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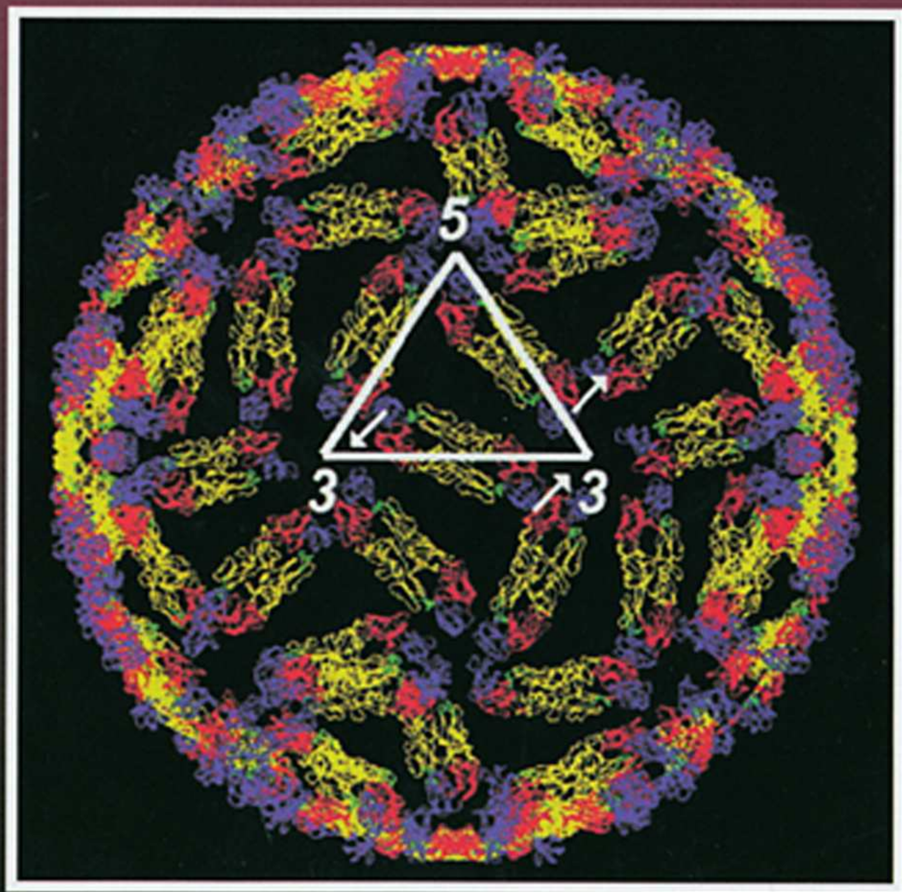
Volume 59

THE FLAVIVIRUSES:

STRUCTURE, REPLICATION AND EVOLUTION



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VOLUME 59

The Flaviviruses: Structure, Replication, and Evolution

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CONTENTS

Taxonomy of the Virus Family *Flaviviridae*

CHARLES H. CALISHER AND ERNEST A. GOULD

I.	Historical Perspective	1
II.	Early Classification	3
III.	Toward an Adequate Taxonomy	5
IV.	Current Taxonomic Placement of Flaviviruses	7
	References	17

STRUCTURE AND REPLICATION 21

Molecular Biology of Flaviviruses

BRETT D. LINDENBACH AND CHARLES M. RICE

I.	Introduction	23
II.	Viral Life Cycle	23
III.	Flavivirus Particles	25
IV.	Genome Structure	28
V.	Virus Proteins	32
VI.	RNA Replication	45
	References	47

Flavivirus Structure and Membrane Fusion

FRANZ X. HEINZ AND STEVEN L. ALLISON

	Preamble	63
I.	Introduction	63
II.	Assembly and Maturation	66
III.	Structure of the E Protein	69
IV.	Architecture of Recombinant Subviral Particles and Virions	72
V.	Fusion Characteristics	74
VI.	Low pH-Induced Structural Changes	75
VII.	Interactions with Membranes	77
VIII.	Function of the prM Protein	80
IX.	Comparative Aspects of the Flavivirus Fusion Machinery	81
	References	84
	Addendum	91
	References for Addendum	97

Kunjin RNA Replication and Applications of Kunjin Replicons

Edwin G. Westaway, Jason M. Mackenzie, and Alexander A. Khromykh

I. Introduction 100
II. Foundations for Developing a Model of KUNV RNA Synthesis 101
III. Extending the Scope of Replication Analyses 103
IV. Ultrastructure of Induced Membranes, Their Association with the Replication Complex (RC), and Their Origins 107
V. Molecular Approaches to Study Formation and Operation of the RC 117
VI. Development of the Kunjin Replicon Expression System for Vaccines and Gene Therapy 129
VII. Concluding Remarks 133
References 134

Antigenic Structure of Flavivirus Proteins

John T. Roehrig

I. Introduction 141
II. Classical Flavivirus Serology 142
III. B-Cell Epitopes 143
IV. T-Cell Epitopes 159
V. Conclusion 163
References 164

5′- and 3′-Noncoding Regions in Flavivirus RNA

Lewis Markoff

I. Introduction 177
II. Conserved Linear Sequence Features of NCRs in Flavivirus RNA 181
III. Secondary Structure of NCRs 203
IV. Mutational Changes in the 5′ or 3′ NCR and the 3′ SL May Result in Attenuation of Virus Virulence 214
V. Hypothesis for Hydrogen Bonding between 5′ and 3′ NCRs 215
VI. Additional Secondary Structure Predictions in the 3′ NCR 216
VII. Summary and Future Directions 219
References 223

Manipulation of Cell Surface Macromolecules by Flaviviruses

Robert Anderson

I. Introduction . . . 230
II. The Flavivirus Receptor Binding Protein . . . 230
III. Cell Targets for Flaviviruses . . . 232
IV. Cell Surface Macromolecules Involved in Flavivirus Attachment . . . 242
V. Cell Surface Macromolecules Modulated by Flavivirus Infection . . . 249
VI. Other Cell Surface Macromolecular Modifications Triggered by Flavivirus Infection . . . 253
VII. Conclusions . . . 255
References . . . 255

FLAVIVIRUS EVOLUTION . . . 275

Origins, Evolution, and Vector/Host Coadaptations Within the Genus *Flavivirus*

Ernest A. Gould, Xavier de Lamballerie, Pablo M. de A Zanotto, and Edward C. Holmes

I. Introduction . . . 278
II. Phylogenetic Analysis of the Genus *Flavivirus* . . . 281
III. Impact of Host/Virus Coadaptation on Flavivirus Evolution . . . 285
IV. What Are the Origins of Flaviviruses? . . . 304
V. Does Recombination Occur between Flaviviruses and, if so, What Is the Likely Impact? . . . 306
References . . . 308

Microevolution and Virulence of Dengue Viruses

Rebeca Rico-Hesse

I. Introduction . . . 316
II. Genetic Variation within Dengue Serotypes . . . 317
III. Phylogenetics of Dengue Viruses . . . 318
IV. Viral Determinants of Disease . . . 331
V. Viral Determinants of Transmission . . . 334
VI. Viral Displacement and Epidemiology . . . 335
References . . . 336

Index . . . 343

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PREFACE TO THE FLAVIVIRUSES

The Flavivirus family continues to provide great fascination for virologists, immunologists, entomologists, epidemiologists, and scientists in various other disciplines. Research during the past few decades has yielded considerable progress in many of these areas, but there remain a number of challenges surrounding our understanding of the behavior of flaviviruses in natural conditions and in the laboratory. At a time when continued global emergence of flaviviruses calls for the development and improvement of vaccines and antiviral agents, it is appropriate that a broad compendium of knowledge be made available that presents recent conceptual advances and that reviews current information on the many different facets of these viruses. Certainly, there have been some noteworthy scientific achievements. For instance, the molecular details of virus structure have been greatly advanced as a result of high-resolution analysis of the envelope protein and its organization at the level of the virion particle, which, together with functional studies, have revealed the uniqueness of this viral protein during replication and pathogenesis. The characterization of an increasing number of flavivirus strains at the sequence level has led to an improved taxonomic classification of the genus, enhanced our understanding of evolution, geographic variation, and epidemiology, and stimulated research on variation in viral virulence. Use of molecular clone technology has advanced from basic studies that have identified the functions and properties of viral proteins during RNA replication and virus assembly to the evaluation of candidate virulence determinants, engineering of live attenuated vaccines, and related applications.

Studies on the immunobiology of flaviviruses have led to the realization that these viruses interact with the host immune system in ways that differ from other small RNA viruses. The importance of neutralizing antibody responses for immunity continues to be an area of focus, and the basis for this protection at the epitope-specific level is gradually being dissected. However, there remain enigmatic aspects, such as the wide cross-reactivity elicited by these viruses and the phenomenon of antibody-dependent enhancement, both of which have important implications for pathogenesis and vaccine development, and

require better molecular characterization. It is becoming clear that T-cell responses to flavivirus infections also have unusual properties that may contribute to pathogenesis through immunopathologic and/or immune-subverting events. Further characterization of these responses and their relationship to immune protection are avenues of research needed to optimize the use of the increasing range of vaccine modalities that are being pursued.

In conjunction with advances in flavivirus molecular virology and immunology, more and more attention is being directed to the investigation of the pathogenesis of flavivirus diseases. Progress in this area has been heralded by the long-awaited identification of the molecular basis for genetic susceptibility of mice to flaviviruses. This will undoubtedly increase interest in the role of innate defenses in these infections and promote research into the genetic basis of flavivirus susceptibility in humans. Together with the use of modern techniques to identify critical target cells of infection, research in this area will expand our understanding of the cellular and molecular basis for flavivirus tropism. In this regard, the cell-surface molecules that interact with these viruses during entry have yet to be fully characterized, but progress continues to be made on this front. It remains somewhat frustrating that suitable animal models for some flavivirus diseases, particularly dengue hemorrhagic fever, are not available. However, data accumulated from human clinical studies are yielding insight into the pathogenesis of this disease, and similar studies with other pathogenic flaviviruses are anticipated in the future.

