ELECTRON MICROSCOPY

In 1957, I took a course in electron microscopy, offered to Rockefeller faculty members by Drs. Keith Porter and George Palade. The course gave me a good knowledge of the fixation procedures, the use of the Porter-Bloom ultramicrotome, the glass, and diamond knives, and so on. When I came to the Boyce Thompson Institute and obtained outside funding from the National Science Foundation and NIH of the US Public Health, I decided to apply for a supplement to my NIH grant to purchase an electron microscope. I consulted Dr. Palade who advised me to specify that I am planning to use the expensive instrument not only for the current grant-funded project but also for long-term studies of vector-borne viruses. Dr. Palade also suggested that I should get a Siemens Elmiscope, and not an RCI electron microscope. I followed both suggestions. My application was approved and the large supplement funded. The advice of Dr. Luria to get a Japanese associate proved excellent. I was fortunate to get Professor Eishiro Shikata from Hokkaido University in Sapporo for 2 years. Dr. Shikata worked as an assistant professor with Professor Teikichi Fukushi, studying the fine structure of leafhopper vectors transmitting the rice stunt virus. He was the first to visualize the rice plant virus not only in diseased plants but also in the invertebrate animal vectors (Shikata *et al.*, 1964). During his 2 years of our collaboration, Dr. Shikata worked 7 days a week, taking no vacation or holidays. Every day he entered the EM room early morning, leaving it late in the afternoon, and taking to the darkened room a sandwich for lunch. He told me that it would have taken him at least 20 min to get his eyes adjusted to the darkened room if he were to leave the room for lunch, and he did not want to lose so much time. During his 2 years at Boyce Thompson, Shikata, as senior author, published a series of articles in *Virology*, *Journal of Virology*, *Nature*, and the *Journal of the National Cancer Institute* (Shikata and Maramorosch, 1965a,b, 1966a,b, 1967a,b, 1969; Shikata *et al.*, 1964, 1966). A few years after

returning to Japan, he succeeded Professor Fukushi as head of the Botany and Plant Pathology Departments at Hokkaido University in Sapporo. Later he was elected to Japan's National Science Academy as the only plant pathologist in this Academy.

IX. BOOKS

In 1960 at the AAAS Annual Meeting, I stopped at the book exhibit of Academic Press where I met Vice President, Kurt Jacoby. We spoke for quite a while about his former work in Germany and the creation of Academic Press in New York. I asked Mr. Jacoby whether symposium papers on biological transmission of animal and plant disease agents could be published by Academic Press. I was organizing a 2-day symposium on this subject, to be held at the Annual Meeting of the Entomological Society of America in Atlantic City, NY. Mr. Jacoby agreed and my first book, of 192 pages, ''Biological Transmission of Disease Agents,'' was published in 1962. As agreed, I received no royalties. Years later, I was told that all 1800 copies were sold when the book went out of print. The idea of publishing the presentations came only after the conference participants had agreed to be symposium speakers. I had considerable difficulty in persuading some authors to submit manuscripts for publication. Foreign participants, Dr. W. C. Willett from Kaduna, Nigeria, and Dr. D. Blascovic from Bratislava, Slovakia, were among the first to send their contributions. The Rockefeller Foundation arranged the travel of these eminent participants through a grant to the Entomological Society of America.

The success of my first book prompted me to again try Academic Press for the publication of a more voluminous volume of 666 pages. The treatise was based on a United States–Japan seminar, which I organized in Tokyo together with Dr. Paul Oman. Mr. Jacoby was not interested this time because as he explained, symposia were not selling well. Wiley Interscience agreed to publish the book when I added several additional authors who did not participate in the Tokyo meeting. I also used the title of this second book, ''Viruses, Vectors, and Vegetation'' (1969) for the title of my current autobiographical chapter.

During the following years several volumes on viruses, edited by me alone or jointly with other virologists, were mainly published by Academic Press. In 1968, Springer published ''Insect Viruses'' (192 pp.). In 1971, ''Comparative Virology,'' edited by me and E. Kurstak, (Academic Press, 584 pp.) was followed by ''Viruses, Evolution, and Cancer'' (813 pp., 1974). In 1975, with R. E. Shope, we edited ''Invertebrate Immunity'' (Academic Press, 365 pp.)

Viruses and virus diseases were included in ''Tropical Diseases of Legumes,'' edited by Julio Bird and me in 1975. In 1977, I edited

the ''Atlas of Insect and Plant Viruses'' as Vol. 8 of Academic Press' ''Ultrastructure in Biological Systems.'' ''Aphids as Virus Vectors,'' edited by K. F. Harris and me in 1977, ''Leafhopper Vectors and Plant Disease Agents,'' by me and Harris, 1979, ''Vectors of Plant Pathogens,'' by Harris and Maramorosch, 1980, ''Plant Diseases and Vectors: Ecology and Epidemiology,'' by Maramorosch and Harris, 1981, and ''Pathogens, Vectors, and Plant Diseases: Approaches to Control,'' by Harris and Maramorosch, 1982, as well as ''Viruses and Environment,'' by Kurstak and Maramorosch, 1978, were all published by Academic Press. ''Vectors of Disease Agents: Interactions with Plants, Animals and Man,'' edited by J. J. McKelvey, Jr. *et al*. was published by Praeger in 1980. The voluminous ''Viral Insecticides for Biological Control,'' by Maramorosch and K. E. Sherman, and ''Subviral Pathogens of Plants and Animals: Viroids and Prions,'' by Maramorosch and McKelvey, were published by Academic Press in 1985. In 1965, Ms. Lore Henlein of Academic Press suggested that I should start a series dealing with ''Methods in Virology.'' Together with Hilary Koprowski, eight volumes of ''Methods in Virology'' were published by Academic Press between 1967 and 1984. Maintenance of ''Animal/Human and Plant Pathogen Vectors,'' by Maramorosch and F. Mahmood, was published by Science Publishers in 1999.

At Rockefeller University Professor William Trager and Dr. Maria Rudzinska gave me excellent suggestions how to attempt the cultivation of leafhopper tissues and cells for virus studies. I was able to maintain leafhopper tissues *in vitro* but not cells or cell layers (Maramorosch, 1956). I continued my attempts and organized several conferences nationally and internationally. Proceedings of the conferences were published by Academic Press in the following volumes: ''Invertebrate Tissue Culture: Research Applications'' (Maramorosch, 1976), ''Invertebrate Tissue Culture: Applications in Medicine Biology and Agriculture'' (E. Kurstak and Maramorosch, 1976), ''Invertebrate Cell Culture Applications'' (Maramorosch and Mitsuhashi, 1982), and ''Biotechnology in Insect Pathology and Cell Culture'' (Maramorosch, 1987). Springer Verlag published ''Invertebrate and Fish Tissue Culture,'' edited by E. Kurstak *et al*. (1988); CRC Press published ''Biotechnology for Biological Control of Pests and Vectors'' (Maramorosch, 1991); ''Arthropod Cell Culture Systems'' (Maramorosch and McIntosh, 1994); ''Insect Cell Biotechnology'' (Maramorosch and McIntosh, 1994). In 1997, ''Invertebrate Cell Culture: Novel Directions and Biotechnology Applications,'' by Maramorosch and Mitsuhashi, was published by Science Publishers. ''Invertebrate Cell Culture: Looking Toward the XXI Century,'' by Maramorosch and M. J. Loeb, was published by SIVB, Columbia, MD (1997).

In 1976, I started the new Academic Press series ''Advances in Cell Culture.'' Volumes I–V appeared between 1981 and 1987. Dr. Gordon Sato

joined me as an editor and Volumes VI and VII were published in 1988 and 1989.

In 1970, Dr. Kenneth M. Smith, whose influence on my decision to become a virologist I described earlier, suggested to Academic Press that I should join him, Drs. Max A. Lauffer, and Frederik B. Bang as an editor of ''Advances in Virus Research (AVR).'' Starting with Vol. 18 till Vol. 27, all four editors worked jointly, but unfortunately in 1981, both Kenneth Smith and Frederik Bang passed away. Dr. Lauffer and I continued editing AVR and after Dr. Lauffer retired, I was able to persuade Drs. Aaron Shatkin and Frederick A. Murphy to join me as AVR editors. Starting with Vol. 30 in 1985, all three of us still continue to edit AVR.

In addition to plant and insect viruses, I worked shortly with other viruses, including Friend murine leukemia virus in experimentally infected mosquitoes, Marek's herpes disease virus, and yellow fever virus.

X. INTERNATIONAL CONNECTIONS

In 1953, shortly after I became a naturalized US citizen, I was invited by Professor H. Thung to come for 3 months to his virology laboratory in Wageningen, the Netherlands. At the same time, Dr. Kenneth M. Smith invited me to a symposium organized by him at the VI International Virology Congress in Rome, Italy. The trip to Europe was only partially reimbursed by Professor Thung and Rockefeller University. The bulk of the expenses required a personal loan from my bank, which I repaid in 24 monthly installments. I never regretted the personal expenses, realizing how important the trips abroad were in making contacts with numerous virologists from different countries. During subsequent years my urge to travel did not subside and my visits to research institutes in Europe, Asia, Australia, and Africa became a constant feature. Contacts with virologists, entomologists, and plant pathologists were made during 28 visits to India. several trips to Japan, China, and Southeast Asia, to West, East, and South Africa, South and Central America, Australia, and several European countries. My knowledge of 7 languages was an important asset during these trips abroad.

In 1962, the New York Academy of Science elected me Vice President and Recording Secretary. In 1970, I was elected to the Leopoldina, oldest European Science Academy. At first, I hesitated to accept the membership in the Academy, located in Germany, but when I found out that among former members were Bohr, Curie Sklodowska, Liebig, Linne, Pavlov, Planck, and Rutherford, I accepted the election and the invitation to give an inaugural lecture in Halle, Germany. A few years later, in 1979, the Indian National Academy of Science elected me an Honorary Fellow, followed by the Indian Virological Society in 1987.

I became a Rockefeller Foundation grantee in 1955 to work on virus diseases of corn with Mexican agricultural scientists. In 1964, the Romanian Academy of Sciences invited me as guest lecturer for 4 weeks to Bucharest. This was followed by the USSR Academy of Medicine, then by the Indian Academy of Sciences, and the Polish Academy. In addition to virology, my special interest became the promoting of international scientific cooperation. As a consultant of the United Nations Development Program, I worked with Indian plant virologists and entomologists at the University of Agricultural Sciences at Hebbal-Bangalore, India in 1978–79, studying virus and phytoplasma diseases of food and fiber plants.

Various honors and awards were received during the past years but I will mention just one, the Wolf Prize in Agriculture, received in 1980 and often called the Nobel Prize in Agriculture. It was received for my studies on interactions between insect vectors, viruses, and plants.

I have listed several lucky events that promoted my scientific career, but the most important and luckiest was—you guessed it—my wife Irene. Without her unwavering support and devotion, I would not have been able to follow my chosen profession. In 1957, when I was invited to succeed Professor Edward Steinhaus as chair of the Entomology Department at the University of California in Berkeley, she was willing to leave her beloved work at the New York Public Library to follow me to California. It took me 4 months before I declined the very tempting offer and we remained in the East. When I accepted the position as Distinguished Professor at Waksman Institute, Irene noticed how the 200 km of daily driving to and from Rutgers University was taken a toll on my energy and health. After 30 years, she sacrificed her own career, resigned, took early retirement, and moved with me to New Jersey (Fig. 6).

I would like to end this biographical sketch by citing my acceptance remarks made in Jerusalem when I received the Wolf Prize:

Mr. President, Members of the Knesset, members of the Wolf Foundation, colleagues and friends. It is difficult for me to find the proper words to express my feelings and emotions on this solemn occasion and this beautiful ceremony. I feel humble and proud of having been selected the recipient of the coveted Wolf Prize in Agriculture and I would like to express my deep appreciation to the Wolf Foundation and to its Founder for their vision and foresight in recognizing agriculture as one of the important fields of modern science. Over the past 30 years numerous associates have contributed significantly to projects carried out in my laboratory and several national and international organizations, and foundations have sponsored my research. Many conferences, surveys, consultancies, and visits to developing and developed nations have been made possible through the excellent international collaboration of scores of scientists and institutions. All of them

FIGURE 6 At home with my wife. Photo by the author.

have contributed to the success of my career and to the honor bestowed on me today.

My only regret is that neither my nor my wife's parents, nor my brother, nor my wife's sister can be with us today. Unfortunately, they perished during the holocaust, together with more than 150 of our closest relatives. Only their names are left here in Jerusalem at Yad Vashem. Let us hope that the ideals that are so aptly expressed by the Wolf Foundation, the fostering of international understanding among scientists throughout the world, will prevent future wars and assure lasting peace on earth. Science recognizes no political, religious, ethnic, or geographic borders and we, scientists, speak only one language—the language of science. I sincerely hope that real peace can be achieved through the efforts of scientists collaborating with each other, irrespective of background and political beliefs. I address my heartiest thanks to the Wolf Foundation for fostering international understanding.''

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I want to express my special thanks to all my past graduate students and postdoctoral associates who worked with me in the Unites States and abroad during the past years. To them I express my sincere thanks and best wishes for their continuous successful research and happy life.

REFERENCES

Bird, J., and Maramorosch, K. (1975). Viruses and virus diseases associated with whiteflies. *Adv. Virus Res.* **22:**55–110.

Black, L. M. (1953). Transmission of plant viruses by Cicadellids. *Adv. Virus Res.* **1:**69–89.

Brakke, M. K., Maramorosch, K., and Black, L. M. (1953). Properties of the wound-tumor virus. *Phytopathology* **43:**387–390.

Corner, G. W. (1964). ''A History of the Rockefeller Institute 1901–1953.'' The Rockefeller Institute Press, New York City.

Fukushi, T. (1935). Multiplication of virus in its insect vector. *Proc. Imp. Acad. Japan* **11:**301–303.

Ginsberg, H. S. (1999). The life and times of adenoviruses. *Adv. Virus Res.* **54:**1–13.

Goodman, R. M. (1977). Single-stranded DNA genome in a whitefly-transmitted virus. *Virology* **83:**171–179.

Granados, R. R., Hirumi, H., and Maramorosch, K. (1967). Electron microscopic evidence for wound-tumor virus accumulation in various organs of an inefficient leafhopper vector, *Agalliopsis novella*. *J. Invertebr. Pathol.* **9:**147–159.

Granados, R. R., Ward, L., and Maramorosch, K. (1968). Insect viremia caused by a plant-pathogenic virus: Electron microscopy of vector hemocytes. *Virology* **34:**790–796.

Hirumi, H., and Maramorosch, K. (1963). Recovery of aster yellows virus from various organs of the insect vector, *Macrosteles fascifrons*. *Contrib. Boyce Thompson Inst.* **22:**165–173.

Hirumi, H., and Maramorosch, K. (1964). The *in vitro* cultivation of embryonic leafhopper tissues. *Exp. Cell Res.* **36:**625–631.

Hirumi, H., and Maramorosch, K. (1968). Electron microscopy of wound-tumor virus in cultured embryonic cells of the leafhopper *Macrosteles fascifrons*. *In* ''Second International Colloquium on Invertebrate Tissue Culture'' (C. Barigozzi, ed.), pp. 203–217. Lombardo Acad. Sci. Lettere, Milano.

Hirumi, H., and Maramorosch, K. (1971). Cell culture of Hemiptera. *In* ''Invertebrate Tissue Culture'' (C. Vago, ed.), Vol. 1, pp. 307–340. Academic Press, New York.

Hirumi, H., Burton, G. J., and Maramorosch, K. (1971). Preliminary studies on electron microscopy of Friend murine leukemia virus in the midgut of experimentally infected mosquitoes. *J. Virol.* **8:**801–804.

Hirumi, H., Frankel, J. W., Prickett, C. O., and Maramorosch, K. (1974). Coexistance of particles resembling herpes virus and Type-C virus in chicken feather follicle epithelium. *J. Natl. Cancer Inst.* **52:**303–306.

Koren, Z., Van Damme, O., Hirumi, H., and Maramorosch, K. (1971). The growth and stimulation of mouse trophoblastic cells in culture by polyoma virus. *Am. J. Obstet. Gynecol.* **111:**846–850.

Maramorosch, K. (1950). Influence of temperature on incubation and transmission of the wound-tumor virus. *Phytopathology* **40:**1071–1093.

Maramorosch, K. (1951a). Handy insect-vector cage. *J. NY Entomol. Soc.* **59:**49–50.

Maramorosch, K. (1951b). A simple needle for micro-injections. *Nature.* **167:**734.

Maramorosch, K. (1952a). Multiplication of aster-yellows virus in its vector. *Nature* **169:**194–195.

Maramorosch, K. (1952b). Toxicity of cellulose acetate sheets to plants and fish. *Science* **115:**236.

Maramorosch, K. (1953). A versatile virus. *Sci. Am.* **188:**78–86.

Maramorosch, K. (1954). Biological transmission of plant viruses by animal vectors. *Trans. NY Acad. Sci.* **16:**189–195.

Maramorosch, K. (1955). Multiplication of plant viruses in insect vectors. *Adv. Virus Res.* **3:**221–249.

Maramorosch, K. (1956). Multiplication of aster yellows virus in *in vitro* preparations of insect tissues. *Virology* **2:**369–376.
Maramorosch, K. (1957). Reversal of virus-caused stunting in plants by gibberellic acid. *Science* **126:**126–651.
Maramorosch, K. (1958). Beneficial effect of virus diseased plants on non-vector insects. *Tijdschr. Plziekt.* **63:**383–391.
Maramorosch, K. (1959). Reversal of virus-caused stunting by gibberellic acid in plants infected with corn stunt, aster yellows and wound tumor. *Proc. 6th Int. Congress of Crop Protection, Hamburg* **I:**271–272.
Maramorosch, K. (1961). Report to the Government of the Philippines on the cadang-cadang disease of coconut. *FAO ETAP Bull.* 1333. Rome.
Maramorosch, K. (1968). Plant pathogenic viruses in insect vectors. *Curr. Top. Microbiol.* **42:**94–107.
Maramorosch, K. (1969). Effects of rice-pathogenic viruses on their insect vectors. *In* ''IRRI Symposium on Rice Virus Diseases,'' pp. 179–203. Johns Hopkins University Press, Baltimore, MD.
Maramorosch, K. (1970). Insect infection caused by a plant tumor virus. *World Rev. Pest Control* **9:**29–41.
Maramorosch, K. (1974). Introduction to the systematics of viruses: Viruses of plants, viruses of invertebrates. *In* ''Handbook of Microbiology'' (Laskin and Lechevalier, eds.), pp. 579–594. CRC Press, Boca Raton, FL.
Maramorosch, K. (1975). Etiology of whitefly-borne diseases. *In* ''Tropical Diseases of Legumes'' (J. Bird and K. Maramorosch, eds.), pp. 71–89. Academic Press, New York.
Maramorosch, K. (1979a). Biological control of insect pests with viruses. *In* ''Practical Tissue Culture Applications'' (K. Maramorosch and H. Hirumi, eds.), pp. 387–398. Academic Press, New York.
Maramorosch, K. (1979b). Leafhopper tissue culture. *In* ''Leafhopper Vectors and Plant Disease Agents'' (K. Maramorosch and K. F. Harris, eds.), pp. 485–514. Academic Press, New York.
Maramorosch, K. (1985). Control of viroid diseases. *In* ''Subviral Pathogens of Plants and Animals: Viroids and Prions'' (K. Maramorosch and J. J. McKelvey, eds.), pp. 151–162. Academic Press, Orlando, New York, London.
Maramorosch, K. (1991). Schadlingsbekampfung mit Mikroorganismen und Viren zur Schonung der Umwelt. *Zeitschrift Pflanzenkrankheiten und Pflanzenschutz* **98:**344–350.
Maramorosch, K. (1993). The threat of cadang-cadang disease. *Principes* **37:**187–196.
Maramorosch, K. (2004). The phantom vector of an emerging viroid. *In* ''Life Sciences for the 21st Century'' (E. Keinan, I. Schechter, and M. Sela, eds.), pp. 297–303. VCH-Wiley, New York.
Maramorosch, K., and Jensen, D. D. (1963). Harmful and beneficial effects of plant viruses in insects. *Annu. Rev. Microbiol.* **17:**495–530.
Maramorosch, K., and Jernberg, N. (1970). An adjustable multiple insect holder for microinjection. *J. Econ. Entomol.* **63:**1216–1218.
Maramorosch, K., Brakke, M. K., and Black, L. M. (1949). Mechanical transmission of a plant tumor virus to an insect vector. *Science* **110:**162–163.
McIntosh, A. H., and Maramorosch, K. (1973). Retention of insect virus infectivity in mammalian cell cultures. *J. NY Entomol. Soc.* **81:**175–182.
McIntosh, A. H., Maramorosch, K., and Riscoe, R. (1979). *Autographa californica* nuclear polyhedrosis virus (NPV) in a vertebrate cell line: Localization by electron microscopy. *J. NY Entomol. Soc.* **87:**55–58.
Mitsuhashi, J., and Maramorosch, K. (1963). Aseptic cultivation of four virus transmitting species of leafhopper (Cicadellidae). *Contrib. Boyce Thompson Inst.* **22:**165–173.

Mitsuhashi, J., and Maramorosch, K. (1964). Inoculation of plant tissue cultures with aster yellows virus. *Virology* **23:**277–279.
Randles, J. W. (1975). Association of two ribonucleic acid species with cadang-cadang disease of coconut palm. *Phytopathology* **65:**163–167.
Shikata, E., and Maramorosch, K. (1965a). Plant tumor virus in arthropod host: Microcrystal formation. *Nature* **288:**507–508.
Shikata, E., and Maramorosch, K. (1965b). Electron microscopic evidence for the systemic invasion of an insect host by a plant pathogenic virus. *Virology* **27:**461–475.
Shikata, E., and Maramorosch, K. (1966a). An electron microscope study of plant neoplasia induced by wound tumor virus. *J. Natl. Cancer Inst.* **36:**97–116.
Shikata, E., and Maramorosch, K. (1966b). Electron microscopy of a pea enation mosaic virus in plant cell nuclei. *Virology* **30:**439–454.
Shikata, E., and Maramorosch, K. (1967a). Electron microscopy of wound tumor virus assembly sites in insect vectors and plants. *Virology* **32:**363–377.
Shikata, E., and Maramorosch, K. (1967b). Electron microscopy of the formation of wound tumor virus in abdominally inoculated insect vectors. *J. Virol.* **1:**1052–1073.
Shikata, E., and Maramorosch, K. (1969). Electron microscopy of insect-borne viruses *in situ*. *In* ''Viruses, Vectors, and Vegetation'' (K. Maramorosch, ed.), pp. 393–415. Interscience, John Wiley & Sons, New York.
Shikata, E., Maramorosch, K., and Granados, R. R. (1966). Electron microscopy of pea enation mosaic virus in plants and aphid vectors. *Virology* **29:**426–436.
Shikata, E., Orenski, S. W., Hirumi, H., Mitsuhashi, J., and Maramorosch, K. (1964). Electron micrographs of wound-tumor virus in an animal host and in a plant tumor. *Virology* **23:**441–444.
Storey, H. H. (1933). Investigations of the mechanism of the transmission of plant viruses by insect vectors. I. *Proc. R. Soc. Lond. Ser. B* **113:**463–485.
Streissle, G., and Maramorosch, K. (1963). Reo virus and wound-tumor virus: Serological cross reactivity. *Science* **140:**996–997.
Szybalski, W. (1999). Maintenance of human-fed live lice in the laboratory and production of Weigl's exanthematous typhus vaccine. *In* ''Maintenance of Human, Animal, and Plant Pathogen Vectors'' (K. Maramorosch and F. Mahmood, eds.), pp. 161–179. Science Publishers, Enfield, NH, USA.
Tokumitsu, T., and Maramorosch, K. (1966). Survival of aphid cells *in vitro*. *Exp. Cell Res.* **44:**652–655.
Tokumitsu, T., and Maramorosch, K. (1967). Cytoplasmic protrusions in insect cells during mitosis *in vitro*. *J. Cell Biol.* **34:**677–683.
Yamada, K., and Maramorosch, K. (1980). Propagation of *Heliothis zea* single embedded baculovirus in a *H. zea* cell line. Abstracts 16th International Entomology Congress Tokyo, Japan, p. 193.
Yamada, K., and Maramorosch, K. (1981). Plaque assay of *Heliothis zea* baculovirus employing a mixed agarose overlay. *Arch. Virol* **67:**187–189.
Yamada, K., Sherman, K. E., and Maramorosch, K. (1981). *In vivo* infectivity of early and late passaged *Heliothis zea* polyhedra produced in tissue culture. *Appl. Entomol. Zool.* **16:**504–505.

BOOKS

K. Maramorosch (ed.) (1962). Biological Transmission of Disease Agents. Academic Press, New York and London.
K. Maramorosch and H. Koprowski (eds.) (1967–1984). Methods in Virology, Vols. 1–8. Academic Press, New York and London.

K. Maramorosch (ed.) (1968). Insect Viruses, Springer Verlag, New York, Berlin.
K. Maramorosch (ed.) (1969). Viruses, Vectors, and Vegetation. Wiley-Interscience, New York, London, Toronto.
K. Maramorosch and E. Kurstak (eds.) (1971). Comparative Virology. Academic Press, New York.
M. A. Lauffer, F. B. Bang, K. Maramorosch, and K. M. Smith (eds.) (1973–1981). Advances in Virus Research, Vols. 22–26. Academic Press, New York.
E. Kurstak and K. Maramorosch (eds.) (1974). Viruses, Evolution, and Cancer. Academic Press, New York, London.
K. Maramorosch and R. E. Shope (eds.) (1975). Invertebrate Immunity. Academic Press, New York and London.
J. Bird and K. Maramorosch (eds.) (1975). Tropical Diseases of Legumes. Academic Press, New York and London.
K. Maramorosch (ed.) (1976). Invertebrate Tissue Culture: Research Applications. Academic Press, New York, London.
E. Kurstak and K. Maramorosch (eds.) (1976). Invertebrate Tissue Culture: Applications in Medicine, Biology, and Agriculture. Academic Press, New York and London.
K. Maramorosch (ed.) (1977). The Atlas of Insect and Plant Viruses. Academic Press, New York and London.
K. Maramorosch and H. Hirumi (eds.) (1979). Practical Tissue Culture Applications. Academic Press, New York.
E. Kurstak, K. Maramorosch, and A. Dubendorfer (eds.) (1977). Invertebrate Systems *In Vitro*. Elsevier-North Holland Biomedical Press.
K. F. Harris and K. Maramorosch (eds.) (1977). Aphids as Virus Vectors. Academic Press, New York and London.
K. Maramorosch and K. F. Harris (eds.) (1979). Leafhopper Vectors and Plant Disease Agents. Academic Press, New York.
K. F. Harris and K. Maramorosch (eds.) (1980). Vectors of Plant Pathogens. Academic Press, New York.
M. A. Lauffer and K. Maramorosch (eds.) (1981–1984). Advances in Virus Research, Vols. 27 and 28. Academic Press, New York.
K. Maramorosch and K. F. Harris (eds.) (1981). Plant Diseases and Vectors: Ecology and Epidemiology. Academic Press, New York.
K. F. Harris and K. Maramorosch (eds.) (1982). Pathogens, Vectors, and Plant Diseases: Approaches to Control. Academic Press, New York.
J. J. McKelvey, Jr., B. F. Eldridge, and K. Maramorosch (eds.) (1980). Vectors of Disease Agents: Interactions with Plants, Animals and Man. Praeger, New York.
E. Kurstak and K. Maramorosch (eds.) (1978). Viruses and Environment. Academic Press, New York.
K. Maramorosch (ed.) (1981–1987). Advances in Cell Culture, Vols. 1–5. Academic Press, New York.
K. Maramorosch and G. H. Sato (eds.) (1988–1989). Advances in Cell Culture, Vols. 6 and 7. Academic Press, New York.
K. Maramorosch and J. Mitsuhashi (eds.) (1982). Invertebrate Cell Culture Applications. Academic Press, New York.
K. Maramorosch and J. J. McKelvey, Jr. (eds.) (1985). Subviral Pathogens of Plants and Animals: Viroids and Prions. Academic Press, New York.
K. Maramorosch, F. A. Murphy, and A. J. Shatkin (eds.) (1985–2007). Advances in Virus Research Vols. 29–70. Academic Press, Elsevier, London, San Diego, Amsterdam etc.
K. Maramorosch and K. E. Sherman (eds.) (1985). Viral Insecticides for Biological Control. Academic Press, Orlando, New York, London, Tokyo.

K. Maramorosch (ed.) (1987). Biotechnology in Insect Pathology and Cell Culture. Academic Press, San Diego, CA.
E. Kurstak, Y. Kuroda, and K. Maramorosch (eds.) (1988). Invertebrate and Fish Tissue Culture. Springer, Berlin and Tokyo.
K. Maramorosch (ed.) (1991). Viroids and Satellites: Molecular Parasites at the Frontier of Life. CRC Press, Boca Raton, FL, USA.
K. Maramorosch (ed.) (1992). Plant Diseases of Viral, Viroid, Mycoplasma, and Uncertain Etiology. Oxford and IBH, New Delhi, INDIA.
K. Maramorosch and A. H. McIntosh (eds.) (1994). Arthropod Cell Culture Systems. CRC Press, Boca Raton, Ann Arbor, London, Tokyo.
K. Maramorosch and A. H. McIntosh (eds.) (1994). Insect Cell Biotechnology. CRC Press, Boca Raton, FL.
K. Maramorosch and J. Mitsuhashi (eds.) (1997). Invertebrate Cell Culture: Novel Directions and Biotechnology Applications. Science Publishers, Enfield, NH, USA.
K. Maramorosch and M. J. Loeb (eds.) (1997). Invertebrate Cell Culture: Looking Toward the XXI Century. SIVB, Columbia, MD.
K. Maramorosch and F. Mahmood (eds.) (1999). Maintenance of Animal/Human and Plant Pathogen Vectors. Science Publishers, Enfield, NH, USA.

CHAPTER 2

Honey Bee Viruses

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Contents

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Abstract

Viruses are significant threats to the health and well-being of the honey bee, *Apis mellifera*. To alleviate the threats posed by these invasive organisms, a better understanding of bee viral infections will be of crucial importance in developing effective and environmentally benign disease control strategies. Although knowledge of honey bee viruses has been accumulated considerably in the past three decades, a comprehensive review to compile the various aspects of bee viruses at the molecular level has not been reported. This chapter summarizes recent progress in the understanding of the morphology, genome organization, transmission, epidemiology, and pathogenesis of honey bee viruses as well as their interactions with their honey bee hosts. The future prospects of research of honey bee viruses are also discussed in detail. The chapter has been designed to provide researchers in the field with updated information about honey bee viruses and to serve as a starting point for future research.

I. INTRODUCTION

The honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), is found all over the world and plays an important role in the global economy by assisting in the pollination of a wide variety of food crops and by producing honey, beeswax, pollen, propolis, royal jelly, and other hive products. To ensure an adequate supply of bees for the pollination of agricultural crops and the production of hive products, a healthy and vigorous population of honey bees will be essential. However, like other animals, honey bees are inevitably subject to infection by a wide variety of pathogens that are responsible for significant colony losses. Among honey bee pathogens, viruses pose one of the major threats to the health and well-being of honey bees and have caused serious concerns for researchers and beekeepers.

Viruses were first identified as a new class of pathogens infecting honey bees when a US scientist, Dr. White, discovered that a filterable agent from diseased bee larvae could cause sacbrood disease in the honey bee (White, 1913). Since then, at least 18 viruses have been reported to infect honey bees worldwide (Allen and Ball, 1996; Ellis and Munn, 2005). Although knowledge of honey bee viruses is still limited compared to that of other well-studied insect viruses, such as baculoviruses, understanding

of virus infections in honey bees has grown considerably over the last three decades and a body of literature dealing with bee virus identification, physiochemical properties, natural history, transmission, incidence, and pathology has been accumulated. In this chapter, we describe recent progress in understanding morphology, genome organization, transmission, epidemiology, and pathogenesis of honey bee viruses as well as their interactions with their honey bee host. Infections of viruses in honey bees have been reviewed previously. The main goal of this chapter is to update previous findings with more recent work relating to the molecular biology of the honey bee viruses, however, some main features of earlier reviews: Bailey, 1976, 1981, 1982a; Bailey and Ball, 1991; Ball, 1996; Ball and Bailey, 1991, 1997.

II. COMMON HONEY BEE VIRUSES

Viruses could attack at different developing stages and castes of the honey bees, including eggs, larvae, pupae, adult worker bees, adult drones, and queen of the colonies. Although bee viruses usually persist as inapparent infections and cause no overt signs of disease, they can dramatically affect honey bee health and shorten the lives of infected bees under certain conditions (Ball and Allen, 1988; Martin, 2001). Of 18 viruses identified to attack honey bees, six viruses, namely, Deformed wing virus (DWV), *Black queen cell virus* (BQCV), *Sacbrood virus* (SBV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV), and Chronic bee paralysis virus (CBPV) are the most common infections and have been objects of active research currently.

A. Deformed wing virus

DWV was first isolated from diseased adult bees in Japan (Bailey and Ball, 1991). The occurrence and distribution of DWV has since been worldwide. Except for Oceania, the infection of DWV so far has been reported in Europe, North America, South America, Africa, Asia, and the Middle East (Allen and Ball, 1996; Antúnez *et al*., 2006; Ellis and Munn, 2005). The infection of DWV has also been identified in *A. cerana* in China (Bailey and Ball, 1991).

DWV is one of a few bee viruses that cause well-defined disease symptoms in infected bees. Typical disease symptoms of DWV infection include shrunken, crumpled wings, decreased body size, and discoloration in adult bees. However, the mechanism by which the DWV causes the morphological deformities of the infected hosts is unclear. Aside from the adult stage, DWV infection is also detected in other stages of

bee development, including egg, larvae, and pupae. When pupae at the normally multiplies slowly and rarely kills the pupae, instead mostly causing deformity and early death in newly emerged adult bees. Adult honey bees infected with DWV usually appear normal but are believed to have a reduction in life span (Bailey and Ball, 1991; Ball and Bailey, 1997; Kovac and Crailsheim, 1988).

DWV appears to be the most prevalent infection in *A. mellifera* in recent years. Our 5-year field survey carried out in Beltsville, MD showed that DWV infection occurred in 100% of the apiaries investigated (Y. P. C., unpublished observation). Similar results were reported previously by Tentcheva *et al*. (2004b) who observed that DWV was detected in over 97% of French apiaries when the adult bee population was examined. A study on the prevalence and distribution pattern of viruses in Austria demonstrated that DWV was present in 91% of tested bee samples (Berényi *et al*., 2006). Although high prevalence of DWV is not geographically related, some seasonal variation in virus incidence was observed and the frequency of DWV infection in both adult bees and pupae increased considerably from summer to autumn during the year (Tentcheva *et al*., 2004a,b). The striking high incidence of DWV infection in honey bees obtained from these studies indicate that DWV is prevalent over a wide range of geographic locations and is likely to become an important cause of mortality in honey bee colonies whenever a viral disease outbreak occurs, and warrants further investigation in the epidemiology and pathogenesis of this pathogen.

Bee colonies infected with DWV are often found to be associated with the infestation of a parasitic mite, *Varroa destructor* (Anderson and Trueman, 2000). Both laboratory and field studies showed that the varroa mite is an effective vector of the DWV (Ball and Allen, 1988; Bowen-Walker *et al*., 1999; Martin *et al*., 1998; Nordström, 2003; Nordström *et al*., 1999; Shen *et al*., 2005b). Varroa mites acquire the virus from infected bees and transmit it to uninfected bees, which either develop morphological deformities or die after the mites feed on them for a period of time. Studies of virus status in varroa mites showed that DWV was present in 100% of varroa mites collected from Thailand (Chantawannakul *et al*., 2006) and that varroa mites appeared to be DWV positive in 100% of French apiaries (Tentcheva *et al*., 2004b). Evaluation of DWV infection in individual bees showed that DWV was detected in 69% of bees collected from mite-infested colonies in Poland (Topolska *et al*., 1995), and in over 90% of bees from mite-infested colonies in England (Ball, 2001). The high frequency of DWV in mites and mite-infested bee colonies suggests that the significant increase in prevalence of DWV infection in recent years is likely associated with the worldwide infestation of varroa mites in honey bees. It also suggests that the varroa mite may play a major role in colony collapse due to the outbreak of viral disease.

B. Sacbrood virus

SBV is the most widely distributed of all honey bee viruses. Since its first identification in the United States in 1913 (White, 1913), infection of SBV has been found on every continent where *A. mellifera* honey bees are present (Allen and Ball, 1996; Bradbear, 1988; Ellis and Munn, 2005).

SBV attacks both brood and adult stages of bees, but larvae about 2-day old are most susceptible to SBV infections (Ball and Bailey, 1997). SBV affects adult bees without causing obvious signs of disease, but the infected adult bees may have a decreased life span (Bailey, 1969; Bailey and Fernando, 1972). The initial spread of SBV within a colony occurs when nurse bees become infected while removing larvae killed by SBV. Virus particles accumulate in the hypopharyngeal glands of the nurse bees and infected nurse bees can then spread the virus throughout the colony by feeding larvae with their glandular secretion and exchanging food with other adult bees including foraging bees. Infected foraging bees spread the virus by passing it from their glandular secretions to the pollen loads as they collect pollen. Young larvae become infected with the virus by ingesting virus-contaminated food. The SBV starts to replicate in the larva, and the infected larva turns pale yellow after the brood cell is capped. As the disease progresses, the skin of the larva becomes leathery and the larva fails to pupate because it cannot digest the old cuticle. A large amount of fluid containing millions of SBV particles accumulates between the body of a diseased larva and its saclike skin. Affected larvae appear to be a water-filled sac when removed from the cell. Sacbrood derives its name from the saclike appearance of the diseased larvae.

Infection of SBV can be readily diagnosed in the field because of the characteristic symptoms produced in diseased brood. Typically, when bee colonies are heavily infected with SBV, there are a number of partially uncapped or completely uncapped brood cells scattered among capped brood that can be found on the brood frame. Dead larva becomes a dark, brittle scale can be easily removed from the brood cell, a characteristic that differs from a bacterium-caused brood disease, American foulbrood.

Prevalence of SBV in honey bees has been found to be prominently seasonal. Frequencies of SBV infection in spring and summer were significantly higher than in autumn (Anderson and Gibbs, 1988; Bailey *et al*., 1981; Tentcheva *et al*., 2004b). The incidence of SBV has been believed to be positively correlated with the number of susceptible brood and young workers in the colonies. During the seasons of spring and summer, the rich sources of pollen and nectar stimulate brood rearing and a great number of new workers hatch from the brood cells, providing opportunities for SBV to attack bees and multiply in the colonies. The seasonal variation in SBV indirectly reflects variable susceptibility of different bee developmental stages to the virus infection.

SBV infection has been associated with varroa mite infestation. SBV was detected in large amount of adult bees from varroa mite–infested colonies (Antúnez *et al*., 2006; Ball, 1989; Berényi *et al*., 2006). Detection of SBV in varroa mites (Chantawannakul *et al*., 2006; Shen *et al*., 2005a; Tentcheva *et al*., 2004b) indicates that varroa mites have the potential to transmit the virus in the bee colonies, although varroa mite as a vector in transmitting SBV has not yet been experimentally demonstrated.

A new strain of SBV has been identified in the eastern honey bee, *A*. *cerana*, from Thailand in 1982. Infection of Thai SBV (TSBV) was also detected in India. TSBV is serologically related to SBV but not physiochemically identical to SBV (Bailey, 1982b).

C. Black queen cell virus

BQCV was first isolated from dead queen larvae and prepupae sealed in their cells that had turned dark brown to black along with the walls of the cell (Bailey and Woods, 1977), hence the designation of the name. The infection of BQCV in bees has been reported in North America, Central America, Europe, Oceania, Asia, Africa, and the Middle East (Allen and Ball, 1996; Ellis and Munn, 2005).

BQCV mainly affects developing queen larvae and pupae in the capped-cell stage. High incidences of the virus infection are observed in queen-rearing colonies in spring and early summer (Laidlaw, 1979). Diseased larvae have a pale yellow appearance and a tough saclike skin, a disease symptom also seen in SBV-infected larvae. BQCV readily multiplies in the pupal stage of the honey bees. Infected pupae turn dark and die rapidly. The wall of the queen cell eventually becomes dark colored, a characteristic symptom of BQCV infection. Worker bees can also be infected by BQCV but normally do not exhibit outward disease symptoms. BQCV does not multiply in bees when the virus particles are ingested.

Our 5-year field survey in Beltsville, MD showed that BQCV was the second most common infection of honey bees in the field after DWV (Y. P. C., unpublished observation). In 1993, Anderson (1993) reported that BQCV was the most common cause of queen larvae mortality in Australia. A study conducted by Tentcheva *et al.* (2004b) indicated that BQCV infection was more prevalent in adult bees than in pupae and that the incidence of BQCV was higher in spring and summer than in autumn. This result was consistent with a previous finding by Laidlaw (1979) that BQCV was more prevalent in spring and summer during the year.

In the field, BQCV disease outbreak has been linked with infection of a protozoan, *Nosema apis*. When the incidence of *N*. *apis* infection was high during the spring and summer, the infection of BQCV was more prevalent in honey bees (Bailey, 1981). It has been observed that BQCV multiplied rapidly in adult bees infected with *N*. *apis* (Bailey, 1982a).

BQCV is believed to be transmitted to queen brood via glandular secretion of nurse bees during the feeding (Bailey, 1982a). *N. apis* infects midgut tissues of the adult bees, increasing the susceptibility of the alimentary tract to infection by BQCV. Bailey *et al.* (1981) reported that honey bees infected with BQCV were found to be infected with *N. apis* simultaneously from all parts of England and Wales during 1979. Field survey of Austrian apiaries showed that *N. apis* was found to be present in 78% of BQCV-positive bee samples and that 75% of *N. apis*–infected colonies were also infected with BQCV (Berényi *et al.*, 2006). Similar results were also obtained from a survey carried out in France (Tentcheva *et al.*, 2004b). Although positive association between the BQCV and *N. apis* infections has been documented in the field observations, definite experimental evidence for deciphering the mechanism of *N. apis* in activation and transmission of BQCV infection remains to be determined.

Varroa mites are thought to sometimes act as a vector for BQCV (Bailey, 1976). Detection of BQCV in varroa mites collected from a Thai honey bee apiary supports this assumption (Chantawannakul *et al.*, 2006). However, an investigation conducted by Tentcheva *et al.* (2004b) yielded a different result; BQCV was never detected in any of the varroa mites they examined. Further studies to confirm the role of varroa mites as a vector in BQCV transmission will be necessary.

D. Kashmir bee virus

The origin of KBV in the bee species is obscure. KBV was first isolated from adult western honey bees, *A. mellifera*, that were experimentally inoculated with an extract prepared from the diseased Asian honey bee (*A. cerana*) in Kashmir, northwestern region of India, hence the name (Bailey and Woods, 1977). Subsequently, KBV has been detected in *A. mellifera* collected from Australia (Bailey *et al.*, 1979). The detection of KBV in the natural population of *A. mellifera* in Australia was unexpected because *A. cerana*, which is assumed to be the original host of KBV, does not exist there. Later, strains of KBV have been found in *A. mellifera* from Canada and New Zealand (Allen and Ball, 1995; Anderson, 1985), Fiji (Anderson, 1990), Spain (Allen and Ball, 1995), and the United States (Bruce *et al.*, 1995; Hung *et al.*, 1995). The unexpected emergence of KBV in the countries such as Australia and New Zealand might be due to the importation of bees from North American or other countries where KBV is endemic. So far, infection of KBV in *A. mellifera* has also been documented in several countries in Europe and Oceania (Allen and Ball, 1996; Ellis and Munn, 2005; Siede *et al.*, 2005).

KBV attacks all stages of the bee life cycle (Hornitzky, 1981, 1982) and commonly persists within brood and adult bees as an inapparent infection (Anderson and Gibbs, 1988; Dall, 1985). The disease and mortality

caused by KBV infection occurs in different developing stages of bees without clearly defined disease symptoms. Among all of the viruses infecting honey bees, KBV is considered to be the most virulent under laboratory conditions. It multiplies quickly once a few viral particles are introduced into the bee hemolymph and can cause bee mortality within 3 days. However, KBV does not cause infection when adult bees are fed with food mixed with KBV particles. The virus probably invades the bees through the cuticle by direct contact between live bees (Bailey *et al.*, 1979).

KBV is genetically, serologically, and pathologically closely related to another bee virus ABPV. Infection of KBV in honey bees resembles infection caused by ABPV in several ways. For example, both viruses usually persist as inapparent infections in bees and replicate readily only when injected into the hemolymph of adult bees (Anderson, 1991). Immunodiffusion tests showed that strains of KBV from Canada and Spain were even more serologically closely related to ABPV than were other KBV strains (Allen and Ball, 1995). Molecular analysis revealed KBV and ABPV share about 70% sequence homology over the entire genome, although there are significant differences in several critical areas of the genomes between the two viruses (De Miranda *et al.*, 2004). Phylogenetic analyses suggest that KBV and ABPV are distinct viruses and can be inferred to be different species, even though there is no clear geographic and ecological separation between the two viruses (De Miranda *et al.*, 2004; Evans, 2001).

Incidence of KBV infection in honey bees is less prevalent, as compared with other highly prevalent bee viruses such as DWV, BQCV, and SBV. Field survey of honey bee viruses on a large geographic scale of France showed that KBV was found in 17% of the apiaries for adult population, and 5% of the apiaries for pupae versus 97% and 94% of the apiaries with DWV infection for adult and pupae, and 86% and 80% of apiaries with SBV infection for adult and pupae, 86% and 23% with SBV infection for adult and pupae, respectively (Tentcheva *et al.*, 2004b). Although KBV has been considered to be more widespread in the United States than in Europe (Allen and Ball, 1996), field survey from 2002 to 2006 in Maryland indicated that the incidence of KBV infection varied significantly from year to year with more than 50% of apiaries with KBV infection in 2002 and about 10–20% of the apiaries with KBV infection for the rest of the years (Y. P. C., unpublished observation).

Although KBV usually persists as an inapparent infection in honey bees, infection of KBV can be activated to a lethal level in the presence of varroa mites (Bailey *et al.*, 1979). A high mite-infestation level could result in high virulence in the bee colonies (Hung *et al.*, 1996b). It has been experimentally proven that varroa mites were effective vectors of KBV. They transmitted KBV in the same way as they transmitted DWV in bee colonies (Chen *et al.*, 2004b). Varroa mites acquired KBV from virus-infected bees and transferred the virus to virus-negative hosts during

feeding. Varroa mites also acquired virus from KBV-positive mites by cohabiting in the same cell with virus-positive mites via a bee host intermediary. A subsequent study conducted by Shen *et al*. (2005b) further supports the role of varroa mites as a vector in transmitting KBV in bees.

E. Acute bee paralysis virus

ABPV was first discovered during laboratory infectivity tests with CBPV (Bailey *et al*., 1963). When bees were experimentally inoculated with purified CBPV particles, the bees remained flightless and trembling for about 5–7 days before they died. In contrast, when healthy bees were injected with extract prepared from a group of apparently healthy bees and incubated for 5–6 days, most of the bees became flightless and died quickly. Virus particles were isolated from the extracts of those apparently healthy bees that caused bee acute paralysis, hence the designation of the name to distinguish it from CBPV (Bailey *et al*., 1963). Since its first identification, the presence of ABPV in honeybees of *A. mellifera* has been reported in North America, Central and South America, Europe, Oceania, Asia, Africa, and the Middle East (Allen and Ball, 1996; Ellis and Munn, 2005).

ABPV can be detected in both brood and adult stages of bee development. In the field, ABPV commonly occurred in apparently healthy adult bees, particularly during the summer, and infection of ABPV was rarely noticed to be associated with disease or mortality of bees (Bailey, 1965b; Bailey *et al*., 1981). Spread of ABPV in the colonies is probably via salivary gland secretion of infected adult bees when glandular secretions are fed to young larvae or mixed in the pollen. Infected larvae either die before they are sealed in brood cell if large amounts of virus particles were ingested, or survive to emerge as inapparently infected adult bees (Bailey and Ball, 1991).

ABPV is considered to be the second most-prevalent virus in Austria (Berényi *et al*., 2006), though it has been a sporadic infection in the United States only for the last 5 years based on our survey results (unpublished observation). ABPV has been identified as a major cause for the decline and collapse of bee colonies that were also infested with varroa mites in Europe and the United States (Antúnez *et al*., 2006; Bakonyi *et al*., 2002; Ball, 1989; Ball and Allen, 1988; Berényi *et al*., 2006; Faucon *et al*., 1992; Hung *et al*., 1996c; Kulincevic *et al*., 1990). The laboratory experiments by Ball (1989) demonstrated that varroa mites can act as a virus vector and transmit ABPV from severely infected bees to healthy adult bees and brood via feeding activities. Detection of ABPV in varroa mites further supports the possible role of varroa mites in the virus transmission (Allen *et al*., 1986; Bakonyi *et al*., 2002; Chantawannakul *et al*., 2006; Tentcheva *et al*., 2004b). In addition to acting as a vector of the virus, the varroa mite is also believed to serve as an activator of ABPV in infected bees.

Detection of large amounts of the virus in diseased or dead bees from colonies heavily infested with varroa mites suggests that infestation of varroa mites may stimulate the virus to replicate to the amounts sufficient to cause bee disease and mortality (Ball and Allen, 1988; Faucon *et al*., 1992; Hung *et al*., 1996c; Kulincevic *et al*., 1990). While varroa mites might activate ABPV replication, replication of the virus in bees can be also induced by some other factors. Previous studies showed that ABPV was present in bees from apiaries where no APBV-positive varroa mites were detected (Tentcheva *et al*., 2004b) and that replication of ABPV can be activated to detectable concentrations by injection of potassium phosphate buffer (Hung *et al*., 1996c), suggesting that the varroa mite is not the sole factor contributing to the disease outbreaks of ABPV infection.

F. Chronic bee paralysis virus

CBPV was identified as a cause of adult bee paralysis by Bailey *et al*. (1963) after long suspicion that the tracheal mite, *Acarapis woodi*, was the culprit of the paralysis. Later, CBPV was extracted from naturally paralyzed bees as one of the first viruses isolated from honey bees (Bailey *et al*., 1968). CBPV has since been detected in adult bees of *A*. *mellifera* from every continent except South America (Allen and Ball, 1996; Ellis and Munn, 2005).

CBPV mainly attacks adult bees and causes two forms of ''paralysis'' symptoms in bees (Bailey, 1975). The most common one is characterized by an abnormal trembling of the body and wings, crawling on the ground due to the flight inability, bloated abdomens, and dislocated wings. The other form is identified by the presence of hairless, shiny, and black-appearing bees that are attacked and rejected from returning to the colonies at the entrance of the hives by guard bees. Both forms of symptoms can be seen in bees from the same colony. The variation in the disease symptoms may reflect differences among individual bees in inherited susceptibility to the multiplication of the virus (Kulincevic and Rothenbuhler, 1975; Rinderer *et al*, 1975).

While CBPV causes the same symptoms of trembling and the inability to fly in infected bees that ABPV does, the two viruses are different in several ways: CBPV is the less virulent of the two viruses, as CBPV takes several days to kill the diseased bees while ABPV takes only 1 day; the shapes of the two viruses are different—CBPV particles are asymmetric and ABPV particles are isometric; there are many more virus particles of CBPV than of ABPV in naturally paralyzed bees (Bailey, 1965a).

Laboratory tests were carried out to investigate the infectivity of CBPV by injecting purified virus particles into the hemolymph of bees, spraying virus preparation on the surfaces of bees, or mixing virus particles with colony food (Bailey and Ball, 1991; Bailey *et al*., 1983). The results showed

that CBPV was readily transmitted to bees by topical application of virus particles after hairs on the surface of the body were denuded. The results also showed that CBPV is not readily replicated to the level sufficient to cause disease when the virus was introduced in bees via food. Accordingly, CBPV naturally spread best among bees when the colonies were the most crowded. The close contact of overcrowded bees breaks hairs from the cuticle, allowing CBPV to spread from diseased bees to healthy bees via their exposed epidermal cytoplasm. It is likely that any factors that result in decreased foraging activities and crowded conditions in the bee colonies may lead to disease outbreaks of CBPV.

It has been reported that CBPV is very widespread in Britain and infects most bees and causes mortality in bee colonies (Bailey *et al*., 1981). The incidence of CBPV in Britain declined from 8% in 1947 to less than 2% by 1963 based on the samples submitted by beekeepers. The decrease in CBPV incidence coincided with the decline in the total number of bee colonies during that period of time (Bailey *et al*., 1983). In Austria, CBPV was found to be present in different geographic regions and infection of CBPV was detected in 10% of bee colonies suffering from various diseases (Berényi *et al*., 2006). A field survey in France showed that CBPV was the least prevalent of all examined viruses and that infection of CBPV was detected only in adult bees with the maximum frequency of 4% in the colonies. Infection of CBPV also did not appear to follow any seasonal pattern (Tentcheva *et al*., 2004b). In the United States, incidence of CBPV has been very sporadic for the last 5 years and less than 1% of bees were identified with CBPV infection in the colonies. Field survey in France and Thailand showed that all examined varroa mites were negative for CBPV. This result suggests that the varroa mite is unlikely a vector of CBPV.

CBPV is often associated with the ''satellite'' virus, chronic paralysis virus associate (CPVA). CPVA is a single-stranded, isometric RNA satellite virus that is of unknown significance. It is serologically unrelated to CBPV but cannot multiply in the absence of CBPV (Ball *et al*., 1985).

III. TAXONOMY

A. Virion properties

Aside from the filamentous virus and the *A. iridescent* virus, all honey bee viruses reported so far share a genome of positive-sense single-stranded RNA; icosahedral, pseudo $T = 3$ structure symmetry; and are free of a lipid-containing envelope although they differ somewhat in their biological properties. The outer shell of the capsid is composed of 60 repeated protomers, each consisting of a single molecule of three subunits VP1, VP2, and VP3. In addition to these three subunits, there is a smaller

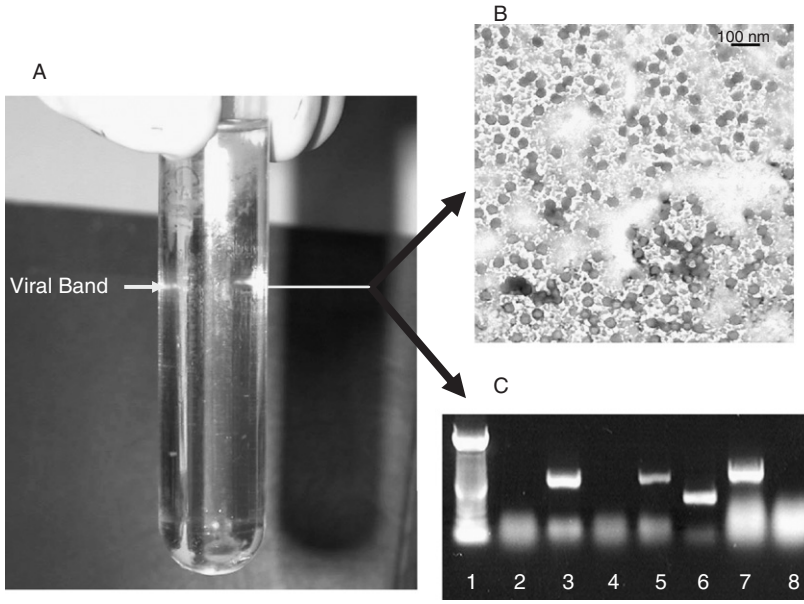

FIGURE 1 (A) Virus band after CsCl density gradient centrifugation. The virus-containing band was collected for subsequent electron micrograph and RT-PCR analyses. (B) Electron micrograph of honey bee virus particles. Bee viruses are spherical to slightly oval particles about 29 nm in diameter as determined from EM. Bar marker represents 0.1 μM. (C) The virus preparation used for this electron micrograph was also examined for the presence of six viruses: ABPV, BQCV, CBPV, DWV, KBV, and SBV by RT-PCR. The primers used in the study were the same as reported earlier (Chen *et al.*, 2005). Four viruses, BQCV, DWV, KBV, and SBV, were detected in the virus preparation. Primer pair specific for BQCV, DWV, KBV, and SBV amplified a PCR fragment of 700, 702, 415, and 824 bp, respectively. Lane 1, 100-bp DNA ladder; Lane 2, ABPV; Lane 3, BQCV; Lane 4, CBPV; Lane 5, DWV; Lane 6, KBV; Lane 7, SBV; and Lane 8, Negative control (previously identified negative sample). As shown in electron micrograph, no significant difference in the virion size and morphology could be observed among the four different virus particles (modified from Chen *et al.*, 2006a).

fourth protein VP4 that is present in the virions of some viruses such as BQCV and ABPV (Govan *et al.*, 2000; Leat *et al.*, 2000). VP4 is not exposed at the surface of the viral particle and is located on the internal surface of the fivefold axis below VP1. The capsid proteins play important roles in the protection of viral RNA from activities of RNases and irregular environments and in the determination of viral host specificity and tissue tropism.

Electron micrographs reveal that honey bee virions are spherical to slightly ovoid in shape, approximately 17–30 nm in diameter. The virions

possess a buoyant density in CsCl ranging from 1.33 to 1.42 g/ml, and a sedimentation coefficient between 100S and 190S (Bailey, 1976; Ball and Bailey, 1991). It is a common phenomenon that several viruses of similar size and shape coexist in natural populations of honey bees (Anderson and Gibbs, 1988; Chen *et al*., 2004c). Purified virus preparations are therefore rarely free of contaminating viruses. As shown in Fig. 1, the virus preparation used for electron microscope analysis was determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis to contain four different viruses BQCV, DWV, KBV, and SBV. No significant differences in virion size and morphology could be observed among the virus particles that comprised the four different viruses (Chen *et al*., 2004c). This is in general agreement with previous EM studies of viruses isolated from bees (Bailey and Ball, 1991; Bailey and Woods, 1977) and bee mites (Kleespies *et al*., 2000).

B. Genome organization and classification

The genomes of the positive-stranded RNA viruses are directly involved in several key viral processes including acting as mRNAs for translation of viral proteins, serving as templates for viral genome replication, and being assembled into progeny of viral particles along with structural proteins. Of course, genomes of honey bee viruses are involved in each of these processes. The replication of viruses occurs entirely in the cytoplasm of the host cell. The virus particle attaches to the surface of the host cell and interacts with a receptor on the host cell membrane and releases its RNA genome into the host cell. No viral enzymes/proteins enter the host cell along with the viral genome. Once inside the host cell, the RNA genome is translated into the protein precursors that undergo a cascade of cleavages to form structural and functional proteins for RNA replication. With the help of RNA-dependent RNA polymerase (RdRp), the positive-stranded RNA genome is copied to a negative-stranded intermediate, which serves as a template for replication of new genomic strands. When sufficient positive-stranded progeny RNAs and structure proteins are generated, they are packed into progeny viral particles. The progeny virions then travel to the cell surface where they are released.

Most honey bee viruses belong to the picorna-like virus superfamily and have the following characteristics in their genomic structure: (1) a single molecule of RNA genome coated with a capsid protein shell; (2) a small protein called VPg (viral *p*rotein *g*enome linked) covalently attached to the 5′ end of the viral RNA genome. VPg is responsible for stabilizing the 5′ end of the RNA genome and serves as a primer for replication and translation, contrary to cellular mRNAs where a methylated G cap is attached at the 5′end; (3) at the 5′ end, a long untranslated region (UTR) containing a ''cloverleaf'' secondary structure, presumably

involved in initiation of translation; (4) a string of adenylic acid residue linked to the 3′ end of the RNA genome and the length of the poly(A) tail is genetically determined and varies in different viruses; and (5) the 3′ terminal sequences of the genomic RNA that can be folded into a stem-loop structure presumably involved in RNA replication.

To date, the complete genome sequences of six honey bee viruses including ABPV (Govan *et al.*, 2000), BQCV (Leat *et al.*, 2000), DWV (Lanzi *et al.*, 2006), KBV (De Miranda *et al.*, 2004), Kakugo virus (KV) (Fujiyuki *et al.*, 2004), and SBV (Ghosh *et al.*, 1999), and partial genome sequences of CBPV (GenBank accession number: AF461061) have been reported. The genomic information of these viruses provides considerable insight into the basic gene structure and organization of honey bee viruses. The genome sizes of honey bee viruses range from 8550 to 10,140 bp, excluding the poly(A) tail. The genomes of bee viruses are enriched in AU (58.97–62.4%), compared to the content of GC (37.6–40.71%) (Table I). Genomes of SBV, DWV, and KV contain one large open reading frame (ORF), while genomes of ABPV, BQCV, and KBV contain two nonoverlapping ORFs. According to the gene order of the proteins, honey bee viruses are divided into two forms of genomic organization. The genomes of ABPV, BQCV, and KBV are monopartite bicistronic with the nonstructural proteins encoded in the 5′-proximal ORF and the structural proteins encoded in the 3′-proximal ORF. In contrast, the genomes of SBV, DWV, and KV are monopartite monocistronic with the structural proteins encoded in the 5′-proximal ORF and the nonstructural proteins encoded in the 3′-proximal ORF (Fig. 2). Based largely on their genomic organization, BQCV, KBV, and ABPV, formerly known as insect picorna-like viruses, are assigned to *Cripavirus*, a genus belonging to family Dicistroviridae. SBV and DWV are assigned to the genus *Iflavirus* which is a ''floating genus'' and not yet assigned to a family (Mayo, 2002).

Phylogenetic analysis using either amino acid sequence alignment of helicase or RdRp of viruses showed that KBV, APBV, and BQCV formed a common lineage with picorna-like viruses that infect plants, insects, and vertebrate. KBV is closely related to ABPV in the phylogenetic tree and BQCV tended to group together with KBV and ABPV but not closely related to them. DWV, KV, and SBV fell into a separate group, with DWV and KV more closely related to one another to SBV in the group (Fig. 3). KV is a novel picorna-like virus isolated from the brains of worker bees and has been associated with aggressive behaviors in worker bees (Fujiyuki *et al.*, 2004, 2005, 2006). Although there are significant differences in the L protein region of the RNA genomes (Lanzi *et al.*, 2006) and in the host pathology (Fujiyuki *et al.*, 2005, 2006; Rortais *et al.*, 2006) between KV and DWV, the species status of KV has not been defined so far because it shares the same host and high nucleotide sequence identity

TABLE I Genome of honey bee viruses

Viruses	Size (bp)	Base composition (%) A	U	G	C	GenBank accession no.	References
ABPV	9470	30.3	30.4	20.5	18.8	AF150629	Govan *et al.*, 2000
BQCV	8550	29.2	30.6	21.6	18.5	AF183905	Leat *et al.*, 2000
DWV	10,140	29.5	32.3	22.4	15.8	NC004830	Lanzi *et al.*, 2006
SBV	8832	29.8	29.4	24.4	16.4	AF092924	Ghosh *et al.*, 1999
KBV	9524	33.8	28.6	20.2	17.4	NC004807	De Miranda *et al.*, 2004

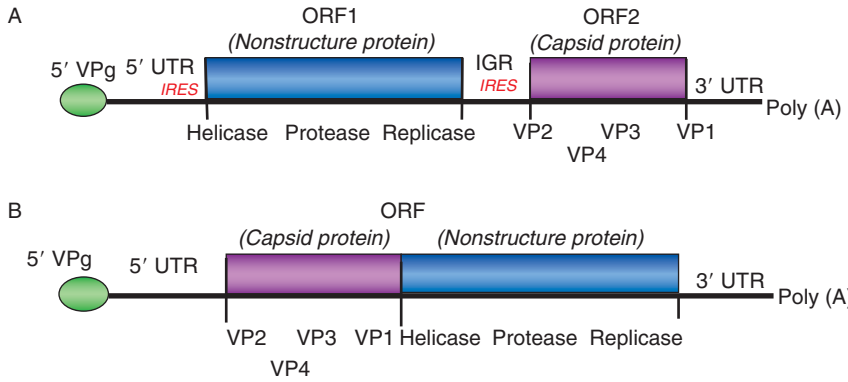

FIGURE 2 Schematic representation of genomes of honey bee viruses. The RNA genome is covalently attached by a genome-linked virion protein (VPg) at the 5′ and a poly(A) tail at 3′ ends. Genomes of honey bee viruses are organized in two different ways. (A) The genomes of ABPV, BQCV, and KBV are monopartite bicistronic with nonstructural genes at the 5′ end and structural genes at the 3′ end. The 5′ UTR and the untranslated intergentic region (IGR) between the two ORFs can initiate efficient translation as the internal ribosomal entry site (IRES). (B) The genome of SBV and DWV are monopartite monocistronic genomes with structural genes at the 5′end and nonstructural genes at the 3′ end (Chen *et al.*, 2006a).

(97%) with DWV. Further investigation of the virus biological properties such as antigenicity, natural cell, and tissue tropism will help to define whether KV is a species distinct from DWV or if KV and DWV are different variants of the same species.

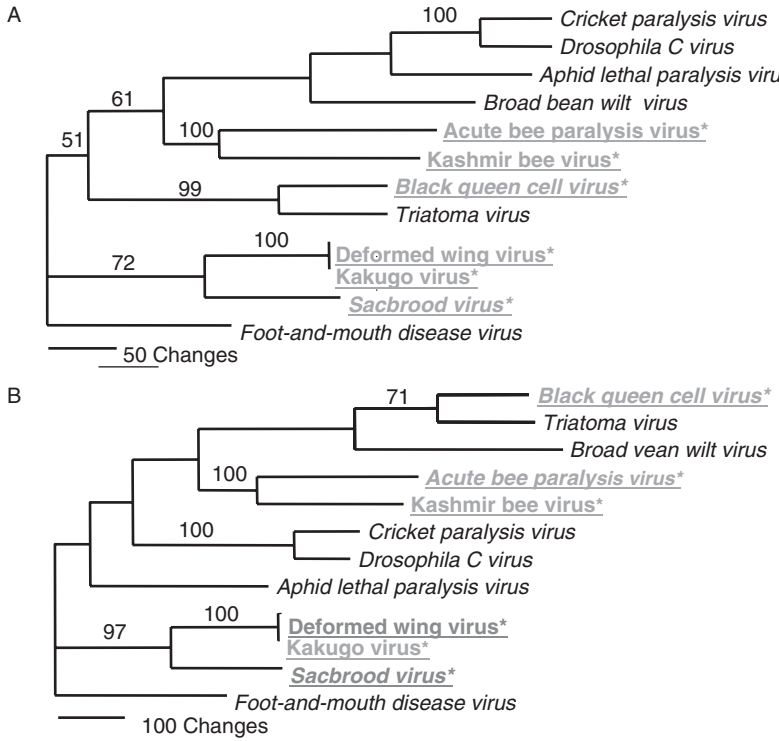

FIGURE 3 Phylogenetic trees derived from the putative helicase (A) and RdRp (B) amino acid sequences of the viruses. For both panels A and B, *Foot-and-mouth disease virus* was used as an out-group to root the trees. Bar lengths represent 50 inferred character state changes for the tree derived from the helicase domain and 100 inferred character state changes for the tree derived from the RdRp domain. Branch lengths are proportional to the number of inferred character state transformations. Numbers at each node represent bootstrap values as percentages of 100 and only bootstrap values greater than 50% are shown. Honey bee viruses are underlined and shown by asterisks. The names of viruses are abbreviated as follows: TV, *Triatoma virus*; ABPV, Acute bee paralysis virus; ALPV, *Aphid lethal paralysis virus*; BBWV, *Broad bean wilt virus*; BQCV, *Black queen cell virus*; CPV, *Cricket paralysis virus*; DCV, *Drosophila C virus*; DWV, Deformed wing virus; FMDV, *Foot-and-mouth disease virus*; KBV, Kashmir bee virus; KV, Kakugo virus; SBV, *Sacbrood virus* (Chen *et al.*, 2004c).

The monopartite bicistronic genomes are also characterized by two ORFs that are separated by an untranslated intergenic region (IGR). Both 5′ UTR and the IGR contain highly structured RNA sequences that function as internal ribosomal entry sites (IRESs) for facilitating the cap-independent translation of the viral proteins, though no sequences and translation initiation mechanisms are the same for two IRES elements.

The 5′ UTR-IRES and IGR-IRES elements were first reported in picornaviruses by Jang *et al*. (1988) and Pelletier and Sonenberg (1988), respectively. Since then, IRES elements have been detected in genomes of several other positive-stranded RNA viruses (Hellen and Sarnow, 2001; Sasaki and Nakashima, 1999). Sequence alignments of the IGR of ABPV, BQCV, and KBV with other positive-stranded RNA viruses that were experimentally identified with IRESs (Sasaki and Nakashima, 1999) revealed a considerable level of sequence similarities and indicated the existence of IRES elements in the IGR of ABPV, BQCV, and KBV. Amino acid sequence analysis revealed that methionine is the initial amino acid in the translation of the capsid proteins of ABPV, BQCV, and KBV, in contrast to the non-AUG codons found in genes of capsid proteins of several other members of the Dicistroviridae (Domier *et al*., 2000; Sasaki and Nakashima, 2000; Wilson *et al*., 2000). There is no evidence that translation of proteins is mediated by IRES for the monopartite monocistronic genome.

IV. TRANSMISSION MODES

Viruses are obligate intracellular parasites that can only multiply inside living host cells utilizing the host cell's metabolic machinery. In order to survive, viruses must have ways to invade hosts and be transmitted from one host to another. Transmission processes determine the persistence and the spread of viruses in a population. In theory, transmission of a virus can occur horizontally or vertically, or both. In horizontal transmission, viruses are transmitted between different individuals of the same generation. In vertical transmission, viruses are passed vertically from mother to offspring via egg during its development through the follicle cells or after completion of egg development. Horizontal transmission of a virus can occur by the following means: foodborne transmission, fecal–oral transmission, venereal (sexual) transmission, airborne transmission, and/or vector-borne transmission. Vertical transmission can be further divided into transovum transmission in which viruses are transmitted on the surface of the egg and/or transovarian transmission in which viruses are transmitted within the egg.

Honey bees are eusocial insects and are characterized by the following traits: (1) they live in colonies consisting of overlapping generations: one mother queen and her successors, 20,000–60,000 workers and several hundred drones; (2) there is a reproductive division of labor, that is, sterile workers contribute their entire lives to support reproduction of a single egg-laying queen in the colony; and (3) each member of the bee colony works together in a highly structured social order and engages in extensive coordinating activities, including rearing brood, defending

against invaders, foraging for food, and constructing the combs. The densely crowded populations and high contact rate between colony members in honey bee colonies provide an ideal environment for transmission of pathogens. Because of the importance of the transmission processes in the dynamics of virus infections, elucidation of virus transmission in honey bees represents one of the rapidly developing research fields. Our understanding of bee virus transmission has markedly advanced, and intricate routes of transmission have been identified and documented in honey bees during the last 5 years.

A. Horizontal transmission

1. Foodborne transmission

Foodborne transmission is a means of spreading infection that occurs after eating virus-contaminated food and is the most common route of virus transmission. Natural food in honey bee colony consists of honey, pollen, and royal jelly. The foraging worker bees collect the nectar from flowers and store it in their stomach ''honey sacs.'' After returning to the colonies, foraging bees regurgitate the nectar and pass it on to nurse bees that add an enzyme to convert the nectar into honey used as an energy component of the bee diet. The worker bees also visit flowers to collect pollen that is brought back to the hive as a load on the hind leg and used as a protein source for bee brood to grow. Both honey and pollen are also stored in the combs of the hive for the winter months when nectar and pollen sources are scarce. Royal jelly is a secretion of the hypopharyngeal and mandibular glands of nurse bees. It is used by the nurse bees to feed the queen bee and young larvae. Although trophallactic chain is an important cohesive force in honey bee colonies, trophallactic activities of honey bees, including processing nectar, packing pollen, feeding the brood, and attending the queen, offer the potential for foodborne transmission of pathogens. It is very likely that contamination of food by viruses can occur during foraging or processing by virus-infected workers and that foodborne infection can take place by eating virus-contaminated food. Under conditions of high population density, high contact rate, and high trophallactic rate, direct foodborne transmission may be a significant route for spreading viruses in bee colonies. Evidence of the foodborne transmission pathway in bees has been provided by detection of viruses in food resources. Early studies demonstrated virus transmission to larvae via brood feeding by the detection of viruses in the thoracic gland and hypopharyngeal gland of honey bees (Bailey, 1969; Bailey and Ball, 1991). A study conducted by Shen *et al*. (2005a) showed that two viruses, KBV and SBV, were detected in colony food including honey, pollen, and royal jelly as well as in all developmental stages of bees, suggesting the involvement of colony food in the spread of virus infections. Similar findings

were reported by Chen *et al*. (2006a) who found that two viruses, BQCV and DWV, were detected in honey and six viruses, including ABPV, BQCV, CBPV, DWV, KBV, and SBV, were detected in pollen samples. The two viruses BQCV and DWV found in the honey were also present in over 80% of the examined brood and adult workers in the bee colonies where the colony food was collected. Although ABPV, CBPV, KBV, and SBV were detected in pollen samples, the same viruses were not detected in the bees and their glandular secretion, royal jelly (Chen *et al*., 2006a). These results suggest that bees ingesting virus-contaminated food such as pollen might not always be necessarily infected. The successful infection of a virus may depend on the amount of the virus introduced into the bees and the pathogenic nature of the virus. When a virus is activated to replicate to the amount sufficient to cross the epithelial barrier of the digestive tract and invade different parts of bee body, infection of the virus will likely be detected in different parts of bee and bee products such as royal jelly.

2. Fecal–oral transmission

Fecal–oral transmission spreads pathogens by transferring feces of diseased hosts to uninfected hosts via ingestion and is strongly suspected in environments with overcrowded conditions. Honey bee colonies with densely crowded populations should be a favorable condition for this transmission route. Evidence of a fecal-borne transmission route of viruses in honey bees has been provided by the detection of viruses in feces and digestive tracts of bees. Chen *et al*. (2006b) demonstrated the presence of two viruses BQCV and DWV in the feces freshly defecated by individual queens. Among samples examined for viruses, 100% of feces samples tested positive for the presence of BQCV, and 90% of feces samples tested positive for the presence of DWV. Findings by Chen *et al*. (2006b) were consistent with previous reports that viruses were found in the feces of worker bees (Bailey and Gibbs, 1964; Hung, 2000). Detection of viruses in feces of bees suggests the possibility of the existence of foodborne transmission in honey bees, where infected bees eliminate viruses in their feces and uninfected bees can be infected by feeding on feces-contaminated food or by cleaning the infected bees' feces accumulated in the hive. Oral infection of viruses by contaminated food can be further traced by examination of the digestive gut for virus infections. The studies showed that the same viruses found in feces were also detected in the digestive tract of the bees, providing further evidence of the ingestion of virus-contaminated food and the existence of foodborne or fecal–oral transmission routes in honey bees (Chen *et al*., 2006b). In addition, quantification of virus load in different bee tissues indicated that virus titer was significantly higher in the digestive tract than other tissues tested (Chen *et al*., 2006b), indicating that the digestive tract was the primary site

of virus accumulation and the epithelial cell lining of the digestive tract may constitute the major portal for the spread of virus infection in bees.

3. Venereal transmission

Venereal transmission is a type of infection in which pathogens are transmitted between two sexes during mating. In honey bees, each virgin queen mates with 10 or more drones and semen acquired from multiple drones is stored in the spermatheca, a special pouch in each queen's body. After mating, queens return to the colonies and release a small amount of sperm at a time to fertilize their eggs. After vitellogenesis and egg maturation are completed, the queens start to lay eggs. If drones in honey bee colonies are infected with viruses, the mating can pose an opportunity for horizontal transmission of viruses from infected drones to queens via semen, which in turn further contributes to the transovarial transmission of viruses from queens to their eggs. The detection of viruses in adult drones (Chen *et al*., 2004a), semen (Chen *et al*., 2006a; Yue *et al*., 2006), and in the spermatheca of queens (Chen *et al*., 2006b) implies the existence of venereal transmission in honey bees. However, it is unclear at this point whether virus infection in queens is a result of foodborne transmission or venereal transmission or both. Further studies will be required to define the role of drones in the spread of virus infections to queens.

4. Airborne transmission

Airborne transmission is a method of spreading infection through aerosol-containing infectious agents that can remain suspended in the air for long periods. Pathogens carried in aerosol are disseminated by air currents and inhaled by susceptible hosts in a localized area. In a honey bee colony, worker bees function as a single unit to maintain a steady temperature within 0.5 °C of 35 °C (Simpson, 1961). During the winter seasons when the ambient temperature is below the temperature range, bees cluster together and raise their metabolic rate to conserve and generate heat. During the summer season when the ambient temperature is above the temperature range, worker bees collect water as well as nectar, evaporate it, and establish air currents through the colony to reduce the internal colony temperature and to prevent the brood nest from overheating. The special thermoregulation mechanism of honey bees creates an active circulating environment within the bee colonies, which might provide opportunities for transmission of viruses via the airborne route. A study carried out by Lighthart *et al*. (2005) reported that honey bees not only absorb airborne bacterial spores but also viruses and showed that honey bees induced to fly in a miniature wind tunnel absorbed aerosol that carried a virus, bacteriophage MS2. Although there is no epidemiological or laboratory data on airborne transmission of honey bee viruses, the results demonstrated in studies of Lighthart *et al*. imply the possibility

that honey bee viruses can be carried by aerosol and spread in the bee colonies through the infected bees to susceptible bees in the colonies. To prove this hypothesis, further studies will be needed.

5. Vector-borne transmission

Vector-borne transmission is an indirect route of horizontal transmission and involves an intermediate biological host, a vector, which acquires and transmits viruses from one host to another. The varroa mite is an obligate parasite of the honey bee attacking different developmental stages and castes of bees and is considered to be the most important pest of honey bees around the world. The entire life cycle of the varroa mite is spent with their honey bee hosts. Female mites feed on the bee larvae and lay eggs of both sexes in the brood cells. Developing mites feed on immature bees. After the mites mature, male and female mites mate inside of the capped brood cell. The male dies after copulation and females emerge from the brood cell along with their bee host and seek another host to repeat the life cycle. The feeding of varroa mites can result in a decline in host vigor, immunity, weight, shorter bee life span, and the eventual destruction of the colonies within a few years (De Jong *et al*., 1982; Korpela *et al*., 1992; Kovac and Crailsheim, 1988; Weinberg and Madel, 1985; Yang and Cox-Foster, 2005). In addition to its direct impact on host health, the feeding of mites on bees provides entry for diseases; both nymph and adult mites feed on bees using their piercing mouthparts to penetrate the body walls of bees to suck the hemolymph. The mites can therefore act as vectors for pathogens during the feeding. The detection of several bee viruses in varroa mites indicates the possible role of varroa mites as vectors in the transmission of viruses among honey bees (Chantawannakul *et al*., 2006; Fujiyuki *et al*., 2006; Hung and Shimanuki, 1999; Ongus *et al*., 2004; Shen *et al*., 2005b; Tentcheva *et al*., 2004a,b; Yue and Genersch, 2005). Previous field investigations reported that viral infections in honey bees have been involved in the collapse of bee colonies also infested with varroa mites (Allen and Ball, 1996; Ball and Allen, 1988; Kulincevic *et al*., 1990). Several viral disease outbreaks including ABPV, CBPV, slow paralysis virus (SPV), BQCV, KBV, Cloudy wing virus (CWV), SBV, and DWV have been documented to be associated with the infestation of varroa mites (Allen and Ball, 1996; Allen *et al*., 1986; Ball and Allen, 1988; Martin, 2001; Martin *et al*., 1998, Tentcheva *et al*., 2004b). The term ''bee parasitic mite syndrome'' has been used to describe a disease complex in which colonies are simultaneously infected with viruses and infested with varroa mites (Shimanuki *et al*., 1994). The observation of positive correlation between the levels of varroa mite infestation and the levels of virus concentration in infected bees suggests that vector-borne transmission exists in honey bees and that the varroa mite is not only a vector but also an activator of bee viruses (Ball and Allen, 1988).

The frequent observations of the association of varroa mite infestation with virus infections in honey bees led to laboratory experiments to further define the role of varroa mites in vectoring virus infections. The fact that varroa mites act as vectors in acquiring and transmitting viruses from severely infected individuals to healthy bees in bee colonies has been experimentally demonstrated in several studies. Bowen-Walker *et al.* (1999) provided the first circumstantial evidence that the varroa mite was an effective vector of DWV in bee colonies. Using serological methods, they demonstrated that varroa mites obtained DWV from infected bees and acted as vectors to transmit the virus to uninfected bees, which consequently developed morphological deformities or died after the mites fed on them for certain periods of time. Subsequent studies conducted by Chen *et al.* (2004b) provided strong evidence that the varroa mite is a vector in transmitting KBV to bees. By collecting mites from the KBV-infected colonies and experimentally introducing variable numbers of mites into the individually sealed brood cells of the KBV-negative colonies, a significant positive relationship between the percentage of pupae becoming virus positive and the number of mites introduced per brood cell were found. The more donor mites that were introduced, the greater the incidence of virus was detected in the recipient brood. Representative results obtained from one transmission experiment showed the following results: in the group with no mite introduction, all brood were virus negative; in the group with one, two, three, and four mites introduced per cell, 20%, 40%, 60%, and 100% brood were KBV positive, respectively. This study definitely showed that varroa mites are capable of transmitting KBV to bee brood. Additional observations were made in the same study. Evaluation of the transmission efficiency of the virus revealed that virus frequency in the mites was directly correlated with the number of mites per cell. The more mites introduced into each brood cell, the higher the chance of all mites becoming KBV positive, as long as at least one mite had KBV. While 37% of mites involved in the single mite introductions were determined to be KBV positive 5 days after their introduction into the cells, this percentage rose to 60% in two-mite introductions, 72% in three-mite introductions, and 94% in four-mite introductions. This result suggests that not only do mites transmit viruses to their bee hosts, but noninfected mites can also acquire viruses by cohabiting in a cell with virus-positive mites, presumably via a honey bee intermediary. Therefore, mites emerging from multiple-infested cells can play a disproportionate role in the spread of viruses within the colony. Shen *et al.* (2005b) provided further evidence for the role of varroa mites in transmitting KBV and DWV in honey bee colonies. In their studies, titers of DWV and KBV were found to be significantly higher in mite-infested bee samples, and the elevated virus titers in mite-infested bees were suggested to be a result of virus replication in infected bees due to the suppression of host

immunity by varroa mite infestation. The laboratory experiments, coupled with the field observations, provide unequivocal evidence of the existence of a vector-borne transmission pathway in honey bees and prove that the varroa mite is an effective vector and activator of honey bee viruses.

Although both field and laboratory studies have confirmed that the varroa mite is an effective vector in transmitting and activating bee virus infections, the mechanism of mite-mediated transmission of bee viruses is uncertain. In general, vector-borne transmission of a pathogen can occur in two ways. Mechanical vector-borne transmission occurs when the vector transmits the pathogen from one host to another but does not support the replication of the pathogen. The pathogen is short-lived in a mechanical vector which is only a carrier of the pathogen and not essential in the life cycle of the pathogen. Biological vector-borne transmission, on the other hand, occurs when a vector is persistently infected with the pathogen and the pathogen multiplies in the body of the vector before it is passed to another host. A biological vector may even be an essential part of the pathogen's life cycle. Ongus *et al.* (2004) reported the discovery of a new virus from varroa mites, namely, *Varroa destructor-1* (VDV-1), and demonstrated that VDV-1 replicates in varroa mites as shown by RT-PCR amplification of the negative strand of VDV-1-specific PCR fragment and by scattered occurrence of paracrystalline structures of viral particles in the cytoplasm of varroa mites in histological sections. Their studies also showed that DWV sharing 83–84% nucleotide sequence identity with VDV-1 and that DWV was found to be replicated in varroa mites. Findings that viruses replicate in the varroa mite and that viruses are present in mite saliva suggest that the varroa mite is likely a biological vector of bee viruses (Ongus *et al.*, 2004; Shen *et al.*, 2005b). Further studies of the pathogenecity of VDV-1 in honey bees would shed more light on the mechanism regulating virus–vector–host interactions and transmission processes of the virus.

B. Vertical transmission

Vertical transmission in which viruses are passed vertically from mother to offspring has long been known to occur in mammals, vertebrates, arthropods, and plants (reviewed in Mims, 1981). Vertical transmission routes of viruses in honey bees were proposed by Fries and Camazine (2001) based on a honey bee disease model. However, it is difficult to demonstrate vertical transmission experimentally by inoculating virus-negative queens with purified viruses and then estimating the filial infection rates or recovering the viruses from the queens' progeny due to the following reasons: (1) most honey bee queens are virus carriers and it is difficult to obtain virus-negative queens for virus inoculation; and

(2) honey bees are often attacked by multiple viral infections, therefore, it is difficult to purify virus particles that contain only a single virus.

Despite limitations, evidence of a vertical transmission pathway has been documented in several reported studies (Chen *et al*., 2005, 2006b; Shen *et al*., 2005a). The detection of multiple viruses in queens suggests that a vertical transmission pathway exists within the bee colony and that eggs have the opportunity to obtain viruses from an infected queen (Chen *et al*., 2005; Shen *et al*., 2005a). Quantification of virus titer in the ovaries of queens showed that virus concentration in ovaries was relatively low when compared to other examined tissues. The weak virus signals detected in ovaries suggests that virus infections in ovaries were retained in a nonreplicate or latent stage so that viruses would not be propagated to the level that would have a deleterious effect on the embryos (Chen *et al*., 2006b).

The detection of virus in eggs, the developmental stage not normally associated with any direct and indirect horizontal transmission routes, provides evidence of vertical transmission in honey bees (Chen *et al*., 2004a; Shen *et al*., 2005a). Further, the detection of viruses in surface-sterilized eggs excludes the possibility of transovum transmission and suggests the existence of a transovarial transmission pathway in which viruses infect ovarian tissues of the queen and disseminate in developing eggs before oviposition. In addition, the detection of a virus-positive signal in larvae and a virus-negative signal in the royal jelly of the same bee colonies excluded the possibility of foodborne transmission contributing to virus infections in the larval stages of bees and suggest possible vertical transmission.

Field surveys of virus status of both mother queens and their offspring showed more evidence of vertical transmission in honey bees. When queens were found to be positive for certain viruses in bee colonies, the same viruses were detected in their eggs, larvae, and adult worker bees, though neither queens nor their offspring exhibited any overt symptoms of disease. Meanwhile, when queens were negative for certain viruses, these viruses could not be detected in their offspring. These data provide an additional line of evidence that vertical transmission of viruses from queens to their progeny is highly likely in honey bees (Chen *et al*, 2006b).

C. Discussion

The mode of transmission is a major determinant of a virus' virulence. Evolution of virulence is governed by competition between two transmission pathways (Ewald, 1983, 1987, 1994; Lipsitch *et al*., 1996). With horizontal transmission, virulence will increase through production of high numbers of pathogens. The greater the number of pathogens produced, the higher the opportunities for host exploitation and thereby the higher

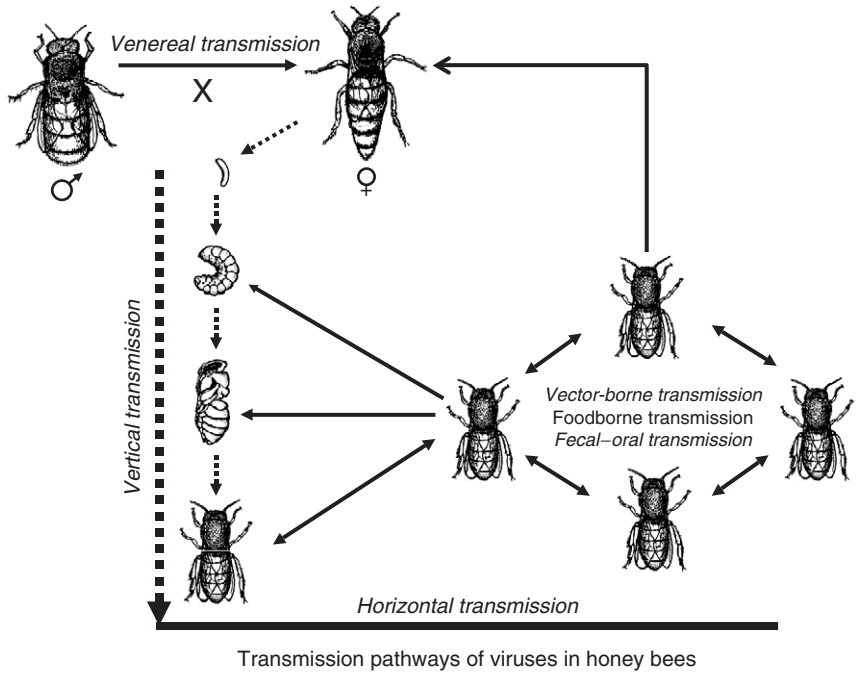

FIGURE 4 Schematic representation of virus transmission routes in honey bees. Virus transmission in honey bees appears to involve both horizontal and vertical transmission pathways. Viruses infect different bee hosts of the same generation by horizontal transmission via the following means: foodborne transmission, fecal–oral transmission, venereal (sexual) transmission, and/or vector-borne transmission. Viruses are also vertically transmitted from infected queen to their offspring. Both transmission pathways are believed to be the important survival strategies for persistence and establishment of viruses in bee population. Solid lines represent horizontal transmission and dotted lines represent vertical transmission (modified from Chen *et al.*, 2006a).

rate of transmission. Hence, selection favors high virulence of pathogens. In contrast, virulence of a pathogen decreases under vertical transmission because the fitness of the pathogen is directly dependent on the survival and reproduction of its hosts and any reduction in host reproductive potential will cause a reduction in the reproduction of pathogen. Hence, vertical transmission is associated with low virulence and latent infection. However, if the replication rates of viruses are too high, high virulence will result in high pathogen-induced host mortality, and hosts will lose fitness before producing enough progeny to infect more hosts. On the other hand, if the replication rate is too low, the pathogen will lose opportunities to infect new hosts and thus will lose fitness. Therefore,

a pathogen's fitness is the result of pathogen–host interactions and trade-offs between horizontal and vertical transmission.

Both horizontal and vertical transmission pathways have been proved to be involved in virus transmission in honey bees. Viruses infect different bee hosts of the same generation via foodborne transmission, fecal–oral transmission, venereal transmission, and vector-borne transmission. Viruses can also infect offspring of the current host via vertical transmission (Fig. 4). Both transmission pathways are important survival strategies for viruses not only for their long-term persistence in bee population but also for their establishment in nature. Viruses choose the appropriate transmission pathway based on the developmental, physiological, ecological, and epidemiological conditions. When colonies are under noncompetitive and healthy conditions, viruses remain in bee colonies via vertical transmission and exist in a persistent or latent state without causing honey bees to show any overt signs of infections. Alternatively, when honey bees live under stressful conditions such as infestation of varroa mites, coinfection of other pathogens such as *N. apis*, or decline in food supply, viruses switch to horizontal transmission and start to replicate. High numbers of produced virions then become much more infectious, leading to the death of hosts and possible collapse of the whole bee colony.

V. PATHOGENESIS

While transmission concerns the spread of viruses in a population, pathogenesis deals with the processes by which viruses infect and cause disease in their target hosts. A virus infection depends on a number of pathogen and host factors as well as environmental factors that affect pathogenesis. The outcomes of the virus infection exhibited in the hosts vary, ranging from inapparent infections to severe infections or acutely lethal diseases. Among the wide spectrum of consequences of the virus infections, latent or persistent infections are the most common and are considered to be a state of balanced pathogenicity where multiplication of viruses is arrested by the host's defense mechanism but the viruses themselves remain in the host for long periods of time without producing a manifesting infection. Viruses in the latent state can be replicated if hosts are put under irregular conditions or other environmental stresses, leading to the outbreak of overt diseases. The asymptomatic virus carriers constitute major sources for the transmission of viruses in a population and have great epidemiological importance.

Elucidation of virus pathogenesis requires investigation of many biological features of the viruses and their respective hosts. While transmission pathways of honey bee viruses have been well studied, not much is known about the pathogenesis of viruses in honey bees. In this section, we focus on the current available information involving pathogenic processes of virus infections in honey bees.

A. Causal relationship between a virus and a disease

One of the biggest challenges of studying virus pathogenesis in honey bees is linking the virus infection with a particular disease and therefore evaluating the economic impact of the virus infection. In the field, honey bees are often infected by multiple viruses simultaneously, most of these viruses usually persisting as latent infections in the bee hosts. In addition, virus infections in honey bees are often associated with the infection of other pathogens and infestation of parasites. Therefore, it is difficult to prove that one disease is indeed caused by a particular virus and not the result of mixed virus infections when hosts harbor multiple viruses. However, studies with DWV have revealed that quantification of virus loads using sequence-based methods provides a new way for proving disease causation in infected bees (Chen, 2005; Chen *et al*., 2004a). Detection of the virus by RT-PCR assay showed that DWV was present not only in 100% of the adult bees with symptoms of wing deformity and reduced body size, but also in 70% of the apparently healthy adult bees. This result fulfills one of Koch's postulates, a scientific standard for causal evidence created by Dr. Robert Koch (1884), that the pathogen is present in every case of the diseased individual. The quantification of virus titers by TaqMan real-time quantitative RT-PCR showed that the DWV concentration in bees with the disease symptoms was 4.4-fold higher than in apparently healthy adult bees and that there was no direct correlation between doses of coexisting viruses other than DWV and the appearance of disease symptoms. These results indicate that DWV titers in infected adult bees are the determinants for the appearance of the disease. This result satisfies the molecular revision of Koch's postulates by Fredericks and Relman (1996) that if sequence detection predicts disease and copy numbers of the pathogen correlates with disease severity, then the relationship between a pathogen and a disease is more likely to be causal. The study with DWV clearly demonstrates that the determination of viral load can link the causal association between a virus and a disease when multiple viruses coexist in the same host. Future efforts to determine the critical threshold of the virus concentration required to induce the disease will help to define viral dose requirements for host pathological responses in order to monitor disease development in honey bee colonies.

B. Tissue tropism

The ability of a virus to invade the tissues of a host is a fundamental requirement for a successful infection. The term ''tissue tropism'' is referred to as the specificity of a virus to infect and replicate in particular cells or tissues. Tissue tropism is determined mostly by (1) the chemical affinity of the virus attachment protein with virus-specific receptors on

the surface of a host cell; (2) the suitability of viral entry sites to support virus replication; and (3) the ability of the virus to escape from the host's immediate immunity and thereby to establish long-term chronic or latent infections. The first step of virus infection is the interaction between the viral capsid protein and the specific receptor on the surface of the susceptible host cell to allow the viral RNA to enter the cell cytoplasm. Despite the fact that virus entry processes have been well established for several family members of the Picornaviridae, such as *Poliovirus* (Basavappa *et al*., 1998; Mendelsohn *et al*., 1989), molecular mechanisms of receptor recognition that determine the tissue tropism of honey bee viruses are currently unknown. Research on studying cellular aspects of the pathogenesis of honey bee viruses is largely impeded by the lack of certified virus-free bees and an *in vitro* cultivation system. While a long-term cultivation of honey bee cells has been reported (Bergem *et al*., 2006), a permanent cell line derived from honey bees is not yet available for bee virus propagation. Studies of the mechanisms of tissue tropism that underlie virus binding and spreading to different host cells require a full understanding of the structural features of a virus particle. The atomic structure of a virus particle by X-ray diffraction offers an opportunity to elucidate the molecular determinants of the virus that are necessary for the recognition of receptors and the specificity of tissue tropism. One essential requirement for the crystallization and determination of a virus atomic structure is that viruses need to be propagated in a cell culture and purified to a very high degree. At present it is very difficult to obtain bee viruses in high purity because bee viruses are usually grown *in vivo* and there is always the chance that preparation of any particular bee virus may be contaminated with one or more additional viruses. In addition, determination of the presence or absence of virus-specific receptor molecules on the surface of host cells and characterization of the interactions between receptors and a particular virus is not even possible without an *in vitro* system. Due to these difficulties, our knowledge of tissue tropism of honey bee viruses is mostly limited to ultrastructural studies of virus cytopathology. There have been attempts to culture embryonic bee cells in a highly nutritive medium (Mitsuhashi, 2001, 2002) and in a classical medium (Bergem *et al*., 2006). Cell migration from the explants was observed. The cells could be maintained for a period of several weeks but passaging of the bee cells failed. Although to date there are no continuous cell lines nor heterogenous cell lines derived from honey bees for the proliferation of bee viruses, the protocols for bee tissue cultures and setup of primary cultures have been developed (Kaatz *et al*., 1985; Kreissl and Bicker 1992; Malun *et al*., 2003; Rachinsky and Hartfelder, 1998).

Bee viruses exhibit some differences in their tissue tropism in their bee hosts. Although bee viruses multiply abundantly and fatally when injected into bee hemolymph, the initial infection site of most honey

bee viruses usually occurs through the cuticle by direct contact between healthy and infected bees or in the alimentary tract when bees ingest virus-contaminated food. For example, KBV, CBPV, and ABPV are most likely transmitted contagiously between crowded live bees via the cytoplasm of broken cuticular hairs, while SBV causes infection in bees when both young adult bees and larvae ingest the virus particles mixed in with their food (Bailey and Ball, 1991; Bailey *et al*., 1979, 1983; Ball and Bailey, 1991). These viruses are then further transmitted to brood via the glandular secretions of infected worker bees during feeding. Although BQCV does not multiply readily when ingested by both worker bees and larvae, it replicates abundantly in adult bees when they are also infected with *N*. *apis* (Bailey, 1982a). Since *N*. *apis* often causes gastrointestinal infections in bees, it is believed that infection of *N*. *apis* increases the susceptibility of the alimentary tract to infection by BQCV, indicative of the alimentary tract as an initial infection site for BQCV.

Honey bee viruses are able to spread their infections systemically from initial sites to secondary target tissues of the host via the blood circulation or nervous systems. KBV infects and replicates in most tissues of an infected bee, including the fore- and hindgut epithelial tissue, alimentary canal musculature, epidermis, tracheal epithelium, hemocytes, oenocytes, and treacheal end cells. However, no evidence of KBV multiplication has been found in the nerve tissues (Dall, 1987). SBV most commonly accumulates in the hypopharyngeal glands of worker bees, but virus particles have also been found in the cytoplasm of fat, muscle, and tracheal-end cells of larvae (Lee and Furgala, 1967). CBPV has a particular tropism for nervous tissues. This is probably why infection of CBPV is often associated with paralysis behavior in infected bees. The CBPV particles can also be found in the alimentary tract, mandibular, and hypopharyngeal glands. However, CBPV does not appear in the cytoplasm of fat or muscle tissues (Giauffret *et al*., 1966, 1970; Lee and Furgala, 1965). ABPV particles have been seen in the cytoplasm of fat body cells, the brain, and hypopharyngeal glands of acutely paralyzed bees (Bailey and Milne, 1969; Furgala and Lee, 1966). Localization of DWV infection in queens and drones by *in situ* hybridization and RT-PCR methods showed that DWV infection is spread throughout the whole body, including the queen ovaries, queen fat body, spermatheca, and drone seminal vesicles (Chen *et al*., 2006b; Fievet *et al*., 2006). Nothing is known about the cytopathological effects of BQCV in honey bees.

C. Host range

A virus' host range is generally referred to as the range of host species that a virus is capable of infecting, although host cell types that a virus infects can also be considered to be a host range in a broad sense. The successful

infection and replication of a virus in a host is a complex process involving the interaction and coevolution of virus and host (Moya *et al*., 2000). Host species specificity is a genetic property of a virus and any changes in host specificity can occur through virus mutation. RNA viruses show the highest mutation rates among all pathogens, roughly one nucleotide per genome is incorrectly reproduced in each replication (Bonhoeffer and Sniegowski, 2002). The high mutation rates of RNA viruses are due to error-prone replication, since there is no proofreading mechanism for RdRps. Error-prone replication along with a short replication time and large population size leads to high levels of genetic diversity of RNA viruses and the formation of viral quasispecies. The viral quasispecies is a population of genetic variants of virus organized in a way that a central master sequence, the most frequent and fittest genotype, is surrounded by a cloud of mutant sequences. Such an organization provides an evolutionary advantage to RNA viruses and allows the viruses to evolve and adapt to new environments and challenges during infection and sometimes to cross species barriers to new hosts. Host expansion is an important evolutionary force for a virus population and allows viruses to expand their ecological niche to a great diversity of resources and to reduce competition among competitors.

Honey bee viruses display a host range that is not restricted to their original *A*. *mellifera* host. Apart from the European honey bee, *A*. *mellifera*, infections of SBV, KBV, and DWV have been reported in the eastern honey bee *A*. *cerana*. Except for CBPV, the other five common bee viruses, DWV, SBV, BQCV, KBV, and ABPV, are found to be harbored by the varroa mite. The host range of ABPV was shown to extend to at least three bumble bee species (Bailey and Gibbs, 1964). KBV also has alternate hosts in nature and infection of KBV has been detected in bumble bees (*Bombus* spp.) from New Zealand and German wasps (*Vespula germanica*) from Australia (Anderson, 1991). Current detection of DWV, in bumble bees, *B*. *terrestris* and *B*. *pascuorum*, demonstrated the ability of DWV to expand its host range (Genersch *et al*., 2006). Evidence that honey bee viruses exploit multiple host species in their habitat reflects the genetic variability and quasispecies nature of bee viruses. When a virus is expanded to a different host or ecological niche, a new variant to adapt to changes of the new environment may already be formed in a viral population. Further, the widespread nature of a mixed virus infection in honey bees implies that viruses sharing the same physiological niches have the potential to undergo genetic changes by recombination or reassortment, leading to the formation of genetic variants or emergence of new viral species. Further studies on genetic variability of honey bee viruses would shed more light on the pathogenesis of bee virus infections.

VI. HOST DEFENSE MECHANISMS

A virus causes infection by invading host cells, multiplying new virions, and exiting the host cell to attack others. As part of their survival strategies, hosts have evolved effective mechanisms to defend against viral invaders by employing multifaceted immune responses. Virulence and pathogenesis are the consequences of the complex interactions between the infecting virus and host immunity. Vertebrates deal with viral infections by two types of immune responses, innate/nonspecific and adaptive/specific responses. The innate immune response is a rapid response to prevent the spread of viruses during the early phase of the invasion. The innate immune response includes synthesis of interferons to inhibit virus replication and the induction of natural killer (NK) cells to lyse virus infected cells. The adaptive immune response has two components, the humoral and cell-mediated responses. The humoral response attacks viruses when they are present in the host's circulation by B-lymphocyte-produced antibodies (immunoglobulins). The cell-mediated response destroys virus-infected cells by T-lymphocyte-produced cytokines once viruses have resided inside of the host cells. The adaptive immune response can also result in the production of ''memory cells'' which endow the immune system with the ability to respond much more rapidly and effectively to a subsequent infection of the same virus, which provides long-term protection against a given virus. In insects, NK cells, antibodies, cytotoxic T cells, and memory cells are all lacking and the entire immune system is innate. In general, insects utilize three lines of defense to combat infections: physical and chemical barriers, humoral immune responses, and cellular immune responses. In insect cellular immune responses, hemocytes confer cellular immunity to insects and hemocytic response is mediated by phygocytosis, nodule formation, and encapsulation of microbes. The insect humoral response is characterized by the activation of the phenoloxidase cascade and biosynthesis of antimicrobial peptides. The hemocytic and phenoloxidase responses are rapid and present the first line of defense behind the physical and chemical barriers, while the synthesis of antimicrobial peptides is much slower and begins to appear some hours after the actual infection has been recognized. Together, these responses constitute an effective defense system to protect insects from challenges by numerous invaders (Schmid-Hempel, 2005).

While the humoral and cellular immune responses to bacterial and fungal infections have been characterized and documented in honey bees, relatively little is known concerning how honey bees recognize and fight viral infections. However, we believe that honey bees do possess effective defense mechanisms that protect them from virus infections.

The commonly observed phenomenon that viruses persist in apparently healthy colonies as latent infections is a good indication that honey bees have the innate ability to resist the multiplication of virus infections.

Recent work has indicated that RNA interference (RNAi) is a natural, conserved mechanism of antiviral immunity in plants, vertebrates, and insects (Keene *et al*., 2004; Li *et al*., 2002; Voinnet, 2001). RNAi is an RNA-dependent gene silencing process triggered by a long double-stranded RNA (dsRNA). When dsRNA is introduced into a cell, a specific RNaseIII endonuclease, Dicer, binds and cleaves dsRNA to produce double-stranded fragments of 20–25 base pairs with 2-nt 3′ overhangs, called small interfering RNAs (siRNAs). The siRNAs are integrated into the RNA-induced silencing complex (RISC) to activate the RISC. Activated RISC bind to homologous mRNA and cause sequence-specific degradation of the target mRNA. Positive-stranded RNA viruses appear to be potentially vulnerable to RNAi because the viruses replicate their genomes through complementary strands resulting in dsRNA replication intermediates that are attractive targets for siRNAs. Since the genomes of most honey bee viruses are positive-stranded RNA molecules, we would expect RNAi to also be an important defense mechanism against viruses in honey bees.

A. Colony-level defense

The honey bee colony is considered to be a superorganism since a bee colony often acts as a single unit to share labor, specialize in tasks, and coordinate efforts. The homogeneous genetic structure, close physical contact, and extensive social interactions among individuals make bee colonies especially vulnerable to the infection and transmission of pathogens. On the other hand, the highly elaborate social organization of bee colonies poses a special advantage for bee immunity to defend against the infection of pathogens and to improve the survival of the colonies (Evans and Pettis, 2005; Fries and Camazine, 2001; Naug and Camazine, 2002). Therefore, it is sometimes necessary to look at the host defense mechanisms at the colony level. Completion of genome sequences of the honey bee shows that *A. mellifera*, compared to *Anopheles* and *Drosophila*, has fewer paralogs for genes related to innate immunity, with about one-third of the total number of genes found in *Anopheles* and *Drosophila* for 17 immune-related gene families (Evans *et al*., 2006; Honey Bee Genome Sequencing Consortium, 2006). Honey bee immunity against intruders is constituted not only by individual-level defense regulated by immune-related genes, but also by the colony-level defense mechanism. Compared to other nonsocial insects, the reduction of immune-related genes in honey bees may be a result of strengthened colony-level defense.

Hygienic behavior is characterized by the rapid detection of diseased and dead brood, uncapping of the brood cell, and removal of the affected brood by worker bees. The hygienic behavior of worker bees is an important aspect of the honey bee's immunity and has been shown to be effective against American foulbrood, chalkbrood, nosema, and varroa mites in colonies (Gilliam *et al.*, 1983; Park *et al.*, 1937; Peng *et al.*, 1987; Rothenbuhler, 1964; Spivak and Reuter, 2001; Woodrow and Holst, 1942). In addition, hygienic activity has been shown to be an effective defensive strategy against virus infections in honey bees. For example, adult worker bees could quickly detect larvae with SBV infection and remove them from the colony to prevent further spread of the infection (Bailey *et al.*, 1964). The cleaning or mutual grooming behaviors displayed by worker bees are believed to be responsible for the spontaneous disappearance of SBV infection in the field during the summer when bee colonies are large and foraging activity is high and the ratio of larvae to adult bees is diminishing (Bailey *et al.*, 1964). The worker bees in the colonies have also been observed to display aggressive behaviors toward bees affected with CBPV (Drum and Rothenbuhler, 1983). The spontaneous disappearance of CBPV infection in bee colonies has also been associated with bee hygienic behavior provoked by the virus infection (Bailey, 1967).

Honey bees have been observed to generate a brood comb fever in response to invasion by the heat-sensitive pathogen *Ascosphaera apis* before larvae are killed (Starks *et al.*, 2000). This fever-producing behavior is a special social defense strategy displayed in honey bees. Brood comb fever can elevate the colony environmental temperature to a level that is above the optimum growing temperature for a microorganism so that the growth and replication of the microorganism are arrested. The higher temperature can also result in the increase of bee metabolism, thereby speeding up the immune activities of individual bees against the microbial infections.

Additionally, honey bees improve their resistance to disease infections by producing antimicrobial substances in their hive products. Propolis is a resinous substance collected from tree sap or other plant sources and then mixed with wax by honey bees. Propolis has been identified to be rich in a group of biologically active antioxidants called flavonoids, which promote natural immunity and cell regeneration (Greeneway *et al.*, 1990). It has been shown that propolis not only functions as a cement to seal nest cracks or cavities but also has antimicrobial properties that help the hive block out viruses, bacteria, and other microorganisms (Kujumgiev *et al.*, 1999; Miorin *et al.*, 2003). Another important feature of honey bees' natural defense is the antimicrobial activity of colony food, including honey, pollen, and royal jelly. The antibiotic agents (also called ''inhibin'') inhibit the development of bacteria and fungi in stored food (Burgett, 1997).

Glucose oxidase is an enzyme known to possess antimicrobial activity against insect pathogens. Glucose oxidase is expressed specifically in the hypopharyngeal gland of honey bees and secreted into the royal jelly, providing protection to bee brood from infection of microorganisms (Ohashi *et al*., 1999; Santos *et al*., 2005). Although the antimicrobial properties of colony food to bacterial and fungal infections have been documented, there have been no reports regarding antiviral activities of the colony food in honey bees. Identification of neopterin which displayed some antiviral properties against Coxsackie B virus, a member of the Picornavirus, in royal jelly implies that colony food may have antiviral effects against viruses (Bratslavska *et al*., 2007; Hamerlinck, 1999). The future identification and characterization of antiviral agents from bees and colony food will be a significant contribution to the management of virus diseases in honey bees.

B. Individual-level defense

1. Physical and chemical barriers

Honey bee viruses usually enter the host through the alimentary tract during feeding or trauma on the body surface, though they can also directly enter the blood circulation via bites by varroa mites or other insects. Like other insects, honey bees can utilize both physical and chemical barriers as a primary line of passive defense to avoid infection. Both physical and chemical barriers confer nonspecific immunity to honey bees. The physical barrier includes the outer cuticle exoskeleton, the chitinous linings of the trachea, the cuticle lining of the foregut and hindgut, and the peritrophic membrane of the midgut. The rigid epidermal cuticle physically separates internal tissues from the external environment and thereby provides protection against microbial invasion. The peritrophic membrane, a chitinous matrix lining of the midgut, constitutes a second interface protecting internal tissues from external environment and also functions as a permeability barrier to keep pathogens that enter the alimentary canal with food from entering the hemocoel through the gut wall. Additionally, the biochemical environment of the gut can form a chemical barrier to inhibit the multiplication and spread of pathogens to other body tissues.

2. Cellular immune response

Although the physical and chemical barriers usually keep pathogens from entering the body, pathogens occasionally break through these defenses and begin to multiply. Whenever physical and chemical barriers are breached, honey bees can actively protect themselves from infection by employing an innate immunity response which represents a second line of defense and occurs immediately on infection. The primary goal of

the immune system is the recognition of pathogens and differentiation of nonself from self molecules. Once a microorganism is recognized as foreign, the immune system is activated to mount a defensive response to kill or eliminate the intruder. Insects lack immunoglobulin-based immune responses. The recognition of nonself is achieved by pattern recognition receptors (PRRs) that are germline-encoded immune proteins that recognize the pathogen-associated molecular patterns (PAMPs) presented on the surface of microorganisms. There are two families of PRRs: the peptidoglycan recognition proteins (PGRPs) and the Gram-negative binding proteins (GNBPs). The binding of PAMPs to PGRPs and GNBPs activates the proteolytic cascades involving serine protease and serpins. These cascades trigger an intracellular humoral pathway that controls antimicrobial peptide expression and a variety of unspecific cell defense reactions including phagocytosis, nodule formation, encapsulation and melanization, which entails immediate action against foreign intruders.

Phagocytosis is the primary response of hemocytes to small microorganisms such as bacteria. It involves the binding of hemocyte proteins to bacterial or fungal polysaccharides, changes in hemocyte number and morphology, and intracellular killing of pathogens. Nodule formation is a multi-hemocyte-cooperated cellular immune response. Hemocytes may entrap a large number of bacteria in hemocyte aggregates called nodules. Nodule formation is an important mechanism for cleaning large doses of microorganism in the hemolymph. When a foreign invader is too large to be phagocytosed or to be formed into a nodule, it becomes encapsulated by a capsule-like envelope that is made of multiple layers of hemocytes or a melanin coat or both. Encapsulation is the most effective cell-mediated immune mechanism in defending against large intruders such as parasitoid. Hemocyte-mediated killing mechanism is often accomplished by phenoloxidase activity and melanization. Melanization is triggered by the activation of a phenoloxidase cascade. A key enzyme, phenoloxidase, converts phenols into quinines, which subsequently polymerize to melanin. Melanin is deposited around a foreign invader before more hemocytes are recruited, leading to the eventual formation of a melanized cell capsule accompanied by elevated levels of nitric oxide, superoxide anion, and hydrogen peroxide in the host. However, there is another sort of encapsulation, cellular encapsulation, that does not depend on oxygen and can occur without any sign of melanization. Killing by cellular encapsulation probably depends on the lysozyme hydrolytic mechanisms (Carton and Nappi, 2001; Dimopoulos, 2003; Dunn, 1986; Lavine and Strand, 2002).

The cell-mediated immune response to fungus infections has been characterized in honey bees (Glinski and Buczek, 2003). Two critical enzymes, phenol oxidase and glucose dehydrogenase that play an important role in melanin synthesis and are necessary for defense against

intruding microorganisms and parasites, are present in the hemolymph of the honey bees (Yang and Cox-Foster, 2005; Zufelato *et al*., 2004). The genes that encode serine protease and serpins, which involve in the binding of PAMPs to PGRPs and GNBPs, have been identified in the genome of the honey bee, suggesting that honey bees have an innate immune system that enable them to defend against various microorganisms and parasites (Honey Bee Genome Sequencing Consortium, 2006; Zou *et al*., 2006). However, how honey bees combat viral infections via cell-mediated defense reaction remains undefined.

3. Humoral immune response

Insect humoral immune responses involve secretion of antimicrobial peptides by fat bodies that is functionally equivalent to the mammalian liver, into the hemolymph in response to challenges to the immune system. Most of our knowledge of the insect humoral immune response is derived from studies of *Drosophila*. To date, seven classes of antimicrobial peptides, including attacin, cecropin, defensin, diptericin, drosocin, drosomycin, and metchnikowin, have been identified in *Drosophila*, and their expression has been found to be regulated by two NF-κB signaling pathways, Toll pathway and immune deficiency (Imd) pathway (reviewed by Bulet *et al*., 2004; Leclerc and Reichhart, 2004). The humoral signaling pathway is also triggered by the binding of PAMPs to PGRPs and GNBPs which is involved in the upstream infection recognition. The Toll pathway has long been recognized to be a critical signaling pathway during Gram-positive bacterial and fungal infections. The Toll transduction cascade is activated when the ligand, Spätzle, binds to the transmembrane Toll receptor and induces the recruitment of a protein complex consisting of MyD88, Tube, and Pelle. The recruitment of the protein complex leads to the proteasome-dependent degradation of cactus. The degradation of cactus allows translocation of two NF-κB transcription factors, Dif and Dorsal, to the nucleus, causing rapid expression of gene-encoding antimicrobial peptides such as defensin, drosomycin, and metchnikowin. Imd signaling pathway, in contrast, is specific for Gram-negative bacteria although it is activated in a similar fashion to the Toll pathway. The Imd pathway activates a transcription factor, Relish, and the processed Relish then enters the nucleus where it regulates the expression of the gene-encoding antibacterial peptides such as attacin, cecropin, diptericin and drosocin. A study by Zambon *et al*. (2005) reported that both the Toll and Imd pathways were activated in *Drosophila* by an infection of *Drosophila X virus* (DXV), a dsRNA virus. Their studies showed that Toll pathway was required for the inhibition of DXV replication and that the inactivation of the Toll pathway could result in increases in virus titer and death in infected flies. This study clearly indicates that the Toll pathway was an essential part of the antiviral response in *Drosophila*.

Another study conducted by Dostert *et al*. (2005) showed that infection of *Drosophila C virus* (DCV), a member of the genus *Cripavirus* and the family Dicistroviridae, that several honey bee viruses also belong to, induced a set of genes distinct from those regulated by the Toll and Imd pathways and triggered a Janus Kinase-signal transducer and activator of transcription (Jak-STAT) DNA-binding activity. Therefore, they suggested that a Jak-STAT signaling pathway is required for an antiviral response in *Drosophila* (Dostert *et al*., 2005). The Jak-STAT pathway is ubiquitous amongst vertebrates. The signaling pathway takes part in the regulation of cellular responses to a variety of cytokines and growth factors to alter gene expression. The binding of a cytokine or growth factor to its receptor activates Jak, a cytoplasmic tyrosine kinase, and triggers it to phosphorylate and stimulate STAT, a gene regulatory protein, to detach from the receptor and translocate to the nucleus. Different STATs accumlated in the nucleus form hetero- and homodimers that induce expression of their target genes. Studies by Dostert *et al.* clearly indicated that in addition to Toll and Imd pathways for defense against bacterial and fungal infections, another evolutionarily conserved innate immune pathway, Jak-STAT pathway, exists in *Drosophila* and participates in the function of antiviral infections.

Several antimicrobial peptides including abaecin, apidaecin, hymenoptaecin, and defensin have been identified in the hemolymph of honey bees on induction of bacterial infections (Casteels *et al*., 1989, 1990; Casteels-Josson *et al*., 1994). These peptides do appear to be involved in the bee immune response to pathogen infections. A recent genome-wide analysis of honey bee immunity indicates that honey bees possess orthologues for the core members involved in different recognition and signaling pathways including Toll, Imd, Jak-STAT, as well as JNK, which is also a pivotal actor in the *Drosophila* immune response and involves the activation of transcriptional factor, Basket, though the functions of most honey bee components in these pathways remain to be validated (Evans *et al*., 2006). The data generated from *Drosophila* studies indicate that insects have an effective innate immune system that is able to respond not only to bacterial and fungal infections but also to viral infections. Knowledge of the antiviral immunity demonstrated in *Drosophila* should provide us with important insight into the relationship between virus infections and host immune responses in honey bees.

VII. MANAGEMENT OF VIRUS INFECTIONS

Viral disease outbreaks as well as inapparent viral infections can seriously affect the profitability of the beekeeping industry. Beekeepers are advised to take measures to limit viral infections, although as with any other

animal and plant viruses, chemotherapies for killing bee viruses are currently not possible. An integrated pest management program for bee diseases caused by viruses should include at least the following three components: (1) accurate diagnosis of diseases that allows rapid development and implementation of control strategies, (2) good beekeeping management practice that enhances honey bees' natural immunity to virus infections, and (3) selecting and breeding of disease-resistant strains of honey bees.

A rapid and accurate diagnosis of virus infections is a critical component of the virus surveillance and control program. It will help to determine the epidemiology of bee viral infections and to monitor honey bee colonies for viruses to prevent the spread of diseases. For many years, the detection and identification of viral infection in honey bees were based largely on serological methods like Ouchterlony gel diffusion, indirect fluorescent antibody (IFA), and enzyme-linked immunosorbent assay (ELISA) tests (Allen and Ball, 1995; Allen *et al*., 1986; Anderson, 1984). The development of molecular methods has revolutionized the diagnosis of viral diseases and provided powerful tools for specific, sensitive, and rapid identification of viruses. The RT-PCR method has become a standard method for detection, quantification, and phylogenetic analysis of honey bee viruses (Bakonyi *et al*., 2002; Benjeddou *et al*., 2001; Evans, 2001; Grabensteiner *et al*., 2001; Hung *et al*., 1996a; Ribiere *et al*., 2002; Stoltz *et al*., 1995; Tentcheva *et al*., 2004a). With increasing genomic information of bee viruses, we would expect that nucleic acid–based methods such as Northern blotting, real-time RT-PCR, microarray analysis, and other emerging methods will continue to serve as predominant tools for the diagnosis of viral diseases in honey bees.

Good bee management practice is fundamental for enhancing honey bees' natural immunity, which is the most useful tool in combating viral diseases. Stressful circumstances can favor outbreaks of viral diseases, thus any efforts that strengthen the colony health are expected to reduce the risk of virus infections. Since the varroa mite has been proven to be an effective vector in transmitting and activating viruses, timely and efficient control of the varroa mite population will reduce the incidence of viral diseases. A mathematical model proposed by Sumpter and Martin (2004) predicts that virus-associated winter collapses can be avoided if bee colonies are treated with varroacides in the summer to decrease the ABPV and DWV loads below a critical level. In addition to controlling the vector population, effective management of bee viral diseases can be achieved by maintaining good sanitation practices, feeding bees with the proper quantity and quality of food, and replacing combs and queens when the problem is serious.

Selection and breeding of disease resistant bee strains are an effective way to defend against viral attacks in honey bees. Several traits of honey bees, such as hygienic behavior and suppressed mite reproduction (SMR), are important behavioral mechanisms of disease resistance (Harbo and Harris, 2005; Lapidge *et al*., 2002). The highly hygienic bees can efficiently suppress the virus infection and *V*. *destructor* infestation by quickly recognizing and removing the diseased brood and varroa mites from combs. Nonhygienic bee lines show a slower removal response to diseased bee brood than bee stocks selected for hygienic traits (Spivak and Gilliam, 1998). Such hygienic behavior strongly depends on gene effects and has been the basis for breeding programs. The development of an integrated program to select bee populations with desirable traits, to preserve honey bee germplasm, and to arrange the mating of queens and drones will provide an important tool to breed for disease-resistant genotypes and hold great promise for colony-level disease resistance. In addition, with the completion of the honey bee genomic sequence, it becomes possible to conduct gene-based selection for genotypes with defensive and hygienic behaviors and to characterize the genes that confer disease resistance and to genetically manipulate the genes to enhance the disease resistance in honey bees.

VIII. FUTURE DIRECTIONS

In recent years, progress in honey bee virus research has been impressive. However, infections of viruses in honey bees have not been fully characterized at the molecular level and there are many gaps in our knowledge of the key processes underlying the dynamics of virus transmission, epidemiology, pathogenesis, and host immunity to virus infections. For example, what mechanisms regulate the virus transmission process, how viral gene expression contributes to disease pathogenesis, and how host immune responses regulate virus survival and replication? In addition, recent progress in the understanding of bee virus infections is limited to the aforementioned six honey bee viruses; the other previously identified honey bee viruses such as Filamentous virus, *A*. *iridescent* virus, Cloudy wing virus, Bee virus X, Bee virus Y, Arkansas bee virus, Egypt bee virus, slow paralysis virus, Thai Sacbrood, and Berkeley bee picornavirus remain poorly characterized. Moreover, identified viruses can act in new and unexpected ways and new viruses keep emerging, forming additional challenges in the elucidation of viral infections. The availability of the bee genome sequence in conjunction with new technologies opens exciting possibilities for exploring new aspects of virus life in the host and foretells future advances in bee virus research.

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REFERENCES

Allen, M., and Ball, B. (1995). Characterisation and serological relationships of strains of Kashmir bee virus. *Ann. Appl. Biol.* **126:**471–484.

Allen, M., and Ball, B. (1996). The incidence and world distribution of honey bee viruses. *Bee World* **77:**141–162.

Allen, M., Ball, B., White, R. F., and Antoniw, J. F. (1986). The detection of acute paralysis virus in *Varroa jacobsoni* by the use of a simple indirect ELISA. *J. Apic. Res.* **25:**100–105.

Anderson, D. L. (1984). A comparison of serological techniques for detecting and identifying honeybee viruses. *J. Invertebr. Pathol.* **44:**233–243.

Anderson, D. L. (1985). Viruses of New Zealand honey bees. *New Zealand Beekeeper* **188:**8–10.

Anderson, D. L. (1990). Pests and pathogens of the honeybee (*Apis mellifera* L.) in Fiji. *J. Apic. Res.* **29:**53–59.

Anderson, D. L. (1991). Kashmir bee virus – a relatively harmless virus of honey bee colonies. *Am. Bee J.* **131:**767–770.

Anderson, D. L. (1993). Pathogen and queen bees. *Australasian Beekeeper* **94:**292–296.

Anderson, D. L., and Gibbs, A. J. (1988). Inapparent virus infections and their interactions in pupae of the honey bee (*Apis mellifera* Linnaeus) in Australia. *J. Gen. Virol.* **69:**1617–1625.

Anderson, D. L., and Trueman, J. W. H. (2000). *Varroa jacobsoni* (Acari:Varroidae) is more than one species. *Exp. Appl. Acarol.* **24:**165–189.

Antúnez, K., D'Alessandro, B., Corbella, E., Ramalllo, G., and Zunino, P. (2006). Honeybee viruses in Uruguay. *J. Invertebr. Pathol.* **93:**67–70.

Bailey, L. (1965a). Paralysis of the honey bee Apis mellifera Linnaeus. *J. Invertebr. Pathol.* **7:**132–140.

Bailey, L. (1965b). The occurrence of chronic and acute bee paralysis viruses in bees outside Britain. *J. Invertebr. Pathol.* **7:**167–169.

Bailey, L. (1967). The incidence of virus diseases in the honey bee. *Ann. Appl. Biol.* **60:**43–48.

Bailey, L. (1969). The multiplication and spread of sacbrood virus of bees. *Ann. Appl. Biol.* **63:**483–491.

Bailey, L. (1975). Recent research on honey bee viruses. *Bee World* **56:**55–64.

Bailey, L. (1976). Viruses attacking the honey bee. *Adv. Virus Res.* **20:**271–304.

Bailey, L. (1981). ''Honey Bee Pathology.'' Academic Press, London.

Bailey, L. (1982a). Viruses of honeybees. *Bee World* **63:**165–173.

Bailey, L. (1982b). A strain of sacbrood virus from *Apis cerana*. *J. Invertebr. Pathol.* **39:**264–265.

Bailey, L., and Ball, B. V. (1991). ''Honey Bee Pathology.'' Academic Press, San Diego, CA.

Bailey, L., and Fernando, E. F. W. (1972). Effects of sacbrood virus on adult honey bees. *Ann. Appl. Biol.* **72:**27–35.

Bailey, L., and Gibbs, A. J. (1964). Acute infection of bees with paralysis virus. *J. Insect. Pathol.* **6:**395–407.

Bailey, L., and Milne, R. G. (1969). The multiplication regions and interaction of acute and chronic bee-paralysis viruses in adult honey bees. *J. Gen. Virol.* **4:**9–14.

Bailey, L., and Woods, R. D. (1977). Two more small RNA viruses from honey bees and further observations on sacbrood and acute bee-paralysis viruses. *J. Gen. Virol.* **37:**175–182.

Bailey, L., Gibbs, A. J., and Woods, R. D. (1963). Two viruses from adult honey bees (*Apis mellifera* Linnaeus). *Virology* **21:**390–395.
Bailey, L., Gibbs, A. J., and Woods, R. D. (1964). Sacbrood virus of the larval honey bee (*Apis mellifera* Linnaeus). *Virology* **23:**425–429.
Bailey, L., Gibbs, A. J., and Woods, R. D. (1968). The purification and properties of chronic bee-paralysis virus. *J. Gen. Virol.* **2:**251–260.
Bailey, L., Carpenter, J. M., and Woods, R. D. (1979). Egypt bee virus and Australian isolates of Kashmir bee virus. *J. Gen. Virol.* **43:**641–647.
Bailey, L., Ball, B. V., and Perry, J. N. (1981). The prevalence of viruses of honey bees in Britain. *Ann. Appl. Biol.* **97:**109–118.
Bailey, L., Ball, B. V., and Perry, J. N. (1983). Honeybee paralysis: Its natural spread and its diminished incidence in England and Wales. *J. Apic. Res.* **22:**191–195.
Bakonyi, T., Farkas, R., Szendröi, A., Dobos-Kovács, M., and Rusvai, M. (2002). Detection of acute bee paralysis virus by RT-PCR in honey bee and *Varroa destructor* field samples: Rapid screening of representative Hungarian apiaries. *Apidologie* **33:**63–74.
Ball, B. (1996). Honeybee viruses: A cause for concern? *Bee World* **77:**117–119.
Ball, B. V. (1989). *Varroa jacobsoni* as a virus vector. *In* ''Present Status of Varroatosis in Europe and Progress in the Varroa Mite Control'' (R. Cavalloro, ed.), pp. 241–244. ECSC-EEC-EAEC, Luxembourg.
Ball, B. V. (2001). Viruses and varroa. *Bee Craft* **83:**20–23.
Ball, B. V., and Allen, M. F. (1988). The prevalence of pathogens in honey bee (*Apis mellifera*) colonies infested with the parasitic mite *Varroa jacobsoni*. *Ann. Appl. Biol.* **113:**237–244.
Ball, B. V., and Bailey, L. (1991). Viruses of honey bees. *In* ''Atlas of Invertebrate Viruses'' (J. R. Adams and J. R. Bonami, eds.), pp. 525–551. CRC press, Boca Raton, FL.
Ball, B. V., and Bailey, L. (1997). Viruses. *In* ''Honey Bee Pest, Predators, & Diseases'' (R. A. Morse and K. Flottum, eds.), pp. 11–31. The A. I. Root Co., Medina, OH.
Ball, B. V., Overton, H. A., Buck, K. W., Bailey, L., and Perry, J. N. (1985). Relationships between the multiplication of chronic bee-paralysis virus and its associate particle. *J. Gen. Virol.* **66:**1423–1429.
Basavappa, R., Gómez-Yafal, A., and Hogle, J. M. (1998). The poliovirus empty capsid specifically recognizes the poliovirus receptor and undergoes some, but not all, of transitions associated with cell entry. *J. Virol.* **72:**7551–7556.
Benjeddou, M., Leat, N., Allsopp, M., and Davison, S. (2001). Detection of acute bee paralysis virus and black queen cell virus from honeybees by reverse transcriptase PCR. *Appl. Environ. Microbiol.* **67:**2384–2387.
Berényi, O., Bakonyi, T., Derakhshifar, I., Köglberger, H., and Nowotny, N. (2006). Occurrence of six honey bee viruses in diseased Austrian Apiaries. *Appl. Environ. Microbiol.* **72:**2414–2420.
Bergem, M., Norberg, K., and Aamodt, R. M. (2006). Long-term maintenance of *in vitro* cultured honeybee (*Apis mellifera*) embryonic cells. *BMC Dev. Biol.* **6:**17.
Bonhoeffer, S., and Sniegowski, P. (2002). Virus evolution: The importance of being erroneous. *Nature* **420:**367–369.
Bowen-Walker, P. L., Martin, S. J., and Gunn, A. (1999). The transmission of deformed wing virus between honey bees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. *J. Invertebr. Pathol.* **73:**101–106.
Bradbear, N. (1988). World distribution of major honeybee diseases and pests. *Bee World* **69:**15–39.
Bratslavska, O., Platace, D., Miklaševičs, E., Fuchs, D., and Martinsons, A. (2007). Influence of neopterin and 7,8-dihydroneopterin on the replication of Coxsackie type B5 and influenza A viruses. *Med. Microbiol. Immunol.* **196**(1)**:**23–29.
Bruce, W. A., Anderson, D. L., Calderone, N. W., and Shimanuki, H. (1995). A survey for Kashmir bee virus in honey bee colonies in the United States. *Am. Bee. J.* **135:**352–354.

Bulet, P., Stocklin, R., and Menin, L. (2004). Anti-microbial peptides: From invertebrates to vertebrates. *Immunol. Rev.* **198:**169–184.

Burgett, D. M. (1997). Antibiotic systems in nectar, honey, and pollen. *In* ''Honey Bee Pests, Predators, and Diseases'' (R. A. Morse and K. Flottum, eds.), pp. 455–468. The A. I. Root Co., Medina, OH.

Casteels, P., Ampe, C., Jacobs, F., Vaeck, M., and Tempst, P. (1989). Apidaecins: Antibacterial peptides from honeybees. *EMBO J.* **8:**2387–2391.

Casteels, P., Ampe, C., Riviere, L., Van Damme, J., Ellicone, C., Fleming, M., Jacobs, F., and Tempst, P. (1990). Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*). *Eur. J. Biochem.* **187:**381–386.

Casteels-Josson, K., Zhang, W., Capaci, T., Casteels, P., and Tempst, P. (1994). Acute transcriptional response of the honeybee peptide-antibiotics gene repertoire and required post-transcriptional conversion of the precursor structures. *J. Biol. Chem.* **269:**28569–28575.

Carton, Y., and Nappi, A. J. (2001). Immunogenetic aspects of the cellular immune response of *Drosophila* against parasitoids. *Immunogenetics* **52:**157–164.

Chantawannakul, P., Ward, L., Boonham, N., and Brown, M. (2006). A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in Varroa mites collected from a Thai honeybee (*Apis mellifera*) apiary. *J. Invertebr. Pathol.* **91:**69–73.

Chen, Y. P. (2005). Koch's postulates and quantitative RT-PCR as a new approach for proving viral disease causation in the honeybee, *Apis mellifera* L. *Proceedings of the Bee Research and Virus in Europe (BRAVE) Meeting,* pp. 161–173.

Chen, Y. P., Higgins, J. A., and Feldlaufer, M. F. (2004a). Quantitative analysis of deformed wing virus infection in the honey bee, *Apis mellifera* L. by real-time RT-PCR. *Appl. Environ. Microbiol.* **71:**436–441.

Chen, Y. P., Pettis, J. S., Evans, J. D., Kramer, M., and Feldlaufer, M. F. (2004b). Transmission of Kashmir bee virus by the ectoparasitic mite, *Varroa destructor*. *Apidologie* **35:**441–448.

Chen, Y. P., Zhao, Y., Hammond, J., Hsu, H. T., Evans, J. D., and Feldlaufer, M. F. (2004c). Multiple virus infections in the honey bee and genome divergence of honey bee viruses. *J. Invertebr. Pathol.* **87:**84–93.

Chen, Y. P., Pettis, J. S., and Feldlaufer, M. F. (2005). Detection of multiple viruses in queens of the honey bee, *Apis mellifera* L. *J. Invertebr. Pathol.* **90:**118–121.

Chen, Y. P., Evans, J. D., and Feldlaufer, M. F. (2006a). Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. *J. Invertebr. Pathol.* **92:**152–159.

Chen, Y. P., Pettis, J. S., Collins, A., and Feldlaufer, M. F. (2006b). Prevalence and transmission of honey bee viruses. *Appl. Environ. Microbiol.* **72:**606–611.

Dall, D. J. (1985). Inapparent infection of honeybee pupae by Kashmir and sacbrood bee viruses in Australia. *Ann. Appl. Biol.* **106:**461–468.

Dall, D. J. (1987). Multiplication of Kashmir bee virus in pupae of the honeybee, *Apis mellifera*. *J. Invertebr. Pathol.* **49:**279–290.

De Jong, D., De Jong, P. H., and Goncalves, L. S. (1982). Weight loss and other damage to developing worker honey bees from infestation with *Varroa jacobsoni*. *J. Apic. Res.* **21:**165–167.

De Miranda, J. R., Drebot, M., Tylor, S., Shen, M., Cameron, C. E., Stolz, D. B., and Camazine, S. M. (2004). Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. *J. Gen. Virol.* **85:**2263–2270.

Dimopoulos, G. (2003). Insect immunity and its implication in mosquito-malaria interactions. *Cell. Microbiol.* **5:**3–14.

Domier, L. L., McCoppin, N. K., and D'Arcy, C. J. (2000). Sequence requirements for translation initiation of *Rhopalosiphum padi* virus ORF2. *Virology* **268:**264–271.

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J. A., and Imler, J.-L. (2005). The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nat. Immunol.* **6:**946–953.

Drum, N. H., and Rothenbuhler, W. C. (1983). Non stinging aggressive responses of worker honey-bees to hivemates, intruder bees and bees affected with chronic bee paralysis. *J. Apic. Res.* **22:**256–260.

Dunn, P. E. (1986). Biochemical aspects of insect immunity. *Annu. Rev. Entomol.* **31:**321–339.

Ellis, J. D., and Munn, P. A. (2005). The worldwide health status of honey bees. *Bee World* **86:**88–101.

Evans, J. D. (2001). Genetic evidence for coinfection of honey bees by acute bee paralysis and kashmir bee viruses. *J. Invertebr. Pathol.* **78:**189–193.

Evans, J. D., and Pettis, J. S. (2005). Colony-level effects of immune responsiveness in honey bees, *Apis mellifera*. *Evolution* **59:**2270–2274.

Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J.-L., Jiang, H., Kanost, M., Thompson, G. J., Zou, Z., and Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect. Mol. Biol.* **15:**645–656.

Ewald, P. W. (1983). Host-parasite relations, vectors and the evolution of disease severity. *Annu. Rev. Ecol. Syst.* **14:**465–485.

Ewald, P. W. (1987). Transmission modes and evolution of the parasitism-mutualism continuum. *Ann. JVT Acad. Sci.* **503:**295–306.

Ewald, P. W. (1994). ''Evolution of Infectious Diseases.'' Oxford University Press, Oxford, UK.

Faucon, J. P., Vitu, C., Russo, P., and Vignoni, M. (1992). Diagnosis of acute paralysis: Application to epidemic honeybee diseases in France in 1990. *Apidologie* **23:**139–146.

Fievet, J., Tentcheva, D., Gauthier, L., de Miranda, J., Cousserans, F., Colin, M. E., and Bergoin, M. (2006). Localization of deformed wing virus infection in queen and drone *Apis mellifera* L. *Virol. J.* **3:**16.

Fredericks, D. N., and Relman, D. A. (1996). Sequence-based identification of microbial pathogens: A reconsideration of Koch's postulates. *Clin. Microbiol. Rev.* **9:**18–33.

Fries, I., and Camazine, S. (2001). Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. *Apidologie* **32:**199–214.

Fujiyuki, T., Takeuchi, H., Ono, M., Ohka, S., Sasaki, T., Nomoto, A., and Kubo, T. (2004). Novel insect picorna-like virus identified in the brains of aggressive worker honeybees. *J. Virol.* **78:**1093–1100.

Fujiyuki, T., Takeuchi, H., Ono, M., Ohka, S., Sasaki, T., Nomoto, A., and Kubo, T. (2005). Kakugo virus from brains of aggressive worker honeybees. *Adv. Virus Res.* **65:**1–27.

Fujiyuki, T., Ohka, S., Takeuchi, H., Ono, M., Nomoto, A., and Kubo, T. (2006). Prevalence and phylogeny of Kakugo virus, a novel insect picorna-like virus that infects the honeybee (*Apis mellifera* L.), under various colony conditions. *J. Virol.* **80:**11528–11538.

Furgala, B., and Lee, P. E. (1966). Acute bee paralysis virus, a cytoplasmic insect virus. *Virology* **29:**346–348.

Genersch, E., Yue, C., Fries, I., and de Miranda, J. R. (2006). Detection of deformed wing virus, a honey bee viral pathogen, in bumble bee (*Bombus terrestris* and *Bombus pascuorum*) with wing deformities. *J. Invertebr. Pathol.* **91:**61–63.

Ghosh, R. C., Ball, B. V., Willcocks, M. M., and Carter, M. J. (1999). The nucleotide sequence of sacbrood virus of the honey bee: An insect picorna-like virus. *J. Gen. Virol.* **80:** 1541–1549.

Giauffret, A., Duthoit, J. L., and Caucat, M. J. (1966). Histological study of nervous tissue from honey bees infected with ''black disease.'' *Bull. Apic. Doc. Sci. Tech. Inf.* **9:**221–228.

Giauffret, A., Duthoit, J. L., and Tostain-Caucat, M. J. (1970). Ultrastructure of cells of bees infected with the virus of black disease paralysis. Study of the cellular inclusions. *Bull. Apic. Doc. Sci. Tech. Inf.* **13:**115–126.

Gilliam, M., Taber, S., III, and Richardson, G. V. (1983). Hygienic behavior of honey bees in relation to chalkbrood disease. *Apidologie* **14:**29–39.

Glinski, Z., and Buczek, K. (2003). Response of the Apoidea to fungal infections. *Apiacta.* **38:**183–189.

Govan, V. A., Leat, N., Allsopp, M., and Davison, S. (2000). Analysis of the complete genome sequence of acute bee paralysis virus shows that it belongs to the novel group of insect-infecting RNA viruses. *Virology* **277:**457–463.

Grabensteiner, E., Ritter, W., Carter, M. J., Davison, S., Pechhacker, H., Kolodziejek, J., Boecking, O., Derakshhifar, I., Moosbeckhofer, R., Licek, E., and Nowotny, N. (2001). Sacbrood virus of the honeybee (*Apis mellifera*): Rapid identification and phylogenetic analysis using reverse transcription-PCR. *Clin. Diagn. Lab. Immunol.* **8:**93–104.

Greeneway, W., Scaysbroock, T., and Whatley, F. R. (1990). The composition and plant origins of propolis: A report work at Oxford. *Bee World* **71:**107–118.

Hamerlinck, F. F. (1999). Neopterin: A review. *Exp. Dermatol.* **8:**167–176.

Harbo, J. R., and Harris, J. W. (2005). Suppressed mite reproduction explained by the behavior of adult bees. *J. Apic. Res.* **44:**21–23.

Hellen, C. U., and Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev.* **15:**1593–1612.

Honey Bee Genome Sequencing Consortium (2006). Insights into social insects from the genome of the honey bee *Apis mellifera*. *Nature* **443:**931–949.

Hornitzky, M. (1981). The examination of honey bee virus in New South Wale. *Australasian Beekeeper* **82:**261–262.

Hornitzky, M. (1982). Bee diseases research. *Australasian Beekeeper* **84:**7–10.

Hung, A. C. F. (2000). PCR detection of Kashmir bee virus in honey bee excreta. *J. Apic. Res.* **39:**103–106.

Hung, A. C. F., and Shimanuki, H. (1999). A scientific note on the detection of Kashmir bee virus in individual honeybees and *Varroa jacobsoni* mites. *Apidologie* **30:**353–354.

Hung, A. C. F., Adams, J. R., and Shimanuki, H. (1995). Bee parasitic mite syndrome (II): The role of Varroa mite and viruses. *Am. Bee. J.* **135:**702–704.

Hung, A. C. F., Ball, B. V., Adams, J. R., Shimanuki, H., and Knox, D. A. (1996a). A scientific note on the detection of American strain of acute paralysis virus and Kashmir bee virus in dead bees in one US honey bee (*Apis mellifera* L.) colony. *Apidologie* **27:**55–56.

Hung, A. C. F., Shimanuki, H., and Knox, D. A. (1996b). The role of viruses in bee parasitic mite syndrome. *Am. Bee J.* **136:**731–732.

Hung, A. C. F., Shimanuki, H., and Knox, D. A. (1996c). Inapparent infection of acute paralysis virus and Kashmir bee virus in the U.S. honey bees. *Am. Bee J.* **136:**874–876.

Jang, S. K., Kräusslich, H. G., Nicklin, M. J. H., Duke, G. M., Palmenberg, A. C., and Wimmer, E. (1988). A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during *in vitro* translation. *J. Virol.* **62:**2636–2643.

Kaatz, H. H., Hagedorn, H. H., and Engels, W. (1985). Culture of honey bee organs: Development of a new medium and the importance of tracheation. *In vitro Cell. Dev. Biol.* **21:** 347–352.

Keene, K. M., Foy, B. D., Sanchez-Vargas, I., Beaty, B. J., Blair, C. D., and Olson, K. E. (2004). RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of *Anopheles gambiae*. *Proc. Natl. Acad. Sci. USA* **107:** 17240–17245.

Kleespies, R. G., Radtke, J., and Bienefeld, K. (2000). Virus-like particles found in the ectoparasitic bee mite *Varoa jacobsoni* Oudemans. *J. Invertebr. Pathol.* **75:**87–90.

Koch, R. (1884). Die Aetiologie der Tuberkulose. *Mitt. Kais Gesundheitsamt.* **2:**1–88.

Korpela, S., Aarhus, A., Fries, I., and Hansen, H. (1992). *Varroa jacobsoni* Oud. in cold climates: Population growth, winter mortality and influence on the survival of honey bee colonies. *J. Apic. Res.* **31:**157–164.

Kovac, H., and Crailsheim, K. (1988). Life span of *Apis mellifera Carnica* Pollm. Infested by *Varroa jacobsoni* in relation to season and extent of infestation. *J. Apic. Res.* **27:**230–238.
Kreissl, S., and Bicker, G. (1992). Dissociated neurons of the pupal honeybee brain in cell culture. *J. Neurocytol.* **21:**545–556.
Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., and Popov, S. (1999). Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *J. Ethnopharmacol.* **64:**235–240.
Kulincevic, J. M., and Rothenbuhler, W. C. (1975). Selection for resistance and susceptibility to hairless-black syndrome in the honeybee. *J. Invertebr. Pathol.* **25:**289–295.
Kulincevic, J., Ball, B., and Mladjan, V. (1990). Viruses in honey bee colonies infested with *Varroa jacobsoni*: First findings in Yugoslavia. *Acta Vet.* **40:**37–42.
Laidlaw, H. H. (1979). Contemporary queen rearing. Dadant and Sons, Hamilton, IL.
Lanzi, G., de Miranda, J. R., Boniotti, M. B., Cameron, C. E., Lavazza, A., Capucci, L., Camazine, S. M., and Rossi, C. (2006). Molecular and biological characterization of deformed wing virus of honey bees (*Apis mellifera* L.). *J. Virol.* **80:**4998–5009.
Lapidge, K. L., Oldroyd, B. P., and Spivak, M. (2002). Seven suggestive quantitative trait loci influence hygienic behaviour of honey bees. *Naturwissenschaften* **89:**565–568.
Lavine, M. D., and Strand, M. R. (2002). Insect hemocytes and their role in immunity. *Insect. Biochem. Mol. Biol.* **32:**1295–1309.
Leat, N., Ball, B., Govan, V., and Davison, S. (2000). Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. *J. Gen. Virol.* **81:**2111–2119.
Leclerc, V., and Reichhart, J. M. (2004). The immune response of *Drosophila melanogaster*. *Immunol. Rev.* **198:**59–71.
Lee, P. E., and Furgala, B. (1965). Chronic bee paralysis virus in the nerve ganglia of the adult honey bee. *J. Invertebr. Pathol.* **7:**170–174.
Lee, P. E., and Furgala, B. (1967). Electron microscopic observations on the localization and development of sacbrood virus. *J. Invertebr. Pathol.* **9:**178–187.
Li, H., Li, W. X., and Ding, S. W. (2002). Induction and suppression of RNA silencing by an animal virus. *Science* **296:**1319–1321.
Lighthart, B., Prier, K. R. S., and Bromenshenk, J. J. (2005). Flying honey bees absorb airborne viruses. *Aerobiologia* **21:**147–149.
Lipsitch, M., Siller, S., and Nowak, M. A. (1996). The evolution of virulence in pathogens with vertical and horizontal transmission. *Evolution* **50:**1729–1741.
Malun, D., Moseleit, A. D., and Grünewald, B. (2003). 20-Hydroxyecdysone inhibits the mitotic activity of neuronal precursors in the developing mushroom bodies of the honeybee, *Apis mellifera*. *J. Neurobiol.* **57:**1–14.
Martin, S., Hogarth, A., van Breda, J., and Perrett, J. (1998). A scientific note on *Varroa jacobsoni* Oudemans and the collapse of *Apis mellifera* L. colonies in the United Kingdom. *Apidologie* **29:**369–370.
Martin, S. J. (2001). The role of Varroa and viral pathogens in the collapse of honey bee colonies: A modelling approach. *J. Appl. Ecol.* **38:**1082–1093.
Mayo, M. A. (2002). Virus Taxonomy—Houston 2002. *Arch. Virol.* **147:**1071–1076.
Mendelsohn, C. L., Wimmer, E., and Racaniello, V. R. (1989). Cellular receptor for poliovirus: Molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell* **56:**855–865.
Mims, C. A. (1981). Vertical transmission of viruses. *Microbiol. Rev.* **45:**267–286.
Miorin, P. L., Levy Junior, N. C., Custodio, A. R., Bretz, W. A., and Marcucci, M. C. (2003). Antibacterial activity of honey and propolis from *Apis mellifera* and *Tetragonisca angustula* against *Staphylococcus aureus*. *J. Appl. Microbiol.* **95:**913–920.
Mitsuhashi, J. (2001). Development of highly nutritive culture media. *In Vitro Cell. Dev. Biol.* **37:**330–337.

Mitsuhashi, J. (2002). ''Invertebrate Tissue Culture Methods.'' Springer Lab Manual, Spring-Verlag, Tokyo.

Moya, A., Elena, S. F., Bracho, A., Miralles, R., and Barrio, E. (2000). The evolution of RNA viruses: A population genetic view. *Proc. Natl. Acad. Sci. USA* **97:**6967–6973.

Naug, D., and Camazine, S. (2002). The role of colony organization on pathogen transmission in social insects. *J. Theor. Biol.* **215:**427–439.

Nordström, S. (2003). Distribution of deformed wing virus within honey (Apis mellifera) brood cells infected with the ectoparasitic mite *Varroa destructor*. *Exp. Appl. Acarol.* **29:**293–302.

Nordström, S., Fries, I., Aarhus, A., Hansen, H., and Korpela, S. (1999). Virus infections in Nordic honey bee colonies with no, low or severe *Varroa jacobsoni* infections. *Apidologie* **30:**475–484.

Ohashi, K., Natori, S., and Kubo, T. (1999). Expression of amylase and glucose oxidase in the hypopharyngeal gland with an age-dependent role change of the worker honeybee (*Apis mellifera* L.). *Eur. J. Biochem.* **265:**127–133.

Ongus, J. R., Peters, D., Bonmatin, J.-M., Bengsch, E., Vlak, J. M., and Van Oers, M. M. (2004). Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite *Varroa destructor*. *J. Gen. Virol.* **85:**3747–3755.

Park, O. W., Pellett, F. C., and Paddock, F. B. (1937). Disease resistance and American foulbrood. *Am. Bee J.* **77:**20–25.

Pelletier, J., and Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334:**320–325.

Peng, Y. S., Fang, Y., Xu, S., Ge, L., and Nasr, M. E. (1987). Response of foster Asian Honey bee (*Apis cerana* Fabr.) colonies to the brood of European honey bee (*Apis mellifera* L.) infested with parasitic mite *Varroa jacobsoni* Oudemanns. *J. Invertebr. Pathol.* **49:**259–264.

Rachinsky, A., and Hartfelder, K. (1998). *In vitro* biosynthesis of juvenile hormone in larval honey bees: Comparison of six media. *In Vitro Cell. Dev. Biol. Anim.* **34:**646–648.

Ribiere, M., Triboulot, C., Mathieu, L., Aurieres, C., Faucon, J.-P., and Pepin, M. (2002). Molecular diagnosis of chronic bee paralysis virus infection. *Apidologie* **33:**339–351.

Rinderer, T. E., Rothenbuhler, W. C., and Kulincevic, J. M. (1975). Responses of three genetically different stocks of the honey bee to a virus from bees with hairless-black syndrome. *J. Inverteb. Pathol.* **25:**297–300.

Rothenbuhler, W. C. (1964). Behaviour genetics of nest cleaning in honey bees. IV. Responses of F1 and backcross generations to disease-killed brood. *Am. Zool.* **4:**111–123.

Rortais, A., Tentcheva, D., Papachristoforou, A., Gauthier, L., Arnold, G., Colin, M. E., and Bergoin, M. (2006). Deformed wing virus is not related to honey bees' aggressiveness. *Virol. J.* **3:**61.

Santos, K. S., dos Santos, L. D., Mendes, M. A., de Souza, B. M., Malaspina, O., and Palma, M. S. (2005). Profiling the proteome complement of the secretion form hypopharyngeal gland of Africanized nurse-honeybees (*Apis mellifera* L.). *Insect. Biochem. Mol. Biol.* **35:**85–91.

Sasaki, J., and Nakashima, N. (1999). Translation initiation at the CUU codon is mediated by the internal ribosome entry site of an insect picorna-like virus *in intro*. *J. Virol.* **73:**1219–1226.

Sasaki, J., and Nakashima, N. (2000). Methionine-independent initiation of translation in the capsid protein of an insect RNA virus. *Proc. Natl. Acad. Sci. USA* **97:**1512–1515.

Schmid-Hempel, P. (2005). Evolutionary ecology of insect immune defense. *Annu. Rev. Entomol.* **50:**529–551.

Shen, M. Q., Cui, L. W., Ostiguy, N., and Cox-Foster, D. (2005a). Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. *J. Gen. Virol.* **86:**2281–2289.

Shen, M. Q., Yang, X. L., Cox-Foster, D., and Cui, L. W. (2005b). The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. *Virology* **342:**141–149.

Shimanuki, H., Calderone, N. W., and Knox, D. A. (1994). Parasitic mite syndrome: The symptoms. *Am. Bee J.* **134:**827–828.

Siede, R., Derakhshifar, I., Otten, C., Berényi, O., Bakonyi, T., Köglberger, H., and Büchler, R. (2005). Prevalence of Kashmir bee virus in Europe. *J. Apic. Res.* **44:**131–132.

Simpson, J. (1961). Nest climate regulation in honeybee colonies. *Science* **133:**1327–1333.

Spivak, M., and Gilliam, M. (1998). Hygienic behaviour of honey bees and its application for control of brood diseases and varroa—Part II. Studies on hygienic behaviour since the Rothenbuhler era. *Bee World* **79:**169–186.

Spivak, M., and Reuter, G. S. (2001). Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. *Apidologie* **32:**555–565.

Starks, P. T., Blackie, C. A., and Seeley, T. D. (2000). Fever in honeybee colonies. *Naturwissenschaften* **87:**229–231.

Stoltz, D., Shen, X.-R., Boggis, C., and Sisson, G. (1995). Molecular diagnosis of Kashmir bee virus infection. *J. Apic. Res.* **34:**153–160.

Sumpter, D., and Martin, S. (2004). The dynamics of virus epidemics in Varroa-infested honey bee colonies. *J. Anim. Ecol.* **73:**51–63.

Tentcheva, D., Gauthier, L., Jouve, S., Canabady-Rochelle, L., Dainat, B., Cousserants, F., Colin, M. E., Ball, B. V., and Bergoin, M. (2004a). Polymerase chain reaction detection of deformed wing virus (DWV) in *Apis mellifera* and *Varroa destructor*. *Apidologie* **35:**431–439.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M. E., and Bergoin, M. (2004b). Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. *Appl. Environ. Microbiol.* **70:**7185–7191.

Topolska, G., Ball, B. V., and Allen, M. (1995). Identification of viruses in bees from two Warsaw apiaries. *Medycyna Weterynaryina* **51:**145–147.

Voinnet, O. (2001). RNA silencing as a plant immune system against viruses. *Trends Genet.* **17:**449–459.

Weinberg, K. P., and Madel, G. (1985). The influence of the mite *Varroa jacobsoni* Oud. on the protein concentration and haemolymph volume of the brood of the worker bees and drones of the honey bee, *Apis mellifera*. *Apidologie* **16:**421–436.

White, G. F. (1913). Sacbrood, a Disease of Bees. *US Department of Agriculture, Bureau of Entomology*. Circular No. 169.

Wilson, J. E., Powell, M. J., Hoover, S. E., and Sarnow, P. (2000). Naturally occurring dicistronic cricket paralysis virus RNA is regulated by two internal ribosome entry sites. *Mol. Cell. Biol.* **20:**4990–4999.

Woodrow, A. W., and Holst, E. C. (1942). The mechanism of colony resistance to American foulbrood. *J. Econ. Entomol.* **35:**327–330.

Yang, X., and Cox-Foster, D. L. (2005). Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. *Proc. Nat. Acad. Sci. USA* **102:**7470–7475.

Yue, C., and Genersch, E. (2005). RT-PCR analysis of deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa desctructor*). *J. Gen. Virol.* **86:**3419–3424.

Yue, C., Schröder, M., Bienefeld, K., and Genersch, E. (2006). Detection of viral sequences in semen of honeybees (*Apis mellifera*): Evidence for vertical transmission of viruses through drones. *J. Invertebr. Pathol.* **92:**105–108.

Zambon, R. A., Nandakumar, M., Vakharia, V. N., and Wu, L. P. (2005). The Toll pathway is important for an antiviral response in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **102:**7257–7262.

Zou, Z., Lopez, D. L., Kanost, M. R., Evans, J. D., and Jiang, H. (2006). Comparative analysis of serine protease-related genes in the honey bee genome: Possible involvement in embryonic development and innate immunity. *Insect. Mol. Biol.* **15:**603–614.

Zufelato, M. S., Lourenco, A. P., Simões, Z. L. P., Jorge, J. A., and Bitondi, M. M. (2004). Phenoloxidase activity in *Apis mellifera* honey bee pupae, and ecdysteroid-dependent expression of the prophenoloxidase mRNA. *Insect. Biochem. Mol. Biol.* **34:**1257–1268.

CHAPTER 3

Use of Functional Genomics to Understand Influenza–Host Interactions

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Contents

Abstract

Infection with influenza typically results in mild-to-moderate illness in healthy individuals; however, it is responsible for 30,000–40,000 deaths each year in the United States. In extreme cases, such as the influenza pandemic of 1918, tens of millions of people have died from the infection. To prepare for future influenza outbreaks, it is necessary to understand how the virus interacts with the host and to determine what makes certain strains of influenza highly pathogenic. Functional genomics provides a unique approach to this effort by allowing researchers to examine the effect of influenza infection on global host mRNA levels. Researchers are making

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increasing use of this approach to study virus–host interactions using a variety of model systems. For example, data obtained using microarray technology, in combination with mouse and macaque infection models, is providing exciting new insights into the pathogenicity of the 1918 virus. These studies suggest that the lethality associated with this virus is in part due to an aberrant and unchecked immune response. Progress is also being made toward using functional genomics in the diagnosis and prognosis of acute lung infections and in the development of more effective influenza vaccines and antivirals.

I. INTRODUCTION

For centuries, influenza virus has plagued humankind. While influenza infection typically causes mild-to-moderate illness in healthy individuals, it still results in 30,000–40,000 deaths per year in the United States. Those most susceptible to influenza infection are infants, the elderly, and those individuals that are immunocompromised due to HIV/AIDS infection or organ/tissue transplant (CDC, 2006). In extreme cases, such as the 1918 pandemic, it is estimated that 50 million people died as a result of influenza infection (Taubenberger and Morens, 2006). What was unique about this pandemic is that the most susceptible to this disease were young, otherwise healthy, individuals. Since 1918, multiple influenza pandemics have occurred, although none nearly as deadly. Another influenza pandemic is inevitable and much effort is being placed on disease surveillance and monitoring of transmission across species (Pandemic Flu, 2007; Subbarao and Joseph, 2007).

Of particular concern is the H5N1 family of avian influenza viruses (Horimoto and Kawaoka, 2005). While the transmission rate of H5N1 viruses from birds to humans is extremely low, the case mortality rate in humans is greater than 50% (WHO, 2007). Fortunately, human-to-human transmission is extremely rare (WHO, 2005). It is difficult to predict for how long this will be the case and there is increasing concern that H5N1 viruses will recombine with human viruses. This could result in an H5N1 virus with the capacity for human-to-human transmission and perhaps generate a catastrophic pandemic (Subbarao and Joseph, 2007).

Understanding the ways in which influenza interacts with the host is an important component of preparing for the next pandemic. It is necessary to understand these interactions in order to improve existing vaccines, to develop new and more efficacious vaccines that will provide protection against multiple strains and subtypes, and to develop new antiviral therapeutics (Subbarao and Joseph, 2007). Because of its ability to provide a global view, functional genomics is one of the most useful approaches for studying virus–host interactions. Our laboratory is using

functional genomics to study a variety of viruses, including HCV, SIV/HIV, Ebola virus, HSV, SARS coronavirus, West Nile virus, and influenza virus (Baas *et al.*, 2006a,b; Baskin *et al.*, 2004; Fredericksen *et al.*, 2004; Geiss *et al.*, 2000, 2001, 2002, 2003; Kash *et al.*, 2004, 2006a,b; Kobasa *et al.*, 2007; Lederer *et al.*, 2006; Pasieka *et al.*, 2006; Smith *et al.*, 2003a,b, 2006; Thomas *et al.*, 2006; Walters *et al.*, 2006, 2006a,b). This chapter will focus on how microarray technology is being utilized to uncover the mysteries of influenza pathogenesis. We will explore increasingly complex models for studying influenza–host interactions using functional genomics, including cell culture systems, murine models of infection, and nonhuman primates (Fig. 1). Finally, we will discuss the promise of using genomics to define molecular signatures of the disease that could lead to the evolution of the microarray as a diagnostic tool.

II. MODEL SYSTEMS OF INFLUENZA A INFECTION USED IN FUNCTIONAL GENOMICS

A. Cell culture models

Initial functional genomic endeavors in our laboratory utilized established cell lines to understand the ways in which influenza virus disrupts cellular processes. We first performed a series of experiments to determine replication-dependent and -independent events during influenza infection. HeLa cells were mock infected or infected with either active or heat-inactivated A/WSN/33 (H1N1). Using the dual-labeling technique, cDNA arrays were hybridized with RNA from mock versus active or heat-inactivated virus or with RNA from heat-inactivated versus active virus, allowing us to determine which genes were regulated by actively replicating virus (Geiss *et al.*, 2001).

We found that while there are distinct subsets of genes whose regulation is replication dependent or independent, more gene expression changes were observed in the presence of replicating virus. Further analysis revealed that these genes could be classified in five major categories: protein synthesis, cytokine and growth factor signaling, transcription factors and DNA-binding proteins, processing and export of mRNA, and the ubiquitin pathway. In contrast, genes whose regulation was replication independent were grouped representative of the following categories: metallothioneins, cell cycle related, transcriptional regulators, part of the ubiquitin pathway, or cellular kinases (Geiss *et al.*, 2001). Although specific aspects of influenza replication-independent and -dependent events could have been assessed using conventional laboratory techniques, our gene expression studies allowed us to examine numerous gene expression changes at the same time. From this data, it was possible to

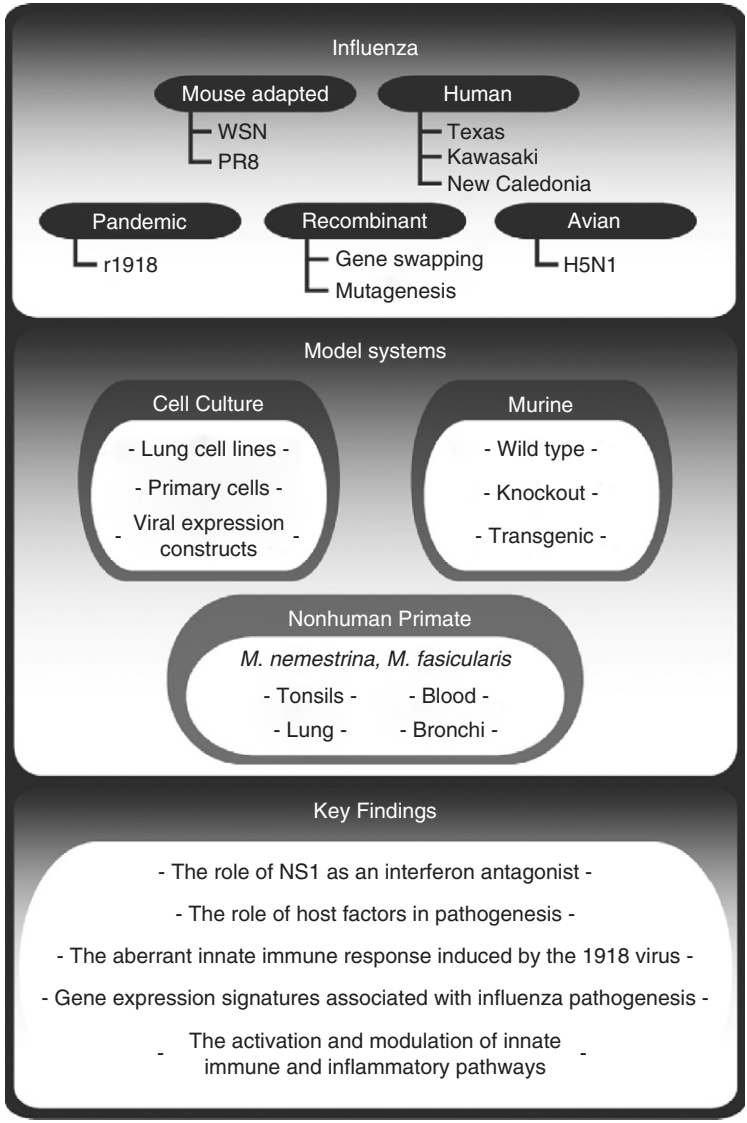

FIGURE 1 Representation of the range of viruses and experimental systems we have used to evaluate influenza virus–host interactions using genomic technologies. Highlights of experiments related to the use of these experimental systems are summarized in this chapter. WSN: A/WSN/33; PR8: A/PR/8/34; Texas: A/Texas/36/91; Kawasaki: A/Kawasaki/173/01; New Caledonia: A/New Caledonia/99; r1918: reconstructed 1918 virus.

identify specific processes that are related to influenza replication-independent and -dependent events and speculate on how these events work together in influenza pathogenesis.

Further studies using a cell culture system were aimed at discerning viral determinants of virulence. Of particular interest was the viral NS_1 protein. NS_1 appears to play a role in subverting the host response to the virus. It has been suggested that NS_1 attenuates the interferon response to the virus through its double-stranded (ds) RNA-binding domain (García-Sastre *et al.*, 1999). It has also been suggested that the dsRNA-binding domain of NS_1 functions to inhibit the 2′5′-OAS/RNaseL antiviral response (Min and Krug, 2006). This may indicate that NS_1 from different influenza viruses plays distinct roles in subverting the host response to the virus.

To better understand the effect of NS_1 on virus–host interactions, we infected an established human lung epithelial cell line, A549, with A/PR/8/34 (H1N1), A/PR/8/34 in which NS_1 was deleted, or with A/PR/8/34 in which NS_1 contained a deletion in the C-terminus (Geiss *et al.*, 2002). The latter two viruses were reconstructed using plasmid-based reverse genetics (Fodor *et al.*, 1999). These studies allowed us to examine the global host response to influenza infection in the absence of NS_1 or in response to infection with a virus exhibiting attenuated NS_1 function.

Numerous genes were preferentially upregulated in response to infection with the mutant viruses compared to the parental strain. Many of these genes were related to the antiviral and interferon responses. These data suggest a role for the NS_1 of A/PR/8/34 as an antagonist of the interferon response to the virus (Geiss *et al.*, 2002). Antagonism of this crucial defense response to influenza most likely contributes to the lethality of this virus in mice. Therefore, these initial studies from our laboratory were crucial in understanding the importance of the interferon response in the host response to influenza.

This study was also the first to use functional genomics to examine the role of specific genes from the 1918 strain. In addition to the viruses mentioned above, A549 cells were infected with A/WSN/33 or a recombinant in which the NS_1 of A/WSN/33 was replaced with the NS_1 from the 1918 virus. We noted that there was greater suppression of interferon-stimulated genes in cells infected with the 1918 NS_1 recombinant virus than in cells infected with the parental strain. The host response to A/WSN/33 virus containing the 1918 NS_1 was also compared with the response to wild-type A/PR/8/34 and with the A/PR/8/34 NS_1 mutant viruses. From these analyses, we determined that the expression of numerous interferon-stimulated genes was anti-correlated between these viruses and A/WSN/33 containing the 1918 NS_1. For example, NMI and STAT1 expression were upregulated in cells infected with the A/PR/8/34 viruses but downregulated in cells infected with A/WSN/33

containing the NS_1 from the 1918 virus. These studies suggest that the NS_1 from the 1918 virus is more adept at suppressing key interferon responses. It will be interesting to use functional genomics to compare the effect of the NS_1 from the 1918 virus on host–virus interactions to that of the NS_1s from modern day low pathogenicity human influenza viruses and both low and high pathogenicity avian H5N1 viruses. Such comparisons will lend a global view into how different influenza NS_1s affect the host response and lead to important observations as to the role of NS_1 in influenza pathogenicity (Geiss *et al.*, 2002).

Interestingly, mice infected with a virus containing the NS_1 of 1918 and the other seven genes from A/WSN/33 did not succumb to the infection. In contrast, all mice infected with A/WSN/33 died by 10 days postinfection (Basler *et al.*, 2001). These data, in conjunction with the array studies described above, suggest that the NS_1 of the 1918 virus is an important virulence factor, but it is not solely responsible for the high lethality of the 1918 virus. Therefore, it was imperative to study the effect of other 1918 genes on mortality and examine the critical interplay of all of the 1918 genes. Such studies will be discussed in the following section covering murine models of influenza infection.

Using cell culture systems in the application of functional genomics is crucial to the understanding of how influenza infection affects antiviral responses on the cellular level. However, these systems are limited in that the data obtained from them can only lead to inferences as to what is occurring in the host as a whole. For this reason, it is necessary to study influenza infection in the context of the whole organism. The use of functional genomics in conjunction with various mammalian models of infection, and in humans, will be discussed in the next sections.

B. Murine models

In order to study the effects of influenza in the context of the whole organism, many scientists have utilized mouse models of infection. Although mice are not a natural host for influenza virus, their accessibility and the vast repertoire of genetically altered species makes them a useful tool in many areas of research, including functional genomics. Since laboratory strains of mice are inbred, this reduces host variation, making it easier to clarify how influenza is affecting the host. Of particular interest to our laboratory is how the host response induced by highly pathogenic influenza infection differs from that induced by viruses with lower pathogenicity.

Of all the influenza viruses that have surfaced in the last century, very few have caused as much intrigue as the 1918 pandemic strain. Among the most perplexing questions surrounding the influenza pandemic of 1918 is what made this virus so deadly. Environmental, biological, or

demographic factors could have contributed to its virulence; however, the most pertinent factors may be related to how this virus interacts with the host innate immune response. As mentioned in the previous section, we used functional genomics to study the effect of the 1918 NS_1 on global gene expression using a cell culture system. While this study provided an important first step in understanding this deadly virus, it only hints at what might be occurring in the whole host.