The interactions between flaviviruses and their arthropod hosts have been the subject of many classical studies that have now progressed to the molecular level as well. There are many secrets to these interactions that must be discovered to understand the process of virus persistence in molecular terms. These will be forthcoming with the use of modern technologies by creative investigators interested in vector biology. The improvement in molecular technologies has had concomitant impact on the ability to conduct molecular epidemiology at the "macro" and "micro" levels. In response to progressive emergence in recent years of dengue, Japanese encephalitis, West Nile, and tick-borne viruses, the application of such technologies for detection and surveillance in arthropod and vertebrate reservoirs has provided insight into the factors that support the global movements of flaviviruses. Yet, there is a tremendous amount of such data concerning virus evolution in the natural environment that is still needed to understand this process and possibly predict future

trends. Additional molecular studies of these viruses as they are transmitted among vectors, reservoirs, and humans are needed to further our conceptual understanding of virus emergence.

The development of vaccines for flaviviruses has also benefited greatly from the availability of modern technologies, and new as well as next-generation vaccines for some viruses are on the horizon. As a better understanding of the immune responses to these viruses in the context of disease as well as vaccine-induced protection becomes available, the ability to control the growing worldwide burden of disease from these agents will likely be improved.

Clearly a comprehensive research approach in many scientific disciplines is needed to unravel the complexities of the virus-host interactions that these viruses have had the benefit of manipulating for centuries. In this three-volume edition on the flaviviruses, our goal has been to assemble a base of knowledge that encompasses these complexities, describes technologies that have contributed to this knowledge, and identifies the major problems faced in attempting to further understand the virus-host interactions that result in disease, and in using vaccine strategies for preventing them. We are grateful to the many contributors who have generously assisted in the preparation of this book series. We must also acknowledge that there are many other colleagues who are active in the field whose expertise has not been represented here.

Thomas J. Chambers,
St. Louis, Missouri, 2003

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Cambridge, Massachusetts, 2003

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PREFACE TO VOLUME 59
THE FLAVIVIRUS: STRUCTURE, REPLICATION, AND EVOLUTION

It has been some 18 years since the first genomic sequence of a flavivirus (yellow fever 17D) was reported. The ensuing decades led to a wealth of sequence data for other flaviviruses and fostered many molecular studies, which collectively have established the common structural organization and mode of replication of these viruses and the development of a genome-based taxonomy. Yet the diversity of flaviviruses and their complex ecology and epidemiology introduce various factors that still thwart definitive classification based on genetic relationships alone. Efforts have continued to define the roles of viral proteins and RNA structures in the flavivirus life cycle with the use of novel tools, such as infectious clones, subgenomic replicons, and chimeric viruses. Great progress has been made in understanding the structural and functional properties of the viral envelope (E) protein, in terms of the mechanics of its remarkably efficient fusion activity, the spectrum of antigenic determinants displayed on its surface, and its relationship to neutralization and antibody-mediated enhancement of infection. In contrast, comparatively little is known about the cell surface proteins with which E interacts during virus entry. Many functional activities of the nonstructural proteins have also been identified, and increasing data are accumulating to implicate the untranslated regions of the genome in virus-host interactions that may be relevant for pathogenesis. Ultrastructural imaging and molecular genetic studies provide an ever more-sophisticated model of viral replication and its unusual association with intracellular membranes. However, our understanding of this process is far from complete.

This volume reviews current knowledge of the basic aspects of flavivirus classification, structure, replication, and evolution. Questions that remain for future research include: What new viruses will evolve, and what natural forces and human factors will facilitate their emergence? What are the critical molecules that subserve virus entry, how do they differ among the viruses in this genus, and to what extent do these alone determine virus tropism? What molecular events drive

viral RNA synthesis, including the formation of the replication complex, the regulation of its template preference, and the sequestering of its activity to membrane compartments? What molecular interactions are required among viral structural proteins to drive virus assembly, and what components of the host secretory pathway are involved? Answers to these fundamental questions are expected to provide greater insight into how flaviviruses have achieved such a broad host range and disease potential, and also yield ideas on new strategies for interrupting their replication and spread.

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ADVANCES IN VIRUS RESEARCH, VOL 59

TAXONOMY OF THE VIRUS FAMILY *FLAVIVIRIDAE*

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I. Historical Perspective
II. Early Classification
III. Toward an Adequate Taxonomy
IV. Current Taxonomic Placement of Flaviviruses
References

I. Historical Perspective

In 1848, when the cause of yellow fever was still unknown, Josiah Clark Nott, a physician in Mobile, Alabama, made note of the erratic distribution of yellow fever cases along the Mississippi River during the epidemic of that year. Observing that places not visited by steamboats had been uniformly exempt from the disease, he stated: "A disease may not be contagious in the proper acceptation of the term, that is, communicable from one human body to another like smallpox, and still it does not follow that the germ or *materies morbi* may not be transported from one place to another in a vessel or baggage car, and there be propagated" (cited in Bloom, 1993). Contrary to conventional wisdom of the time, Nott suggested mosquitoes as the possible source of yellow fever virus (YFV). However, it was not until Carlos Finlay, a Havana, Cuba, physician, proposed his more scientific theory of mosquito transmission of this virus in 1881, at a time when most other theories had been disproven, that he was taken seriously (Reed, 1901). This was the first indication that viruses could be transmitted by arthropods, opening the field of arbovirology.

It was not until about 1930 that the laboratory mouse was used as a test animal in virology. At that time Max Theiler, who was later awarded a Nobel prize for devising an effective vaccine against yellow fever, reported that newborn mice inoculated intraperitoneally with YFV died of encephalitis (Theiler, 1930). Shortly thereafter, with the

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discovery of the etiologic agent of Louping ill, a virus distantly related to YFV, it was shown that ticks could also transmit viruses and that these viruses could be isolated in mice (Greig *et al.*, 1931). From this time and for many years after, arboviruses were nearly always isolated in mice. The first classification of these viruses was based on their ability to replicate in and be transmitted by arthropods, but it was later shown that certain viruses related to YFV and Louping ill virus were not transmitted by arthropods.

Five years after Theiler's finding of the utility of mice for arbovirus isolation, Webster and Dawson (1935) reported that laboratory mice were suitable test animals for diagnosing rabies. In ensuing years it was reported that suckling mice could be used as hosts for detecting neutralizing antibodies to eastern equine encephalitis, western equine encephalitis, and Venezuelan equine encephalitis viruses (Lennette and Koprowski, 1944).

An outbreak of encephalitis in Massachusetts in 1938 drew the attention of Webster and his associate Jordi Casals, both of the Rockefeller Institute in New York City. They were interested in comparing the etiologic agent of that outbreak, eastern equine encephalitis virus, with other viruses known to cause human encephalitis, including western equine encephalitis virus, Russian spring–summer encephalitis virus, Louping ill virus, rabies virus, and poliomyelitis virus. Casals, a meticulous and methodical worker, applied to these viruses the complement-fixation (CF) technique taught to him by Jules Freund (the discoverer of the well-known adjuvant that still carries his name), who was studying tuberculosis, and so the torch was passed between generations.

Casals and Webster (1944; Casals, 1944) found that whereas antibodies to rabies, poliomyelitis, and other viruses did not cross-react with the heterologous viruses, antibodies to Russian spring–summer encephalitis and Louping ill viruses did. Casals also detected antigenic cross–reactivity between these two viruses and St. Louis encephalitis, Japanese encephalitis, and Murray Valley encephalitis viruses but noted that antigenic proximity between them varied; i.e., whereas all were related, some were more distantly related and some more closely related one to another (Casals, 1957).

The hemagglutination technique, developed at the Rockefeller laboratories by George Hirst working with influenza A virus (Hirst, 1941), was modified by Casals and Brown (1954) and shown to be useful for studies of arboviruses. Albert Sabin produced hemagglutinins of YFV and dengue viruses and showed by hemagglutination inhibition (HI) with antisera that these viruses were related to each other and, with Edward Buescher, demonstrated that the HI test could

be applied in the same way to other arboviruses (Sabin and Buescher, 1950). When Theiler suggested using HI as a serologic test for evidence of infection by arboviruses, an exquisitely sensitive tool became available not only for diagnosis, at that time the *sine qua non* of serologic testing, but also provided a method for simply and rapidly assessing antigenic similarities as well as differences between arboviruses.

High-titer viruses, antigens, and antibodies are essential for detecting distant antigenic relationships between viruses. Therefore, the discovery by Gilbert Dalldorf and Joseph Melnick in early 1950 that coxsackieviruses replicate to high titer in newborn mice (summarized in Dalldorf and Melnick, 1965) led Casals and others to replace adult mice with newborn mice for studies with arboviruses. By 1951 Casals and co-workers had adapted human poliomyelitis virus type 2 virus to replicate in newborn mice and had developed a CF test to detect it (Casals *et al.*, 1951). Similar applications of newborn mice to studies of arboviruses resulted in shorter incubation periods, higher titers, and more satisfactory polyclonal antibodies. Newborn mice and serologic tests became invaluable tools for studies of the many insect and vertebrate viruses being sent to the New York laboratories by investigators at Rockefeller Institute outposts in Africa, South America, and elsewhere. Casals, Loring Whitman, Robert Shope, and their co-workers were in the unique position of having available not only this plethora of viruses, but superb reagents with which to compare them. They thus began the classical investigations so integral to our knowledge of the interrelationships among arboviruses (Theiler and Downs, 1973).

Casals, Shope, and others began attempts to categorize these scores of viruses and many hundreds of isolates, and from the results Casals formulated three dicta (personal communication to CHC, 1967), paraphrased as follows: "(1) No virus can belong to two antigenic groups. (2) If two viruses cross-react antigenically, they are related. (3) If viruses of different groups cross-react, they do not belong to different groups." These simple premises have never been disproved. Their significance can best be understood in the context of phenotypic expression of genotypes and in terms of phylogeny. However, for many years after, all that was possible was classification, not taxonomy, of arboviruses.

II. Early Classification

By 1960 it was recognized that eastern equine encephalitis, western equine encephalitis, and certain other arboviruses were related to each other. These were categorized as "Group A arboviruses," now known as

the genus *Alphavirus* of the family *Togaviridae*. As YFV, Japanese encephalitis, Murray Valley encephalitis, St. Louis encephalitis, West Nile, Louping ill, Russian spring–summer encephalitis, and other viruses were also known to be interrelated antigenically, they were termed "Group B arboviruses," now known as the genus *Flavivirus* (from Latin *flavus* = "yellow," for yellow fever virus) of the family *Flaviviridae*. Although alphaviruses and flaviviruses initially were considered members of the same family, the *Togaviridae*, (cf. genus *Togavirus*, for what is now the genus *Alphavirus*, and genus *Flavivirus* for what is still the genus *Flavivirus*), it later became clear that differences between them warranted the establishment of two families: *Togaviridae* for the alphaviruses and certain other viruses and *Flaviviridae* for the flaviviruses and certain other viruses. Along with improved recognition of the antigenic relationships of viruses now recognized to be members of the family *Bunyaviridae* or other virus families, some semblance of order was beginning to take place with respect to the classification of arboviruses (reviewed in detail by Theiler and Downs, 1973).