With the sequencing and reconstruction of the 1918 virus using reverse genetics (Tumpey *et al*., 2005a), our laboratory and others have been able to study the effects of various genes from this virus and the fully reconstructed virus on the host (Basler *et al*., 2001; Reid *et al*., 1999, 2000, 2002, 2004; Taubenberger, 1998; Taubenberger *et al*., 1997). In initial studies, we infected mice with the lethal, A/WSN/33 stain or with a recombinant of this virus containing the HA and NA from the 1918 virus. A recombinant A/WSN/33 virus containing the HA and NA of a contemporary human strain (A/New Caledonia/99) was also included in these studies. Both HA and NA are major virulence factors and HA is the major viral factor against which host antibodies are produced (Lamb and Krug, 1996) and evidence suggests that the HA of the 1918 virus is necessary for transmission (Tumpey *et al*., 2007). Gene expression profiling was then performed on lungs isolated from these mice. Increased gene expression in the lungs of mice infected with either A/WSN/33 or the recombinant virus containing the HA and NA from the 1918 virus was observed at 24 h postinfection. In contrast, relatively few gene expression changes were observed in the lungs of mice infected with the A/WSN/33 recombinant strain containing the HA and NA from A/New Caledonia/33. By 72 h postinfection, gene expression changes were similar between the two infection groups, indicating that the HA and NA of the 1918 virus were sufficient to accelerate the host response to the virus (Kash *et al*., 2004).

A subset of genes was preferentially upregulated in mice infected with the A/WSN/33 recombinant virus containing the 1918 HA and NA. Among this group were genes that are indicative of T cell activation, macrophage activation, and cell death (Kash *et al*., 2004). In support of these findings, Tumpey *et al*. demonstrated that mice infected with A/Texas/36/91 containing the HA and NA from the 1918 virus or with A/WSN/33 containing these genes developed severe lung pathology, including varying degrees of necrotizing bronchitis, alveolitis, and pulmonary edema. Strikingly, there was also an increase in neutrophils and alveolar macrophages in the lungs of these animals. To analyze the importance of these immune cells in the context of A/Texas/36/91 recombinant virus, animals in which neutrophils and/or alveolar macrophages had been depleted were infected with a sublethal dose of the virus. Infected neutrophil-depleted mice had a 60% survival rate.

In contrast, all animals in which alveolar macrophages or both alveolar macrophages and neutrophils were depleted died by 9 days postinfection with the recombinant virus (Tumpey *et al.*, 2005b). Taken together, these data emphasize the importance of certain immune mediators in combating infection with a recombinant virus containing the HA and NA from the 1918 virus. However, as discussed below, these findings also suggest that an inappropriate activation of the host response to the virus may contribute to its pathogenicity.

We have also used functional genomics to analyze the host response of mice infected with the fully reconstructed 1918 virus. These studies, led by John Kash, revealed that genes related to various immune cells, notably NK cells, neutrophils, macrophages, and T helper 1 (Th1) cells, were upregulated in mice infected with the fully reconstructed 1918 virus as early as 1 day postinfection. These genes were persistently activated in the lungs of r1918-infected mice throughout the course of the experiment (5 days) (Kash *et al.*, 2006b). Key mediators of the immune response to influenza virus are cytokines and chemokines that are responsible for the activation of and recruitment of immune cells into the infected tissue (Julkunen *et al.*, 2001). In agreement with early and persistent activation of immune cells in r1918-infected mice, this same gene expression pattern was observed for genes related to pro-inflammatory cytokines and chemokines such as Tnf, Il6, and Ccl5 (Kash *et al.*, 2006b). It is crucial that a delicate balance of immune responses is maintained during infection in order to limit excessive damage to the host. If these responses go unchecked, or are insufficient, it can result in dire consequences for the host (La Gruta *et al.*, 2007). Our data suggest that a hyperactive and persistent host response is associated with the 1918 virus and that this is a key contributor to the high mortality associated with this virus.

Another important aspect of our study was an examination of what effect the full constellation of genes from the 1918 virus had on gene expression and virus-induced morbidity and mortality. In order to accomplish this, mice were infected with the fully reconstructed 1918 virus (r1918), with A/Texas/91/36 containing the HA and NA from 1918 (2:6 1918), or with A/Texas/91/36 containing the HA, NA, M, NP, and NS_1 genes from the 1918 virus (5:3 1918) (Fig. 2). Compared with the response of mice infected with the r1918 virus, which exhibited early and persistent upregulation of genes related to NK cells, neutrophils, macrophages, and T helper 1 (Th1) cells, mice infected with either the 5:3 1918 or 2:6 1918 virus exhibited a delay in the upregulation of these genes. However, expression levels of genes related to these immune cells was similar in all three 1918 recombinant viruses by day 5 postinfection.

Interestingly, animals infected with either the 5:3 1918 virus or the 2:6 1918 virus exhibited lung pathology intermediate to mice infected with A/Texas/91/36 and those infected with r1918 at day 3 postinfection.

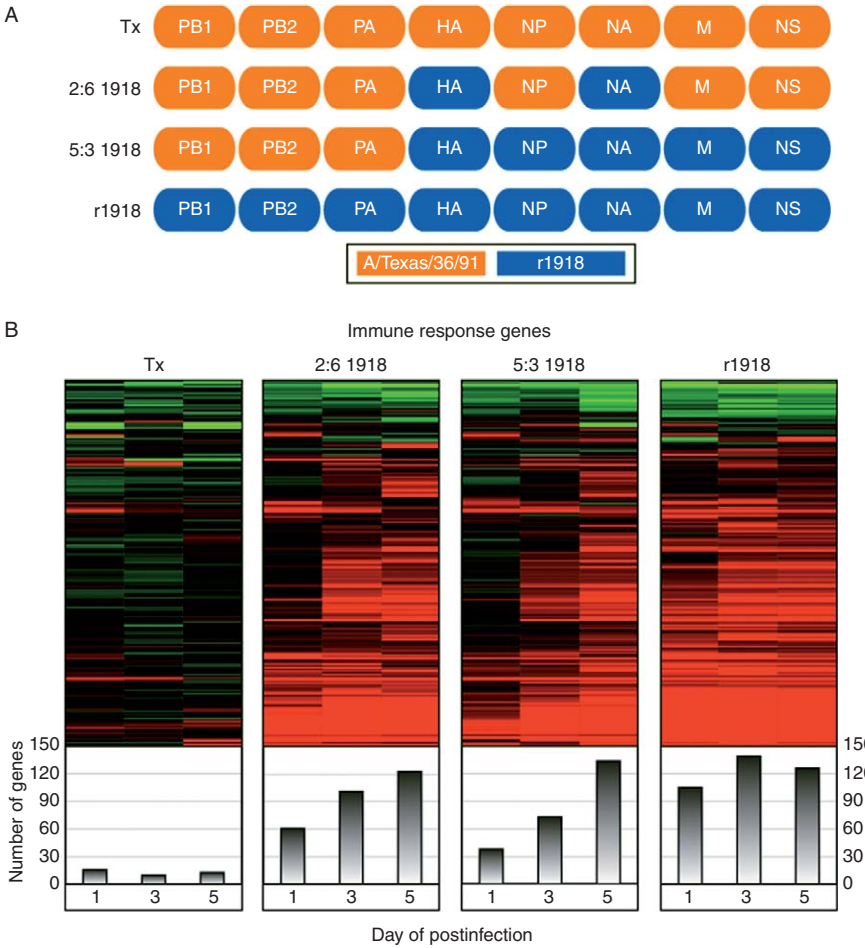

FIGURE 2 Effect of the full constellation of genes from the 1918 virus on gene expression. (A) Mice were infected with A/Texas/91/36, with A/Texas/91/36 containing the HA and NA from 1918 (2:6 1918), with A/Texas/91/36 containing the HA, NA, M, NP, and NS_1 genes from the 1918 virus (5:3 1918), or with the fully reconstructed 1918 virus (r1918). (B) Global gene expression profiles and number of differentially regulated genes for mice infected with each virus at days 1, 3, and 5 postinfection.

The differences in gene expression not only correlated with lung pathology, but also with viral titers and morbidity, demonstrating the usefulness of gene expression profiling in understanding molecular mechanisms of disease and disease outcome (Kash *et al.*, 2006b).

Studies in our laboratory are now focusing on combining genomics with the use of knockout or transgenic mice to further understand the complex host–virus interactions that occur in response to infection with the 1918 virus. We are also interested in using genomics to examine the effects of H5N1 infection on mice and to determine if there are molecular signatures of disease that are present as a consequence of infection with highly pathogenic strains of influenza. In addition, we are taking advantage of the vast repertoire of transgenic and knockout mice available to gain further insight into key regulators of the innate and/or adaptive immune response to influenza infection in general. For example, in collaboration with Michael Gale, we are working to understand the role of the pattern recognition receptor, retinoic acid inducible gene I (RIG-I), during influenza infection. RIG-I plays an important role in the interferon response to many viruses, including influenza (Foy *et al.*, 2005; Fredericksen and Gale, 2006; Kato *et al.*, 2005, 2006; Liu *et al.*, 2007; Sumpter *et al.*, 2005; Yoneyama *et al.*, 2005, 2004). We recently examined the gene expression profiles in RIG-I deficient mouse embryonic fibroblasts (MEFs) infected with A/PR/8/34. Global gene expression profiles revealed significant differences in gene expression between wildtype and RIG-I deficient MEFs. We are in the process of furthering analyzing data, but preliminary analyses have revealed an important role for RIG-I in the host response to the virus (Loo *et al.*, submitted).

C. Nonhuman primate models

Even though mouse models have provided critical insights into the pathogenesis of influenza, the information gained from these studies is limited since mice are not natural hosts for the virus. For this reason, data obtained from mouse studies can be difficult to translate to human infection. Numerous studies have utilized nonhuman primate models to study influenza pathogenesis (Berendt, 1974; Grizzard *et al.*, 1978; Liu *et al.*, 1997; Rimmelzwaan *et al.*, 2001; van Riel *et al.*, 2006). Unlike mouse models of infection, influenza infection in nonhuman primate models mimics human infection. For example, nonhuman primates can be infected with human influenza strains without prior adaptation and the virus is transmissible between animals. In addition, nonhuman primates and humans are close evolutionary relatives. As a consequence, nonhuman primates are increasingly being utilized to examine influenza pathogenesis, and with the sequencing of the rhesus macaque genome (Rhesus macaque genome sequencing and analysis consortium, 2007), genomic and proteomic resources for working with these animals are becoming progressively more available (Magness *et al.*, 2005; Spindel *et al.*, 2005; Wallace *et al.*, 2007).

Although there are numerous advantages to using nonhuman primates in influenza research, certain considerations must be taken before working with them. Nonhuman primates exhibit host variation similar to that in humans, a factor that needs to be taken into account when analyzing genomics data. Additionally, the numbers of nonhuman primates available for research are limited. Therefore, most nonhuman primate studies are restricted in their sample size. Lastly, considerable ethical concerns must be taken into account when using nonhuman primates in research studies. Our laboratory, along with others, has diligently worked to address these concerns and yet still obtain the insights into influenza–host interactions that only studies in nonhuman primates can provide.

In a seminal study led by Carole Baskin, pigtailed macaques (*Macaca nemestrina*) were infected with the reconstructed H1N1 human influenza strain, A/Texas/36/91. Physical symptoms, such as throat inflammation, loss of appetite, and weight loss, correlated with the upregulation of interferon-stimulated genes at days 4 and 7 postinfection. Gene expression profiling also revealed the upregulation of genes related to neutrophil and monocyte/macrophage function. Accordingly, an influx of neutrophils and macrophages into the lungs of infected monkeys was observed. Although not the first to use nonhuman primates as a model of influenza infection, this study was unique for two reasons. It was the first to use pigtailed macaques and it was the first in which functional genomics was used to examine influenza infection in nonhuman primates (Baskin *et al.*, 2004).

To expand upon the above study, we have also employed functional genomics to assess the effect of influenza infection on the early innate immune response in the lungs of pigtailed macaques, how genes related to this response were regulated over time, and whether gene expression signatures of infection could also be detected in the blood. Finally, we examined the correlation between genomic and proteomic data collected for both lung and PBMC samples. Significantly, this study was also the first to use macaque-specific oligonucleotide arrays, which were developed in our laboratory (Wallace *et al.*, 2007).

As in the previous study, animals were infected with A/Texas/36/91. Subsequent analysis focused on gene expression changes present at day 2 postinfection in order to determine molecular correlates of early influenza infection. In lesions where viral mRNA was present, there was increased expression of interferon-stimulated genes and antiviral-related genes. Notably, the majority of these genes were significantly upregulated, suggesting a robust host response against the virus. Differential expression of cytokine, chemokine, and immune-related genes was also present in samples isolated at 7 days postinfection (Baas *et al.*, 2006a). These data indicate a robust and sustained host response in the lungs of influenza-infected pigtail macaques.

We also compared the signatures of infection in the lung with those found in whole blood. This analysis identified numerous genes whose expression was upregulated in the lung and in the blood throughout the time course of the infection. There was an upregulation in interferon-stimulated genes and antiviral-related genes such as IRF7, IFIT2, OAS1, and OAS3. Our findings suggest that there are common signatures of influenza infection between the lung and whole blood, indicating that gene expression profiling of blood may eventually prove useful for diagnostic or prognostic applications. This subject is further discussed in the following section.

We also worked with Richard Smith's group at Pacific Northwest National Laboratory to perform the first ever global proteomic analyses on macaque lung samples. Side-by-side comparison of genomic and proteomic data from infected macaque lung samples revealed that there were many correlations between the two sets of data. Of particular interest, were the correlations observed for interferon-stimulated genes and antiviral-related genes. Members of these families, such as IFIT1, IFIT2, STAT1, and MX1, were identified by both genomics and proteomics. In further support for the use of whole blood as a surrogate marker of influenza pathogenesis in the lungs, gene expression data for the above markers and others correlated with the lung genomics and proteomics data (Baas *et al*., 2006a). We would like to further these studies by determining if similar proteomic results are observed in whole blood.

From these analyses, we also identified an increase in the abundance of certain proteins in influenza-infected lung that would not have been predicted by our genomics data. This observation points to the need for the integration of genomics and proteomics data to gain a more complete understanding of influenza pathogenesis. Furthermore, integration of genomic and proteomic data will enhance our understanding of the differences between mRNA levels and protein abundance.

We have also recently used functional genomics and a macaque infection model to study the pathogenesis of the 1918 virus. For these studies, cynomolgus macaques (*Macaca fasicularis*) were infected with the human H1N1 virus, A/Kawasaki/173/01, or with the reconstructed 1918 virus (Kobasa *et al*., 2007). Microarray analysis on bronchi from infected animals revealed a robust activation of numerous pro-inflammatory chemokine and cytokine genes in both A/Kawasaki/173/01 and 1918-infected animals at day 3 postinfection. Additionally, there was an increased activation of genes related to the interferon-α response in response to infection with either of these viruses at this time-point. Strikingly, many of the genes related to these responses exhibited a more robust upregulation in the A/Kawasaki/173/01-infected animals at day 3 postinfection, but returned to baseline levels or were downregulated later in infection compared with r1918-infected animals. In contrast, animals infected with

the r1918 virus exhibited an increased and robust upregulation of expression of interferon-stimulated genes and chemokines and cytokines through the study endpoint.

In support of our genomics observations, CCL2, CCL5, IL-8, and IL-6 levels were increased in the serum of r1918-infected macaques compared with the levels present in A/Kawasaki/173/01-infected animals at days 3 and 6 postinfection. Viral titers were substantially greater in both the upper and lower respiratory tracts of macaques infected with r1918 at all three time-points postinfection. r1918-infected animals also exhibited severe lung pathology at 8 days postinfection (Kobasa *et al*., 2007). Taken together, these data agree with data obtained using mouse models, suggesting that the pathogenesis induced by infection with the 1918 virus is associated with, and potentially caused by, an aberrant and unchecked immune response to the virus. As a consequence, this response turns from one that is beneficial to the host to one that is extremely detrimental.

The studies described above demonstrate how nonhuman primate models can be used in combination with functional genomics to understand influenza–host interactions. Our studies, in conjunction with those of others, firmly demonstrate that nonhuman primate models of influenza provide crucial information into disease progression and pathogenesis. Currently, we are focused on using functional genomics to assess the effectiveness of influenza vaccination in nonhuman primates (Baskin *et al.*, submitted). These studies illustrate a novel use for functional genomics in influenza vaccine development. Genomic analyses during vaccine trials may reveal gene expression markers of protective immunity or gene expression changes that are indicative of a predisposition to a particular response to immunization and subsequent challenge.

D. New diagnostic approaches

As mentioned previously, a major promise of genomics is the capacity to use this technology in the more precise and efficient diagnosis of disease. Of major interest, is the use of functional genomics to ascertain molecular signatures of infection that permit the distinction among diseases. Discussed below is how this technology is being tested for influenza diagnosis.

To identify the gene expression signatures induced by various pathogens, Chaussabel *et al*. examined peripheral blood mononuclear cells (PBMCs) obtained from pediatric patients presenting with various illnesses (2005). Specifically, they examined diseases with distinct immunological components such as systemic lupus erythematosus (SLE), influenza A, *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus pneumoniae*. They also examined adult patients who received liver transplants with immunosuppressive therapy or patients who received bone marrow transplants

and experienced graft versus host disease. These samples were compared with PBMCS from healthy volunteers. The authors were able to identify unique gene expression patterns for patients presenting with influenza and SLE. They then determined expression profiles common to all of the diseases using genes that were either up- or downregulated in patients infected with influenza or SLE. Analyses also demonstrated that the genes whose expression was regulated in a similar manner in both influenza and SLE patients fell into distinct categories such as defense response, interferon induction, and heavy metal binding. Furthermore, the authors were able to determine how many genes related to these processes were expressed in individual patients (Chaussabel *et al.*, 2005).

In an extension of the above studies, the authors examined the gene expression profiles of PBMCs from young patients presenting with acute infections including influenza A, *S. aureus*, *S. pneumoniae*, and *E. coli* (Ramilo *et al.*, 2007). Analysis of these samples was performed in a methodical manner using statistical comparison, sample classification, validation of classifier genes using a test set, and validation of microarray platforms and chips. The authors were able to identify subsets of genes that distinguished patients with influenza (viral infection) from those that presented with either *E. coli* or *S. pneumoniae* (Gram-negative and Gram-positive bacterial infections, respectively). The same was found for patients infected with influenza compared to those infected with *S. aureus* (Gram-positive bacterial infection). Distinct expression patterns were also present in PBMCs from patients infected with *E. coli* or *S. aureus*.

Using sets of classifier genes obtained from the above analyses, the authors examined the gene expression profiles of PBMCs isolated from patients presenting with lower respiratory infections the same as those listed above or from healthy volunteers. From these analyses, the authors were able to classify the samples from these new patients into the correct disease categories. In addition, the authors tested a separate set of samples using a different array platform. These studies also demonstrated that patients presenting with these illnesses could be accurately classified into distinct groups based on gene expression profiles (Ramilo *et al.*, 2007). Through these painstaking efforts, the authors convincingly used functional genomics to discriminate between patients with a variety of acute infections, including influenza.

While these studies provide evidence that genomics can be used to define molecular signatures of disease associated with certain pathogens, they also have significant limitations. For example, these studies were performed on samples that had been taken from patients that had already been diagnosed with a particular illness and genomic analyses only had to distinguish between a relatively few possibilities. However, in order to be effective in a clinical setting, gene expression profiling will need to provide a high degree of accuracy and overcome numerous confounding

factors such as age, race, gender, immune status, and co-infection with more than one pathogen. All of these issues must be addressed before functional genomics can function in disease diagnosis. However, once these challenges have been met, genomic diagnosis may decrease the amount of elapsed time between sample collection and disease diagnosis thereby allowing doctors to treat patients more quickly. This is particularly important for patients presenting with acute infections. Additionally, the use of microarrays in this manner may eliminate the need for patients to undergo certain painful and potentially dangerous diagnostic procedures, such as tissue biopsies.

III. CONCLUSIONS

Functional genomics has clearly provided critical information regarding virus–host interactions and has made significant contributions to influenza research. As we have described, functional genomics has been utilized to study influenza infection in a variety of model systems including cell culture, mice, and macaques. Researchers are also utilizing functional genomics to study influenza infections in chickens, but these endeavors are still in their infancy (Degen *et al.*, 2006). It will also be desirable to use functional genomics to examine influenza infection in ferrets. Unlike mice, ferrets can be productively infected with human influenza viruses and ferret-to-ferret transmission occurs. Due to these characteristics, ferrets provide a useful and unique model for influenza infection studies. Unfortunately, genomic studies using ferrets are currently limited due to the lack of ferret nucleotide sequence information. We therefore strongly recommend that the ferret genome be sequenced and that ferret-specific microarrays be developed.

From the functional genomics experiments published so far, we have been able to gain invaluable insight into influenza pathogenesis. Perhaps the most critical use of this technology has been in the study of the virus responsible for the deadly 1918 influenza pandemic. In regards to highly pathogenic influenza, future experiments should also focus on the effect of avian H5N1 infection on global gene expression, using multiple model systems such as those that are being used to study the 1918 virus.

Functional genomics has provided us with numerous insights into influenza–host interactions. In particular, we have utilized this technology to discern how low and high pathogenicity viruses affect host responses. However, there are many challenges facing our laboratory and others that use functional genomics. Of utmost concern, is the integration of the vast amounts of genomics data that is available and has yet to be generated. Among the major obstacles are microarray platform differences, species differences, cell type differences, and annotation differences

(Wallace *et al.*, 2006). Data from microarray studies also needs to be integrated with conventional biological approaches and with data that will be obtained from the burgeoning field of proteomics. As demonstrated throughout this manuscript, our laboratory has worked tirelessly to achieve these goals. We firmly believe that functional genomics will be crucial to the development of novel therapies necessary for the prevention of influenza infection and spread.

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REFERENCES

Baas, T., Baskin, C. R., Diamond, D. L., Garcia-Sastre, A., Bielefeldt-Ohmann, H., Tumpey, T. M., Thomas, M. J., Carter, V. S., Teal, T. H., Van Hoeven, N., Proll, S., Jacobs, J. M., *et al.* (2006a). Integrated molecular signature of disease: Analysis of influenza virus-infected macaques through functional genomics and proteomics. *J. Virol.* **80:**10813–10828.

Baas, T., Taubenberger, J. K., Chong, P. Y., Chui, P., and Katze, M. G. (2006b). SARS-CoV virus-host interactions and comparative etiologies of acute respiratory distress syndrome as determined by transcriptional and cytokine profiling of formalin-fixed paraffin-embedded tissues. *J. Interferon Cytokine Res.* **26:**309–317.

Baskin, C. R., Garcia-Sastre, A., Tumpey, T. M., Bielefeldt-Ohmann, H., Carter, V. S., Nistal-Villan, E., and Katze, M. G. (2004). Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (*Macaca nemestrina*). *J. Virol.* **78:**10420–10432.

Basler, C. F., Reid, A. H., Dybing, J. K., Janczewski, T. A., Fanning, T. G., Zheng, H., Salvatore, M., Perdue, M. L., Swayne, D. E., García-Sastre, A., Palese, P., Taubenberger, J. K., *et al.* (2001). Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. *Proc. Natl. Acad. Sci. USA* **98:**2746–2751.

Berendt, R. F. (1974). Simian model for the evaluation of immunity to influenza. *Infect. Immun.* **9:**101–105.

CDC (2006). CDC Flu Shot. www.cdc.gov/flu/about/qa/flushot.htm

Chaussabel, D., Allman, W., Mejias, A., Chung, W., Bennett, L., Ramilo, O., Pascual, V., Palucka, A. K., and Banchereau, J. (2005). Analysis of significance patterns identifies ubiquitous and disease-specific gene-expression signatures in patient peripheral blood leukocytes. *Ann. N. Y. Acad. Sci.* **1062:**146–154.

Degen, W. G. J., Smith, J., Simmelink, B., Glass, E. J., Burt, D. W., and Schijns, V. E. J. C. (2006). Molecular immunophenotyping of lungs and spleens in naive and vaccinated

chickens early after pulmonary avian influenza A (H9N2) virus infection. *Vaccine* **24:**6096–6109.

Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G., and García-Sastre, A. (1999). Rescue of influenza A virus from recombinant DNA. *J. Virol.* **73:**9679–9682.

Foy, E., Li, K., Sumpter, R., Jr., Loo, Y. M., Johnson, C. L., Wang, C., Fish, P. M., Yoneyama, M., Fujita, T., Lemon, S. M., and Gale, M., Jr. (2005). Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proc. Natl. Acad. Sci. USA* **102:**2986–2991.

Fredericksen, B. L., and Gale, M., Jr. (2006). West Nile virus evades activation of interferon regulatory factor 3 through RIG-I-dependent and -independent pathways without antagonizing host defense signaling. *J. Virol.* **80:**2913–2923.

Fredericksen, B. L., Smith, M., Katze, M. G., Shi, P. Y., and Gale, M., Jr. (2004). The host response to West Nile virus infection limits viral spread through the activation of the interferon regulatory factor 3 pathway. *J. Virol.* **78:**7737–7747.

García-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P., and Muster, T. (1999). Influenza A virus lacking the NS_1 gene replicates in interferon-deficient systems. *Virology* **252:**324–330.

Geiss, G. K., Bumgarner, R. E., An, M., Agy, M. B., van't Wout, A., Hammersmark, E., Carter, V. S., Upchurch, D., Mullins, J. I., and Katze, M. G. (2000). Large-scale monitoring of host cell gene expression during HIV-1 infection using cDNA microarrays. *Virology* **266:**8–16.

Geiss, G. K., An, M. C., Bumgarner, R. E., Hammersmark, E., Cunningham, D., and Katze, M. G. (2001). Global impact of influenza virus on cellular pathways is mediated by both replication-dependent and -independent events. *J. Virol.* **75:**4321–4331.

Geiss, G. K., Salvatore, M., Tumpey, T. M., Carter, V. S., Wang, X., Basler, C. F., Taubenberger, J. K., Bumgarner, R. E., Palese, P., Katze, M. G., and García-Sastre, A. (2002). Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: The role of the nonstructural NS_1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. *Proc. Natl. Acad. Sci. USA* **99:**10736–10741.

Geiss, G. K., Carter, V. S., He, Y., Kwieciszewski, B. K., Holzman, T., Korth, M. J., Lazaro, C. A., Fausto, N., Bumgarner, R. E., and Katze, M. G. (2003). Gene expression profiling of the cellular transcriptional network regulated by alpha/beta interferon and its partial attenuation by the hepatitis C virus nonstructural 5A protein. *J. Virol.* **77:**6367–6375.

Grizzard, M. B., London, W. T., Sly, D. L., Murphy, B. R., James, W. D., Parnell, W. P., and Chanock, R. M. (1978). Experimental production of respiratory tract disease in cebus monkeys after intratracheal or intranasal infection with influenza A/Victoria/3/75 or influenza A/New Jersey/76 virus. *Infect. Immun.* **21:**201–205.

Horimoto, T., and Kawaoka, Y. (2005). Influenza: Lessons from past pamdemics, warnings from current incidents. *Nat. Rev. Microbiol.* **3:**591–600.

Julkunen, I., Sareneva, T., Pirhonen, J., Ronni, T., Melen, K., and Matikainen, S. (2001). Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine Growth Factor Rev.* **12:**171–180.

Kash, J. C., Basler, C. F., García-Sastre, A., Carter, V., Billharz, R., Swayne, D. E., Przygodzki, R. M., Taubenberger, J. K., Palese, P., Katze, M. G., and Tumpey, T. M. (2004). Global host immune response: Pathogenesis and transcriptional profiling of type A influenza viruses expressing the hemagglutinin and neuraminidase genes from the 1918 pandemic virus. *J. Virol.* **78:**9499–9511.

Kash, J. C., Muhlberger, E., Carter, V., Grosch, M., Perwitasari, O., Proll, S. C., Thomas, M. J., Weber, F., Klenk, H. D., and Katze, M. G. (2006a). Global suppression of the host

antiviral response by Ebola- and Marburgviruses: Increased antagonism of the type I interferon response is associated with enhanced virulence. *J. Virol.* **80:**3009–3020.

Kash, J. C., Tumpey, T. M., Proll, S. C., Carter, V., Perwitasari, O., Thomas, M. J., Basler, C. F., Palese, P., Taubenberger, J. K., Garcia-Sastre, A., Swayne, D. E., Katze, M. G., *et al.* (2006b). Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* **443:**578–581.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O., and Akira, S. (2005). Cell type-specific involvement of RIG-I in antiviral response. *Immunity* **23:**19–28.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., *et al.* (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441:**101–105.

Kobasa, D., Jones, S. M., Shinya, K., Kash, J. C., Copps, J., Ebihara, H., Hatta, Y., Hyun Kim, J., Halfmann, P., Hatta, M., Feldmann, F., Alimonti, J. B., *et al.* (2007). Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* **445:**319–323.

La Gruta, N. L., Kedzierska, K., Stambas, J., and Doherty, P. C. (2007). A question of self-preservation: Immunopathology in influenza virus infection. *Immunol. Cell Biol.* **85:**85–92.

Lamb, R. A., and Krug, R. M. (1996). Orthomyxoviridae: The viruses and their replication. *In* ''Fields Virology'' (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), pp. 1353–1395. Lippincott-Raven, Philadelphia.

Lederer, S., Walters, K. A., Proll, S., Paeper, B., Robinzon, S., Boix, L., Fausto, N., Bruix, J., and Katze, M. (2006). Distinct cellular responses differentiating alcohol- and hepatitis C virus-induced liver cirrhosis. *Virol. J.* **3:**98.

Liu, M. A., McClements, W., Ulmer, J. B., Shiver, J., and Donnelly, J. (1997). Immunization of non-human primates with DNA vaccines. *Vaccine* **15:**909–912.

Liu, P., Jamaluddin, M., Li, K., Garofalo, R. P., Casola, A., and Brasier, A. R. (2007). Retinoic acid-inducible gene I mediates early antiviral response and toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J. Virol.* **81:**1401–1411.

Magness, C., Fellin, P. C., Thomas, M., Korth, M., Agy, M., Proll, S., Fitzgibbon, M., Scherer, C., Miner, D., Katze, M., and Iadonato, S. (2005). Analysis of the macaca mulatta transcriptome and the sequence divergence between macaca and human. *Genome Biol.* **6:**R60.

Min, J. Y., and Krug, R. M. (2006). The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2′-5′ oligo (A) synthetase/RNase L pathway. *Proc. Natl. Acad. Sci. USA* **103:**7100–7105.

Pandemic Flu (2007). www.pandemicflu.gov/.

Pasieka, T. J., Baas, T., Carter, V. S., Proll, S. C., Katze, M. G., and Leib, D. A. (2006). Functional genomic analysis of herpes simplex virus type 1 counteraction of the host innate response. *J. Virol.* **80:**7600–7612.

Ramilo, O., Allman, W., Chung, W., Mejias, A., Ardura, M., Glaser, C., Wittkowski, K. M., Piqueras, B., Banchereau, J., Palucka, A. K., and Chaussabel, D. (2007). Gene expression patterns in blood leukocytes discriminate patients with acute infections. *Blood* **109:** 2066–2077.

Reid, A. H., Fanning, T. G., Hultin, J. V., and Taubenberger, J. K. (1999). Origin and evolution of the 1918 ''Spanish'' influenza virus hemagglutinin gene. *Proc. Natl. Acad. Sci. USA* **96:** 1651–1656.

Reid, A. H., Fanning, T. G., Janczewski, T. A., and Taubenberger, J. K. (2000). Characterization of the 1918 ''Spanish'' influenza virus neuraminidase gene. *Proc. Natl. Acad. Sci. USA* **97:**6785–6790.

Reid, A. H., Fanning, T. G., Janczewski, T. A., McCall, S., and Taubenberger, J. K. (2002). Characterization of the 1918 ''Spanish'' influenza virus matrix gene segment. *J. Virol.* **76:**10717–10723.

Reid, A. H., Fanning, T. G., Janczewski, T. A., Lourens, R. M., and Taubenberger, J. K. (2004). Novel origin of the 1918 pandemic influenza virus nucleoprotein gene. *J. Virol.* **78:**12462–12470.

Rhesus macaque genome sequencing and analysis consortium (2007). The rhesus macaque genome sequence informs biomedical and evolutionary analyses. *Science.* **316:**222–234.

Rimmelzwaan, G. F., Kuiken, T., van Amerongen, G., Bestebroer, T. M., Fouchier, R. A. M., and Osterhaus, A. D. M. E. (2001). Pathogenesis of influenza A (H5N1) virus infection in a primate model. *J. Virol.* **75:**6687–6691.

Smith, M. W., Yue, Z. N., Geiss, G. K., Sadovnikova, N. Y., Carter, V. S., Boix, L., Lazaro, C. A., Rosenberg, G. B., Bumgarner, R. E., Fausto, N., Bruix, J., and Katze, M. G. (2003a). Identification of novel tumor markers in hepatitis C virus-associated hepatocellular carcinoma. *Cancer Research* **63:**859–864.

Smith, M. W., Yue, Z. N., Korth, M. J., Do, H. A., Boix, L., Fausto, N., Bruix, J., Carithers, R. L., Jr., and Katze, M. G. (2003b). Hepatitis C virus and liver disease: Global transcriptional profiling and identification of potential markers. *Hepatology* **38:**1458–1467.

Smith, M. W., Walters, K. A., Korth, M. J., Fitzgibbon, M., Proll, S., Thompson, J. C., Yeh, M. M., Shuhart, M. C., Furlong, J. C., Cox, P. P., Thomas, D. L., Phillips, J. D., *et al.* (2006). Gene expression patterns that correlate with Hepatitis C and early progression to fibrosis in liver transplant recipients. *Gastroenterology* **130:**179–187.

Spindel, E., Pauley, M., Jia, Y., Gravett, C., Thompson, S., Boyle, N., Ojeda, S., and Norgren, R. (2005). Leveraging human genomic information to identify nonhuman primate sequences for expression array development. *BMC Genomics* **6:**160.

Subbarao, K., and Joseph, T. (2007). Scientific barriers to developing vaccines against avian influenza viruses. *Nat. Rev. Immunol.* **7:**267–278.

Sumpter, R., Jr., Loo, Y. M., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S. M., and Gale, M., Jr. (2005). Regulating intracellular antiviral aefense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J. Virol.* **79:**2689–2699.

Taubenberger, J. K. (1998). Influenza virus hemagglutinin cleavage into HA1, HA2: No laughing matter. *Proc. Natl. Acad. Sci. USA* **95:**9713–9715.

Taubenberger, J. K., and Morens, D. M. (2006). 1918 Influenza: The mother of all pandemics. *Emerg. Infect. Dis.* **12:**15–22.

Taubenberger, J. K., Reid, A. H., Krafft, A. E., Bijwaard, K. E., and Fanning, T. G. (1997). Initial genetic characterization of the 1918 ''Spanish'' influenza virus. *Science* **275:**1793–1796.

Thomas, M. J., Agy, M. B., Proll, S. C., Paeper, B. W., Li, Y., Jensen, K. L., Korth, M. J., and Katze, M. G. (2006). Functional gene analysis of individual response to challenge of SIVmac239 in M. mulatta PBMC culture. *Virology* **348:**242–252.

Tumpey, T. M., Basler, C. F., Aguilar, P. V., Zeng, H., Solorzano, A., Swayne, D. E., Cox, N. J., Katz, J. M., Taubenberger, J. K., Palese, P., and Garcia-Sastre, A. (2005a). Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* **310:**77–80.

Tumpey, T. M., Garcia-Sastre, A., Taubenberger, J. K., Palese, P., Swayne, D. E., Pantin-Jackwood, M. J., Schultz-Cherry, S., Solorzano, A., Van Rooijen, N., Katz, J. M., and Basler, C. F. (2005b). Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: Functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. *J. Virol.* **79:**14933–14944.

Tumpey, T. M., Maines, T. R., Van Hoeven, N., Glaser, L., Solorzano, A., Pappas, C., Cox, N. J., Swayne, D. E., Palese, P., Katz, J. M., and Garcia-Sastre, A. (2007). A

two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. *Science* **315:**655–659.

van Riel, D., Munster, V. J., de Wit, E., Rimmelzwaan, G. F., Fouchier, R. A. M., Osterhaus, A. D. M. E., and Kuiken, T. (2006). H5N1 virus attachment to lower respiratory tract. *Science* **312:**399.

Wallace, J. C., Korth, M. J., Diamond, D. L., Proll, S. C., and Katze, M. G. (2006). Virology in the 21st century: Finding function with functional genomics. *Future Virol.* **1:**47–53.

Wallace, J., Korth, M., Paeper, B., Proll, S., Thomas, M., Magness, C., Iadonato, S., Nelson, C., and Katze, M. (2007). High-density rhesus macaque oligonucleotide microarray design using early-stage rhesus genome sequence information and human genome annotations. *BMC Genomics* **8:**28.

Walters, K. A., Joyce, M. A., Thompson, J. C., Smith, M. W., Yeh, M. M., Proll, S., Zhu, L. F., Gao, T. J., Kneteman, N. M., Tyrell, D. L., and Katze, M. G. (2006). Host-specific response to HCV infection in the chimeric SCID-beige/Alb-uPA mouse model: Role of the innate antiviral immune response. *PLOS Pathog.* **2:**e59.

Walters, K. A., Joyce, M., Thompson, J., Proll, S., Wallace, J., Smith, M., Furlong, J., Tyrrell, D. L., and Katze, M. (2006a). Application of functional genomics to the chimeric mouse model of HCV infection: Optimization of microarray protocols and genomics analysis. *Virol. J.* **3:**37.

Walters, K. A., Smith, M. W., Pal, S., Thompson, J. C., Thomas, M. J., Yeh, M. M., Thomas, D. L., Fitzgibbon, M., Proll, S., Fausto, N., Gretch, D. R., Carithers, J., *et al.* (2006b). Identification of a specific gene expression pattern associated with HCV-induced pathogenesis in HCV- and HCV/HIV-infected individuals. *Virol.* **350:**453–464.

WHO (2005). Avian Flu Facts www.who.int/csr/disease/avian_influenza/avian_faqs/en/index.html#isit.

WHO (2007). Avian Flu Timeline www.who.int/csr/disease/avian_influenza/Timeline_2007_03_20.pdf.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* **5:**699–701.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y. M., Gale, M., Jr., Akira, S., Yonehara, S., Kato, A., *et al.* (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.* **175:**2851–2858.

CHAPTER 4

A Guide to Viral Inclusions, Membrane Rearrangements, Factories, and Viroplasm Produced During Virus Replication

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Abstract

Virus replication can cause extensive rearrangement of host cell cytoskeletal and membrane compartments leading to the "cytopathic effect" that has been the hallmark of virus infection in tissue culture for many years. Recent studies are beginning to redefine these signs of viral infection in terms of specific effects of viruses on cellular processes. In this chapter, these concepts have been illustrated by describing the replication sites produced by many different viruses. In many cases, the cellular rearrangements caused during virus infection lead to the construction of sophisticated platforms in the cell that concentrate replicase proteins, virus genomes, and host proteins required for replication, and thereby increase the efficiency of replication. Interestingly, these same structures, called virus factories, virus inclusions, or virosomes, can recruit host components that are associated with cellular defences against infection and cell stress. It is possible that cellular defence pathways can be subverted by viruses to generate sites of replication. The recruitment of cellular membranes and cytoskeleton to generate virus replication sites can also benefit viruses in other ways. Disruption of cellular membranes can, for example, slow the transport of immunomodulatory proteins to the surface of infected cells and protect against innate and acquired immune responses, and rearrangements to cytoskeleton can facilitate virus release.

I. INTRODUCTION

Viruses are obligate intracellular parasites. Unlike their hosts, they cannot replicate by growth or division but use their genomes to redirect host cell processes to produce all the components needed to make new viruses. Virus replication and assembly are often confined within specific intracellular compartments called virus factories, viroplasm, or viral inclusions. These are thought to provide a physical platform to concentrate new genomes and proteins involved in replication and assembly, and this is likely to increase the efficiency of virus production. The formation of specialized sites of replication can involve extensive reorganization of cellular cytoskeleton and membrane compartments. This can lead to cell rounding and swelling and a ''cytopathic effect'' that has been documented for many years (Reissig *et al*., 1956; Robbins *et al*., 1950). Recent advances in microscopy, such as live cell imaging and tomography, combined with the power of reverse genetics, are now allowing the cytopathic effect to be redefined in terms of specific effects of viral proteins on specific cellular processes rather than an overwhelming assault on the cell in preparation for cell lysis.

There is considerable interest in understanding how virus infection leads to the large changes in cellular organization required to produce complex replication sites. In the simplest model, virus replication sites would form passively through self-association of viral components and exclusion of host organelles. Viruses, however, require a considerable number of host proteins to facilitate replication, and there is increasing evidence that these are specifically transported to sites of replication. Host proteins may move to replication sites because they are actively recruited by binding to specific viral proteins. Alternatively, viruses may transport viral and host material to replication sites by subverting host defences against infection [reviewed by Kirkegaard *et al*. (2004) and Wileman (2006)]. The large scale changes in cellular membrane and cytoskeletal organization, which occur during the formation of replication sites, can offer further benefit to viruses. Rearrangement of the cytoskeleton can, for example, facilitate virus release, and the block in the secretory pathway seen during infection with positive-stranded RNA viruses can reduce release of inflammatory mediators and protect against innate and acquired immune responses. This is a broad subject of considerable interest to virologists and cell biologists, and we have benefited from excellent reviews that have been published (Mackenzie, 2005; Novoa *et al*., 2005). In writing this chapter, we have concentrated on describing sites of virus replication in the context of the cell in which its replication takes place. We have illustrated these concepts with reference to replication sites

produced by many different viruses and, where possible, described how virus replication impacts on the functioning of the host cell.

II. VIROPLASM, VIROSOMES, FACTORIES, AND INCLUSIONS

Virus replication sites have been studied for many years and have evolved their own terminology. Early studies of poxvirus replication (Dales and Siminovitch, 1961; Morgan *et al.*, 1954) describe electron-dense aggregates and amorphous material induced early during infection called viroplasm. Viroplasm has also been used to describe similar structures induced during infection with *Poliovirus* (Dales *et al.*, 1965a). Viroplasm is often concentrated within perinuclear areas that exclude host organelles. Viroplasm is thought to indicate sites of virus replication, and concentrations of viroplasm have been called virosomes, or virus factories, to reflect an organelle involved in virus production. Virus infection also produces inclusion bodies. As a working definition, these can be considered to form later during infection. They can form virus factories once virus production has peaked, and/or at other sites in the cell they probably arise from an accumulation of viral proteins that do not become incorporated into viruses.

III. MEMBRANE REARRANGEMENTS OCCURRING DURING THE REPLICATION OF THE POSITIVE-STRANDED RNA VIRUSES

The positive-stranded RNA viruses encode nonstructural proteins (NSP) that cause proliferation and modification of membranes of the host secretory pathway. The membranes are thought to provide a physical framework or ''replication complex'' that concentrates the cellular and viral components required for virus replication (Bienz *et al.*, 1987; Egger *et al.*, 2002; Froshauer *et al.*, 1988; Gazina *et al.*, 2002; Magliano *et al.*, 1998; Schlegel *et al.*, 1996; van der Meer *et al.*, 1998). Assembly of the replicase on membranes, rather than the cytosol, may also help viruses evade host defence pathways that monitor cells for double-stranded RNA (dsRNA) intermediates indicative of virus replication. The replicase complexes of all the positive-stranded RNA viruses contain an RNA-dependent RNA polymerase (RdRp), a protein with NTPase and helicase activity, and in many cases a methyl transferase to cap viral RNA. These proteins are generated from the viral polyproteins by viral proteases, and are then targeted to membranes in ways that differ depending on virus family (Fig. 1).

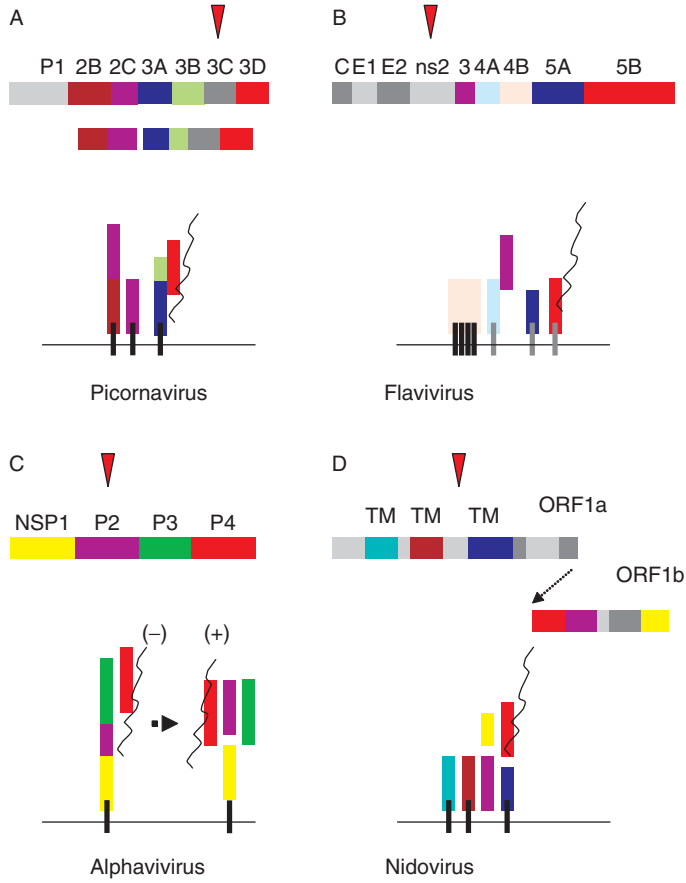

FIGURE 1 The replicase proteins of positive-stranded RNA viruses are directed to membranes by NSP with membrane-targeting information. (A) Picornavirus. The replication complex contains 3D, the RdRp (red), and 2C which has NTPase and helicase motifs (purple). The 3D polymerases do not have membrane-targeting information but are synthesized as a 3ABCD precursor. 3ABCD is processed to 3AB by the 3C protease (red triangle) and a hydrophobic domain in 3A targets 3AB to the cytoplasmic face of ER membranes. 3AB binds directly to 3D and this targets the polymerase to the replication complex. The replication complex also requires 2BC and 2C proteins that are targeted to membranes via their own hydrophobic domains (black lines). (B) Flavivirues. The replication complex is encoded at the C-terminus of a polyprotein that is processed by the NS2 protease (red triangle). NS5B is the RNA-dependent polymerase (red), and NS3 acts as helicase (purple). NS4B is a polytopic membrane protein inserted into the ER cotranslationally. NS4A, 5A, and 5B have hydrophobic domains (gray lines) that allow posttranslational insertion into the cytoplasmic face of the ER membrane. NS3 is recruited into the complex by associating with NS4A. (C) Alphavirus. The NSP1234 polyprotein is processed by a protease activity in the C-terminus of P2 (red triangle). The polyprotein is anchored to the cytoplasmic face of endosome and lysosome membranes

A. Regulation of membrane traffic in the early secretory pathway

Membrane rearrangements by the positive-stranded RNA viruses arise from modifications of membrane compartments in the early secretory pathway. The secretory pathway is carefully regulated in cells, and subversion of this pathway by viruses involves interactions between viral proteins and the host proteins that control membrane traffic. For some viruses we are beginning to understand how this is achieved. It is therefore useful to review briefly what is known about the control of membrane traffic at the start of the secretory pathway. Membrane proteins and proteins secreted by cells are synthesized by ribosomes attached to the cytoplasmic face of the endoplasmic reticulum (ER). Proteins destined for transport to the Golgi apparatus, or the plasma membrane, are folded by chaperones and assembled in the lumen of the ER, and transport to the Golgi apparatus and beyond involves a series of transport vesicles. The formation of these vesicles is controlled by coat proteins that are recruited from the cytosol. They select cargos for transport into the secretory pathway and facilitate vesicle formation by inducing membrane curvature (Bonifacino and Glick, 2004).

Movement from the ER involves a coat made from COPII proteins that localize to specific domains of the ER called ER exit sites (ERES), or transitional ER. Vesicle budding from ERES requires the small GTPase, Sar1p. Binding of GTP to Sar1p translocates Sar1p from the cytosol onto ER membranes. Here, Sar1p-GTP recruits cargo proteins into ERES and seeds polymerization of the COPII coat containing Sec13–Sec31p proteins and production of 60- to 80-nm-diameter vesicles. Movement of vesicles from the ER to the Golgi apparatus requires microtubules and the dynein/dynactin motor protein. The vesicles fuse with a series of

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by a hydrophobic region at the N-terminus of P1. P1 also acts as the methyltransferase (yellow). P2 encodes the helicase (purple) and P4 is the RdRp (red). The P123 precursor associates with P4 and generates negative-stranded RNA. Further processing produces a complex of separate P1, 2, 3, and 4 proteins that produce positive-stranded RNA.
(D) Nidoviruses. The Nidovirales order comprises the Arteriviridae, Coronaviridae, and Roniviridae families. The replicase gene is composed of two open reading frames termed ORF1a and ORF1b, both of which encode complex polyproteins. Arterivirus ORF1b encodes NSPs 9–12 including the RdRp (NSP9, red), helicase (NSP10, purple). The ORF1b reading frame lacks hydrophobic domains able to target the replicase to membranes. Proteins necessary for membrane targeting (brown and blue) are encoded by ORF1a (NSP2, 3, and 5). For the CoVs, for example, MHV and SARS-CoV transmembrane domains are located in NSP3, 4, and 6, and helicase and polymerase proteins are NSP12 and 13, respectively. ORF1b also contains a methyltransferase (NSP16, yellow).

membranes that lie between the ER and the Golgi apparatus called the *E*R-Golgi *i*ntermediate *c*ompartment (ERGIC), or tubulovesicular structures, and specific fusion with ERGIC membranes is determined by a complex of proteins called *t*ransport *p*rotein *p*article 1 (TRAPP1). TRAPP1 proteins tether the vesicles on ERGIC and Golgi membranes, allowing interactions between vesicle and target SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins to facilitate membrane fusion. The SNARE interactions are controlled by vesicle-specific small GTPases called rab proteins (Fig. 2).

Further sorting events in the ERGIC and early Golgi involve a second complex of coat proteins called COPI. The COPI complex contains seven proteins (α, β, β′, γ, δ, ε, and ζ COP proteins), which generate vesicles that take proteins from the ERGIC and Golgi apparatus back to the ER through a retrieval pathway (Fig. 2). The COPI proteins are recruited from the cytosol by the Arf1-GTPase. Activation of Arf1 requires binding to GTP and is facilitated by GTP exchange protein, Arf-GEF. Arf1-GTP inititates coat assembly while hydrolysis of GTP by Arf1 leads to coat disassembly. This disassembly is stimulated by an Arf1-GTP-activating protein (Arf-GAP) that promotes GTP hydrolysis by Arf1. A possible role for Arf1 in the generation of vesicles during picornavirus replication has been the focus of much work following the observation that *Poliovirus* replication is blocked by brefeldin-A (BFA), a drug that inhibits the recruitment of Arf1 onto membranes (Maynell *et al.*, 1992).

Membrane vesicles are also produced in cells in response to starvation. This pathway, known as autophagy, is used as a part of a quality control system that removes long-lived proteins and damaged organelles from the cytoplasm and has been shown to provide a defence against intracellular pathogens (Deretic, 2005; Kirkegaard *et al.*, 2004; Levine and Klionsky, 2004; Shintani and Klionsky, 2004). The origins of the membranes formed during autophagy are unclear but may be derived from the ER (Reggiori and Klionsky, 2005). Autophagy is suppressed by the *t*arget *o*f *r*apamycin (TOR) kinase and is activated by conditions that lead to inactivation of TOR. This leads to the production of membrane crescents in the cytoplasm, called isolation membranes, which mature into double-membraned vesicles of 500- to 1000-nm diameter called autophagosomes. This maturation engulfs small quantities of cytoplasm, and any organelles or pathogens present at sites of autophagy become trapped within autophagosomes. The autophagosomes ultimately fuse with lysosomes resulting in degradation of their content. Autophagosomes are of interest because infection of cells with picornaviruses and coronaviruses (CoVs) can generate double-membraned vesicles that may be related to autophagosomes.

In addition to supplying membrane and proteins to the secretory pathway, the ER acts as a major site of lipid synthesis. As a consequence, the ER contains a large quantity of membrane, and this is organized into a

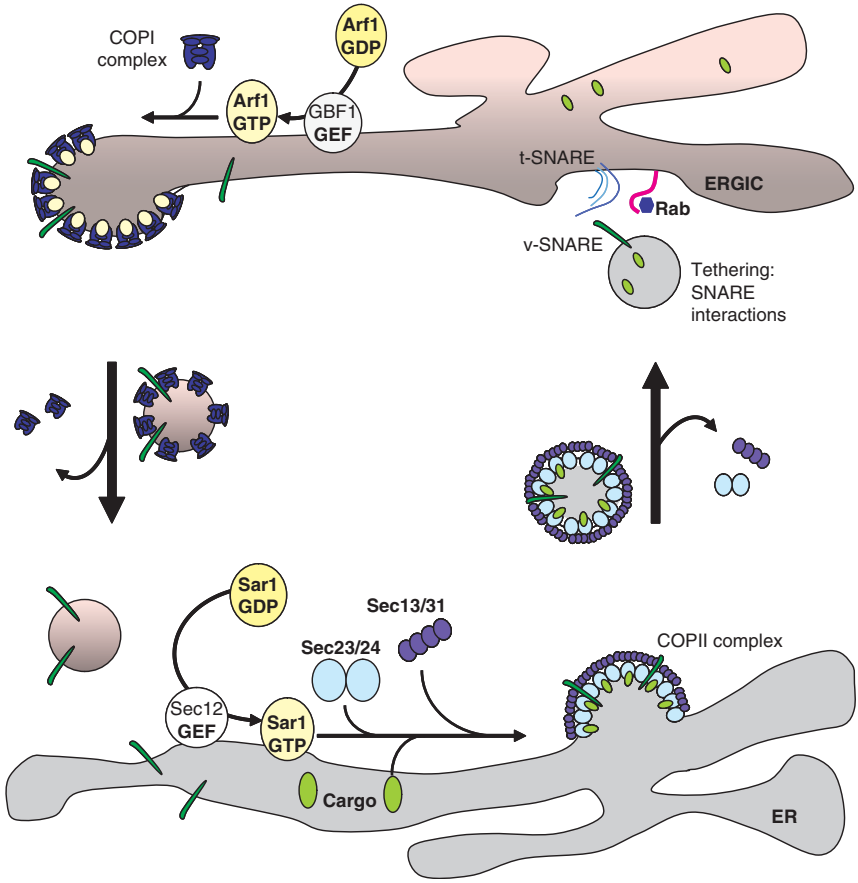

FIGURE 2 Protein trafficking in the early secretory pathway. 1. Anterograde transport from the ER to the ERGIC is mediated by COPII-coated vesicles. Formation of COPII coats is regulated by the Sar1p-GTPase. Binding of Sar1p to the ER requires binding of GTP and this is facilitated by the Sec12p-GTP exchange protein. Sar1p-GTP recruits the Sec23–Sec24p subcomplex (light blue) of the COPII coat and this recruits cargo proteins (light green) to ERES. The Sec23–Sec24p subcomplex then recruits the Sec13–Sec31p proteins (purple) that induce membrane curvature and formation of a vesicle. Hydrolysis of GTP on Sar1p by Sec23p results in coat disassembly. The vesicle docks with ERGIC membranes by binding tethering proteins and interactions between v-SNAREs and t-SNAREs results in vesicle fusion. 2. Retrograde transport from the ERGIC to the ER provides a pathway to retrieve proteins from the ERGIC and Golgi apparatus and is mediated by COPI-coated vesicles. Formation of COPI coats is regulated by the Arf1-GTPase. Binding of Arf1 to the ERGIC requires binding of GTP and this is facilitated by the GBF1 and BIG1/2 GTP exchange proteins. Arf1-GTP recruits the COPI coat complex (dark blue), which induces membrane curvature and formation of a vesicle that returns to the ER.

complex reticulum made from tubular and lamella structures (Borgese *et al.*, 2006). The smooth ER increases in response to a buildup of ER membrane proteins and can be organized into lamellae or concentric whorls called organized smooth ER (OSER). Structures similar to OSER are also seen during virus replication.

B. Picornavirus replication induces numerous membrane vesicles

1. The picornavirus replicase

Picornaviruses are nonenveloped positive-stranded RNA viruses. The genome encodes a large polyprotein that is processed to generate capsid proteins from the P1 region and nonstructural replicase proteins from the P2 and P3 regions. Picornavirus 3D contains the RdRp, while 2C has NTPase and helicase motifs. The 3D polymerase does not have membrane-targeting information but is synthesized as a 3ABCD precursor. 3ABCD is processed to 3AB by the 3C protease, and a hydrophobic domain in 3A targets 3AB to the cytoplasmic face of the ER. 3D binds directly to 3AB, and this targets the polymerase to the replication complex. The 3D polymerase of *Poliovirus* is believed to self-assemble into a large ordered array on membranes, which is critical for binding RNA and RNA elongation (Lyle *et al.*, 2002). The replication complex also requires 2BC and 2C proteins that are targeted to membranes via their own hydrophobic domains (Fig. 1A).

2. Membrane rearrangements induced by picornaviruses provide sites for replication

The accumulation of large numbers of densely packed membrane vesicles in the cytoplasm is characteristic of a picornavirus infection (Bienz *et al.*, 1983, 1987; Cho *et al.*, 1994; Dales *et al.*, 1965a; Schlegel *et al.*, 1996; Stuart and Fogh, 1961; Suhy *et al.*, 2000). Studies have suggested that vesicles induced by *Poliovirus* are derived from the ER, either from COPII-coated vesicles or from ER-derived autophagic double-membraned vacuoles (Bienz *et al.*, 1987; Jackson *et al.*, 2005; Rust *et al.*, 2001; Schlegel *et al.*, 1996; Suhy *et al.*, 2000). However, the detection of ER, Golgi, and lysosomal markers in membranes induced at later stages of infection by *Poliovirus* suggests that more than one organelle may contribute membranes to the replication complex (Schlegel *et al.*, 1996). In interpreting these studies, it is important to consider if the vesicles observed are involved in replication, or if they represent a bystander response to virus infection. Evidence for a role of specific membranes in replication is provided by the presence of replicase proteins, or better still dsRNA or negative-stranded intermediate viral RNA (Egger and Bienz, 2005). Examination of cells infected with *Poliovirus* for the first appearance of

negative-stranded RNA suggests that this initial stage of replication starts on the ER. This is consistent with high-resolution immunofluorescence microscopy (Rust *et al.*, 2001) showing the *Poliovirus* 2B protein associated with ERES containing the Sec13–Sec31p proteins of the COPII complex. These sites exclude resident ER proteins, suggesting colocalization of 2B with COPII-coated transport vesicles. Replication complexes containing negative-stranded RNA then move on microtubules to a perinuclear area to initiate synthesis of positive-stranded RNA (Egger and Bienz, 2005).

3. Membrane rearrangements can be induced by expression of nonstructural proteins

Membrane rearrangements have been studied by expressing individual, or combinations, of picornavirus proteins in cells. Most of this work has involved studies of *Poliovirus* proteins, and membrane rearrangements are reported for the 2B, 2C, 2BC, 3A, and 3AB proteins. *Poliovirus* 2B causes fragmentation of the Golgi complex (Sandoval and Carrasco, 1997). The 2BC and 2C proteins lead to vesiculation and tubulation and sometimes myelin-like swirls of ER-derived membranes (Aldabe *et al.*, 1996; Cho *et al.*, 1994). Similar structures are induced by 2C and 2BC of hepatitis A virus (Teterina *et al.*, 1997). Expression of the *Poliovirus* 3A protein causes swelling of ER cisternae (Doedens *et al.*, 1997) and the disappearance of vesicles budding from the ER, while the 3AB protein also induces myelin-like swirls of ER (Egger *et al.*, 2000). The membrane rearrangements induced by expression of single proteins do not, however, mirror those observed in infected cells, and since myelin-like modifications to the ER are also seen following overexpression of ER proteins [reviewed by Borgese *et al.* (2006)], their relevance to viral replication is unclear. Importantly for *Poliovirus*, it is a combination of 2BC and 3A protein expression that induces membrane structures morphologically similar to those seen in infected cells (Suhy *et al.*, 2000).

4. Membrane rearrangements may vary between different picornavirus families

Gazina *et al.* (2002) have studied replication complexes formed by several different picornaviruses. *Encephalomyocarditis virus* (EMCV), parechovirus 1, and echovirus 11 induce clustered vesicles containing dsRNA in the perinuclear region of the cell. The precise nature of the vesicles varied with virus. Parechovirus 1 produced homogeneous vesicles of 70–100 nm, while membranes produced by EMCV and echovirus 11 were heterogeneous but more compact and associated with electron-dense material. Differences for parechovirus 1 have also been reported by Krogerus *et al.* (2003) who suggest that replication may occur on membranes derived from the late Golgi rather than early ER and ERGIC compartments. All three viruses, however, cause loss of ribosomes from the

ER and lack of visible Golgi apparatus. The COPI coat protein β-COP was found to colocalize with echovirus 11 replication complexes, but not with replication complexes produced by EMCV, again suggesting that vesicles produced by different picornaviruses may differ. Infection with *Foot-and-mouth disease virus* (FMDV) also results in loss of ribosomes from the ER and an accumulation of heterogeneous vesicles to one side of the nucleus (Monaghan *et al*., 2004).

High-pressure freezing can be used to increase the preservation of cellular ultrastructure during processing for electron microscopy. Such analysis of cells infected with *Poliovirus* shows that the vesicles have two membranes suggestive of autophagosomes (Jackson *et al*., 2005; Suhy *et al*., 2000). Double-membraned structures containing electron-dense material, and possibly viruses, were also revealed by the early work on *Poliovirus* (Dales *et al*., 1965a). High-pressure freezing has been used to compare FMDV and *Bovine enterovirus* (BEV). BEV produced heterogeneous membrane clusters similar to the rosettes described for *Poliovirus* (Egger *et al*., 1996). Many of the vesicle membranes have high electron density suggestive of double membranes and lie adjacent to accumulations of virus-like particles. Clusters of FMD viruses were also associated with vesicles and electron-dense material, but there were fewer double-membraned vesicles (Monaghan *et al*., 2004). Immunofluorescence analysis of *Poliovirus* vesicles shows colocalization of replicase protein 3A and autophagy marker LC3, suggesting assembly of the replicase on autophagosomes. Similar work suggesting the use of autophagosomes during replication of CoVs will be described below. For *Poliovirus*, expression of 3A and 2BC, which produces vesicles similar to those seen in infected cells (Suhy *et al*., 2000), can induce autophagy (Jackson *et al*., 2005), and inhibition of autophagy reduces yields of extracellular virus. The results suggest that the autophagy pathway may facilitate the release of *Poliovirus* from cells, and it will be interesting to see if this is true for other enteroviruses that are resistant to the low pH and proteases present in lysosomes and autophagosomes.

5. Vesicle coat proteins may play a role during picornavirus replication

Evidence that different members of the picornavirus family vary in the way that they interact with host membranes is provided by studies of virus sensitivity to BFA. BFA completely inhibits *Poliovirus* and echovirus 11 replication (Cuconati *et al*., 1998; Gazina *et al*., 2002; Irurzun *et al*., 1992; Maynell *et al*., 1992) and partially inhibits parechovirus 1 replication (Gazina *et al*., 2002) but not other picornaviruses such as EMCV (Gazina *et al*., 2002) or FMDV (Monaghan *et al*., 2004; O'Donnell *et al*., 2001). BFA prevents assembly of COPI coats and this has generated considerable interest in understanding how COPI and COPII coats contribute to

formation of the replication complex, and how BFA inhibits picornavirus replication. In cells infected with the highly BFA-sensitive virus echovirus 11, β-COP was recruited into the replication complex; in contrast, the replication complex formed by the BFA-resistant EMCV did not contain β-COP. This correlation suggests that BFA-sensitive viruses may require COPI coats for replication (Gazina *et al.*, 2002; Mackenzie, 2005). Since COPII coats are resistant to BFA (Lippincott-Schwartz *et al.*, 2000; Orci *et al.*, 1993; Ward *et al.*, 2001), it is suggested that COPII coats may provide the membranes for replication complexes formed by BFA-insensitive viruses. The observation that *Poliovirus* replicase 2B protein is seen in ERES containing COPII proteins, but *Poliovirus* is sensitive to BFA, can be reconciled if this association of 2B with ERES is considered to be an early step in generation of membrane for the replication complex that precedes recruitment of COPI coat proteins. This is supported by work showing the movement of *Poliovirus* replication complexes containing negative-stranded RNA from the ER to perinuclear sites (Egger and Bienz, 2005).

Direct evidence that COPI coat proteins are required for picornavirus replication comes from studies of *Drosophila C virus* (DCV). DCV is a positive-stranded RNA dicistronic virus that is similar to *Poliovirus* and replicates in a cytoplasmic compartment containing virus-induced membrane vesicles. A genome-wide RNA silencing screen identified six (α, β, β′, γ, δ, and ζ) of the seven COPI coat proteins as essential for virus replication. Furthermore, the formation of virus-induced vesicles required β-COP, but not COPII protein, Sec23p. Notably, small interfering RNAs against α-COP, but not Sec23p, also slowed *Poliovirus* replication (Cherry *et al.*, 2006).

6. Arf proteins and Brefeldin-A can modulate poliovirus and coxsackievirus replication

The formation of COPI-coated vesicles is regulated by the Arf1-GTPase. The observation that BFA inhibits the replication of enteroviruses such as *Poliovirus*, and also inhibits the function of the Arf1-GTPase, provides a second link between virus replication and COPI coats. Arf proteins are regulated by Arf-GEFs that facilitate binding of GTP by removing GDP, and by Arf-GAPs that increase hydrolysis of GTP by Arfs. Arf1-GEFs are inhibited by BFA, and BFA therefore reduces levels of Arf1-GTP in cells. The GEFs affected by picornavirus infection are *G*olgi-associated *BF*A-resistant protein (GBF1) and *B*FA-*i*nhibited protein (BIG1/2). Work by Belov *et al.* (2005, 2007) indicates that infection of cells with *Poliovirus* increases intracellular Arf-GTP levels fourfold, suggesting increased activity of Arf1-GEFs or inhibition of Arf1-GAP proteins. In the absence of virus, Arf1 is concentrated in the Golgi apparatus, but during infection with *Poliovirus* Arf1 staining fragments and colocalizes with replicase

protein 2C. This suggests that infection leads to a redistribution of Arf proteins from the Golgi apparatus to the replication complex. The binding of Arf proteins to membranes is dynamic, with Arf-GDP being released from membranes following hydrolysis of GTP. Cytosolic Arf1-GDP would redistribute naturally to membranes enriched for the Arf1-GEFs that facilitate loading of new GTP. Significantly, *Poliovirus* infection causes enrichment of GEFs in membranes containing replicase proteins, and this would provide a mechanism for increasing levels of Arf1-GTP at sites of virus replication.

Translation of *Poliovirus* RNA on membranes *in vitro* provides an alternative means of studying the role of Arf proteins in virus replication. Replication is inhibited by BFA and peptides that function as competitive inhibitors of Arf (Cuconati *et al.*, 1998), and for the most part, the assay mimics what is observed in infected cells. Translation *in vitro* leads to recruitment of Arf3 and Arf5 but not Arf6 (Belov *et al.*, 2007) onto membranes. Suitable antibodies recognizing the ER-associated Arf1 were not available for these experiments, so it is not known if Arf1 is also recruited to membranes during translation. Membrane recruitment of Arf proteins can be reconstituted by translation and expression of *Poliovirus* 3A or 3CD. *Poliovirus* proteins do not show intrinsic GEF activity, but 3A and 3CD will induce association of GBF1 and BIG1/2, respectively, with membranes *in vitro*. This raises the possibility that recruitment of 3A and 3CD to the replication complex during infection targets Arf-GEF to virus-induced membranes, which in turn increases local levels of Arf1-GTP. This is thought to be necessary for replication because inhibition of Arf1-GEF by BFA blocks replication, and replication can be rescued by overexpression of GBF1 (Belov *et al.*, 2007). High levels of Arf1-GTP would also increase recruitment of COPI proteins and be consistent with the work on DCV showing that COPI proteins are required for replication and vesicle production (Cherry *et al.*, 2006). A *Poliovirus* 3A mutant with a serine insertion at position 16 is unable to cause translocation of Arf to membranes (Belov *et al.*, 2005). *Poliovirus* carrying the 3A mutation does not, however, show defects in replication, suggesting that Arf1-GEF recruitment to membranes by 3A is not essential for replication. It is possible that during infection the defect in 3A is compensated for by 3CD. Interestingly, a BFA-insensitive *Poliovirus* with mutations in the 2C and 3A proteins (Crotty *et al.*, 2004) induces vesicles and dispersal of the Golgi apparatus, which begs the questions, does this mutant use a different process for forming the replication complex, or do the mutations in 3A allow the proteins to compete with BFA for GBF1 recruitment?

The role of Arf proteins during coxsackievirus infection has also been studied. In common with *Poliovirus*, coxsackieviruses are enteroviruses and their replication is inhibited by BFA. Expression of coxsackievirus 3A causes loss of COPII coats from ERES, and an accumulation of 3A,

COPII and a model secreted protein in both the ER, and tubular-vesicular post-ER structures containing ERGIC marker proteins. These effects closely resemble the effects of adding BFA to cells, suggesting coxsackievirus 3A may affect the function of Arf proteins. Coxsackievirus 3A affects the regulation of Arf proteins (Wessels *et al.*, 2006b). Interestingly, the process differs to that described by Belov *et al.* (2005, 2007) for *Poliovirus* 3A translated *in vitro*. Expression of coxsackievirus 3A in cells caused loss of COPI and Arf1 from membranes, and there was redistribution of BIG1/2 and GBF1 from the Golgi apparatus into the cytoplasm. This suggests that coxsackievirus 3A reduces, rather than enhances, levels of Arf1-GTP. Coxsackievirus 3A also caused redistribution of Arf1-GAP to punctate structures suggestive of the ERGIC. A block in Arf1-GEF activity, combined with recruitment of Arf1-GAP, would reduce the levels of Arf-GTP and inhibit membrane recruitment of COPI. Wessels *et al.* (2006a) examined the effects of the 3A proteins of other picornaviruses and found that only the 3A proteins of enteroviruses bound GEFs. Intriguingly, Wessels' work contrasts with Belov in that they found the interaction of 3A with GEFs lead to a loss of Arf proteins from membranes. Why these differences are seen is, as yet, unknown but may be due to differences in cell type/methods used or differences in levels of 3A protein expression.

7. Picornavirus replication blocks protein secretion

Poliovirus and coxsackievirus slow protein movement through the secretory pathway (Doedens and Kirkegaard, 1995; Wessels *et al.*, 2005). Expression of 2B, 2BC, and 3A individually were all able to slow secretion (Cornell *et al.*, 2006; Doedens and Kirkegaard, 1995; Doedens *et al.*, 1997; van Kuppeveld *et al.*, 1997; Wessels *et al.*, 2005, 2006a), but for both viruses the 3A protein was found to have the greatest impact on ER-to-Golgi transport. *Poliovirus* infection, and the 3A protein expressed alone in cells, reduces surface expression of MHC class I, the TNF receptor, and secretion of β-IFN, IL-6, and IL-8 (Choe *et al.*, 2005; Deitz *et al.*, 2000; Dodd *et al.*, 2001; Neznanov *et al.*, 2001), and this may offer an immune evasion strategy to the picornaviruses. This is consistent with the observation that the ability of the coxsackievirus 3A protein to slow secretion may be important for virulence (Wessels *et al.*, 2006b) and has led to studies of the mechanism of action of 3A in blocking ER-to-Golgi transport.

Deletion analysis has identified residues in the unstructured N-terminal region of *Poliovirus* and coxsackievirus 3A as important for the block in host protein secretion (Choe *et al.*, 2005). An N-terminal proline-rich region (particularly Pro19) is important for coxsackievirus block in trafficking (Wessels *et al.*, 2005). In *Poliovirus*, Lys9 appears important, and in the triple-proline motif (positions 16–18), only the Pro18 is indispensable for inhibition of protein secretion (Choe *et al.*, 2005). A serine insertion in 3A protein between Thr14 and Ser15, creating the 3A-2 mutant virus

(Berstein and Baltimore, 1988), was found to abolish the ER-to-Golgi inhibition of protein trafficking but has little effect on virus replication or membrane rearrangements (Dodd *et al.*, 2001; Doedens *et al.*, 1997). This important observation shows that the ability of 3A to inhibit protein secretion is separate from its role in membrane rearrangements and viral replication.

There is continuing interest in understanding how picornavirus proteins block secretion. *Poliovirus* 3A and 3CD, and coxsackievirus 3A, can interact with Arf-GEF, but the downstream events are unclear. The recruitment of Arf-GEF by *Poliovirus* 3A and 3CD would increase recruitment of Arf-GTP to membranes of the replication complex. This would increase recruitment of COPI coat proteins into sites of virus replication and reduce the pool of COPI proteins available to the ERGIC and Golgi apparatus. Alternatively, inhibition of Arf-GEF and recruitment of Arf-GAP onto ERGIC membranes by enterovirus 3A would decrease membrane association of Arf-GTP and again reduce recruitment of COPI onto ERGIC and Golgi membranes. Both mechanisms would reduce the formation of COPI vesicles, and as seen for BFA, block secretion. *Poliovirus* 3A also binds and inactivates L1S1, a component of the dynein–dynactin motor complex (Kondratova *et al.*, 2005), which is required to move COPII-derived vesicles from ERES to the ERGIC. As seen for expression of 3A, mutant L1S1 leads to disruption of the ER-to-Golgi traffic and reduction in plasma membrane receptors such as TNF receptor. It is possible that 3A may also slow ER-to-Golgi transport by binding L1S1.

a. Picornaviruses differ in the use of nonstructural proteins to block secretion

The ability of 3A to inhibit ER-to-Golgi trafficking has not been conserved in all picornaviruses (Choe *et al.*, 2005; Cornell *et al.*, 2006; Deitz *et al.*, 2000; Moffat *et al.*, 2005). For example FMDV infection leads to reduced surface expression of MHC class I (Sanz-Parra *et al.*, 1998), but the FMDV 3A protein does not inhibit ER-to-Golgi transport (Moffat *et al.*, 2005). A lack of inhibition of secretion has also been reported for 3A proteins of human rhinovirus, hepatitis A, Theiler's virus, human enterovirus, and EMCV (Choe *et al.*, 2005; Wessels *et al.*, 2006a). The 3A protein of human rhinovirus is unable to bind GBF1, or inhibit COPI recruitment to membranes, and this may explain its inability to slow secretion. Importantly, studies on FMDV have shown that the 2BC protein, or a combination of the processed products, 2B and 2C, inhibits protein movement from the ER to the Golgi apparatus (Moffat *et al.*, 2005, 2007), and this may be similar for other picornaviruses with 3A proteins that do not block ER-to-Golgi transport.

A lack of effect of FMDV 3A on secretion does not result from an inability to bind membranes. FMDV 3A is recovered from postnuclear membrane fractions, and when expressed alone in cells it colocalizes with resident ER proteins. In common with 3A, picornavirus 2B, 2C, and 2BC proteins also contain membrane-binding sequences. Sequence alignment

of the 2B, 2C (2BC), and 3A proteins of different picornaviruses showed a high level of conservation between the 2C proteins, which contain an NTP-binding site and predicted helicase motifs (Gorbalenya *et al.*, 1990) but large variations in the sequences of the 2B and 3A proteins (Choe *et al.*, 2005; Moffat *et al.*, 2005), and these may explain their different abilities to block secretion. The FMDV 3A protein is, for example, much longer than 3A of enteroviruses, such as *Poliovirus*, and it does not contain the N-terminal sequences thought important for *Poliovirus* 3A to block the secretory pathway.

The 2B protein of FMDV also locates to ER membranes but shows a more reticular pattern than the FMDV 3A protein (Moffat *et al.*, 2005) and can be seen in punctate structures aligned along the ER suggestive of ERES (Fig. 3). This is similar to the 2B of *Poliovirus* that colocates with both

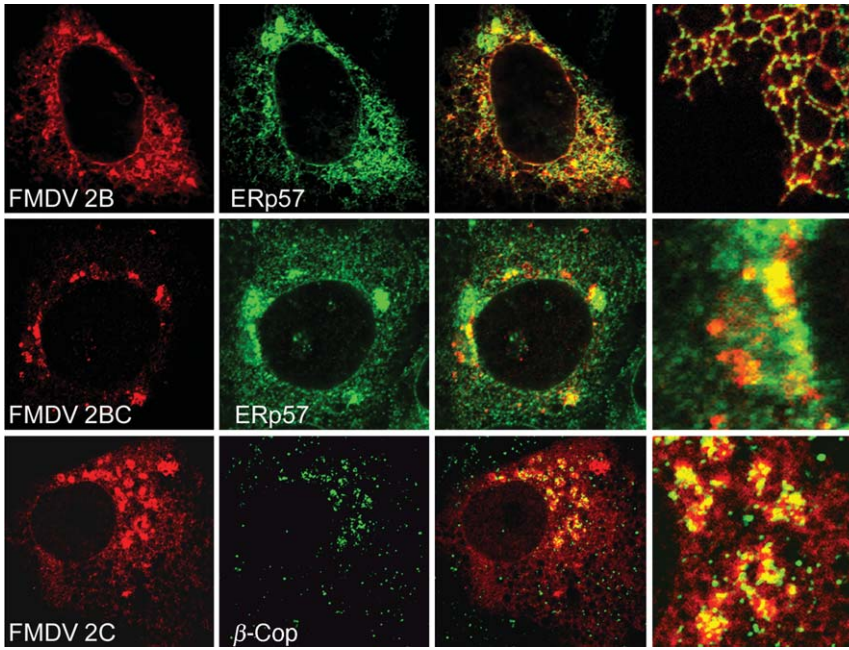

FIGURE 3 Subcellular location of Foot-and-mouth disease NSP encoded in the P2 region of the FMDV genome. Vero cells expressing FMDV 2B (top), 2BC (middle), or 2C (bottom) were fixed and permeabilized and processed for immunofluorescence. 2C and 2BC were located using antibodies specific for 2C (3F7) and 2B was located using an antibody raised against an epitope tag in 2B. Cells were counterstained using antibodies against ER luminal protein ERP57 (top and middle panels), or COPI protein β-COP (bottom). Merged images are shown at higher magnification on the far left. See Moffat *et al.* (2005) for more details. Reprinted from Moffat *et al.* (2005) with permission from American Society for Microbiology.

Sec13p and Sec31p of the COPII coat. As expected, FMDV 2C is also membrane associated. When expressed in cells, 2C produces faint ER staining, but mainly locates to bright punctate structures in a perinuclear region close to β-COP, reminiscent of Golgi staining. The β-COP staining is, however, fragmented suggesting dispersal of the Golgi apparatus, and there is not complete colocalization since 2C structures negative for β-COP protein can also be seen (Moffat *et al.*, 2007). A similar location of FMDV NSP within the area of the cell occupied by the Golgi apparatus is seen in cells infected with FMDV, and again they do not colocalize with Golgi markers (Knox *et al.*, 2005). The 2BC protein of FMDV is also recovered in postnuclear membrane fractions, but when expressed in cells, 2BC staining differs from that seen for the processed products, 2B and 2C (Fig. 3). FMDV 2BC locates to punctate cytoplasmic structures and larger structures surrounding the nucleus that contain ER markers suggesting swelling of the ER. 2BC shows partial overlap with luminal ER markers but, unlike *Poliovirus* 2BC, does not colocate with the COPII marker Sec13p. The ER markers also appeared punctate in cells expressing 2BC, suggesting disruption of the ER (Moffat *et al.*, 2005). Interestingly, coexpression of 2B and 2C blocks secretion within post-ER compartments, similar to those containing 2C. The site of block therefore seems to be determined by the subcellular location of 2C (Moffat *et al.*, 2007) and is consistent with the observation that the block in the presence of 2B can be redirected to the ER, if 2C is tethered to the ER by an ER retention sequence.

C. Alphaviruses produce membrane invaginations and spherules

Sindbis virus (SbV) and *Semliki Forest virus* (SFV) are the best studied examples of alphavirus replication in mammalian cells [reviewed by Salonen *et al.* (2005)]. Early electron microscopy studies showed that vesicular structures called cytopathic vacuoles between 600- and 2000-nm diameter, accumulated in infected cells. The vacuoles contained 50-nm-diameter vesicles called spherules, many of which were aligned along the inside face of the vacuole and attached by a neck to the limiting membrane. The neck was often seen connected to an electron-dense matrix extending into the cytoplasm. The observation that the cytopathic vacuoles contained NSPs required for RNA replication, cofractionated with lysosomal enzymes, and could be labeled with endocytic markers (Froshauer *et al.*, 1988), led to the conclusion that they are sites of viral replication derived from endosomes and lysosomes. In many cases, the vacuoles were also connected to the rough ER by filaments and granular material containing the RNA polymerase.

1. The alphavirus replicase is located Within invaginations in cellular membranes

Alphavirus NSPs are synthesized in the cytoplasm and bind to endosomes and lysosomes to generate a replication complex. The replicase proteins are synthesized as a polyprotein (P1234). The P4 domain is the RdRp while P2 has NTPase and helicase activities, and P1 is the methytransferase required to cap RNA (Fig. 1C). The P1234 polyprotein locates to endosome or lysosome membranes via an amphipathic peptide sequence in P1 (Salonen *et al.*, 2003). At this stage the P4 polymerase is cleaved from the polyprotein and functions with the remaining P123 protein to generate negative-stranded RNA. Interestingly, once the P123 is processed to individual NSPs, the polymerase preferentially produces positive-stranded RNA. Expression of individual NSPs does not lead to the formation of a cytopathic vacuoles or spherules. Formation of spherules requires interactions between NSP P1, P3, and P4 and the P123 polyprotein precursor complex (Salonen *et al.*, 2003).

Rubella virus is a member of the Togaviridae family within the *Alphavirus* genus. Cells infected with *Rubella virus* also contain vacuoles containing spherules and these colocalize with lysosomal markers, suggesting use of lysosomes for replication. A fibrous material connects the vacuoles to the ER (Lee *et al.*, 1994; Magliano *et al.*, 1998), again suggesting strong similarities with SFV and SbV. Members of the alphavirus superfamily share homologies between proteins required for RNA replication, and this extends to plant viruses. *Alfalfa mosaic virus* replicase proteins colocalize with the plant vacuole (van der Heijden *et al.*, 2001), and *Turnip yellow mosaic virus* uses the chloroplast outer envelope as a site for replication. Replication of *Tobacco mosaic virus*, a tobamovirus, is dependent on *Arabdopsis* proteins TOM1 and TOM2A that are integral membrane proteins of the tonoplast (Hagiwara *et al.*, 2003). The tonoplast is a membrane compartment within plants that surrounds the vacuole/lysosome, suggesting plant alphaviruses also use the endosome/lysosome system as a site of replication. Infection of plants with alphavirus-like superfamily viruses can also induce the formation of spherules (Prod'homme *et al.*, 2001). There is evidence that *Tobacco mosaic virus* also uses the ER as a site of replication because the replicase enzyme and viral RNA are located on the ER of infected cells, and infection causes major changes in ER morphology (Reichel and Beachy, 1998), including ER aggregation and formation of lamella structures.

Flock house virus replicates in spherules in the outer membrane of mitochondria. The RNA polymerase (protein A) of *Flock house virus* is the only protein required for RNA replication and is targeted directly to the mitochondrial outer membrane by hydrophobic amino acids at the N-terminus. This sequence contains a mitochondrial localization signal and transmembrane domain that leaves the bulk of the protein exposed to

the cytoplasm (Miller and Ahlquist, 2002). *Brome mosaic virus* replicates in yeast and has been studied extensively. The 1a and 2a replicase proteins are produced from separate viral RNAs. The 1a protein contains a C-terminal helicase domain and an N-terminus required for RNA capping. 1a is targeted to the cytoplasmic face of ER membranes and recruits the 2a polymerase to the replication complex (Schwartz *et al.*, 2002). Importantly, replication of *Brome mosaic virus* on the cytoplasmic face of the ER in yeast induces membrane invaginations of 50 nm that are very similar to the spherules produced in endosomes and lysosomes during alphavirus infection of mammalian cells.

2. Membrane invaginations and spherules induced by alphviruses share similarity with virus budding

It has been suggested that the active formation of spherules to separate viral RNA from host responses is analogous to the coordinated assembly of viral proteins, which leads to capsid assembly, genome packaging, and budding (Ahlquist, 2006; Schwartz *et al.*, 2002). The *Brome mosaic virus* replication complex contains viral 1a and $2a^{Pol}$ proteins within spherules. Expression of 1a alone produces a shell containing hundreds of copies of 1a on the inside of 50-nm spherules. In a capsid assembly model (Schwartz *et al.*, 2002), vesicles of uniform size would arise if the 1a protein first made a planar lattice with hexameric symmetry on membranes and achieved curvature by localized rearrangement of 1a into pentamers. Interestingly, the formation of spherules is dependent on the relative levels of 1a and $2a^{Pol}$. When levels of $2a^{Pol}$ are high, the spherules are lost, and 1a and $2a^{Pol}$ assemble into flat lamella structures associated with the ER (Schwartz *et al.*, 2004). One explanation for a failure to achieve curvature is that high levels of $2a^{Pol}$ may interfere with this hexamer to pentamer transition. This is supported by the observation that when domains that allow association of 1a and $2a^{Pol}$ are deleted, the $2a^{Pol}$ is unable to alter the structure of spherules formed by 1a. The correct ratio of 1a and $2a^{Pol}$ is clearly important for replication complex assembly and may be maintained during infection through inhibition of translation initiation of the 2a RNA.