Elegant electron microscopic studies by Frederick Murphy and co-workers demonstrated that all arboviruses placed in a given serologic group also shared similar morphologies and morphogenetic characteristics, providing partial but confirmatory evidence for the earlier antigenic studies and a basis for understanding the pathogenetic mechanisms of these viruses (Murphy *et al.*, 1973; Murphy, 1980).

Inclusive serologic comparisons of flaviviruses by de Madrid and Porterfield (1974), Porterfield (1980), and Calisher *et al.* (1989) extended the original studies done at the Rockefeller Institute. On the basis of cross-reactivity in plaque reduction neutralization tests, and also by taking into account the arthropod host with which each virus was most commonly associated, Porterfield and associates distinguished the flaviviruses as mosquito-borne, tick-borne, and nonvectored viruses within the genus *Flavivirus*. Moreover, it was proposed by Porterfield (1980) that nonarthropod-borne flaviviruses may provide an evolutionary link between arthropod-borne and nonarthropod-borne viruses in the family *Flaviviridae*. Classifications based on phenotypic characteristics are never optimal, as demonstrated by the fact that some principally mosquito-borne flaviviruses have been isolated from ticks and some principally tick-borne flaviviruses have been isolated from mosquitoes.

III. Toward an Adequate Taxonomy

Subsequent studies of flaviviruses with monoclonal antibodies against the envelope protein supported the previously held belief that flaviviruses had evolved from a single ancestral virus (Gould *et al.*, 1985). However, it was not until the appropriate computer programs and molecular biological techniques became widely available that extensive nucleotide sequencing could be exploited to reveal more precise genetic relationships between the viruses. For the first time, a rational basis for flaviviral taxonomy was put in place. Numerous comparative assessments of flaviviral genomes have contributed to our understanding of the flavivirus genome strategy, of the structure and function of its various genes (Rice *et al.*, 1985), and of the phylogenetic relationships among these viruses (Gaunt *et al.*, 2001; Kuno *et al.*, 1998; Marin *et al.*, 1995; Zanotto *et al.*, 1995, 1996). It is worth emphasizing that a good parallel exists between the relationships of these viruses when compared using either phylogenetic or antigenic methods. In neutralization tests, Russian spring–summer encephalitis, Central European encephalitis, Louping ill, Langat, Kyasanur Forest disease, Royal Farm, Karshi, and Powassan viruses showed significant antigenic cross-reactivity, whereas Phnom Penh bat and Carey Island viruses did not cross-react antigenically with the tick-borne encephalitis (TBE) complex viruses (Calisher *et al.*, 1989). These relationships are reflected quite accurately in the phylogenetic tree presented in the chapter by Rico-Hesse. In general, this principle holds for most but not all flaviviruses, i.e., viruses that are phylogenetically closely related are more likely to cross-react in neutralization tests using hyperimmune antisera. More details of the antigenic cross-reactions between these viruses can be found in the International Catalogue of Arboviruses (Karabatsos, 1985). There are one or two notable exceptions to these rules. For example, St. Louis encephalitis virus (SLEV) has always been included in the Japanese encephalitis virus (JEV) antigenic group but is placed phylogenetically among the New World *Culex* spp.–associated viruses (which include Ntaya virus and others) rather than in the JEV clade. Such apparent discrepancies can be attributed partly to the restricted range of cross-neutralization tests that have been performed in the past. However, they largely reflect the imprecision of the neutralization test, which is highly sensitive to virus strain variation and the quality of the antiserum used for the analysis. Neutralization is dependent on antibody binding to a limited number of conformational domains within the viral envelope protein, thus

reflecting a very small portion of the genetic properties of the virus. In contrast, the phylogenetic relationships of these viruses reflect the properties of several entire genes and are therefore more likely to represent the true genetic relationships. Nevertheless, the value of the neutralization test must not be underestimated as, despite its limitations at the finest level of differentiation, it is still proving to be a robust system for primary comparisons.

Details of the complex characteristics and peculiarities of individual flaviviruses are presented elsewhere in this volume, but it can be stated with some assurance that a parallel exists between tree topology and virus classification based on ecological and epidemiological characteristics. Because recombination occurs among the dengue viruses (Holmes *et al.*, 1999; Tolou *et al.*, 2001; Uzcategui *et al.*, 2001; Worobey *et al.*, 1999), it is likely that forthcoming evidence will show that recombination is an accessory evolutionary mechanism of the flaviviruses. Indeed, recombination may have profound evolutionary consequences for many viral populations and adds an extra dimension of complexity to phylogenetic analyses and interpretations of virus dispersal patterns. The impact of recombination on the evolution of virus taxa will be dependent to a large extent on the level of difference between the viruses that recombine. All current evidence is that recombination between flaviviruses occurs only between very closely related viruses. Therefore, from the point of view of phylogenetic analysis, trees for entire genera are basically sound, whereas trees based on analyses only of very closely related viruses may not necessarily reflect their true evolutionary relationships. Appropriate corrections in the tree-building algorithms need to be made to account for this. Despite these qualifications, evidence for recombination between a South American and an Asian genotype dengue 2 virus (Uzcategui *et al.*, 2001) could have important implications for our understanding of dengue 2 virus pathogenicity, even though the parent viruses are closely related. Only Asian genotype dengue viruses have thus far been directly associated with dengue hemorrhagic fever. If recombinants of the type mentioned earlier become established, the introduction of such phenotypes will inevitably lead to different epidemiological patterns and possibly to strains with different pathogenicities. It is also worth noting that among viruses of the genus *Alphavirus*, considerable evidence has been obtained showing that Western equine encephalitis virus is a recombinant of Eastern equine encephalitis virus (EEEV) and a Sindbis-related virus. If, in the future, equivalent recombination events occur between more distantly related flaviviruses, such as different dengue virus serotypes, or West Nile

virus and SLEV, or a dengue virus and YFV, or even more extreme combinations such as a dengue virus and West Nile virus, the resulting phenotypes could display properties significantly different from their parents/progenitors (e.g., having a much wider range of suitable vectors for transmission and dispersal).

IV. Current Taxonomic Placement of Flaviviruses

According to the International Committee on Taxonomy of Viruses, a subgroup of the Division of Virology of the International Union of Microbiology Societies, flaviviruses comprise one of three genera within the virus family *Flaviviridae* (Heinz *et al.*, 2000). The other two are the genera *Pestivirus* (from Latin pestis = "plague") and *Hepacivirus* (from Greek hepar, hepatos = "liver"). Although pestiviruses and hepaciviruses have genome replication strategies similar to that of flaviviruses, they are antigenically distinct from the flaviviruses and are not arthropod–borne and may represent lineages that diverged early in the evolution of the family. An insect virus known as Cell-Fusing Agent had also been placed informally and tentatively in the genus *Flavivirus* on the basis of its similar genome strategy and partial homology with these viruses (Cammissa Parks *et al.*, 1992), but this placement is not clear-cut. Virions of each of these viruses are 40–60 nm in diameter (50 in the case of flaviviruses), are spherical, and contain a lipid envelope. The capsid is composed of a single protein (C), and the envelope contains either two or three virus-encoded membrane proteins. Flaviviruses and hepaciviruses have two membrane-associated proteins, whereas pestiviruses have three. The nonstructural proteins of these viruses contain sequence motifs characteristic of a serine protease, RNA helicase, and RNA-dependent RNA polymerase, encoded at similar locations along the genome in all three genera. The genome of each virus in the family is positive sense ssRNA, for flaviviruses 10.6 kb, for pestiviruses 12.3 kb, and for hepaciviruses 9.6 kb, and these genomes lack a 3′-terminal poly (A) tract; flaviviruses contain a 5′-terminal type I cap. Viruses of this family contain lipids derived from host cell membranes, and virions contain carbohydrates in the form of glycolipids and glycoproteins.

Genomic RNA of viruses of the three genera is similarly organized and is the only mRNA found in infected cells. It contains a single long open reading frame flanked by 5′- and 3′-terminal noncoding regions that form specific secondary stem–loop structures required for genome replication. In flaviviruses, translation initiation is cap–dependent,

TABLE I

Alphabetical List of Flaviviruses by Year, Location, and Source of Initial Isolation; Geographic Distribution; Principal Vector Species; Principal Host Species; and Disease Associations

Virus or serotype	Year of Isolation	Location	Source	Geographic Distribution	Principal Vector Species	Principal Host Species	Human Disease
Alfuy	1956	Australia	*Centropus phasianus*	Australia	Mosquitoes?	Unknown	None
Apoi	1954	Japan	Pooled rodents	Japan	?	Rodents?	Encephalitis
Aroa	1975	Venezuela	Sentinel hamster	Venezuela	Sole source	Unknown	None
Bagaza	1966	Central African Republic	Culex spp.	Africa	Culex spp.	Unknown	Fever
Banzi	1956	South Africa	Human	Africa	*Culex* spp.	Unknown	Fever
Bouboui	1967	Central African Republic	*An. paludis*	Africa	?	?	None
Bussuquara	1956	Brazil	*Alouatta belzebul*	Brazil, Colombia, Panama	*Culex* spp.?	Unknown	Fever
Cacipacore	1977	Brazil	*Formicarius analis*	Brazil	Unknown	Unknown	None
Carey Island	1970	Malaysia	*Cynopterus brachyotis*	Malaysia	?	Bats?	None
(Central European encephalitis)	1951	Russia	Human	Europe	*Ixodes* spp.	Unknown	Encephalitis
Cowbone Ridge	1965	USA	*Sigmodon hispidus*	USA	?	?	None