D. The Flaviviridae replicate in vesicular packets and membraneous webs

1. The Flavivirus Replicase

In the Flaviviridae family, which includes the *Flavivirus*, *Pestivirus*, and *Hepacivirus* genera, the RNA genome encodes a polyprotein precursor that is cleaved by viral proteases to produce structural proteins from the N-terminal region. The replicase of the Flaviviridae is made from NSPs, NS5A, NS5B, NS4B, and NS3–4A, found at the C-terminus.

With the exception of the polytopic NS4B membrane protein, which is inserted cotranslationally into the ER, the membrane-anchored components of the complex are inserted into the cytoplasmic face of the ER after translation (Fig. 1B). The NS5B is the RdRp, and a C-terminal stretch of 21 hydrophobic amino acids directs NS5B to the cytoplasmic face of the ER (Dubuisson *et al.*, 2002; Moradpour *et al.*, 2004). The NS3 protein has NTPase/helicase activity. NS3 is not a membrane protein but is recruited to the complex through association with membrane-anchored NS4A. NS5A is also membrane associated, and association is mediated via 31 amino acids at the N-terminus that form an amphipathic α-helix (Brass *et al.*, 2002; Elazar *et al.*, 2003).

2. Membranes used for flavivirus replication are provided by the *trans*-Golgi network

Replication of flaviviruses (e.g., Dengue, West Nile, and Yellow Fever viruses) takes place in membrane invaginations. For historical reasons, these are called vesicular packets [reviewed in Mackenzie (2005)]. They are larger (80- to 100-nm diameter) than the 50-nm alphavirus spherules, and form from the limiting membrane of the *trans*-Golgi network (TGN) (Uchil and Satchidanandam, 2003; Westaway *et al.*, 1997b). Infection by Kunjin virus leads to unique membrane structures thought to be derived from both the early and late secretory pathways. These include convoluted membranes and paracrystalline arrays derived from the rough ER and ERGIC, and vesicle packets derived from the TGN (Mackenzie *et al.*, 1999; Ng, 1987; Roosendaal *et al.*, 2006; Westaway *et al.*, 1997b). The detection of dsRNA and viral NSPs (NS1, NS2A, NS3, and NS4A) within the vesicle packets points strongly to this being the site of RNA replication (Mackenzie *et al.*, 1998; Westaway *et al.*, 1997b). The vesicle packets associate closely with the convoluted membranes and paracrystalline arrays, which are thought to be the sites of proteolytic processing of NS3 and NS2B (Westaway *et al.*, 1997b). These modified membranes are linked with the ER, and ultrastructural studies have shown virions present in the ER, cytoplasmic vesicles, Golgi cisternae, and vacuoles. The results suggest that membranes containing the spherules responsible for replication may become associated with the ER to facilitate delivery of genomes to viruses, budding into early compartments of the secretory pathway (Mackenzie and Westaway, 2001).

3. Hepacivirus replication occurs in association with the ER

Hepatitis C virus (HCV) is closely related to the flaviviruses, and its importance as a human pathogen has generated great interest in its mechanism of replication. Until, recently infection models have not been available to study the replication complex of HCV, and the studies discussed here have focussed on the expression of the entire polyprotein from replicons (Egger

et al., 2002; Gosert *et al.*, 2003). However, the recent production of a HCV that replicates efficiently both *in vivo* and in cell culture (Lindenbach *et al.*, 2006; Wakita *et al.*, 2005; Zhong *et al.*, 2005) will expand the possibilities for studying and understanding the viral replication cycle. HCV replication is thought to occur on membranes derived from the ER as all studies of NSPs have found them localized to this organelle (Dubuisson *et al.*, 2002; Hugle *et al.*, 2001; Kim *et al.*, 1999; Wolk *et al.*, 2000). Studies have also identified a ''membraneous web'' of membrane vesicles of ~85-nm diameter associated with the ER and a population of irregular double-membraned vesicles. The web resembled the ''sponge-like inclusions'' seen in the liver of chimpanzees infected with HCV, suggesting it is physiologically relevant. Interestingly, the great majority of the NSP synthesized by full–length genomes or subgenomic replicons may not be involved in RNA replication (Quinkert *et al.*, 2005). The bulk of the NSPs associated with membranes isolated from cells expressing replicons is sensitive to protease, while *in vitro* replicase activity is resistant to protease and nuclease activity (El-Hage and Luo, 2003; Quinkert *et al.*, 2005). The results suggest that replication of HCV takes place within membrane vesicles, rather than on the surface of the membraneous web. These vesicles may be associated with the membraneous web, but the similarity between HCV and the flaviviruses leaves open the possibility that the membrane invaginations responsible for replication may also form in the TGN but be closely associated with the ER.

4. Flavivirus nonstructural proteins can induce membrane rearrangements

Studies have investigated which viral proteins are responsible for membrane rearrangements seen in cells infected with flaviviruses. The NS4A of Kunjin virus induces the characteristic convoluted membranes and paracrystalline arrays seen in flavivirus infections. The NS4A-B protein also causes membrane rearrangement, but the highly condensed structures seen in infected cells are not produced until the NS2B-3 protease cleaves NS4A free from NS4B (Roosendaal *et al.*, 2006). The NS4B then translocates to the nucleus (Westaway *et al.*, 1997a). Interestingly, this contrasts with HCV where NS4B (and NS4A-B) (Egger *et al.*, 2002; Konan *et al.*, 2003) rather than NS4A is able to induce the membranous structures.

5. Flaviviruses can modulate the secretory pathway

Flaviviruses have been found to upregulate cell surface expression of MHC class I and II in response to interferon (King and Kesson, 1988; Liu *et al.*, 1989; Lobigs *et al.*, 2004). This is not caused by effects of the NS4A or NS4B proteins on membrane traffic; instead flavivirus infection increases expression of the ER peptide transporter, TAP1. This increases the supply of peptides that are necessary for the folding and export of

newly synthesized MHC proteins from the ER. Increased TAP expression is mediated by increased transcriptional activity of p53 and can be induced in liver HepG2 cells by expression of the HCV core/capsid protein alone (Herzer *et al.*, 2003; Momburg *et al.*, 2001).

While the capsid/core protein is able to increase cell surface expression of MHC class I through increase expression of TAP1, expression of the HCV polyprotein has been shown to slow the movement of proteins through the secretory pathway of host cells (Konan *et al.*, 2003). The rate of delivery of MHC class I to the plasma membrane in cells infected with HCV was reduced three- to fivefold relative to cured control cells. Expression of the precursor NS4A-B was found to reduce ER-to-Golgi traffic two- to threefold (Konan *et al.*, 2003), while the other NS proteins of HCV including NS4A and NS4B, individually or combined, were unable to interfere with the trafficking pathway. NS4B alone induces a membraneous web in cells (Egger *et al.*, 2002), and both NS4A-B and NS4B induce, and locate to, clustered and aggregated membranes looking very similar to the membraneous web seen in cells expressing replicons. In addition to aggregated membranes, NS4A/B also induces, but does not colocalize with, swollen vesicular structures. Theses swollen vesicles have a similar morphology to the vesicles induced by the 3A protein of *Poliovirus*, which swells ER membranes and blocks secretion between the ER and the Golgi apparatus (Doedens *et al.*, 1997). Konan *et al.* (2003) hypothesize that the NS4A/B could be functioning in a similar manner to *Poliovirus* 3A.

E. The Nidovirales replicate in association with double-membraned vesicles

1. The Nidovirus replicase is generated from two polyproteins

The Nidovirales order comprises the Arteriviridae, Coronaviridae, and Roniviridae families. The replicase gene is composed of two open reading frames termed ORF1a and ORF1b. ORF1b is generated from a frameshift in 1a, and both reading frames encode complex polyproteins processed by viral proteases (Gorbalenya *et al.*, 2006; Ziebuhr, 2006). The arterivirus ORF1b encodes NSPs 9–12, including the RdRp (NSP9) and helicase (NSP10). The ORF1b, however, lacks hydrophobic domains able to target the replicase to membranes. Interestingly, the hydrophobic domains necessary for membrane targeting are encoded by ORF1a in NSP2, 3, and 5, suggesting that ORF1a proteins produce a scaffold to locate the viral replication–transcription complex to membranes (Fig. 1D) (Pedersen *et al.*, 1999; van der Meer *et al.*, 1998). A similar strategy is used by CoV, for example mouse hepatitis virus (MHV) and severe acute respiratory syndrome-CoV (SARS-CoV) (Prentice *et al.*, 2004a,b), where transmembrane domains are located in NSP3, 4, and 6, and helicase and polymerase proteins are NSP12 and 13, respectively, and NSP16 encodes the methytransferase. The Nidovirales have the largest coding capacity of the single-stranded

RNA viruses, and not all the 16 NSPs have been studied in detail. It is possible that other proteins encoded by ORFs1a and 1b, such as RNA processing enzymes, are incorporated into the replication complex.

2. Sites of arterivirus and CoV replication are separate from sites of envelopment and budding

Several studies have investigated the intracellular sites of replication of equine arterivirus (EAV), MHV, and SARS-CoV. Such studies are difficult because during nidovirus infection, the processes of replication and envelopment occur on different membranes, and these may merge during encapsidation. Furthermore, late during infection cells infected with MHV can form syncitia. Newly synthesized MHV viral RNA has been found in perinuclear sites colocalized with the RdRp (Shi *et al.*, 1999), and depending on whether human or murine cells were infected, these sites colocalized with Golgi or ER membranes, respectively. Similar studies in mouse L cells report that the polymerase and newly synthesized RNA locate to late endosomes and endocytic carrier vesicles (van der Meer *et al.*, 1999). This discrepancy is in part reconciled by later work showing that the subcellular distribution of the replicase proteins can change during the course of infection, since replicase proteins move to sites of envelopment in the ERGIC (Bost *et al.*, 2001). This is supported by the finding that individual replicase proteins distribute differently following cell membrane fractionation (Sims *et al.*, 2000). Membrane fractionation has also been carried out by Gosert *et al.* (2002), who showed that several proteins encoded by ORF1a and b were associated with membranes, and when observed by immunogold electron microscopy, these were associated with rosettes of double-membraned vesicles 200–350 nm in diameter. The role of these vesicles in viral RNA replication was confirmed by *in situ* hybridization of labeled riboprobes. Double-membraned vesicles are also seen in cells infected with EAV (Pedersen *et al.*, 1999). EAV replicase proteins accumulate in perinuclear regions containing ERGIC and ER markers and colocalize with newly synthesized viral RNA, again suggesting sites of genome replication. Notably, similar structures can be produced by expression of arterivirus ORF1a-encoded proteins NSP2–7, which contain the membrane proteins thought to tether the replicase to membranes.

3. The double-membraned vesicles induced by arteriviruses and CoVs may be related to autophagosomes

Double-membraned vesicles are usually rare in cells but are induced during autophagy. A role for autophagy during MHV infection is suggested because autophagy is induced in cells infected with MHV. Furthermore, in cells lacking Atg5, a protein required for the formation of autophagosomes, there is a 99% reduction in virus yield and MHV fails to induce double-membraned vesicles (Prentice *et al.*, 2004a). Electron micrographs show that the double-membraned vesicles induced by

SARS-CoV extend from the ER and can be labeled with antibodies specific for replicase proteins. This suggests that, in common with MHV, the vesicles are a site of replication (Snijder *et al.*, 2006). Even though all SARS-CoV replicase proteins tested colocalize to punctate structures that accumulate near the nucleus, there are conflicting reports about their relationship with autophagosomes. In monkey Vero cells, the replicase proteins colocalize with autophagosomes identified using antibodies against LC3 (Prentice *et al.*, 2004a). However, when autophagosomes are identified by expression of GFP-LC3, the replicase proteins do not colocalize with the GFP signal (Snijder *et al.*, 2006). The vesicles induced by SARS-CoV are smaller at 100- to 300-nm diameter than autophagosomes (500–1000 nm) and are labeled with ER markers. This has lead Snijder and colleagues to suggest that they are virus-induced extensions to the ER, rather than *bona fide* autophagosomes (Pedersen *et al.*, 1999; Snijder *et al.*, 2006). The precise origins of the membrane crescents that form at the start of autophagy are unclear, and a number of studies have suggested they may form from the ER. This makes it possible that the double-membraned structures may be autophagosomes that have been modified by an accumulation of viral protein. Determining if autophagy is beneficial to SARS-CoV replication will have to await studies in cells where key proteins in the autophagy pathway have been removed or suppressed by gene silencing.

IV. VIRUS FACTORIES AND INCLUSION BODIES GENERATED BY LARGE DNA VIRUSES

A. Cytoplasmic virus factories formed by large cytoplasmic DNA viruses

The asfiviruses, poxviruses, iridoviruses, and the phycodnaviruses are large DNA viruses encoding hundreds of proteins from genomes ranging between 150 and 350 kbp. A comparison of protein sequences encoded by these viruses has suggested that they should be grouped together in a family of viruses called the *n*ucleo*c*ytoplasmic *l*arge *D*NA *v*iruses (NCLDV) (Iyer *et al.*, 2001). Sequence similarities are seen in the major capsid proteins, redox enzymes that maintain disulphide bonds in the cytosol, and proteins that regulate apoptosis; and the family has been extended to include the giant mimivirus isolated from the ameba *Acanthamoeba polyphaga* (La Scola *et al.*, 2003). Even though these viruses infect a diverse range of hosts from different phyla, including vertebrates [poxviruses, *African swine fever virus* (ASFV)], arthropods (entomopox, ASFV, chloriridoviruses), amphibians and fish (*Ranavirus*, *Megalocytivirus*, and *Lymphocystivirus* genera of the Iridoviridae family), marine algae (phycodnaviruses), and protozoa (mimivirus), they all generate cytoplasmic

factories as major sites of virus assembly and replication (illustrated in Fig. 4). The factories share many similarities with one another, again suggesting that this diverse group of viruses may be related and that the need to produce a virus factory in the cytoplasm was generated early in virus evolution.

1. ASFV factories form next to the microtubule organizing center

ASFV is the sole member of the *Asfivirus* genus, family Asfarviridae but shares striking icosahedral similarity with the iridoviruses, phycodnaviruses, and mimivirus. ASFV is a large double-stranded DNA (dsDNA) virus with a genome size ranging from 170 to 190 kbp. Gene expression is a regulated cascade and immediate early, early, early/late, intermediate, and true late gene types have been characterized to date. The virion has multiple concentric layers with an electron-dense core at the center that contains the viral genome. A protein matrix surrounds the core, which in turn is enclosed by a lipid bilayer. Finally, the bilayer is surrounded by a protein capsid layer. ASFV can gain a third envelope when it buds from the plasma membrane at the tip of actin-rich projections that resemble filopodia (Jouvenet *et al.*, 2006). ASFV probably enters cells by receptor-mediated endocytosis, but the steps following entry are poorly understood. It is possible that a viral core is delivered into the cytoplasm intact; alternatively, cores may dissociate in endosomes requiring some mechanism of genome delivery across the endosome membrane. Genome replication occurs both in the nucleus and cytoplasmic factories. Transfer to the nucleus may involve microtubule transport since late gene expression is inhibited by agents that depolymerize microtubules and the dominant-negative dynein motor protein p50-dynamitin (Alonso *et al.*, 2001; Heath *et al.*, 2001). ASFV does not produce nuclear inclusions analogous to those seen in herpesvirus and adenovirus infection, but there is evidence that small fragments of viral DNA are synthesized in the nucleus. The major site of ASFV DNA replication is, however, the virus factory (Rojo *et al.*, 1999).

a. Cytoplasmic factories formed during ASFV infection are assembled at the microtubule organizing center ASFV induces one principal factory in the cytoplasm during infection. Electron microscopy shows that the virus factory excludes obvious cellular organelles and contains mostly viral DNA, viral proteins, virus-induced membranes, and partially and fully assembled virions (Table I; Fig. 5A; Brookes *et al.*, 1996; Moura Nunes *et al.*, 1975; Rouiller *et al.*, 1998). The mechanisms that target viral proteins, virus-induced membranes, and viral DNA to the ASFV factories are poorly understood. Immunofluorescence staining for viral structural proteins generally reveals a strong signal at the factory and a weaker signal in the cytoplasm. The B602Lp protein (CAP80), which is a viral chaperone

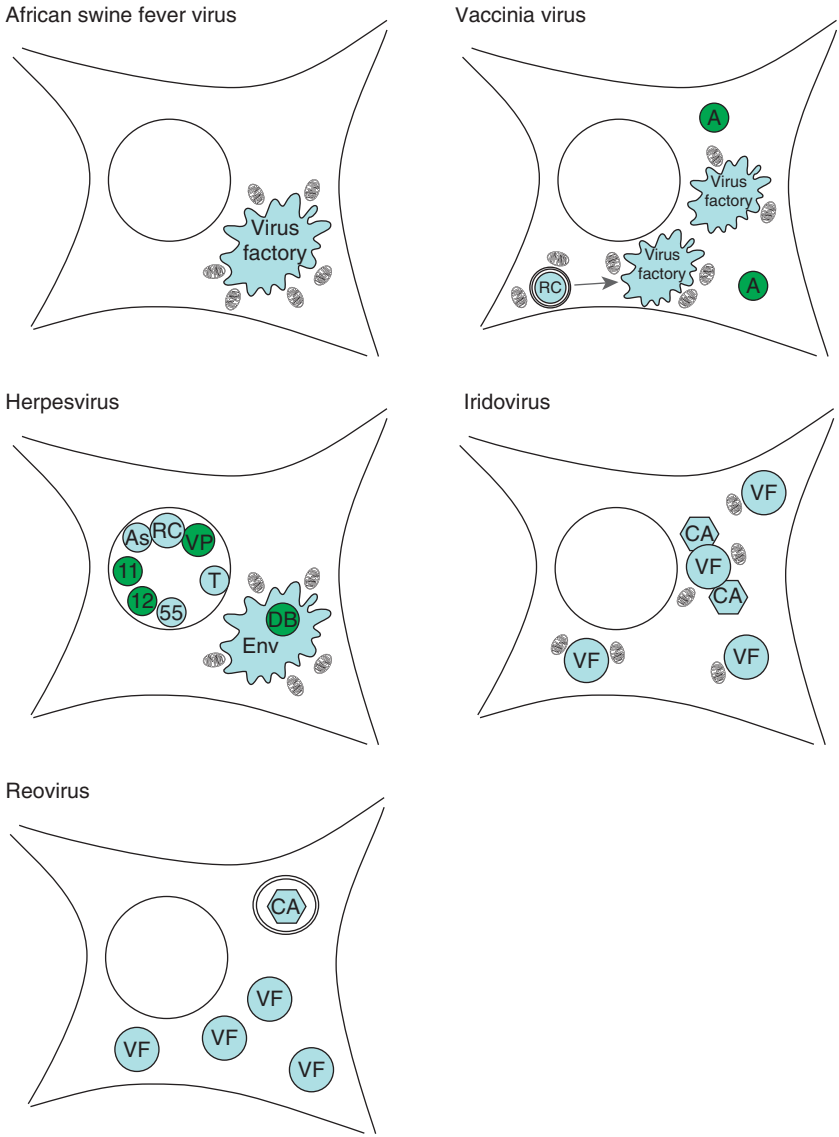

FIGURE 4 Schematics of inclusions induced during virus infection. ASFV induces single large perinuclear factories surrounded by mitochondria. *Vaccinia virus* induces multiple factories derived from membrane-enclosed replication complexes (RC) both of which are associated with mitochondria. Certain poxviruses also induces electron-dense A-type inclusions (A). Human herpesvirus 1 induces capsid assembly sites, or assemblons (As), replication compartments (RC), inclusions of tegument proteins VP13/14 and VP22 (VP), and electron-dense bodies of UL11 and UL12 gene products (11 and 12) in the nucleus. Human herpesvirus 2 also induces nuclear inclusions of UL55 gene product (55) and

involved in folding and membrane recruitment of the major capsid protein, p73, is, for example, absent from the virus factories (Cobbold *et al.*, 2001; Epifano *et al.*, 2006). This suggests that p73 is synthesized and folded in the cytoplasm and then recruited to factories. Similarly, the viral dUTPase, which is necessary for efficient replication, is excluded from the viral factory (Oliveros *et al.*, 1999). Since the bulk of viral DNA synthesis occurs in the factory (García-Beato *et al.*, 1992), it is not easy to explain how the viral dUTPase edits uracil from progeny viral genomes, without being present at the site of viral DNA synthesis and encapsidation. ASFV factories disperse when cells are incubated with drugs that depolymerize microtubules (Heath *et al.*, 2001) suggesting their formation involves microtubule motors. This may involve dynein motor proteins since p50-dynamitin, a dominant-negative version of the dynein motor, prevents both late ASFV gene expression (Heath *et al.*, 2001) and vimentin recruitment to factories (see below and Stefanovic *et al.*, 2005). Yeast-two-hybrid screens and *in vitro* pull-down experiments show that one ASFV structural protein, p54/j13Lp, interacts with dynein (Alonso *et al.*, 2001). While direct binding of p54/j13Lp to the motor protein has not been observed in infected cells, it is possible that the protein is involved in transporting some viral proteins into factories. The protein locates to virus factories and deletion of the E183L gene encoding p54/j13Lp generates factories that lack viral membranes, the major capsid protein p73, and the polyprotein precursors (pp220, and pp62) of the viral matrix (Epifano *et al.*, 2006; Rodríguez *et al.*, 2004). P54/j13Lp is a membrane protein with the bulk of the protein, including the dynein-binding motif, exposed to the cytosol. The p73 capsid protein and pp220 polyprotein associate with membranes before assembly into viruses (Cobbold and Wileman, 1998; Cobbold *et al.*, 1996; Heath *et al.*, 2003). If these membranes contain p54/j13Lp, it would provide a means of allowing recruitment to factories by retrograde transport along microtubules.

b. ASFV factories recruit intermediate filaments and resemble aggresomes

The formation and morphology of ASFV factories closely resemble the formation of aggresomes (Heath *et al.*, 2001), a cellular response to accumulation of misfolded protein aggregates (Johnston *et al.*, 1998). Aggresomes are microtubule-dependent inclusions containing protein aggregates that

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human herpesvirus 6 induces nuclear tegusomes (T). Herpesviruses induce cytoplasmic assembly sites where envelopment and some tegument are acquired (Env) in human herpesvirus 5, these sites include electron-dense bodies (DB). Iridoviruses induce multiple cytoplasmic virus factories (VF) and crystalline arrays (CA), both of which associate with mitochondria. Reoviruses also induce multiple cytoplasmic virus factories (VF) and crystalline arrays (CA) that are enclosed within lysosomal membranes.

TABLE I Known contents of viral inclusions induced by different virus families. Each section includes a brief description of the viral inclusion and lists both viral and host-cell proteins confirmed to localize within, or associate with, the specified structure

Asfarviridae, Asfivirus African swine fever virus	References
Cytoplasmic virus factory	
Appearance and contents of viral origin[a]	
Viral membranes, assembling and complete particles, electron dense condensations, viral DNA, A224L IAP apoptosis inhibitor, A104R (5AR) DNA binding histone like, A137R p11.5, B119L Erv1p homologue, B438L p49, B646L p73 major capsid protein, CP2475L pp220 precursor to p150; p37; p34 and p14 CP530R pp62 precursor to p35 and p15, O61R p12 attachment, D117L (i1L) transmembrane, S273R (i6R) cysteine protease, H108R (j5R) membrane, E183L p54 (j13L) dynein interacting, E199L (j18L) membrane, E120R (k3R) p14.5 DNA binding necessary for viral exit from factory	Alcamí *et al.*, 1993; Alonso *et al.*, 2001; Andrés *et al.*, 1997, 2001; Borca *et al.*, 1996; Brookes *et al.*, 1998a,b; Carrascosa *et al.*, 1986; Chacón *et al.*, 1995; Cobbold *et al.*, 1996; Galindo *et al.*, 2000; García-Beato *et al.*, 1992; Heath *et al.*, 2001; Hingamp *et al.*, 1992; Jouvenet and Wileman, 2005; Jouvenet *et al.*, 2004; Martinez-Pomares *et al.*, 1997; Moura Nunes *et al.*, 1975; Rodríguez *et al.*, 2006; Rouiller *et al.*, 1998; Sanz *et al.*, 1985; Simón-Mateo *et al.*, 1997; Sun *et al.*, 1996; Vigário *et al.*, 1967
Contents of cellular origin	
Ubiquitin, hsp70 chaperone, γ-tubulin, Pericentrin, p21, mdm1 Surrounded by: ER membranes, vimentin, p230 Golgin, mitochondria, and tubulin.	Granja *et al.*, 2004; Heath *et al.*, 2001; Hingamp *et al.*, 1992; Jouvenet and Wileman, 2005; Netherton *et al.*, 2004, 2006; Rojo *et al.*, 1998; Rouiller *et al.*, 1998; Stefanovic *et al.*, 2005
Poxviridae, Chordopoxvirinae, *Orthopoxvirus Vaccinia virus*	
Cytoplasmic A-type inclusion	
Contents[b]	
Electron dense, IMV, A26L (WR148 and WR149) myristylated	Patel *et al.*, 1986

Cytoplasmic B-type inclusion, virosome, or virus factory

Appearance and contents of viral origin[b]

Electron dense viroplasm, viral crescents, IV and IMV, viral DNA, A2.5L (WR121) redox, A3L (WR122) p4b core, A4L (WR123) p39 core, A9L (WR128) membrane, A10L (WR129) p4a core, A11R (WR130) phosphoprotein, A13L (WR132) membrane ERGIC, A14L (WR133) membrane ERGIC, A14.5L (WR134) membrane virulence, A15L (WR135) viroplasm/membrane association, A16L (WR136) cell-fusion/entry, A17L (WR137) membrane assembly, A18R (WR138), A30L (WR153) viroplasm/membrane association, A35R (WR158) virulence, A40R (WR165) SUMO-1 modified, A45R (WR171) virion superoxide dismutase homologue, B1R (WR183) protein kinase, D4R (WR109) uracil DNA glycolase, D8L (WR113) p32, D13L (WR118) p65 scaffold, E3L (WR059) dsRNA binding E5R (WR061) E8R (WR064) ER protein, surrounds virosome E10R (WR066), F10L (WR049) protein kinase viroplasm/membrane association, F17R (WR056) actin tail formation G7L (WR085) viroplasm/membrane association, H3L (WR101) p35 core membrane, H5R (WR103) transcription factor VLTF-4, I3L (WR072) ssDNA binding, I4L (WR073) ribonucleotide reducatase large subunit, J1R (WR093) core viroplasm/membrane association, L1R (WR088) myristylated, L4R (WR091) p25K core; ssDNA/ssRNA binding, Ectromelia zinc finger binding protein (absent in Copenhagen, fragment in WR), Cowpox CP77 host range factor, WR011 E3-ubiquitin ligase.

Almazán *et al.*, 2001; Beaud and Beaud, 1997; Betakova *et al.*, 2000; Chiu *et al.*, 2005; Cudmore *et al.*, 1996; da Fonseca *et al.*, 2000; Davis and Mathews, 1993; De Silva and Moss, 2005; Domi and Beaud, 2000; Krijnse-Locker *et al.*, 1996; Murcia-Nicolas *et al.*, 1999; Nerenberg *et al.*, 2005; Ojeda *et al.*, 2006; Palacios *et al.*, 2005; Pedersen *et al.*, 2000; Reckmann *et al.*, 1997; Resch *et al.*, 2005; Risco *et al.*, 1999; Roper, 2006; Salmons *et al.*, 1997; Senkevich *et al.*, 2002; Sodeik *et al.*, 1995; Szajner *et al.*, 2004a,b,c; Tolonen *et al.*, 2001; Vanslyke and Hruby, 1994; Welsch *et al.*, 2003; Wolffe *et al.*, 1995; Yeh *et al.*, 2000; Yuwen *et al.*, 1993

(*continued*)

TABLE I (*continued*)

Contents of cellular origin	
HMG20A viral genome binding protein, hSP90; transient association, Ubiquitin, ying-yang 1 transcription factor, TBP transcription factor, SP1 transcription factor, RNA polymerase II, SUMO-1, ERGIC-53[c] Surrounded by: vimentin and mitochondria.	Broyles *et al.*, 1999; Dales and Siminovitch, 1961; Hsiao *et al.*, 2006; Hung *et al.*, 2002; Husain and Moss, 2003; Nerenberg *et al.*, 2005; Oh and Broyles, 2005; Palacios *et al.*, 2005; Risco *et al.*, 2002; Wilton and Dales, 1989
Iridoviridae, *Ranavirus*	
Cytoplasmic virus factories	
Appearance and contents[f]	
Electron lucent, virus, viral DNA, 108K early protein, 57K, 55K major capsid protein (ORF 90R in FV3), 38K, 17K, 16K *Rana grylio* virus dUTPase (ORF 63R in FV3). Surrounded by vimentin, rough ER, mitochondria and polysomes.	Chinchar *et al.*, 1984; Darlington *et al.*, 1966; Huang *et al.*, 2006; Murti and Goorha, 1983, 1989; Zhao *et al.*, 2007
Herpesviridae, *alphaherpesvirinae, simplexvirus* and *varicellovirus*	
Nuclear replication compartment	
Contents of viral origin[d]	
UL3, UL4 virion, UL5 helicase-primase, UL6 DNA cleavage/packaging, UL8 helicase-primase, UL15 DNA packaging, UL17 tegument DNA packaging, UL18 DNA packaging, UL19 ICP5 major capsid protein, UL26.5 ICP35 DNA packaging, UL29 ICP8 single strand binding UL30 DNA polymerase, UL32 DNA packaging, UL33 DNA packaging, UL35 VP26 p12 capsid, UL42 65K DNA polymerase accessory, UL49 VP22 tegument, UL52 helicase-primase UL54 ICP27 regulatory, α0 UL57 ICP0 transactivator, α4 ICP4 regulatory, α22 US1 ICP22 regulatory, US1.5 truncated, US1 regulatory.	Barnard *et al.*, 1997; de Bruyn Kops *et al.*, 1998; Everett and Maul, 1994; Goodrich *et al.*, 1990; Jahedi *et al.*, 1999; Knipe *et al.*, 1987; Lamberti and Weller, 1998; Leopardi *et al.*, 1997; Liptak *et al.*, 1996; Markovitz and Roizman, 2000; Olivo *et al.*, 1989; Randall and Dinwoodie, 1986; Reynolds *et al.*, 2000; Taus *et al.*, 1998; Ward *et al.*, 1996

Contents of cellular origin	
RNA polymerase II, EAP ribosome component, proliferating cell antigen, retinoblastoma protein, p53, DNA ligase 1, DNA polymerase α, promyelocytic leukemia (PML), DNA-PKcs, Ku86 nonhomologous end joining, Bloom syndrome gene product, breast cancer-associated gene 1 protein, MSH2, Rad50, WRN RecQ helicase family member, BRG1 or BRM-associated factor 155, brahma-related gene-1 protein, brahma protein, histone deacetylase 2, hSNF2H, mSin3a, TATA binding protein (TBP), TBP-associated factors.	Leopardi *et al.*, 1997; Lukonis *et al.*, 1997; Quadt *et al.*, 2006; Taylor and Knipe, 2004; Wilcock and Lane, 1991
Nuclear sites of capsid assembly or assemblons	
Contents of viral origin[d]	
UL7 (HHV-2), UL14 (HHV-2) tegument, UL16 capsid, UL19 ICP5 major capsid protein, UL26.5 ICP35 DNA packaging, UL27 DNA packaging, UL35 VP26 p12 capsid, UL38 VP19c capsid assembly, UL43.5, UL55.	de Bruyn Kops *et al.*, 1998; Goshima *et al.*, 1998; Nalwanga *et al.*, 1996; Nozawa *et al.*, 2002; Wada *et al.*, 1999; Ward *et al.*, 1996a,b; Yamada *et al.*, 1998
Contents of cellular origin	
Actin, myosin 5a actin motor	Feierbach *et al.*, 2006
Cytoplasmic assembly and envelopment site	
Contents of viral origin[d]	
Membranes, vacuoles, capsids and enveloped virus UL19 (HHV-2) VP5 major capsid protein UL27 (HHV-2) gB VP7 UL36 (HHV-2) ICP1–2, tegument UL46 (HHV-2) tegument, UL48 (HHV-2) tegument	Kato *et al.*, 2000; Murata *et al.*, 2000; Nozawa *et al.*, 2004; Watanabe *et al.*, 2000
Contents of cellular origin	
Mitochondria, γ-tubulin, hsp40 chaperone, hsp70 chaperone, GM130 Golgi marker	Murata *et al.*, 2000; Nozawa *et al.*, 2004

(*continued*)

TABLE I (*continued*)

Herpesviridae, Betaherpesvirinae, *Cytomegalovirus* Human herpesvirus 5	
Cytoplasmic assembly sites	
Appearance and contents[e]	
Membranes, vacuoles, capsids, enveloped virus and dense bodies (see below) UL23 tegument, UL24 tegument, UL25, UL32 pp150, UL43 tegument, UL53, UL55 gB, UL73 gN, UL75 gH, UL80 p38, UL83 pp65–69 UL99 pp28, gp65.	Adair *et al*., 2002; Battista *et al*., 1999; Dal Monte *et al*., 2002; Landini *et al*., 1991; Pignatelli *et al*., 2002; Sanchez *et al*., 2000
Cytoplasmic dense bodies	
Appearance and contents[e]	
Homogenous electron dense material, UL73 gN, UL83 p65–69.	Craighead *et al*., 1972; Pignatelli *et al*., 2002
Herpesviridae, Betaherpesvirinae, *Roseolovirus* Human herpesvirus 6	
Nuclear/cytoplasmic tegusome	
Appearance	
Enveloped nucleocapsids, virus with tegument in cytoplasmic invagination of nucleus	Roffman *et al*., 1990
Adenoviridae, *Mastadenovirus*	
Nuclear small fibrillar masses, ssDNA accumulation sites or early replicative sites	
Appearance and contents	
Viral ssDNA replication, 72kDa ssDNA binding protein, viral RNA (early)	Puvion-Dutilleul and Puvion, 1990; Puvion-Dutilleul *et al*., 1992

Nuclear fibrillogranular matrix or peripheral replicative sites

Appearance and contents

Viral RNA (late), E1A oncogenic proteins, E4-ORF3, 72kDa ssDNA-binding protein, DNA polymerase, terminal protein, PML, splicesomes, sp100, hsp70, nuclear factor 1	Bosher *et al.*, 1992; Carvalho *et al.*, 1995; Murti *et al.*, 1990; Puvion-Dutilleul, 1991; Puvion-Dutilleul *et al.*, 1994

Nuclear virus-induced compact ring

Appearance and contents

Viral RNA (late), pIVa2 DNA packaging	Lutz *et al.*, 1996

Nuclear clear amorphous inclusion

Appearance and contents

pIX, PML, PKR, CK2α	Lutz *et al.*, 1996; Rosa-Calatrava *et al.*, 2001, 2003; Souquere-Besse *et al.*, 2002

Nuclear electron-translucent area

Appearance and contents

Virus, protein crystals, pentons, hexons, fiber protein, pIX, L1 52 kDa, L1 55 kDa, PML, PKR, CK2β	Puvion-Dutilleul *et al.*, 1995, 1999; Souquere-Besse *et al.*, 2002

Other pIVa2 positive nuclear structures induced during adenovirus 5 infection

Name

Nuclear irregular electron-dense amorphous inclusion, Nuclear regular electron-dense amorphous inclusion, nucleolus electron-dense virus-induced globules, nucleous irregular amorphous inclusion.	Lutz *et al.*, 1996

(*continued*)

TABLE I (*continued*)

Reoviridae, *Orthoreovirus*	
Cytoplasmic virus factories	
Appearance and contents	
Filamentous or globular dependent on μ2, phase and electron dense, viral RNA, virus, σNS nonstructural, μ1 outer-capsid, μ2 nonstructural, μNS nonstructural, λ1 core surface, λ2 core surface, λ3 RNA polymerase, σ2 core surface, σ3 structural, ubiquitin, microtubules, vimentin (association with).	Becker *et al*., 2001, 2003; Broering *et al*., 2004; Cashdollar, 1994; Dales *et al*., 1965b; Miller *et al*., 2004; Sharpe *et al*., 1982; Silverstein and Schur, 1970
Reoviridae, *rotavirus*	
Cytoplasmic virus factories	
Appearance and contents	
Electron-dense viroplasm, assembling and complete double-shelled particles VP2, VP6, VP9, NSP2, NSP5, NSP6	Altenburg *et al*., 1980; González *et al*., 2000; Petrie *et al*., 1982, 1984; Silvestri *et al*., 2004, 2005

[a] *African swine fever virus* gene nomenclature is based on that for the Badajoz 1971 vero adapted strain with that of the Malawi Lil 20/1 strain in parentheses.
[b] *Vaccinia virus* gene nomenclature is based on that for the Copenhagen strain with that of the western reserve strain in parentheses.
[c] One report places in ERGIC-53 within the virosome (Risco *et al*., 2002), one report places it outside (Husain and Moss, 2003).
[d] Open reading frames from human herpesvirus 1 (herpes simplex virus 1) unless specified otherwise.
[e] Open reading frames from human herpesvirus 5 (human cytomegalovirus) unless specified.
[f] Proteins specified by frog virus 3 unless indicated otherwise.

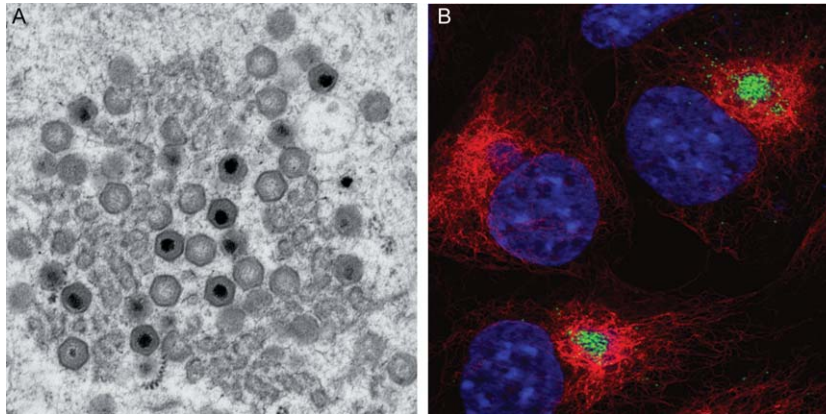

FIGURE 5 (A) Electron micrograph of an ASFV factory showing partially assembled, empty and fully mature capsids as well as electron-dense viroplasm accumulating around viral membranes. Image courtesy of P. Hawes, J. Simpson, and P. Monaghan, Bioimaging Group, IAH-Pirbright. (B) Confocal micrograph of ASFV-infected cells immunolabeled with antimajor capsid protein (green) and vimentin (red) and stained with a DNA dye (blue). Note vimentin cages enclosing ASFV factories. Reprinted from Monaghan *et al.* (2003) with permission from Blackwell Publishing, Inc.

form next to the microtubule-organizing center (MTOC). Aggresomes recruit cellular components needed to deal with the problems associated with a buildup of aggregated misfolded protein. These include cellular chaperones and proteasomes to facilitate protein folding and/or degradation and mitochondria that may provide the ATP required for folding and proteolysis. The most striking structural changes seen during aggresome formation are the collapse of the intermediate filament protein, vimentin, into a cage surrounding the protein aggregates and the gross fragmentation of the Golgi apparatus. ASFV factory formation shows many similarities with this response to protein aggregation. Factory formation is preceded by clearance of cytoplasmic proteins from perinuclear areas around the MTOC. Vimentin then concentrates at the MTOC where it forms an aster aligned along microtubules (Stefanovic *et al.*, 2005). Following the onset of virus DNA replication and synthesis of late structural proteins, the vimentin aster is rearranged into a cage around the factory (Fig. 5B; Heath *et al.*, 2001; Monaghan *et al.*, 2003; Stefanovic *et al.*, 2005). During this period, mitochondria and cellular chaperones are recruited to the factory (Heath *et al.*, 2001; Rojo *et al.*, 1998). Formation of vimentin cages in ASFV-infected cells is linked to phosphorylation of vimentin at serine 82 by calcium calmodulin-dependent protein kinase II (CamKinase II) (Stefanovic *et al.*, 2005), and drugs that inhibit CamKinase II activity block late gene expression and

vimentin rearrangement. As will be discussed for poxviruses and iridoviruses, the vimentin cage may form a physical scaffold within the factory, or act as a cage to prevent movement of viral components into the cytoplasm. Chaperones recruited to the factory may facilitate folding of viral structural proteins during assembly, as has been shown for other viruses. The proximity of mitochondria to viral factories may provide the ATP that is required for ASFV assembly (Cobbold *et al.*, 2000) or be indicative of an antiviral response as mitochondria are effectors of apoptosis. Taken together these results suggest that a cellular response originally designed to deal with the buildup of protein aggregates in cells is used by ASFV to generate a site specialized for virus assembly. As will be described later, similarities between aggresomes and virus assembly sites are also seen for the iridoviruses and poxviruses.

Following the onset of ASFV DNA replication, the microtubule network becomes disorganized. Microtubules are partially excluded from virus factories and form bundles and concentric rings in the cytoplasm (Jouvenet and Wileman, 2005). ASFV infection leads to disassembly of γ-tubulin and pericentrin from the centrosome, and the centrosome becomes less able to nucleate microtubules. At the same time microtubules are stabilized by acetylation (Jouvenet *et al.*, 2004). Since pericentrin and γ-tubulin play key roles in microtubule organization and nucleation at the MTOC, their loss from the centrosome, coupled with acetlylation of tubulin, may explain the rearrangement of microtubules induced by ASFV. The reasons for these profound effects on microtubules are not known but they may facilitate disruption of the virus factory allowing release of assembled viruses into the cytoplasm.

c. Membrane rearrangements caused by ASFV infection perturb the secretory pathway Current models for ASFV envelopment in virus factories predict that viral membranes are obtained from the ER. The major structural proteins are recruited from the cytoplasm onto the cytoplasmic face of the ER, and after which protein–protein interactions between these, and possibly viral proteins targeted to the ER lumen, lead to constriction of ER cisternae and clearance of host proteins from the ER lumen prior to envelopment (Andrés *et al.*, 1998; Netherton *et al.*, 2004, 2006; Rouiller *et al.*, 1998). This is consistent with low levels of ER proteins observed at ASFV assembly sites by immunoelectronmicroscopy (Rouiller *et al.*, 1998) and standard fluorescence microscopy where ER proteins appear to be actively excluded from areas of viral replication (Andrés *et al.*, 1998; Netherton *et al.*, 2004). In addition to effects on the ER, ASFV also affects the structure and function of later Golgi compartments of the secretory pathway (McCrossan *et al.*, 2001; Netherton *et al.*, 2006). Golgi structure is linked to microtubule organization and the changes seen during infection may in part be related to effects of ASFV infection on

centrosome and microtubule function listed above. ASFV infection causes dispersal of ERGIC marker protein ERGIC-53, the peripheral Golgi protein GM130, and late Golgi protein GalNac-T2 transferase, suggesting disruption of ERGIC and Golgi membrane compartments. Most striking is the complete loss of the TGN. TGN loss is dependent on microtubules and involves dispersal of the TGN into separate vesicle populations containing either peripheral Golgi proteins or the integral membrane protein, TGN46. Not surprisingly, this dispersal slows the transport of proteins through the secretory pathway. ASFV slows the delivery of newly synthesized lysosomal enzymes to lysosomes (McCrossan *et al.*, 2001), and in macrophages reduces transport of newly synthesized MHC class I to the plasma membrane (Netherton *et al.*, 2006). Thus, in common with picornaviruses, disruption of the secretory pathway by ASFV has the potential to slow the transport of important immunomodulatory proteins to the surface of infected cells and may mask them from immune surveillance.

2. Poxviruses generate virus factories and inclusions

Poxviruses are large dsDNA viruses with genomes ranging from 130 to 375 kbp. Poxvirus gene expression follows the regulated cascade of other large dsDNA viruses with early, intermediate, and late transcripts described. Poxvirus progeny genomes are replicated exclusively in the cytoplasm in virus factories. The virus encodes all the enzymes necessary for transcription and replication of its genome. Genetic analysis has identified a minimum of five viral genes necessary for genome replication, these are A20R, B1R, D4R, D5R, and E9L encoding the DNA polymerase processivity factor, serine/threonine protein kinase, uracil DNA glycosylase, DNA-independent nucleoside triphosphatase, and the DNA polymerase, respectively (De Silva and Moss, 2005; Evans *et al.*, 1995; Millns *et al.*, 1994; Punjabi *et al.*, 2001; Rempel *et al.*, 1990; Sridhar and Condit, 1983). Only the product of the D4R gene, encoding the viral DNA glycosylase, has been confirmed to localize to the site of genome synthesis (De Silva and Moss, 2005), and it would be interesting to discover the subcellular location of the other members of the minimum replicase. When viewed by electron microscopy, infectious virions have a striking brick-shaped morphology, and different forms of virus are documented which vary in degree of complexity [for review, see Condit *et al.* (2006)]. The interior of all poxvirus particles contains the virus core which houses the viral genome. Cores are enveloped in virus factories to produce the intracellular mature virus (IMV), which is fully infectious. Additional envelope layers gained at the TGN give rise to intracellular enveloped viruses (IEV), which after budding through the plasma membrane form cell-associated and extracellular enveloped viruses (CEV and EEV). Poxviruses induce two principal inclusions during infection, the A-type inclusion that is nonreplicative and the B-type inclusion where virus

replication and assembly occur in the virus factory (Fig. 4; Kato *et al.*, 1959).

a. Poxvirus A-type inclusions contain the mature intracellular virus but not enveloped viruses A-type inclusions are cytoplasmic bodies of dense homogeneous matter that contain mature virus particles and are studded with polyribosomes (Fig. 6A) (Ichihashi *et al.*, 1971). A-type inclusions are extremely rare in vaccinia, variola, and rabbit pox infections but are prominent in cowpox, ectromelia, fowlpox, and canarypox infections where they are also referred to as Downie, Marchal, Bollinger, and Burnet bodies, respectively (Kato *et al.*, 1959). The major component of A-type inclusions is the product of the A26L gene or its equivalents. In vaccinia, A26 is truncated and produces a protein of 92–94 kDa whereas the full-length gene in cowpox encodes a protein of 160 kDa (Patel *et al.*, 1986), both versions are myristylated (Martin *et al.*, 1999). Immunfluorescence analysis of cells infected with *Vaccinia virus* with antibodies raised against A26 does reveal multiple A-type inclusions in the cytoplasm, but they are much smaller than those seen in cells infected with cowpox, and do not contain virus particles (Patel *et al.*, 1986). In cells infected with wild-type cowpox, only IMV particles were observed within A-type inclusions, but treatment with rifampicin, a drug that blocks poxvirus maturation at an early stage in morphogenesis, caused aberrant immature virus particles to integrate into the inclusions (Ichihashi *et al.*, 1971). The factor necessary for occlusion of viral particles in A-type inclusions has been identified as the 4c core protein (McKelvey *et al.*, 2002; Shida *et al.*, 1977; Ulaeto *et al.*, 1996). It has been hypothesized that 4c retains vaccinia virions within the cell as IMVs in A-type inclusions preventing their transport to the TGN for envelopment and maturation to the IEV types of virion (McKelvey *et al.*, 2002). A-type inclusions are predicted to protect IMVs during transport between hosts akin to that of the polyhedra that occlude entomopox and baculoviruses (Rohrmann, 1986). Therefore, EEVs may be important for cell-to-cell spread, while IMVs (whether occluded or not) may be more important for host-to-host spread (McKelvey *et al.*, 2002).

b. Poxvirus B-type inclusions are factories and are the main sites of replication and assembly B-type inclusions originally called Guarnieri bodies (Guarnieri, 1893) are the primary replication centers of the poxviruses, now generally referred to as virosomes or virus factories (Fig. 6C). Electron microscopic analysis of B-type inclusions revealed a granular matrix that was denser than the surrounding cellular material and in a defined area of the cytoplasm called viroplasm (Dales and Siminovitch, 1961; Higashi, 1973). The factories also contain viral crescents consisting of membrane and viral proteins associated with viroplasm, spherical immature virus,

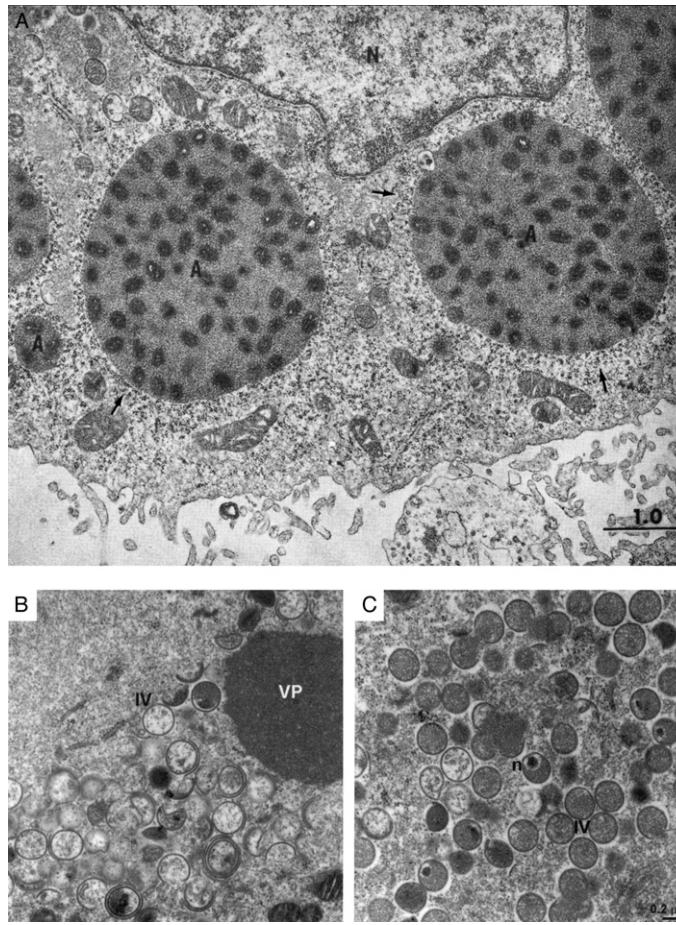

FIGURE 6 (A) Electron micrograph of A-type inclusions from cowpox-infected cells, showing intracellular mature virus in electron-dense inclusions (A) surrounded by polyribosomes (arrows). Reprinted from Ichihashi *et al.* (1971) with permission from Elsevier. (B and C) Electron micrographs of factories of recombinant *Vaccinia virus* encoding the A15L gene under the control of the lac operon under nonpermissive (B) and permissive (C) conditions. Note empty immature virus particles (IV), viral crescents in an electron-lucent environment, and a separate homogenous viroplasm (VP) in panel B and compare to wild-type like conditions in panel C, which include immature virus with electron-dense centers and particles containing nucleoids (n). Reprinted from Szajner *et al.* (2004a) with permission from Elsevier.

and IMVs (Dales and Siminovitch, 1961). Factories are surrounded by mitochondria, increase in number and size during the replication cycle and can occupy the majority of the cytoplasm at late times of infection (Dales and Siminovitch, 1961).

The assembly and envelopment of *Vaccinia virus* within virus factories has been the subject of many studies and is discussed in papers and reviews (Griffiths *et al.*, 2001; Heuser, 2005; Hollinshead *et al.*, 1999; Sodeik and Krijnse-Locker, 2002). Here, we will review some of the early steps that lead up to the start of genome replication and factory production. These have also been described in a review (Schramm and Krijnse-Locker, 2005). It is generally believed that infection results in the delivery of viral cores into the cytoplasm. Cores are seen associated with microtubules (Carter *et al.*, 2003; Mallardo *et al.*, 2001; Ploubidou *et al.*, 2000) and may use microtubules to reach perinuclear sites that will eventually house the virus factories. Viral cores can transcribe as many as 100 early mRNAs before the onset of DNA replication, and these early mRNAs appear in discrete foci that associate with microtubules, contain polyribosomes and other translational machinery. It is unlikely that foci involved in transcribing early RNAs mature into viral replication sites because they do not initiate DNA synthesis (Mallardo *et al.*, 2002). It is likely that each infecting virus can induce its own replication center (Cairns, 1960), but it is not clear where in the cell the cores initiate DNA synthesis. It has been suggested that the onset of DNA synthesis may occur at peripheral sites and therefore precedes delivery to the perinuclear region of the cell. When cells are incubated with hydroxyurea to prevent the onset of viral DNA replication, it is possible to localize viral DNA released into the cytoplasm. Under these conditions, viral genomes are seen at several discrete sites that contain B1 protein kinase, E8 membrane protein, I3 ssDNA-binding protein, and H5 late transcription factor (Domi and Beaud, 2000; Welsch *et al.*, 2003). After removal of hydroxyurea, these foci begin to make new viral DNA, showing that they are sites of DNA replication. Live cell imaging studies have shown that these initial sites of DNA replication form in the cell periphery and then move toward the nucleus where they coalesce into large structures (Schramm and Krijnse-Locker, 2005).

Electron micrographs suggest that sites of DNA release from cores are intimately associated with ER membranes and become completely enclosed by them during the initial stages of DNA replication (Mallardo *et al.*, 2002). This process is likely facilitated by the E8R gene product which is a membrane protein localized to the ER and early Golgi membranes, has DNA-binding activity, and is able to capture viral genomes (Doglio *et al.*, 2002; Tolonen *et al.*, 2001). These ER-enclosed genomes are short-lived structures because they are not seen once viral crescents, IV and IMVs, appear in factories (Tolonen *et al.*, 2001). The sites of DNA replication are also separate from the foci involved in transcribing early RNAs, and it is interesting to consider how the cores are separated from newly transcribed RNA. Viral cores and sites of RNA transcription both align on microtubules and partially colocalize with the L4 core

DNA-binding protein (Mallardo *et al.*, 2001). The L4 protein is able to bind microtubules (Ploubidou *et al.*, 2000) and may be involved in separating RNA from cores along microtubule tracks (Mallardo *et al.*, 2001).

Inducible recombinants or temperature-sensitive mutants grown under nonpermissive conditions can give further insight into the early stages of inclusion formation. Electron micrographic analysis of the factories formed under these conditions yield striking images of distinct inclusions of homogeneous electron-dense viroplasm next to empty spherical immature virions (Fig. 6B and C) (Szajner *et al.*, 2001, 2003, 2004a). A seven-protein complex comprising the gene products of the A15L, A30L, D2L, D3L, F10L G7L, and J1R open reading frames has been identified as being necessary for association of viral membranes with the viroplasm (Szajner *et al.*, 2004a). Consistent with this role, all of these proteins are known to localize to the virus factory except D2 and D3 (Table I); however, these have been identified as core proteins (Dyster and Niles, 1991) so are likely to reside at viral assembly sites. Localization of D13L to the virus factory is sensitive to the antibiotic rifampicin (Miner and Hruby, 1989), and treatment with this drug induces irregular shaped viral membranes instead of the well-defined hemispherical viral crescents seen in natural infection (Moss *et al.*, 1969; Pennington *et al.*, 1970). Therefore, it was suggested that D13L may act as a scaffold on which the viral membrane is shaped, allowing correct association with the viroplasm (Mohandas and Dales, 1995). Deep etch electron microscopy has confirmed this role for D13L, as it forms the honeycomb lattice identified as the outer coat of the viral membrane of immature virions (Heuser, 2005; Szajner *et al.*, 2005). Interestingly, D13L shares a structural similarity with structural proteins from many other virus families, including those of the other large dsDNA viruses (Benson *et al.*, 2004). It will be interesting to see if the structural similarities to D13L translate to functional similarities in the assembly strategies of other viruses.

c. Poxvirus infection recruits host proteins into factories and rearranges cellular organelles *Vaccinia virus* recruits a number of cellular proteins to the viral factory. Ying-Yang 1 (YY1), TBP, SP1 transcription factors, and RNA polymerase II are recruited from the nucleus to the factory (Broyles *et al.*, 1999; Oh and Broyles, 2005; Wilton and Dales, 1989). YY1 is a nuclear transcription factor that can activate late viral promoters and although poxviruses encode most of the genes necessary for transcription, there is evidence that cellular factors may be required for intermediate and late gene expression (Lackner and Condit, 2000; Rosales *et al.*, 1994; Wright *et al.*, 2001). The function of the other transcription factors in viral replication is unknown. They may be necessary for viral transcription like YY1, or perhaps they are sequestered into the factory to divert them from their

normal roles in the nucleus, or their presence may represent an antiviral response by the cell. The presence of RNA polymerase II in the viral factory is a surprise because the virus encodes its own RNA polymerase activity which accounts for at least 9 ORFs and ~7% of the genome capacity [Western Reserve (WR) strain]. Another cellular protein recruited from the nucleus to the cytoplasm is the HMG20A protein. This protein can bind the viral genome and has been implicated in host range restriction of *Vaccinia virus* in Chinese hamster ovary cells (Hsiao *et al.*, 2006). During unproductive infection by *Vaccinia virus*, HMG20A is recruited from the nucleus to the factory where it binds viral DNA. If the cowpox host range gene CP77 is artificially introduced into *Vaccinia virus* then CP77 also enters the virus factory and binds to HMG20A; the cellular protein then dissociates from the viral genome and replication proceeds (Hsiao *et al.*, 2006).

As seen for iridovirus and ASFV replication sites, vaccinia factories are surrounded by a vimentin cage (Risco *et al.*, 2002; Schepis *et al.*, 2006) and recruit molecular chaperones (Hung *et al.*, 2002), suggesting similarity with aggresomes. Many proteins targeted to aggresomes are ubiqutinated, and most poxviruses encode a RING protein that is both a functional ubiquitin ligase and a virulence factor (Nerenberg *et al.*, 2005). Exceptions to this are the two most common laboratory strains of vaccinia, Copenhagen and WR. The RING protein from the IHD-W strain of vaccinia is capable of directing transfected tagged ubiquitin to WR virus factories (Nerenberg *et al.*, 2005); however, it is unknown if native ubiquitin is localized to WR factories. The product of the A40R gene of vaccinia is tagged with the ubiquitin-like protein SUMO-1, and this modification is necessary for A40 targeting to viral factories, where it associates with ER membranes and may play a role in the formation of I3 sites (Palacios *et al.*, 2005). It is not known if movement of SUMOlyated A40 and ubiquitinated protein is directed along microtubules in a manner analogous to HDAC6-mediated targeting of misfolded proteins to aggresomes (Kawaguchi *et al.*, 2003). As reported for ASFV (see above) and cells infected with herpes simplex virus (Avitabile *et al.*, 1995), infection of cells with *Vaccinia virus* also leads to disruption of microtubule organization and centrosome function and dispersal of the Golgi apparatus (Ploubidou *et al.*, 2000). Whether these are bystander effects of the production of virus factories close to the centrosome or induced deliberately to facilitate virus egress is not known. IMV exit from the factory and transport to envelopment sites at the TGN is nonetheless dependent on microtubules (Sanderson *et al.*, 2000) and has been reported to be dependent on the A4L and A27L gene products (Sanderson *et al.*, 2000; Ward, 2005). Following envelopment, the A35L and F12L gene products then regulate microtubule-dependent movement of intracellular enveloped viruses from the TGN to the plasma membrane (Herrero-Martínez *et al.*, 2005; Ward and Moss, 2001).

3. The iridoviruses generate cytoplasmic factories and crystalline arrays

a. Iridoviruses Iridoviruses are large dsDNA viruses with genomes ranging from 100 to 210 kbp in length encoding between 100 and 230 proteins (Williams *et al*., 2005). Much of the work on iridovirus replication has been carried out on the ranavirus frog virus 3 (FV3). FV3 genome synthesis occurs in the nucleus and cytoplasm. No nuclear inclusions have been reported during FV3 infection, and as such it is unclear how the nuclear replication stage is mediated. However, viral DNA is initially synthesized as units that are 1–2 genomes in length and then transported to the cytoplasm where multiple length concatemers are produced (Goorha, 1982).

b. Cytoplasmic factories formed during iridovirus infection resemble aggresomes Infection induces two cytoplasmic inclusions. Viral factories form in the cytoplasm and become the major site of viral DNA replication. FV3 also induces large crystalline arrays of viral particles which give rise to the iridescent coloring of purified virus, and hosts, that are characteristic of iridovirus infections. Virus factories are electron lucent relative to the cytoplasm and contain viral membranes, partially assembled viruses, and are surrounded by rough ER membranes and polysomes. FV3 factories also resemble aggresomes since they recruit intermediate filaments (Fig. 7A) and mitochondria, some of which show

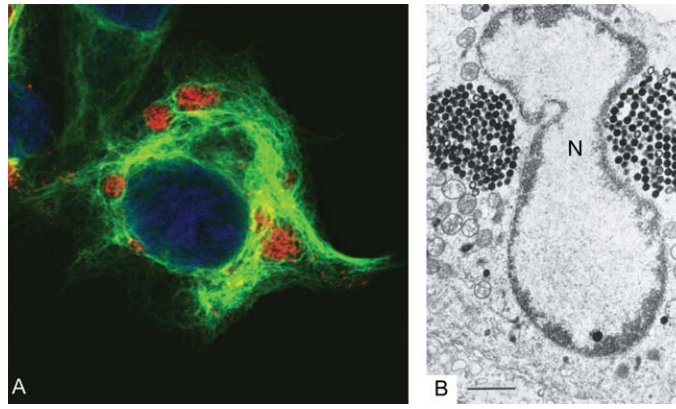

FIGURE 7 (A) Confocal micrograph of frog virus 3-infected cell showing relationship between the major capsid protein (red), vimentin (green), and DNA (blue). Note multiple viral inclusions in the cytoplasm, each associated with an individual vimentin cage. Authors own. (B) Electron micrograph of a frog virus 3-infected cell showing two crystalline arrays that appear to induce a kidney-shaped nucleus (N). Reprinted from Darlington *et al*. (1966) with permission from Elsevier.

signs of damage (Darlington *et al.*, 1966; Granoff *et al.*, 1966; Huang *et al.*, 2006; Tripier *et al.*, 1977). Crystalline arrays of virus are associated with virus factories and can induce nuclear deformations that lead to kidney-shaped nuclei similar to those seen in ASFV infection (Fig. 7B) and after aggresome formation (Darlington *et al.*, 1966; Heath *et al.*, 2001; Johnston *et al.*, 1998). As seen for ASFV and poxviruses, the intermediate filament vimentin plays an important role in replication (Murti and Goorha, 1983). Vimentin is phosphorylated during FV3 infection, prior to factory formation (Chen *et al.*, 1986; Willis *et al.*, 1979), and temperature-sensitive mutants that are unable to phosphorylate vimentin do not form vimentin cages and are unable to proceed to late gene expression. Drug treatment with taxol or colchicine (Murti *et al.*, 1988) showed that recruitment of vimentin to assembly sites requires dynamic, but not polymerizing microtubules, and microinjection of anti-vimentin antibody prevented recruitment of vimentin to factories. This allowed intrusion of cell components into assembly sites and reduced virus growth by 70–80% (Murti *et al.*, 1988). Vimentin may therefore provide a scaffold for iridovirus replication, maintaining a barrier between the cytoplasm and the contents of the virus factory. Consistent with this hypothesis is the observation that during infection, polyribosomes and most newly synthesized viral proteins associate with intermediate filaments (Murti and Goorha, 1989). FV3 factory formation may also be dependent on the early 108K protein, as it is recruited to factories in the absence of late protein synthesis (Chinchar *et al.*, 1984).

4. Phycodnavirus and mimivirus replicate in cytoplasmic factories

Phycodnaviruses and the recently described giant virus mimivirus (La Scola *et al.*, 2003) induce replication complexes in the cytoplasm of infected ameba (Meints *et al.*, 1986; Raoult *et al.*, 2007). The factories of the phycodnavirus Paramecium bursaria Chlorella virus 1 (PBCV-1) are electron translucent areas of the cytoplasm and contain viral membranes, electron-dense viroplasm, and assembling viruses. Unlike many viral factories, a distinct order appears to be present in PBCV-1 virosomes. The assembling viruses are arranged at the periphery of the virosome/factory, giving the appearance of a rosette (Meints *et al.*, 1986). Phycodnavirus replication and factory formation are not affected by a wide range of pharmacological disruptors of the cytoskeleton, including microtubule depolymerization by nocodazole and taxol, and depolymerization of actin by cytochalasin D (Nietfeldt *et al.*, 1992). In this way, they differ from factories formed by large DNA viruses such as ASFV, vaccinia, and FV3. The successful cultivation of algae in the laboratory has allowed studies of the intracellular sites of replication of large icosahedral MclaV-1 and HincV-1 viruses (Wolf *et al.*, 1998, 2000). These viruses produce a latent

infection that becomes apparent once the algae produce reproductive organs that become host to millions of virus particles. Replication of these viruses begins in the nucleus, but the first evidence for virus assembly is provided by the appearance of electron-dense bodies next to the nucleus at sites of breakdown in the nuclear envelope. Infection leads to stacking of ER cisternae that may provide membranes for virus envelopment. The dense bodies remain next to the nucleus in large inclusions, and take on the angular shape characteristic of capsid assembly seen for iridoviruses and ASFV. The nucleus eventually disintegrates, and the virus factory occupies most of the cytoplasm.

V. HERPESVIRUSES INDUCE NUCLEAR INCLUSIONS AND CYTOPLASMIC ASSEMBLY SITES

A. Herpesviruses

Herpesviruses are large dsDNA viruses with genomes ranging in size from 120 to 250 kbp. Herpesvirus genes are expressed in a regulated cascade starting with the immediate early α genes, then early β genes, and finally two subsets of late γ genes, γ_1 and γ_2. Complete herpesvirus particles have four main layers, the core containing DNA, an icosahedral capsid, a poorly defined layer of protein called tegument, and finally the viral envelope containing several glycoproteins. Genome synthesis and packaging and capsid assembly occur in inclusions in the nucleus. Nucleocapsids then obtain tegument in either the nucleus or the cytoplasm, or both, and the viral envelope is acquired exclusively in the cytoplasm [see Mettenleiter (2002) and Mettenleiter *et al.* (2006) for more thorough analysis]. The transfer of virus from the nucleus to the cytoplasm and acquisition of tegument appears well defined for human herpesvirus 6 (HHV-6) (Roffman *et al.*, 1990) but is controversial for the alphaherpesviruses (Campadelli-Fiume and Roizman, 2006; Mettenleiter and Minson, 2006). The subcellular organization of herpesvirus replication complexes formed in the nucleus during the early stages of productive infection has been described in considerable detail. The inclusions function as sites of virus replication and contain the virally encoded proteins and host proteins needed for virus replication. Interestingly, nuclear inclusions formed during herpes virus replication also contain cellular proteins involved in the control of DNA damage and repair. These may be recruited into inclusions in response to virus genome replication, and whether they are beneficial or detrimental to virus replication is a subject of considerable interest [reviewed by Everett (2006)].

B. Herpesvirus replication generates inclusions in the nucleus

Herpesviruses enter the cell by fusing their envelopes with the plasma membrane, whereon the naked nucleocapsids migrate to nuclear pores, possibly along microtubules (Granzow *et al.*, 1997; Sodeik *et al.*, 1997) [reviewed by Smith and Enquist (2002)]. Nuclear inclusions housing herpesvirus DNA replication are globular and can occupy the majority of the nucleus (de Bruyn Kops and Knipe, 1988; Randall and Dinwoodie, 1986; Taylor *et al.*, 2003). They are identified through the presence of the viral DNA-binding protein encoded by the UL29 gene, which is also known as infected cell protein 8 (ICP8). A minimum set of seven genes, UL5, UL8, UL9, UL29, UL30, UL42, and UL52, has been identified as necessary for viral DNA replication (Challberg, 1991). A plasmid transfection system has shown *in vitro* these can form globular nuclear compartments that are sites of 5-bromo-2′-deoxyuridine (BrdU) incorporation and visually are similar to those formed during infection (Lukonis and Weller, 1997; Zhong and Hayward, 1997). Nuclear inclusions organizing viral DNA replication have been followed in real time by a recombinant virus expressing a GFP-ICP8 fusion protein. Small inclusions merge with adjacent replication complexes and increase in size to form globular replication complexes, which eventually fill most of the nucleus (Randall and Dinwoodie, 1986; Taylor *et al.*, 2003).

1. Nuclear inclusions associated with herpesvirus replication are linked to ND10/PML Bodies

Replication compartments are formed from a number of different discrete foci that are induced early in infection and whose interrelatedness is not fully understood. The initial stages of productive herpesvirus infection are, however, intimately linked with nuclear structures called ND10 bodies (illustrated in Fig. 8) [Ishov and Maul (1996), Maul *et al.* (1996), review by Borden (2002)]. Live cell studies have shown that the immediate early regulatory protein ICP4, which binds viral DNA, forms discrete foci as early as 30-min postinfection (Fig. 8A). These initially appear close to the nuclear envelope, possibly at sites where the genome first enters the nucleus following capsid disassembly at nuclear pores (Everett and Murray, 2005), and are then seen throughout the nucleus (Everett *et al.*, 2004). ICP4 foci are seen juxtaposed to the ND10 marker promyelocytic leukemia protein (PML) some 60-min later. The early and late regulatory protein ICP27 is recruited to ICP4 foci 2-h postinfection and facilitates efficient early gene expression (Everett *et al.*, 2004). During the same period, the immediate early regulatory protein, ICP0, colocalizes with ND10 bodies, some of which are likely juxtaposed to ICP4 bodies (Everett *et al.*, 2003). ICP0 mediates the ubiquitin and/or SUMO-1-targeted proteasomal degradation of ND10 components (Chelbi-Alix and de Thé, 1999;

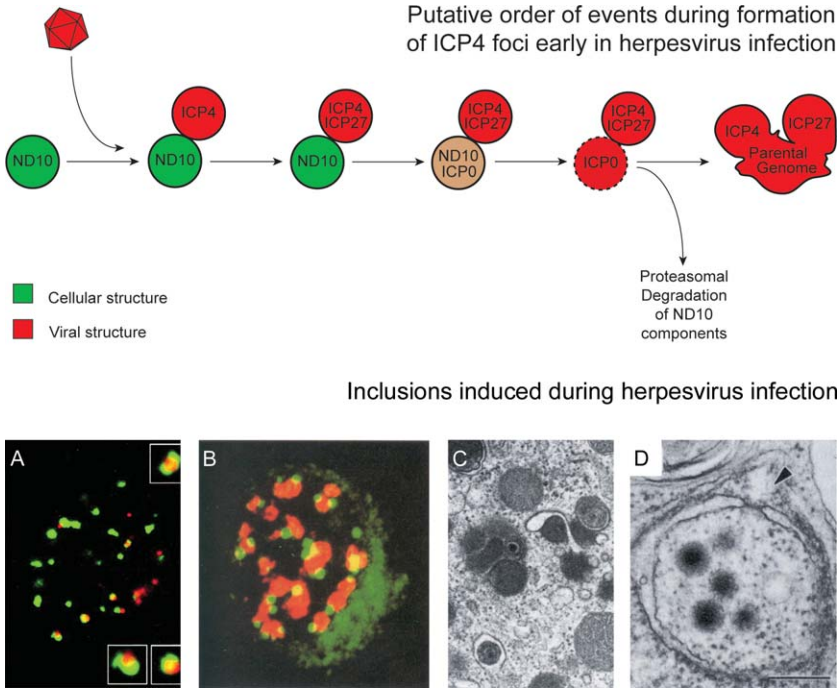

FIGURE 8 Schematic representing interaction of herpesvirus foci with ND10 bodies. (A) Cell expressing PML-ECFP (green) and infected with human herpesvirus-1-encoding ICP4-EYFP (red) 115-min postinfection. Boxes show zoomed sections demonstrating juxtaposition of ND10 and ICP4 bodies early during virus infection. Reprinted from Everett *et al.* (2003) with permission from American Society for Microbiology. (B) Cell infected with human herpesvirus 2 showing assemblons immunolabeled with ICP35 (red) and UL55 inclusions (green). Note juxtaposition of the two compartments. Reprinted from Yamada *et al.* (1998) with permission from Society for General Microbiology. (C) Electron micrograph of human herpesvirus 5-infected cell showing a section of a cytoplasmic assembly site. Note complete virus particle within a vacuole in bottom left-hand corner, dense bodies in center of image, including one budding into a membrane. Reprinted from Craighead *et al.* (1972) with permission from American Society for Microbiology. (D) Electron micrograph of a tegusome within a nucleus of a human herpesvirus-6-infected cell, note apparent continuity between tegusome and cytoplasm (arrowed). Reprinted from Roffman *et al.* (1990) with permission from American Society for Microbiology.

Everett, 2000; Everett and Maul, 1994; Everett *et al.*, 2004). Finally, parental genomes localize to ICP4 foci (Everett and Murray, 2005), and the ICP4 foci enlarge into structures that resemble early ICP8 replication compartments (Everett and Murray, 2005; Everett *et al.*, 2003). Formation of ICP8 replication compartments (Taylor and Knipe, 2004) is also known to

involve redistribution of ND10 bodies (Burkham *et al.*, 1998). The relationship between the early ICP4 structures associated with parental genome and the later ICP8 compartments associated with replication and production of progeny genome is not clear; however, ICP4 and ICP8 both localize to late replication compartments (Knipe *et al.*, 1987). A description of the relative and temporal distribution of the two proteins at early times awaits live cell studies following both proteins simultaneously.

2. Nuclear inclusions also form as sites of herpesvirus assembly: The assemblon

A second prominent nuclear inclusion induced by herpesvirus infection is the assemblon (Ward *et al.*, 1996b). This is the site where capsid proteins accumulate and assemble into nucleocapsids (Fig. 8B). The assembly of herpesvirus nucleocapsids has been researched in great detail at the ultrastructural level facilitated by a cell-free system for reconstituting the particles (Heymann *et al.*, 2003; Newcomb *et al.*, 1994, 1996). The mature herpesvirus capsid is icosahedral with a $T = 16$ symmetry and is composed of 150 hexons and 11 pentons of the major capsid protein UL19. The place of the remaining penton is taken by a 12-mer of the portal protein UL6, which by analogy with bacteriophage may be the site of genome entry. Nucleocapsids mature from fragile procapsids, through B capsids that lack DNA and contain the internal scaffold protein UL26.5, to C capsids that contain the viral genome.

The relationship between assemblons and sites of viral DNA replication has been a topic of some controversy as some reports show direct colocalization (Taus *et al.*, 1998), whereas others have shown a proximity (Nalwanga *et al.*, 1996; Ward *et al.*, 1996b), similar to that seen between ND10 bodies and ICP4 foci during the initial stages of infection. Clearly, the DNA has to reach the capsid in order to complete assembly, and it is likely that the different results are indicative of the dynamic interactions between different herpesvirus nuclear inclusions. The DNA cleavage and packaging proteins encoded by the UL17 and UL32 genes are required for colocalization of viral DNA and capsids (Lamberti and Weller, 1998; Taus *et al.*, 1998). Cells infected with a virus encoding a faulty UL32 gene exhibit nuclear localization of the capsid protein VP5 that is separate from replication sites (Lamberti and Weller, 1998). Similarly, in cells infected with mutants that lack functional UL17, the ICP8 protein fails to colocalize with ICP5 and ICP35 (Taus *et al.*, 1998). Actin also plays an important role in the correct nuclear subcompartmentalization of viral proteins. Infection with HHV-1[1] or suid herpesvirus-1[2] causes actin filaments to assemble in the nucleus, prior to the accumulation of capsid proteins

[1] Human herpesvirus 1 is herpes simplex virus 1 and human herpesvirus 2 is herpes simplex virus 2.
[2] Suid herpesvirus 1 is pseudorabiesvirus or Aujesky's disease virus.

(Feierbach *et al.*, 2006). Depolymerization of actin with latrunculin A inhibited correct nuclear compartmentalization of a representative capsid protein (VP26). VP26 colocalizes with the actin motor myosin Va (Feierbach *et al.*, 2006), and capsid movement within the nucleus is inhibited by the myosin motor inhibitor 2,3-butanedione monoxime (Forest *et al.*, 2005). This suggests that the organization of nuclear inclusions involved in herpesvirus assembly is dependent on cellular actin filaments, and it will be interesting to see if the organization of inclusions housing viral DNA replication sites is similarly dependent.

Other inclusion bodies have been reported in the nucleus of cells infected with herpesvirus. The tegument proteins VP22 and VP13/14 localize to inclusion bodies that align closely but do not overlap ICP0/ND10/ICP8 pre-replication complexes or assemblon inclusions (Hutchinson *et al.*, 2002). UL55 also localizes to structures that overlap but are distinct from assemblons and DNA replication complexes (Fig. 8B) (Yamada *et al.*, 1998). UL11 localizes to type IV and type V intranuclear dense bodies as well as virions and cytoplasmic ribbon structures (Baines *et al.*, 1995). The alkaline DNase encoded by the UL12 gene localizes to discrete electron-dense bodies within the nucleus that also contain B-36 nucleolar protein (Lopez-Iglesias *et al.*, 1988; Puvion-Dutilleul and Pichard, 1986). It is unknown whether these different structures are related to each other, whether they are homogenous accumulations of the individual herpesvirus protein(s), or if they are simply dead-end accumulations of protein.

3. Nuclear inclusions contain both viral and host proteins

A large number and variety of cellular proteins accumulate at nuclear sites of herpesvirus replication and assembly. A comprehensive proteomic analysis of ICP8 interacting proteins revealed more than 50 viral and cellular proteins that maybe recruited to DNA replication sites (Taylor and Knipe, 2004). A number of these interacting proteins were confirmed to localize to replication sites by microscopy experiments (Taylor and Knipe, 2004), and these as well as proteins identified in other studies (Leopardi *et al.*, 1997; Lukonis *et al.*, 1997; Quadt *et al.*, 2006; Wilcock and Lane, 1991) reveal that at least 23 cellular proteins are known to localize to nuclear inclusions involved in DNA replication during herpesvirus infection (Table I). The functions of these proteins span the expected functions of nuclear genes, including DNA replication, transcription, chromatin remodeling, DNA repair, recombination, and nonhomologous end joining. Of particular importance is the recruitment of RNA polymerase II, which is required to transcribe the viral genome. RNA polymerase II is phosphorylated during viral infection by ICP22 and ICP27, and the latter modification is required for targeting to replication complexes (Dai-Ju *et al.*, 2006).

The role of all of these cellular genes in the viral replication cycle is poorly understood; however, cells deficient in WRN, a recQ helicase

family member, produced reduced virus yields while cells lacking Ku86, part of a nonhomologous end-joining protein complex, produced increased yields of virus (Taylor and Knipe, 2004). The implication therefore is that some cellular proteins may be actively recruited to replication complexes to aid viral replication, and some may be recruited by the cell as part of an antiviral response or sequestered by the virus in inclusions to subvert their antiviral nature. PML is induced by interferon, suggesting an antiviral role. Many of the genes shown to be required for recruitment of PML to viral pre-replication sites are part of the minimal set of genes required to synthesize viral DNA. Recruitment of PML to viral replication sites is, for example, dependent on the viral DNA polymerase (UL30), the origin binding protein (UL9 gene) and the helicase–primase complex (UL5, UL8, and UL52) (Burkham *et al.*, 2001). Recent evidence has suggested that this may be the reason why ICP0 causes dispersal of PML early in infection. PML knockdown by short interfering RNAs (siRNA) facilitates productive replication of ICP0 null mutants of herpesvirus (Everett *et al.*, 2004, 2006); moreover, ICP0 null mutants are hypersensitive to interferon in a manner dependent on PML (Chee *et al.*, 2003). This is of particular importance because ICP0 plays a role in determining whether herpesvirus induces a quiescent or a productive, lytic infection (Mossman and Smiley, 2002).

C. Cytoplasmic inclusions form during late stages of herpesvirus tegumentation: The cytoplasmic assembly compartment

The tegument layer of alphaherpesviruses is composed of at least 15 different proteins (Mettenleiter, 2002). US11, UL17, UL47, UL48, and UL49 are components of the tegument, and all are localized to the nucleus (if not exclusively) during the productive life cycle of the virus (Fuchs *et al.*, 2002; Hutchinson *et al.*, 2002; Kopp *et al.*, 2002; Roller and Roizman, 1992; Taus *et al.*, 1998). UL48 may play a role in egress from the nucleus, though this has not been unequivocally established (Mossman *et al.*, 2000). Therefore, it is likely that some tegument proteins are acquired in or during viral egress from the nuclear inclusions. Recently, cytoplasmic aggresome-like structures have been described in cells infected with HHV-2.[1] These contain the major capsid protein, tegument proteins, envelope glycoproteins, and markers for the Golgi complex (Nozawa *et al.*, 2004). The latter finding is particularly interesting because herpesvirus envelopment involves membranes from the TGN (Mettenleiter *et al.*, 2006; Turcotte *et al.*, 2005). HHV-5[3] is a betaherpesvirus and late during infection produces a juxtanuclear ''assembly compartment'' that again contains tegument proteins (pp150, pp28, and pp68), the major capsid protein, and viral envelope

[3] Human herpesvirus 5 is human cytomegalovirus.

proteins (gB, gH, and gp65), suggesting a cytoplasmic site specialized for tegumentation and envelopment (Fig. 8C); (Adair *et al.*, 2002; Sanchez *et al.*, 2000). The precise role of the cytoplasmic assembly compartment is unclear. On the one hand, the concentration of glycoproteins and tegument proteins in one site may facilitate final stages of assembly prior to release from the cell. Interestingly, in common with aggresomes induced by ASFV and misfolded proteins, the cytoplasmic assembly compartment recruits chaperones and mitochondria and is dependent on microtubules and localizes to the microtubule organizing center.

At present, the assembly compartments are not considered to be *bona fide* aggresomes because they are not surrounded by a collapsed cage of intermediate filaments (Nozawa *et al.*, 2004; Sanchez *et al.*, 2000). It is nevertheless possible that these structures are related to aggresomes and are produced in response to a buildup of products resulting from nonproductive assembly pathways that occur late during infection. They may also contribute to the cytopathic effect seen in cells infected with HHV-5. HHV-5 infection results in cytomegaly characterized by increased cell size and intracellular water content. Cytomegaly and virus replication are both dependent on the presence of extracellular Na^+, and infection results in sequestration of the plasma membrane Na-K-Cl-cotransporter protein in large perinuclear structures that resemble the assembly compartment/viral aggresome (Maglova *et al.*, 2004). Electron-dense bodies can be seen by electron microscopy within the cytoplasmic assembly compartments induced during HHV-5 infection (Craighead *et al.*, 1972). Dense bodies are enveloped and obtain viral glycoproteins but do not contain DNA and are noninfectious. As can be seen in Fig. 8C, dense bodies bud into membranes and appear as oversized enveloped viral particles without a DNA containing core. Dense bodies exit the cell to become extracellular dense bodies (Craighead *et al.*, 1972). Interestingly, HHV-5 immediate early IE1 proteins also become associated with extracellular dense bodies despite no reported localization to their intracellular relations (Tsutsui and Yamazaki, 1991). Purified extracellular dense bodies are mostly composed of UL83 but have a full complement of viral glycoproteins (Irmiere and Gibson, 1983). The function of dense bodies remain unclear, and they may represent the end point of a nonproductive assembly pathway resulting from attempts to envelope capsids lacking genomes or may be used to deliver viral components to neighboring cells.

Interestingly, for human herpesvirus 6 (HHV-6), the tegument layer appears to be acquired within a dedicated structure that has been dubbed the tegusome (Roffman *et al.*, 1990). This work is based on electron microscopy of cells infected with HHV-6 and shows tegusomes as intranuclear membrane compartments that abut the nuclear envelope (Fig. 8D). Tegusomes may be cytoplasmic invaginations of the nuclear envelope into the nucleus because they appear to contain ribosomes and

are sometimes in continuity with the cytoplasm. Nucleocapsids appear to bud into the tegusome, capsids obtain a tegument layer, and then bud into cytoplasmic vacuoles where they acquire envelopes and exit the cell.

VI. NUCLEAR INCLUSIONS ARE FORMED BY SMALL DNA VIRUSES

A. Adenovirus

Adenovirus are medium-sized, nonenveloped dsDNA viruses with genomes ranging from 26 to 45 kbp in length and virions of the order of 70–100 nm in diameter. Like other DNA viruses, they have an ordered cascade of transcripts, early, delayed early, and late types having been described. Adenovirus transcripts are spliced to generate multiple transcripts from a given transcriptional unit. Viral replication occurs in the nucleus, and adenovirus infection was utilized extensively as a model system for exploring different nuclear subcompartments. A productive infection of lytic adenovirus induces profound rearrangement of existing subcompartments and the induction of several new ones within the host nucleus. A study on the localization of the human adenovirus5 IVa2 protein described 10 distinct nuclear and nucleolar subcompartments induced or associated with virus replication (Lutz *et al.*, 1996), and these are listed in Table I.

1. Structure and location of nuclear inclusions formed during adenovirus replication

Earlier studies carried out before markers for specific nuclear subcompartments were available have described the structures in terms of shape and location (see Table I). During the initial stage of infection, viral RNA (Puvion-Dutilleul *et al.*, 1992), single-stranded DNA (ssDNA), and dsDNA (Puvion-Dutilleul and Pichard, 1992) are all synthesized in small fibrillar regions termed early replication sites. By the intermediate stage of replication, the ssDNA is deposited in the center of these structures, while transcription and dsDNA synthesis occur on the outside and begin to form an inclusion. The inclusion has a characteristic doughnut shape, and has been called the fibrogranular network. At late stages of infection, dsDNA, viruses, and trace amounts of ssDNA appear in large viral inclusions (Besse and Puvion-Dutilleul, 1994; Puvion-Dutilleul and Pichard, 1992). Targeting of the initial replicon is dependent on a dCMP modification of the preterminal protein (pTP), which enables pTP to form a complex with the DNA polymerase and the genome (Temperley and Hay, 1992). PTP mediates targeting of the heterotrimeric complex to the nuclear matrix

(Fredman and Engler, 1993), possibly through an interaction with CAD (carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase) (Angeletti and Engler, 1998). Transcription and splicing are mediated by host proteins and viral RNA, and non-SNP RNA splicing factor, hnRNP C proteins, and RNA polymerase II all colocalize with viral RNA in nuclear inclusions. Splicing small nuclear ribonucleoproteins (snRNPs) colocalize with viral RNA but not replication foci (Pombo *et al.*, 1994), and snRNPs then move to interchromatin granules late in infection, which is blocked by mutations in E4 (Bridge *et al.*, 2003).

a. Rearrangement of host nuclear compartments during adenovirus replication

Like herpesvirus described above, adenovirus infection redistributes the components of ND10 bodies. Prior to infection, PML is associated with interchromatin granules but is redistributed to the fibrillogranular matrix within the nucleus along with SP100, another ND10 component (Carvalho *et al.*, 1995). Later in infection, PML is redistributed once again from the fibrillogranular matrix to clear amorphous inclusions and protein crystals (Puvion-Dutilleul *et al.*, 1995). Another study reported that SP100 and NDP55, but not PML, were relocated from ND10 bodies to viral inclusions (Doucas *et al.*, 1996). While this is confusing, it is clear that adenovirus employs multiple mechanisms to reorganize PML. The initial movement of PML, SP100, and NDP55 to the fibrillogranular matrix occurs prior to viral DNA synthesis and is dependent on the E4-ORF3 11-kDa protein (Carvalho *et al.*, 1995; Doucas *et al.*, 1996). It may also be mediated by E1A proteins that colocalize with PML (Carvalho *et al.*, 1995). E1B-55-kDa protein also colocalizes with PML early on in infection, then associates with the periphery of replication centers; these interactions are mediated by the ORF6 protein of the E4 transcriptional unit (Lethbridge *et al.*, 2003). Interestingly, E1B-55K and E4-ORF3 target the MRE11-RAD50-NBS1 (MRN) complex to aggresomes for degradation (Araujo *et al.*, 2005; Liu *et al.*, 2005). The MRN complex causes concatenation of viral DNA and inhibits packaging. Transport of MRN to the cytoplasm for degradation in aggresomes relieves this inhibition and facilitates production of infectious viruses. The later movement of PML from the fibrillogranular matrix to clear amorphous inclusions also appears important for replication. Movement is mediated by the IX gene product, and adenovirus encoding mutant IX do not create clear amorphous inclusions, have reduced growth, and are sensitive to PML overexpression (Rosa-Calatrava *et al.*, 2003). Interestingly, PKR is also redistributed to clear amorphous inclusions (Souquere-Besse *et al.*, 2002) during infection, as are pentons and hexons in the absence of the fiber gene; this suggests these structures may represent sites for sequestering excess viral proteins, and cellular proteins with potentially deleterious effects on the virus (Puvion-Dutilleul *et al.*, 1999).

B. Nuclear inclusions formed during polyomavirus and papillomavirus infection

Polyoma- and papillomaviruses are small double-stranded tumorigenic DNA viruses with genomes of 5 and 8 kbp, respectively. Replication and assembly of these two viruses follow similar strategies, and both involve ND10 bodies. The VP1 capsid protein of human polyomavirus JC is targeted to ND10 domains by VP2, VP3, and agnoprotein where they are assembled into virions (Shishido-Hara *et al.*, 2004). A similar process occurs during papillomavirus infection where the minor capsid protein, L2, is responsible for targeting capsomeres of the major capsid protein, L1, to ND10 domains (Florin *et al.*, 2002a). This process involves L2-induced redistribution of ND10 bodies by targeting SP100 for proteasomal degradation. At this point the cellular Daxx protein is recruited (Florin *et al.*, 2002b). Daax has multiple functions in the nucleus including transcriptional activation and modulating Fas-mediated apoptosis [reviewed by Salomoni and Khelifi (2006)]. Its role in virus replication is at present unclear.

One characteristic of papillomavirus infections is the appearance of nuclear and cytoplasmic inclusions in cells contained within warts. The size and number of inclusions is dependent on the type of papillomavirus and the site of infection. *Human papillomavirus 1* (HPV-1), for example, induces many small inclusions while HPV-4 induces one single inclusion that takes over most of the cytoplasm (Croissant *et al.*, 1985). *In vivo* these structures label strongly with antiserum raised against E4 gene products which are the 17-kDa E1^E4 and 16-kDa E4 proteins (Doorbar *et al.*, 1986; Rogel-Gaillard *et al.*, 1993). Inclusions can be induced in certain cell types *in vitro* by expressing E4 gene products. HPV-1 E4 staining reveals an initial association with the intermediate filament keratin and subsequent formation of inclusion bodies in the cytoplasm and nucleus (Roberts *et al.*, 2003; Rogel-Gaillard *et al.*, 1993). The HPV-1 cytoplasmic inclusions retain their association with keratin and appear to induce small cages surrounding E4 protein that are interconnected by keratin filaments (Roberts *et al.*, 2003). The E4 gene gives rise to two proteins, the 17-kDa E1^E4 which can induce cytoplasmic and nuclear inclusions whereas the 16-kDa E4 can induce inclusions solely in the cytoplasm (Rogel-Gaillard *et al.*, 1993). Interestingly, expression of E1^E4 gene product from HPV-16 induces the complete collapse of the keratin network, but not that of the microtubule or actin networks (Doorbar *et al.*, 1991). It is unclear what the role of the inclusions is in viral replication or the pathology of infection. However, HPV-1 E4 expression induces the redistribution of ND10 to the periphery of nuclear inclusions in cells in culture, and similar signals are seen *in vivo* (Roberts *et al.*, 2003). The temporal and functional connection between E4 and L1 redistribution of PML is unknown.

VII. VIRUS FACTORIES AND INCLUSIONS FORMED BY RNA VIRUSES

A. Reoviruses

Members of the Reoviridae family are dsRNA viruses with segmented genomes and include the clinically important rotavirus and orbiviruses that cause diseases in human and animals. Reoviruses are nonenveloped viruses with genome segments contained inside a virion ~80 nm in diameter. The genome is encapsidated by two protein shells, an outer capsid and an inner core shell. The core contains the RdRp, capping enzymes, and the dsRNA genome segments [reviewed in Yue and Shatkin (1998) and Furuichi and Shatkin (2000)]. Viruses are taken up by receptor-mediated endocytosis, the outer capsid is lost and the core is delivered into the cytoplasm. The core does not disassemble on entering cells and imports ribonucleoside triphosphates and *S*-adenosyl-l-methionine from the cytosol to synthesize and then export viral mRNAs. In this way the core particle functions as a self-contained transcriptional unit and as such represents the replication complex. Viral mRNA transcribed in the cytoplasm make viral proteins that eventually form large perinuclear inclusions, called virus factories that function as sites of further virus replication and assembly. The Reoviridae family contains 13 genera, and this chapter will concentrate on the two best characterized of these, the orthoreoviruses and rotaviruses.

1. Formation of factories during orthoreoviruses replication and assembly

a. The shape of orthoreovirus factories is determined by association with the cytoskeleton Orthoreoviruses contain 10 genome segments which are classed by size and then numbered, that is L1 is large segment 1. Large segments encode λ genes, medium (M) segments encode μ genes, and small (S) segments encode σ genes. Virus replication occurs in the cytoplasm in virus factories, and the majority of the virus-encoded proteins have been shown to localize completely or partially with factories (Table I). Early observations revealed that different strains of orthoreoviruses induced factories with different appearances; orthoreovirus type 1 Lang factories were filamentous while the factories of the Dearing isolate of orthoreovirus type 3 were globular (Fig. 9A and B) (Parker *et al.*, 2002). This difference maps precisely to a serine–proline switch at residue 208 of the μ2 core protein (Parker *et al.*, 2002). Control of the localization of orthoreovirus factories reflects the degree of association μ2 has with the microtubule network. Filamentous virus μ2 stabilizes microtubules to a greater relative degree than globular virus μ2, and depolymerizing microtubules with nocodazole convert filamentous factories to globular ones.

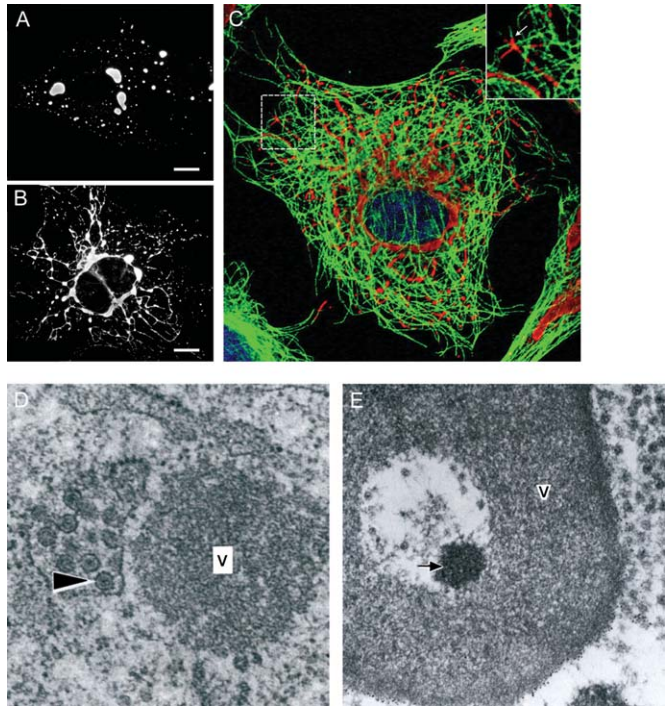

FIGURE 9 (A and B) Confocal images of orthoreovirus type 3 Dearing (A)- and type 1 Lang (B)-infected cells labeled with showing difference between globular and filamentous types of viral factories. (C) Confocal image of an infected cell immunolabeled with (red) and α-tubulin (green) showing relationship between filamentous factories and microtubules. Reprinted from Parker *et al.* (2002) with permission from American Society for Microbiology. (D) Electron micrograph showing rotavirus viroplasm (V) next to TLP within membranes derived from the ER (arrow). Reprinted from Petrie *et al.* (1984) with permission from Elsevier. (E) Doughnut-shaped rotavirus factory labeled with anti-NSP2 antibody showing electron-lucent center with electron-dense core (arrow) surrounded by viroplasm (V). Reprinted from Altenburg *et al.* (1980) with permission from Society for General Microbiology.

b. Virus nonstructural proteins determine orthoreovirus factory organization

Many of the events of orthoreovirus factory formation have been successfully reconstituted *in vitro*. A screen of orthoreovirus proteins revealed that μNS, σNS, and σ3 were the first viral proteins to localize with viral mRNA prior to the synthesis of progeny dsRNA (Antczak and Joklik, 1992). Subsequently, it was discovered that expression of the μNS protein of isolate Dearing in the absence of other viral proteins induced a phase-dense structure that was indistinguishable in appearance from that observed during wild-type infection (Broering *et al.*, 2002). The shape of

the artificial μNS inclusion could be altered from globular to filamentous by coexpressing a μ2 protein from a filamentous virus (Broering *et al.*, 2002). Similar experiments showed that coexpression of λ1, λ2, and σ2 core surface proteins with μNS caused them to localize to the μNS inclusion (Broering *et al.*, 2004). Furthermore, the shape of the μNS structure that the core proteins colocalized to could be altered to filamentous by coexpressing μ2 from a filamentous virus (Broering *et al.*, 2004). μNS can also recruit σNS, but not σ3, to artificial inclusions (Becker *et al.*, 2003), so other factors or conditions are necessary for complete assembly of an orthoreovirus factory. The precise domains involved in initiating factory formation are beginning to be elucidated. The minimal region of μNS necessary for inclusion like body formation *in vitro* is the region composed of 250 C-terminal amino acids of the 721 residue proteins (Broering *et al.*, 2005). Residues 1–11 of σNS are important for the interaction between σNS and RNA (Gillian and Nibert, 1998), and treatment with RNase dissociates a proportion of μNS from σNS in coimmunoprecipiation experiments (Miller *et al.*, 2003). Interaction between μNS and μ2 is dependent on residues 1–40 or 41 of μNS (Broering *et al.*, 2002) and residues 1–13 are necessary for interaction between μNS and σNS (Miller *et al.*, 2003). It is likely that factory formation occurs through an interaction between μNS and a σNS-RNA complex; this can then recruit μ2 that will determine the globular or filamentous localization of the factory and hence the localization of the other viral proteins.

Orthoreovirus factories are clearly intimately associated with the microtubule network (Fig. 9C) and have also been suggested to interact with intermediate filaments. Orthoreovirus type 3 infection induces a redistribution of vimentin and viral inclusions reported to contain filamentous structures (Sharpe *et al.*, 1982). It will be interesting to see if the *in vitro* factories induced by μNS can also alter the distribution of the intermediate filament network. Orthoreovirus factories are also ubiquitinated, and interestingly the nature of the factory determined the degree of ubiquitination; globular factories are prone to contain more ubiquitinated protein than filamentous ones (Miller *et al.*, 2004). Ubiquitination of orthoreovirus factories has been mapped to the μ2 protein but is independent of the filamentous/globular factory determinate of μ2, that is converting a filamentous factory to a globular factory does not lead to an increase in ubiquitinated μ2.

2. Formation of factories during rotavirus replication and assembly

a. Virus nonstructural proteins organize factory formation and virus assembly

Rotaviruses contain 11 genome segments of dsRNA and like the orthoreoviruses replicate in cytoplasmic factories. Rotavirus virions are composed of three protein layers. These are the core which contains the genome and polymerase, an inner capsid layer, and an outer capsid layer.

The core and inner capsid layer comprise the double-layered particle (DLP), while the addition of the third capsid layer forms the mature triple-layered particle (TLP). The acquisition of the third capsid layer occurs after the virus buds into the ER, and in doing so obtains a transient envelope. Rotavirus factories are composed of electron-dense viroplasm often in proximity to membranes derived from the ER (Fig. 9D) (Altenburg *et al.*, 1980). Viroplasm contains high levels of NSP2 (Fig. 9E) and NSP5 which are thought to coordinate assembly of the factory and recruitment of structural proteins such as the inner core protein VP2 and viral polymerase VP1. The factory also contains double-layered rotaviruses, whereas the ER membranes associated with the factory contain enveloped intermediates and TLP (arrowed in Fig. 9D). Virus factories grow in size and decrease in number during the course of infection as neighboring factories merge (Eichwald *et al.*, 2004). Rotavirus factories appear to have an internal structure, as their centers occasionally appear more electron lucent than the periphery, giving a doughnut-like appearance (Fig. 9E). Electron microscopy shows DLP at the periphery of the factory and this is (Altenburg *et al.*, 1980) consistent with fluorescent microscopy showing that the nonstructural protein NSP2 localizes to the center of the virus factory, whereas NSP5 and inner capsid protein VP6 localize to the periphery (Eichwald *et al.*, 2004; González *et al.*, 2000). These different localizations could have functional relevance because VP6 binds the ER-targeted NSP4 membrane protein and is implicated in the budding of DLPs into ER membranes associated with factories (Silvestri *et al.*, 2005). Therefore, a localization to the exterior of the factory may represent an organized progression of virus maturation from the interior of the viroplasm to the exterior. However, things are probably not that straightforward because VP6 is also part of the viral RNA complex along with NSP2 (Aponte *et al.*, 1996) which, as noted above, is localized to the center of the viroplasm.

Virus factory-like structures can be introduced *in vitro* by coexpressing NSP2 and NSP5 (Fabbretti *et al.*, 1999), and this is regulated by domains in the N- and C-termini (Fabbretti *et al.*, 1999) as well as the central portion of NSP5 (Eichwald *et al.*, 2002). The process is also dependent on phosphorylation of NSP5, possibly by cellular casein kinase II (Eichwald *et al.*, 2002). Structures similar to factories can also be induced by expressing the inner capsid protein VP6 *in vitro* (Nilsson *et al.*, 1998). These structures look similar to factories in the electron microscope but lack electron-lucent areas and DLPs. Interestingly, expression of VP6 of group A simian rotavirus SA-11 induced globular structures, whereas expression of VP6 from group C porcine rotavirus Cowden/AmC-1 induced filamentous structures (Nilsson *et al.*, 1998) analogous to the difference between type 1 and type 3 orthoretroviruses. It is not clear if the difference in factory shape is solely determined by VP6 and if this involves differences in association of the factory components with microtubules.

b. Virus factories organize viral RNA replication and translation The factory does provide the virus with a mechanism to organize viral RNAs. Positive-stranded viral RNA is utilized as the template for synthesizing progeny dsRNA genomes and as mRNA for translating viral proteins. Interestingly, siRNA-targeted degradation of NSP1 RNA blocks translation of the protein but does not block genome synthesis (Silvestri *et al.*, 2004). Furthermore, RNA synthesis occurs in factories, but viral RNA transcribed *in vitro* and introduced to infected cells after infection does not localize to factories. The implication of these experiments are that the factory enables rotavirus to sort viral RNA into separate pools, one within the factory to act as a template for the RNA polymerase and genome replication, and the other outside the factory where it translated on ribosomes to make viral proteins. It likely that this organization allows the virus factory to protect dsRNA genomes from antiviral responses.

B. Inclusions formed during arenavirus infection

Arenaviruses are negative-stranded RNA viruses that have two single-stranded genome segments which are packaged into 60- to 200-nm-diameter enveloped virions. Lassa, Junín, and Manchupo viruses are responsible for emerging hemorraghic fevers in humans. Arenaviruses induce moderately electron-dense inclusions in the cytoplasm that are composed of 20- to 25-nm-diameter granules identical to those seen within virus particles in the electron microscope (Murphy *et al.*, 1970). The granules represent host ribosomes and between 2 and 10 are packaged into virions (Pedersen, 1979). The inclusions increase in size and density during infection until cytopathic effects are observed in cells (Buckley, 1965; Buckley and Casals, 1970) and stain positive for viral antigens (Young *et al.*, 1987); however, it is unclear if they represent true virus factories. Arenavirus replication is believed to occur in the cytoplasm but also requires a nuclear step as limited growth is observed in enucleated cells (Banerjee *et al.*, 1975). The viral Z protein may play a role in this as it is sufficient *in vitro* to shuttle PML from the nucleus to cytoplasmic inclusion bodies as occurs *in vivo* (Borden *et al.*, 1998). N protein also localizes to discrete nuclear foci, as well as in the cytoplasm (Young *et al.*, 1987), but the relationship to ND10 bodies and Z protein is unknown.

C. Inclusions formed during rabies virus infection

Rabies virus is a neurotropic lyssavirus of the rhabdovirus family. Rhabdovirus virions are bullet-shaped $180 \times 75\,\text{nm}^2$ particles containing a single negative strand of RNA. Rabies induces two types of inclusion body *in vitro*, neither of which have been proven as replication sites. Negri bodies are induced by street rabies viruses in infected neurons of

the brain (Negri, 1903) and are a good indicator for the presence of an infection site in tissue (Jackson *et al.*, 2001). Different neuronal cell types appear to be more prone to Negri bodies (Jackson *et al.*, 2001). Negri bodies contain innerbodies (Negri, 1909) and electron microscopic studies suggest the subcompartments may be cytoplasmic material engulfed by the coalescence of several smaller Negri bodies (Matsumoto, 1970). The role of Negri bodies in infection is poorly understood. Initial EM observations showed virions localized to some bodies in some cells (Matsumoto *et al.*, 1974), and cytological staining show they contain genetic material, indicating they may be replication complexes. However, ^{3}H-thymidine or ^{3}H-uridine fail to label the structures, arguing against this conclusion (Matsumoto, 1970). Fixed (brain-adapted laboratory strains) rabies can infect nonneuronal cell lines and in these cell types induce fuchsin-stained cytoplasmic structures (FCPS) as well as Negri-like bodies (Ni *et al.*, 1996). FCPS increase in size during infection that correlates with cytopathic effects and are composed of rabies glycoprotein and matrix protein, whereas Negri bodies contain nucleocapsid (Ni *et al.*, 1996).

VIII. CONCLUSIONS

This chapter has described the changes to cell architecture that are induced during virus replication. We have focused on viruses that induce new cellular structures, such as inclusion bodies, virus factories, or replication complexes, to concentrate virus and host factors necessary for replication and assembly. Much progress has been made in identifying which cellular components are used to generate these structures, and in some cases specific virus proteins have been identified that are able to induce them. Virus inclusions often result in rearrangement of cellular membrane compartments and/or cytoskeleton. The functions of these organelles are carefully regulated in cells, and it is a challenge for the future to determine how viruses disrupt them for use as sites of replication and assembly. Changes in cellular architecture may represent bystander responses to the stress associated with virus infection, and some viruses may replicate perfectly well without them. Alternatively, viruses may have evolved to target key stages in the regulatory pathways that control organelle structure and function to generate sites that are essential for replication and assembly. Given the coevolution of viruses with the cells that carry them, changes in cell structure induced during infection are likely to involve a combination of the two. It is also important to appreciate that many of the structures that have been studied to date have been generated by infecting tissue culture cells with attenuated viruses, often with disregard to the host range and tropism. It is possible that in the natural setting, changes in cell structure induced by viruses

will be more subtle, particulary during persistent infections that occur without inflammation or cell lysis.

REFERENCES

Adair, R., Douglas, E. R., Maclean, J. B., Graham, S. Y., Aitken, J. D., Jamieson, F. E., and Dargan, D. J. (2002). The products of human cytomegalovirus genes UL23, UL24, UL43 and US22 are tegument components. *J. Gen. Virol.* **83:**1315–1324.

Ahlquist, P. (2006). Parallels among positive-strand RNA viruses, reverse-transcribing viruses and double-stranded RNA viruses. *Nat. Rev. Microbiol.* **4:**371–382.

Alcamí, A., Angulo, A., and Viñuela, E. (1993). Mapping and sequence of the gene encoding the African swine fever virion protein of M_r 11500. *J. Gen. Virol.* **74:**2317–2324.

Aldabe, R., Barco, A., and Carrasco, L. (1996). Membrane permeabilization by poliovirus proteins 2B and 2BC. *J. Biol. Chem.* **271:**23134–23137.

Almazán, F., Tscharke, D. C., and Smith, G. L. (2001). The vaccinia virus superoxide dismutase-like protein (A45R) is a virion component that is nonessential for virus replication. *J. Virol.* **75:**7018–7029.

Alonso, C., Miskin, J., Hernáez, B., Fernandez-Zapatero, P., Soto, L., Cantó, C., Rodríguez-Crespo, I., Dixon, L., and Escribano, J. M. (2001). African swine fever virus protein p54 interacts with the microtubular motor complex through direct binding to light-chain dynein. *J. Virol.* **75:**9819–9827.

Altenburg, B. C., Graham, D. Y., and Estes, M. K. (1980). Ultrastructural study of rotavirus replication in cultured cells. *J. Gen. Virol.* **46:**75–85.

Andrés, G., Simón-Mateo, C., and Viñuela, E. (1997). Assembly of African swine fever virus: Role of polyprotein pp220. *J. Virol.* **71:**2331–2341.

Andrés, G., García-Escudero, R., Simón-Mateo, C., and Viñuela, E. (1998). African swine fever virus is enveloped by a two-membraned collapsed cisterna derived from the endoplasmic reticulum. *J. Virol.* **72:**8988–9001.

Andrés, G., Alejo, A., Simón-Mateo, C., and Salas, M. L. (2001). African swine fever virus protease, a new viral member of the SUMO-1-specific protease family. *J. Biol. Chem.* **276:**780–787.

Angeletti, P. C., and Engler, J. A. (1998). Adenovirus preterminal protein binds to the CAD enzyme at active sites of viral DNA replication on the nuclear matrix. *J. Virol.* **72:** 2896–2904.

Antczak, J. B., and Joklik, W. K. (1992). Reovirus genome segment assortment into progeny genomes studied by the use of monoclonal antibodies directed against reovirus proteins. *Virology* **187:**760–776.

Aponte, C., Poncet, D., and Cohen, J. (1996). Recovery and characterization of a replicase complex in rotavirus-infected cells by using a monoclonal antibody against NSP2. *J. Virol.* **70:**985–991.

Araujo, F. D., Stracker, T. H., Carson, C. T., Lee, D. V., and Weitzman, M. D. (2005). Adenovirus type 5 E4orf3 protein targets the Mre11 complex to cytoplasmic aggresomes. *J. Virol.* **79:**11382–11391.

Avitabile, E., Digaeta, S., Torrisi, M. R., Ward, P. L., Roizman, B., and Campadellifiume, G. (1995). Redistribution of microtubules and Golgi-apparatus in herpes-simplex virus-infected cells and their role in viral exocytosis. *J. Virol.* **69:**7472–7482.

Baines, J. D., Jacob, R. J., Simmerman, L., and Roizman, B. (1995). The herpes simplex virus 1 UL11 proteins are associated with cytoplasmic and nuclear membranes and with nuclear bodies of infected cells. *J. Virol.* **69:**825–833.

Banerjee, S. N., Buchmeier, M., and Rawls, W. E. (1975). Requirement of cell nucleus for the replication of an arenavirus. *Intervirology* **6:**190–196.

Battista, M. C., Bergamini, G., Boccuni, M. C., Campanini, F., Ripalti, A., and Landini, M. P. (1999). Expression and characterization of a novel structural protein of human cytomegalovirus, pUL25. *J. Virol.* **73:**3800–3809.

Beaud, G., and Beaud, R. (1997). Preferential virosomal location of underphosphorylated H5R protein synthesized in vaccinia virus-infected cells. *J. Gen. Virol.* **78:**3297–3302.

Becker, M. M., Goral, M. I., Hazelton, P. R., Baer, G. S., Rodgers, S. E., Brown, E. G., Coombs, K. M., and Dermody, T. S. (2001). Reovirus σNS protein is required for nucleation of viral assembly complexes and formation of viral inclusions. *J. Virol.* **75:**1459–1475.

Becker, M. M., Peters, T. R., and Dermody, T. S. (2003). Reovirus σNS and μNS proteins form cytoplasmic inclusion structures in the absence of viral infection. *J. Virol.* **77:**5948–5963.

Belov, G. A., Fogg, M. H., and Ehrenfeld, E. (2005). Poliovirus proteins induce membrane association of GTPase ADP-ribosylation factor. *J. Virol.* **79:**7207–7216.

Belov, G. A., Altan-Bonnet, N., Kovtunovych, G., Jackson, C. L., Lippincott-Schwartz, J., and Ehrenfeld, E. (2007). Hijacking components of the secretory pathway for replication of poliovirus RNA. *J. Virol.* **81:**558–567.

Benson, S. D., Bamford, J. K. H., Bamford, D. H., and Burnett, R. M. (2004). Does common architecture reveal a viral lineage spanning all three domains of life? *Mol. Cell* **16:**673–685.

Barnard, E. C., Brown, G., and Stow, N. D. (1997). Deletion mutants of the herpes simplex virus type 1 UL8 protein: Effect on DNA synthesis and ability to interact with and influence the intracellular localization of the UL5 and UL52 proteins. *Virology* **237:**97–106.

Berstein, H. D., and Baltimore, D. (1988). Poliovirus mutant that contains a cold-sensitive defect in viral RNA synthesis. *J. Virol.* **62:**2922–2928.

Besse, S., and Puvion-Dutilleul, F. (1994). High resolution localization of replicating viral genome in adenovirus-infected HeLa cells. *Eur. J. Cell. Biol.* **63:**269–279.

Betakova, T., Wolffe, E. J., and Moss, B. (2000). The vaccinia virus A14.5L gene encodes a hydrophobic 53-amino-acid virion membrane protein that enhances virulence in mice and is conserved among vertebrate poxviruses. *J. Virol.* **74:**4085–4092.

Bienz, K., Egger, D., Rasser, Y., and Bossart, W. (1983). Intracellular distribution of poliovirus proteins and the induction of virus-specific cytoplasmic structures. *Virology* **131:**39–48.

Bienz, K., Egger, D., and Pasamontes, L. (1987). Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography. *Virology* **160:**220–226.

Bonifacino, J. S., and Glick, B. S. (2004). The mechanisms of vesicle budding and fusion. *Cell* **116:**153–166.

Borca, M. V., Irusta, P. M., Kutish, G. F., Carrillo, C., Afonso, C. L., Burrage, T., Neilan, J. G., and Rock, D. L. (1996). A structural DNA binding protein of African swine fever virus with similarity to bacterial histone-like proteins. *Arch. Virol.* **141:**301–313.

Borden, K. L. B. (2002). Pondering the promyelocytic leukemia protein (PML) puzzle: Possible functions for PML nuclear bodies. *Mol. Cell Biol.* **22:**5259–5269.

Borden, K. L. B., Campbell Dwyer, E. J., and Salvato, M. S. (1998). An arenavirus RING (zinc-binding) protein binds the oncoprotein promyelocyte leukemia protein (PML) and relocates PML nuclear bodies to the cytoplasm. *J. Virol.* **72:**758–766.

Borgese, N., Francolini, M., and Snapp, E. (2006). Endoplasmic reticulum architecture: Structures in flux. *Curr. Opin. Cell Biol.* **18:**358–364.

Bosher, J., Dawson, A., and Hay, R. T. (1992). Nuclear factor I is specifically targeted to discrete subnuclear sites in adenovirus type 2-infected cells. *J. Virol.* **66:**3140–3150.

Bost, A. G., Prentice, E., and Denison, M. R. (2001). Mouse hepatitis virus replicase protein complexes are translocated to sites of M protein accumulation in the ERGIC at late times of infection. *Virology* **285:**21–29.

Brass, V., Bieck, E., Montserret, R., Wolk, B., Hellings, J. A., Blum, H. E., Penin, F., and Moradpour, D. (2002). An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. *J. Biol. Chem.* **277:**8130–8139.

Bridge, E., Mattsson, K., Aspegren, A., and Sengupta, A. (2003). Adenovirus early region 4 promotes the localization of splicing factors and viral RNA in late-phase interchromatin granule clusters. *Virology* **311:**40–50.

Broering, T. J., Parker, J. S., Joyce, P. L., Kim, J., and Nibert, M. L. (2002). Mammalian reovirus nonstructural protein μNS forms large inclusions and colocalizes with reovirus microtubule-associated protein μ2 in transfected cells. *J. Virol.* **76:**8285–8297.

Broering, T. J., Kim, J., Miller, C. L., Piggott, C. D., Dinoso, J. B., Nibert, M. L., and Parker, J. S. (2004). Reovirus nonstructural protein μNS recruits viral core surface proteins and entering core particles to factory-like inclusions. *J. Virol.* **78:**1882–1892.

Broering, T. J., Arnold, M. M., Miller, C. L., Hurt, J. A., Joyce, P. L., and Nibert, M. L. (2005). Carboxyl-proximal regions of reovirus nonstructural protein μNS necessary and sufficient for forming factory-like inclusions. *J. Virol.* **79:**6194–6206.

Brookes, S. M., Dixon, L. K., and Parkhouse, R. M. E. (1996). Assembly of African swine fever virus: Quantitative ultrastructural analysis *in vitro* and *in vivo*. *Virology* **224:**84–92.

Brookes, S. M., Hyatt, A. D., Wise, T., and Parkhouse, R. M. E. (1998a). Intracellular virus DNA distribution and the acquisition of the nucleoprotein core during African swine fever virus particle assembly: Ultrastructural *in situ* hybridisation and DNase-gold labelling. *Virology* **249:**175–188.

Brookes, S. M., Sun, H., Dixon, L. K., and Parkhouse, R. M. E. (1998b). Characterization of African swine fever virion proteins j5R and j13L: Immuno-localization in virus particles and assembly. *J. Gen. Virol.* **79:**1179–1188.

Broyles, S. S., Liu, X., Zhu, M., and Kremer, M. (1999). Transcription factor YY1 is a vaccinia virus late promoter activator. *J. Biol. Chem.* **274:**35662–35667.

Buckley, S. M. (1965). Junín and Tacaribe work in HeLa cells. *Am. J. Trop. Med. Hyg.* **14:**792–794.

Buckley, S. M., and Casals, J. (1970). Lassa fever, a new virus disease of man from West Africa. 3. Isolation and characterization of the virus. *Am. J. Trop. Med. Hyg.* **19:**680–691.

Burkham, J., Coen, D. M., and Weller, S. K. (1998). ND10 protein PML is recruited to herpes simplex virus type 1 prereplicative sites and replication compartments in the presence of viral DNA polymerase. *J. Virol.* **72:**10100–10107.

Burkham, J., Coen, D. M., Hwang, C. B., and Weller, S. K. (2001). Interactions of herpes simplex virus type 1 with ND10 and recruitment of PML to replication compartments. *J. Virol.* **75:**2353–2367.

Cairns, J. (1960). The initiation of vaccinia infection. *Virology* **11:**603–623.

Campadelli-Fiume, G., and Roizman, B. (2006). The egress of herpesviruses from cells: The unanswered questions. *J. Virol.* **80:**6716–6717.

Carrascosa, J. L., González, P., Carrascosa, A. L., Garciá-Barenno, B., Enjuanes, L., and Viñuela, E. (1986). Localization of structural proteins in African swine fever virus particles by immunoelectron microscopy. *J. Virol.* **58:**377–384.

Carter, G. C., Rodger, G., Murphy, B. J., Law, M., Krauss, O., Hollinshead, M., and Smith, G. L. (2003). Vaccinia virus cores are transported on microtubules. *J. Gen. Virol.* **84:**2443–2458.

Carvalho, T., Seeler, J. S., Ohman, K., Jordan, P., Pettersson, U., Akusjarvi, G., Carmo-Fonseca, M., and Dejean, A. (1995). Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. *J. Cell Biol.* **131:**45–56.

Cashdollar, L. W. (1994). Characterization and structural localization of the reovirus λ3 protein. *Res. Virol.* **145:**277–285.

Chacón, M. R., Almazán, F., Nogal, M. L., Viñuela, E., and Rodríguez, J. F. (1995). The African swine fever virus IAP homolg is a late structural polypeptide. *Virology* **214:**670–674.

Challberg, M. D. (1991). Herpes simplex virus DNA replication. *Semin. Virol.* **2:**247.

Chee, A. V., Lopez, P., Pandolfi, P. P., and Roizman, B. (2003). Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. *J. Virol.* **77:**7101–7105.

Chelbi-Alix, M. K., and de Thé, H. (1999). Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. *Oncogene* **18:**935–941.

Chen, M., Goorha, R., and Murti, K. G. (1986). Interaction of frog virus 3 with the cytomatrix. IV. Phosphorylation of vimentin precedes the reorganization of intermediate filaments around the virus assembly sites. *J. Gen. Virol.* **67:**915–922.

Cherry, S., Kunte, A., Wang, H., Coyne, C., Rawson, R. B., and Perrimon, N. (2006). COPI activity coupled with fatty acid biosynthesis is required for viral replication. *PLoS Pathog.* **2:**e102.

Chinchar, V. G., Goorha, R., and Granoff, A. (1984). Early proteins are required for the formation of frog virus 3 assembly sites. *Virology* **135:**148–156.

Chiu, W. L., Szajner, P., Moss, B., and Chang, W. (2005). Effects of a temperature sensitivity mutation in the J1R protein component of a complex required for vaccinia virus assembly. *J. Virol.* **79:**8046–8056.

Cho, M. W., Teterina, N., Egger, D., Bienz, K., and Ehrenfeld, E. (1994). Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells. *Virology* **202:**129–145.

Choe, S. S., Dodd, D. A., and Kirkegaard, K. (2005). Inhibition of cellular protein secretion by picornaviral 3A proteins. *Virology* **337:**18–29.

Cobbold, C., and Wileman, T. (1998). The major structural protein of African swine fever virus, p73, is packaged into large structures, indicative of viral capsid or matrix precursors, on the endoplasmic reticulum. *J. Virol.* **72:**5215–5223.

Cobbold, C., Whittle, J. T., and Wileman, T. (1996). Involvement of the endoplasmic reticulum in the assembly and envelopment of African swine fever virus. *J. Virol.* **70:**8382–8390.

Cobbold, C., Brookes, S. M., and Wileman, T. (2000). Biochemical requirements of virus wrapping by the endoplasmic reticulum calcium store during envelopment of African swine fever virus. *J. Virol.* **74:**2151–2160.

Cobbold, C., Windsor, M., and Wileman, T. (2001). A virally encoded chaperone specialized for folding of the major capsid protein of African swine fever virus. *J. Virol.* **75:**7221–7229.

Condit, R. C., Moussatche, N., Traktman, P., and Karl Maramorosch, A. J. S. (2006). In a nutshell: Structure and assembly of the vaccinia virion. *In* ''Advances in Virus Research,'' Vol. 66, pp. 31–124. Academic Press, San Diego.

Cornell, C. T., Kiosses, W. B., Harkins, S., and Whitton, J. L. (2006). Inhibition of protein trafficking by coxsackievirus b3: Multiple viral proteins target a single organelle. *J. Virol.* **80:**6637–6647.

Craighead, J. E., Kanich, R. E., and Almeida, J. D. (1972). Nonviral microbodies with viral antigenicity produced in cytomegalovirus-infected cells. *J. Virol.* **10:**766–775.

Croissant, O., Breitburd, F., and Orth, G. (1985). Specificity of cytopathic effect of cutaneous human papillomaviruses. *Clin. Dermatol.* **3:**43–55.

Crotty, S., Saleh, M. C., Gitlin, L., Beske, O., and Andino, R. (2004). The poliovirus replication machinery can escape inhibition by an antiviral drug that targets a host cell protein. *J. Virol.* **78:**3378–3386.

Cuconati, A., Molla, A., and Wimmer, E. (1998). Brefeldin A inhibits cell-free, *de novo* synthesis of poliovirus. *J. Virol.* **72:**6456–6464.

Cudmore, S., Blasco, R., Vincentelli, R., Esteban, M., Sodeik, B., Griffiths, G., and Krijnse Locker, J. (1996). A vaccinia virus core protein, p39, is membrane associated. *J. Virol.* **70:**6909–6921.

da Fonseca, F. G., Wolffe, E. J., Weisberg, A., and Moss, B. (2000). Characterization of the vaccinia virus H3L envelope protein: Topology and posttranslational membrane insertion via the C-terminal hydrophobic tail. *J. Virol.* **74:**7508–7517.

Dai-Ju, J. Q., Li, L., Johnson, L. A., and Sandri-Goldin, R. M. (2006). ICP27 interacts with the C-terminal domain of RNA polymerase II and facilitates its recruitment to herpes simplex

virus 1 transcription sites, where it undergoes proteasomal degradation during infection. *J. Virol.* **80:**3567–3581.

Dal Monte, P., Pignatelli, S., Zini, N., Maraldi, N. M., Perret, E., Prevost, M. C., and Landini, M. P. (2002). Analysis of intracellular and intraviral localization of the human cytomegalovirus UL53 protein. *J. Gen. Virol.* **83:**1005–1012.

Dales, S., and Siminovitch, L. (1961). The development of vaccinia virus in Earle's L strain cells as examined by electron microscopy. *J. Biophys. Biochem. Cytol.* **10:**475–503.

Dales, S., Eggers, H. J., Tamm, I., and Palade, G. E. (1965a). Electron microscopic study of the formation of poliovirus. *Virology* **26:**379–389.

Dales, S., Gomatos, P. J., and Hsu, K. C. (1965b). The uptake and development of reovirus in strain L cells followed with labeled viral ribonucleic acid and ferritin-antibody conjugates. *Virology* **25:**193–211.

Darlington, R. W., Granoff, A., and Breeze, D. C. (1966). Viruses and renal carcinoma of Rana pipiens: II. Ultrastructural studies and sequential development of virus isolated from normal and tumor tissue. *Virology* **29:**149–156.

Davis, R. E., and Mathews, C. K. (1993). Acidic C terminus of vaccinia virus DNA-binding protein interacts with ribonucleotide reductase. *Proc. Natl. Acad. Sci. USA* **90:**745–749.

de Bruyn Kops, A., and Knipe, D. M. (1988). Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. *Cell* **55:**857–868.

de Bruyn Kops, A., Uprichard, S. L., Chen, M., and Knipe, D. M. (1998). Comparison of the intranuclear distributions of herpes simplex virus proteins involved in various viral functions. *Virology* **252:**162–178.

De Silva, F. S., and Moss, B. (2005). Origin-independent plasmid replication occurs in vaccinia virus cytoplasmic factories and requires all five known poxvirus replication factors. *Virol J.* **2:**23–34.

Deitz, S. B., Dodd, D. A., Cooper, S., Parham, P., and Kirkegaard, K. (2000). MHC I-dependent antigen presentation is inhibited by poliovirus protein 3A. *Proc. Natl. Acad. Sci. USA* **97:**13790–13795.

Deretic, V. (2005). Autophagy in innate and adaptive immunity. *Trends Immunol.* **26:**523–528.

Dodd, D. A., Giddings, T. H., Jr., and Kirkegaard, K. (2001). Poliovirus 3A protein limits interleukin-6 (IL-6), IL-8, and beta interferon secretion during viral infection. *J. Virol.* **75:**8158–8165.

Doedens, J. R., and Kirkegaard, K. (1995). Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A. *EMBO J.* **14:**894–907.

Doedens, J. R., Giddings, T. H., Jr., and Kirkegaard, K. (1997). Inhibition of endoplasmic reticulum-to-Golgi traffic by poliovirus protein 3A: Genetic and ultrastructural analysis. *J. Virol.* **71:**9054–9064.

Doglio, L., De Marco, A., Schleich, S., Roos, N., and Krijnse Locker, J. (2002). The vaccinia virus E8R gene product: A viral membrane protein that is made early in infection and packaged into the virions' core. *J. Virol.* **76:**9773–9786.

Domi, A., and Beaud, G. (2000). The punctate sites of accumulation of vaccinia virus early proteins are precursors of sites of viral DNA synthesis. *J. Gen. Virol.* **81:**1231–1235.

Doorbar, J., Campbell, D., Grand, R. J., and Gallimore, P. H. (1986). Identification of the human papilloma virus-1a E4 gene products. *EMBO J.* **5:**355–362.

Doorbar, J., Ely, S., Sterling, J., McLean, C., and Crawford, L. (1991). Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature* **352:**824–827.

Doucas, V., Ishov, A. M., Romo, A., Juguilon, H., Weitzman, M. D., Evans, R. M., and Maul, G. G. (1996). Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. *Genes Dev.* **10:**196–207.

Dubuisson, J., Penin, F., and Moradpour, D. (2002). Interaction of hepatitis C virus proteins with host cell membranes and lipids. *Trends Cell Biol.* **12:**517–523.

Dyster, L. M., and Niles, E. G. (1991). Genetic and biochemical characterization of vaccinia virus genes D2L and D3R which encode virion structural proteins. *Virology* **182:**455–467.
Egger, D., and Bienz, K. (2005). Intracellular location and translocation of silent and active poliovirus replication complexes. *J. Gen. Virol.* **86:**707–718.
Egger, D., Pasamontes, L., Bolten, R., Boyko, V., and Bienz, K. (1996). Reversible dissociation of the poliovirus replication complex: Functions and interactions of its components in viral RNA synthesis. *J. Virol.* **70:**8675–8683.
Egger, D., Teterina, N., Ehrenfeld, E., and Bienz, K. (2000). Formation of the poliovirus replication complex requires coupled viral translation, vesicle production, and viral RNA synthesis. *J. Virol.* **74:**6570–6580.
Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H. E., Moradpour, D., and Bienz, K. (2002). Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76:**5974–5984.
Eichwald, C., Vascotto, F., Fabbretti, E., and Burrone, O. R. (2002). Rotavirus NSP5: Mapping phosphorylation sites and kinase activation and viroplasm localization domains. *J. Virol.* **76:**3461–3470.
Eichwald, C., Rodriguez, J. F., and Burrone, O. R. (2004). Characterization of rotavirus NSP2/NSP5 interactions and the dynamics of viroplasm formation. *J. Gen. Virol.* **85:**625–634.
El-Hage, N., and Luo, G. (2003). Replication of hepatitis C virus RNA occurs in a membrane-bound replication complex containing nonstructural viral proteins and RNA. *J. Gen. Virol.* **84:**2761–2769.
Elazar, M., Cheong, K. H., Liu, P., Greenberg, H. B., Rice, C. M., and Glenn, J. S. (2003). Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. *J. Virol.* **77:**6055–6061.
Epifano, C., Krijnse-Locker, J., Salas, M. L., Rodríguez, J. M., and Salas, J. (2006). The African swine fever virus non-structural protein pB602L is required for the formation of the icosahedral capsid of the virus particle. *J. Virol.* **80:**12260–12270.
Evans, E., Klemperer, N., Ghosh, R., and Traktman, P. (1995). The vaccinia virus D5 protein, which is required for DNA replication, is a nucleic acid-independent nucleoside triphosphatase. *J. Virol.* **69:**5353–5361.
Everett, R. D. (2000). ICP0 induces the accumulation of colocalizing conjugated ubiquitin. *J. Virol.* **74:**9994–10005.
Everett, R. D. (2006). Interactions between DNA viruses, ND10 and the DNA damage response. *Cell. Microbiol.* **8:**365–374.
Everett, R. D., and Maul, G. G. (1994). HSV-1 IE protein Vmw110 causes redistribution of PML. *EMBO J.* **13:**5062–5069.
Everett, R. D., and Murray, J. (2005). ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection. *J. Virol.* **79:**5078–5089.
Everett, R. D., Sourvinos, G., and Orr, A. (2003). Recruitment of herpes simplex virus type 1 transcriptional regulatory protein ICP4 into foci juxtaposed to ND10 in live, infected cells. *J. Virol.* **77:**3680–3689.
Everett, R. D., Sourvinos, G., Leiper, C., Clements, J. B., and Orr, A. (2004). Formation of nuclear foci of the herpes simplex virus type 1 regulatory protein ICP4 at early times of infection: Localization, dynamics, recruitment of ICP27, and evidence for the *de novo* induction of ND10-like complexes. *J. Virol.* **78:**1903–1917.
Everett, R. D., Rechter, S., Papior, P., Tavalai, N., Stamminger, T., and Orr, A. (2006). PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. *J. Virol.* **80:**7995–8005.
Fabbretti, E., Afrikanova, I., Vascotto, F., and Burrone, O. R. (1999). Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm-like structures *in vivo*. *J. Gen. Virol.* **80:**333–339.

Feierbach, B., Piccinotti, S., Bisher, M., Denk, W., and Enquist, L. W. (2006). Alpha-herpesvirus infection induces the formation of nuclear actin filaments. *PLoS Pathog.* **2:**e85.

Florin, L., Sapp, C., Streeck, R. E., and Sapp, M. (2002a). Assembly and translocation of papillomavirus capsid proteins. *J. Virol.* **76:**10009–10014.

Florin, L., Schafer, F., Sotlar, K., Streeck, R. E., and Sapp, M. (2002b). Reorganization of nuclear domain 10 induced by papillomavirus capsid protein l2. *Virology* **295:**97–107.

Forest, T., Barnard, S., and Baines, J. D. (2005). Active intranuclear movement of herpesvirus capsids. *Nat. Cell. Biol.* **7:**429–431.

Fredman, J. N., and Engler, J. A. (1993). Adenovirus precursor to terminal protein interacts with the nuclear matrix *in vivo* and *in vitro*. *J. Virol.* **67:**3384–3395.

Froshauer, S., Kartenbeck, J., and Helenius, A. (1988). Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J. Cell. Biol.* **107:**2075–2086.

Fuchs, W., Granzow, H., Klupp, B. G., Kopp, M., and Mettenleiter, T. C. (2002). The UL48 tegument protein of pseudorabies virus is critical for intracytoplasmic assembly of infectious virions. *J. Virol.* **76:**6729–6742.

Furuichi, Y., and Shatkin, A. J. (2000). Viral and cellular mRNA capping: Past and prospects. *Adv. Virus Res.* **55:**135–184.

Galindo, I., Viñuela, E., and Carrascosa, A. L. (2000). Characterization of the African swine fever virus protein p49: A new late structural polypeptide. *J. Gen. Virol.* **81:**59–65.

García-Beato, R., Salas, M. L., Viñuela, E., and Salas, J. (1992). Role of the host cell nucleus in the replication of African swine fever virus DNA. *Virology* **188:**637–649.

Gazina, E. V., Mackenzie, J. M., Gorrell, R. J., and Anderson, D. A. (2002). Differential requirements for COPI coats in formation of replication complexes among three genera of Picornaviridae. *J. Virol.* **76:**11113–11122.

Gillian, A. L., and Nibert, M. L. (1998). Amino terminus of reovirus nonstructural protein σNS is important for ssRNA binding and nucleoprotein complex formation. *Virology* **240:**1–11.

González, R. A., Espinosa, R., Romero, P., López, S., and Arias, C. F. (2000). Relative localization of viroplasmic and endoplasmic reticulum-resident rotavirus proteins in infected cells. *Arch. Virol.* **145:**1963–1973.

Goodrich, L. D., Schaffer, P. A., Dorsky, D. I., Crumpacker, C. S., and Parris, D. S. (1990). Localization of the herpes simplex virus type 1 65-kilodalton DNA-binding protein and DNA polymerase in the presence and absence of viral DNA synthesis. *J. Virol.* **64:**5738–5749.

Goorha, R. (1982). Frog virus 3 DNA replication occurs in two stages. *J. Virol.* **43:**519–528.

Gorbalenya, A. E., Koonin, E. V., and Wolf, Y. I. (1990). A new superfamily of putative NTP-binding domains encoded by genomes of small DNA and RNA viruses. *FEBS Lett.* **262:**145–148.

Gorbalenya, A. E., Enjuanes, L., Ziebuhr, J., and Snijder, E. J. (2006). Nidovirales: Evolving the largest RNA virus genome. *Virus Res.* **117:**17–37.

Gosert, R., Kanjanahaluethai, A., Egger, D., Bienz, K., and Baker, S. C. (2002). RNA replication of mouse hepatitis virus takes place at double-membrane vesicles. *J. Virol.* **76:**3697–3708.

Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H. E., Bienz, K., and Moradpour, D. (2003). Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J. Virol.* **77:**5487–5492.

Goshima, F., Daikoku, T., Yamada, H., Oshima, S., Tsurumi, T., and Nishiyama, Y. (1998). Subcellular localization of the US3 protein kinase of herpes simplex virus type 2. *Arch. Virol.* **143:**613–622.

Granja, A. G., Nogal, M. L., Hurtado, C., Salas, J., Salas, M. L., Carrascosa, A. L., and Revilla, Y. (2004). Modulation of p53 cellular function and cell death by African swine fever virus. *J. Virol.* **78:**7165–7174.

Granoff, A., Came, P. E., and Breeze, D. C. (1966). Viruses and renal carcinoma of Rana pipiens. I. The isolation and properties of virus from normal and tumor tissue. *Virology* **29:**133–148.

Granzow, H., Weiland, F., Jöns, A., Klupp, B. G., Karger, A., and Mettenleiter, T. C. (1997). Ultrastructural analysis of the replication cycle of pseudorabies virus in cell culture: A reassessment. *J. Virol.* **71:**2072–2082.

Griffiths, G., Roos, N., Schleich, S., and Locker, J. K. (2001). Structure and assembly of intracellular mature vaccinia virus: Thin-section analyses. *J. Virol.* **75:**11056–11070.

Guarnieri, G. (1893). Recherches sur la pathologie et étiologie de l'infection vaccinique et varioleuse. *Arch. Ital. de Biol.* **19:**195.

Hagiwara, Y., Komoda, K., Yamanaka, T., Tamai, A., Meshi, T., Funada, R., Tsuchiya, T., Naito, S., and Ishikawa, M. (2003). Subcellular localization of host and viral proteins associated with tobamovirus RNA replication. *EMBO J.* **22:**344–353.

Heath, C. M., Windsor, M., and Wileman, T. (2001). Aggresomes resemble sites specialized for virus assembly. *J. Cell Biol.* **153:**449–456.

Heath, C. M., Windsor, M., and Wileman, T. (2003). Membrane association facilitates the correct processing of pp220 during production of the major matrix proteins of African swine fever virus. *J. Virol.* **77:**1682–1690.

Herrero-Martínez, E., Roberts, K. L., Hollinshead, M., and Smith, G. L. (2005). Vaccinia virus intracellular enveloped virions move to the cell periphery on microtubules in the absence of the A36R protein. *J. Gen. Virol.* **86:**2961–2968.

Herzer, K., Falk, C. S., Encke, J., Eichhorst, S. T., Ulsenheimer, A., Seliger, B., and Krammer, P. H. (2003). Upregulation of major histocompatibility complex class I on liver cells by hepatitis C virus core protein via p53 and TAP1 impairs natural killer cell cytotoxicity. *J. Virol.* **77:**8299–8309.

Heuser, J. (2005). Deep-etch EM reveals that the early poxvirus envelope is a single membrane bilayer stabilized by a geodetic ''honeycomb'' surface coat. *J. Cell Biol.* **169:**269–283.

Heymann, J. B., Cheng, N., Newcomb, W. W., Trus, B. L., Brown, J. C., and Steven, A. C. (2003). Dynamics of herpes simplex virus capsid maturation visualized by time-lapse cryo-electron microscopy. *Nat. Struct. Biol.* **10:**334–341.

Higashi, N. (1973). Electron microscopy of viruses in thin sections of cells grown in culture. *Prog. Med. Virol.* **15:**331–379.

Hingamp, P. M., Arnold, J. E., Mayer, R. J., and Dixon, L. K. (1992). A ubiquitin conjugating enzyme encoded by African swine fever virus. *EMBO J.* **11:**361–366.

Hollinshead, M., Vanderplasschen, A., Smith, G. L., and Vaux, D. J. (1999). Vaccinia virus intracellular mature virions contain only one lipid membrane. *J. Virol.* **73:**1503–1517.

Hsiao, J. C., Chao, C. C., Young, M. J., Chang, Y. T., Cho, E. C., and Chang, W. (2006). A poxvirus host range protein, CP77, binds to a cellular protein, HMG20A, and regulates its dissociation from the vaccinia virus genome in CHO-K1 cells. *J. Virol.* **80:**7714–7728.

Huang, X. H., Huang, Y. H., Yuan, X. P., and Zhang, Q. Y. (2006). Electron microscopic examination of the viromatrix of Rana grylio virus in a fish cell line. *J. Virol. Methods* **133:**117–123.

Hugle, T., Fehrmann, F., Bieck, E., Kohara, M., Krausslich, H. G., Rice, C. M., Blum, H. E., and Moradpour, D. (2001). The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. *Virology* **284:**70–81.

Hung, J. J., Chung, C. S., and Chang, W. (2002). Molecular chaperone Hsp90 is important for vaccinia virus growth in cells. *J. Virol.* **76:**1379–1390.

Husain, M., and Moss, B. (2003). Evidence against an essential role of COPII-mediated cargo transport to the endoplasmic reticulum-Golgi intermediate compartment in the formation of the primary membrane of vaccinia virus. *J. Virol.* **77:**11754–11766.

Hutchinson, I., Whiteley, A., Browne, H., and Elliott, G. (2002). Sequential localization of two herpes simplex virus tegument proteins to punctate nuclear dots adjacent to ICP0 domains. *J. Virol.* **76:**10365–10373.

Ichihashi, Y., Matsumoto, S., and Dales, S. (1971). Biogenesis of poxviruses: Role of A-type inclusions and host cell membranes in virus dissemination. *Virology* **46:**507–532.

Irmiere, A., and Gibson, W. (1983). Isolation and characterization of a noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus. *Virology* **130:**118–133.

Irurzun, A., Perez, L., and Carrasco, L. (1992). Involvement of membrane traffic in the replication of poliovirus genomes: Effects of brefeldin A. *Virology* **191:**166–175.

Ishov, A. M., and Maul, G. G. (1996). The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. *J. Cell Biol.* **134:**815–826.

Iyer, L. M., Aravind, L., and Koonin, E. V. (2001). Common origin of four diverse families of large eukaryotic DNA viruses. *J. Virol.* **75:**11720–11734.

Jackson, A. C., Ye, H., Ridaura-Sanz, C., and Lopez-Corella, E. (2001). Quantitative study of the infection in brain neurons in human rabies. *J. Med. Virol.* **65:**614–618.

Jackson, W. T., Giddings, Jr., T. H., Taylor, M. P., Mulinyawe, S., Rabinovitch, M., Kopito, R. R., and Kirkegaard, K. (2005). Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol.* **3:**e156.

Jahedi, S., Markovitz, N. S., Filatov, F., and Roizman, B. (1999). Colocalization of the herpes simplex virus 1 UL4 protein with infected cell protein 22 in small, dense nuclear structures formed prior to onset of DNA synthesis. *J. Virol.* **73:**5132–5138.

Johnston, J. A., Ward, C. L., and Kopito, R. R. (1998). Aggresomes: A cellular response to misfolded proteins. *J. Cell Biol.* **143:**1883–1898.

Jouvenet, N., and Wileman, T. (2005). African swine fever virus infection disrupts centrosome assembly and function. *J. Gen. Virol.* **86:**589–594.

Jouvenet, N., Monaghan, P., Way, M., and Wileman, T. (2004). Transport of African swine fever virus from assembly sites to the plasma membrane is dependent on microtubules and conventional kinesin. *J. Virol.* **78:**7990–8001.

Jouvenet, N., Windsor, M., Rietdorf, J., Hawes, P., Monaghan, P., Way, M., and Wileman, T. (2006). African swine fever virus induces filopodia-like projections at the plasma membrane. *Cell. Microbiol.* **8:**1803–1811.

Kato, S., Takahashi, M., Kameyama, S., and Kamahora, J. (1959). A study on the morphological and cyto-immunological relationship between the inclusions of variola, cowpox, rabbitpox, vaccinia (variola origin) and vaccinia IHD and a consideration of the term ''Guarnieri body.'' *Biken's J.* **2:**353.

Kato, K., Daikoku, T., Goshima, F., Kume, H., Yamaki, K., and Nishiyama, Y. (2000). Synthesis, subcellular localization and VP16 interaction of the herpes simplex virus type 2 UL46 gene product. *Arch. Virol.* **145:**2149–2162.

Kawaguchi, Y., Kovacs, J. J., McLaurin, A., Vance, J. M., Ito, A., and Yao, T. P. (2003). The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell* **115:**727–738.

Kim, J. E., Song, W. K., Chung, K. M., Back, S. H., and Jang, S. K. (1999). Subcellular localization of hepatitis C viral proteins in mammalian cells. *Arch. Virol.* **144:**329–343.

King, N. J., and Kesson, A. M. (1988). Interferon-independent increases in class I major histocompatibility complex antigen expression follow flavivirus infection. *J. Gen. Virol.* **69:**2535–2543.

Kirkegaard, K., Taylor, M. P., and Jackson, W. T. (2004). Cellular autophagy: Surrender, avoidance and subversion by microorganisms. *Nat. Rev. Microbiol.* **2:**301–314.

Knipe, D. M., Senechek, D., Rice, S. A., and Smith, J. L. (1987). Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. *J. Virol.* **61:**276–284.

Knox, C., Moffat, K., Ali, S., Ryan, M., and Wileman, T. (2005). Foot-and-mouth disease virus replication sites form next to the nucleus and close to the Golgi apparatus, but exclude marker proteins associated with host membrane compartments. *J. Gen. Virol.* **86:**687–696.

Konan, K. V., Giddings, T. H., Jr., Ikeda, M., Li, K., Lemon, S. M., and Kirkegaard, K. (2003). Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus. *J. Virol.* **77:**7843–7855.

Kondratova, A. A., Neznanov, N., Kondratov, R. V., and Gudkov, A. V. (2005). Poliovirus protein 3A binds and inactivates LIS1, causing block of membrane protein trafficking and deregulation of cell division. *Cell Cycle* **4:**1403–1410.

Kopp, M., Klupp, B. G., Granzow, H., Fuchs, W., and Mettenleiter, T. C. (2002). Identification and characterization of the pseudorabies virus tegument proteins UL46 and UL47: Role for UL47 in virion morphogenesis in the cytoplasm. *J. Virol.* **76:**8820–8833.

Krijnse-Locker, J., Schleich, S., Rodriguez, D., Goud, B., Snijder, E. J., and Griffiths, G. (1996). The role of a 21-kDa viral membrane protein in the assembly of vaccinia virus from the intermediate compartment. *J. Biol. Chem.* **271:**14950–14958.

Krogerus, C., Egger, D., Samuilova, O., Hyypia, T., and Bienz, K. (2003). Replication complex of human parechovirus 1. *J. Virol.* **77:**8512–8523.

La Scola, B., Audic, S., Robert, C., Jungang, L., de Lamballerie, X., Drancourt, M., Birtles, R., Claverie, J.-M., and Raoult, D. (Scola 2003). A giant virus in amoebae. *Science* **299:**2033.

Lackner, C. A., and Condit, R. C. (2000). Vaccinia virus gene A18R DNA helicase is a transcript release factor. *J. Biol. Chem.* **275:**1485–1494.

Lamberti, C., and Weller, S. K. (1998). The herpes simplex virus type 1 cleavage/packaging protein, UL32, is involved in efficient localization of capsids to replication compartments. *J. Virol.* **72:**2463–2473.

Landini, M. P., Severi, B., Cenacchi, G., Lazzarotto, T., Lindenmeier, W., and Necker, A. (1991). Human cytomegalovirus structural components: Intracellular and intraviral localization of p38. *Virus Res.* **19:**189–198.

Lee, J. Y., Marshall, J. A., and Bowden, D. S. (1994). Characterization of rubella virus replication complexes using antibodies to double-stranded RNA. *Virology* **200:**307–312.

Leopardi, R., Ward, P. L., Ogle, W. O., and Roizman, B. (1997). Association of herpes simplex virus regulatory protein ICP22 with transcriptional complexes containing EAP, ICP4, RNA polymerase II, and viral DNA requires posttranslational modification by the U(L)13 proteinkinase. *J. Virol.* **71:**1133–1139.

Lethbridge, K. J., Scott, G. E., and Leppard, K. N. (2003). Nuclear matrix localization and SUMO-1 modification of adenovirus type 5 E1b 55K protein are controlled by E4 Orf6 protein. *J. Gen. Virol.* **84:**259–268.

Levine, B., and Klionsky, D. J. (2004). Development by self-digestion: Molecular mechanisms and biological functions of autophagy. *Dev. Cell* **6:**463–477.

Lindenbach, B. D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A. J., McKeating, J. A., Lanford, R. E., Feinstone, S. M., Major, M. E., Leroux-Roels, G., and Rice, C. M. (2006). Cell culture-grown hepatitis C virus is infectious *in vivo* and can be recultured *in vitro*. *Proc. Natal. Acad. Sci. USA* **103:**3805–3809.

Lippincott-Schwartz, J., Roberts, T. H., and Hirschberg, K. (2000). Secretory protein trafficking and organelle dynamics in living cells. *Annu. Rev. Cell Dev. Biol.* **16:**557–589.

Liptak, L. M., Uprichard, S. L., and Knipe, D. M. (1996). Functional order of assembly of herpes simplex virus DNA replication proteins into prereplicative site structures. *J. Virol.* **70:**1759–1767.

Liu, Y., King, N., Kesson, A., Blanden, R. V., and Mullbacher, A. (1989). Flavivirus infection up-regulates the expression of class I and class II major histocompatibility antigens on and enhances T cell recognition of astrocytes in vitro. *J. Neuroimmunol.* **21:**157–168.

Liu, Y., Shevchenko, A., Shevchenko, A., and Berk, A. J. (2005). Adenovirus exploits the cellular aggresome response to accelerate inactivation of the MRN complex. *J. Virol.* **79:**14004–14016.

Lobigs, M., Mullbacher, A., and Lee, E. (2004). Evidence that a mechanism for efficient flavivirus budding upregulates MHC class I. *Immunol. Cell Biol.* **82:**184–188.

Lopez-Iglesias, C., Puvion-Dutilleul, F., Cebrian, J., and Christensen, M. E. (1988). Herpes simplex virus type 1-induced modifications in the distribution of nucleolar B-36 protein. *Eur. J. Cell Biol.* **46:**259–269.

Lukonis, C. J., and Weller, S. K. (1997). Formation of herpes simplex virus type 1 replication compartments by transfection: Requirements and localization to nuclear domain 10. *J. Virol.* **71:**2390–2399.

Lukonis, C. J., Burkham, J., and Weller, S. K. (1997). Herpes simplex virus type 1 prereplicative sites are a heterogeneous population: Only a subset are likely to be precursors to replication compartments. *J. Virol.* **71:**4771–4781.

Lutz, P., Puvion-Dutilleul, F., Lutz, Y., and Kedinger, C. (1996). Nucleoplasmic and nucleolar distribution of the adenovirus IVa2 gene product. *J. Virol.* **70:**3449–3460.

Lyle, J. M., Bullitt, E., Bienz, K., and Kirkegaard, K. (2002). Visualization and functional analysis of RNA-dependent RNA polymerase lattices. *Science* **296:**2218–2222.

Mackenzie, J. (2005). Wrapping things up about virus RNA replication. *Traffic* **6:**967–977.

Mackenzie, J. M., and Westaway, E. G. (2001). Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. *J. Virol.* **75:**10787–10799.

Mackenzie, J. M., Khromykh, A. A., Jones, M. K., and Westaway, E. G. (1998). Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. *Virology* **245:**203–215.

Mackenzie, J. M., Jones, M. K., and Westaway, E. G. (1999). Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. *J. Virol.* **73:**9555–9567.

Magliano, D., Marshall, J. A., Bowden, D. S., Vardaxis, N., Meanger, J., and Lee, J. Y. (1998). Rubella virus replication complexes are virus-modified lysosomes. *Virology* **240:**57–63.

Maglova, L. M., Crowe, W. E., and Russell, J. M. (2004). Perinuclear localization of Na-K-Cl-cotransporter protein after human cytomegalovirus infection. *Am. J. Physiol. Cell Physiol.* **286:**C1324–C1334.

Mallardo, M., Schleich, S., and Krijnse Locker, J. (2001). Microtubule-dependent organization of vaccinia virus core-derived early mRNAs into distinct cytoplasmic structures. *Mol. Biol. Cell* **12:**3875–3891.

Mallardo, M., Leithe, E., Schleich, S., Roos, N., Doglio, L., and Krijnse Locker, J. (2002). Relationship between vaccinia virus intracellular cores, early mRNAs, and DNA replication sites. *J. Virol.* **76:**5167–5183.

Markovitz, N. S., and Roizman, B. (2000). Small dense nuclear bodies are the site of localization of herpes simplex virus 1 U(L)3 and U(L)4 proteins and of ICP22 only when the latter protein is present. *J. Virol.* **74:**23–28.

Martin, K. H., Franke, C. A., and Hruby, D. E. (1999). Novel acylation of poxvirus A-type inclusion proteins. *Virus Res.* **60:**147–157.

Martinez-Pomares, L., Simon-Mateo, C., Lopez-Otin, C., and Viñuela, E. (1997). Characterization of the African swine fever virus structural protein p14.5 a DNA binding protein. *Virology* **229:**201–211.

Matsumoto, S. (1970). Rabies virus. *Adv. Virus Res.* **16:**257–301.

Matsumoto, S., Schneider, L. G., Kawai, A., and Yonezawa, T. (1974). Further studies on the replication of rabies and rabies-like viruses in organized cultures of mammalian neural tissues. *J. Virol.* **14:**981–996.

Maul, G. G., Ishov, A. M., and Everett, R. D. (1996). Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. *Virology* **217:**67–75.

Maynell, L. A., Kirkegaard, K., and Klymkowsky, M. W. (1992). Inhibition of poliovirus RNA synthesis by brefeldin A. *J. Virol.* **66:**1985–1994.

McCrossan, M., Windsor, M., Ponnambalam, S., Armstrong, J., and Wileman, T. (2001). The *trans* Golgi network is lost from cells infected with African swine fever virus. *J. Virol.* **75:**11755–11765.

McKelvey, T. A., Andrews, S. C., Miller, S. E., Ray, C. A., and Pickup, D. J. (2002). Identification of the orthopoxvirus p4c gene, which encodes a structural protein that directs intracellular mature virus particles into A-type inclusions. *J. Virol.* **76:**11216–11225.

Meints, R. H., Lee, K., and Van Etten, J. L. (1986). Assembly site of the virus PBCV-1 in a Chlorella-like green alga: Ultrastructural studies. *Virology* **154:**240–245.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J. Virol.* **76:**1537–1547.

Mettenleiter, T. C., and Minson, T. (2006). Egress of alphaherpes viruses. *J. Virol.* **80:**1610–1611.

Mettenleiter, T. C., Klupp, B. G., and Granzow, H. (2006). Herpesvirus assembly: A tale of two membranes. *Curr. Opin. Microbiol.* **9:**423–429.

Miller, D. J., and Ahlquist, P. (2002). Flock house virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. *J. Virol.* **76:**9856–9867.

Miller, C. L., Broering, T. J., Parker, J. S., Arnold, M. M., and Nibert, M. L. (2003). Reovirus σNS protein localizes to inclusions through an association requiring the μNS amino terminus. *J. Virol.* **77:**4566–4576.

Miller, C. L., Parker, J. S., Dinoso, J. B., Piggott, C. D., Perron, M. J., and Nibert, M. L. (2004). Increased ubiquitination and other covariant phenotypes attributed to a strain- and temperature-dependent defect of reovirus core protein μ2. *J. Virol.* **78:**10291–10302.

Millns, A. K., Carpenter, M. S., and DeLange, A. M. (1994). The vaccinia virus-encoded uracil DNA glycosylase has an essential role in viral DNA replication. *Virology* **198:**504–513.

Miner, J. N., and Hruby, D. E. (1989). Rifampicin prevents virosome localization of L65, an essential vaccinia virus polypeptide. *Virology* **170:**227–237.

Moffat, K., Howell, G., Knox, C., Belsham, G. J., Monaghan, P., Ryan, M. D., and Wileman, T. (2005). Effects of foot-and-mouth disease virus nonstructural proteins on the structure and function of the early secretory pathway: 2BC but not 3A blocks endoplasmic reticulum-to-Golgi transport. *J. Virol.* **79:**4382–4395.

Moffat, K., Knox, C., Howell, G., Clark, S. J., Yang, Y. G., Belsham, G. J., Ryan, M., and Wileman, T. (2007). Inhibition of the secretory pathway by the Foot-and-Mouth disease virus 2BC protein is reproduced by co-expression of 2B with 2C and the site of inhibition is determined by the subcellular location of 2C. *J. Virol.* **81:**1129–1139.

Mohandas, A. R., and Dales, S. (1995). Involvement of spicules in the formation of vaccinia virus envelopes elucidated by a conditional lethal mutant. *Virology* **214:**494–502.

Momburg, F., Mullbacher, A., and Lobigs, M. (2001). Modulation of transporter associated with antigen processing (TAP)-mediated peptide import into the endoplasmic reticulum by flavivirus infection. *J. Virol.* **75:**5663–5671.

Monaghan, P., Cook, H., Hawes, P., Simpson, J., and Tomley, F. (2003). High-pressure freezing in the study of animal pathogens. *J. Microsc.* **212:**62–70.

Monaghan, P., Cook, H., Jackson, T., Ryan, M., and Wileman, T. (2004). The ultrastructure of the developing replication site in foot-and-mouth disease virus-infected BHK-38 cells. *J. Gen. Virol.* **85:**933–946.

Moradpour, D., Brass, V., Bieck, E., Friebe, P., Gosert, R., Blum, H. E., Bartenschlager, R., Penin, F., and Lohmann, V. (2004). Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. *J. Virol.* **78:**13278–13284.

Morgan, C., Ellison, S. A., Rose, H. M., and Moore, D. H. (1954). Structure and development of viruses observed in the electron microscope. II. Vaccinia and fowl pox viruses. *J. Exp. Med.* **100:**301.

Moss, B., Rosenblum, E. N., Katz, E., and Grimley, P. M. (1969). Rifampicin: A specific inhibitor of vaccinia virus assembly. *Nature* **224:**1280–1284.

Mossman, K. L., and Smiley, J. R. (2002). Herpes simplex virus ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication. *J. Virol.* **76:**1995–1998.

Mossman, K. L., Sherburne, R., Lavery, C., Duncan, J., and Smiley, J. R. (2000). Evidence that herpes simplex virus VP16 is required for viral egress downstream of the initial envelopment event. *J. Virol.* **74:**6287–6299.

Moura Nunes, J. F., Vigário, J. D., and Terrinha, A. M. (1975). Ultrastructural study of African swine fever virus replication in cultures of swine bone marrow cells. *Arch. Virol.* **49:**59–66.

Murata, T., Goshima, F., Daikoku, T., Inagaki-Ohara, K., Takakuwa, H., Kato, K., and Nishiyama, Y. (2000). Mitochondrial distribution and function in herpes simplex virus-infected cells. *J. Gen. Virol.* **81:**401–406.

Murcia-Nicolas, A., Bolbach, G., Blais, J. C., and Beaud, G. (1999). Identification by mass spectroscopy of three major early proteins associated with virosomes in vaccinia virus-infected cells. *Virus Res.* **59:**1–12.

Murphy, F. A., Webb, P. A., Johnson, K. M., Whitfield, S. G., and Chappell, W. A. (1970). Arenoviruses in Vero cells: Ultrastructural studies. *J. Virol.* **6:**507–518.

Murti, K., and Goorha, R. (1983). Interaction of frog virus-3 with the cytoskeleton. I. Altered organization of microtubules, intermediate filaments, and microfilaments. *J. Cell Biol.* **96:**1248–1257.

Murti, K., and Goorha, R. (1989). Synthesis of frog virus 3 proteins occurs on intermediate filament-bound polyribosomes. *Biol. Cell* **65:**205–214.

Murti, K. G., Goorha, R., and Klymkowsky, M. W. (1988). A functional role for intermediate filaments in the formation of frog virus 3 assembly sites. *Virology* **162:**264–269.

Murti, K. G., Davis, D. S., and Kitchingman, G. R. (1990). Localization of adenovirus-encoded DNA replication proteins in the nucleus by immunogold electron microscopy. *J. Gen. Virol.* **71:**2847–2857.

Nalwanga, D., Rempel, S., Roizman, B., and Baines, J. D. (1996). The UL 16 gene product of herpes simplex virus 1 is a virion protein that colocalizes with intranuclear capsid proteins. *Virology* **226:**236–242.

Negri, A. (1903). Beitrag zum Studium de Aetiologie der Tollwuth. *Z. Hyg. Infektionskr.* **43:**507–528.

Negri, A. (1909). Über die morphologie und den entwicklungszyklus des parasiten der tollwut. *Z. Hyg. Infektionskr.* **63:**421–443.

Nerenberg, B. T., Taylor, J., Bartee, E., Gouveia, K., Barry, M., and Fruh, K. (2005). The poxviral RING protein p28 is a ubiquitin ligase that targets ubiquitin to viral replication factories. *J. Virol.* **79:**597–601.

Netherton, C., Rouiller, I., and Wileman, T. (2004). The subcellular distribution of multigene family 110 proteins of African swine fever virus is determined by differences in C-terminal KDEL endoplasmic reticulum retention motifs. *J. Virol.* **78:**3710–3721.

Netherton, C. L., McCrossan, M. C., Denyer, M., Ponnambalam, S., Armstrong, J., Takamatsu, H. H., and Wileman, T. E. (2006). African swine fever virus causes microtubule dependent dispersal of the trans-Golgi network and slows delivery of membrane protein to the plasma membrane. *J. Virol.* **80:**11385–11392.

Newcomb, W. W., Homa, F. L., Thomsen, D. R., Ye, Z., and Brown, J. C. (1994). Cell-free assembly of the herpes simplex virus capsid. *J. Virol.* **68:**6059–6063.

Newcomb, W. W., Homa, F. L., Thomsen, D. R., Booy, F. P., Trus, B. L., Steven, A. C., Spencer, J. V., and Brown, J. C. (1996). Assembly of the herpes simplex virus capsid: Characterization of intermediates observed during cell-free capsid formation. *J. Mol. Biol.* **263:**432–446.

Neznanov, N., Kondratova, A., Chumakov, K. M., Angres, B., Zhumabayeva, B., Agol, V. I., and Gudkov, A. V. (2001). Poliovirus protein 3A inhibits tumor necrosis factor (TNF)-induced apoptosis by eliminating the TNF receptor from the cell surface. *J. Virol.* **75:**10409–10420.

Ng, M. L. (1987). Ultrastructural studies of Kunjin virus-infected Aedes albopictus cells. *J. Gen. Virol.* **68:**577–582.

Ni, Y., Iwatani, Y., Morimoto, K., and Kawai, A. (1996). Studies on unusual cytoplasmic structures which contain rabies virus envelope proteins. *J. Gen. Virol.* **77:**2137–2147.

Nietfeldt, J. W., Lee, K., and Van Etten, J. L. (1992). Chlorella virus PBCV-1 replication is not affected by cytoskeletal disruptors. *Intervirology* **33:**116–120.

Nilsson, M., von Bonsdorff, C. H., Weclewicz, K., Cohen, J., and Svensson, L. (1998). Assembly of viroplasm and virus-like particles of rotavirus by a Semliki Forest virus replicon. *Virology* **242:**255–265.

Novoa, R. R., Calderita, G., Arranz, R., Fontana, J., Granzow, H., and Risco, C. (2005). Virus factories: Associations of cell organelles for viral replication and morphogenesis. *Biol. Cell* **97:**147–172.

Nozawa, N., Daikoku, T., Yamauchi, Y., Takakuwa, H., Goshima, F., Yoshikawa, T., and Nishiyama, Y. (2002). Identification and characterization of the UL7 gene product of herpes simplex virus type 2. *Virus Genus* **24:**257–266.

Nozawa, N., Yamauchi, Y., Ohtsuka, K., Kawaguchi, Y., and Nishiyama, Y. (2004). Formation of aggresome-like structures in herpes simplex virus type 2-infected cells and a potential role in virus assembly. *Exp. Cell Res.* **299:**486–497.

O'Donnell, V. K., Pacheco, J. M., Henry, T. M., and Mason, P. W. (2001). Subcellular distribution of the foot-and-mouth disease virus 3A protein in cells infected with viruses encoding wild-type and bovine-attenuated forms of 3A. *Virology* **287:**151–162.

Oh, J., and Broyles, S. S. (2005). Host cell nuclear proteins are recruited to cytoplasmic vaccinia virus replication complexes. *J. Virol.* **79:**12852–12860.

Ojeda, S., Senkevich, T. G., and Moss, B. (2006). Entry of vaccinia virus and cell-cell fusion require a highly conserved cysteine-rich membrane protein encoded by the A16L gene. *J. Virol.* **80:**51–61.

Oliveros, M., García-Escudero, R., Alejo, A., Viñuela, E., Salas, M. L., and Salas, J. (1999). African Swine Fever Virus dUTPase is a highly specific enzyme required for efficient replication in Swine macrophages. *J. Virol.* **73:**8934–8943.

Olivo, P. D., Nelson, N. J., and Challberg, M. D. (1989). Herpes simplex virus 1 gene products required for DNA replication: Identification and overexpression. *J. Virol.* **63:**196–204.

Orci, L., Perrelet, A., Ravazzola, M., Wieland, F. T., Schekman, R., and Rothman, J. E. (1993). ''BFA bodies'': A subcompartment of the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **90:**11089–11093.

Palacios, S., Perez, L. H., Welsch, S., Schleich, S., Chmielarska, K., Melchior, F., and Locker, J. K. (2005). Quantitative SUMO-1 modification of a vaccinia virus protein is required for its specific localization and prevents its self-association. *Mol. Biol. Cell.* **16:**2822–2835.

Parker, J. S., Broering, T. J., Kim, J., Higgins, D. E., and Nibert, M. L. (2002). Reovirus core protein μ2 determines the filamentous morphology of viral inclusion bodies by interacting with and stabilizing microtubules. *J. Virol.* **76:**4483–4496.

Patel, D. D., Pickup, D. J., and Joklik, W. K. (1986). Isolation of cowpox virus A-type inclusions and characterization of their major protein component. *Virology* **149:**174–189.

Pedersen, I. R. (1979). Structural components and replication of arenaviruses. *Adv. Virus. Res.* **24:**277–330.

Pedersen, K. W., van der Meer, Y., Roos, N., and Snijder, E. J. (1999). Open reading frame 1a-encoded subunits of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. *J. Virol.* **73:**2016–2026.

Pedersen, K., Snijder, E. J., Schleich, S., Roos, N., Griffiths, G., and Locker, J. K. (2000). Characterization of vaccinia virus intracellular cores: Implications for viral uncoating and core structure. *J. Virol.* **74:**3525–3536.

Pennington, T. H., Follett, E. A., and Szilagyi, J. F. (1970). Events in vaccinia virus-infected cells following the reversal of the antiviral action of rifampicin. *J. Gen. Virol.* **9:**225–237.

Petrie, B. L., Graham, D. Y., Hanssen, H., and Estes, M. K. (1982). Localization of rotavirus antigens in infected cells by ultrastructural immunocytochemistry. *J. Gen. Virol.* **63:**457–467.

Petrie, B. L., Greenberg, H. B., Graham, D. Y., and Estes, M. K. (1984). Ultrastructural localization of rotavirus antigens using colloidal gold. *Virus Res.* **1:**133–152.

Pignatelli, S., Dal Monte, P., Zini, N., Valmori, A., Maraldi, N. M., and Landini, M. P. (2002). Immunoelectron microscopy analysis of HCMV gpUL73 (gN) localization. *Arch. Virol.* **147:**1247–1256.

Ploubidou, A., Moreau, V., Ashman, K., Reckmann, I., González, C., and Way, M. (2000). Vaccinia virus infection disrupts microtubule organization and centrosome function. *EMBO J.* **19:**3932–3944.

Pombo, A., Ferreira, J., Bridge, E., and Carmo-Fonseca, M. (1994). Adenovirus replication and transcription sites are spatially separated in the nucleus of infected cells. *EMBO J.* **13:**5075–5085.

Prentice, E., Jerome, W. G., Yoshimori, T., Mizushima, N., and Denison, M. R. (2004a). Coronavirus replication complex formation utilizes components of cellular autophagy. *J. Biol. Chem.* **279:**10136–10141.

Prentice, E., McAuliffe, J., Lu, X., Subbarao, K., and Denison, M. R. (2004b). Identification and characterization of severe acute respiratory syndrome coronavirus replicase proteins. *J. Virol.* **78:**9977–9986.

Prod'homme, D., Le Panse, S., Drugeon, G., and Jupin, I. (2001). Detection and subcellular localization of the turnip yellow mosaic virus 66K replication protein in infected cells. *Virology* **281:**88–101.

Punjabi, A., Boyle, K., DeMasi, J., Grubisha, O., Unger, B., Khanna, M., and Traktman, P. (2001). Clustered charge-to-alanine mutagenesis of the vaccinia virus A20 gene: Temperature-sensitive mutants have a DNA-minus phenotype and are defective in the production of processive DNA polymerase activity. *J. Virol.* **75:**12308–12318.

Puvion-Dutilleul, F. (1991). Simultaneous detection of highly phosphorylated proteins and viral major DNA binding protein distribution in nuclei of adenovirus type 5-infected HeLa cells. *J. Histochem. Cytochem.* **39:**669–680.

Puvion-Dutilleul, F., and Pichard, E. (1986). Viral alkaline nuclease in intranuclear dense bodies induced by herpes simplex infection. *Biol. Cell* **58:**15–22.

Puvion-Dutilleul, F., and Pichard, E. (1992). Segregation of viral double-stranded and single-stranded DNA molecules in nuclei of adenovirus infected cells as revealed by electron microscope *in situ* hybridization. *Biol. Cell* **76:**139–150.

Puvion-Dutilleul, F., and Puvion, E. (1990). Replicating single-stranded adenovirus type 5 DNA molecules accumulate within well-delimited intranuclear areas of lytically infected HeLa cells. *Eur. J. Cell Biol.* **52:**379–388.

Puvion-Dutilleul, F., Roussev, R., and Puvion, E. (1992). Distribution of viral RNA molecules during the adenovirus type 5 infectious cycle in HeLa cells. *J. Struct. Biol.* **108:**209–220.

Puvion-Dutilleul, F., Bachellerie, J. P., Visa, N., and Puvion, E. (1994). Rearrangements of intranuclear structures involved in RNA processing in response to adenovirus infection. *J. Cell Sci.* **107:**1457–1468.

Puvion-Dutilleul, F., Chelbi-Alix, M. K., Koken, M., Quignon, F., Puvion, E., and de Thé, H. (1995). Adenovirus infection induces rearrangements in the intranuclear distribution of the nuclear body-associated PML protein. *Exp. Cell Res.* **218:**9–16.

Puvion-Dutilleul, F., Legrand, V., Mehtali, M., Chelbi-Alix, M. K., de Thé, H., and Puvion, E. (1999). Deletion of the fiber gene induces the storage of hexon and penton base proteins in PML/Sp100-containing inclusions during adenovirus infection. *Biol. Cell* **91:**617–628.