Dakar bat	1962	Senegal	Pooled bats	Africa	?	Bats?	Fever
Dengue 1	1944	Hawaii	Human	Essentially worldwide	*Aedes aegypti*	Humans	Fever, rash, HF[a]
Dengue 2	1944	New Guinea	Human	Essentially worldwide	*Ae. aegypti*	Humans	Fever, rash, HF
Dengue 3	1957	Philippines	Human	Essentially worldwide	*Ae. aegypti*	Humans	Fever, rash, HF
Dengue 4	1957	Philippines	Human	Essentially worldwide	*Ae. aegypti*	Humans	Fever, rash, HF
Edge Hill	1961	Australia	*Aedes vigilax*	Australia	Mosquitoes	?	None
Gadgets Gully	1977	Australia	*Ixodes uriae*	Australia	Sole source	Unknown	None
Iguape	1979	Brazil	Sentinel mice	Brazil	Unknown	Birds?	None
Ilheus	1944	Brazil	Pooled *Aedes* and *Psorophora*	South and Central America	Mosquitoes	Birds	Fever
Israel turkey meningoencephalitis	1959	Israel	*Meleagris gallopavo*	Israel, South Africa	?	?	None
Japanese encephalitis	1935	Japan	Human	Asia	*Culex tritaeniorhynchus*	Birds	Encephalitis
Jugra	1969	Malaysia	*Aedes* spp.	Malaysia	Mosquitoes	?	None
Jutiapa	1969	Guatemala	*S. hispidus*	Guatemala	Sole source	Rodents?	None
Kadam	1967	Uganda	*Rhipicephalus pravus*	Uganda, Saudi Arabia	*R. pravus*	Unknown	None
Karshi	1972	Uzbekistan	*Ornithodoros papillipes*	Uzbekistan, Kazahkistan	Sole source	Unknown	None

(*continues*)

TABLE I (*continued*)

Virus or serotype	Year of Isolation	Location	Source	Geographic Distribution	Principal Vector Species	Principal Host Species	Human Disease
Kedougou	1972	Senegal	*Aedes minutus*	Senegal, Central African Republic	*Aedes* spp.	Unknown	None
Kokobera	1960	Australia	*Culex annulirostris*	Australia, New Guinea	*C. annulirostris*	Macropods	None
Koutango	1969	Senegal	*Tatera kempi*	Senegal, Central African Republic	Unknown	Rodents?	Fever, rash
Kunjin	1960	Australia	*Culex annulirostris*	Australia, Asia	*Culex* spp.	Birds	Fever, rash
Kyasanur Forest disease	1957	India	*Presbytis entellus*	India	*Haemophysalis spinigera*	Monkeys	HF
Langat	1956	Malaysia	*Ix. granulatus*	Malaysia, Thailand, Siberia	*Ixodes granulatus*	Unknown	Fever
Louping ill	1929	Scotland	*Ovis aries*	UK	*Ixodes* spp.	Sheep	Encephalitis
Irish subtype	1968	Ireland	*Bos* spp.	Ireland	*Ixodes* spp.	Sheep	Encephalitis
Spanish subtype	1987	Spain	*Ovis* spp.	Spain	*Ixodes* spp.	Sheep	Encephalitis
Turkish subtype	1969	Turkey	*Ovis* spp.	Turkey	*Ixodes* spp.	Sheep	Encephalitis
Meaban	1981	France	*Ornithodoros maritimus*	France	*O. maritimus*	Birds	None
Modoc	1958	USA	*Peromyscus maniculatus*	USA	?	*P. maniculatus*	Encephalitis?

Montana myotis leukoencephalitis	1958	USA	*Myotis lucifugus*	USA	?	Bats?	None
Murray Valley encephalitis	1951	Australia	Human	Australia, New Guinea	*C. annulirostris*	Birds	Encephalitis
Naranjal	1976	Ecuador	Sentinel hamster	Ecuador	*Culex* spp.	Unknown	None
Ntaya	1943	Uganda	Mosquitoes	Africa	Mosquitoes	?	Fever
Omsk hemorrhagic fever	1947	USSR	Human	Western Siberia	*Dermacentor pictus*	Muskrats	HF
Phnom Penh bat	1969	Cambodia	*Cynopterus brachiotis*	Cambodia	?	Bats?	None
Powassan	1958	Canada	Human	Canada, USA, USSR	*Ixodes* spp.	Small mammals	Encephalitis
Rio Bravo	1954	USA	*Tadanida braziliensis mexicana*	USA, Mexico	?	*T. braziliensis mexicana*	Fever
Rocio	1975	Brazil	Human	Brazil	Mosquitoes	Birds	Encephalitis
Royal Farm	1968	Afghanistan	*Argas hermanni*	Afghanistan	*Argas* spp.	Birds?	None
Russian spring–summer encephalitis	1937	USSR	Human	Europe, Asia	*Ixodes* spp.	Rodents	Encephalitis
Saboya	1968	Senegal	*Tatera kempi*	Senegal	?	*T. kempi*	None
St. Louis encephalitis	1933	USA	Human	The Americas	*Culex* spp.	Birds	Encephalitis
Sal Vieja	1978	USA	*Peromyscus leucopus*	USA	Sole source	Rodents?	None
San Perlita	1972	USA	*S. hispidus*	USA	Sole source	Rodents?	None
Saumarez Reef	1974	Australia	*Ornithodoros capensis*	Australia	Bird ticks	Birds?	None

(*continues*)

TABLE I (*continued*)

Virus or serotype	Year of Isolation	Location	Source	Geographic Distribution	Principal Vector Species	Principal Host Species	Human Disease
Sepik	1966	New Guinea	*Mansonia septempunctata*	New Guinea	Mosquitoes	?	Fever
Sokoluk	1970	USSR	*Vespertilio pipistrellus*	Kirghizia	?	?	None
Spondweni	1955	South Africa	*Mansonia uniformis*	Africa	*Aedes circumluteolus*	Unknown	Fever
Stratford	1961	Australia	*Ae. vigilax*	Australia	*Ae. vigilax*	Unknown	None
Tembusu	1955	Malaysia	*Culex tritaeniorhynchus*	Malaysia, Thailand	*Culex* spp.	?	None
Tyuleniy	1969	USSR	*Ixodes uriae*	Russia, USA	*Ixodes* spp.	Birds?	None
Uganda S	1947	Uganda	*Aedes* spp.	Africa	Mosquitoes	Birds?	None
Usutu	1959	South Africa	*Culex neavei*	Africa	Mosquitoes	Birds?	Fever, rash
Wesselsbron	1955	South Africa	*Ovis* spp.	Africa, *Asia*	*Aedes* spp.	Unknown	Fever
West Nile	1937	Uganda	Human	Worldwide	Mosquitoes	Birds	Fever, rash
Yaounde	1968	Cameroon	*Cx. nebulosus*	Cameroon	*Culex* spp.	Unknown	None
Yellow fever	1927	Ghana	Human	Africa, South America	*Aedes aegypti*	Monkeys	Pantropic
Zika	1947	Uganda	*Macaca mulatta*	Africa, Asia	*Aedes* spp.	Monkeys?	Fever, rash

[a] Hemorrhagic fever.

TABLE II

TAXONOMY OF VIRUSES OF THE FAMILY *FLAVIVIRIDAE*, GENERA *FLAVIVIRUS*, *PESTIVIRUS*, AND *HEPACIVIRUS*, AS WELL AS VIRUSES (SPECIES) UNASSIGNED TO A GENUS WITHIN THE FAMILY[a,b]

	Abbreviation
Genus *Flavivirus*	
Tick-borne viruses	
Mammalian tick-borne virus complex[c]	
Gadgets Gully virus	GGYV
Kadam virus	KADV
Kyasanur Forest disease virus	KFDV
Langat virus	LGTV
Omsk hemorrhagic fever virus	OHFV
Powassan virus	POWV
Royal Farm virus	RFV
Karshi virus	KSIV
Tick-borne encephalitis virus	(TBEV[d])
European subtype	
Far Eastern subtype	
Siberian subtype	
Louping ill virus	LIV
Irish subtype	
British subtype	
Spanish subtype	
Turkish subtype	
Seabird tick-borne virus complex	
Meaban virus	MEAV
Saumarez Reef virus	SREV
Tyuleniy virus	TYUV
Mosquito-borne viruses	
Aroa virus complex	
Aroa virus	AROAV
Bussuquara virus	BSQV
Iguape virus	IGUV
Naranjal virus	NJLV
Dengue virus complex	
Dengue virus	
Dengue virus type 1	DENV-1
Dengue virus type 2	DENV-2
Dengue virus type 3	DENV-3
Dengue virus type 4	DENV-4

(*continues*)

TABLE II (*continued*)

	Abbreviation
Kedougou virus	KEDV
Japanese encephalitis virus complex	
Cacipacore virus	CPCV
Koutango virus	KOUV
Japanese encephalitis virus	JEV
Murray Valley encephalitis virus	MVEV
Alfuy virus	ALFV
St. Louis encephalitis virus	SLEV
Usutu virus	USUV
West Nile virus	WNV
Kunjin virus	KUNV
Yaounde virus	YAOV
Kokobera virus complex	
Kokobera virus	KOKV
Stratford virus	STRV
Ntaya virus complex	
Bagaza virus	BAGV
Ilheus virus	ILHV
Rocio virus	ROCV
Israel turkey meningoencephalitis virus	ITV
Ntaya virus	NTAV
Tembusu virus	TMUV
Spondweni virus complex	
Zika virus	ZIKV
Spondweni virus	SPOV
Yellow fever virus complex	
Banzi virus	BANV
Bouboui virus	BOUV
Edge Hill virus	EHV
Jugra virus	JUGV
Saboya virus	SABV
Potiskum virus	POTV
Sepik virus	SEPV
Uganda S virus	UGSV
Wesselsbron virus	WESSV
Yellow fever virus	YFV
Viruses with no known arthropod vector	
Entebbe bat virus complex	
Entebbe bat virus	ENTV

(*continues*)

TABLE II (*continued*)

	Abbreviation
Sokoluk virus	SOKV
Yokose virus	YOKV
Modoc virus complex	
Apoi virus	APOIV
Cowbone Ridge virus	CRV
Jutiapa virus	JUTV
Modoc virus	MODV
Sal Vieja virus	SVV
San Perlita virus	SPV
Rio Bravo virus complex	
Bukalasa bat virus	BBV
Carey Island virus	CIV
Dakar bat virus	DBV
Montana myotis leukoencephalitis virus	MMLV
Phnom Penh bat virus	PPBV
Batu Cave virus	BCV
Rio Bravo virus	RBV
Tentative species/subtypes in the genus	
Alkhurma virus (Charrel *et al.*, 2001)	
Genus *Pestivirus*	
Border disease virus (two serotypes)	BDV
Bovine viral diarrhea virus 1 (four serotypes)	BVDV-1
Bovine viral diarrhea virus 2 (two serotypes)	BVDV-2
Classical swine fever virus (Hog cholera virus) (four serotypes)	CSFV
Tentative species in the genus	
Pestivirus of giraffe	
Genus *Hepacivirus*	
Hepatitis C virus (six genotypes)	HCV
Unassigned viruses in the family	
Tamana bat virus	TABV
Cell-fusing agent	CFAV
GB virus A	GBV-A
GBV A-like agents	GBV-A-like
GB virus B	GBV-B
GB virus C/Hepatitis G virus	GBV-C/HGV-1

[a] Virus species names are shown in italics. Tentative virus species names, alternative names ( ), and strains or serotypes are not italicized.
[b] Modified from Heinz *et al*. (2000).