Quadt, I., Günther, A. K., Voß, D., Schelhaas, M., and Knebel-Mörsdorf, D. (2006). TATA-binding protein and TBP-associated factors during herpes simplex virus type 1 infection: Localization at viral DNA replication sites. *Virus Res.* **115:**207–213.

Quinkert, D., Bartenschlager, R., and Lohmann, V. (2005). Quantitative analysis of the hepatitis C virus replication complex. *J. Virol.* **79:**13594–13605.

Randall, R. E., and Dinwoodie, N. (1986). Intranuclear localization of herpes simplex virus immediate-early and delayed-early proteins: Evidence that ICP 4 is associated with progeny virus DNA. *J. Gen. Virol.* **67:**2163–2177.

Raoult, D., La Scola, B., and Birtles, R. (2007). The discovery and characterization of mimivirus, the largest known virus and putative pneumonia agent. *Clin. Infect. Dis.* **45:**95–102.

Reckmann, I., Higley, S., and Way, M. (1997). The vaccinia virus F17R protein interacts with actin. *FEBS Lett.* **409:**141–146.

Reggiori, F., and Klionsky, D. J. (2005). Autophagosomes: Biogenesis from scratch? *Curr. Opin. Cell Biol.* **17:**415–422.

Reichel, C., and Beachy, R. N. (1998). Tobacco mosaic virus infection induces severe morphological changes of the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **95:**11169–11174.

Reissig, M., Howes, D. W., and Melnick, J. D. (1956). Sequence of morphological changes in epithelial cell cultures infected with poliovirus. *J. Exp. Med.* **104:**289–304.

Rempel, R. E., Anderson, M. K., Evans, E., and Traktman, P. (1990). Temperature-sensitive vaccinia virus mutants identify a gene with an essential role in viral replication. *J. Virol.* **64:**574–583.

Resch, W., Weisberg, A. S., and Moss, B. (2005). Vaccinia virus nonstructural protein encoded by the A11R gene is required for formation of the virion membrane. *J. Virol.* **79:**6598–6609.

Reynolds, A. E., Fan, Y., and Baines, J. D. (2000). Characterization of the UL33 gene product of herpes simplex virus 1. *Virology* **266:**310–318.

Risco, C., Rodriguez, J. R., Demkowicz, W., Heljasvaara, R., Carrascosa, J. L., Esteban, M., and Rodriguez, D. (1999). The vaccinia virus 39-kDa protein forms a stable complex with the p4a/4a major core protein early in morphogenesis. *Virology* **265:**375–386.

Risco, C., Rodríguez, J. R., López-Iglesias, C., Carrascosa, J. L., Esteban, M., and Rodriguez, D. (2002). Endoplasmic reticulum-Golgi intermediate compartment membranes and vimentin filaments participate in vaccinia virus assembly. *J. Virol.* **76:**1839–1855.

Robbins, F. C., Enders, J. F., and Weller, T. H. (1950). Cytopathogenic effect of poliomyelitis viruses '*in vitro*' on human embryonic tissues. *Proc. Soc. Exp. Biol. Med.* **75:**370–374.

Roberts, S., Hillman, M. L., Knight, G. L., and Gallimore, P. H. (2003). The ND10 component promyelocytic leukemia protein relocates to human papillomavirus type 1 E4 intranuclear inclusion bodies in cultured keratinocytes and in warts. *J. Virol.* **77:**673–684.

Rodríguez, J. M., García-Escudero, R., Salas, M. L., and Andrés, G. (2004). African swine fever virus structural protein p54 is essential for the recruitment of envelope precursors to assembly sites. *J. Virol.* **78:**4299–4313.

Rodríguez, I., Redrejo-Rodríguez, M., Rodríguez, J. M., Alejo, A., Salas, J., and Salas, M. L. (2006). African swine fever virus pB119L protein is a flavin adenine dinucelotide-linked sulfhydryl oxidase. *J. Virol.* **80:**3157–3166.

Roffman, E., Albert, J. P., Goff, J. P., and Frenkel, N. (1990). Putative site for the acquisition of human herpesvirus 6 virion tegument. *J. Virol.* **64:**6308–6313.

Rogel-Gaillard, C., Pehau-Arnaudet, G., Breitburd, F., and Orth, G. (1993). Cytopathic effect in human papillomavirus type 1-induced inclusion warts: *In vitro* analysis of the contribution of two forms of the viral E4 protein. *J. Investig. Dermatol.* **101:**843–851.

Rohrmann, G. F. (1986). Polyhedrin structure. *J. Gen. Virol.* **67:**1499–1513.

Rojo, G., Chamorro, M., Salas, M. L., Viñuela, E., Cuezva, J. M., and Salas, J. (1998). Migration of mitochondria to viral assembly sites in African swine fever virus-infected cells. *J. Virol.* **72:**7583–7588.

Rojo, G., García-Beato, R., Viñuela, E., Salas, M. L., and Salas, J. (1999). Replication of African swine fever virus DNA in infected cells. *Virology* **257:**524–536.

Roller, R. J., and Roizman, B. (1992). The herpes simplex virus 1 RNA binding protein US11 is a virion component and associates with ribosomal 60S subunits. *J. Virol.* **66:**3624–3632.

Roosendaal, J., Westaway, E. G., Khromykh, A., and Mackenzie, J. M. (2006). Regulated cleavages at the West Nile virus NS4A-2K-NS4B junctions play a major role in rearranging cytoplasmic membranes and Golgi trafficking of the NS4A protein. *J. Virol.* **80:** 4623–4632.

Roper, R. L. (2006). Characterization of the vaccinia virus A35R protein and its role in virulence. *J. Virol.* **80:**306–313.

Rosa-Calatrava, M., Grave, L., Puvion-Dutilleul, F., Chatton, B., and Kedinger, C. (2001). Functional analysis of adenovirus protein IX identifies domains involved in capsid stability, transcriptional activity, and nuclear reorganization. *J. Virol.* **75:**7131–7141.

Rosa-Calatrava, M., Puvion-Dutilleul, F., Lutz, P., Dreyer, D., de Thé, H., Chatton, B., and Kedinger, C. (2003). Adenovirus protein IX sequesters host-cell promyelocytic leukaemia protein and contributes to efficient viral proliferation. *EMBO Rep.* **4:**969–975.

Rosales, R., Sutter, G., and Moss, B. (1994). A cellular factor is required for transcription of vaccinia viral intermediate-stage genes. *Proc. Natl. Acad. Sci. USA* **91:**3794–3798.

Rouiller, I., Brookes, S. M., Hyatt, A. D., Windsor, M., and Wileman, T. (1998). African swine fever virus is wrapped by the endoplasmic reticulum. *J. Virol.* **72:**2373–2387.

Rust, R. C., Landmann, L., Gosert, R., Tang, B. L., Hong, W., Hauri, H. P., Egger, D., and Bienz, K. (2001). Cellular COPII proteins are involved in production of the vesicles that form the poliovirus replication complex. *J. Virol.* **75:**9808–9818.

Salmons, T., Kuhn, A., Wylie, F., Schleich, S., Rodriguez, J. R., Rodriguez, D., Esteban, M., Griffiths, G., and Locker, J. K. (1997). Vaccinia virus membrane proteins p8 and p16 are cotranslationally inserted into the rough endoplasmic reticulum and retained in the intermediate compartment. *J. Virol.* **71:**7404–7420.

Salomoni, P., and Khelifi, A. F. (2006). Daxx: Death or survival protein? *Trends Cell Biol.* **16:**97–104.

Salonen, A., Vasiljeva, L., Merits, A., Magden, J., Jokitalo, E., and Kaariainen, L. (2003). Properly folded nonstructural polyprotein directs the semliki forest virus replication complex to the endosomal compartment. *J. Virol.* **77:**1691–1702.

Salonen, A., Ahola, T., and Kaariainen, L. (2005). Viral RNA replication in association with cellular membranes. *Curr. Top. Microbiol. Immunol.* **285:**139–173.

Sanchez, V., Greis, K. D., Sztul, E., and Britt, W. J. (2000). Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: Characterization of a potential site of virus assembly. *J. Virol.* **74:**975–986.

Sanderson, C. M., Hollinshead, M., and Smith, G. L. (2000). The vaccinia virus A27L protein is needed for the microtubule-dependent transport of intracellular mature virus particles. *J. Gen. Virol.* **81:**47–58.

Sandoval, I. V., and Carrasco, L. (1997). Poliovirus infection and expression of the poliovirus protein 2B provoke the disassembly of the Golgi complex, the organelle target for the antipoliovirus drug Ro-090179. *J. Virol.* **71:**4679–4693.

Sanz-Parra, A., Sobrino, F., and Ley, V. (1998). Infection with foot-and-mouth disease virus results in a rapid reduction of MHC class I surface expression. *J. Gen. Virol.* **79:**433–436.

Sanz, A., García-Barreno, B., Nogal, M. L., Viñuela, E., and Enjuanes, L. (1985). Monoclonal antibodies specific for African swine fever virus proteins. *J. Virol.* **54:**199–206.

Schepis, A., Schramm, B., de Haan, C. A. M., and Krijnse-Locker, J. (2006). Vaccinia virus-induced microtubule-dependent cellular rearrangements. *Traffic* **7:**308–323.

Schlegel, A., Giddings, T. H., Jr., Ladinsky, M. S., and Kirkegaard, K. (1996). Cellular origin and ultrastructure of membranes induced during poliovirus infection. *J. Virol.* **70:** 6576–6588.

Schramm, B., and Krijnse-Locker, J. (2005). Cytoplasmic organization of poxvirus DNA replication. *Traffic* **6:**839–846.

Schwartz, M., Chen, J., Janda, M., Sullivan, M., den Boon, J., and Ahlquist, P. (2002). A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol. Cell* **9:**505–514.

Schwartz, M., Chen, J., Lee, W. M., Janda, M., and Ahlquist, P. (2004). Alternate, virus-induced membrane rearrangements support positive-strand RNA virus genome replication. *Proc. Natl. Acad. Sci. USA* **101:**11263–11268.

Senkevich, T. G., White, C. L., Weisberg, A., Granek, J. A., Wolffe, E. J., Koonin, E. V., and Moss, B. (2002). Expression of the vaccinia virus A2.5L redox protein is required for virion morphogenesis. *Virology* **300:**296–303.

Sharpe, A. H., Chen, L. B., and Fields, B. N. (1982). The interaction of mammalian reoviruses with the cytoskeleton of monkey kidney CV-1 cells. *Virology* **120:**399–411.

Shi, S. T., Schiller, J. J., Kanjanahaluethai, A., Baker, S. C., Oh, J. W., and Lai, M. M. (1999). Colocalization and membrane association of murine hepatitis virus gene 1 products and *de novo*-synthesized viral RNA in infected cells. *J. Virol.* **73:**5957–5969.

Shida, H., Tanabe, K., and Matsumoto, S. (1977). Mechanism of virus occlusion into A-type inclusion during poxvirus infection. *Virology* **76:**217–233.

Shintani, T., and Klionsky, D. J. (2004). Autophagy in health and disease: A double-edged sword. *Science* **306:**990–995.

Shishido-Hara, Y., Ichinose, S., Higuchi, K., Hara, Y., and Yasui, K. (2004). Major and minor capsid proteins of human polyomavirus JC cooperatively accumulate to nuclear domain 10 for assembly into virions. *J. Virol.* **78:**9890–9903.

Silverstein, S. C., and Schur, P. H. (1970). Immunofluorescent localization of double-stranded RNA in reovirus-infected cells. *Virology* **41:**564–566.

Silvestri, L. S., Taraporewala, Z. F., and Patton, J. T. (2004). Rotavirus replication: Plus-sense templates for double-stranded RNA synthesis are made in viroplasms. *J. Virol.* **78:**7763–7774.

Silvestri, L. S., Tortorici, M. A., Vasquez-Del Carpio, R., and Patton, J. T. (2005). Rotavirus glycoprotein NSP4 is a modulator of viral transcription in the infected cell. *J. Virol.* **79:**15165–15174.

Simón-Mateo, C., Andrés, G., Almazán, F., and Viñuela, E. (1997). Proteolytic processing in African swine fever virus: Evidence for a new structural polyprotein pp62. *J. Virol.* **71:**5799–5804.

Sims, A. C., Ostermann, J., and Denison, M. R. (2000). Mouse hepatitis virus replicase proteins associate with two distinct populations of intracellular membranes. *J. Virol.* **74:**5647–5654.

Smith, G. A., and Enquist, L. W. (2002). Break ins and break outs: Viral interactions with the cytoskeleton of mammalian cells. *Annu. Rev. Cell Dev. Biol.* **18:**135–161.

Snijder, E. J., van der Meer, Y., Zevenhoven-Dobbe, J., Onderwater, J. J., van der Meulen, J., Koerten, H. K., and Mommaas, A. M. (2006). Ultrastructure and origin of membrane vesicles associated with the severe acute respiratory syndrome coronavirus replication complex. *J. Virol.* **80:**5927–5940.

Sodeik, B., and Krijnse-Locker, J. (2002). Assembly of vaccinia virus revisited: *De novo* membrane synthesis or acquisition from the host? *Trends Microbiol.* **10:**15–24.

Sodeik, B., Cudmore, S., Ericsson, M., Esteban, M., Niles, E. G., and Griffiths, G. (1995). Assembly of vaccinia virus: Incorporation of p14 and p32 into the membrane of the intracellular mature virus. *J. Virol.* **69:**3560–3574.

Sodeik, B., Ebersold, M. W., and Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J. Cell. Biol.* **136:**1007–1021.

Souquere-Besse, S., Pichard, E., Filhol, O., Legrand, V., Rosa-Calatrava, M., Hovanessian, A. G., Cochet, C., and Puvion-Dutilleul, F. (2002). Adenovirus infection targets the cellular

protein kinase CK2 and RNA-activated protein kinase (PKR) into viral inclusions of the cell nucleus. *Microsc. Res. Tech.* **56:**465–478.

Sridhar, P., and Condit, R. C. (1983). Selection for temperature-sensitive mutations in specific vaccinia virus genes: Isolation and characterization of a virus mutant which encodes a phosphonoacetic acid-resistant, temperature-sensitive DNA polymerase. *Virology* **128:**444–457.

Stefanovic, S., Windsor, M., Nagata, K.-I., Inagaki, M., and Wileman, T. (2005). Vimentin rearrangement during African swine fever virus infection involves retrograde transport along microtubules and phosphorylation of vimentin by calcium calmodulin kinase II. *J. Virol.* **79:**11766–11775.

Stuart, J. D. C., and Fogh, J. (1961). Micromorphology of FL cells infected with polio and Coxsackie viruses. *Virology* **13:**177–190.

Suhy, D. A., Giddings, T. H., Jr., and Kirkegaard, K. (2000). Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: An autophagy-like origin for virus-induced vesicles. *J. Virol.* **74:**8953–8965.

Sun, H., Jenson, J., Dixon, L. K., and Parkhouse, R. M. E. (1996). Characterization of the African swine fever virion protein j18L. *J. Gen. Virol.* **77:**941–946.

Szajner, P., Weisberg, A. S., Wolffe, E. J., and Moss, B. (2001). Vaccinia virus A30L protein is required for association of viral membranes with dense viroplasm to form immature virions. *J. Virol.* **75:**5752–5761.

Szajner, P., Jaffe, H., Weisberg, A. S., and Moss, B. (2003). Vaccinia virus G7L protein interacts with the A30L protein and is required for association of viral membranes with dense viroplasm to form immature virions. *J. Virol.* **77:**3418–3429.

Szajner, P., Jaffe, H., Weisberg, A. S., and Moss, B. (2004a). A complex of seven vaccinia virus proteins conserved in all chordopoxviruses is required for the association of membranes and viroplasm to form immature virions. *Virology* **330:**447–459.

Szajner, P., Weisberg, A. S., and Moss, B. (2004b). Evidence for an essential catalytic role of the F10 protein kinase in vaccinia virus morphogenesis. *J. Virol.* **78:**257–265.

Szajner, P., Weisberg, A. S., and Moss, B. (2004c). Physical and functional interactions between vaccinia virus F10 protein kinase and virion assembly proteins A30 and G7. *J. Virol.* **78:**266–274.

Szajner, P., Weisberg, A. S., Lebowitz, J., Heuser, J., and Moss, B. (2005). External scaffold of spherical immature poxvirus particles is made of protein trimers, forming a honeycomb lattice. *J. Cell Biol.* **170:**971–981.

Taus, N. S., Salmon, B., and Baines, J. D. (1998). The herpes simplex virus 1 UL 17 gene is required for localization of capsids and major and minor capsid proteins to intranuclear sites where viral DNA is cleaved and packaged. *Virology* **252:**115–125.

Taylor, T. J., and Knipe, D. M. (2004). Proteomics of herpes simplex virus replication compartments: Association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. *J. Virol.* **78:**5856–5866.

Taylor, T. J., McNamee, E. E., Day, C., and Knipe, D. M. (2003). Herpes simplex virus replication compartments can form by coalescence of smaller compartments. *Virology* **309:**232–247.

Temperley, S. M., and Hay, R. T. (1992). Recognition of the adenovirus type 2 origin of DNA replication by the virally encoded DNA polymerase and preterminal proteins. *EMBO J.* **11:**761–768.

Teterina, N. L., Bienz, K., Egger, D., Gorbalenya, A. E., and Ehrenfeld, E. (1997). Induction of intracellular membrane rearrangements by HAV proteins 2C and 2BC. *Virology* **237:**66–77.

Tolonen, N., Doglio, L., Schleich, S., and Krijnse Locker, J. (2001). Vaccinia virus DNA replication occurs in endoplasmic reticulum-enclosed cytoplasmic mini-nuclei. *Mol. Biol. Cell* **12:**2031–2046.

Tripier, F., Braunwald, J., Markovic, L., and Kirn, A. (1977). Frog virus 3 morphogenesis: Effect of temperature and metabolic inhibitors. *J. Gen. Virol.* **37:**39–52.

Tsutsui, Y., and Yamazaki, Y. (1991). Subcellular distribution of the major immediate early proteins of human cytomegalovirus changes during infection. *J. Gen. Virol.* **72:**1415–1419.

Turcotte, S., Letellier, J., and Lippe, R. (2005). Herpes simplex virus type 1 capsids transit by the trans-Golgi network, where viral glycoproteins accumulate independently of capsid egress. *J. Virol.* **79:**8847–8860.

Uchil, P. D., and Satchidanandam, V. (2003). Architecture of the flaviviral replication complex. Protease, nuclease, and detergents reveal encasement within double-layered membrane compartments. *J. Biol. Chem.* **278:**24388–24398.

Ulaeto, D., Grosenbach, D., and Hruby, D. E. (1996). The vaccinia virus 4c and A-type inclusion proteins are specific markers for the intracellular mature virus particle. *J. Virol.* **70:**3372–3377.

van der Heijden, M. W., Carette, J. E., Reinhoud, P. J., Haegi, A., and Bol, J. F. (2001). Alfalfa mosaic virus replicase proteins P1 and P2 interact and colocalize at the vacuolar membrane. *J. Virol.* **75:**1879–1887.

van der Meer, Y., van Tol, H., Locker, J. K., and Snijder, E. J. (1998). ORF1a-encoded replicase subunits are involved in the membrane association of the arterivirus replication complex. *J. Virol.* **72:**6689–6698.

van der Meer, Y., Snijder, E. J., Dobbe, J. C., Schleich, S., Denison, M. R., Spaan, W. J., and Locker, J. K. (1999). Localization of mouse hepatitis virus nonstructural proteins and RNA synthesis indicates a role for late endosomes in viral replication. *J. Virol.* **73:**7641–7657.

van Kuppeveld, F. J., Melchers, W. J., Kirkegaard, K., and Doedens, J. R. (1997). Structure-function analysis of Coxsackie B3 virus protein 2B. *Virology* **227:**111–118.

Vanslyke, J. K., and Hruby, D. E. (1994). Immunolocalization of vaccinia virus structural proteins during virion formation. *Virology* **198:**624–635.

Vigário, J. D., Relvas, M. E., Ferraz, F. P., Riberio, J. M., and Pereira, C. G. (1967). Identification and localization of genetic material of African swine fever virus by autoradiography. *Virology* **33:**173–175.

Wada, K., Goshima, F., Takakuwa, H., Yamada, H., Daikoku, T., and Nishiyama, Y. (1999). Identification and characterization of the UL14 gene product of herpes simplex virus type 2. *J. Gen. Virol.* **80:**2423–2431.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Kräusslich, H. G., Mizokami, M., Bartenschlager, R., Liang, T. J., *et al.* (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11:**791–796.

Ward, B. M. (2005). Visualization and characterization of the intracellular movement of vaccinia virus intracellular mature virions. *J. Virol.* **79:**4755–4763.

Ward, B. M., and Moss, B. (2001). Vaccinia virus intracellular movement is associated with microtubules and independent of actin tails. *J. Virol.* **75:**11651–11663.

Ward, P. L., Barker, D. E., and Roizman, B. (1996a). A novel herpes simplex virus 1 gene, UL43.5, maps antisense to the UL43 gene and encodes a protein which colocalizes in nuclear structures with capsid proteins. *J. Virol.* **70:**2684–2690.

Ward, P. L., Ogle, W. O., and Roizman, B. (1996b). Assemblons: Nuclear structures defined by aggregation of immature capsids and some tegument proteins of herpes simplex virus 1. *J. Virol.* **70:**4623–4631.

Ward, T. H., Polishchuk, R. S., Caplan, S., Hirschberg, K., and Lippincott-Schwartz, J. (2001). Maintenance of Golgi structure and function depends on the integrity of ER export. *J. Cell Biol.* **155:**557–570.

Watanabe, D., Ushijima, Y., Goshima, F., Takakuwa, H., Tomita, Y., and Nishiyama, Y. (2000). Identification of nuclear export signal in UL37 protein of herpes simplex virus type 2. *Biochem. Biophys. Res. Commun.* **276:**1248–1254.

Welsch, S., Doglio, L., Schleich, S., and Krijnse Locker, J. (2003). The vaccinia virus I3L gene product is localized to a complex endoplasmic reticulum-associated structure that contains the viral parental DNA. *J. Virol.* **77:**6014–6028.

Wessels, E., Duijsings, D., Notebaart, R. A., Melchers, W. J., and van Kuppeveld, F. J. (2005). A proline-rich region in the coxsackievirus 3A protein is required for the protein to inhibit endoplasmic reticulum-to-golgi transport. *J. Virol.* **79:**5163–5173.

Wessels, E., Duijsings, D., Lanke, K. H., van Dooren, S. H., Jackson, C. L., Melchers, W. J., and van Kuppeveld, F. J. (2006a). Effects of picornavirus 3A proteins on protein transport and GBF1-dependent COP-I recruitment. *J. Virol.* **80:**11852–11860.

Wessels, E., Duijsings, D., Niu, T. K., Neumann, S., Oorschot, V. M., de Lange, F., Lanke, K. H., Klumperman, J., Henke, A., Jackson, C. L., Melchers, W. J., and van Kuppeveld, F. J. (2006b). A viral protein that blocks Arf1-mediated COP-I assembly by inhibiting the guanine nucleotide exchange factor GBF1. *Dev. Cell* **11:**191–201.

Westaway, E. G., Khromykh, A. A., Kenney, M. T., Mackenzie, J. M., and Jones, M. K. (1997a). Proteins C and NS4B of the flavivirus Kunjin translocate independently into the nucleus. *Virology* **234:**31–41.

Westaway, E. G., Mackenzie, J. M., Kenney, M. T., Jones, M. K., and Khromykh, A. A. (1997b). Ultrastructure of Kunjin virus-infected cells: Colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. *J. Virol.* **71:**6650–6661.

Wilcock, D., and Lane, D. P. (1991). Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. *Nature* **349:**429–431.

Wileman, T. (2006). Aggresomes and autophagy generate sites of virus replication. *Science* **312:**875–878.

Williams, T., Barbosa-Solomieu, V., and Chinchar, V. G. (2005). A decade of advances in iridovirus research. *Adv. Virus Res.* **65:**173–248.

Willis, D. B., Goorha, R., and Granoff, A. (1979). Macromolecular synthesis in cells infected by frog virus 3. XI. A ts mutant of frog virus 3 that is defective in late transcription. *Virology* **98:**328–335.

Wilton, S., and Dales, S. (1989). Relationship between RNA polymerase II and efficiency of vaccinia virus replication. *J. Virol.* **63:**1540–1548.

Wolf, S., Maier, I., Katsaros, C., and Muller, D. G. (1998). Virus assembly in Hincksia hincksiae (Ectocarpales, Phaeophyceae) an electron and fluorescence microscopic study. *Protoplasma* **203:**153–167.

Wolf, S., Muller, D. G., and Maier, I. (2000). Assembly of a large icosahedral DNA virus, MclaV-1, in the marine alga Myriotrichia clavaeformis (Dictyosiphonales, Phaeophyceae). *Eur. J. Phycol.* **35:**163–171.

Wolffe, E. J., Vijaya, S., and Moss, B. (1995). A myristylated membrane protein encoded by the vaccinia virus L1R open reading frame is the target of potent neutralizing monoclonal antibodies. *Virology* **211:**53–63.

Wolk, B., Sansonno, D., Krausslich, H. G., Dammacco, F., Rice, C. M., Blum, H. E., and Moradpour, D. (2000). Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J. Virol.* **74:**2293–2304.

Wright, C. F., Oswald, B. W., and Dellis, S. (2001). Vaccinia virus late transcription is activated *in vitro* by cellular heterogeneous nuclear ribonucleoproteins. *J. Biol. Chem.* **276:**40680–40686.

Yamada, H., Jiang, Y. M., Oshima, S., Daikoku, T., Yamashita, Y., Tsurumi, T., and Nishiyama, Y. (1998). Characterization of the UL55 gene product of herpes simplex virus type 2. *J. Gen. Virol.* **79:**1989–1995.

Yeh, W. W., Moss, B., and Wolffe, E. J. (2000). The vaccinia virus A9L gene encodes a membrane protein required for an early step in virion morphogenesis. *J. Virol.* **74:**9701–9711.

Young, P. R., Chanas, A. C., Lee, S. R., Gould, E. A., and Howard, C. R. (1987). Localization of an arenavirus protein in the nuclei of infected cells. *J. Gen. Virol.* **68:**2465–2470.

Yue, Z., and Shatkin, A. J. (1998). Enzymatic and control functions of reovirus structural proteins. *Curr. Top. Microbiol. Immunol.* **233:**31–56.

Yuwen, H., Cox, J. H., Yewdell, J. W., Bennink, J. R., and Moss, B. (1993). Nuclear localization of a double-stranded RNA-binding protein encoded by the vaccinia virus E3L gene. *Virology* **195:**732–744.

Zhao, Z., Ke, F., Gui, J., and Zhang, Q. (2007). Characterization of an early gene encoding for dUTPase in *Rana grylio* virus. *Virus Res.* **123**(2)**:**128–137; doi:10.1016/j.virusres.2006.08.007.

Zhong, L., and Hayward, G. S. (1997). Assembly of complete, functionally active herpes simplex virus DNA replication compartments and recruitment of associated viral and cellular proteins in transient cotransfection assays. *J. Virol.* **71:**3146–3160.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T., and Chisari, F. V. (2005). Robust hepatitis C virus infection *in vitro*. *Proc. Natl. Acad. Sci. USA* **102:**9294–9299.

Ziebuhr, J. (2006). The coronavirus replicase: Insights into a sophisticated enzyme machinery. *Adv. Exp. Med. Biol.* **581:**3–11.

CHAPTER 5

Parvoviral Host Range and Cell Entry Mechanisms

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Abstract

Parvoviruses elaborate rugged nonenveloped icosahedral capsids of ~260 Å in diameter that comprise just 60 copies of a common core structural polypeptide. While serving as exceptionally durable shells, capable of protecting the single-stranded DNA genome from environmental extremes, the capsid also undergoes sequential conformational changes that allow it to translocate the genome from its initial host cell nucleus all the way into the nucleus of its subsequent host. Lacking a duplex transcription template, the virus must then wait for its host to enter S-phase before it can initiate transcription and usurp the cell's synthetic pathways. Here we review cell entry mechanisms used by parvoviruses. We explore two apparently distinct modes of host cell specificity, first that used by Minute virus of mice, where subtle glycan-specific interactions between host receptors and residues surrounding twofold symmetry axes on the virion surface mediate differentiated cell type target specificity, while the second involves novel protein interactions with the canine transferrin receptor that allow a mutant of the feline leukopenia serotype, Canine parvovirus, to bind to and infect dog cells. We then discuss conformational shifts in the virion that accompany cell entry, causing exposure of a capsid-tethered phospholipase A2 enzymatic core that acts as an endosomolytic agent to mediate virion translocation across the lipid bilayer into the cell cytoplasm. Finally, we discuss virion delivery into the nucleus, and consider the nature of transcriptionally silent DNA species that, escaping detection by the cell, might allow unhampered progress into S-phase and hence unleash the parvoviral Trojan horse.

I. INTRODUCTION TO THE VIRUSES

A. The family Parvoviridae

All small nonenveloped viruses with ~5-kb linear, self-priming, single-stranded DNA genomes are grouped in the taxonomic family Parvoviridae (from *Parvus*—Latin for ''small''), and share a common evolutionary history as assessed by DNA sequence. This broad group is divided into two subfamilies, superficially on the basis of host range: the Parvovirinae, infecting vertebrate hosts and the Densovirinae, infecting insects and other arthropods. While species and genera within the Parvovirinae appear to be derived from a single common ancestor, the arthropod genera are separated by massive evolutionary distances, probably reflecting divergence coincident with that of their hosts (Tattersall *et al.*, 2005). Thus,

this is an ancient and widely dispersed virus family with, apparently, a single evolutionary branch that became adapted to vertebrate hosts.

Members of the subfamily Parvovirinae have been divided into five genera on the basis of DNA and protein sequence-based phylogenetic analyses: these are the *Parvoviruses*, which are the subject of this chapter, and the *Amdoviruses*, *Bocaviruses*, *Dependoviruses*, and *Erythroviruses*. While all genera contain viruses that can replicate independently of helper viruses (commonly described as ''autonomously replicating'' viruses), the *Dependovirus* genus is so called because it includes a large number of agents that depend for their own productive replication on coinfection with a more complex helper virus from a different taxonomic family. This association with adenoviruses is reflected in the name, ''adeno-associated viruses'' (AAVs), although these same viruses may also derive help from herpesviruses, papillomaviruses, or vaccinia viruses. In the absence of such help, AAVs establish a latent interaction with their vertebrate host, and this nondisruptive, but persistent, lifestyle has engendered significant interest in them as gene therapy transfer vectors. Accordingly, they have been the focus of much recent research, so that emerging data from viruses in this genus does much to complement our current knowledge of entry processes used by their *Parvovirus* cousins, and is cited accordingly in this chapter.

The biology of the Parvovirinae is dominated by their small physical size. With nonenveloped protein capsids of around 260 Å diameter, constructed in the simplest icosahedral form ($T = 1$), these remarkably dense and rugged particles deliver their enclosed genomes into the cell, traverse the cytoplasm, and penetrate the nucleus while still comprising a structurally intact, albeit somewhat rearranged, capsid (Farr *et al.*, 2006; Sonntag *et al.*, 2006; Vihinen-Ranta *et al.*, 2002). Encapsidation within such a small virion is possible because parvoviruses typically encode just two gene cassettes, and are unique among known microorganisms in having DNA genomes that are both single stranded and linear, which makes their chromosome optimally small and flexible. This single DNA strand is inserted vectorially into a preformed capsid, using energy provided by a viral helicase, and packed in such a way that bases in the outer DNA shell bond with side chains from amino acids lining the icosahedral threefold axis of the capsid, creating a virion of remarkable density and stability (Agbandje-McKenna and Chapman, 2006; Chapman and Agbandje-McKenna, 2006). Inevitably, such minimalism has some apparently negative biological consequences. Parvoviruses not only lack accessory proteins that might induce resting cells to enter S-phase, they also lack a duplex transcription template so that they are generally unable to express their genes until the DNA synthetic machinery of the host cell, activated at the start of a cell-directed S-phase, coincidentally provides them with a complementary-sense DNA strand. Consequently,

these viruses have had to become masters of stealth, apparently avoiding triggering many of the cellular responses that commonly accompany cell entry by viruses of other families. As a result, although relatively inert, they are able to become sequestered within resting cells without inhibiting the cell's program of transit through the cell cycle. Indeed, this suggests an entry strategy in which the disadvantages of being single stranded are outweighed by the ability to package a relatively complex genome in a particle small enough to be imported intact into the host cell's nucleus.

B. The genus *Parvovirus*

Much of our knowledge of the molecular biology and pathogenic potential of the family Parvoviridae has been derived by studying members of the genus *Parvovirus*, which typically grow efficiently in cell culture, are open to reverse and forward genetic analysis, and predominantly infect host species that are readily susceptible to experimental manipulation. This genus contains four distinct subgroups: (1) a broadly related, but serologically diverse cluster of ''rodent virus'' species that contains three distinct clades [Minute virus of mice (MVM), the type species of the genus, Mouse parvovirus 1 (MPV1), and a rat virus group that includes Rat minute virus 1 (RMV1), H1 virus and Kilham rat virus (KRV)], and LuIII, an ''orphan'' virus; (2) an outlying Rat parvovirus 1 (RPV1) branch; (3) the Feline panleukopenia virus/Canine parvovirus (FPV/CPV) serotype, strains of which infect various members of the *Carnivora*; and (4) Porcine parvovirus (PPV). As seen in Table I, the NS1 genes of species within this genus vary by up to 30%, whereas their VP2 genes vary by up to 50%, this wider range reflecting the fact that the members of each species represent a serologically distinct group. In contrast to these broad interspecies values, the intraspecies homologies for the NS1 and VP2 proteins specified by the prototype MVM strain, MVMp, and those of the ''immunosuppressive'' strain, MVMi, are both 97.8%, and for the NS1 and VP2 proteins of FPV and CPV are 99.0% and 98.6%, respectively.

Patterns of parvovirus-induced disease are largely determined by the fact that these viruses cannot induce resting cells to enter S-phase, and hence only replicate productively in actively mitotic host cell populations. They also commonly exhibit finely tuned tissue specificity, only infecting cells of particular differentiated phenotypes, although such preferences can vary profoundly even within virus strains of a single serotype. Accordingly, pathogenic or lethal infections typically occur in fetal or neonatal hosts, which have many dividing cell populations, or involve adult tissues that remain actively dividing in later life such as cells of the gut epithelium or leukocyte lineages. Acute clinical infections are typically resolved rapidly by development of a predominantly humoral

TABLE I Comparison of NS1 and VP2 protein sequences within the genus *Parvovirus*

	MVM	MPV1	KRV	RMV1	H1	LuIII	RPV1	FPV	PPV	
MVM	100	97.5	91.8	91.5	91.4	89.6	81.4	71.9	67.7	MVM
MPV1	71.2	100	91.5	91.2	91.2	90.3	81.4	72.1	68.0	MPV1
KRV	68.0	66.2	100	99.3	98.1	88.0	81.9	72.0	68.1	KRV
RMV1	69.3	71.9	73.2	100	97.9	87.8	81.9	72.1	68.3	RMV1
H1	63.1	61.7	72.3	67.7	100	88.0	82.2	71.7	67.7	H1
LuIII	71.2	83.5	66.3	71.9	64.5	100	79.2	70.8	67.5	LuIII
RPV1	58.0	58.6	60.5	60.3	57.2	59.1	100	77.9	70.2	RPV1
FPV	48.6	49.0	50.5	48.9	47.3	47.0	48.2	100	64.5	FPV
PPV	48.1	47.1	49.5	49.5	48.1	49.2	48.8	56.0	100	PPV
	MVM	MPV1	KRV	RMV1	H1	LuIII	RPV1	FPV	PPV	

Percent homology was calculated for each pairwise combination of NS1 (shaded) or VP2 (unshaded) polypeptides, using the Diagonals method (BLOSUM62 alignment score matrix) in DNA Strider 1.4, using a block length of 6 amino acids. Mismatch and gap penalties were set to 1 and 2, respectively.
Protein sequences were derived for a representative of each virus species, using DNA sequences data from the GenBank database, as follows: MVM, Minute virus of mice (prototype strain) [J02275]; MPV1, Mouse parvovirus 1 [U12469]; KRV, Kilham rat virus [AF321230]; RMV1, Rat minute virus 1 [AF332882]; H1, H-1 virus [X01457]; LuIII, LuIII virus [M81888]; RPV1, Rat parvovirus 1 [AF036710]; FPV, Feline panleukopenia virus [M38246]; and PPV, Porcine parvovirus (NADL-2 strain) [L23427]. Double-lined box denotes the closely related ''rodent'' subgroup described in the text.

immune response, but latency often ensues. In their natural host some viruses, most notably members of the rodent groups, are clinically silent, and can establish persistent infections associated with prolonged virus release from reservoirs that are currently unknown.

II. STRUCTURE OF A UNIQUELY DENSE AND COMPACT VIRION

Infectious parvoviral virions are nonenveloped, ~260 Å in diameter, and contain a single-stranded, linear DNA genome of ~5 kb. They comprise between 70% and 80% protein, with the remainder being DNA, and are uniquely dense and compact, with molecular masses in the order of $5.5–6.2 \times 10^6$, sedimentation coefficients of 110S–122S, and buoyant densities of 1.39–1.43 g/cm^3 in cesium chloride. Mature virions are stable in the presence of lipid solvents or on exposure to pH 3–9. They are historically reported to survive prolonged incubation at 56 °C, although this characteristic applies only to concentrated suspensions of particles or in situations where they are protected by animal tissue, since in dilute solution they are metastable, undergoing an inactivating conformational transition in response to heat or denaturants. However, under natural conditions, infectious virions are exceptionally durable, surviving for weeks or months at room temperature or for several years at 4 °C.

A. Rugged 260 Å protein capsids with T = 1 icosahedral symmetry

Parvovirus-infected cells typically generate thousands of copies of both empty capsids and full virions, with almost indistinguishable core X-ray structures. These capsids are constructed from 60 copies of a single polypeptide sequence, and hence exhibit $T = 1$ icosahedral symmetry. Virions generally contain proteins of two or three size classes (VP1–VP3) that constitute a nested set. These share a common C-terminal core sequence but have N-terminal extensions of different lengths. The largest capsid polypeptide, designated VP1, has a molecular mass of ~83,000 and is present at ~10 copies per capsid. It is dispensable for particle assembly, DNA packaging, and virion release, but is essential for infectivity (Tullis *et al.*, 1993), since it carries a series of elements that are required for trafficking through host cell entry pathways. These include a unique phospholipase domain that is deployed to breach the lipid bilayer of an endosomal vesicle. Three-dimensional structures of several wild-type and mutant parvovirus particles have been determined to near-atomic resolution by X-ray crystallography, including forms of CPV, FPV, two strains of MVM, and recombinant virus-like particles (VLPs) of PPV

(reviewed in Chapman and Agbandje-McKenna, 2006). Core structure is based on a classic eight-stranded antiparallel β-barrel, but in parvoviruses these β-strands are connected by elaborate and highly variable loops, which make up most of the viral surface (Chapman and Rossmann, 1993). The N-terminal peptide domains of the larger proteins are submolar and disordered, so their disposition cannot be deduced from X-ray data.

The outer architecture of the parvovirus capsid has a number of structural features, illustrated in Fig. 1. Each asymmetric unit has two ''spike''-like elevations, which surround the 20 threefold symmetry axes of the icosahedron, a deep depression, called the ''dimple,'' at each twofold axis, and a hollow cylinder, surrounding each of the 12 icosahedral fivefold axes, which contains a central pore that connects the inside of the virion with the particle exterior. In full virions, each pore contains a single copy of a glycine-rich sequence from a single VP2 molecule, positioned so that the N-terminal 25 amino acids of the peptide are externalized (Agbandje-McKenna *et al.*, 1998; Tsao *et al.*, 1991). These cylindrical

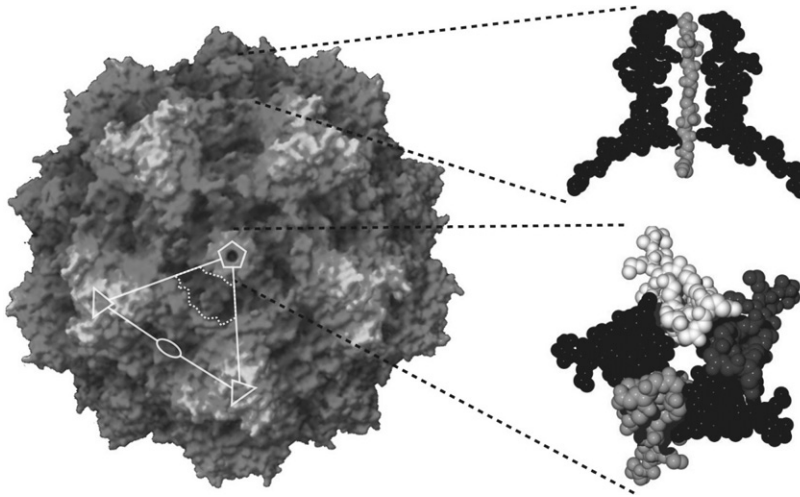


FIGURE 1 Topology of the parvoviral particle. Left—depth-cued, space-filling model of MVM, centered on a fivefold symmetry axis (pentangle). One crystallographic asymmetric unit is indicated by the large triangle, bordered by a fivefold axis, two threefold axes (triangles), and a twofold axis (oval). Topological features seen here that are referred to in the text include the fivefold cylinder surrounded by the canyon (enclosed in dotted line), the threefold spikes and the dimple surrounding the twofold axis. Upper right—cross-section of the fivefold channel, showing two of the five β-ribbons that comprise the cylinder, and residues 28–37 of VP2 in gray. Lower right—view down the fivefold cylinder, with the five β-ribbons differentially shaded.

structures are themselves encircled on the outer virion surface by a deep, canyon-like depression with highly conserved amino acid sequence, but unknown function. Neutralizing antibody binding sites generally map to the threefold spike or to its shoulders, as do protein receptor contacts for those serotypes in which such interactions have been identified. Sequences that determine viral tissue specificity and oligosaccharide recognition lie in the twofold dimple and up the adjacent edge of the threefold spike.

B. Linear single-stranded DNA genomes with palindromic telomeres

Mature virions of most species in this genus contain a single 5-kb DNA strand that is negative sense with respect to transcription, while one virus, LuIII, packages approximately equimolar positive- and negative-sense strands. This remarkable variability illuminates the whole process of strand selectivity, since it is caused by differential rates of initiation from the two viral replication origins rather than by any strand-specific packaging signal or mechanism (Cotmore and Tattersall, 2005b). Since most, but not all, genomes are negative sense with regard to transcription, a unifying convention has been adopted whereby the 3′ terminus of the negative strand is rather called ''the left'' end and the 5′ terminus of this strand ''the right'' end. Within the virions, some of the single-stranded DNA displays icosahedral symmetry, so that about a third of the genome can be visualized by crystallography, abutting the particle shell. This DNA has some limited nucleotide specificity, and is oriented with its bases pointing outward, forming a number of conserved protein–base hydrogen bonds with the inner surface of the capsid (Agbandje-McKenna *et al*., 1998; Xie and Chapman, 1996). Remarkably, not all of the genome is contained within the particle. DNA packaging proceeds in a 3′-to-5′ direction, but the 5′ end of the strand is left projecting through the capsid wall at an unknown location so that ~24 nucleotides (nts), called the ''tether'' sequence, are left outside the particle, covalently attached, at its 5′ end, to a single molecule of the viral replication initiator protein, NS1 (Cotmore and Tattersall, 1989).

At both termini of the linear, nonpermuted genome there are essential palindromic sequences that can fold into self-priming duplex ''hairpin'' telomeres, as illustrated at the top of Fig. 2, which are diagnostic features of this virus family. These provide most of the *cis*-acting information needed for both viral DNA replication and encapsidation. In viruses from the genus *Parvovirus*, these two terminal hairpins differ from one another in both sequence and predicted secondary structure. This disparity allows differential initiation and encapsidation of the two strands, and typically means that infected cells only receive negative-sense DNA.

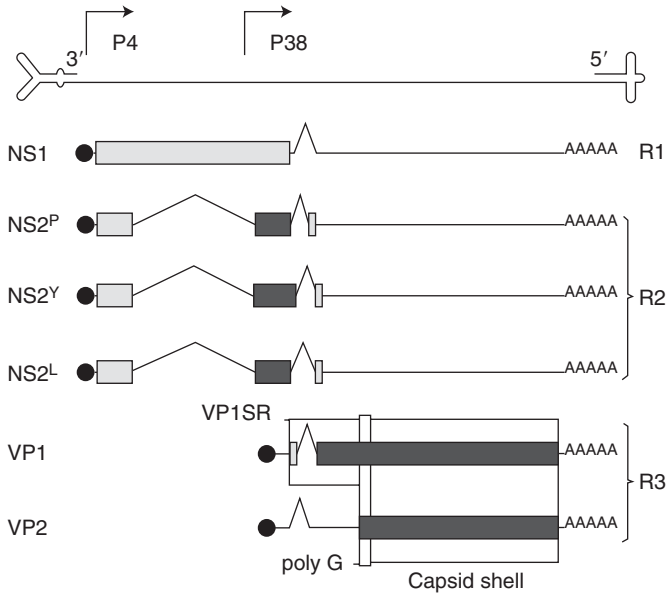


FIGURE 2 Genetic strategy of the prototypic *Parvovirus* MVM. The single-stranded, negative-sense DNA genome is shown as a continuous line, which terminates in folded hairpin structures that are expanded ~20-fold in scale with respect to the coding sequence between them. The two viral promoters, at 4 and 38 map units, are shown by rightward arrows, and the mature, cytoplasmic transcript classes driven by each, R1, R2, and R3, are displayed below, with a black sphere indicating the capped 5′ ends and AAAA denoting their polyadenylated tails. Open reading frames (ORFs) shades specifying the viral gene products, named on the left, are displayed in different shades according to their reading phase, and their spliced-out introns are represented by the thin-lined carets. The boxes denote, from the left, the VP1-specific region involved in entry functions, the glycine rich "spacer" that occupies the fivefold pore, and the common region of the VP polypeptides, 60 copies of which comprise the $T = 1$ protein shell of the capsid.

This, in turn, may dictate the mechanism(s) of latency adopted by the virus. In contrast, members of the *Dependovirus* and *Erythrovirus* genera have inverted terminal repeat (ITR) sequences, and encapsidate strands of both senses with equal efficiency. In the genus *Parvovirus* the left-end telomere usually comprises ~120 nts and can be folded in a Y-shaped configuration, while the right-end palindrome is ~250 nts in length and is predicted to be able to alternate, with little change in free energy, between linear and cruciform configurations. These termini serve as hinges, allowing the ancient single-strand displacement ''rolling circle replication'' strategy, to be adapted for the replication of a linear genome. Protein motifs characteristic of initiator nucleases derived from these ancestral

replicons are conserved in the viral genome, and its modified replication scheme is termed ''rolling hairpin replication.''

C. Creating and expressing transcription templates

When the cell enters S-phase, the viral 3′ hairpin acts as a primer for complementary-strand DNA synthesis, generating a duplex unit-length replicative intermediate that can support viral transcription. This contains two mRNA transcription units, with promoters at map units 4 and 38 and a single functional polyadenylation site at the extreme right-hand end (reviewed in Qiu *et al.*, 2006). These two promoters, P4 and P38, drive expression of a nonstructural gene (NS), encoded in the left half of the genome, and a capsid gene (VP), encoded in the right half, respectively. Alternative splicing events orchestrate gene expression, as shown for MVM in Fig. 2 (Cotmore and Tattersall, 1990; Jongeneel *et al.*, 1986; Morgan and Ward, 1986; Pintel *et al.*, 1983). The R1 transcripts, synthesized from the P4 promoter, contain a single contiguous open reading frame (ORF) that encodes the 83-kDa multifunctional replication initiator protein, NS1, located upstream of a complex alternately spliced small intron region. In a further set of P4-derived transcripts, R2, the NS1 ORF is spliced into an alternate reading frame by removal of the major intron, and these transcripts encode, in order of abundance, NS2P, NS2Y, and NS2L, the extreme C-termini of which are different due to the use of two pairs of alternative 5′ and 3′ splice sites bordering the small intron. In contrast, members of the FPV serotype express a single, shorter NS2 species, whose second exon is encoded in, and terminates within, the alternative reading frame, some 15 codons upstream of the small intron (Wang *et al.*, 1998).

One function of NS1 is to upregulate the P4 promoter itself, and this positive feedback loop appears to be a part of the ''hard-wiring'' of infection that ensures rapid viral takeover of the cell. As infection progresses, the second promoter, at 38 map units, is transactivated by NS1 (Clemens and Pintel, 1988) and drives synthesis of the R3 transcripts, which use the same pair of alternative 5′ and 3′ splice sites present in the small intron region to regulate synthesis of the two primary capsid proteins, VP1 and VP2. In this case, a transcript that uses the downstream 5′ and 3′ splice sites encodes the minor VP1 polypeptide, translation of which initiates at a methionine codon between the two alternate 5′ splice sites. In the more abundant transcripts, which employ the upstream 5′ splice site, this initiation codon is spliced out, and translation of the major coat protein VP2 initiates from a start codon nearly 400 nts further downstream of the splice. Thus, the two primary translation products from the structural gene, VP1 (~83 kDa) and VP2 (~63 kDa), are expressed at a ~1:5 ratio. A third, more-truncated form of the VP2 polypeptide, called VP3 (~60 kDa),

is generated in full, but not in empty, particles by proteolytic cleavage of some 22–25 amino acids from the N-termini of the VP2 polypeptides, following their exposure on the particle surface.

While all parvoviruses encode both NS1 and one or more forms of NS2, only NS1, the replication initiator protein, is absolutely required for virus growth in all cell types (Cater and Pintel, 1992; Naeger *et al.*, 1990). NS1 functions in replication as an ATP-dependent, site-specific DNA-binding protein with DNA nicking and helicase activities, which allows initiation of DNA synthesis at specific viral origin sequences by introducing a site-specific single-strand nick. This provides a base-paired 3′ nt to serve as a primer for successive rounds of strand displacement DNA synthesis (reviewed in Cotmore and Tattersall, 2006a), while the transesterification reaction that creates the nick leaves NS1 covalently attached to the 5′ nt, where it is thought to recruit additional NS1 molecules to form the 3′-to-5′ replicative helicase.

However, parvoviral replication initiators have evolved into highly pleiotropic proteins, playing multiple roles in the viral life cycle. As mentioned above, in addition to their site-specific nicking function, they act as potent transactivators of viral gene transcription, binding to their recognition sequences in viral promoters and activating transcription through acidic C-terminal domains (Legendre and Rommelaere, 1994). In the MVM genome, NS1 binding sites are reiterated so frequently that any sequence of 100 base pairs or more contains a site, and some carry multiple tandem and inverted reiterations (Cotmore *et al.*, 1995). This suggests that NS1 might play a significant role in viral chromatin structure and/or progeny strand packaging. In contrast, NS2 polypeptides play indirect roles in supporting the MVM life cycle, modifying the cells of their natural murine host to support viral replication and mediate efficient capsid assembly. Advances in our knowledge of parvoviral DNA replication and packaging mechanisms have been reviewed extensively elsewhere (Cotmore and Tattersall, 2006a,b).

III. RECOGNIZING THE TARGET: CELL SURFACE RECEPTORS AND VIRAL HOST RANGE

Parvovirus particles are extraordinarily rugged, remaining viable at room temperature for months, or years, and resisting desiccation or exposure to chaotropic agents. However, they also serve as covert delivery vehicles, able to gain access to the host cell cytosol and penetrate into its nucleus, where they lie in wait for it to initiate DNA synthesis as part of its own normal cell cycle. This reliance on the cell's unchecked transit into S-phase therefore suggests that the processes of parvovirus entry and latency remain largely undetected by their host's innate defense mechanisms.

This report focuses on both host range and cell entry mechanisms, since these topics are often intimately linked and informed by each other. Infection initiates through capsid-mediated binding to one or more glycosylated receptor molecule on the cell surface and is followed by virion uptake into the cell via receptor-mediated endocytosis. Transfer across the delimiting lipid bilayer of the entry vesicle into the cytoplasm is then affected by a capsid-borne phospholipase, and this is followed by delivery to, and entry into, the nucleus, where the viral genome is finally released from its protective shell. Thus, parvovirus genomes remain associated with their intact capsid throughout the entire entry process, and possibly even in primary viral transcription complexes, so that host cell–specific interactions with the viral particle could potentially impinge at multiple stages during the initiation of infection. While some parvoviruses exhibit narrowly restricted host ranges, others infect multiple host species and/or many tissues. Although such specificity can operate by disparate mechanisms, and be mediated either during entry or by cell type–specific differences in viral metabolism, two quite distinct patterns of capsid-controlled host range control have arisen in the genus *Parvovirus*, one exemplified by MVM, and the other by the FPV/CPV serotype. Whether these operate by similar mechanisms or even at the same stage in the entry process still remains to be determined.

Rather than interacting with a single cell surface receptor, many virus families employ two more-or-less separate classes of molecules: ''attachment'' receptors, or coreceptors, which simply accumulate virus in the vicinity of the cell surface; and infectious-entry receptors, which critically mediate genome transfer into the cell cytoplasm. Some members of the Parvovirinae are known to bind to a number of different cell surface molecules in ways that potentiate infection, although the extent to which they rely on multiple interactions appears to vary from species to species, and within a species from host cell to host cell, so that few general rules are apparent. Within the genus *Parvovirus*, members of the FPV serotype commonly bind to neuraminidase-sensitive *N*-glycolyl neuraminic acid side chains on some host cell types, but these presumably only function as attachment receptors, since infectious entry is insensitive to neuraminidase and is specifically mediated by binding to host species–specific protein domains on cell surface transferrin receptor (TfR) molecules (Parker *et al.*, 2001; reviewed in Hueffer and Parrish, 2003). In contrast, MVM binds to sialoglycoprotein receptor(s) present at about 5×10^5 copies per cell on murine fibroblasts, and both binding and infection are neuraminidase sensitive, indicating a critical role for specific oligosaccharide side chains in both of these steps. However, at present it is not clear whether one specific cell surface molecule mediates MVM entry, while others effect attachment, or if all 5×10^5 receptors are equipotent.

The clearest example of a receptor interaction dictating parvovirus host range is seen for FPV and its canine-tropic variant CPV, in Chinese hamster ovary (CHO)-derived TRVb cells, which lack any form of TfR. If feline TfR is expressed by transfection in these cells it allows efficient binding of CPV and FPV, leading to infection. In contrast, transfected canine TfR binds CPV capsids poorly, and FPV capsids not at all, and only allows infection by CPV (Hueffer *et al.*, 2003a). In this case, binding is specified by protein determinants on the receptor and involves several critical capsid residues that are arranged some 20–30 Å apart around the threefold spike, suggesting a broad region of receptor–capsid interaction. Remarkably, for CPV this interaction appears to be restricted to as few as one site per capsid rather than occurring at every 60-fold-related position (Hafenstein *et al.*, 2007; Palermo *et al.*, 2006). In contrast, MVM entry does not rely on interactions with the TfR, since MVM infects CHO TRVb cells efficiently without TfR transfection (Cotmore, S. F., and Tattersall, P., unpublished observations), but whether it establishes comparable interactions with other cell surface glycoprotein species is currently unknown. Irrespective of any such protein-mediated interaction, MVM host range is critically regulated by subtle, cell type–specific, interactions with sialic acid-containing oligosaccharides, which bind into the dimple-like depression at the capsid's icosahedral twofold axis. Below, we review details of what is known about receptor binding and host range constraints in these two disparate examples.

A. The MVM model: Glycan-specific interactions around the twofold symmetry axes

MVM exhibits subtle strain-specific variations that allow different isolates to grow productively in murine cells of dissimilar differentiated phenotypes. Two independently isolated strains, termed allotropic variants, were initially identified: the prototype strain, MVMp, which grows productively in culture in fibroblasts such as the A9 cell line; and the hematotropic strain, MVMi, which replicates productively in T lymphocytes and hematopoietic precursors (McMaster *et al.*, 1981; Segovia *et al.*, 1991; Spalholz and Tattersall, 1983). Despite sharing 97% sequence identity and being serologically indistinguishable, these viruses are reciprocally restricted for growth in each other's host cell type (Tattersall and Bratton, 1983). In nonpermissive cells infection is restricted prior to viral gene expression (Antonietti *et al.*, 1988; Gardiner and Tattersall, 1988a), but both virus strains are known to compete for specific binding sites on the surfaces of both cell types (Spalholz and Tattersall, 1983), estimated to be present at 5×10^5 copies per cell on mouse A9 fibroblasts (Linser *et al.*, 1977; Spalholz and Tattersall, 1983). Following intranasal inoculation into newborn mice, MVMp is asymptomatic, and the virus remains confined to

the oropharynx (Kimsey *et al.*, 1986), while MVMi causes a generalized infection in which the main targets are endothelial cells, lymphocytes, and hepatic erythropoietic precursors, but where the pathological outcome varies with host genotype (Brownstein *et al.*, 1992).

The ability of MVMp to grow in fibroblasts was mapped *in vitro* using a selective plaque assay to two specific amino acids at positions 317 and 321 in the VP2 capsid protein sequence (Ball-Goodrich and Tattersall, 1992; Gardiner and Tattersall, 1988b). These lie at or near the particle surface, adjacent to the dimple-like depression that spans the icosahedral twofold axis of the virion (Agbandje-McKenna *et al.*, 1998). When a restriction fragment from MVMp differing at only these two VP2 residues (T317 and G321) was substituted into an infectious plasmid clone of MVMi (A317 and D321), the resulting virus was found to be >100-fold better at infecting fibroblasts than its parent (Gardiner and Tattersall, 1988b). In contrast, when either single change was introduced into MVMi separately, the resulting viruses showed at most a twofold increase in their ability to replicate in fibroblasts (Ball-Goodrich and Tattersall, 1992). This restriction, in turn, allowed the selection of second site mutants that could complement either of these changes (Agbandje-McKenna *et al.*, 1998; López-Bueno *et al.*, 2007). For each of the single mutants, multiple alternative second site mutations were identified, all affecting residues surrounding or extending down the sides of the twofold-related dimple. Surprisingly, if the MVMi backbone already carried the A317T mutation, complementing mutations in D321 were not selected, but instead the additional mutations D399G, D399A, V551A, or D553N were each found to effectively confer fibrotropism. In contrast, when the MVMi backbone already carried the D321G mutation, four of the six second-site mutants identified carried the MVMp A317T change, while in the other two, the coordinated mutations were S460A and Y558H. Thus, in an MVMi backbone, fibrotropism can be conferred by switching the side chains of a number of different residues that surround the twofold depression, suggesting that structural changes in this depression may mediate MVM cell type specificity. While little is know about the control of tissue specificity for most other parvoviruses, it is clear that amino acid changes involved in determining both PPV cell type specificity and virulence are also localized in this depression (Simpson *et al.*, 2002).

Lack of a lymphocyte plaque assay prevented the equivalent analysis of MVMp host range mutants in culture, but this has been effectively accomplished *in vivo* using adult immunodeficient SCID mice (Rubio *et al.*, 2005). Following intravenous injection of MVMp into such mice, this normally apathogenic virus strain was found to evolve through at least two distinct steps, the first of which conferred enhanced virulence, while the second generated complex shifts in host cell specificity and pathogenicity. During the early weeks of subclinical infection, injected MVMp viruses consistently segregated variants that showed altered,

large-plaque, phenotypes when tested *in vitro*, but retained the fibrotropic MVMp host range. However, unlike wild-type MVMp, when these variants were reinoculated into SCID mice via the oronasal route, they spread systemically from the oronasal cavity and were able to access, and replicate in, various major organs such as the brain, kidney, and liver. Genetic analysis of 48 of these clones consistently showed one of three single changes in the VP2 gene, V325M, I362S, or K368R. Both MVMp and the recombinant viruses could be detected in the bloodstream 1- to 2-day postoronasal inoculation and remained infectious when adsorbed to blood cells *in vitro*. However, wild-type MVMp was cleared from the circulation within a few days, while the viremia caused by the mutant viruses was sustained for life, leading to their being described as having higher ''virulence.'' Significantly, attachment of both mutant and wild-type viruses to an abundant receptor on primary mouse kidney epithelial cells could be quantitatively competed by wild-type MVMp capsids, suggesting that this enhanced virulence was not associated with major differences in receptor usage in the target tissues. However, productive adsorption of variants carrying any of the three mutations showed increased sensitivity to neuraminidase, when compared to wild-type virus, suggesting that the particles had a lower affinity for the sialic acid component of the receptor. This diminished affinity for sialic acid–bearing oligosaccharide chains was later confirmed by plasmon surface resonance studies, discussed below. This suggests that the selection of capsids with lower affinity for their cell surface receptors favors systemic infection, which may be a major evolutionary process in the adaptation of parvoviruses to new hosts.

As illustrated in Fig. 3, two of these virulence determinants, residues I362 and K368, are located on the wall of the dimple recess surrounding the icosahedral twofold symmetry axis, while V325 is positioned ~22 Å away in a threefold-related monomer, near the top of the depression. Consistent with this, the X-ray crystal structure of MVMp capsids soaked with sialic acid (*N*-acetyl neuraminic acid) showed the sugar positioned in this depression, immediately adjacent to residues I362 and K368. Thus, this likely identifies the position of the terminal sugar in the infectious receptor attachment site on the viral capsid. However, the equivalent phenotype seen in mutants carrying the V325M mutation suggests that this residue also modulates sialic acid binding in a manner similar to I362 and K368, even though it is physically somewhat distant (López-Bueno *et al.*, 2006). The depression at the twofold icosahedral axis of MVMp does extend toward the loop containing V325 from a threefold-related monomer, which interdigitates with the reference monomer, as shown in Fig. 3C. These observations therefore suggest that although sialic acid is an essential component of the receptor for MVMp infection, and it binds to capsid residues in the icosahedral twofold depression, the carbohydrate component of the surface receptor recognized by the virus may

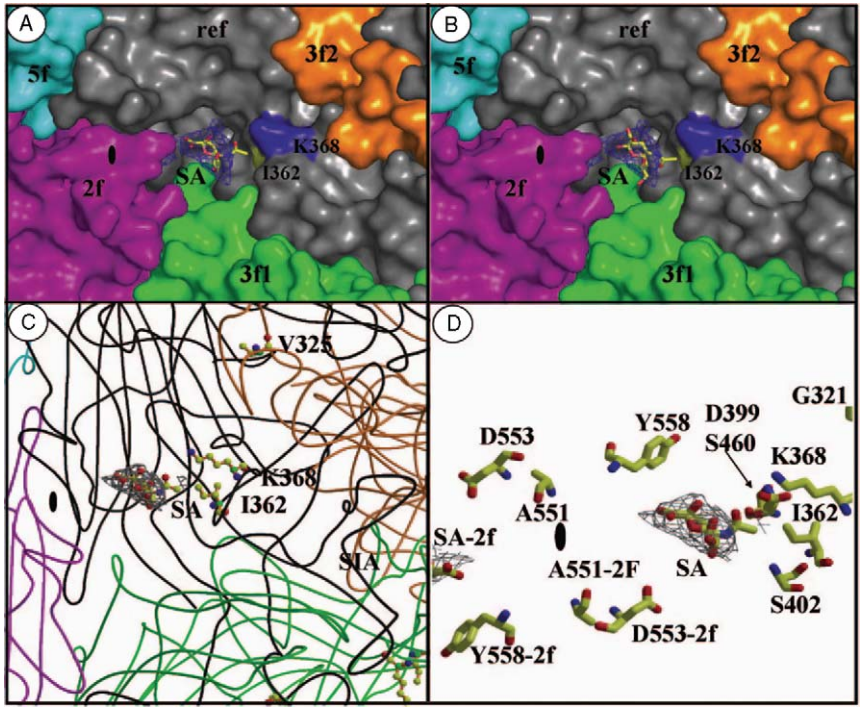


FIGURE 3 Tissue specificity determinants lining the twofold dimple of MVMp: sialic acid (SA) binds in the dimple of the MVMp capsid, surrounded by residues involved in virulence. (A and B) Surface representations of a close-up of the depression at the icosahedral twofold axes of the MVMp capsid showing a reference VP2 monomer (ref, in gray), and icosahedrally related twofold (2f, in magenta), threefold (3f1 and 3f2, in orange and green), and fivefold (5f, in cyan) monomers. The surface positions of residues I362 and K368 are highlighted in yellow and blue, respectively. Residue V325 is not surface accessible. The SA model (colored according to atom type) is shown inside a $2F_O - F_C$ map, in blue, contoured at 1.8σ in the two possible orientations of the carboxyl and *N*-acetyl groups of the SA molecule. (C) Coil representations of the ref, 2f, 3f1, 3f2, and 5f VP2 monomers, colored as in panels A and B. The positions and side chain atoms of residues I362, K368 (in the reference monomer), and V325 (in a threefold-related monomer) are shown colored according to atom type. The SA molecule is shown as in panel A, with the carboxyl group pointing down from the ring and the *N*-acetyl group pointing upward. (D) Close-up of the SA molecule (as in panel C) and residues on the wall of the twofold depression close to the binding pocket that either differ between MVMp and MVMi or confer fibrotropism on MVMi. The approximate location of the icosahedral twofold axes is shown by the filled oval. [Reproduced from López-Bueno *et al.* (2006), with permission. Copyright 2006, the American Society for Microbiology.]

be larger than a single sialic acid residue. Accordingly, a longer oligosaccharide might show additional contacts both within the dimple and adjacent to the loop carrying V325 at the top of this depression.

Evidence for enhanced interactions with longer, sialic acid–bearing oligosaccharides comes from glycan array and surface plasmon resonance studies (Nam *et al.*, 2006). These monitored the interactions of baculovirus-derived VLPs harboring the VP2 protein of MVMi, MVMp, the high-virulence MVMp mutants I362S, and K368R, or the double mutant I362S/K368R, with 180 different glycans. All of the particles bound specifically to oligosaccharide chains carrying terminal sialic acid residues linked 2–3 to a common Gal 1–4GlcNAc moiety. However, binding only occurred when the chains contained at least five saccharide residues and the binding affinity generally increased as a function of chain length. None of the VLPs recognized oligosaccharides with NeuAc α2–6 linked sialic acids, while MVMi was unique in binding efficiently to the four multisialylated glycans with α2–8 linkages that were present in the array, although the MVMp-derived K368R mutant also bound to one of these with lower affinity. This therefore supports a model in which the slight differences in topology and side chain interactions of specific residues lining the dimple, which can be seen in comparisons of the three-dimensional structures of MVMp and MVMi, reflect differences in the abilities of this cleft in each virus to accommodate somewhat different carbohydrate arrangements.

When reintroduced into SCID mice, these high-virulence MVMp mutants subsequently underwent pathogenic tissue-specific evolution, which again involved residues that map to the dimple (López-Bueno *et al.*, 2007). In this case, MVMp viruses carrying the I362S or K368R virulence changes, inoculated via the oronasal route, induced a lethal leukopenia after a 14–18 week delay, reflecting the pattern of disease typically found for MVMi infections within 7 weeks of infection. Sequencing the emerging MVM populations in these leukopenic mice prior to cloning identified consensus sequence changes at G321E and A551V in the I362S infections and at V575A and A551V in the K368R infections. Notably, changes at dimple residues 321 and 551 (indicated in Fig. 3) were among those previously identified in fibrotropic switch mutants selected by plaquing MVMi on mouse fibroblast monolayers. However, clonal analysis of the mutant populations from SCID mice revealed genetic heterogeneity at specific capsid residues, and only a few of these clonal isolates, which retained the parental G321 and V575 residues, were infectious *in vitro*. Rather, consensus genotypes were poorly infectious in culture, even in 324 K cells, an SV40-transformed human cell line that supports both lymphotropic and fibrotropic MVM variants, although virions could be generated following transfection of cloned genomes into these cells, indicating that later stages in the viral life cycle were

conserved. Virions from one such mutant, carrying the consensus mutations A551V and V575A, while unable to initiate infection in culture in a variety of different cell lines, rapidly induced lethal leukopenia when given to SCID mice, suggesting that *in vivo* this virus may exploit a subtly different allotropic interaction. This all suggests that the MVM dimple can be finely adapted to accommodate a range of different oligosaccharides and that, by changing the side chains and interactions of a small number of surface residues, the virus appears to be able to infect diverse repertoires of differentiated host cell types.

Other aspects of the viral life cycle clearly influence MVM's remarkable ability to switch its tissue specificity. In particular, the speed and efficiency with which heterogeneous virus populations are generated during parvoviral disease depend on high viral mutation rates, and resemble the generation of quasispecies typically encountered during the expansion of RNA viruses. Thus, for example, López-Bueno *et al.* (2003) observed that when MVMi-infected SCID mice received passive immunotherapy with a neutralizing monoclonal anti-capsid antibody, escape mutants, harboring single radical amino acid changes at tip of the threefold spike, emerged at high frequency ($2.8 \pm 0.5 \times 10^{-5}$). Such heterogeneity had not been previously expected for this DNA virus, which replicates using the normally high-fidelity DNA synthetic machinery of its host cell. However, similar mutation rates have now been observed for several members of the Parvovirinae (Badgett *et al.*, 2002; Shackelton and Holmes, 2006; Shackelton *et al.*, 2006), although the underlying causes remain conjectural. Thus, during a productive MVM infection, where high mutation rates are coupled with rapid virus expansion, generating up to 10^8 infectious particles per infected mouse, specific virus strains may evolve rapidly, giving rise to host range mutants that are potentially able to infect an alternative set of differentiated cell types.

For MVM there is even further latitude for phenotypic expansion, since the ability of host range mutants to thrive in their new host cell can depend on the sequence, or even the expression level, of NS2, the minor viral nonstructural protein. As discussed above, when MVMi is adapted for growth in fibroblasts, the host range switch typically involves two coordinate mutations in the vicinity of the dimple. However, two host range switch mutants have been characterized that carry a single coding mutation at residue D399 in VP2, to alanine or glycine, together with a second, noncoding, guanine-to-adenine change at nucleotides 1970 or 1967, which influence the splicing patterns of the viral transcripts (D'Abramo *et al.*, 2005). When reconstructed into an infectious molecular clone of MVMi, all single mutants failed to replicate productively in fibroblasts, but viruses carrying a pair of mutations, with one of each type, were highly infectious. Specifically, the single D399 mutations allowed viruses to initiate infection in fibroblasts, but NS2 expression

was low, which led to poor accumulation and release of progeny virus. Mutations at 1967 or 1970 restored the MVMp splicing pattern, enhanced NS2 accumulation, and allowed efficient progeny production and release. Conversely, the D399 mutations destroyed the viruses' ability to initiate infection in EL4 lymphocytes. However, in lymphocyte infections, NS2 was expressed at high ratios even in the absence of upstream mutations, and progeny accumulation was efficient. Choi *et al.* (2005) showed that this requirement for different splicing signals to achieve optimal MVM NS2 levels reflects cell type–specific differences in RNA processing, which can thus impact host range. Exactly why high NS2 levels are required for efficient progeny virus production remains uncertain, and is probably multifactorial, but, in part, it appears to reflect a defect in capsid assembly seen in NS2 depleted cells (Cotmore *et al.*, 1997). This may suggest that it is difficult to assemble the single D399 mutants, but that either a second local capsid modification, such as A317T, or a boost in NS2 levels, eases this constraint. While wild-type NS2 is known to interact with the cellular nuclear export protein, Crm1 (Bodendorf *et al.*, 1999), remarkably, a mutation that promotes higher affinity Crm1 binding than wild type was also able to reverse this progeny production defect, so that even low-level expression of NS2 led to efficient virus expansion (Choi *et al.*, 2005). The high-affinity Crm1 binding mutant used in this study and several other similar mutations were first identified in SCID mice that had been infected with MVMi and exposed to neutralizing polyclonal antisera, in an attempt to protect the mice from leukopenic disease. These single or double amino acids changes in the NS2 Crm1 binding domain increased its ability to sequester Crm1 in a perinuclear locale, leading to an accelerated viral life cycle that somehow allowed the virus to circumvent the effects of neutralizing antibody (López-Bueno *et al.*, 2004). Taken together, this data indicates that mutations in NS2 that promote its efficient interaction with Crm1 can effectively modulate viral host range, by allowing a productive viral cycle to proceed in cells that would normally be nonproductive due to inadequate NS2 expression. Clearly, this provides a second example of how the virus's capacity for rapid evolutionary change can permit shifts in host range *in vivo*. Against this evolutionary force is ranged the extreme conservatism of this intensely compact virus, since most random mutations, or combinations thereof, appear to be incompatible with overall viral viability.

B. The FPV/CPV model: Engaging the transferrin receptor at the threefold symmetry axes

In sharp contrast to the situation in MVM, where research has focused on analyzing changes in specificity for differentiated murine cell types, for viruses of the FPV serotype most attention has been directed at

understanding how the virus switched from being able to infect a number of carnivore species, excluding dogs, to being a potent canine pathogen. This event appears to have occurred early in the 1970s, when a complex virus mutant emerged and spread rapidly through the global dog population, erupting to pandemic status in 1978. This virus, called CPV-2, had lost the ability to infect cats. However, in 1979 an antigenic variant emerged, called CPV-2a, which can infect both host species and has since globally replaced the original virus in both domestic and wild dog populations. Phylogenetic analysis of DNA sequences suggests that all CPV isolates from dogs are derived from a single common ancestor, which only differs by a few nucleotides, some 0.4% of the genome, from the most recent common ancestor among the FPV-like viruses. Most of these changes have been conserved in the CPV variants emerging since 1978. All of the viruses from either cats or dogs replicate efficiently in feline cells in culture, but only CPV isolates infect dogs and cultured dog cells (Truyen and Parrish, 1992). The host range properties of CPV and FPV for both dogs and cats are controlled by multiple residues that map to disparate locales on or around the three-fold spike, as shown in Fig. 4. Primary control of canine host range is determined by residues at VP2 positions 93 and 323, which must be switched coordinately (Chang *et al.*, 1992; Horiuchi *et al.*, 1994; Hueffer *et al.*, 2003b; Llamas-Saiz *et al.*, 1996; Parker and Parrish, 1997; Strassheim *et al.*, 1994). Certain changes at residues 299 and 300 block the ability of the virus to infect dog cells, and changes in that region also appear to control the *in vivo* feline host range of CPV (Truyen *et al.*, 1994). The CPV-2a variant that emerged in 1979, which infects both host species, has additional changes at VP2 residues 87, 101, 300, and 305 (Parrish and Carmichael, 1986; Parrish *et al.*, 1988, 1991), and several other single mutations in CPV-2a have become widely distributed *in vivo* since 1980, including an N426D mutation that is present in the antigenic variant designated CPV-2b, which shares the CPV-2a host range (Strassheim *et al.*, 1994; Truyen *et al.*, 1995).

Some of the host range constraints of CPV and FPV seen in animals are reflected in tissue culture, where it is now clear that the block to infection by FPV in dog cells is primarily due to lack of a functional cell surface receptor. FPV and CPV both bind the feline TfR and use it to infect cat cells, but only CPV can bind to canine TfR. However, although CPV-2, CPV-2a, and CPV-2b all bind the canine TfR and infect dog cells, CPV-2 capsids bind to feline and canine cells much more efficiently and to higher levels than do CPV-2a or CPV-2b capsids, suggesting that CPV-2 forms different interactions with the TfR or binds to additional receptors on those cells (Hueffer *et al.*, 2003a). Thus, while VP2 residues 93 and 323 together control virus binding to the canine TfR (Hueffer *et al.*, 2003a), changes at VP2 residues 87, 300, and 305 in CPV-2a reduce receptor affinity and improve, in some way, the ability of the virus to use this receptor for infection (Hueffer *et al.*, 2003a; Palermo *et al.*, 2006).

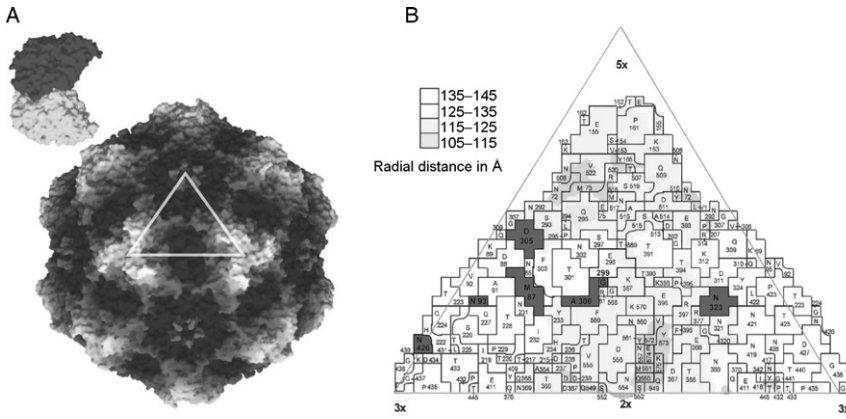


FIGURE 4 Distribution of host-range determinant residues on the surface of CPV. (A) Surface-rendered model of the CPV capsid, viewed from above the dimple that surrounds the twofold symmetry axis, located on the bottom side of the triangle representing a single asymmetric unit, halfway between the threefold spikes. Toward the apex of the triangle lies the canyon surrounding the fivefold cylinder. A model of the ectodomain human transferrin receptor is shown at the same scale to indicate the relative size of the virus and its ligand on feline or canine cells. (B) A road map determined by the method of Rossmann and Palmenberg (1988) showing the surface exposure of VP2 residues in one asymmetric unit of the CPV type 2 capsid. The region shown comprises several symmetry-related VP2 subunits. Residues mentioned in the text that affect receptor binding or host range, and which differ naturally between FPV and CPV strains, are shaded. [Modified from Hueffer *et al.* (2003b) with permission. Copyright 2003, the American Society for Microbiology.]

TfR is a type II membrane protein that protrudes about 30 Å from the cell surface. The structures of canine and feline TfR have yet to be determined, but structural information is available for the human TfR, which is 79% identical to feline TfR at the amino acid sequence level. The human TfR consists of a large, butterfly-shaped, dimeric molecule with a span of about 100 Å and a molecular weight of 180 kDa. Each monomer has an apical domain, a helical domain, and a carboxypeptidase-like domain (Lawrence *et al.*, 1999), and mutagenesis of feline and canine TfRs indicates that both CPV and FPV bind to the apical domain. In confirmation of results from the *in vitro* cell binding assays, both FPV and CPV capsids were found to bind strongly to a recombinant form of the feline TfR ectodomain, while CPV-2b capsids bound much more weakly. In contrast, FPV capsids failed to bind at all to recombinant canine TfR (Palermo *et al.*, 2006), and while CPV-2 capsids bound the canine receptor, they did so only to very low levels, and CPV-2b binding was essentially undetectable. This binding pattern reflects the weak interaction seen in culture when the same receptor was expressed by transfection on otherwise TfR-negative

CHO cells, which nevertheless was sufficient to allow CPV-2b to be taken into and infect the cells. This low level of binding between canine TfR and CPV-2 or CPV-2b capsids, and its inability to bind FPV are in large part determined by minimal differences in the TfR apical domain, since simply changing residues 383 and 385 in canine TfR to their feline TfR counterparts allowed the mutant receptor to bind FPV to levels similar to those seen for the feline TfR, and likewise increased binding of CPV capsids. Residues 383 and 385 create a potential glycosylation site on canine TfR, which appears to be occupied *in vivo*, but the increased binding seen for the mutant is probably due to protein sequence, rather than oligosaccharide, changes, since enzymatic removal of N-linked glycans from the canine receptor did not lead to increased binding (Palermo *et al*., 2006).

The specific binding of CPV to canine TfR is thus controlled by several residues, positioned 20–30 Å apart on the ''high ground'' around the threefold spike, suggesting that a broad surface of the capsid interacts with the receptor (Govindasamy *et al*., 2003; Hueffer *et al*., 2003a,b). While less is known about the capsid residues that are involved in feline TfR binding, capsid mutations reciprocal to those which in CPV prevented canine TfR binding, at positions 93 and 323, did not appear to alter the binding of FPV to the feline TfR expressed on CHO TRVb cells, indicating that the canine and feline receptors make somewhat different contacts with these viruses (Hueffer *et al*., 2003a).

Asymmetric cryo-electron microscopic (cryo-EM) reconstructions, supported by quantitative *in vitro* binding studies, suggest a model in which the canine TfR ectodomain can bind to only one, or a few, of the 60 icosahedrally equivalent sites on empty CPV capsids, suggesting that these either have inherent asymmetry or that binding to their receptor induces asymmetry (Hafenstein *et al*., 2007). When a difference map, calculated by comparing the virus-receptor complex with the native virus, was superimposed on a stereographic projection of the icosahedral CPV surface structure, the known crystal structure of the human TfR ectodomain dimer (Lawrence *et al*., 1999) could be modeled into the additional cryo-EM density such that one of its two apical domains was in contact with the shoulder of one of the CPV spikes. In this model, the projected contact sites on the virus included residues that are known to control specific binding to canine TfR (Hueffer *et al*., 2003a).

Possibly, the restricted binding observed for the CPV–canine TfR interaction is due to inherent asymmetry in the empty particle, with one, or a few, distinct sites that have a conformation capable of binding TfR, whereas the other icosahedrally equivalent sites are slightly different. If so, this asymmetry must exist prior to genome encapsidation, and might indeed direct that process. This might happen if assembly is initiated around a special icosahedral fivefold vertex, which is the site of subsequent genome entry and exit, as is believed to occur in tailed

bacteriophage (Morais *et al.*, 2003). Alternatively, the final subunits to assemble may be sterically hindered from perfectly finishing the icosahedron, thus creating an asymmetric structural element at the capsid surface. However, it is also possible that TfR binding itself might induce asymmetry in an initially icosahedral particle, perhaps priming it for subsequent conformational shifts destined to occur during cell entry, as discussed below.

Thus it appears that after first adapting to dogs by acquiring changes that allowed it to bind canine TfR in a productive way, CPV has continued to evolve *in vivo*, acquiring additional mutations that lower its affinity for this receptor but enhance its ability to infect cells. Use of the TfR as the cellular receptor for these viruses also correlates well with the patterns of tissue specificity seen *in vivo*, as this receptor is highly expressed on crypt cells in the intestinal epithelium and on hematopoietic cells, which are the main target cells of CPV and FPV in animals (Parrish, 1995). However, TfR acts as more than a simple tether, dragging the capsid into the cell, since the precise interactions are important for successful cell infection, and some mutational changes in either the virus or the receptor allow capsid binding and cell uptake without leading to infection (Hueffer *et al.*, 2003b; Palermo *et al.*, 2003).

Transfer of the viral genome across the limiting lipid bilayer of its prospective host cell is one of the most challenging steps encountered during cell entry, and for many nonenveloped viruses this maneuver is so finely orchestrated that critical interactions required with cell surface receptor molecules play a major role in determining viral host cell specificity. To date, it is not clear whether any parvoviruses employ a receptor-orchestrated transfer mechanism of this type, but it is clear that they must undergo a specific structural transition after endocytosis, but before bilayer penetration, which leads to exposure of their VP1-specific ''entry'' peptide, VP1SR, and that inappropriate exposure of this peptide leads to their inactivation. Thus, it is tempting to speculate that interactions with specific receptors could modulate host range by allowing this transition to occur in a controlled way or in a favored locale that would be compatible with the transfer of a viable particle across the lipid bilayer, as we will now discuss.

IV. BREACHING THE OUTER BARRIER: TO THE CYTOSOL AND BEYOND

Viral particles must function as rugged containers that protect the genome from environmental assaults encountered during transmission, but must also recognize and respond to a succession of specific cellular signals that allow them to navigate the complex entry portals of their host cell, and

ultimately deliver their nucleic acid to the appropriate replication compartment. Since parvoviral virions lack any accessory proteins, the component polypeptides of the nonenveloped capsid are the sole mediators of entry. While the capsid shell itself directs certain interactions, many other necessary contacts with cellular pathways rely on signal-rich N-terminal extensions present on VP1 and VP2 molecules. These are initially sequestered within the particle but are sequentially deployed at the virion surface during the cell exit and entry processes by a series of concerted conformational shifts in the capsid structure. Aspects of parvoviral entry have been reviewed by others in recent years (Vihinen-Ranta and Parrish, 2006; Vihinen-Ranta *et al*., 2004), but in this section we attempt to integrate data from a broader range of analyses. Specifically, we will examine the structural flexibility and transitions that viral particles are able to undergo *in vitro*, explore the structure of the VP1SR entry peptide, and finally consider vesicle trafficking and deployment of the entry peptide *in vivo*. This overview suggests that each step in the program of intracellular translocation of the intact particle to the cell nucleus is catalyzed by successively revealed motifs built into the capsid structure itself.