(*continues*)

whereas internal ribosomal entry sites have been demonstrated for pestiviruses and hepaciviruses.

Table I summarizes flaviviruses by year, location, and source of initial isolation, known geographic distribution, principal vector species, principal host species, and disease associations. Table II summarizes the taxonomic placement of viruses (species) in the family *Flaviviridae*, as recognized at this time by the International Committee for Taxonomy of Viruses (Heinz *et al.*, 2000). Viruses in the genus *Flavivirus* (flaviviruses) can be grouped serologically (antigenically) and with regard to their vector associations. Taxonomic placements are based on a variety of replicative and genomic characters, with uniqueness being used to distinguish closely related viruses. However, virus species are defined as "a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecologic niche" (Van Regenmortel *et al.*, 1991). Obviously, the definitions of "lineage" and "particular ecologic niche" allow some flexibility, there is a lack of data in some instances, and different members of taxonomic study groups bring different views to the ongoing discussion so that taxonomic placements should always be considered provisional. Intentionally and justifiably ignored in this scheme are important epidemiologic parameters, such as the presence or absence of cross-protection between viruses or subtypes, pathogenic potential, and other markers. Given that the taxonomy of flaviviruses is genome based, this is logical, but investigators, students, and others interested in these viruses should be aware of possible ambiguities among taxonomic placement, vernacular names, and biological characterization (Drebot *et al.*, 2002).

TABLE II (*continued*)

[c] The International Committee on Taxonomy of Viruses (Heinz *et al.*, 2000) considers certain genetic and antigenic clusters of flaviviruses as "groups." The original nomenclature for the flaviviruses, "group B," seems more inclusive. The word "group" has been replaced here with the word "complex" to indicate close relationships of viruses within the genus. "Group" and "complex" often are used interchangeably, but the more restrictive "complex" is preferred.

[d] The nomenclature of viruses causing "tick-borne encephalitis" is controversial in some quarters. One could argue that, except in the ICTV report (Heinz *et al.*, 2000), there is no virus by the name of "tick-borne encephalitis virus" (a species; an abbreviation for this nonentity, "TBEV," could, therefore, be considered superfluous). Two of the ICTV-recognized subtypes (cf. genetic variants), "European" and "Far Eastern," are near synonyms for the well-known and medically important Central European encephalitis virus (CEEV) and Russian spring–summer encephalitis virus (RSSEV), which are not listed in the ICTV report (Heinz *et al.*, 2000) and so have been omitted here.

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STRUCTURE AND REPLICATION

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ADVANCES IN VIRUS RESEARCH, VOL 59

MOLECULAR BIOLOGY OF FLAVIVIRUSES

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I. Introduction
II. Viral Life Cycle
III. Flavivirus Particles
IV. Genome Structure
  A. 5′-Noncoding Region
  B. 3′-Noncoding Region
V. Virus Proteins
  A. Translation and Polyprotein Processing
  B. Structural Proteins
  C. Nonstructural Proteins
VI. RNA Replication
  References

I. Introduction

The major medical importance of flaviviruses has fostered studies into their molecular biology in research laboratories around the world. Although the molecular biology of flaviviruses has been presented in other reviews (Brinton, 2002; Lindenbach and Rice, 2001), this active research community has provided many new advances in the field over the past few years. Our goal therefore is to summarize the current understanding of the molecular biology of flaviviruses and point out promising avenues for future work. Where appropriate, we have indicated additional chapters in this volume that delve into greater detail on particular subjects.

II. Viral Life Cycle

The molecular biology of flaviviruses is best understood in the context of the viral life cycle, which provides a framework for the organization of this chapter. Flavivirus particles bind to cells via interactions between the viral surface glycoprotein and cellular receptors. Several

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cell surface proteins have been described as putative receptors (Bielefeldt-Ohmann, 1998; Kimura *et al.*, 1994; Kopecky *et al.*, 1999; Marianneau *et al.*, 1996; Munoz *et al.*, 1998; Ramos-Castaneda *et al.*, 1997; Salas-Benito and del Angel, 1997). In addition, opsonization with immunoglobulins enhances virus particle binding and infection of cells expressing immunoglobulin Fc receptors (Peiris and Porterfield, 1979; Schlesinger *et al.*, 1983). Virions are internalized into clathrin-coated pits via receptor-mediated endocytosis (Gollins and Porterfield, 1985, 1986; Ishak *et al.*, 1988; Nawa, 1998; Ng and Lau, 1988). It is thought that virions are brought into a prelysosomal endocytic compartment where low pH induces fusion between the virus and host cell membranes to release the virus nucleocapsid (Gollins and Porterfield, 1985, 1986; Heinz *et al.*, 1994; Nawa, 1998). The viral genome is released into the host cytoplasm by the process of nucleocapsid uncoating, which is not yet fully understood. Translation of the viral genome produces proteins that lead to replication of the viral genome and assembly of new virus particles.

Flavivirus infection induces rearrangement of cytoplasmic membranes in the perinuclear region. The earliest of these events is proliferation of the endoplasmic reticulum (ER) (Murphy *et al.*, 1968; Ng and Hong, 1989), followed by the appearance of smooth membrane structures around the time of early logarithmic virus production. Smooth membrane vesicles appear as clustered vesicles containing electron-dense material (Cardiff *et al.*, 1973; Leary and Blair, 1980; Murphy *et al.*, 1968; Ng and Hong, 1989; Ng *et al.*, 1994b). In samples prepared via cryosectioning, these structures appear as vesicle packets (VP), clusters of vesicles, each 100 to 200 nm in diameter, bound by a smooth outer membrane (Mackenzie *et al.*, 1999). During later times of infection, convoluted membranes (CM) are found adjacent to VPs. CM appear as randomly folded or ordered membranes (sometimes referred to as paracrystalline arrays) that are contiguous with the ER (Leary and Blair, 1980; Murphy *et al.*, 1968). One study showed that 1,4-galactosyltransferase, a marker of *trans*-Golgi, was localized to VPs, whereas the ER–Golgi intermediate compartment–53 protein was localized within the CM, suggesting distinct pathways for the formation of VPs and CMs (Mackenzie *et al.*, 1999). Based on the localization of viral RNAs and replication proteins, it appears that VPs are sites of RNA replication (Mackenzie *et al.*, 1996, 1998; Westaway *et al.*, 1997b, 1999). The viral genome is replicated via a negative-strand intermediate, which serves as a template for additional positive-strand RNAs.

Virus particles are thought to assemble by budding into the ER (Deubel *et al.*, 1981; Hase *et al.*, 1987a,b; Ishak *et al.*, 1988;

Ko *et al.*, 1979; Leary and Blair, 1980; Mackenzie and Westaway, 2001; Matsumura *et al.*, 1977; Murphy *et al.*, 1968; Ohyama *et al.*, 1977; Sriurairatna and Bhamarapravati, 1977; Sriurairatna *et al.*, 1973; Wang *et al.*, 1998). A few studies have shown evidence for budding at the plasma membrane (Hase *et al.*, 1987a; Ng *et al.*, 1994a, 2001; Ohyama *et al.*, 1977; Sriurairatna and Bhamarapravati, 1977). Based on *trans*-complementation studies, it appears that genome packaging is coupled to RNA replication (Khromykh *et al.*, 2001b). Nascent virus particles pass through the host secretory pathway, where virion maturation occurs, and are released (Chambers *et al.*, 1990b; Heinz *et al.*, 1994; Mason, 1989; Nowak *et al.*, 1989).

III. Flavivirus Particles

Flaviviruses are small ($\approx$50 nm), spherical particles containing an electron-dense core of $\approx$30 nm, surrounded by a lipid envelope (Murphy, 1980). Viruses sediment between 170 and 210S and have buoyant densities of 1.19 to 1.23 g/cm^3, depending on the lipid composition, which can vary by host (Russell *et al.*, 1980). Electron micrographs of virus particles are presented in Figs. 1A and 1B. The surface of virus particles contains two viral proteins: E (envelope) and M (membrane). The E glycoprotein is the major antigenic determinant

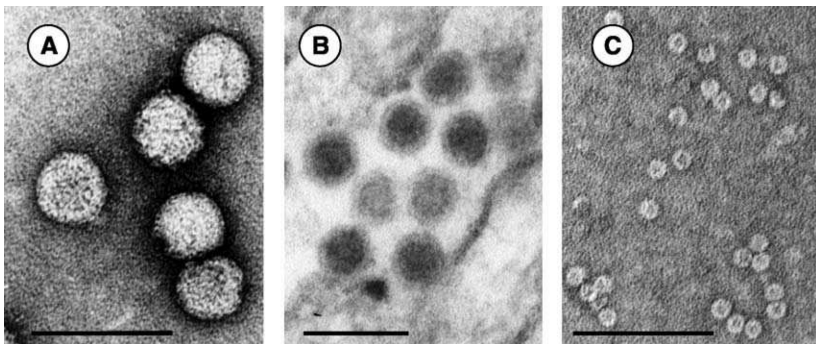


Fig 1. Electron micrographs of flavivirus particles. Bars represent 100 nm. (A) Negatively stained DEN2 virus particles isolated from an infected mouse brain homogenate via rate zonal sedimentation through a sucrose gradient. (B) St. Louis encephalitis virus particles can be seen in this thin section micrograph from an infected suckling mouse brain. (C) SHA particles isolated from the same gradient as in (A). All figures courtesy of Dr. F. A. Murphy.

on virus particles and mediates binding and fusion during virus entry. The M protein, produced during the maturation of nascent virus particles within the secretory pathway, is a small proteolytic fragment of the precursor prM protein. Removal of the lipid envelope with nonionic detergents reveals discrete nucleocapsids (120 to 140S; 1.30 to 1.31 g/cm^3), which consist of C (capsid) protein and genomic RNA (Russell *et al.*, 1980).