A. Structural transitions in the virion induced *in vitro*

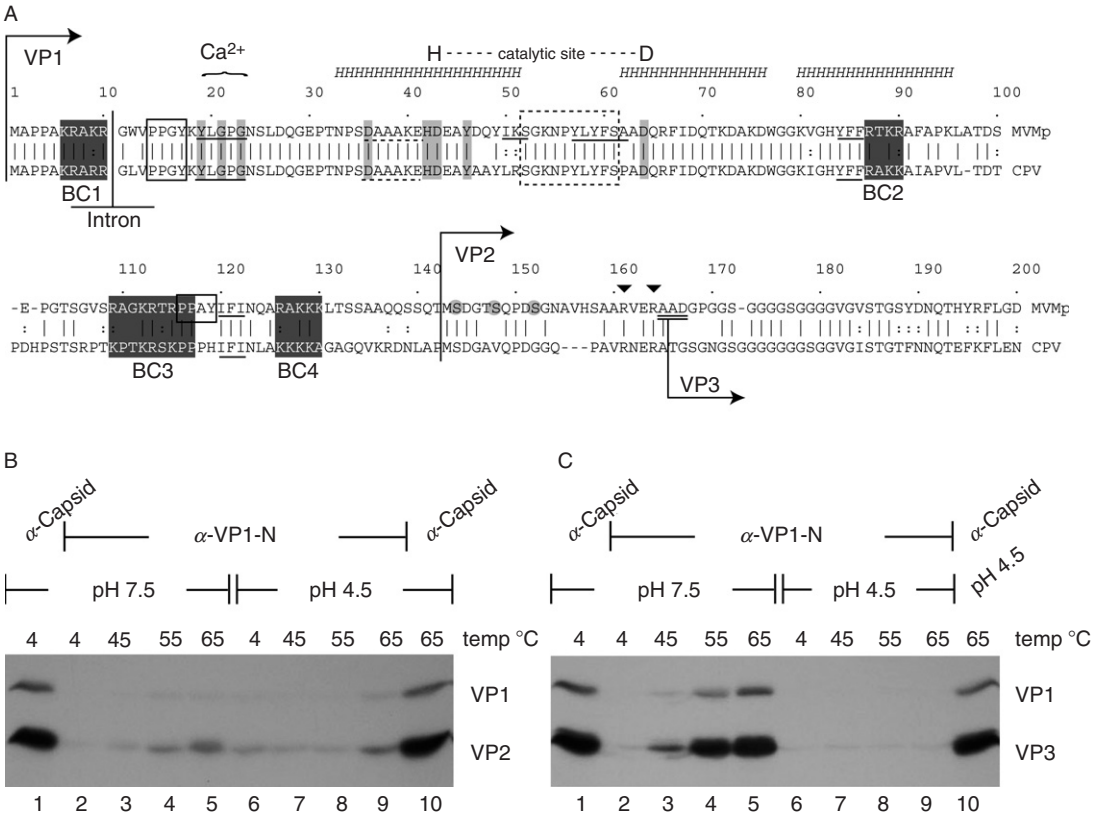
Empty parvovirus capsids are constructed from 60 copies of the capsid polypeptides, comprising, on average, 50 copies of VP2 and 10 copies of VP1. As diagrammed in Fig. 2, VP1 contains all of the VP2 sequence but has an extra, basically charged, 142-amino acid N-terminal extension, termed the VP1-specific region, VP1SR. The VP1 extension, shown in detail in Fig. 5A, is dispensable for both capsid assembly and DNA packaging, but is absolutely required for infectious entry, since it carries a phospholipase A2 (PLA2) active site essential for endosomal exit, as well as various clusters of basic amino acids and signaling motifs that may function at subsequent steps during nuclear localization. However, in MVM, only 547 amino acids from the C-terminus of the VP polypeptides are ordered, and therefore visible in the crystal structure, while the signal-rich N-terminal extensions, of 39 residues for VP2 and 181 residues for VP1, resist 60-fold averaging. These N-terminal regions are sequestered within the empty particle, but become sequentially externalized at specific steps in its life cycle, to modulate particle stability and to mediate successive interactions with the host cell.

In the viral particle, a cylindrical projection surrounds each of the 12 fivefold symmetry axes, and is itself encircled by a 15 Å-deep exterior depression, of unknown function, called the canyon. The cylinder is created by the juxtaposition of antiparallel β-hairpins from each of the fivefold-related capsid proteins. These β-hairpins are not interdigitated within the upper part of the resulting ''turret'' and so are potentially

flexible, and their organization in the crystal structure creates a narrow, 8 Å, central pore that penetrates through the virion shell to the particle interior. The tightest constriction in this pore is formed at its inner end by the juxtaposition of leucine side chains from VP2 residue 172 of five independent VP2 molecules. The phenotypic analysis of a complete set of amino acid substitution mutants at this highly conserved residue strongly suggests that L172 modulates the extrusion of VP1 N-termini (VP1NT) (Farr and Tattersall, 2004). All but one of these mutants produced DNA-containing virions, but only two, L172V and L172I, were infectious, the others being blocked for assembly, packaging, or viral entry. Several of the mutants were significantly defective for assembly at 39 °C, but not at 32 °C, and, while tryptic cleavage of their VP2 N-termini was normal, VP1 was degraded during *in vitro* proteolysis of mutant, but not wild-type, virions. The L172W substitution, while not significantly affecting assembly, effectively abrogated genome encapsidation, contributing to the emerging genetic evidence for both the *Parvovirus* and *Dependovirus* genera suggesting that one of these fivefold pores mediates encapsidation of the viral genome late in infection. For this step, the presumptive portal acts in concert with a viral helicase complex, which has been shown for AAV to be a small Rep protein, but, for the autonomous parvoviruses, is derived from NS1 in an unknown manner. It is currently not clear whether the packaging portal is physically distinct from the other 11 cylinders prior to being selected as the encapsidation point.

X-ray crystallography of MVM virions revealed ordered structure beginning at VP2 residue 40, which is on the inside of the shell, forming part of the basal structure that supports the cylinder. In full virions, but not in empty particles, the pore contains additional weak density, into which has been modeled a single copy of a conserved glycine-rich peptide that spans VP2 residues 28–38 (VP2 residue 28-GGSGGGGSGGG-38), shown in Figs. 1 and 5A. Additional density, corresponding to residues 36–39 from the remaining capsid proteins, extends back into the particle interior. Since, in the crystal structure, each pore accommodates a single glycine-rich peptide, only one of the five locally available VP N-termini can be externalized at any time. However, almost all of the VP2 N-terminal peptides become surface-exposed during entry, or during proteolytic digestion *in vitro*, suggesting that there are dynamic fluctuations in pore structure. Since the pore is only 8 Å in diameter, but must accommodate the passage of amino acids with bulky side chains during these extrusion events, this implies that the cylinder is an inherently dynamic structure. Indeed, one function of the canyon might be to provide space for the β-hairpins of the cylinder to move outward, thus allowing the pore to expand.

Viral genomes are packaged into some sort of preassembled empty particle, but evidence from AAV2 suggests that such particles are


A
VP1
Ca2+
H ----- catalytic site ----- D
BC1
Intron
BC2
VP2
BC3
BC4
VP3
B
α-Capsid
α-VP1-N
α-Capsid
pH 7.5
pH 4.5
4 4 45 55 65 4 45 55 65 65 temp °C
VP1
VP2
1 2 3 4 5 6 7 8 9 10
C
α-Capsid
α-VP1-N
α-Capsid
pH 7.5
pH 4.5
pH 4.5
4 4 45 55 65 4 45 55 65 65 temp °C
VP1
VP3
1 2 3 4 5 6 7 8 9 10

somewhat specialized since they have to be assembled in the presence of the Rep proteins, which are the functional equivalent of the MVM NS1 polypeptide (Wu *et al.*, 2000). Both VP1 and VP2 N-termini are completely sequestered inside these empty capsids, but a structural shift occurs in the packaging complex prior to, or concomitant with, the beginning of DNA translocation, which allows a cohort of VP2 N-terminal peptides to emerge at the virion surface (Cotmore and Tattersall, 2005a). Whether these termini play a role in the packaging process remains uncertain, but they do appear to stabilize the final structure, as discussed below. These N-terminal extensions carry phosphoserine-rich export signals, which in some cell types direct packaged virions to be trafficked out of the nucleus prior to cell lysis (Maroto *et al.*, 2004). Full particles are thus released from the parental cell with all of their VP2 N-termini intact, but a third structural protein, VP3, is subsequently generated from most VP2 molecules by a proteolytic cleavage that removes 22–25 amino acids from its N-terminus. VP2 to VP3 cleavage can occur in the extracellular environment following release, but, if not, invariably occurs during entry into a new host cell (Clinton and Hayashi, 1975; Paradiso, 1984; Ros *et al.*, 2002). This cleavage can be mimicked *in vitro* by incubating virions with a broad variety of proteases, but the cleavage site appears flexible, and very accessible, so that it has been essentially impossible to totally ablate cleavage in MVM by mutagenesis or to stop it occurring *in vivo* using combinations of protease inhibitors (Clinton and Hayashi, 1975; Tullis *et al.*, 1992; Farr, G. A., Cotmore, S. F., and Tattersall, P., unpublished results). Since each pore can only accommodate one N-terminal peptide at a time, it is suggested that following proteolytic cleavage the residual

FIGURE 5 Properties of the VP1 specific region. (A) Landmarks of the MVM VP1 N-terminus aligned with that of CPV, showing the basic clusters (shaded black), SH2 ligand motifs (single underline), SH3 ligand motif (dashed underline), PPXY motifs (open boxes), and individual PLA2 active site residues of the Ca^{2+} binding and catalytic sites (shaded gray). The position of the minor splice intron is shown as an inverted "T," and the starts of VP1, VP2, and VP3 are indicated by arrows, and, in the latter case, potential N-terminal residues are double underlined. The serine residues phosphorylated in the VP2 N-terminus are circled in gray and the tryptic sites upstream of the VP3 N-terminus denoted by inverted carets (▼). Open, dashed box denotes conserved sequences unique to parvoviral PLA2s, between the predicted helices (*HHH*) bearing the catalytic histidine [H] and aspartic acid residues [D]. (B) Wild-type virions with intact VP2 N-termini (VP1/VP2 virions) were incubated for 10 min at the temperatures and pHs indicated, before buffer conditions were normalized and samples immunoprecipitated with antibodies that only react with intact virions (lanes 1 and 10), or with the VP1 N-terminal peptide (lanes 2–9). (C) Wild-type virions with cleaved VP2 N-termini (VP1/VP3 virions) were treated as in panel B. [Panels B and C reproduced with permission from Farr *et al.* (2006). Copyright 2006, the American Society for Microbiology. All rights reserved.]

glycine-rich sequence that is left in the pore is in some way retracted into the particle interior, and replaced by the intact terminus of a fivefold-related VP2. However, as mentioned above, the fivefold pores are quite narrow, and could not accommodate the bulky side chains that would need to be threaded through the cylinder from the particle interior, suggesting that each cylinder may be metastable. Remarkably, MVM virions carrying the single point mutations V40A, N149A, N170A, L172F, or L172T, located in the base of the cylinder, are stable as long as their VP2 N-termini remain intact, but become unstable when their VP2 N-termini are cleaved, disgorging their VP1SRs and genomic DNA at neutral pH (Farr *et al*., 2006; S. F. C. and P. T., unpublished results). This suggests a model in which the exposed VP2 N-termini act as ''guy-ropes,'' stabilizing the virion by preventing the metastable cylinder from undergoing a major structural rearrangement that is required for VP1SR extrusion, and which normally occurs at a later stage in entry. These point mutations apparently promote instability by lowering the activation energy required for this final transition. In this model, externally tethered VP2 N-terminal peptides stabilize the full virion, but cleavage of the resident cohort results in a transient conformational instability that allows concerted replacement of the cleaved peptides by a subsequent cohort of intact VP2 N-termini, which in turn restabilize the virion. Thus, the MVM structure would undergo several successive waves of destabilization and restabilization, until all of the available VP2 N-termini were cleaved, at which point the cylinders would exist permanently in the metastable state, poised to undergo the more drastic rearrangement that leads to extrusion of the VP1SR.

Although VP1 contains the same proteolytic cleavage site that is found in VP2, this is not accessible to digestion, and the VP1SR remains totally sequestered within the capsid during the early stages of entry. However, *in vitro*, the particle is capable of undergoing its second, more-extensive, rearrangement in response to controlled heating, discussed above, which allows exposure of the VP1SR without causing virion disassembly (Cotmore *et al*., 1999; Vihinen-Ranta *et al*., 2000; Weichert *et al*., 1998). In accord with the ''guy-rope'' model, freshly harvested, VP2-intact, virions are substantially refractory to this transition, but it is greatly facilitated, and rendered almost quantitative at neutral pH, by extensive proteolysis of VP2 N-termini to yield VP3, as documented in Figs. 5B and C, respectively, where transitioned particles are quantified by precipitation with antibodies directed against the VP1SR. Remarkably, this VP2 cleavage also renders the capsid transition highly pH dependent, so that it is impossible to induce under acidic conditions, at least just by heating. However, such pH-induced stabilization is entirely reversible, because once returned to a neutral environment, particles transition in response to

heat as if they had never experienced low pH (Farr *et al*., 2006). The VP2 cleavage thus resembles an activation cleavage step seen in a number of other nonenveloped virus families, where a previously stable virion is potentially compromised by a specific proteolytic event that facilitates subsequent exposure of a protein known to be essential for membrane penetration (Bubeck *et al*., 2005; Chandran *et al*., 2003). This allows the particle to exist in a metastable state, where the lowest energy form of the cleaved product is sequestered by the energy barrier between the two forms (Hogle, 2002). During entry, such viruses encounter some form of catalyst, such as low pH or an interaction with a specific receptor, which releases the metastable configuration, allowing the *de novo* exposure of sequences required for membrane penetration. Extensive proteolysis of the VP2 N-termini thus appears to play a comparable global role for MVM, in that it has a major effect on the stability of most particles in the population, strongly suggesting that it is likely part of a programmed entry mechanism. However, this cleavage has an unexpected outcome: it renders subsequent exposure of the entry peptide highly pH dependent, such that it occurs readily at neutral pH, but is effectively, but transiently, suspended in acidic environments. The structural basis for this enhanced stability at low pH remains to be detailed, and it may be that *in vivo* it is constrained by, for example, receptor interactions. Otherwise, it appears to indicate that the virion must access a neutral locale before it can undergo the type of programmed transition that is needed to expose its bilayer-penetrating PLA2 activity, and that this occurs as part of an authentic, and highly controlled, unfolding process, ultimately leading to productive infection.

In support of this model, heat-induced transition *in vitro* typically results in exposure of both the VP1SR and the viral genome (Cotmore *et al*., 1999; Farr *et al*., 2006; Vihinen-Ranta *et al*., 2002; Weichert *et al*., 1998), either of which would be irreversibly damaged within an obligate late endosomal/lysosomal entry compartment by exposure to hydrolases or depurinating acidic conditions. Enhanced virion stability at low pH could thus serve to protect these sensitive elements as the particle is trafficked through hazardous entry compartments into a more favorable vacuolar microenvironment. Alternatively, although apparently closely linked *in vitro*, exposure of the VP1SR and viral genome might be part of a multistep process *in vivo*, triggered sequentially by different stimuli in the entry pathway.

Suikkanen *et al.* (2003b) drew substantially different conclusions concerning the significance of particle acidification during CPV entry. They observed that CPV particles exposed to pH 4–6 *in vitro* developed measurable PLA2 activity, which persisted when virions were returned to neutral pH. Accordingly, they suggested that low pH could provide

an essential activation step in virion maturation preparatory to cytoplasmic entry, which correlated with immunofluorescence studies of virion uptake, discussed later, that show exposure of VP1NT in a cellular lysosome-like compartment. However, the study does not report what proportion of CPV particles became structurally rearranged, or whether they remained infectious. It is possible, therefore, that this observation corresponds to the enhanced VP1 accessibility seen for a small proportion of MVM VP2-intact virions following exposure to pH 4.5 (compare lanes 2 and 6 of Fig. 5B), and which is not seen in VP2-cleaved particles (compare lanes 2 and 6 of Fig. 5C). According to the alternative, ''low pH-stabilization model,'' developed here, any particles in which these sequences were exposed prematurely would be unlikely to progress correctly through the rest of the programmed sequence, and any particle in which they became exposed in an acidic environment, would, in any case, be inactivated. Such low-pH-induced activation would also be surprising, and counterintuitive, in any virus that, like CPV, transits through the gastrointestinal tract of its host. However, further experiments will be needed to clarify whether these disparate findings represent a significant biological difference between CPV and MVM.

Ultimately, the genome may well be extruded *in vivo*, as it is *in vitro*, but still remain attached to, and possibly sequestered by, the particle. Prolonged storage of VP2-cleaved MVM virions at 4 °C does lead to exposure of both VP1SR and the genome in an increasing proportion of otherwise intact particles. However, strong interactions between the left-end hairpin of the DNA and the transitioned particle keep these two elements together. Attempts to recapitulate this type of measured transition *in vitro*, just using heating steps, have proven equivocal, but it is possible to bind the left-end hairpins of MVM to intact particles *in vitro* (Willwand and Hirt, 1991), so that perhaps physiologically induced transitions might preserve such interactions.

B. Essential elements in the VP1-specific entry peptide

During infection, VP1 molecules are transported into the nucleus as part of a trimeric assembly intermediate, comprising one VP1 and two VP2 molecules, which are then further assembled into empty particles (Riolobos *et al*., 2006; Valle *et al*., 2006). However, whether these heterotrimers are distributed throughout the particle so that there is one VP1SR at 10 of the 12 fivefold symmetry axes, or are clustered in some other way, remains uncertain. The sequence of the MVM and CPV VP1 N-terminal regions are shown in Fig. 5A, with the positions of the VP2 start sites and the predominant VP2-to-VP3 cleavage sites indicated. The 142-amino acid VP1SR contains at least three distinct elements: (1) a short N-terminal peptide that contains a consensus nuclear localization sequence (NLS)

dubbed BC1, (2) a PLA2 domain of around 70–80 amino acids that is highly conserved among the Parvoviridae, and (3) a second stretch of some 70 amino acids, which carries a series of basic amino acid clusters (BC2–BC4) that resemble conventional NLS, and, in MVM, also contains a PPXY motif that is essential for infectious entry. As can be seen in Fig. 5A, the VP1SR also contains several putative *src* homology (SH) interaction domains, to which no function has yet been ascribed. Unfortunately, to date we have no structural data for the VP1SR positioned either inside the particle or following its extrusion to the virion surface. It is quite likely that this peptide domain may need to unfold and refold during transit, to navigate its exit portal, and while the conserved PLA2 module is clearly essential for infection, the exact limits of this functional unit have not been determined. It is thus possible that the inboard ~70-amino acid peptide, which spaces the PLA2 sequences from the VP core, may also play a structural role in the folding and disposition of this essential enzyme, or may function as a ''stem'' to position the PLA2 active site at an optimal orientation and distance from the virion surface.

1. The PLA2 domain

The conserved PLA2 domain, containing a sequence of ~60 amino acids that can be modeled into a characteristic PLA2 helical fold, is present in most Parvoviridae, generally occupying a region near the extreme N-terminus of VP1. First identified by Zadori *et al.* (2001), this element is expressed in seven out of the nine genera in the family Parvoviridae, while no other virus families are currently known to possess such an activity (Tijssen *et al.*, 2006). The exceptions within the Parvoviridae are *Aleutian mink disease virus*, the single member of the genus *Amdovirus*, and members of the *Brevidensovirus* genus of insect parvoviruses. Phospholipases are classified according to the position of the ester bond they hydrolyze in the glycerol backbone of their phospholipid substrate, with PLA2 enzymes cleaving fatty acids at the sn-2 position. Parvovirus PLA2s require millimolar Ca^{2+}concentrations for catalysis, which groups them with a large class of extracellular or secretory enzymes (sPLA2s) rather than with intracellular species. Parvoviral PLA2s comprise a novel subfamily, Type XIII, of the secreted PLA2 (sPLA2) superfamily (Balsinde *et al.*, 2002; Brown *et al.*, 2003), which contain a YxGxG Ca^{2+} binding site and a histidine/aspartic acid active site, as shown in Fig. 5A. Where structural details are known, the active site H and D residues in sPLA2s are situated on apposing α-helices, which are usually held in a parallel orientation by a number of disulfide bonds (Berg *et al.*, 2001). Indeed, these small proteins are remarkable for the number of cysteine residues they contain—that is, except for the parvoviral enzymes, which contain none. It seems likely that this absence of disulfide bonds reflects the extraordinary requirement for parvoviral PLA2s to be translocated from

the inside to the outside of the virion. The viral enzymes are also distinguished by being more compact than other subtypes, particularly in the loop between the two α-helices carrying the active site residues, which is normally 20–40 residues long, but is truncated to ~10 residues in the parvoviral PLA2s, with several of these being highly conserved across the parvoviral genera. Remarkably, PLA2s from different parvovirus genera can vary in specific activity by 1000-fold, but all exhibit resistance to most specific sPLA2 inhibitors and low phospholipid polar head group specificity (Canaan *et al.*, 2004), perhaps as a consequence of the relative lack of rigidity predicted from the absence of disulfide cross-links. Accordingly, parvoviral PLA2s exhibit broad substrate specificity *in vitro*, hydrolyzing phosphatidyl-glycerol, phosphatidyl-choline, and phosphatidic acid with high efficiency, phosphatidyl-ethanolamine and phosphatidyl serine somewhat less well, and phosphatidyl-inositol poorly. These enzymes can therefore attack the outer leaflet of mammalian cell bilayers (Tijssen *et al.*, 2006). They have pH optima between 6.0 and 7.0, and require concentrations of calcium that are typically ~10,000 times those found in the cytosol (the $^{Ca}K_d$ for the PPV enzyme is 1 mM), suggesting that they are unlikely to function in this environment. How the apparently globular viral PLA2 polypeptide transits an ~8Å channel in order to function in endosomal escape remains enigmatic. Given that these enzymes lack disulfide bridges, it may be that this feat is achieved by both the directional unfolding and refolding of the enzyme, as well as by the opening of the pore at the fivefold vertex. This would allow the bulkier side groups of the random coil form of the polypeptide to reach the exterior of the virion, where it could refold to an enzymatically active form.

2. Nuclear localization motifs, basic clusters, and PPXY motifs

Capsid proteins must be transported into the cell nucleus twice during the viral life cycle, first as trimeric assembly intermediates following synthesis, and then again during cell entry, to traffic the incoming viral genome into the nucleus. Lombardo *et al.* (2002) identified four clusters of basic amino acids in the VP1SR of MVM that conform to conventional NLS sequences, as shown in Fig. 5A, and showed that two of these, BC1 and BC2, as well as a nonconventional structural domain in VP2 referred to as a nuclear localization motif (NLM), were able to target individually expressed VP proteins to the nucleus. Peptides containing the BC1 equivalent from CPV (6-KRARR-10) could also transport foreign proteins into the cell nucleus, while changing individual basic residues to glycine, impaired such transport (Vihinen-Ranta *et al.*, 1997). Introducing these mutations into an infectious plasmid clone gave virus with diminished infectivity, suggesting that BC1 might also be involved in transporting

incoming CPV virions to the nuclear pore (Vihinen-Ranta *et al.*, 2002). However, direct associations with members of the cellular karyopherin family of shuttling transport factors, which would be expected to mediate such processes, have yet to be demonstrated, and the BC1 motif is positioned immediately next to conserved PLA2 sequences, so that major substitutions in the MVM BC1 do compromise PLA2 activity (Farr, G. and Tattersall, P., unpublished results), and hence impair virion infectivity for a different reason. Thus, at present, the trafficking role of BC1 during virion entry remains uncertain. In contrast, while the entire region between BC2 and BC4 could not be deleted without destroying infectivity (Lombardo *et al.*, 2002), BC3 and BC4 did not behave like NLS as part of microinjected peptides (Vihinen-Ranta *et al.*, 1997), and did not show transport activity for VP1 either expressed alone or in the context of the MVM genome (Lombardo *et al.*, 2002), so that their role in the viral life cycle remains obscure.

Comparisons with the VP1 and VP2 N-termini of AAV2, which together constitute a region equivalent to the parvovirus VP1SR, highlight the complex nature of this region. Thus, while AAV2 similarly deploys these sequences to mediate virion entry, the peptides have no NLS activity in the position of BC1 (Sonntag *et al.*, 2006). However, they do retain both the PLA2 module and a 70-amino acid sequence containing three basic clusters in approximately the positions of the parvovirus BCs 2 through four motifs. Notably, the last two motifs are represented in the virion both as part of approximately five PLA2-bearing VP1NT, and also as part of five VP2 N-termini, which, like VP1NT, can become exposed at the virion surface *in vitro* during a heat-induced transition (Grieger *et al.*, 2006; Sonntag *et al.*, 2006). While VP2 forms are not absolutely essential for AAV2 infectivity, the expression of these two basic motifs on a discrete extension suggests that the sequence does not merely serve as scaffolding for the PLA2 domain. Rather, it appears to perform some specific function, as it would if, for example, it provided additional signals that enhance VP1-mediated nuclear trafficking. Alanine scanning mutagenesis directed at the three basic motifs effectively impaired the infectivity of the resulting particles (Wu *et al.*, 2000), as did substitution of glutamic acid for three of the basic residues in each motif (Sonntag *et al.*, 2006), but substituting asparagine for two basic residues in the first two motifs gave infectious virus, indicating that these two motifs may not individually constitute critical nuclear homing signals. In contrast the third motif (166-PARKRLNF-173) in AAV2, called BR3 by Grieger *et al.* (2006), was inactivated by the double asparagine substitution. Significantly, this mutant retained functional PLA2 activity, and simply substituting the BC1 NLS from CPV (4-PAKRARR-10) for the mutated sequence, restored infectivity, suggesting that this cluster might well be implicated in virion

trafficking. However, Sonntag *et al*. (2006) could not detect transport activity associated with this peptide or with the longer sequence, 160-GKAGQQPARKRLNF-173, following microinjection, although peptides representing the first two basic clusters did mediate nuclear transport. Thus the available evidence is conflicted, but the strong conservation of spaced basic clusters, together with the negative effects of mutations and deletions in this complex region, indicate that it has some sort of essential role(s) in entry, possibly involving nuclear trafficking.

In MVM there are also two PPXY motifs in the VP1SR, one (6-KRAKRGWVPPGY-17) positioned just downstream of BC1 and the other (109-RAGKRTRPPAY-119) overlapping BC3. PPXY motifs could potentially influence trafficking because this sequence binds a subgroup of cellular ''WW'' domain-containing proteins. WW domains form a large family of interaction modules, which mediate a wide range of protein–protein interactions in complex regulatory networks in the cell. For example, PPXY motifs control the trafficking of some cell surface receptors following endocytosis and orchestrate interactions with the class E vacuolar protein sorting machinery, which directs trafficking to the multivesicular body. This motif can also mediate ''late domain'' functions in enveloped viruses, directing the final pinching-off step during progeny virus budding. Such interactions are generally inactivated by mutating the PPXY tyrosine to alanine, and when introduced into either of the MVM sites this mutation severely impaired virion infectivity (Farr, G. and Tattersall, P., in preparation). However, the BC1-proximal mutation also inactivated the viral phospholipase so that its significance for other steps in entry is hard to assess. In contrast, the BC3-proximal mutant retained PLA2 activity, and could complement PLA2 knockout virions for entry, but could not itself be complemented, even by wild-type virus. This suggests that it is likely required *in cis* with the incoming virion and operates at a point in the entry process that is downstream of the PLA2 function. All other members of the genus *Parvovirus* lack a perfect VP1SR PPXY motif in the BC3-equivalent position, for instance, in both FPV and CPV this sequence is 117-PPPH-120. However, the exact sequence constraints that operate on the consensus remain uncertain, so that this region of the VP1SR may also play motif-driven trafficking or interaction roles in other parvovirus entry pathways.

C. Endocytosis, vacuolar trafficking, and structural transitions *in vivo*

Relatively little is known about parvovirus infectious entry pathways, in part because productive and nonproductive routes are difficult to differentiate. Particle-to-infectivity ratios are in the order of 300:1 for

MVMp and ~1000:1 for CPV, with most of the incoming particles failing to navigate the entry compartments successfully, so that ultimate translocation into the nucleus is a rare event. Vesicle trafficking is complex, and its study is further complicated by the observation that particles are delivered to many different cell locations, including both recycling and degradative compartments. Many studies typically compound these problems by using high multiplicities of input virus to facilitate signal detection, but trafficking pathways within the cell can be modified by cargo overload or drug treatments. Consequently, much of the internalized virus appears to enter dead-end pathways that cannot provide the genome access to the nucleus, perhaps becoming structurally modified and/or inactivated *en route*. In such situations, comparison with mutant viruses that have specific, known, entry defects can be illuminating. For example, Fig. 6 shows by immunofluorescence confocal microscopy that 8 h after binding the intracellular distribution of wild-type MVMp appears identical to that of a PLA2 mutant known to be unable to exit from its vacuolar entry portal. Thus, essentially all observable wild-type viruses appear to be retained within the cell's vacuolar network, and

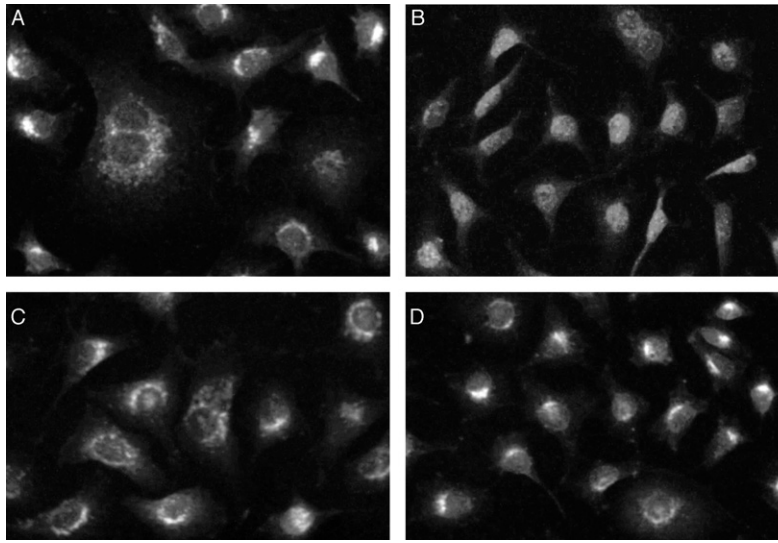


FIGURE 6 Intracellular distribution of incoming virions. A9 cells infected with 500,000 wild-type (A), H42R (C), or ΔVP1 (D) virions per cell, fixed 8 h postinfection and stained with a monoclonal antibody specific for intact capsids. Cells shown in panel B were infected with wild type as in panel A, except that Type V neuraminidase (100 μg/ml) was added to the medium during infection. Images (1-μm sections) were acquired on a Zeiss LSM 510 laser scanning confocal microscope. (Reproduced with permission from Farr, Ph. D. thesis, 2005.)

could be earmarked for recycling or degradation rather than infection. To be biologically significant, entry steps must be seen to lead to productive infection, but for parvoviruses the first readily measurable indicator of successful initiation is the expression of NS1, a significantly late event that requires prior viral DNA synthesis and transcription. Moreover, for these viruses to initiate infection, the host cell must enter S-phase of its own volition so that any experimental intervention that slows or inhibits progress through the cell cycle may artifactually appear to interrupt the entry process. Accordingly, studies involving inhibitory drugs that are more-or-less specific for particular cellular interactions, or the delivery of mutant or overexpressed cellular control proteins, represent an area of considerable interpretive challenge.

Following receptor binding, all parvoviruses are rapidly internalized from the cell surface by receptor-mediated endocytosis, predominantly via clathrin-coated pits, and enter an endosome compartment that is sensitive to lysosomotropic agents such as bafilomycin A, indicating that low endosomal pH is somehow essential for infection. However, members of the genus *Parvovirus* remain sensitive to bafilomycin A for many hours after internalization, indicating that the required trafficking scheme may be complex and/or the penetration process inefficient. Such exposure to low pH during entry might be required because it induces essential conformational changes in the virion, because the viruses specifically need to transit a hydrolase-rich late endosomal/lysosomal compartment to accomplish an essential cleavage event, or because the ability to generate low pH compartments is absolutely required for the cell to sustain the required endosomal trafficking patterns. How many of these possibilities pertain is currently unclear and could vary between viral species. For MVM, endosomal proteases are known to generate VP3 polypeptides from VP2 molecules following engulfment (Mani *et al*., 2006; Ros and Kempf, 2004), which is likely important because it both removes the nuclear export signals in the VP2 N-termini (Maroto *et al*., 2004) and primes the virion for its subsequent conformational transition (Farr *et al*., 2006). The need for this modification thus supports immunofluorescence analysis of internalized particles and studies with inhibitory drugs (Ros *et al*., 2002; Suikkanen *et al*., 2002) in suggesting that infectious entry probably occurs via a late endosomal or lysosomal route, since these compartments are rich in proteases and nucleases. Such exposure would also explain how genomes lose their covalently linked NS1 molecules, and the nucleotides of the ''tether'' DNA sequence, prior to arrival in the nucleus. Exposure to acidic conditions *in vitro* also influences particle stability, as discussed in detail in Section IV.A , perhaps protecting essential acid or hydrolase-sensitive viral structures within an obligate late entry compartment or mediating other required rearrangements. Finally, vesicle trafficking is a protracted and potentially flexible process that leads to

particle delivery to many different cell locations. This complexity is illustrated by studies with CPV, which appears to remain physically associated with its receptor, TfR, for at least 4 h after internalization, since infectious entry can be blocked throughout this period by intracytoplasmic injection of antibodies directed against the cytoplasmic tail of the receptor (Parker *et al.*, 2001). The normal cellular uptake and complex recycling patterns of TfRs have been well characterized, and are known to depend on the presence of a YTRF (Tyr-Thr-Arg-Phe) motif in its cytoplasmic tail. However, when these sequences were deleted or mutated, or polar residues introduced into the TfR transmembrane domain, which vastly increased receptor degradation, virus infection efficiency was unaffected (Hueffer *et al.*, 2004), suggesting that infectious entry for CPV may involve a minority of TfRs that take-or are induced by bound virions to take-a rare pathway.

For MVM, the kinetics of intracellular VP2-to-VP3 cleavage, and of VP1SR and DNA exposure for the bulk particle population, have been tracked within cellular entry vesicles by immunofluorescent staining and *in situ* hybridization (Mani *et al.*, 2006). These changes were not conspicuously triggered by interactions with cell surface receptors, but became detectable, apparently simultaneously, within minutes of internalization in early endosomes, and could be blocked by preventing endosomal acidification with chloroquine or bafilomycin A. Remarkably, these authors observed VP1SR extrusion from both empty and full virus populations, occurring with similar kinetics. Since such VP1SR exposure is never seen if empty particles are heated *in vitro*, this might suggest that prior interactions with the cell had modified their structure, or led to their fragmentation. Suikkanen *et al.* (2003b) observed that VP1SR exposure from CPV virions increased with time between 1- and 8-h postinfection, and such forms colocalized with intact capsids in perinuclear lysosomes, whether or not the cells were treated with acidification-blocking drugs. While relatively few TfRs were detected in lysosomal vesicles in uninfected cells, throughout the course of CPV infection intact virions colocalized with TfRs, first in vesicles that resembled recycling endosomes, but later, by 8-h postinfection, in perinuclear lysosomes, perhaps suggesting that the virus had modified the cycling pathway of its receptor. Signal from exposed MVM genomes also colocalized with intact capsids in successive endosomal compartments, progressively accumulating for around 8 h after internalization, but by 21 h, although perinuclear vesicles remained loaded with intact capsid particles, little exposed viral DNA remained. Unfortunately, the fraction of the total endosomal virion population that contributes to the colocalizing signals cannot be assessed in this kind of microscopic analysis, and may be quite limited. It also seems likely that, given the relative inefficiency with which infection is initiated, the great majority of observed shifts in particle structure may reflect their degradation rather than their participation in a

productive infectious entry pathway. Nevertheless, such observations do illustrate that some transitions that have been documented *in vitro*, do also occur *in vivo*.

Within a few hours of internalization, both infectious virions and entry-defective mutants or empty particles are similarly trafficked to large, extranuclear, crescent-shaped clusters of vesicles that are focused on one side of the cell nucleus, as seen in Fig. 6. These resemble, and likely are, microtubule organizing centers since many of the vesicles appear to be lysosomes, and the processing of early endosomes to late endosomes/lysosomes requires their movement along microtubule networks. Accordingly, Parker and Parrish (2000) showed that overexpression of a dominant interfering mutant of dynamin altered trafficking of CPV-containing vesicles such that the concentration of input virus in perinuclear vesicles was significantly inhibited. Likewise, Vihinen-Ranta and colleagues (Suikkanen *et al.*, 2002; Vihinen-Ranta *et al.*, 1998), showed that the microtubule-depolymerizing drug nocodazole inhibits productive infection and leaves vesicular structures containing CPV near the cell periphery. In a classic study of transcytosolic vacuole trafficking, Heuser showed that shifting the extracellular medium of fibroblasts from pH 7.5 to 6.8 caused many perinuclear late endosomes/lysosomes to return to the cell periphery, in a nocodazole-dependent reaction, that could be reversed, mediated again by microtubules, by returning the cells to neutral pH (Heuser, 1989). Similarly, we have found that perinuclear clusters of MVM virions can be disrupted and scattered toward the cell periphery by exposing cells to low pH, in a reaction that can be blocked by nocodazole, but these return rapidly to their original location if the extracellular pH is returned to neutral, again in a nocodazole-dependent fashion. Thus many of the virus-filled vacuoles that occupy these perinuclear crescents appear to be typical late endosomes/lysosomes, pursuing their normal trafficking pathways. Microinjection of antibodies to dynein caused CPV vesicles to remain peripheral, supporting a model in which this motor protein drives microtubular transport of CPV entry vesicles.

MVM virions in perinuclear crescents disperse with time and much internalized virus is recycled back to the cell surface, where it can be released by the receptor-destroying enzyme, neuraminidase. Thus, for example, in one quantitative multiplex PCR analysis, populations of synchronized A9 cells infected for 6 h at 37 °C with 500 genomes per cell of either wild-type or a PLA2-negative mutant MVM, and then incubated in neuraminidase for an hour to ensure removal of all cell surface–bound virus, subsequently recycled approximately two-third of the remaining, intracellular, genomes back into the neuraminidase-supplemented medium during an overnight incubation, without evidence of accompanying cell death (S. F. C. and P. T., unpublished results). Clearly, these virus particles had failed to navigate the necessary infectious entry pathway.

Exactly where infecting viruses penetrate the endosomal bilayer is uncertain, but CPV infectivity can be blocked by the intracytoplasmic injection of antibodies directed against structural epitopes on the capsid or VP1SR-specific sequences, indicating that there must be an essential, capsid-associated, cytoplasmic phase, and that exposure of the VP1SR must accompany or precede infectious entry into the cytoplasm (Vihinen-Ranta *et al.*, 2000, 2002). Labeled dextrans with molecular radii of ~3000 were progressively released into the cytosol 8–20 h after they are codelivered to the cell with CPV virions, while dextrans of Mr 10,000 were retained in vesicles. This may suggest that CPV infection does not lead to disruption of the endosomal vesicles, but does induce a permeability change in their membranes (Suikkanen *et al.*, 2003b). Thus, although the effects of co-uptake with PLA2-defective virions were not explored in this study, the observed permeability increase might reflect viral enzyme activity. Complementation analysis between wild-type and mutant particles has been used to show that bilayer penetration does require deployment of this lipolytic PLA2 function (Farr *et al.*, 2005). These studies used an MVM mutant with a single H42D amino acid substitution in its PLA2 active site, which severely impaired its enzymatic activity and abrogated its infectivity. However, the mutant phenotype could be complemented in *trans* by coinfection with wild-type or mutant virions, provided they expressed functional PLA2, but not by wild-type empty particles, even though these carry sequestered VP1SR sequences. The H42R mutant was also complemented by polyethyleneimine-induced endosome rupture or by coinfection with adenovirus, as long as uptake of the two viruses was simultaneous and the adenovirus was capable of deploying pVI, a capsid protein with endosomolytic activity. Thus MVM, and likely other members of the genus, appears to use its capsid-tethered phospholipase activity to penetrate the endosomal membrane. If this event is successful, infection with the H42R mutant then proceeds normally, suggesting that, for MVM at least, transiting the endosomal membrane is the only step during infection that requires such potent phospholipase activity. However, since the PLA2 activity of the mutant virus was compromised, rather than destroyed, it remains possible that this diminished activity could be sufficient to support additional roles for the enzyme in the viral life cycle.

D. From cytosol to nucleus

Since CPV infectivity can be blocked by the intracytoplasmic injection of antibodies directed against both intact capsids and the VP1-specific sequences (Vihinen-Ranta *et al.*, 2000, 2002), genomes associated with such forms must at least enter the cytoplasm during the normal course of infection. For AAV2 it has also been shown that monoclonal antibodies with equivalent specificities injected into the cell nucleus similarly block

infection, providing the first functional evidence that, at least for this virus, a transitioned capsid is present throughout the cytoplasmic and nuclear translocation phases, and is implicated in nuclear functions (Sonntag *et al*., 2006). In support of this interpretation, CPV virions microinjected into the cytoplasm were found to translocate into the nucleus intact, as demonstrated by their reactivity with structure specific antibodies, where they successfully initiated NS1 expression within 24 h (Suikkanen *et al*., 2003a; Vihinen-Ranta *et al*., 2000). Microinjected empty capsids were similarly transported, but whether any of this movement was VP1SR-driven remains uncertain. Notably, whereas karyopherin-mediated entry via the nuclear pores is typically rapid, viral transport in the CPV studies was slow, with few particles entering the nucleus within 3 h of cytoplasmic injection, although these became apparent in 40–50% of injected cells by 6 h. However, the injected particles did not initially carry exposed VP1SR, suggesting that they had to undergo protracted structural rearrangements before they were recognized as cargo (Vihinen-Ranta *et al*., 2000).

Although very few viral capsids are ever observed in the nuclei of infected cells (Mani *et al*., 2006; Suikkanen *et al*., 2003a), microinjection of full virions into the cytoplasm allows potential transport mechanisms to be explored. Thus, microtubule-depolymerizing drugs have been shown to block the nuclear transport of injected CPV virions, as have anti-dynein antibodies, suggesting that such free particles may be transported along microtubules. Electron micrographs of cells taken 10–12 h after infection with CPV, in which the capsids were detected by immunolabeling with nanogold particles, identified virus lying next to, and in some cases apparently associated with, the nuclear membrane, which appeared intact (Suikkanen *et al*., 2003a). However, whether these virions were associated with nuclear pores is unclear. The ~260 Å diameter of the virion means that it could, theoretically, be transported, Trojan horse-like, into nuclei via the nuclear pores, using normal cellular trafficking mechanisms, and the potential for karyopherin-mediated interactions with motifs in the VP1SR has been already been discussed at length. However, compelling evidence for such transport is lacking, and an alternate nuclear entry strategy, involving partial disruption of the nuclear membrane, has been proposed (Cohen and Pante, 2005; Cohen *et al*., 2006). These authors showed that between 1 and 4 h after infection with MVM there were dramatic changes in the shape and morphology of A9 cell nuclei, alterations in nuclear lamin immunostaining, and breaks in the nuclear envelope that increased in severity with time (Cohen *et al*., 2006). Addition of bafilomycin at hourly intervals following similar infections (Ros *et al*., 2002; S. F. C. and P. T., unpublished results) suggests that by 4-h postinfection many potentially infecting virions must still remain inside acidified vesicles, so that it will be interesting to see if damage to

the nuclear membrane becomes even more pronounced at later time points. Nevertheless, by 4 h, the lamin changes are reported to have occurred in ~20% of infected cells, although whether such changes heralded productive infection or cell death remains uncertain, and will be important to assess. Theoretically, it is difficult to envision how such mechanisms could be compatible with the subsequent unchecked entry of these damaged cells into S-phase.

Finally, both MVM and CPV infections are reported to be disrupted by various proteosome inhibitors, such as MG132, lactacystin, or epoxomicin (Ros and Kempf, 2004; Ros *et al.*, 2002), although analysis of capsid proteins during internalization in these studies showed no evidence of particle ubiquitination or degradation. Specifically, the chymotrypsin-like, but not the trypsin-like, activity of the proteosome appeared necessary, but whether this operates in the cytoplasm or nucleus, or what step in infection it might influence, remains to be determined.

However, as discussed earlier, there is a major caveat that must be considered when interpreting experiments involving drugs or other treatments that appear to interfere with parvovirus entry. Specifically, until methods are developed for directly demonstrating the arrival of the genome in the nucleus, such experiments inevitably rely on NS1 expression or the replication of viral DNA as the earliest indicator(s) of successful infection, but these events depend on the infected cell entering S-phase as part of its own replicative program. Thus, it follows that any intervention that delays or arrests the cell cycle will score as one that interferes with virus entry, whether or not it really does. Thus it is of paramount importance, for the correct interpretation of parvoviral entry experiments, to determine that the experimental approach does not itself perturb the normal cell cycle.

E. Waiting for S-phase: Cryptic versus productive infection

Once inside the nucleus, parvoviruses must wait for the cell to enter S-phase before they can commandeer its synthetic machinery for their own preferential replication. Moreover, protracted latency occurs in parvovirus-infected animals and in noncycling cells in culture, but the location and physical state of the viral genome during this phase of the life cycle is uncertain. However, several lines of evidence suggest that it may remain sequestered within its intact particle. As discussed previously, heat-induced transitions that expose the VP1SR also expose the 3′ end of the viral DNA to polymerases, so that it is possible that the genome is ultimately extracted from the particle by the progress of the fork during complementary-strand DNA synthesis, leaving it physically attached to the capsid via interactions involving the left-end hairpin telomere. *In vitro* replicating DNA is not released from the particle until the rolling-hairpin

mechanism proceeds through a dimer intermediate, which cannot occur in the absence of the major virally coded nonstructural protein, NS1, since this mediates the necessary hairpin transitions. Thus, capsid-associated duplexes may even serve as the initial viral transcription templates, providing the NS1 necessary for their own subsequent release. Initial transcription of MVM also depends on the availability of the host transcription factor E2F, which activates its P4 promoter (Deleu *et al.*, 1999) so that viral transcription is optimized for expression during early S-phase.

While little is known of the latency strategies employed by members of the genus *Parvovirus*, AAV persistence has been explored in greater depth, and is known to involve several alternative mechanisms. In cycling human cells, but in the absence of a helper virus, genomes capable of expressing competent Rep proteins can integrate into a specific site on chromosome 13qter, although this appears to be a rare event *in vivo* (Schnepp *et al.*, 2005). Recombinant AAV (rAAV) vectors, in which genomes with viral hairpins flanking a foreign promoter-driven transgene are packaged into virions, provide additional insight into possible mechanisms of persistence in the absence of Rep expression. Because these vectors generally have identical ITRs, they give rise to virion populations with equal numbers of plus- and minus-sense genomes. When delivered *in vivo* to postmitotic cell populations at high input multiplicity, some of these genomes are able to escape from their capsids and integrate into the host genome in a site-independent manner, predominantly at the position of preexisting double-strand breaks (Miller *et al.*, 2004). More commonly, the genomes appear to emerge as unit-length episomal duplexes, perhaps by progressive annealing between complementary strands or by some sort of extensive DNA repair-driven pathway, and their ITRs then undergo intramolecular recombination, generating duplex circles (Duan *et al.*, 1998; Nakai *et al.*, 2000). These can concatamerize with time, possibly due to the recombinogenic characteristics of their ITRs, generating stable multimeric episomes. When formed *in vivo* from rAAV vector genomes, such circles can support transcription over extended periods, since they typically contain constitutive promoters and express nontoxic products.

Whether similar patterns of episomal stabilization and maintenance can occur during the life cycle of members of the *Parvovirus* genus is unknown, but seems unlikely for several reasons. First, the autonomously replicating parvoviruses almost invariably package predominantly one strand, therefore cannot give rise to duplexes in the absence of significant DNA replication. Second, each of their termini is distinct from the other, both in sequence and predicted structure, making it much less likely that they would readily undergo intramolecular recombination to form stable circular episomes. Third, the AAV2 P5 promoter drives expression of the Rep proteins, which in the absence of the helper adenovirus E1A

protein, downregulate P5, resulting in a negative feedback loop. In contrast, the parvovirus P4 promoter upregulates expression of its cytocidal product, NS1. Thus, for the parvoviruses, it appears likely that only cells arrested somewhere in the cell cycle, presumably mostly in G1, could sustain viral persistence without succumbing to the cytocidal effects of infection. This type of persistence has been termed cryptic infection (Tattersall and Gardiner, 1990) in order to distinguish it from the types of latent infection enjoyed by AAV, described above. Since infected, quiescent cell populations are difficult to maintain as such under culture conditions and cannot, by definition, be expanded, this aspect of the viral life cycle has proven difficult to explore, but it has been possible to show that autonomously replicating parvoviruses will persist in noncycling cells *in vitro*, emerging again once quiescence is broken (Paul *et al*., 1979; Tattersall, 1972). The presence of unreplicated, single-stranded DNA in the nucleus, even in quiescent cells, would be expected to strongly activate DNA damage responses through the ATM–ATR pathway, leading in normal cells to the suspension of subsequent entry into S-phase. The simplest solution to this conundrum would be for the virus to persist in the nucleus in a capsid-sequestered form, but this has yet to be explored experimentally. Once cryptically infected cells enter S-phase, however, viral DNA could be uncoated and converted to a transcriptionally competent duplex form, allowing viral gene expression to be unleashed. Since NS1 expression is concomitant with the cessation of host cell DNA synthesis, its further progress through the cell cycle must then be impeded, leaving the cell's DNA synthetic machinery at the disposal of the replicating invader.

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REFERENCES

Agbandje-Mckenna, M., and Chapman, M. S. (2006). Correlating structure with function in the viral capsid. *In* ''The Parvoviruses'' (J. Kerr, S. F. Cotmore, M. E. Bloom, R. M. Linden, and C. R. Parrish, eds.), Chap. 10, pp. 125–140. Hodder Arnold, London.

Agbandje-McKenna, M., Llamas-Saiz, A. L., Wang, F., Tattersall, P., and Rossmann, M. G. (1998). Functional implications of the structure of the murine parvovirus, minute virus of mice. *Structure* **6:**1369–1381.

Antonietti, J. P., Sahli, R., Beard, P., and Hirt, B. (1988). Characterization of the cell type-specific determinant in the genome of minute virus of mice. *J. Virol.* **62:**552–557.

Badgett, M. R., Auer, A., Carmichael, L. E., Parrish, C. R., and Bull, J. J. (2002). Evolutionary dynamics of viral attenuation. *J. Virol.* **76:**10524–10529.

Ball-Goodrich, L. J., and Tattersall, P. (1992). Two amino acid substitutions within the capsid are coordinately required for acquisition of fibrotropism by the lymphotropic strain of minute virus of mice. *J. Virol.* **66:**3415–3423.

Balsinde, J., Winstead, M. V., and Dennis, E. A. (2002). Phospholipase A2 regulation of arachidonic acid mobilization. *FEBS Lett.* **531:**2–6.

Berg, O. G., Gelb, M. H., Tsai, M. D., and Jain, M. K. (2001). Interfacial enzymology: The secreted phospholipase. *Chem. Rev.* **101:**2613–2653.

Bodendorf, U., Cziepluch, C., Jauniaux, J. C., Rommelaere, J., and Salome, N. (1999). Nuclear export factor CRM1 interacts with nonstructural proteins NS2 from parvovirus minute virus of mice. *J. Virol.* **73:**7769–7779.

Brown, W. J., Chambers, K., and Doody, A. (2003). Phospholipase A2 (PLA2) enzymes in membrane trafficking: Mediators of membrane shape and function. *Traffic* **4:**214–221.

Brownstein, D. G., Smith, A. L., Johnson, E. A., Pintel, D. J., Naeger, L. K., and Tattersall, P. (1992). The pathogenesis of infection with minute virus of mice depends on expression of the small nonstructural protein NS2 and on the genotype of the allotropic determinants VP1 and VP2. *J. Virol.* **66:**3118–3124.

Bubeck, D., Filman, D. J., Cheng, N., Steven, A. C., Hogle, J. M., and Belnap, D. M. (2005). The structure of the poliovirus 135S cell entry intermediate at 10-angstrom resolution reveals the location of an externalized polypeptide that binds to membranes. *J. Virol.* **79:**7745–7755.

Canaan, S., Zadori, Z., Ghomashchi, F., Bollinger, J., Sadilek, M., Moreau, M. E., Tijssen, P., and Gelb, M. H. (2004). Interfacial enzymology of parvovirus phospholipases A2. *J. Biol. Chem.* **279:**14502–14508.

Cater, J. E., and Pintel, D. J. (1992). The small non-structural protein NS2 of the autonomous parvovirus minute virus of mice is required for virus growth in murine cells. *J. Gen. Virol.* **73:**1839–1843.

Chandran, K., Parker, J. S., Ehrlich, M., Kirchhausen, T., and Nibert, M. L. (2003). The delta region of outer-capsid protein micro 1 undergoes conformational change and release from reovirus particles during cell entry. *J. Virol.* **77:**13361–13375.

Chang, S. F., Sgro, J. Y., and Parrish, C. R. (1992). Multiple amino acids in the capsid structure of canine parvovirus coordinately determine the canine host range and specific antigenic and hemagglutination properties. *J. Virol.* **66:**6858–6867.

Chapman, M. S., and Agbandje-McKenna, M. (2006). Atomic structure of viral particles. *In* ''The Parvoviruses'' (J. Kerr, S. F. Cotmore, M. E. Bloom, R. M. Linden, and C. R. Parrish, eds.), Chap. 9, pp. 107–124. Hodder Arnold, London.

Chapman, M. S., and Rossmann, M. G. (1993). Structure, sequence, and function correlations among parvoviruses. *Virology* **194:**491–508.

Choi, E. Y., Newman, A. E., Burger, L., and Pintel, D. (2005). Replication of minute virus of mice DNA is critically dependent on accumulated levels of NS2. *J. Virol.* **79:**12375–12381.

Clemens, K. E., and Pintel, D. J. (1988). The two transcription units of the autonomous parvovirus minute virus of mice are transcribed in a temporal order. *J. Virol.* **62:** 1448–1451.

Clinton, G. M., and Hayashi, M. (1975). The parvovirus MVM: Particles with altered structural proteins. *Virology* **66:**261–263.

Cohen, S., and Pante, N. (2005). Pushing the envelope: Microinjection of minute virus of mice into Xenopus oocytes causes damage to the nuclear envelope. *J. Gen. Virol.* **86:**3243–3252.

Cohen, S., Behzad, A. R., Carroll, J. B., and Pante, N. (2006). Parvoviral nuclear import: Bypassing the host nuclear-transport machinery. *J. Gen. Virol.* **87:**3209–3213.

Cotmore, S. F., and Tattersall, P. (1989). A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. *J. Virol.* **63:**3902–3911.

Cotmore, S. F., and Tattersall, P. (1990). Alternate splicing in a parvoviral nonstructural gene links a common amino-terminal sequence to downstream domains which confer radically different localization and turnover characteristics. *Virology* **177:**477–487.

Cotmore, S. F., and Tattersall, P. (2005a). Encapsidation of minute virus of mice DNA: Aspects of the translocation mechanism revealed by the structure of partially packaged genomes. *Virology* **336:**100–112.

Cotmore, S. F., and Tattersall, P. (2005b). Genome packaging sense is controlled by the efficiency of the nick site in the right-end replication origin of parvoviruses minute virus of mice and LuIII. *J. Virol.* **79:**2287–2300.

Cotmore, S. F., and Tattersall, P. (2006a). Parvoviruses. *In* ''DNA Replication and Human Disease'' (M. L. DePamphilis, ed.), pp. 593–608. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Cotmore, S. F., and Tattersall, P. (2006b). A rolling hairpin strategy: Basic mechanisms of DNA replication in the parvoviruses. *In* ''The Parvoviruses'' (J. Kerr, S. F. Cotmore, M. E. Bloom, R. M. Linden, and C. R. Parrish, eds.), Chap. 14, pp. 5–16. Hodder Arnold, London.

Cotmore, S. F., Christensen, J., Nuesch, J. P., and Tattersall, P. (1995). The NS1 polypeptide of the murine parvovirus minute virus of mice binds to DNA sequences containing the motif (ACCA)2–3. *J. Virol.* **69:**1652–1660.

Cotmore, S. F., D'Abramo, A. M., Jr., Carbonell, L. F., Bratton, J., and Tattersall, P. (1997). The NS2 polypeptide of parvovirus MVM is required for capsid assembly in murine cells. *Virology* **231:**267–280.

Cotmore, S. F., D'Abramo, A. M., Jr., Ticknor, C. M., and Tattersall, P. (1999). Controlled conformational transitions in the MVM virion expose the VP1 N-terminus and viral genome without particle disassembly. *Virology* **254:**169–181.

D'Abramo, A. M., Jr., Ali, A. A., Wang, F., Cotmore, S. F., and Tattersall, P. (2005). Host range mutants of minute virus of Mice with a single VP2 amino acid change require additional silent mutations that regulate NS2 accumulation. *Virology* **340:**143–154.

Deleu, L., Pujol, A., Faisst, S., and Rommelaere, J. (1999). Activation of promoter P4 of the autonomous parvovirus minute virus of mice at early S phase is required for productive infection. *J. Virol.* **73:**3877–3885.

Duan, D., Sharma, P., Yang, J., Yue, Y., Dudus, L., Zhang, Y., Fisher, K. J., and Engelhardt, J. F. (1998). Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J. Virol.* **72:**8568–8577.

Farr, G. A., and Tattersall, P. (2004). A conserved leucine that constricts the pore through the capsid fivefold cylinder plays a central role in parvoviral infection. *Virology* **323:**243–256.

Farr, G. A. (2005). The capsid five-fold cylinder and the VP1 N-terminal unique region are critical components of the parvoviral entry machine. PhD Thesis, Yale University.

Farr, G. A., Zhang, L. G., and Tattersall, P. (2005). Parvoviral virions deploy a capsid-tethered lipolytic enzyme to breach the endosomal membrane during cell entry. *Proc. Natl. Acad. Sci. USA* **102:**17148–17153.

Farr, G. A., Cotmore, S. F., and Tattersall, P. (2006). VP2 cleavage and the leucine ring at the base of the fivefold cylinder control pH-dependent externalization of both the VP1 N terminus and the genome of minute virus of mice. *J. Virol.* **80:**161–171.

Gardiner, E. M., and Tattersall, P. (1988a). Evidence that developmentally regulated control of gene expression by a parvoviral allotropic determinant is particle mediated. *J. Virol.* **62:**1713–1722.

Gardiner, E. M., and Tattersall, P. (1988b). Mapping of the fibrotropic and lymphotropic host range determinants of the parvovirus minute virus of mice. *J. Virol.* **62:**2605–2613.

Govindasamy, L., Hueffer, K., Parrish, C. R., and Agbandje-McKenna, M. (2003). Structures of host range-controlling regions of the capsids of canine and feline parvoviruses and mutants. *J. Virol.* **77:**12211–12221.

Grieger, J. C., Snowdy, S., and Samulski, R. J. (2006). Separate basic region motifs within the adeno-associated virus capsid proteins are essential for infectivity and assembly. *J. Virol.* **80:**5199–5210.

Hafenstein, S., Palermo, L. M., Kostyuchenko, V. A., Xiao, C., Morais, M. C., Nelson, C. D. S., Bowman, V. D., Battisti, A. J., Chipman, P. R., Parrish, C. R., and Rossmann, M. G. (2007). Asymmetric binding of transferrin receptor to parvovirus capsids. *Proc. Natl. Acad. Sci. USA* **104**(16)**:**6585–6589.

Heuser, J. (1989). Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. *J. Cell Biol.* **108:**855–864.

Hogle, J. M. (2002). Poliovirus cell entry: Common structural themes in viral cell entry pathways. *Annu. Rev. Microbiol.* **56:**677–702.

Horiuchi, M., Goto, H., Ishiguro, N., and Shinagawa, M. (1994). Mapping of determinants of the host range for canine cells in the genome of canine parvovirus using canine parvovirus/mink enteritis virus chimeric viruses. *J. Gen. Virol.* **75:**1319–1328.

Hueffer, K., and Parrish, C. R. (2003). Parvovirus host range, cell tropism and evolution. *Curr. Opin. Microbiol.* **6:**392–398.

Hueffer, K., Govindasamy, L., Agbandje-McKenna, M., and Parrish, C. R. (2003a). Combinations of two capsid regions controlling canine host range determine canine transferrin receptor binding by canine and feline parvoviruses. *J. Virol.* **77:**10099–10105.

Hueffer, K., Parker, J. S., Weichert, W. S., Geisel, R. E., Sgro, J. Y., and Parrish, C. R. (2003b). The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. *J. Virol.* **77:**1718–1726.

Hueffer, K., Palermo, L. M., and Parrish, C. R. (2004). Parvovirus infection of cells by using variants of the feline transferrin receptor altering clathrin-mediated endocytosis, membrane domain localization, and capsid-binding domains. *J. Virol.* **78:**5601–5611.

Jongeneel, C. V., Sahli, R., McMaster, G. K., and Hirt, B. (1986). A precise map of splice junctions in the mRNAs of minute virus of mice, an autonomous parvovirus. *J. Virol.* **59:**564–573.

Kimsey, P. B., Engers, H. D., Hirt, B., and Jongeneel, C. V. (1986). Pathogenicity of fibroblast- and lymphocyte-specific variants of minute virus of mice. *J. Virol.* **59:**8–13.

Lawrence, C. M., Ray, S., Babyonyshev, M., Galluser, R., Borhani, D. W., and Harrison, S. C. (1999). Crystal structure of the ectodomain of human transferrin receptor. *Science* **286:**779–782.

Legendre, D., and Rommelaere, J. (1994). Targeting of promoters for trans activation by a carboxy-terminal domain of the NS-1 protein of the parvovirus minute virus of mice. *J. Virol.* **68:**7974–7985.

Linser, P., Bruning, H., and Armentrout, R. W. (1977). Specific binding sites for a parvovirus, minute virus of mice, on cultured mouse cells. *J. Virol.* **24:**211–221.

Llamas-Saiz, A. L., Agbandje-McKenna, M., Parker, J. S., Wahid, A. T., Parrish, C. R., and Rossmann, M. G. (1996). Structural analysis of a mutation in canine parvovirus which controls antigenicity and host range. *Virology* **225:**65–71.

Lombardo, E., Ramirez, J. C., Garcia, J., and Almendral, J. M. (2002). Complementary roles of multiple nuclear targeting signals in the capsid proteins of the parvovirus minute virus of mice during assembly and onset of infection. *J. Virol.* **76:**7049–7059.

López-Bueno, A., Mateu, M. G., and Almendral, J. M. (2003). High mutant frequency in populations of a DNA virus allows evasion from antibody therapy in an immunodeficient host. *J. Virol.* **77:**2701–2708.

López-Bueno, A., Valle, N., Gallego, J. M., Perez, J., and Almendral, J. M. (2004). Enhanced cytoplasmic sequestration of the nuclear export receptor CRM1 by NS2 mutations developed in the host regulates parvovirus fitness. *J. Virol.* **78:**10674–10684.

López-Bueno, A., Rubio, M. P., Bryant, N., McKenna, R., Agbandje-McKenna, M., and Almendral, J. M. (2006). Host-selected amino acid changes at the sialic acid binding pocket of the parvovirus capsid modulate cell binding affinity and determine virulence. *J. Virol.* **80:**1563–1573.

López-Bueno, A., Segovia, J. C., Bueren, J. A., O'Sullivan, G. M., Wang, F., Tattersall, P., and Almendral, J. M. (2007). Rapid evolution to pathogenicity of a DNA virus in an immunodeficient host targets tropism determinant residues in the capsid. (In preparation).

Mani, B., Baltzer, C., Valle, N., Almendral, J. M., Kempf, C., and Ros, C. (2006). Low pH-dependent endosomal processing of the incoming parvovirus minute virus of mice virion leads to externalization of the VP1 N-terminal sequence (N-VP1), N-VP2 cleavage, and uncoating of the full-length genome. *J. Virol.* **80:**1015–1024.

Maroto, B., Valle, N., Saffrich, R., and Almendral, J. M. (2004). Nuclear export of the non-enveloped parvovirus virion is directed by an unordered protein signal exposed on the capsid surface. *J. Virol.* **78:**10685–10694.

McMaster, G. K., Beard, P., Engers, H. D., and Hirt, B. (1981). Characterization of an immunosuppressive parvovirus related to the minute virus of mice. *J. Virol.* **38:**317–326.

Miller, D. G., Petek, L. M., and Russell, D. W. (2004). Adeno-associated virus vectors integrate at chromosome breakage sites. *Nat. Genet.* **36:**767–773.

Morais, M. C., Kanamaru, S., Badasso, M. O., Koti, J. S., Owen, B. A., McMurray, C. T., Anderson, D. L., and Rossmann, M. G. (2003). Bacteriophage phi29 scaffolding protein gp7 before and after prohead assembly. *Nat. Struct. Biol.* **10:**572–576.

Morgan, W. R., and Ward, D. C. (1986). Three splicing patterns are used to excise the small intron common to all minute virus of mice RNAs. *J. Virol.* **60:**1170–1174.

Naeger, L. K., Cater, J., and Pintel, D. J. (1990). The small nonstructural protein (NS2) of the parvovirus minute virus of mice is required for efficient DNA replication and infectious virus production in a cell-type-specific manner. *J. Virol.* **64:**6166–6175.

Nakai, H., Storm, T. A., and Kay, M. A. (2000). Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver *in vivo*. *J. Virol.* **74:**9451–9463.

Nam, H. J., Gurda-Whitaker, B., Yee Gan, W., Ilaria, S., McKenna, R., Mehta, P., Alvarez, R. A., and Agbandje-McKenna, M. (2006). Identification of the sialic acid structures recognized by minute virus of mice and the role of binding affinity in virulence adaptation. *J. Biol. Chem.* **281:**25670–25677.

Palermo, L. M., Hueffer, K., and Parrish, C. R. (2003). Residues in the apical domain of the feline and canine transferrin receptors control host-specific binding and cell infection of canine and feline parvoviruses. *J. Virol.* **77:**8915–8923.

Palermo, L. M., Hafenstein, S. L., and Parrish, C. R. (2006). Purified feline and canine transferrin receptors reveal complex interactions with the capsids of canine and feline parvoviruses that correspond to their host ranges. *J. Virol.* **80:**8482–8492.

Paradiso, P. R. (1984). Identification of multiple forms of the noncapsid parvovirus protein NCVP1 in H-1 parvovirus-infected cells. *J. Virol.* **52:**82–87.

Parker, J. S. L., and Parrish, C. R. (1997). Canine parvovirus host range is determined by the specific conformation of an additional region of the capsid. *J. Virol.* **71:**9214–9222.

Parker, J. S. L., and Parrish, C. R. (2000). Cellular uptake and infection by canine parvovirus involves rapid dynamin-regulated clathrin-mediated endocytosis, followed by slower intracellular trafficking. *J. Virol.* **74:**1919–1930.

Parker, J. S. L., Murphy, W. J., Wang, D., O'Brien, S. J., and Parrish, C. R. (2001). Canine and feline parvoviruses can use human or feline transferrin receptors to bind, enter, and infect cells. *J. Virol.* **75:**3896–3902.

Parrish, C. R. (1995). Pathogenesis of feline panleukopenia virus and canine parvovirus. *Baillieres Clin. Haematol.* **8:**57–71.

Parrish, C. R., and Carmichael, L. E. (1986). Characterization and recombination mapping of an antigenic and host range mutation of canine parvovirus. *Virology* **148:**121–132.

Parrish, C. R., Aquadro, C. F., and Carmichael, L. E. (1988). Canine host range and a specific epitope map along with variant sequences in the capsid protein gene of canine parvovirus and related feline, mink, and raccoon parvoviruses. *Virology* **166:**293–307.

Parrish, C. R., Aquadro, C. F., Strassheim, M. L., Evermann, J. F., Sgro, J. Y., and Mohammed, H. O. (1991). Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. *J. Virol.* **65:**6544–6552.

Paul, P. S., Mengeling, W. L., and Brown, T. T., Jr. (1979). Replication of porcine parvovirus in peripheral blood lymphocytes, monocytes, and peritoneal macrophages. *Infect. Immun.* **25:**1003–1007.

Pintel, D., Dadachanji, D., Astell, C. R., and Ward, D. C. (1983). The genome of minute virus of mice, an autonomous parvovirus, encodes two overlapping transcription units. *Nucleic Acids Res.* **11:**1019–1038.

Qiu, J., Yoto, Y., Tullis, G., and Pintel, D. J. (2006). Parvovirus RNA processing strategies. *In* ''The Parvoviruses'' (J. Kerr, S. F. Cotmore, M. E. Bloom, R. M. Linden, and C. R. Parrish, eds.), Chap. 18, pp. 253–274. Hodder Arnold, London.

Riolobos, L., Reguera, J., Mateu, M. G., and Almendral, J. M. (2006). Nuclear transport of trimeric assembly intermediates exerts a morphogenetic control on the icosahedral parvovirus capsid. *J. Mol. Biol.* **357:**1026–1038.

Ros, C., and Kempf, C. (2004). The ubiquitin-proteasome machinery is essential for nuclear translocation of incoming minute virus of mice. *Virology* **324:**350–360.

Ros, C., Burckhardt, C. J., and Kempf, C. (2002). Cytoplasmic trafficking of minute virus of mice: Low-pH requirement, routing to late endosomes, and proteasome interaction. *J. Virol.* **76:**12634–12645.

Rossmann, M. G., and Palmenberg, A. C. (1988). Conservation of the putative receptor attachment site in picornaviruses. *Virology* **164:**373–382.

Rubio, M. P., López-Bueno, A., and Almendral, J. M. (2005). Virulent variants emerging in mice infected with the apathogenic prototype strain of the parvovirus minute virus of mice exhibit a capsid with low avidity for a primary receptor. *J. Virol.* **79:**11280–11290.

Schnepp, B. C., Jensen, R. L., Chen, C. L., Johnson, P. R., and Clark, K. R. (2005). Characterization of adeno-associated virus genomes isolated from human tissues. *J. Virol.* **79:**14793–14803.

Segovia, J. C., Real, A., Bueren, J. A., and Almendral, J. M. (1991). *In vitro* myelosuppressive effects of the parvovirus minute virus of mice (MVMi) on hematopoietic stem and committed progenitor cells. *Blood* **77:**980–988.

Shackelton, L. A., and Holmes, E. C. (2006). Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. *J. Virol.* **80:**3666–3669.

Shackelton, L. A., Parrish, C. R., and Holmes, E. C. (2006). Evolutionary basis of codon usage and nucleotide composition bias in vertebrate DNA viruses. *J. Mol. Evol.* **62:**551–563.

Simpson, A. A., Hebert, B., Sullivan, G. M., Parrish, C. R., Zadori, Z., Tijssen, P., and Rossmann, M. G. (2002). The structure of porcine parvovirus: Comparison with related viruses. *J. Mol. Biol.* **315:**1189–1198.

Sonntag, F., Bleker, S., Leuchs, B., Fischer, R., and Kleinschmidt, J. A. (2006). Adeno-associated virus type 2 capsids with externalized VP1/VP2 trafficking domains are generated prior to passage through the cytoplasm and are maintained until uncoating occurs in the nucleus. *J. Virol.* **80:**11040–11054.

Spalholz, B. A., and Tattersall, P. (1983). Interaction of minute virus of mice with differentiated cells: Strain-dependent target cell specificity is mediated by intracellular factors. *J. Virol.* **46:**937–943.

Strassheim, M. L., Gruenberg, A., Veijalainen, P., Sgro, J. Y., and Parrish, C. R. (1994). Two dominant neutralizing antigenic determinants of canine parvovirus are found on the threefold spike of the virus capsid. *Virology* **198:**175–184.

Suikkanen, S., Saajarvi, K., Hirsimaki, J., Valilehto, O., Reunanen, H., Vihinen-Ranta, M., and Vuento, M. (2002). Role of recycling endosomes and lysosomes in dynein-dependent entry of canine parvovirus. *J. Virol.* **76:**4401–4411.

Suikkanen, S., Aaltonen, T., Nevalainen, M., Valilehto, O., Lindholm, L., Vuento, M., and Vihinen-Ranta, M. (2003a). Exploitation of microtubule cytoskeleton and dynein during parvoviral traffic toward the nucleus. *J. Virol.* **77:**10270–10279.

Suikkanen, S., Antila, M., Jaatinen, A., Vihinen-Ranta, M., and Vuento, M. (2003b). Release of canine parvovirus from endocytic vesicles. *Virology* **316:**267–280.

Tattersall, P. (1972). Replication of the parvovirus MVM I. Dependence of virus multiplication and plaque formation on cell growth. *J. Virol.* **10:**586–590.

Tattersall, P., and Bratton, J. (1983). Reciprocal productive and restrictive virus-cell interactions of immunosuppressive and prototype strains of minute virus of mice. *J. Virol.* **46:**944–955.

Tattersall, P., and Gardiner, E. M. (1990). Autonomous parvovirus-host cell interactions. *In* ''Handbook of Parvoviruses; Volume I'' (P. Tjissen, ed.), pp. 111–122. CRC Press, Boca Raton, FL.

Tattersall, P., Bergoin, M., Bloom, M. E., Brown, K. E., Linden, R. M., Muzyczka, N., Parrish, C. R., and Tijssen, P. (2005). Parvoviridae. *In* ''Virus Taxonomy, VIIIth Report of the ICTV'' (C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball, eds.), pp. 353–369. Elsevier/Academic Press, London.

Tijssen, P., Szelei, J., and Zadori, Z. (2006). Phospholipase A2 domains in structural proteins of parvoviruses. *In* ''The Parvoviruses'' (J. Kerr, S. F. Cotmore, M. E. Bloom, R. M. Linden, and C. R. Parrish, eds.), Chap. 8, pp. 95–105. Hodder Arnold, London.

Truyen, U., and Parrish, C. R. (1992). Canine and feline host ranges of canine parvovirus and feline panleukopenia virus: Distinct host cell tropisms of each virus *in vitro* and *in vivo*. *J. Virol.* **66:**5399–5408.

Truyen, U., Agbandje, M., and Parrish, C. R. (1994). Characterization of the feline host range and a specific epitope of feline panleukopenia virus. *Virology* **200:**494–503.

Truyen, U., Gruenberg, A., Chang, S. F., Obermaier, B., Veijalainen, P., and Parrish, C. R. (1995). Evolution of the feline-subgroup parvoviruses and the control of canine host range *in vivo*. *J. Virol.* **69:**4702–4710.

Tsao, J., Chapman, M. S., Agbandje, M., Keller, W., Smith, K., Wu, H., Luo, M., Smith, T. J., Rossmann, M. G., Compans, R. W., and Parrish, C. R. (1991). The three-dimensional structure of canine parvovirus and its functional implications. *Science* **251:**1456–1464.

Tullis, G. E., Burger, L. R., and Pintel, D. J. (1992). The trypsin-sensitive RVER domain in the capsid proteins of minute virus of mice is required for efficient cell binding and viral infection but not for proteolytic processing *in vivo*. *Virology* **191:**846–857.

Tullis, G. E., Burger, L. R., and Pintel, D. J. (1993). The minor capsid protein VP1 of the autonomous parvovirus minute virus of mice is dispensable for encapsidation of progeny single-stranded DNA but is required for infectivity. *J. Virol.* **67:**131–141.

Valle, N., Riolobos, L., and Almendral, J. M. (2006). Synthesis, post-translational modification and trafficking of the parvovirus structural polypeptides. *In* ''The Parvoviruses'' (J. Kerr, S. F. Cotmore, M. E. Bloom, R. M. Linden, and C. R. Parrish, eds.), Chap. 20, pp. 291–304. Hodder Arnold, London.

Vihinen-Ranta, M., and Parrish, C. R. (2006). Cell infection processes of the autonomous parvoviruses. *In* ''The Parvoviruses'' (J. Kerr, S. F. Cotmore, M. E. Bloom, R. M. Linden, and C. R. Parrish, eds.), Chap. 12, pp. 157–163. Hodder Arnold, London.

Vihinen-Ranta, M., Kakkola, L., Kalela, A., Vilja, P., and Vuento, M. (1997). Characterization of a nuclear localization signal of canine parvovirus capsid proteins. *Eur. J. Biochem.* **250:**389–394.

Vihinen-Ranta, M., Kalela, A., Makinen, P., Kakkola, L., Marjomaki, V., and Vuento, M. (1998). Intracellular route of canine parvovirus entry. *J. Virol.* **72:**802–806.

Vihinen-Ranta, M., Yuan, W., and Parrish, C. R. (2000). Cytoplasmic trafficking of the canine parvovirus capsid and its role in infection and nuclear transport. *J. Virol.* **74:**4853–4859.

Vihinen-Ranta, M., Wang, D., Weichert, W. S., and Parrish, C. R. (2002). The VP1 N-terminal sequence of canine parvovirus affects nuclear transport of capsids and efficient cell infection. *J. Virol.* **76:**1884–1891.

Vihinen-Ranta, M., Suikkanen, S., and Parrish, C. R. (2004). Pathways of cell infection by parvoviruses and adeno-associated viruses. *J. Virol.* **78:**6709–6714.

Wang, D., Yuan, W., Davis, I., and Parrish, C. R. (1998). Nonstructural protein-2 and the replication of canine parvovirus. *Virology* **240:**273–281.

Weichert, W. S., Parker, J. S., Wahid, A. T., Chang, S. F., Meier, E., and Parrish, C. R. (1998). Assaying for structural variation in the parvovirus capsid and its role in infection. *Virology* **250:**106–117.

Willwand, K., and Hirt, B. (1991). The minute virus of mice capsid specifically recognizes the 3′ hairpin structure of the viral replicative-form DNA: Mapping of the binding site by hydroxyl radical footprinting. *J. Virol.* **65:**4629–4635.

Wu, P., Xiao, W., Conlon, T., Hughes, J., Agbandje-McKenna, M., Ferkol, T., Flotte, T., and Muzyczka, N. (2000). Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J. Virol.* **74:**8635–8647.

Xie, Q., and Chapman, M. S. (1996). Canine parvovirus capsid structure, analysed at 2.9 Å resolution. *J. Mol. Biol.* **264:** 497–520.

Zadori, Z., Szelei, J., Lacoste, M. C., Li, Y., Gariepy, S., Raymond, P., Allaire, M., Nabi, I. R., and Tijssen, P. (2001). A viral phospholipase A2 is required for parvovirus infectivity. *Dev. Cell* **1:**291–302.

CHAPTER 6

Viral Stress-Inducible Genes

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Contents

Abstract

Virus-infection of mammalian cells causes transcriptional induction of many cellular genes, collectively called as "viral stress-inducible genes." The proteins encoded by these genes are essential to maintain cell-virus homeostasis, which is required for both virus replication and host survival. Many viral products, including RNA, DNA, and proteins, can induce these genes by using distinct, but partially overlapping, signaling pathways. Type I interferons, direct products of virus infection, can also induce many of these genes, thus providing a positive feedback loop. Double-stranded RNA, a common by-product of virus replication, can induce them by multiple signaling pathways initiated by Toll-like receptor 3 or RIG-I/Mda-5. Several viral stress-inducible proteins inhibit protein

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synthesis. Proteins of the P56 family bind to the translation initiation factor, eIF-3, and block translation initiation. PKR, a protein kinase, phosphorylates a different initiation factor, eIF-2, and inhibits translation initiation. However, unlike P56, PKR needs to be first activated by dsRNA or PACT, another cellular protein. Another family of enzymes, the 2′-5′ oligoadenylate synthetases, synthesizes 2′-5′ linked oligoadenylates [2–5(A)] in the presence of dsRNA; 2–5(A) activates the latent ribonuclease, RNase L, which degrades mRNA. Many viruses have evolved mechanisms to evade these genes by blocking their induction or actions; often more than one strategy is used by the same virus to achieve this goal. Thus, in an infected cell, equilibrium is reached between the virus and the cell with regards to the viral stress-inducible genes.

LIST OF ABBREVIATIONS

VSIG	viral stress-inducible gene
IFN	interferon
ds	double-stranded
TLR	toll-like receptor
RIG-I	retinoic acid-inducible gene
Mda-5	melanoma differentiation associated gene
ISRE	interferon-stimulated response element
IRF	interferon regulatory factor
JAK	Janus tyrosine kinase
STAT	signal transducers and activators of transcription
TRIF	TIR-domain-containing adapter-inducing interferon-β
IPS-1	IFN-β promoter stimulator 1
JNK	c-jun N-terminal kinase
TBK-1	TANK-binding kinase-1
PI3K	phosphatidylinositol 3-kinase
eIF3	eukaryotic initiation factor 3
TPR	tetratricopeptide repeat
PCI	proteasome COP9 signalosome
IRES	internal ribosomal entry site
2-5 (A)	2′-5′ oligoadenylate
OAS	2′-5′-oligoadenylate synthetase
PKR	double-stranded RNA-activated protein kinase
PACT	human PKR protein activator
RAX	mouse PKR activator X
dsRBM	double-stranded RNA binding motif
eIF2α	the α subunit of eukaryotic initiation factor 2

I. INTRODUCTION

Infection of mammalian cells by viruses causes rapid induction of many cellular genes. The proteins encoded by these genes mediate cell-virus homeostasis. Some of them impair virus replication by interfering with specific steps in viral gene expression; others are antiviral cytokines, such as interferons (IFNs), which are secreted from the infected cells and act on as yet uninfected neighboring cells to protect them from oncoming viral infection. IFNs are not direct antivirals; they render the host cell inhospitable for viruses by inducing many antiviral genes. A subset of these genes, the viral stress-inducible genes (VSIGs), is induced by not only IFNs but also viral proteins, RNAs, and DNAs and most notably double-stranded (ds) RNA, a common by-product of virus replication. In some cell types, they can also be induced by other microbial products, such as bacterial lipopolysaccharides, indicating a broad functional spectrum of this family of genes.

Viruses, dsRNA, and the IFN system are interconnected at many levels. Viruses induce IFN synthesis as does dsRNA, added to cell cultures or transfected into cells. Because some viruses are known to produce dsRNA during their replication, it was originally thought to be an obligatory intermediate for viral induction of IFNs and VSIGs. As it turned out, many other viral gene products, such as single-stranded RNA, dsDNA, glycoproteins, and lipoproteins, can also induce these genes. Nonetheless, dsRNA remains a major player in host–virus interaction. In addition to its role in gene induction, it is a cofactor for two important VSIG-encoded enzymes, dsRNA-activated protein kinase (PKR) and 2′-5′-oligoadenylate synthetase (OAS). It is also the substrate of the RNA-editing enzyme ADAR, another VSIG-product. Finally, induction and action of VSIG-products are often subjected to regulation by constitutively expressed cellular and viral proteins and RNAs, including dsRNA and dsRNA-binding proteins.

A variety of viral stresses causing VSIG induction use signaling pathways that have both common and distinct elements. The most important common element is the *cis*-element in the promoters of the induced genes that receives the signal. This element, the IFN-stimulated response element (ISRE), is recognized by members of the IFN regulatory factor (IRF) family of transcription factors. These factors are present in the cytoplasm of uninduced cells, they get activated and translocated to the nucleus, bind to the ISRE, and stimulate transcription either by themselves or in conjunction with other transcription factors. Three major members of this family are IRF-9, IRF-3, and IRF-7; the first two are expressed widely, whereas IRF-7 is expressed primarily in the cells of the immune system. IRF-9 is a component of interferon stimulated gene factor 3 (ISGF3), a transcription complex of activated STAT1 (signal tranducers and activation of

transcription 1), STAT2, and IRF-9; it is activated and used by type I IFNs to induce transcription of VSIGs. In contrast, IRF-3 and IRF-7 act by themselves, after activation by phosphorylation and dimerization. These factors are activated by all relevant pathways other than the IFN-signaling pathway. In this article, we discuss how VSIGs are induced by different signaling pathways, how proteins encoded by selected VSIGs function, and how different viruses try to evade these processes.

II. SIGNALING PATHWAYS LEADING TO VSIG INDUCTION

The IRF-ISRE connection is the common link between all signaling pathways leading to VSIG induction. In addition, these are inducer-specific pathways. For example, viruses and dsRNA can activate the transcription factors NF-κB and AP-1; consequently genes regulated by these transcription factors are induced by them but not by IFNs. In contrast, IFNs can activate STAT1 dimers which recognize interferon-gamma-activated sequence (GAS) elements and are effective transcription factors. Transcription of the IFN-β gene requires combined action of several transcription factors, such as NF-κB, AP-1, and IRF-3/IRF-7. Consequently, IFNs cannot induce IFN synthesis. There are opportunities for extensive cross talks among the various signaling pathways, and many genes that encode components of the signaling pathways are themselves induced by them. For example, genes encoding Toll-like receptor 3 (TLR-3), a receptor for dsRNA and STAT1, a component of the IFN-signaling pathway, are strongly induced by IFN. Conversely, some of the VSIG products, such as suppressors of cytokine signaling (SOCS) or A20, are negative regulators of specific signaling pathways. Thus, VSIG products can affect their own expression and reinforce, either positively or negatively, the relevant signaling pathways.

The IFN-signaling pathways are the most well-understood and the majority of the VSIGs had been originally identified as IFN-stimulated genes. The two major types of IFNs, types I and type II, act through different cell surface receptors and are structurally unrelated. Type I IFNs consist of IFN-α, which has many subspecies, IFN-β, IFN-ω and IFN-τ. IFN-γ is the sole member of the type II family. Type I IFNs use the heterodimeric receptor complex IFNAR whose ligand-induced conformational change causes cross-activation of the two receptor-associated Janus protein tyrosine kinases, Jak1 and Tyk2. Activated Jaks phosphorylate themselves, the receptor subunits, and the transcription factors called signal tranducers and activation of transcription (STAT). Tyrosine phosphorylated STAT1 and STAT2 form a trimeric complex with IRF-9 (P48). This complex translocates to the nucleus, binds to ISRE, and activates transcription of the cognate genes. IFN-γ binds to its own receptor,

IFNGR, and initiates a similar signaling process by causing dimerization of the receptor, phosphorylation of Jak1 and Jak2, and phosphorylation of STAT1. Phosphorylated STAT1 forms dimers, translocates to the nucleus, and binds to the GAS elements in the promoters of IFN-γ-activated genes. These genes are usually not induced by viruses and dsRNA because their transcription is not IRF-driven. But, IFN-γ can also induce some genes through ISRE, using a transcription complex containing STAT1 and IRF-9.