The structure of purified dengue 2 virus (DEN2) particles has been determined at a resolution of 24 Å by reconstruction from cryoelectron microscopic images (Kuhn *et al.*, 2002). These studies revealed a relatively smooth outer surface (Fig. 2A). This is consistent with the arrangement of E protein dimers in a head-to-tail configuration lying parallel to the lipid bilayer, as predicted from X-ray crystallographic data on the structure of the tick-borne encephalitis (TBE) virus E protein (Rey *et al.*, 1995). Fitting of the E protein structure into this model predicts a tight packing of 90 E dimers in an unusual "herring-bone" pattern with icosahedral symmetry (Fig. 2B). These results suggest that E dimers may undergo rotational rearrangements around three- and five-fold axes of symmetry to form fusogenic trimeric complexes (Kuhn *et al.*, 2002), as shown in Fig. 2C. Interestingly, E trimerization has been observed during the conversion of E to the fusogenic form by low pH (Allison *et al.*, 1995; Heinz *et al.*, 1994; Stiasny *et al.*, 1996). Within the native virion, the M protein presumably fits within an electron-dense space in E dimers near the fusogenic domain (Kuhn *et al.*, 2002). Electron densities corresponding to the nucleocapsid lie just below the lipid bilayer and reveal a less ordered, cage-like arrangement of C protein surrounding the viral genome (Kuhn *et al.*, 2002). Thus, the icosahedral symmetry of virions is thought to be imposed by interactions between the surface proteins, rather than the nucleocapsid.

In addition to mature virions, smaller noninfectious particles are released from flavivirus-infected cells (Smith *et al.*, 1970). These particles are termed slowly sedimenting (70S) hemagglutinin (SHA) because like virions, they can agglutinate red blood cells at low pH. SHA are smaller than virions ($\approx$14 nm diameter, compare Figs. 1A and 1C). These particles contain E and M proteins, but lack nucleocapsids (Smith *et al.*, 1970). A related or identical type of particle, recombinant subviral particles (RSP), can be produced by cells expressing prM and E alone (Allison *et al.*, 1995; Konishi and Mason, 1993; Mason *et al.*, 1991; Schalich *et al.*, 1996; Sugrue *et al.*, 1997). RSPs are 30 nm in diameter, less dense than virus particles (1.14 g/cm^3), and can undergo acid-catalyzed fusion similar to virions (Schalich *et al.*, 1996).

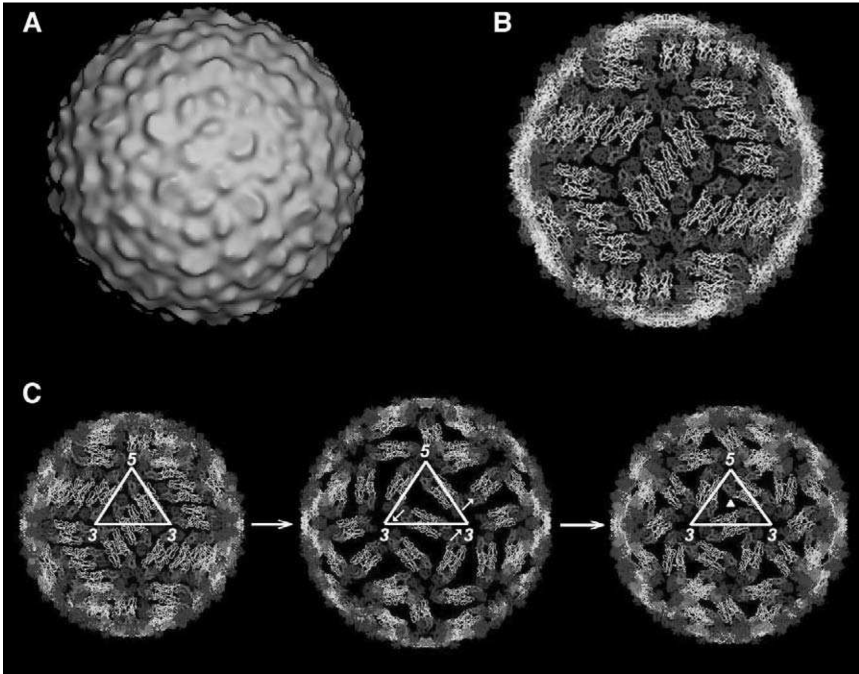


FIG 2. DEN2 virion structure determined by cryoelectron microscopy. (A) The relatively smooth surface of the DEN2 virion is visible in this model based on image reconstruction. (B) The unusual "herringbone" arrangement of E protein dimers fitted into the electron density map. In this image, E protein domains I, II, III, and the fusion peptide are colored red, yellow, blue, and green, respectively. (C) Proposed acid-catalyzed rearrangement of E protein dimers into the fusogenic state. An icosahedral asymmetric unit is represented by the triangle, and three- and fivefold axes of symmetry are illustrated. Small arrows indicate the proposed rotation of the E protein. Images adapted from Kuhn *et al*. (2002) and used with permission. Composite image kindly provided by Dr. R. J. Kuhn. (See Color Insert.)

Cryoimage reconstruction of TBE RSPs suggests a T=1 icosahedral arrangement of 30 E protein dimers (Ferlenghi *et al*., 2001), which is markedly different from native virions but qualitatively similar to their proposed fusogenic form (Kuhn *et al*., 2002).

Much progress has been made in understanding the structure of flavivirus particles. Future work will involve understanding the structure of M and C proteins to high resolution, the orientation of these proteins within virions, how the native glycoprotein arrangement forms during virus assembly and maturation, and confirmation of the proposed rearrangement of E dimers to a fusogenic form.

IV. Genome Structure

Flavivirus genomes consist of a single positive-strand RNA of ≈11 kb (sedimentation, 42S) with a type I 5′ cap, $m^7GpppAmpN_2$ (Cleaves and Dubin, 1979; Wengler *et al.*, 1978). Additional methylation of the N_2 residue (type II cap) has also been detected in RNA from infected cells (Cleaves and Dubin, 1979; Wengler *et al.*, 1978). Unlike cellular mRNAs, flavivirus genomes are not 3′ polyadenylated (Wengler *et al.*, 1978). Genomes encode a single long open reading frame flanked by 5′- and 3′-noncoding regions (NCR) of ≈100 and 400 to 700 nucleotides, respectively.

As for all positive-stranded RNA viruses, flavivirus genomic RNA is infectious (Peleg, 1969). Following the advent of molecular cloning it became possible to produce recombinant flavivirus genomes that can initiate replication and virus production in appropriately transfected host cells (Gritsun and Gould, 1995; Gualano *et al.*, 1998; Hurrelbrink *et al.*, 1999; Kapoor *et al.*, 1995a; Khromykh and Westaway, 1994; Kinney *et al.*, 1997; Lai *et al.*, 1991; Mandl *et al.*, 1997; Polo *et al.*, 1997; Rice *et al.*, 1989; Shi *et al.*, 2002; Sumiyoshi *et al.*, 1992; Yamshchikov *et al.*, 2001). The ability to do reverse genetics on flaviviruses has proven to be a valuable research tool for dissecting the function of viral genes (see, e.g., Khromykh *et al.*, 1999a; Lindenbach and Rice, 1997; Lindenbach and Rice, 1999; Muylaert *et al.*, 1996, 1997), understanding the virulence of natural virus isolates (Gualano *et al.*, 1998; Mandl *et al.*, 1997; Shi *et al.*, 2002), providing stable genetic stocks of live-attenuated vaccines (Kinney *et al.*, 1997; Rice *et al.*, 1989), and developing novel vaccine strategies (Bray *et al.*, 1996; Chambers *et al.*, 1999; Guirakhoo *et al.*, 1999, 2000; Mandl *et al.*, 1998a; Pletnev and Men, 1998). Nevertheless, the quasi-species nature of RNA virus sequences and the instability of cloned flavivirus cDNAs remain hurdles that must be overcome. For more information on the construction of infectious flavivirus molecular clones, we refer readers to Ruggli and Rice (1999).

A. 5′-Noncoding Region

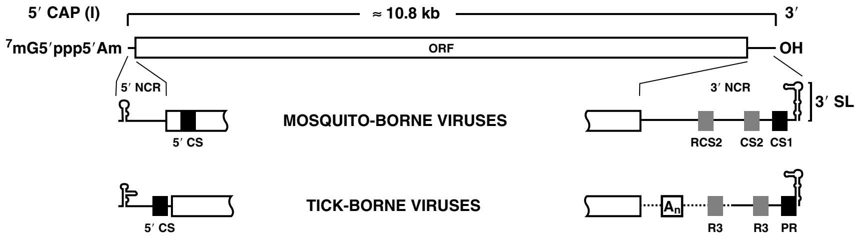
The sequence of the 5′ NCR is not well conserved among different flaviviruses, although common secondary structures have been found within this region (Brinton and Dispoto, 1988; Cahour *et al.*, 1995; Hahn *et al.*, 1987). It is likely that these structures influence translation of the genome. Another major function of the 5′ NCR probably resides in the complementary region of the negative strand, which

serves as a site of initiation for positive strand synthesis during RNA replication. A stem–loop that can form near this terminus of either strand is an important determinant for genome replication: deletions engineered into this structure were lethal for DEN4 replication, although they had minimal effects on translation of the mutant RNAs (Cahour *et al.*, 1995). One of the viable mutants exhibited a limited host-range growth phenotype, suggesting that this RNA region interacts with host-specific factors. Indeed, four hamster cell proteins of 42, 50, 60, and 108 kDa were found to bind to this region of the West Nile virus (WN) 3′-negative strand RNA (Shi *et al.*, 1996b). Purification of the 42-kDa protein revealed it to be TIAR, an RNA-binding protein containing three RNA recognition motifs (RRM) (Li *et al.*, 2002). Further studies showed that TIAR, as well as a related protein, TIA-1, bound to this region of WN negative-strand RNA with reasonably high affinity ($K_d \approx 10^{-8}$ and 10^{-7} *M*, respectively) via their second RRM. The growth of WN was inhibited in a TIAR knockout cell line, indicating the functional importance of the TIAR interaction (Li *et al.*, 2002).