Many IFN-independent signaling pathways can cause VSIG induction. They initiate differently but converge at various points and activate IRF-3 or IRF-7, the common denominators (Fig. 1). The Toll-like receptors are major sensors of viral components. The nucleic acid recognizing members, TLR-3, TLR-7, TLR-8, and TLR-9, reside primarily in the endosomal membranes. TLR-3 recognizes dsRNA (Alexopoulou *et al.*, 2001), TLR-7 and TLR-8 recognize ssRNA (Beutler and Crozat, 2004), and TLR-9 recognizes dsDNA (Hemmi *et al.*, 2000). Some viral glycoproteins are recognized by the cell surface receptors TLR-2 and TLR-4 (Bieback *et al.*, 2002). The two other major receptors are the cytoplasmic RNA-helicases, retinoic acid-inducible gene (RIG-I) and melanoma differentiation-associated gene (Mda-5). They recognize uncapped viral RNAs, probably dsRNA (Hornung *et al.*, 2006; Pichlmair *et al.*, 2006). A similar pathway may exist for recognizing cytoplasmic DNA. The adaptor proteins for the different receptors connect them to the common protein kinase, TANK-binding kinase-1 (TBK1), which phosphorylates and activates IRF-3 and IRF-7 (Fitzgerald *et al.*, 2003). For TLR-3, the critical adaptor protein is TIR-domain-containing adapter-inducing interferon-*β* (TRIF), which is also recruited by TLR-4 using an intermediate adaptor called trif-related adaptor molecule (TRAM). TLR-7, TLR-8, TLR-9 and TLR-4, all use the major adaptor of TLR pathways, MyD88; TLR-3 is the only TLR that does not use myeloid differentiation factor 88 (MyD88). RIG-I and Mda-5 use the adaptor protein, IFN-*β* promoter stimulator 1 (IPS-1), which is a mitochondrial membrane-anchored protein. Different viruses use different pathways to trigger VSIG induction. The primary pathway is not only virus dependent, but cell-type dependent as well. TLR-7 or TLR-8 is the major receptor for vesicular stomatitus virus (VSV), influenza virus and HIV-mediated innate immune response (Diebold *et al.*, 2004; Lund *et al.*, 2004), whereas TLR-3 is used by murine cytomegalovirus (MCMV), influenza virus, reovirus, and mouse mammary tumor virus (MMTV) (Guillot *et al.*, 2005; Hoebe *et al.*, 2003). TLR-2 is used by human cytomegalovirus (HCMV) and measles virus (Bieback *et al.*, 2002; Compton *et al.*, 2003), TLR-9 by MCMV and herpes simplex virus (HSV) (Krug *et al.*, 2004; Tabeta *et al.*, 2004). Paramyxoviruses, hepatitis C virus (HCV), and picornaviruses use primarily the RIG-I/Mda-5 system. TLR-4 recognizes respiratory syncytial virus (RSV) and MMTV. For some viruses, the exact viral components responsible for the response have been identified. For RSV, it

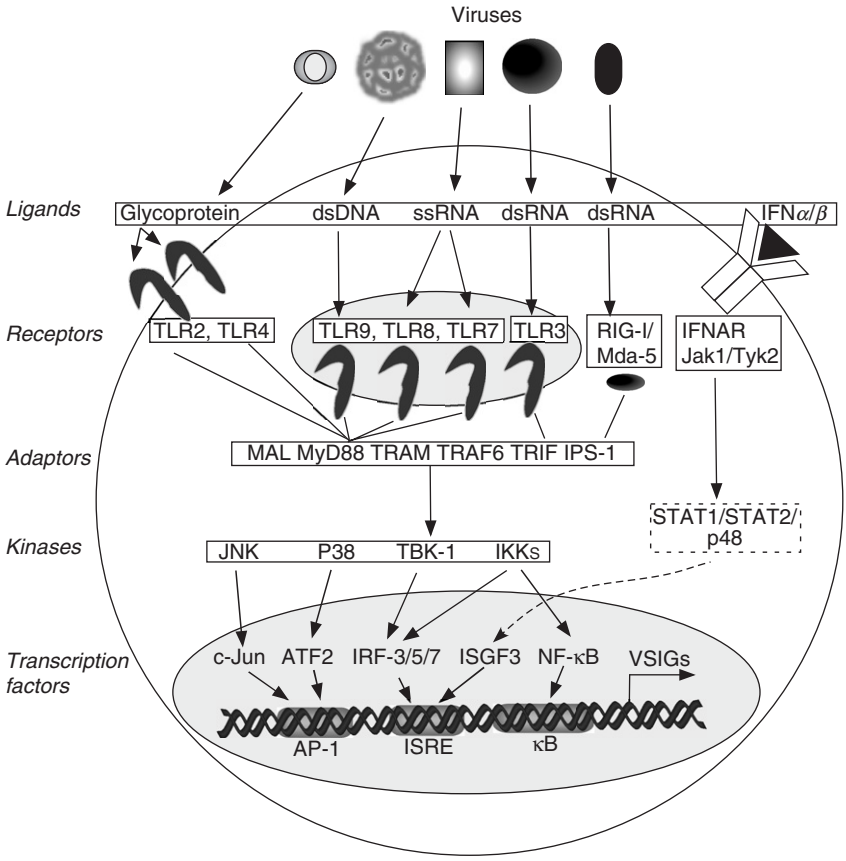

FIGURE 1 Signaling leading to VSIG induction. Major signaling pathways are initiated differently by different viruses and IFNs that converge at various points and cause VSIG induction. The Toll-like receptors are major sensors of viral components. Nucleic acid sensing TLRs are all located in endosomal membranes except for RIG-I which is cytoplasmic, while proteins, sugars, and lipids are recognized by TLR receptors at the cell surface. Adaptors used by the various TLRs are needed to transmit signals to kinases that activate transcription factors, which are transported to the nucleus. The transcription factors bind to response elements and induce VSIGs.

is the F protein and for MMTV it is the envelope protein, both of which activate TLR-4. HCMV gB protein and MV H protein activate TLR-2, and CpG DNA of HSV-1, HSV-2, and MCMV can activate TLR-9. dsRNA produced by MCMV, influenza virus, NDV, and LCMV are implicated in activating TLR-3, and ssRNA of influenza virus and VSV can activate TLR-7. In specific viral infection models, TLRs may trigger not only host defense but pathogenesis as well.

A. Signaling by dsRNA

Among the TLRs, TLR-3, present in the endosomal membrane, recognizes dsRNA (Alexopoulou *et al*., 2001). The recognition is by ionic interaction between the negatively charged ligand and positively charged amino acid residues present along both sides of a groove that accommodates dsRNA (Bell *et al*., 2005; Choe *et al*., 2005). Extracellular dsRNA has to be endocytosed to reach TLR-3; however, many viruses enter the cell through endocytosis and their genomic RNAs may encounter TLR-3 in the endosome. Ligand binding leads to TLR-3 dimerization, presumably causing a conformational change of its cytoplasmic domain to initiate the signaling process. A novel aspect of TLR-3 signaling is the need for its tyrosine phosphorylation; among the TLRs, this feature is unique to TLR-3. Human TLR-3 contains five tyrosine residues in its cytoplasmic domain. Only two of them, Tyr759 and Tyr858, are absolutely needed for complete signaling by the receptor (Sarkar *et al*., 2003). These residues get phosphorylated on ligand binding to the receptor, and phosphorylation is essential for signaling because their mutation to Phe or the presence of a tyrosine kinase inhibitor blocks signaling. The two phosphotyrosines recruit the signaling complex. The adaptor proteins include TRIF and TNF receptor-associated factor 3 (TRAF3), and the two main branches of signaling bifurcate from TRIF. The Ser/Thr protein kinases, c-jun N-terminal kinase (JNK), P38, and IκB Kinase (IKK) are activated by a complex containing TRAF6, TAB1, TAB2, and TAK1. JNK, P38, and IKK activate the transcription factors c-Jun, ATF2, and NF-κB, respectively. Another adaptor protein, RIP1, is also required for NF-κB activation. The second independent branch of signaling from TRIF is mediated by the recruitment of the protein kinase TBK1 or IKKε, which directly phosphorylates the transcription factor IRF-3. Its phosphorylation causes dimerzation and nuclear translocation. In the nucleus, it binds to the ISRE elements in the promoters of target genes and induces their transcription. The histone deacetylase, HDAC6, is required for the transcriptional activity of IRF-3.

Activation of both NF-κB and IRF-3 by TLR-3 signaling is a two-step process, and the two phosphotyrosines of TLR-3 initiate each of the two steps (Fig. 2). If Tyr759 is mutated, TBK1 is still recruited to the receptor, and IRF-3 activation occurs up to the stage of its nuclear translocation (Sarkar *et al*., 2004). However, although in the nucleus, it cannot interact with co-activators and form a stable transcription complex; consequently, genes are not induced under these conditions. The biochemical defect of the incompletely activated IRF-3 was traced to its partial phosphorylation. Full activation requires its further phosphorylation by a PI3 kinase/Akt-mediated pathway. This pathway is initiated by the recruitment of PI3 kinase to the TLR-3 complex via phosphotyrosine 759. The tyrosine kinase, Src, which is known to be activated by Akt, may be a participant in

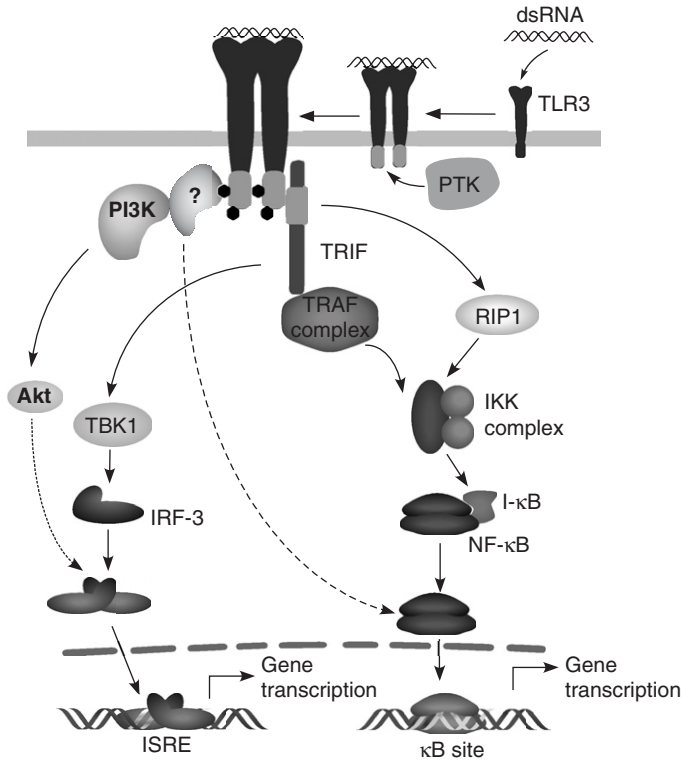

FIGURE 2 TLR signaling: Two-step activation model for IRF-3 and NF-κB. On binding dsRNA, TLR3 dimerizes and is activated, being phosphorylated at Tyr 759 and Tyr 858 (small black dots) by an unknown protein tyrosine kinase (PTK). In the case of IRF-3 activation, PI3 kinase is recruited by Tyr 759 and activated. Activated TLR3 also activates TBK1 via TRIF, which phosphorylates IRF-3 (step 1). To activate transcription, IRF-3 requires further phosphorylation by the PI3 kinase/Akt pathway (step 2). In the case of NF-κB activation, PI3K is not needed. Here, a TRAF complex or RIP1 linked to activated TLR3 via TRIF mediates IKK activation. IκB is phosphorylated by activated IKK and NF-κB is released (step 1). To activate gene transcription, the RelA (p65) subunit of NF-κB needs to be further phosphorylated (step 2) which requires phosphorylation of TLR residues Tyr 759 and Tyr 858.

this pathway because Src is activated by TLR-3 signaling, and its presence is needed for gene induction (our unpublished observation). Inhibition of PI3 kinase, Akt, and Src has the same effect as TLR-3 Tyr759 mutation; IRF-3 is incompletely phosphorylated and transcriptionally inactive.

The details of NF-κB activation are somewhat different, but the same two-step activation principle is operative here as well (Sarkar *et al.*, 2007). In the first step, binding of dsRNA to TLR-3 causes IKK activation, IκB phosphorylation and the release of NF-κB from the NF-κB–IκB complex.

TLR-3 Tyr759 is not required for the above process or for the phosphorylation of NF-κB P65 protein in Ser276 and Ser546 residues. However, full activation of NF-κB needs further phosphorylation of P65 which is initiated by phosphotyrosine 759 of TLR-3. Under-phosphorylated NF-κB goes to the nucleus but cannot bind to the promoters of the target genes tightly enough to induce transcription. Thus, the roles of Tyr759 and Tyr858 of TLR-3 are distinct, but complementary, in the activation pathways of NF-κB and IRF-3.

dsRNA can induce gene transcription by using the cytoplasmic sensors RIG-I and Mda-5 as well (Yoneyama *et al.*, 2004). These pathways are used by many RNA viruses to induce VSIGs. We compared the characteristics of gene induction by the dsRNA/TLR-3 pathway and the RIG-I pathway activated by Sendai virus (SeV) (Elco *et al.*, 2005). We used microarray analyses of gene induction by the two agents as the primary tool, and suitable cell mutants were used to investigate the role of specific proteins in the signaling pathways. These studies revealed that SeV does not use TLR-3 at all, and the repertoires of genes induced by SeV/RIG-I and dsRNA/TLR-3 are partially overlapping, many VSIGs being common to both of them. IRF-3, NF-κB, and IFN signaling were required for the induction of different subsets of gene by SeV. This study produced an unexpected result, namely, a cross talk between the IRF-3 pathway and the NF-κB pathway, increasing levels of IRF-3 block gene induction by the NF-κB pathway. But, this inhibition is selective; induction of only some NF-κB-driven genes is impaired. The underlying molecular mechanism remains to be delineated.

III. INHIBITION OF TRANSLATION BY PROTEINS ENCODED BY VSIGS

Although the biochemical and cellular functions of the majority of VSIGs' products are unknown, several of them block protein synthesis. These proteins inhibit a variety of steps in mRNA translation, some acting constitutively and others requiring enzymatic activation by binding to dsRNA (Fig. 3). The first class of proteins, members of the P56 family, can block translation initiation as such and their induction by viral and other stresses leads to inhibition of cellular protein synthesis. By necessity, these proteins are not expressed at all in unstressed cells. Moreover they are often induced robustly, but only transiently, and both the proteins and the mRNAs turn over rapidly. Thus, these proteins are designed to block cellular translation efficiently but temporarily. Viral-stress inducible proteins of the second class, which block protein synthesis, are enzymes. They are often expressed at low levels in uninfected cells, the levels are further increased on cellular exposure to viral stresses. These proteins are enzymatically inactive as such and they need to be activated by

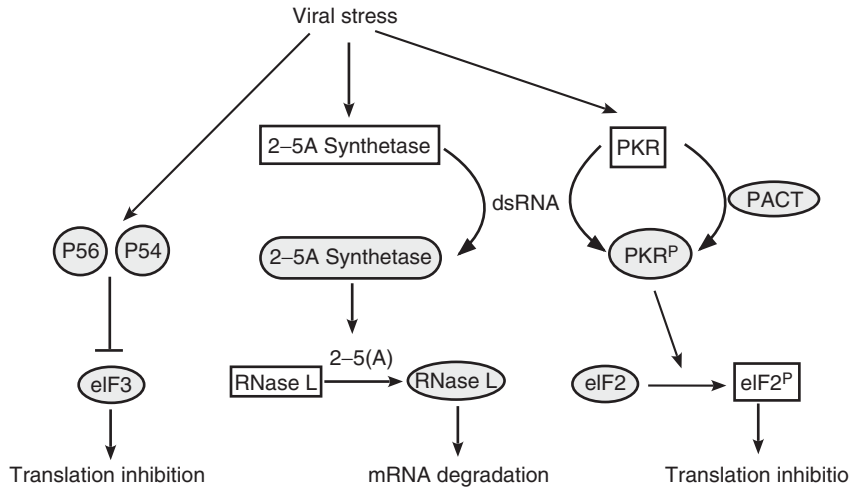

FIGURE 3 Translational inhibition pathways induced by viral stress. Members of the P56 family are robustly and transiently induced by viral stresses and bind to eIF3 to cause translation inhibition. 2′-5′ oligoadenylate synthetases and PKR levels are increased by viral stresses and activated by cofactor dsRNA, which result in an inhibition of protein synthesis. Phosphorylated proteins are indicated by the letter "P"; active proteins are shaded.

conformational changes induced by binding to their activators. One family of such enzymes, the OAS, is activated by dsRNA and synthesizes 2′-5′ linked oligoadenylates [2–5 (A)]. The 2–5 (A) molecules, in turn, activate the latent ribonuclease, RNase L, causing mRNA degradation. The other VSIG-encoded latent enzyme is the serine-threonine protein kinase, PKR. PKR is activated by its binding to either of two activators, dsRNA or PACT, a cellular protein. Activation of PKR leads to its autophosphorylation and phosphorylation of its most well-characterized substrate, the α subunit of the translation initiation factor, eukaryotic initiation factor 2 (eIF-2), which leads to inhibition of protein synthesis. The characteristics and the modes of action of these selected VSIG products are described below in more detail.

A. The P56 family of proteins

Human P56 is encoded by ISG56 (1FIT-1), a gene whose transcription is strongly induced by type I IFNs, dsRNA, or virus infection (Guo *et al.*, 2000a). There are three other members of this family of human genes: ISG54 (IFIT-2) encodes P54, ISG58 (IFIT-5) encodes P58, and ISG60 (IFIT-4) encodes P60 (deVeer *et al.*, 1998; Niikura *et al.*, 1997; Wathelet *et al.*, 1988; Yu *et al.*, 1997). In mouse, there are three genes: ISG56 (IFIT-1), ISG54

(IFIT-2), and ISG49 (IFIT-3) (Bluyssen *et al.*, 1994). Among the human and mouse proteins, the cognate members of the two species are more structurally related than two members from the same species. For example, human P56 and mouse P56 have 50% sequence identity and human P54 and mouse P54 have 73% sequence identity. In contrast, human P56 and P54 have only 42% identical residues.

Viral stresses, that trigger different signaling pathways leading to the activation of the IRF transcription factors, induce synthesis of the P56 family of proteins. The corresponding genes appear at or near the top of microarray charts of cellular genes induced by many viruses, IFNs, and dsRNA (Der *et al.*, 1998; Geiss *et al.*, 2001). Different members of this gene family are usually induced coordinately both in tissue culture and in mice. Recently, Wacher *et al.* (2007) examined their induction in the brains of mice infected with either lymphocytic choriomeningitis virus or West Nile virus. ISG49, ISG56, and to lesser extent, ISG54, were induced widely in the neuronal population. Their induction patterns in STAT1−/− and STAT2−/− mice, interpreted as IFN-independent induction, were lower, delayed, and restricted to cells in the choroids plexus, meninges, and endothelium. These results demonstrated that infection of the central nervous system of mice by an arena virus or a flavivirus causes robust induction of the P56 family members. Their induction in some cells, especially neurons, is IFN-signaling dependent, whereas in other cell types, it is IFN independent.

Although these genes usually induced coordinately, interesting exceptions have recently been noted. In human fibrosarcoma HT1080 cells, IFN-β strongly induces both ISG56 and ISG54 mRNAs; the level of ISG54 mRNA, but not ISG56 mRNA, declines quickly (Terenzi *et al.*, 2006). In contrast, the induction kinetics of both mRNAs, in response to dsRNA, are very similar. In these cells, Sendai virus induces both mRNAs strongly but transiently, whereas in HEK293 cells the induction is both strong and sustained. Thus, there are both inducer-specific and cell-specific differences in the induction patterns of human ISG56 and ISG54. Interesting differences in the *in vivo* induction patterns of the two corresponding mouse genes have been observed as well (Terenzi *et al.*, unpublished observations). In this study, IFN-α, IFN-β, dsRNA, or vesicular stomatitis virus was administered to mice by tail-vein injections, and the expression of P56 and P54 in different tissues was examined. Both proteins were induced by all inducers in many tissues, but there were intriguing exceptions. For example, in spleenic B cells, only P54 was induced in response to any inducer. In the liver, both proteins were induced well by IFN-β and dsRNA, but IFN-α and VSV induced primarily P56. The observed differences of the protein level were reflected at the mRNA level as well, indicating that the differences are in the gene induction patterns. Future investigation of the molecular basis of the above observations

should lead to the discovery of novel features of regulation of expression of this class of genes.

The P56 proteins are related in their sequences, but quite distinct. Their one common feature is that they all contain multiple tetratricopeptide repeat (TPR) motifs (D'Andrea and Regan, 2003). These are degenerate protein–protein interaction motifs that function in combinations. The cognate members of the human and the mouse P56 family contain the same number of TPR motifs distributed at similar distances (Sarkar and Sen, 2004). For example, although human and mouse P56 proteins have only 50% sequence identities, they both contain six TPR motifs located at similar positions in the linear sequences of the proteins. In contrast, the P54 proteins of both species have four TPR motifs. The TPR motifs adopt helix-turn-helix structures, and adjacent motifs pack in parallel forming spirals of repeating anti-parallel helices (D'Andrea and Regan, 2003). The TPR motifs allow binding of these proteins to multi-protein complexes and regulate their functions. In the case of the P56 family of proteins, the most characterized binding partner is the translation initiation factor, eIF-3. eIF-3 is a 12-subunit protein complex that catalyzes many steps in the initiation of protein synthesis. The P56 proteins interact with specific subunits of eIF-3 and stay bound to the whole eIF-3 complex. The human P56 binds to the ''e'' subunit of eIF-3 (also known as Int-6 or P48) (Guo *et al*., 2000b). Whereas human P54 binds to both the ''e'' and the ''c'' subunits; P60 and P58 do not bind to either of the subunits. Both mouse P56 and P54 bind to the ''c'' but not the ''e'' subunit of eIF-3. The carboxyl terminal regions of the ''c'' and ''e'' subunits of eIF-3 are responsible for the interactions (Guo *et al*., 2000b). These regions contain a motif called the proteasome COP9 signalosome (PCI) motif, a long α-helix, that is present in different subunits of three large protein complexes: the regulatory subunit of proteasome (P), the CoP9/signalsome (C) complex, and translation initiation (I) factor 3 (Hofmann and Bucher, 1998). Some P56-family proteins can interact with more than one region of eIF3c. HuP54 interacts with both the N-terminal and the C-terminal regions of eIF3c. Using its own N-terminal region, mouse P54 interacts with only the PCI domain of eIF3c, whereas mouse P56 interacts with its N-terminal region.

Recombinant human and mouse P56 and P54 proteins inhibit *in vitro* translation of mRNAs in rabbit reticulocyte lysate system (Guo *et al*., 2000b; Hui *et al*., 2005; Terenzi *et al*., 2005, 2006). The observed inhibition is due to their ability to block specific function of eIF-3 because exogenously added excess eIF-3 can reverse the inhibition and a mutant P56, which cannot bind to eIF3, does not inhibit translation. Detailed investigation of the nature of the eIF-3 functions that are impaired by the binding of P56 has produced interesting results. Among the many functions of eIF-3 in initiating protein synthesis, all but two are unaffected. One of the two affected functions is the ability of eIF-3 to stabilize the ternary

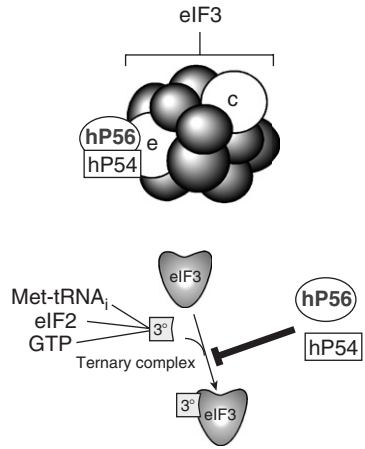

FIGURE 4 Model of inhibition of protein synthesis by human P56 and P54. Different P56 family members block translation at different steps of the initiation pathway. (Top panel) Human P56 and P54 bind to the eIF3e subunit. (Bottom panel) Binding to eIF3 blocks formation of a stable eIF3-ternary complex.

complex of eIF-2.GTP.tRNA$_i$ Met. Human P56 and P54, which can bind to the ''e'' subunit of eIF-3, block the above function (Fig. 4; Hui *et al.*, 2003). In contrast, mouse P56 and P54, which bind to the ''c'' subunit, do not affect it. Instead, binding of the P56 proteins to the ''c'' subunit blocks a different function of eIF-3, namely, its ability to facilitate the formation of the 48S pre-initiation complex (Fig. 5; Hui *et al.*, 2005; Terenzi *et al.*, 2006), composed of the 40S ribosomal subunit and the 20S complex containing eIF-3, ternary complex, eIF4F, and mRNA. Mouse P56 and P54 and human P54, but not P56, block 48S complex formation although the 20S complex forms normally. Thus, there is a perfect correlation between the subunit of eIF-3 to which a P56-family protein binds and the specific function of eIF-3 that is inhibited. P56 can inhibit initiation of translation of not only capped mRNAs but also of those that use internal ribosomal entry sites (IRES). For example, translation of HCV mRNA is strongly inhibited by human P56 and this mechanism is thought to be one of the major arms of IFN's antiviral effects on HCV replication (Wang *et al.*, 2003). Interestingly, translation initiated by the IRES of encephalomyocarditis virus mRNA is hardly blocked by P56, reinforcing the concept that different IRES elements may use distinct mechanisms to initiate translation and depending on the specific role of eIF-3 in a particular mechanism, P56 may or may not have any effect (Hui *et al.*, 2003). These observations suggest that the P56 family of proteins can be used as important tools to probe the nature of the specific functions of eIF-3 that are needed in different models of translation initiation. Beyond translation initiation,

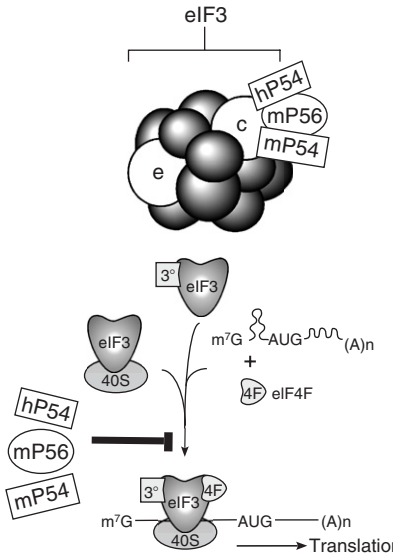

FIGURE 5 Model of inhibition of protein synthesis by mouse P56 and human and mouse P54. Different P56 family members block translation at different steps of the initiation pathway. (Top panel) Human P54 and mouse P54 and P56 bind to the eIF3c subunit. (Bottom panel) Binding to eIF3c interferes with the formation of the 48S pre-initiation complex, consisting of the ternary complex, eIF3, the 40S ribosomal subunit, eIF4F, and mRNA.

P56 proteins may affect other putative functions of the eIF-3 subunits. Because many of these subunits are present in the nucleus as well, they are anticipated to have additional cellular functions. For example, eIF3e is a shuttle protein that contains both a nuclear localization signal and a nuclear export signal. This protein was originally discovered as the product of the mammalian Int-6 gene, whose disruption in mice by the integration of the mouse mammary tumor virus genome causes breast cancer in mice (Marchetti *et al.*, 1995). Although, the biochemical basis of that pathogenesis is unknown, it will be interesting to investigate whether the P56 proteins have any effect on it.

B. 2′-5′ Oligoadenylate synthetases

Proteins of the OAS family are present in low quantities in most cells, but their levels are increased on viral stresses. These are latent enzymes that are activated by conformational changes induced by binding to their activator, dsRNA. Once activated, they catalyze the synthesis of 2′-5′ linked oligoadenylates [2–5 (A)], which, in turn, activate the latent ribonuclease, RNase L, by promoting its dimerization (Hovanessian, 1991;

Lengyel, 1987; Silverman and Cirino, 1997). Three human genes, OAS1, OAS2, and OAS3, encode enzymatically active proteins; in addition there are several OAS-like genes. The OAS genes produce alternatively spliced mRNAs encoding multiple isozymes with different carboxyl terminal regions. The OAS1 isozymes are 40–46 kDa and form tetramers; the OAS2 isozymes are 69–71 kDa and form dimers, whereas the OAS3 enzyme is a monomer of 100 kDa. Some of these isoforms are posttranslationally modified by lipids and sugars causing their translocation to different subcellular sites (Marie *et al.*, 1990; Sarkar *et al.*, 1999a).

There are notable differences in the enzymatic properties of the three classes of OAS. The OAS1 isozymes synthesize up to hexamers of 2–5(A) and the OAS2 isozyme can synthesize up to 30 mer of 2–5 (A) (Sarkar *et al.*, 1999a). In contrast, OAS3 can make only dimeric 2–5 (A) which cannot activate RNase L (Rebouillat *et al.*, 1999). Extensive structure-function studies of OAS1 and OAS2 have led to the identification of their oligomerization site, the catalytic site (C), the substrate acceptor-binding site (A), and the substrate donor-binding site (D) (Sarkar *et al.*, 1999b, 2002a). The latter three sites of OAS2 P69 are located near its C-terminal, whereas the dimerization domain is at the very C-terminal. Dimerization of P69 is essential for its enzymatic activity because of the crisscross nature of catalysis (Sarkar *et al.*, 2002b). The donor bound to the ''D'' site of subunit 1 is transferred to the acceptor bound to the ''A'' site of subunit 2 by the action of the ''C'' site of subunit 2. Thus, each enzyme molecule simultaneously synthesizes two chains of 2–5 (A). By mutating one or more of the sites in one subunit, but not the other, the catalytic activity could be reduced by half.

Solution of the crystal structure of OAS1 revealed that it is a U-shaped protein with two major domains (Hartmann *et al.*, 2003). One domain from the N-terminus consists of five-stranded antiparallel β-sheets and two α-helices. The other domain from the C-terminus consists of a four-helix bundle. The two lobes are linked by a 35-residue stretch containing two α-helices. Moreover, the first 20 residue at the N-terminus of the protein pack tightly against the C-terminal domain. The structure of the catalytic site, composed of three Asp residues, is similar to that of poly(A) polymerases, which suggests that the way the substrates become accessible to be active centers of the two classes of enzymes, determines the nature of the bond formed, 2′-5′ or 3′-5′ (Hartmann *et al.*, 2002). The structure of OASA1 shed light on its mechanism of activation by dsRNA as well. Among the dsRNA-binding proteins, the OAS proteins are like TLR-3 and unlike PKR, they do not have any defined dsRNA-binding motif. Instead, OAS1 contains on the opposite side of the catalytic center, a positively charged groove in which negatively charged dsRNA fits perfectly. Mutagenesis and enzyme kinetics studies suggest that binding of dsRNA to this groove causes a domain–domain shift that widens the active site cleft of the protein.

Some OAS isoforms and OAS-related proteins have nonenzymatic activities as well. Because of alternative splicing, the human OAS1 E18 isozyme acquires a Bcl2-homology 3 domain in its C-terminus. Through this domain, E18 interacts with members of the Bcl-2 family and causes cellular apoptosis (Ghosh *et al.*, 2001). This action of E18 does not require its enzymatic activity, dimerization or activation by dsRNA. Thus, E18, an IFN-inducible protein, has dual functions, to synthesize 2–5 (A) and to promote apoptosis. The enzymatically inactive OAS-like protein, P59 OASL, can confer antiviral activity probably through its C-terminal region which contains ubiquitin-like sequences (Hartmann *et al.*, 2001). The mouse OASL1 gene, which encodes an enzymatically inactive protein, has been implicated to confer resistance to West Nile virus by an unknown mechanism (Mashimo *et al.*, 2002). These recent observations of nonenzymatic functions of some OAS and OAS-related proteins have generated new interest in their biological roles.

C. PKR/PACT

PKR (protein kinase RNA regulated) is a latent protein kinase (Meurs *et al.*, 1990). Like OAS, PKR is expressed ubiquitously at a low level but its expression is induced by viral stresses. The enzyme is activated by autophosphorylation which requires its conformational change on binding to its activator (Galabru and Hovanessian, 1987). In the presence of ATP, it can be activated by dsRNA or the cellular protein PACT. Once activated, it can phosphorylate only a limited set of proteins, the most characterized of which is the α subunit of the translation initiation factor, eIF-2, whose phosphorylation causes inhibition of translation initiation (Samuel, 1993). In virus-infected cells, viral dsRNA, a common by-product of viral replication, causes PKR activation, eIF-2 phosphorylation, and inhibition of viral protein synthesis. To counteract the PKR-mediated antiviral effect, many viruses encode proteins or RNAs that interfere with PKR activation or action. In addition to its role in translation regulation and cell growth, PKR has been shown to be an important element in the transcriptional signaling pathways activated by specific cytokines, growth factors, dsRNA, and extracellular stresses (reviewed in Williams, 2001). In addition, PKR has been implicated in cell differentiation, apoptosis, and oncogenic transformation.

PKR contains two structurally and functionally distinct domains connected by a linker region. At its N-terminus, is the dsRNA binding domain containing two dsRNA-binding motifs (Green and Mathews, 1992; Patel and Sen 1992) and at the C-terminus, is the catalytic domain containing eleven kinase subdomains (Meurs *et al.*, 1990). The two dsRNA-binding motifs, dsRBM1 and dsRBM2, also mediate direct protein–protein interactions (Patel *et al.*, 1995, 1996). Consequently, members of the PKR

family of dsRNA-binding proteins can homodimerize and heterodimerize. The structure of the dsRNA-binding domain has been solved by nuclear magnetic resonance (NMR) (Nanduri *et al.*, 1998) and that of the kinase domain has been solved by x-ray crystallography (Dar *et al.*, 2005; Dey *et al.*, 2005). However, the structures of the linker region and the whole protein remain undetermined.

PACT, the protein activator of PKR, is also a dsRNA-binding protein (Patel and Sen, 1998). Like PKR, it contains two dsRNA-binding motifs, domains 1 and 2, at its N-terminus. But these domains do not activate PKR, nor are they required for PKR activation *in vitro*. Domain 3, a 66 residue stretch near the C-terminus, by itself, can activate PKR (Huang *et al.*, 2002; Peters *et al.*, 2001). Domain 3 does not bind dsRNA and its mode of activation of PKR is quite distinct form that used by dsRNA; domain 3 has low affinity for the linker region of PKR, whereas domains 1 and 2 bind strongly to the dsRBD of PKR. *In vivo*, PKR activation by PACT requires not only domain 3 but also either domain 1 or domain 2. Presumably, the need of the latter domains is for anchoring PACT tightly to PKR, because they can be replaced by PKR's own dimerization domains. Domains 1 and 2 of PACT, not only do not activate PKR but when expressed without domain 3 they inhibit PKR activation. Consequently, eIF-2 phosphorylation is diminished and protein synthesis is enhanced.

The inactive conformation of PKR is maintained by an intramolecular interaction between dsRBM2 and the linker/kinase domain (Nanduri *et al.*, 2000; Li *et al.*, 2006b). DsRNA binds to dsRBM1 with higher affinity which promotes cooperative binding to dsRBM2 and disruption of the intramolecular protein–protein interaction (Nanduri *et al.*, 2000). The motif in the linker region of PKR, with which dsRBM2 interacts, has recently been identified (Li *et al.*, 2006b). To this motif (PBM), PACT domain 3 also binds. Biochemical, genetic, and NMR analysis showed that PBM binds to PACT domain 3 as well as dsRBM2 of PKR. Thus, the PKR activation mechanisms used by dsRNA and PACT domain 3 are reciprocal: dsRNA binds to dsRBM2 and PACT domain 3 binds to PBM and either binding leads to disruption of the intramolecular interaction and consequent activation of PKR (Fig. 6). This model of PKR activation has been further confirmed genetically and biochemically; critical residues in PBM that are required for the interaction with dsRBM2 have been identified. Their mutations in PKR led to disruption of the intramolecular interaction and constitutive activation of the protein. Expression of such a mutant, but not the Wt PKR, in cells caused enhanced phosphorylation of eIF-2α. Moreover, a decoy peptide containing PBM could activate PKR by interfering with the intramolecular interaction that maintains the inactive conformation of the protein.

In virus-infected cells, viral dsRNA is the likely activator of PKR, whereas in uninfected cells PACT is probably the more relevant activator.

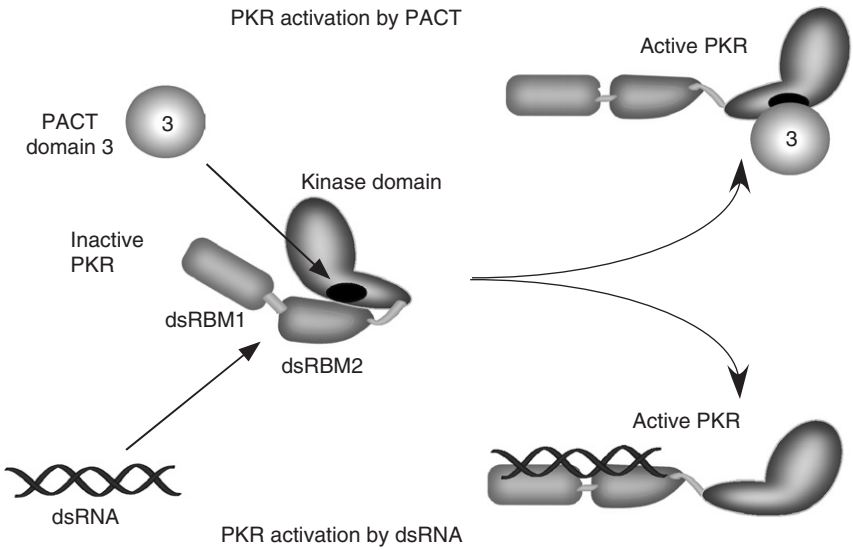

FIGURE 6 Model for PKR activation by PACT or dsRNA. The intramolecular inhibitory interaction between the PACT domain 3 binding motif (residues 328–335 of PKR, represented as a small black circle) in the kinase domain and dsRBM2 maintains PKR in an inactive conformation. Binding of PACT domain 3 to PBM or binding of dsRNA to dsRBM1 and dsRBM2 disrupts the intramolecular interaction in latent PKR and induces similar conformational changes leading to its activation.

Activation of PKR by PACT *in vivo* requires exposure of the cells to extracellular stresses that can be provided by withdrawal of growth factors or treatment of cells with a low dose of actinomycin D, arsenite, thapsigargin, or hydrogen peroxide (Ito *et al.*, 1999; Patel *et al.*, 2000; Peters *et al.*, 2001). Under these conditions, PACT is phosphorylated at specific serine residues, which makes it a better activator of PKR. These residues have recently been identified as Ser246 and Ser287 in domain 3 of PACT (Peters *et al.*, 2006). Alanine scanning mutagenesis of domain 3 revealed the identity of the 10 residues that were essential for activating PKR and causing cellular apoptosis. Among them were Ser246 and Ser287; they could be substituted by Thr, but not Ala, suggesting that they may be targets of phosphorylation. In support of this notion, their substitution with the phosphoserine mimetic Asp was also tolerated. Biochemical analyses revealed that Ser246 was constitutively phosphorylated in cells, whereas Ser287 was phosphorylated on the application of stress. Moreover, phosphorylation of Ser287 did not take place if Ser246 was mutated, indicating that its constitutive phosphorylation is a prerequisite for Ser287 phosphorylation. As anticipated, the S246D, S287D mutant was constitutively active and needed no stress-activated signals for its

ability to activate PKR. The S246A, S287D mutant was inactive indicating that phosphorylation of both residues were essential. *In vitro* analysis revealed that the constitutively active mutant of PACT could bind PKR more strongly and hence activate it more efficiently.

Physiological functions of PACT have been explored by generating Pact–/– mice (Rowe *et al.*, 2006). These mice have major phenotypes that are not shared by Pkr–/– mice (Abraham *et al.*, 1999; Yang *et al.*, 1995). Most notably, Pact–/– mice are smaller in size, have major fertility problems, and display microtia defects in ear development. These mice suffer from congenital abnormality of both outer and middle ears; consequently, they are hearing impaired. PACT is expressed in all three parts of the ear in adult and embryonic wild-type mice suggesting a direct role of the protein in ear development. This is the first example of a developmental role of a mammalian dsRNA-binding protein, although in *Drosophila* such a role of a similar protein, Staufen, is well documented (Micklem *et al.*, 2000; St Johnston *et al.*, 1989, 1991). The infertility problems of male and female Pact–/– mice were explained by the observation that they have reduced sex hormone levels. Similarly, the smaller size is most probably due to a lower level of growth hormone. The lower levels of various hormones could be traced back to a problem in pituitary development; Pact–/– mouse has a much smaller anterior pituitary because of a defect in the development of this tissue after birth (Peters *et al.*, unpublished observations). The pituitary defect can account for all developmental defects of Pact–/– mice other than microtia. These observations indicate that PACT and possibly other mammalian dsRNA-binding proteins have major roles in development. The challenge now is to delineate the molecular basis of their functions in this context.

IV. VIRAL EVASION OF VSIG EXPRESSION AND FUNCTION

Because unchecked induction of the VSIGs is detrimental to efficient virus replication, many viruses employ a variety of strategies to block either their induction and/or their functions. These viral evasion strategies are integral components of the host–virus equilibrium maintained in nature. In the laboratory, viral evasion of the innate response can be studied effectively by expressing the relevant viral protein or RNA in cells in isolation and measuring the ability of those cells to effectively mount an antiviral response. The knowledge gathered by employing the above strategy is often complemented with experiments using mutant viruses that do not express the ''evasion'' genes. However, the latter experiments are often difficult to interpret on their own because of the direct need of the products of the same genes for virus replication itself. For sorting out this additional complication, the replication efficiencies of the mutant

virus need to be compared in normal cells and in cells that are defective in the relevant feature of the antiviral response. Almost all of our knowledge on viral evasion is based on the results of studies that used one or more of the above experimental strategies. These investigations have revealed that viruses can inhibit all stages of cellular antiviral response: induction of VSIG by blocking TLR, RIG-I, Mda-5 signaling, blocking IFN synthesis and its action by inhibiting receptor interaction and signaling, and blocking the functions of proteins encoded by VSIGs (Fig. 7). Often more than one of the above processes are blocked by the same virus, thus ensuring effective inhibition of the entire pathway of cellular response to viral infection. Specific examples of such inhibitions are presented below. Some RNA viruses evade the IFN system by global shut-off of host RNA synthesis or processing. Poliovirus, VSV, bunya viruses, and influenza viruses are known to use these strategies.

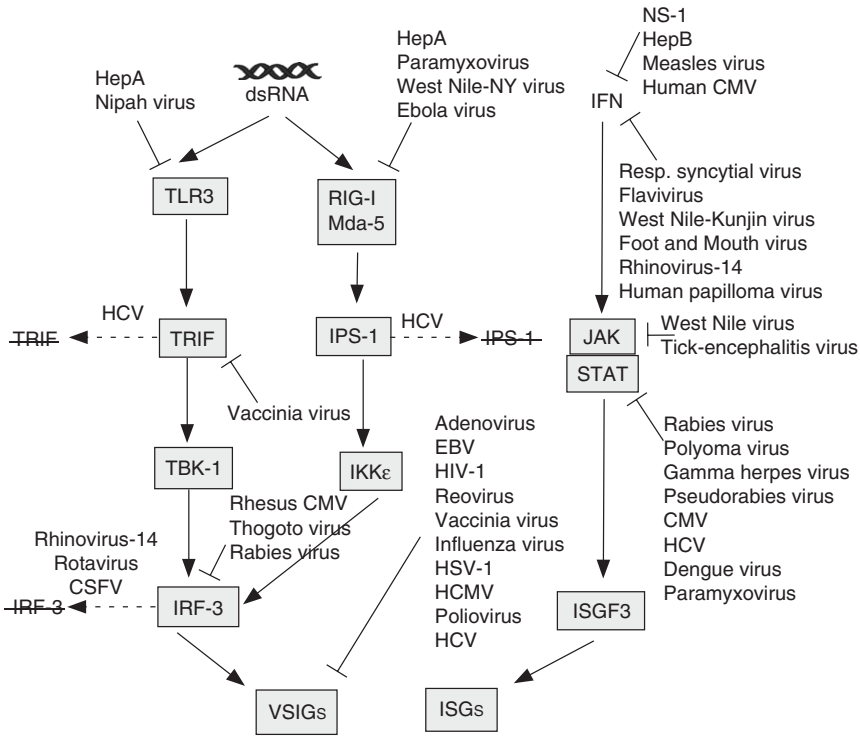

FIGURE 7 Viral strategies to interfere with VSIG induction and function. Many viruses block key signaling proteins or their cofactors/ligands by directly binding them, and some disrupt signaling by cleaving the proteins or leading them to degradation. Some viruses have the ability to block multiple steps in the cellular response to infection.

A. Inhibition of IFN synthesis and VSIG induction

HCV blocks signaling, by both TLR-3 and RIG-I/Mda-5, through the degradation of the key adaptor proteins of the two pathways. Consequently, neither IFNs nor VSIGs are efficiently induced. The key viral protein that brings about these inhibitions is the NS3/4A protease (Freundt and Lenardo, 2005), although NS3-independent blockage has also been noted (Cheng *et al*., 2006). Inhibition of TLR-3-mediated signaling is accomplished by NS3-mediated cleavage of the adaptor protein TRIF, which is also required for TLR-4 signaling (Breiman *et al*., 2005; Li *et al*., 2005a). Recognition of TRIF by NS3 employs a mechanism slightly different from that used for the recognition of the viral substrates by the same protease (Ferreon *et al*., 2005). The RIG-I-mediated cytoplasmic pathway of VSIG induction is blocked by HCV NS3/4A protease by cleaving the critical adaptor protein IPS-1 (Foy *et al*., 2005; Li *et al*., 2005b; Loo *et al*., 2006).

Paramyxoviruses block intracellular signaling leading to VSIG induction by using several viral proteins. The ''V'' proteins bind to Mda-5 and inhibit its signaling ability (Andrejeva *et al*., 2004). Nipah virus V protein, a nuclear protein, can block TLR-3 signaling (Shaw *et al*., 2005). Specific strains of respiratory syncytial virus and measles virus can block TLR-7 and TLR-9-mediated IFN production by plasmacytoid dendritic cells (Schlender *et al*., 2005). These effects may be mediated by the viral nonstructural proteins NS1/ NS2 (Spann *et al*., 2005). In addition, the secreted form of the viral ''F'' protein can block TLR-4 signaling by the membrane-bound viral ''F'' protein or other agonists of TLR-4 (Polack *et al*., 2005). Among rhabdoviruses, rabies virus ''P'' protein can block IRF-3 activation by an unknown mechanism (Brzozka *et al*., 2006). Flaviviruses can also block IFN synthesis. Classical swine fever virus (CSFV) achieves this by degrading IRF-3 through the action of the viral protease N^{Pro} (La Rocca *et al*., 2005). West Nile virus – NY can block the early host response mediated by RIG-I (Fredericksen and Gale, 2006). The NS2A protein of the Kunjin subtype of the same virus can block IFN induction, an activity that is abolished by the substitution of a single amino acid of the protein. A virus carrying the NS2A mutation can replicate as well as the wild-type virus in cells that cannot produce IFN but not in IFN-competent cells. The mutant virus is attenuated not only in wild-type mice but also in IFN-α/β receptor-null mice indicating that IFN-independent antiviral pathways are also blocked by NS2A (Liu *et al*., 2006). Ebola virus VP35 protein can block RIG-I signaling probably by binding dsRNA (Cardenas *et al*., 2006). A virus encoding a mutant VP35 that cannot block IRF-3 activation is attenuated in cell culture (Hartman *et al*., 2006).

Rotavirus NSP1 protein binds to IRF-3 using its own C-terminal domain and induces rapid degradation of IRF-3 by a proteasomal pathway.

Silencing of NSP1 blocks viral spreading (Barro and Patton, 2005). Among the picornaviruses, hepatitis A virus can block both TLR-3 and RIG-I-mediated signaling pathways (Fensterl *et al.*, 2005). The leader proteinase, L^{Pro}, of foot-and-mouth disease virus can block induction of IFN and VSIGs by virus infection (de Los Santos *et al.*, 2006); rhinovirus-14 can also block IRF-3 activation and IFN production. For the prototypic arenavirus, lymphocytic choriomeningitis virus, the nucleoprotein is responsible for blocking the above processes (Martinez-Sobrido *et al.*, 2006). Among the orthomyxoviruses, thogoto virus ML protein blocks a nuclear function of IRF-3 (Jennings *et al.*, 2005). For influenza viruses, the NS1 protein appears to affect the efficacy of IFN production although the underlying mechanism remains unclear (Donelan *et al.*, 2004; Fernandez-Sesma *et al.*, 2006; Marcus *et al.*, 2005).

The DNA viruses remain to be investigated more rigorously for their ability to block IFN synthesis. Hepatitis B virus ORF-C product and the viral terminal protein and human papilloma virus E6 protein can block IFN syntheses (Biron and Sen, 2001). Vaccinia virus A46R protein can bind to the TIR domains of TLRs and TLR adaptor proteins, such as MyD88, MAL, TRIF, TRAM, and TLR-4, and block their functions (Stack *et al.*, 2005). HHV8 RTA protein, a ubiquitous E3 ligase, targets IRF7 for degradation (Yu *et al.*, 2005), and ORF45 protein blocks IRF7 activation (Zhu *et al.*, 2002). Human CMV IE86 protein can block IFN induction (Taylor and Bresnahan, 2005) and Rhesus CMV can block IRF-3 activation very efficiently using a virion-associated factor (DeFilippis and Fruh, 2005).

B. Inhibition of IFN signaling

The Jak-STAT signaling pathways activated by IFNs are often the targets of inhibition by viral gene products. Consequently, the second wave of VSIG induction mediated through IFN is negatively regulated. Many poxviruses encode secreted proteins that mimic IFN-receptors and neutralize circulating IFNs. Among other DNA viruses, Adenovirus EIA protein, EBV EBNA1 protein, and Polyoma virus large T protein block the Jak-STAT signaling pathways by interacting with one or more of the signaling proteins (Biron and Sen, 2001). The murine gamma herpesvirus 68 M2 protein downregulates the expression of both STAT1 and STAT2 proteins and thus affects IFN signaling (Liang *et al.*, 2004). Pseudorabies virus infection can differentially downregulate the induction of specific VSIGs by affecting the level of STAT1 phosphorylation (Brukman and Enquist, 2006). For human cytomegalovirus (CMV), blockage is achieved through the interaction of the viral IE1 protein with activated STAT1 and STAT2 in the nucleus (Paulus *et al.*, 2006).

HCV core protein can bind to STAT1 and block its phosphorylation and dimerization (Lin *et al.*, 2006). The nonstructural proteins of West Nile virus,

on the other hand, block Jak1 and Tyk2, the Janus kinases needed for IFN signaling (Guo *et al.*, 2005; Liu *et al.*, 2005). Dengue virus nonstructural proteins reduce the levels of STAT2 (Jones *et al.*, 2005), whereas NS5 protein of the tick-borne encephalitis virus blocks Jak1 and Tyk2 phosphorylation (Best *et al.*, 2005). Paramyxoviruses have been extensively studied for their ability to knockdown IFN signaling (Horvath, 2004). Their ''V'' proteins bind to STATs and often target them for degradation. Different paramyxoviral V proteins target different STAT proteins, but they all assemble large multi-protein complexes containing the STAT proteins and the V protein. The rabies virus ''P'' protein uses a different strategy for blocking IFN signaling; it binds to activated STAT1 and STAT2 and retains them in the cytoplasm (Brzozka *et al.*, 2006).

Among the proteins encoded by VSIGs, PKR is a common target of inhibition by viruses (reviewed in Gale and Katze, 1998). A variety of viral RNAs and proteins can block the activation or the action of PKR. Although extensive *in vitro* and cell-based investigations have determined the underlying mechanisms in most cases, the impact of PKR evasion on virus replication *in vivo* and the consequent effects on pathogenesis have not been studied to the same extent. One general strategy exploited by several viruses is to block PKR activation by dsRNA, through the action of specific viral RNAs that can compete with authentic dsRNA for binding to PKR. Adenovirus VAI RNA, EBV EBER RNA, and HIV-1 TAR RNA can all bind to PKR, but they do not activate it. Other viral proteins can bind RNA and sequester dsRNA from PKR. Reovirus sigma 3 protein, vaccinia virus E3L protein, and influenza virus NS1 protein fall in this category. However, a closer examination of influenza A virus NS1 protein has revealed that its RNA-binding property is not required for its ability to block PKR activation. A mutant NS1 protein, that is defective in RNA binding, can still inhibit PKR activation by either dsRNA or PACT. This inhibitory property is dependent on the ability of NS1 to directly interact with PKR (Li *et al.*, 2006a). When cells are infected with an influenza virus encoding a mutant NS1 that is defective in PKR interaction, but not RNA binding, PKR is strongly activated and consequently protein synthesis is inhibited (Min, Li, Sen and Krug, unpublished observation). The HSV-1 protein US11, another RNA-binding protein, can also block PKR activation, and like influenza virus NS1 protein, the blocking action of US11 is mediated by direct PKR binding, not by RNA binding (Peters *et al.*, 2002). Another HSV-1 protein, γ34.5, blocks PKR action by promoting de-phosphorylation of eIF-2, the substrate of PKR (He *et al.*, 1997). A mutant virus, lacking the corresponding gene, replicates poorly in mice and is less pathogenic compared to the wild-type virus. In contrast, in Pkr−/− mice, both the wild-type and the mutant viruses are equally pathogenic (Leib *et al.*, 2000). HCMV TRS1 and IRS1 proteins can block PKR (Cassady, 2005; Child

et al., 2004). Although TRS1 can bind RNA, the effect on PKR is independent of that property; TRS1 binds to PKR and translocates it to the nucleus from the cytoplasm, thus sequestering it from both its activator and its substrate (Hakki *et al.*, 2006). In addition to E3L, vaccinia virus encodes another PKR-inhibitory protein, K3L, which functions as a decoy of eIF-2. Poliovirus and HIV-1 promotes degradation of PKR, whereas HCV NS5A and the influenza virus-activated cellular protein P58IPK blocks PKR activation probably by blocking its dimerization. The above examples of novel mechanisms by which different viruses evade PKR activation and action underscore the importance of this enzyme in determining the outcome of host response to virus infection.

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REFERENCES

Abraham, N., Stojdl, D. F., Duncan, P. I., Methot, N., Ishii, T., Dube, M., Vanderhyden, B. C., Atkins, H. L., Gray, D. A., McBurney, M. W., Koromilas, A. E., Brown, E. G., *et al.* (1999). Characterization of transgenic mice with targeted disruption of the catalytic domain of the double-stranded RNA-dependent protein kinase, PKR. *J. Biol. Chem.* **274**(9)**:**5953–5962.

Andrejeva, J., Childs, K. S., Young, D. F., Carlos, T. S., Stock, N., Goodbourn, S., and Randall, R. E. (2004). The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc. Natl. Acad. Sci. USA* **101**(49)**:**17264–17269.

Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**(6857)**:** 732–738.

Barro, M., and Patton, J. T. (2005). Rotavirus nonstructural protein 1 subverts innate immune response by inducing degradation of IFN regulatory factor 3. *Proc. Natl. Acad. Sci. USA* **102**(11)**:**4114–4119.

Bell, J. K., Botos, I., Hall, P. R., Askins, J., Shiloach, J., Segal, D. M., and Davies, D. R. (2005). The molecular structure of the Toll-like receptor 3 ligand-binding domain. *Proc. Natl. Acad. Sci. USA* **102**(31)**:**10976–10980.

Best, S. M., Morris, K. L., Shannon, J. G., Robertson, S. J., Mitzel, D. N., Park, G. S., Boer, E., Wolfinbarger, J. B., and Bloom, M. E. (2005). Inhibition of interferon-stimulated JAK-STAT signaling by a tick-borne flavivirus and identification of NS5 as an interferon antagonist. *J. Virol.* **79**(20)**:**12828–12839.

Bieback, K., Lien, E., Klagge, I. M., Avota, E., Schneider-Schaulies, J., Duprex, W. P., Wagner, H., Kirschning, C. J., Ter Meulen, V., and Schneider-Schaulies, S. (2002). Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. *J. Virol.* **76**(17)**:**8729–8736.

Biron, C., and Sen, G. C. (2001). Interferons and other cytokines. *In* ''Fields Virology'' 4th Ed. (D. Knipe, P. Howley, D. Griffin, R. Lamb, M. Martin, and S. Straus, eds.), Vol. 1, pp. 321–351. Lippincott, Williams & Wilkins, Philadelphia.

Bluyssen, H. A., Vlietstra, R. J., Faber, P. W., Smit, E. M., Hagemeijer, A., and Trapman, J. (1994). Structure, chromosome localization, and regulation of expression of the interferon-regulated mouse Ifi54/Ifi56 gene family. *Genomics* **24**(1)**:**137–148.

Breiman, A., Grandvaux, N., Lin, R., Ottone, C., Akira, S., Yoneyama, M., Fujita, T., Hiscott, J., and Meurs, E. F. (2005). Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. *J. Virol.* **79**(7)**:**3969–3978.

Brukman, A., and Enquist, L. W. (2006). Suppression of the interferon-mediated innate immune response by pseudorabies virus. *J. Virol.* **80**(13)**:**6345–6356.

Brzozka, K., Finke, S., and Conzelmann, K. K. (2006). Inhibition of interferon signaling by rabies virus phosphoprotein P: Activation-dependent binding of STAT1 and STAT2. *J. Virol.* **80**(6)**:**2675–2683.

Cardenas, W. B., Loo, Y. M., Gale, M., Jr., Hartman, A. L., Kimberlin, C. R., Martinez-Sobrido, L., Saphire, E. O., and Basler, C. F. (2006). Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. *J. Virol.* **80**(11)**:**5168–5178.

Cassady, K. A. (2005). Human cytomegalovirus TRS1 and IRS1 gene products block the double-stranded-RNA-activated host protein shutoff response induced by herpes simplex virus type 1 infection. *J. Virol.* **79**(14)**:**8707–8715.

Cheng, G., Zhong, J., and Chisari, F. V. (2006). Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. *Proc. Natl. Acad. Sci. USA* **103**(22)**:**8499–8504.

Child, S. J., Hakki, M., De Niro, K. L., and Geballe, A. P. (2004). Evasion of cellular antiviral responses by human cytomegalovirus TRS1 and IRS1. *J. Virol.* **78**(1)**:**197–205.

Choe, J., Kelker, M. S., and Wilson, I. A. (2005). Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. *Science* **309**(5734)**:**581–585.

Compton, T., Kurt-Jones, E. A., Boehme, K. W., Belko, J., Latz, E., Golenbock, D. T., and Finberg, R. W. (2003). Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. *J. Virol.* **77**(8)**:**4588–4596.

Crozat, K., and Beutler, B. (2004). TLR7: A new sensor of viral infection. *Proc. Natl. Acad. Sci. USA* **101**(18)**:**6835–6836.

D'Andrea, L. D., and Regan, L. (2003). TPR proteins: The versatile helix. *Trends Biochem. Sci* **28**(12)**:**655–662.

Dar, A. C., Dever, T. E., and Sicheri, F. (2005). Higher-order substrate recognition of eIF2alpha by the RNA-dependent protein kinase PKR. *Cell* **122**(6)**:**887–900.

DeFilippis, V., and Fruh, K. (2005). Rhesus cytomegalovirus particles prevent activation of interferon regulatory factor 3. *J. Virol.* **79**(10)**:**6419–6431.

de Los Santos, T., de Avila Botton, S., Weiblen, R., and Grubman, M. J. (2006). The leader proteinase of foot-and-mouth disease virus inhibits the induction of beta interferon mRNA and blocks the host innate immune response. *J. Virol.* **80**(4)**:**1906–1914.

Der, S. D., Zhou, A., Williams, B. R., and Silverman, R. H. (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* **95**(26)**:**15623–15628.

de Veer, M. J., Sim, H., Whisstock, J. C., Devenish, R. J., and Ralph, S. J. (Veer 1998). IFI60/ISG60/IFIT4, a new member of the human IFI54/IFIT2 family of interferon-stimulated genes. *Genomics* **54**(2)**:**267–277.

Dey, M., Cao, C., Dar, A. C., Tamura, T., Ozato, K., Sicheri, F., and Dever, T. E. (2005). Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell* **122**(6)**:**901–913.

Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**(5663)**:**1529–1531.

Donelan, N. R., Dauber, B., Wang, X., Basler, C. F., Wolff, T., and Garcia-Sastre, A. (2004). The N- and C-terminal domains of the NS1 protein of influenza B virus can independently inhibit IRF-3 and beta interferon promoter activation. *J. Virol.* **78**(21)**:**11574–11582.

Elco, C. P., Guenther, J. M., Williams, B. R., and Sen, G. C. (2005). Analysis of genes induced by Sendai virus infection of mutant cell lines reveals essential roles of interferon regulatory factor 3, NF-kappaB, and interferon but not toll-like receptor 3. *J. Virol.* **79**(7)**:**3920–3929.

Fensterl, V., Grotheer, D., Berk, I., Schlemminger, S., Vallbracht, A., and Dotzauer, A. (2005). Hepatitis A virus suppresses RIG-I-mediated IRF-3 activation to block induction of beta interferon. *J. Virol.* **79**(17)**:**10968–10977.

Fernandez-Sesma, A., Marukian, S., Ebersole, B. J., Kaminski, D., Park, M. S., Yuen, T., Sealfon, S. C., Garcia-Sastre, A., and Moran, T. M. (2006). Influenza virus evades innate and adaptive immunity via the NS1 protein. *J. Virol.* **80**(13)**:**6295–6304.

Ferreon, J. C., Ferreon, A. C., Li, K., and Lemon, S. M. (2005). Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3/4A protease. *J. Biol. Chem.* **280**(21)**:** 20483–20492.

Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., and Maniatis, T. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol.* **4**(5)**:**491–496.

Foy, E., Li, K., Sumpter, R., Jr., Loo, Y. M., Johnson, C. L., Wang, C., Fish, P. M., Yoneyama, M., Fujita, T., Lemon, S. M., and Gale, M., Jr. (2005). Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proc. Natl. Acad. Sci. USA* **102**(8)**:**2986–2991.

Fredericksen, B. L., and Gale, M., Jr. (2006). West Nile virus evades activation of interferon regulatory factor 3 through RIG-I-dependent and -independent pathways without antagonizing host defense signaling. *J. Virol.* **80**(6)**:**2913–2923.

Freundt, E. C., and Lenardo, M. J. (2005). Interfering with interferons: Hepatitis C virus counters innate immunity. *Proc. Natl. Acad. Sci. USA* **102**(49)**:**17539–17540.

Galabru, J., and Hovanessian, A. (1987). Autophosphorylation of the protein kinase dependent on double-stranded RNA. *J. Biol. Chem.* **262**(32)**:**15538–15544.

Gale, M., Jr., and Katze, M. G. (1998). Molecular mechanisms of interferon resistance mediated by viral-directed inhibition of PKR, the interferon-induced protein kinase. *Pharmacol. Ther.* **78**(1)**:**29–46.

Geiss, G., Jin, G., Guo, J., Bumgarner, R., Katze, M. G., and Sen, G. C. (2001). A comprehensive view of regulation of gene expression by double-stranded RNA-mediated cell signaling. *J. Biol. Chem.* **276**(32)**:**30178–30182.

Ghosh, A., Sarkar, S. N., Rowe, T. M., and Sen, G. C. (2001). A specific isozyme of 2′-5′ oligoadenylate synthetase is a dual function proapoptotic protein of the bcl-2 family. *J. Biol. Chem.* **276**(27)**:**25447–25455.

Green, S. R., and Mathews, M. B. (1992). Two RNA-binding motifs in the double-stranded RNA-activated protein kinase, DAI. *Genes Dev.* **6**(12B)**:**2478–2490.

Guillot, L., Le Goffic, R., Bloch, S., Escriou, N., Akira, S., Chignard, M., and Si-Tahar, M. (2005). Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J. Biol. Chem.* **280**(7)**:**5571–5580.

Guo, J., Peters, K. L., and Sen, G. C. (2000a). Induction of the human protein P56 by interferon, double-stranded RNA, or virus infection. *Virology* **267**(2)**:**209–219.

Guo, J., Hui, D. J., Merrick, W. C., and Sen, G. C. (2000b). A new pathway of translational regulation mediated by eukaryotic initiation factor 3. *EMBO J.* **19**(24)**:**6891–6899.

Guo, J. T., Hayashi, J., and Seeger, C. (2005). West Nile virus inhibits the signal transduction pathway of alpha interferon. *J. Virol.* **79**(3)**:**1343–1350.

Hakki, M., Marshall, E. E., De Niro, K. L., and Geballe, A. P. (2006). Binding and nuclear relocalization of protein kinase R by human cytomegalovirus TRS1. *J. Virol.* **80**(23)**:** 11817–11826.

Hartman, A. L., Dover, J. E., Towner, J. S., and Nichol, S. T. (2006). Reverse genetic generation of recombinant Zaire Ebola viruses containing disrupted IRF-3 inhibitory domains results in attenuated virus growth *in vitro* and higher levels of IRF-3 activation without inhibiting viral transcription or replication. *J. Virol.* **80**(13)**:**6430–6440.

Hartmann, R., Rebouillat, D., Justesen, J., Sen, G. C., and Williams, B. R. (2001). The P59 oligoadenylate synthetase like protein (P59OASL) does not display oligoadenylate synthetase activity but posses anti-viral properties conferred by an ubiquitin-like domain. *J. Interferon Cytokine Res.* **21**(9)**:**S–18.

Hartmann, R., Justesen, J., Sen, G. C., and Yee, V. C. (2002). The crystal structure of 2′-5′ oligoadenylate synthetase provides structural evidence for a common catalytic mechanism for 3′ and 2′ specific nucleotidyl transferases. *J. Interferon Cytokine Res.* **22**(9)**:**S–143.

Hartmann, R., Justesen, J., Sarkar, S. N., Sen, G. C., and Yee, V. C. (2003). Crystal structure of the 2′-specific and double-stranded RNA-activated interferon-induced antiviral protein 2′-5′-oligoadenylate synthetase. *Mol. Cell* **12**(5)**:**1173–1185.

He, B., Gross, M., and Roizman, B. (1997). The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. *Proc. Natl. Acad. Sci. USA* **94**(3)**:**843–848.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature* **408**(6813)**:**740–745.

Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., Goode, J., Lin, P., Mann, N., Mudd, S., Crozat, K., Sovath, S., *et al.* (2003). Identification of Lps2 as a key transducer of MyD88-independent TIR signaling. *Nature* **424**(6950)**:**743–748.

Hofmann, K., and Bucher, P. (1998). The PCI domain: A common theme in three multiprotein complexes. *Trends Biochem. Sci* **23**(6)**:**204–205.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S., and Hartmann, G. (2006). 5′-Triphosphate RNA is the ligand for RIG-I. *Science* **314**(5801)**:**994–997.

Horvath, C. M. (2004). Silencing STATs: Lessons from paramyxovirus interferon evasion. *Cytokine Growth Factor Rev.* **15**(2–3)**:**117–127.

Hovanessian, A. G. (1991). Interferon-induced and double-stranded RNA-activated enzymes: A specific protein kinase and 2′,5′-oligoadenylate synthetases. *J. Interferon Res.* **11**(4)**:**199–205.

Huang, X., Hutchins, B., and Patel, R. C. (2002). The C-terminal, third conserved motif of the protein activator PACT plays an essential role in the activation of double-stranded-RNA-dependent protein kinase (PKR). *Biochem. J.* **366**(Pt. 1)**:**175–186.

Hui, D. J., Bhasker, C. R., Merrick, W. C., and Sen, G. C. (2003). Viral stress-inducible protein p56 inhibits translation by blocking the interaction of eIF3 with the ternary complex eIF2. GTP.Met-tRNAi. *J. Biol. Chem.* **278**(41)**:**39477–39482.

Hui, D. J., Terenzi, F., Merrick, W. C., and Sen, G. C. (2005). Mouse p56 blocks a distinct function of eukaryotic initiation factor 3 in translation initiation. *J. Biol. Chem.* **280**(5)**:** 3433–3440.

Ito, T., Yang, M., and May, W. S. (1999). RAX, a cellular activator for double-stranded RNA-dependent protein kinase during stress signaling. *J. Biol. Chem.* **274**(22)**:**15427–15432.

Jennings, S., Martinez-Sobrido, L., Garcia-Sastre, A., Weber, F., and Kochs, G. (2005). Thogoto virus ML protein suppresses IRF3 function. *Virology* **331**(1)**:**63–72.

Jones, M., Davidson, A., Hibbert, L., Gruenwald, P., Schlaak, J., Ball, S., Foster, G. R., and Jacobs, M. (2005). Dengue virus inhibits alpha interferon signaling by reducing STAT2 expression. *J. Virol.* **79**(9)**:**5414–5420.

Krug, A., Luker, G. D., Barchet, W., Leib, D. A., Akira, S., and Colonna, M. (2004). Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. *Blood* **103**(4)**:**1433–1437.

La Rocca, S. A., Herbert, R. J., Crooke, H., Drew, T. W., Wileman, T. E., and Powell, P. P. (Rocca 2005). Loss of interferon regulatory factor 3 in cells infected with classical swine fever virus involves the N-terminal protease, Npro. *J. Virol.* **79**(11)**:**7239–7247.

Leib, D. A., Machalek, M. A., Williams, B. R., Silverman, R. H., and Virgin, H. W. (2000). Specific phenotypic restoration of an attenuated virus by knockout of a host resistance gene. *Proc. Natl. Acad. Sci. USA* **97**(11)**:**6097–6101.

Lengyel, P. (1987). Double-stranded RNA and interferon action. *J. Interferon Res* **7**(5)**:**511–519.

Li, S., Min, J. Y., Krug, R. M., and Sen, G. C. (2006a). Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology* **349**(1)**:**13–21.

Li, S., Peters, G. A., Ding, K., Zhang, X., Qin, J., and Sen, G. C. (2006b). Molecular basis for PKR activation by PACT or dsRNA. *Proc. Natl. Acad. Sci. USA* **103**(26)**:**10005–10010.

Li, K., Foy, E., Ferreon, J. C., Nakamura, M., Ferreon, A. C., Ikeda, M., Ray, S. C., Gale, M., Jr., and Lemon, S. M. (2005a). Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. USA* **102**(8)**:**2992–2997.

Li, X. D., Sun, L., Seth, R. B., Pineda, G., and Chen, Z. J. (2005b). Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc. Natl. Acad. Sci. USA* **102**(49)**:**17717–17722.

Liang, X., Shin, Y. C., Means, R. E., and Jung, J. U. (2004). Inhibition of interferon-mediated antiviral activity by murine gammaherpesvirus 68 latency-associated M2 protein. *J. Virol.* **78**(22)**:**12416–12427.

Lin, W., Kim, S. S., Yeung, E., Kamegaya, Y., Blackard, J. T., Kim, K. A., Holtzman, M. J., and Chung, R. T. (2006). Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. *J. Virol.* **80**(18)**:**9226–9235.

Liu, W. J., Wang, X. J., Mokhonov, V. V., Shi, P. Y., Randall, R., and Khromykh, A. A. (2005). Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. *J. Virol.* **79**(3)**:**1934–1942.

Liu, W. J., Wang, X. J., Clark, D. C., Lobigs, M., Hall, R. A., and Khromykh, A. A. (2006). A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. *J. Virol.* **80**(5)**:**2396–2404.

Loo, Y. M., Owen, D. M., Li, K., Erickson, A. K., Johnson, C. L., Fish, P. M., Carney, D. S., Wang, T., Ishida, H., Yoneyama, M., Fujita, T., Saito, T., *et al.* (2006). Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. *Proc. Natl. Acad. Sci. USA* **103**(15)**:**6001–6006.

Lund, J. M., Alexopoulou, L., Sato, A., Karow, M., Adams, N. C., Gale, N. W., Iwasaki, A., and Flavell, R. A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* **101**(15)**:**5598–5603.

Marchetti, A., Buttitta, F., Miyazaki, S., Gallahan, D., Smith, G. H., and Callahan, R. (1995). Int-6, a highly conserved, widely expressed gene, is mutated by mouse mammary tumor virus in mammary preneoplasia. *J. Virol.* **69**(3)**:**1932–1938.

Marcus, P. I., Rojek, J. M., and Sekellick, M. J. (2005). Interferon induction and/or production and its suppression by influenza A viruses. *J. Virol.* **79**(5)**:**2880–2890.

Marie, I., Svab, J., Robert, N., Galabru, J., and Hovanessian, A. G. (1990). Differential expression and distinct structure of 69- and 100-kDa forms of 2–5A synthetase in human cells treated with interferon. *J. Biol. Chem.* **265**(30)**:**18601–18607.

Martinez-Sobrido, L., Zuniga, E. I., Rosario, D., Garcia-Sastre, A., and de la Torre, J. C. (2006). Inhibition of the type I interferon response by the nucleoprotein of the prototypic arenavirus lymphocytic choriomeningitis virus. *J. Virol.* **80**(18)**:**9192–9199.

Mashimo, T., Lucas, M., Simon-Chazottes, D., Frenkiel, M. P., Montagutelli, X., Ceccaldi, P. E., Deubel, V., Guenet, J. L., and Despres, P. (2002). A nonsense mutation in the gene encoding 2′-5′-oligoadenylate synthetase/L1 isoform is associated with West Nile virus susceptibility in laboratory mice. *Proc. Natl. Acad. Sci. USA* **99**(17)**:**11311–11316.

Micklem, D. R., Adams, J., Grunert, S., and St. Johnston, D. (2000). Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation. *EMBO J.* **19**(6)**:**1366–1377.

Meurs, E., Chong, K., Galabru, J., Thomas, N. S., Kerr, I. M., Williams, B. R., and Hovanessian, A. G. (1990). Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* **62**(2)**:**379–390.

Nanduri, S., Carpick, B. W., Yang, Y., Williams, B. R., and Qin, J. (1998). Structure of the double-stranded RNA-binding domain of the protein kinase PKR reveals the molecular basis of its dsRNA-mediated activation. *EMBO J.* **17**(18)**:**5458–5465.

Nanduri, S., Rahman, F., Williams, B. R., and Qin, J. (2000). A dynamically tuned double-stranded RNA binding mechanism for the activation of antiviral kinase PKR. *EMBO J.* **19**(20)**:**5567–5574.

Niikura, T., Hirata, R., and Weil, S. C. (1997). A novel interferon-inducible gene expressed during myeloid differentiation. *Blood Cells Mol. Dis* **23**(3)**:**337–349.

Patel, C. V., Handy, I., Goldsmith, T., and Patel, R. C. (2000). PACT, a stress-modulated cellular activator of interferon-induced double-stranded RNA-activated protein kinase, PKR. *J. Biol. Chem.* **275**(48)**:**37993–37998.

Patel, R. C., and Sen, G. C. (1992). Identification of the double-stranded RNA-binding domain of the human interferon-inducible protein kinase. *J. Biol. Chem.* **267**(11)**:**7671–7676.

Patel, R. C., and Sen, G. C. (1998). PACT, a protein activator of the interferon-induced protein kinase, PKR. *EMBO J.* **17**(15)**:**4379–4390.

Patel, R. C., Stanton, P., McMillan, N. M., Williams, B. R., and Sen, G. C. (1995). The interferon-inducible double-stranded RNA-activated protein kinase self-associates *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA* **92**(18)**:**8283–8287.

Patel, R. C., Stanton, P., and Sen, G. C. (1996). Specific mutations near the amino terminus of double-stranded RNA-dependent protein kinase (PKR) differentially affect its double-stranded RNA binding and dimerization properties. *J. Biol. Chem.* **271**(41)**:**25657–25663.

Paulus, C., Krauss, S., and Nevels, M. (2006). A human cytomegalovirus antagonist of type I IFN-dependent signal transducer and activator of transcription signaling. *Proc. Natl. Acad. Sci. USA* **103**(10)**:**3840–3845.

Peters, G. A., Hartmann, R., Qin, J., and Sen, G. C. (2001). Modular structure of PACT: Distinct domains for binding and activating PKR. *Mol. Cell Biol.* **21**(6)**:**1908–1920.

Peters, G. A., Khoo, D., Mohr, I., and Sen, G. C. (2002). Inhibition of PACT-mediated activation of PKR by the herpes simplex virus type 1 Us11 protein. *J. Virol.* **76**(21)**:**11054–11064.

Peters, G. A., Li, S., and Sen, G. C. (2006). Phosphorylation of specific serine residues in the PKR activation domain of PACT is essential for its ability to mediate apoptosis. *J. Biol. Chem.* **281**(46)**:**35129–35136.

Pichlmair, A., Schulz, O., Tan, C. P., Naslund, T. I., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2006). RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* **314**(5801)**:**997–1001.

Polack, F. P., Irusta, P. M., Hoffman, S. J., Schiatti, M. P., Melendi, G. A., Delgado, M. F., Laham, F. R., Thumar, B., Hendry, R. M., Melero, J. A., Karron, R. A., Collins, P. L., *et al.* (2005). The cysteine-rich region of respiratory syncytial virus attachment protein inhibits innate immunity elicited by the virus and endotoxin. *Proc. Natl. Acad. Sci. USA* **102**(25)**:** 8996–9001.

Rebouillat, D., Hovnanian, A., Marie, I., and Hovanessian, A. G. (1999). The 100-kDa 2′, 5′-oligoadenylate synthetase catalyzing preferentially the synthesis of dimeric pppA2′p5′A molecules is composed of three homologous domains [In Process Citation]. *J. Biol. Chem.* **274**(3)**:**1557–1565.

Rowe, T. M., Rizzi, M., Hirose, K., Peters, G. A., and Sen, G. C. (2006). A role of the double-stranded RNA-binding protein PACT in mouse ear development and hearing. *Proc. Natl. Acad. Sci. USA* **103**(15)**:**5823–5828.

Samuel, C. E. (1993). The eIF-2 alpha protein kinases, regulators of translation in eukaryotes from yeasts to humans. *J. Biol. Chem.* **268**(11)**:**7603–7606.

Sarkar, S. N., and Sen, G. C. (2004). Novel functions of proteins encoded by viral stress-inducible genes. *Pharmacol. Ther.* **103**(3)**:**245–259.

Sarkar, S. N., Bandyopadhyay, S., Ghosh, A., and Sen, G. C. (1999a). Enzymatic characteristics of recombinant medium isozyme of 2′-5′ oligoadenylate synthetase. *J. Biol. Chem.* **274**(3)**:**1848–1855.

Sarkar, S. N., Ghosh, A., Wang, H. W., Sung, S. S., and Sen, G. C. (1999b). The nature of the catalytic domain of 2′-5′-oligoadenylate synthetases. *J. Biol. Chem.* **274**(36)**:**25535–25542.

Sarkar, S. N., Miyagi, M., Crabb, J. W., and Sen, G. C. (2002a). Identification of the substrate-binding sites of 2′-5′-oligoadenylate synthetase. *J. Biol. Chem.* **277**(27)**:**24321–24330.

Sarkar, S. N., Pal, S., and Sen, G. C. (2002b). Crisscross enzymatic reaction between the two molecules in the active dimeric P69 form of the 2′-5′ oligodenylate synthetase. *J. Biol. Chem.* **277**(47)**:**44760–44764.

Sarkar, S. N., Smith, H. L., Rowe, T. M., and Sen, G. C. (2003). Double-stranded RNA signaling by toll-like receptor 3 requires specific tyrosine residues in its cytoplasmic domain. *J. Biol. Chem.* **278**(7)**:**4393–4396.

Sarkar, S. N., Peters, K. L., Elco, C. P., Sakamoto, S., Pal, S., and Sen, G. C. (2004). Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. *Nat. Struct. Mol. Biol* **11**(11)**:**1060–1067.

Sarkar, S. N., Elco, C. P., Peters, K. L., Chattopadhyay, S., and Sen, G. C. (2007). Two tyrosine residues of toll-like receptor 3 trigger different steps of NF-kappa B activation. *J. Biol. Chem.* **282**(6)**:**3423–3427.

Schlender, J., Hornung, V., Finke, S., Gunthner-Biller, M., Marozin, S., Brzozka, K., Moghim, S., Endres, S., Hartmann, G., and Conzelmann, K. K. (2005). Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. *J. Virol.* **79**(9)**:** 5507–5515.

Shaw, M. L., Cardenas, W. B., Zamarin, D., Palese, P., and Basler, C. F. (2005). Nuclear localization of the Nipah virus W protein allows for inhibition of both virus- and toll-like receptor 3-triggered signaling pathways. *J. Virol.* **79**(10)**:**6078–6088.

Silverman, R. H., and Cirino, N. M. (1997). mRNA metabolism and post-translational gene regulation. *In* ''Gene Regulation'' (D. R. Morris and J. B. Hartford, eds.), pp. 295–309. Wiley & Sons, New York.

Spann, K. M., Tran, K. C., and Collins, P. L. (2005). Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. *J. Virol.* **79**(9)**:**5353–5362.

St Johnston, D., Driever, W., Berleth, T., Richstein, S., and Nusslein-Volhard, C. (1989). Multiple steps in the localization of bicoid RNA to the anterior pole of the *Drosophila* oocyte. *Development* **107**(Suppl.)**:**13–19.

St Johnston, D., Beuchle, D., and Nusslein-Volhard, C. (1991). Staufen, a gene required to localize maternal RNAs in the *Drosophila* egg. *Cell* **66**(1)**:**51–63.

Stack, J., Haga, I. R., Schroder, M., Bartlett, N. W., Maloney, G., Reading, P. C., Fitzgerald, K. A., Smith, G. L., and Bowie, A. G. (2005). Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. *J. Exp. Med* **201**(6)**:**1007–1018.

Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J., Alexopoulou, L., Flavell, R. A., *et al.* (2004). Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA* **101**(10)**:**3516–3521.

Taylor, R. T., and Bresnahan, W. A. (2005). Human cytomegalovirus immediate-early 2 gene expression blocks virus-induced beta interferon production. *J. Virol.* **79**(6)**:**3873–3877.

Terenzi, F., Pal, S., and Sen, G. C. (2005). Induction and mode of action of the viral stress-inducible murine proteins, P56 and P54. *Virology* **340**(1)**:**116–124.

Terenzi, F., Hui, D. J., Merrick, W. C., and Sen, G. C. (2006). Distinct induction patterns and functions of two closely related interferon-inducible human genes, ISG54 and ISG56. *J. Biol. Chem.* **281**(45)**:**34064–34071.

Wacher, C., Muller, M., Hofer, M. J., Getts, D. R., Zabaras, R., Ousman, S. S., Terenzi, F., Sen, G. C., King, N. J., and Campbell, I. L. (2007). Coordinated regulation and widespread cellular expression of interferon-stimulated genes (ISG) ISG-49, ISG-54, and ISG-56 in the central nervous system after infection with distinct viruses. *J. Virol.* **81**(2)**:**860–871.

Wang, C., Pflugheber, J., Sumpter, R., Jr., Sodora, D. L., Hui, D., Sen, G. C., and Gale, M., Jr. (2003). Alpha interferon induces distinct translational control programs to suppress hepatitis C virus RNA replication. *J. Virol.* **77**(7)**:**3898–3912.

Wathelet, M. G., Clauss, I. M., Content, J., and Huez, G. A. (1988). The IFI-56K and IFI-54K interferon-inducible human genes belong to the same gene family. *FEBS Lett* **231**(1)**:** 164–171.

Williams, B. R. (2001). Signal integration via PKR. *Sci STKE* **2001**(89)**:**RE2.

Yang, Y. L., Reis, L. F., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B. R., Aguet, M., and Weissmann, C. (1995). Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J.* **14**(24)**:**6095–6106.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* **5**(7)**:**730–737.

Yu, M., Tong, J. H., Mao, M., Kan, L. X., Liu, M. M., Sun, Y. W., Fu, G., Jing, Y. K., Yu, L., Lepaslier, D., Lanotte, M., Wang, Z. Y., *et al.* (1997). Cloning of a gene (RIG-G) associated with retinoic acid-induced differentiation of acute promyelocytic leukemia cells and representing a new member of a family of interferon-stimulated genes. *Proc. Natl. Acad. Sci. USA* **94**(14)**:**7406–7411.

Yu, Y., Wang, S. E., and Hayward, G. S. (2005). The KSHV immediate-early transcription factor RTA encodes ubiquitin E3 ligase activity that targets IRF7 for proteosome-mediated degradation. *Immunity* **22**(1)**:**59–70.

Zhu, F. X., King, S. M., Smith, E. J., Levy, D. E., and Yuan, Y. (2002). A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation. *Proc. Natl. Acad. Sci. USA* **99**(8)**:**5573–5578.

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