B. 3′-Noncoding Region

The 3′ NCRs of flavivirus genomes exhibit great variability, although several conserved features and secondary structures have been elucidated and are illustrated in Fig. 3A. The greatest structural similarity among this region of flavivirus genomes lies near the 3′ terminus, which is predicted to form a conserved, long (90 to 120 nucleotides) stem–loop (3′ SL) that has been supported by secondary structure predictions, covariation analyses, and biochemical probing (Brinton *et al.*, 1986; Grange *et al.*, 1985; Hahn *et al.*, 1987; Mandl *et al.*, 1989, 1991; Proutski *et al.*, 1997; Rauscher *et al.*, 1997; Rice, 1985; Shi *et al.*, 1996b; Sumiyoshi *et al.*, 1987; Takegami *et al.*, 1986; Wengler and Castle, 1986; Zhao *et al.*, 1986). Mutational analysis of the DEN2 3′ SL revealed an essential role of this structure for virus replication (Zeng *et al.*, 1998). Chimeric DEN2-WN 3′ SL structures were used to map virus-specific regions to the bottom half of the DEN2 3′ SL (Zeng *et al.*, 1998). One interesting substitution mutant grew well in monkey kidney cells but poorly in mosquito cells, suggesting an involvement of host-specific factors (Zeng *et al.*, 1998).

While the precise role of the 3′ SL is unknown, it has been shown to interact with several proteins of functional relevance. The viral replicase proteins NS3 and NS5 have been shown to bind this structure *in vitro* (Chen *et al.*, 1997; Cui *et al.*, 1998). UV cross-linking and gel



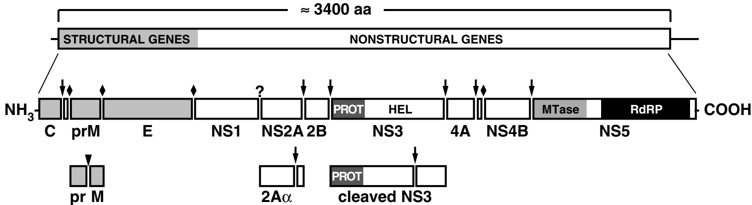


FIG 3. Flavivirus genome organization. (A) RNA elements of the 5′-capped, ≈10.8-kb flavivirus genome are illustrated. Similar structual and sequence elements within the mosquito- and tick-borne virus groups are shown. These include stem–loop structures within the 5′ NCR, 5′ CS elements near the beginning of the open reading from (ORF), and 3′SL at the 3′ end of the genome. Mosquito-borne viruses also contain CS1 and one or two CS2 elements (CS2 and RCS2) in the 3′ NCR. The PR element, which may base pair with a 5′ CS in tick-borne viruses, overlaps the 3′ SL. A conserved element (R3) is found in the conserved region of the tick-borne virus 3′ NCRs and is often duplicated within the variable region (dashed line), which sometimes also contains a polyadenylate sequence. (B) The protein expression and polyprotein processing strategy of flaviviruses is illustrated. Viral serine protease cleavage sites are indicated by a downward arrow. Diamonds and a triangle indicate sites of cleavage with signal peptidase and furin or a furin-like enzyme, respectively. Processing of NS1-2A occurs by an unknown ER resident host enzyme, which is indicated by a question mark. Internal serine protease cleavage sites within NS2A and NS3 are also illustrated. In addition, the serine protease (PROT) and helicase (HEL) domains of NS3 and methyltransferase (MTase) and RdRP domains of NS5 are indicated.

mobility shift assays were used to show specific interaction of three hamster cell proteins (52, 84, and 105 kDa) with the 3′ SL of WN RNA (Blackwell and Brinton, 1995). The 52-kDa protein was later purified chromatographically and was identified as translation elongation factor 1A (EF1A) (Blackwell and Brinton, 1997). The normal function of EF1A is to transport charged tRNAs into the ribosome, and one mechanism of translational control is through phosphorylation

of this protein. Binding of EF1A to the WN 3′ SL was inhibited by dephosphorylation of this protein with calf intestine alkaline phosphatase (Blackwell and Brinton, 1997). This interaction with EF1A was confirmed by another group using the DEN4 3′ SL (De Nova-Ocampo *et al.*, 2002). These results are intruiging, as EF1A has also been shown to bind to the poliovirus 5′ NCR RNA (Harris *et al.*, 1994) and to the tRNA-like 3′ ends of turnip yellow mosaic virus and tobacco mosaic virus (Joshi *et al.*, 1986; Zeenko *et al.*, 2002). Furthermore, the bacterial homologue of this protein, EF-Tu, is also a component of the bacteriophage Qβ replicase (Blumenthal and Carmichael, 1979). Thus, in addition to its role in the translation of numerous positive-strand RNA viruses, this protein seems to play an important, albeit obscure, role in RNA replication. In addition to EF1A, the human La autoantigen (De Nova-Ocampo *et al.*, 2002) and mouse Mov34 protein (Ta and Vrati, 2000) were found to bind to the 3′ SL of DEN4 and Japanese encephalitis (JE) virus, respectively, although the functional relevance of these interactions is presently unknown.

Despite sequence heterogeneity within the 3′ NCR, similar patterns of conserved sequences and structures have been found among mosquito-borne flaviviruses that are not shared by tick-borne viruses (Hahn *et al.*, 1987; Olsthoorn and Bol, 2001; Proutski *et al.*, 1997). Just upstream of the 3′ SL, a well-conserved 25 nucleotide region (CS1) was identified in the genomes of yellow fever (YF), DEN2, WN, and Murray Valley encephalitis (MVE) viruses (Hahn *et al.*, 1987). CS1 has been predicted to base pair with a complementary sequence (5′ CS) in the beginning of the capsid gene, greater than 10 kb upstream (Hahn *et al.*, 1987). A deletion in the 3′ NCR that included the CS1 was lethal for DEN4 virus replication (Men *et al.*, 1996). Further, complementarity of the proposed cyclization sequences was shown to be required for the efficient utilization of exogenous templates added to *in vitro* RNA-dependent RNA polymerase (RdRP) assays (You and Padmanabhan, 1999). More recently, the replication of Kunjin virus (KUN) replicons was shown to be dependent on this interaction (Khromykh *et al.*, 2001a). Mutation of either cyclization motif destroyed replication, whereas compensatory mutations restored it (Khromykh *et al.*, 2001a). Thus, the base pairing ability of these distant regions, and not their sequence per se, is an important determinant for replication. The latter half of the WN CS1 was also shown by thermal melting and ribonuclease probing to be capable of base pairing with an internal loop in the stalk of the 3′ SL, forming a small pseudoknot (Shi *et al.*, 1996a). Similar structures have also been predicted for DEN3 and YF (Shi *et al.*, 1996a). It is presently unclear how these different interactions of CS1

are related, although it is tempting to speculate that they may function in regulating the competing processes of genome translation vs genome replication.

One or two copies of a second conserved sequence (CS2 and RCS2) are also found among mosquito-borne flaviviruses (Hahn *et al.*, 1987). For several viruses, these regions were predicted to fold into dumbbell-shaped secondary structures that potentially form pseudoknots with adjacent sequences (Olsthoorn and Bol, 2001). Additional repeated sequence elements that are conserved among mosquito-borne flavivirus subgroups have also been noted (Proutski *et al.*, 1997; Rauscher *et al.*, 1997; Rice, 1985; Wallner *et al.*, 1995; Wang *et al.*, 1996). DEN4 genomes containing deletions in the 3′ NCR that encompass CS2 and RCS2 were shown to be viable in cell culture (Men *et al.*, 1996). Thus the functional importance of these structures remains to be established.

As mentioned, tick-borne flaviviruses share a unique 3′ NCR organization, with a 350 nucleotide 3′-conserved region preceded by a variable domain (Wallner *et al.*, 1995). Although not conserved in primary sequence with mosquito-borne viruses, the 3′-terminal 100 nucleotides of TBE genomic RNA is also predicted to fold into a 3′ SL (Wallner *et al.*, 1995). The conserved region contains conserved sequence elements (R3 and PR in Fig. 3A) and retains a capacity for long-distance base pairing between PR and a 5′ CS (Khromykh *et al.*, 2001a; Mandl *et al.*, 1993). Interestingly, a polyadenylate sequence is found in the variable region of some TBE isolates (Wallner *et al.*, 1995). Sequence comparison and deletion analysis indicated that the variable region is dispensible for virus replication (Mandl *et al.*, 1998b; Wallner *et al.*, 1995), whereas deletions that extended into the conserved region were progressively deleterious (Mandl *et al.*, 1998b).

A great deal of information has been learned about the structure and functions of the flavivirus 5′ and 3′ NCRs. Additional information on their role in replication and virulence is covered in the chapter by Dr. Lewis Markoff.

V. Virus Proteins

A. Translation and Polyprotein Processing

Like all positive-strand RNA viruses, the flavivirus genome serves as mRNA for the translation of viral proteins. Translation of the single long open reading frame produces a large polyprotein that is cleaved co- and posttranslationally into at least 10 proteins (Fig. 3B). The

N-terminal one fourth of the polyprotein encodes structural proteins (C-prM-E), followed by nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) (Rice *et al.*, 1985). Host signal peptidase is responsible for cleavage among C-prM, prM-E, E-NS1, and near the C terminus of NS4A. A virus-encoded serine protease, discussed later, is responsible for cleavages among NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B, and NS4B/NS5. The enzyme responsible for NS1-2A cleavage is presently unknown.

According to our best structural model, virions contain 180 copies of the E protein, an unknown amount of prM and C, and a single copy of the viral genome. Thus, translation of the viral genome must occur at least 180 times for every nascent genome that is produced and packaged. As discussed later, a single input genome can give rise to multiple daughter genomes. Thus, utilization of the flavivirus genome as a template for translation is probably more efficient than its use as a template for replication. Because translation occurs in a 5′ to 3′ direction, whereas replication occurs in a 3′ to 5′ direction, it is thought that these processes must occur sequentially, rather than simultaneously, for all positive-strand RNA viruses. Future research may help to understand how these two competing processes are regulated for flaviviruses.

B. Structural Proteins

1. Capsid Protein

Capsid is a highly basic protein of ≈11 kDa (Boege *et al.*, 1983; Rice *et al.*, 1985; Trent, 1977). Charged residues, which presumably mediate the RNA interaction, are clustered at the N and C termini (Khromykh and Westaway, 1996), separated by a short internal hydrophobic domain that mediates membrane association (Markoff *et al.*, 1997). Nascent C (anchC) also contains a C-terminal hydrophobic anchor that serves as a signal peptide for ER translocation of prM. This hydrophobic domain is cleaved from mature C by the viral serine protease (Amberg *et al.*, 1994; Lobigs, 1993; Yamshchikov and Compans, 1994). While the structure of C is unknown, TBE C protein has been predicted to be rich in α helix, including the central hydrophobic domain (Kofler *et al.*, 2002). Interestingly, progressive internal deletions at the N terminus of this central hydrophobic helix led to decreased TBE virus release and increased RSP production (Kofler *et al.*, 2002). Nevertheless, deletions of up to 16 amino acids were tolerated. Additional work is required to better understand the structure of this protein as well as its interaction with the viral envelope and surface proteins.

2. *Membrane Protein*

As just mentioned, the glycoprotein precursor of M protein, prM (≈26 kDa), is translocated into the ER by the C-terminal hydrophobic domain of C. Cleavage by the host signal peptidase is delayed until this signal sequence is removed from the capsid (Amberg *et al.*, 1994; Lobigs, 1993; Yamshchikov and Compans, 1994). This strategy seems to be due to the combination of a fairly short (14 to 22 amino acid) signal sequence, suboptimal residues at the signalase cleavage site, and downstream regions of prM (Stocks and Lobigs, 1998). The rate of signalase cleavage can also be influenced by the expression of E (Lorenz *et al.*, 2002). One explanation for this unusual processing strategy could be to delay structural protein processing and, consequently, virus production until late in the infection, when viral protease levels are high. Surprisingly, optimization of the YF prM signalase cleavage site was lethal for virus production but could be suppressed by second site mutations in the signal sequence (Lee *et al.*, 2000). However, revertant viruses retained the improved anchC-prM cleavage (Lee *et al.*, 2000), and our understanding of this processing mechanism remains incomplete.

The N-terminal region of pr contains one to three *N*-linked glycosylation sites (Chambers *et al.*, 1990a) and six conserved cysteine residues, all of which are disulfide linked (Nowak and Wengler, 1987). Pulse-chase analyses of TBE structural glycoproteins indicate that the folding of prM is rapid, that prM and E form a heterodimeric complex shortly after synthesis, and that prM is needed for the proper folding of E (Lorenz *et al.*, 2002). These data are consistent with a chaperone-like activity for prM in the folding of E protein (Konishi and Mason, 1993; Lorenz *et al.*, 2002). The conversion of immature virus particles to mature virions occurs in the secretory pathway and coincides with cleavage of prM into pr and M by the Golgi-resident furin or a furin-like enzyme (Stadler *et al.*, 1997). Thus, the pr segment of prM is thought to stabilize E and keep it from undergoing rearrangement to the fusogenic form in the reduced pH environment of the early secretory pathway (Guirakhoo *et al.*, 1991, 1992). Following cleavage, the M protein is found in mature virions, whereas the pr fragment is secreted (Murray *et al.*, 1993).

3. *Envelope Protein*

The E protein (≈53 kDa) is the major virion surface protein and mediates binding and membrane fusion. Much recent progress has been made in understanding the structure and function of E, which

is covered in the chapter by Franz X. Heinz and Steven L. Allison. E is synthesized as a type I membrane protein containing 12 conserved cysteines that form disulfide bonds (Nowak and Wengler, 1987) and, for some viruses, E is *N*-glycosylated (Chambers *et al.*, 1990b; Winkler *et al.*, 1987). As just mentioned, proper folding and stabilization of E are dependent on coexpression with prM (Konishi and Mason, 1993; Lorenz *et al.*, 2002).

The crystal structure of a soluble tryptic fragment of the TBE E protein was determined at a resolution of 2.0 Å (Rey *et al.*, 1995). This model revealed an elongated structure rich in β sheets, forming head-to-tail homodimers that most likely lie parallel with the virus envelope. Each E protein subunit is composed of three domains: I, which forms a β barrel; II, which projects along the virus surface between the transmembrane regions of the homodimer subunits; and III, which maintains an immunoglobulin constant region-like fold, thought to be the receptor-binding region (Mandl *et al.*, 2000; Rey *et al.*, 1995). The putative fusion peptide, which mediates insertion into the target cell membrane, is located at the tip of domain II, distal to the transmembrane region (Allison *et al.*, 2001; Rey *et al.*, 1995). Surprisingly, the E1 glycoprotein of a distantly related positive-strand RNA virus, Semliki Forest virus (SFV; family *Togaviridae*, genus alphavirus) was found to fold in a remarkably similar way to the TBE E protein, with three β sheet-rich domains lying parallel to the membrane as head-to-tail homodimers and containing a distal fusion peptide on domain II (Lescar *et al.*, 2001). An interesting twist is that the SFV E1 dimer interface is opposite that of the TBE E dimers. Nevertheless, these similarities have led to the classification of both proteins into a new group of virus fusion proteins, class II, which are distinct from previously characterized fusion proteins such as influenza HA. A common feature of class II proteins is that low pH converts them from a hetero- or homomultimeric native state into a trimeric fusogenic complex, whereas class I fusion proteins are trimeric coiled–coil "hairpin" structures that, upon triggering, reveal their fusion peptides much like a switchblade (Skehel and Wiley, 1998).

C. Nonstructural Proteins

1. NS1

NS1 ($\approx$46 kDa) is retained within cells, but is also found on the cell surface and is secreted slowly from mammalian cells (Mason, 1989; Post *et al.*, 1991; Smith *et al.*, 1970; Winkler *et al.*, 1988). NS1 is translocated into the ER and released from the E protein by host signal

peptidase (Chambers *et al.*, 1990b; Falgout *et al.*, 1989; Falgout and Markoff, 1995). This glycoprotein contains two or three *N*-linked glycosylation sites and 12 conserved cysteines that form disulfide bonds (Lee *et al.*, 1989; Mason, 1989; Smith *et al.*, 1970). An analysis of tryptic digests of MVE NS1 indicated that the Cys_4–Cys_{15}, Cys_{55}–Cys_{143}, and Cys_{179}–Cys_{223} pairs were disulfide bonded (Blitvich *et al.*, 2001). Proteolytic processing at the NS1/2A junction occurs by an unknown ER resident host enzyme (Falgout and Markoff, 1995). The eight C-terminal residues of NS1 and greater than 140 amino acids of NS2A are required for this processing event (Chambers *et al.*, 1990b; Falgout and Markoff, 1995; Hori and Lai, 1990; Pethel *et al.*, 1992). In addition, truncated and elongated forms of NS1, which presumably differ in their C-terminal cleavage sites, have been observed for JE and Murray Valley encephalitis (MVE) viruses (Blitvich *et al.*, 1999; Mason, 1989). Around 30 min after synthesis, NS1 forms homodimers that are highly stable, resistant to denaturation with 6 *M* urea or 5 *M* guanidinium–HCl, but are unstable at high temperatures or low pH (Winkler *et al.*, 1989). A mutation in KUN NS1 that destabilized dimers, Pro_{250} to Leu, was found to cause a virus growth defect in culture and was severely attenuating in mice (Hall *et al.*, 1999). However, because the assay used to detect dimers was their stability in SDS–PAGE, it is unclear whether this mutant only makes monomeric NS1 or whether SDS-sensitive NS1 dimers are formed.

Coincident with dimerization, NS1 acquires a partially hydrophobic character, as demonstrated by sedimentation with cellular membrane fractions or extraction with Triton X-114 (Winkler *et al.*, 1988, 1989). As this protein is largely hydrophilic in amino acid content and contains no putative transmembrane domains, the nature of this membrane association remains controversial. One possibility is that dimerization creates a hydrophobic surface for peripheral association with membranes. An alternate proposal has been put forward that NS1 could be glycosyl-phosphatidylinositol (GPI) anchored (Winkler *et al.*, 1989). This posttranslational modification involves removal of a C-terminal GPI-anchoring signal peptide followed by the covalent addition of a glycolipid membrane anchor to the nascent C terminus by an ER resident enzyme (Low, 1989). Thus, this hypothesis also seemed to provide a convenient explanation for the mechanism of NS1-2A cleavage and could explain why NS1 is found in discrete foci on the cell surface (Cardiff and Lund, 1976; Westaway and Goodman, 1987), which is a common feature of GPI-anchored proteins. One group found that DEN2 NS1 could be labeled metabolically with [^{3}H]ethanolamine or [^{3}H]inositol, which are precursors of GPI anchors (Jacobs

et al., 2000). Furthermore, a preparation of phosphatidylinositol-specific phosphatidyl lipase C (PI-PLC), which can cleave GPI anchors, led to a decreased cell surface staining of NS1 and its recovery in the supernatant (Jacobs *et al.*, 2000). These results are interesting, as we were unsuccessful at metabolically labeling YF NS1 with [^{3}H]inositol, and labeling with [^{3}H]ethanolamine or [^{3}H]palmitate was extremely inefficient compared to a well-characterized GPI-anchored protein, human decay accelerating factor (DAF) (Lindenbach, 1999). Due to the extended labeling incubations, it was unclear whether the low level of labeling seen represented *bona fide* incorporation into a GPI anchor on only a small fraction of NS1 or whether the isotope was recycled metabolically into other precursors of NS1. Moreover, efficient GPI anchoring of this protein seems inconsistent with the C-terminal peptide sequencing data of WNV NS1 (Wengler *et al.*, 1990). The possibility remains that NS1 could usurp the GPI anchoring pathway to affect NS1-2A cleavage, while largely avoiding GPI anchoring. This would be quite unusual, as inhibitor studies have shown that removal of the GPI-anchoring signal and anchor addition are tightly coupled. Clearly it will be of interest to see the GPI-anchoring results confirmed.

It is not yet clear what functions are performed by the extracellular forms of NS1. During infection, strong humoral responses are made against this protein (Falgout *et al.*, 1990; Jacobs *et al.*, 1992; Lin *et al.*, 1998; Qu *et al.*, 1993; Schlesinger *et al.*, 1993, 1985; Timofeev *et al.*, 1998), and antibodies against the cell surface form can direct the complement-mediated lysis of flavivirus-infected cells (Henchal *et al.*, 1988; Schlesinger *et al.*, 1985, 1993). In addition, antibodies against NS1 can induce tyrosine phosphorylation of several unidentified proteins in DEN2-infected cells (Jacobs *et al.*, 2000), and it has been proposed that cross-reactive anti-NS1 antibodies may cause immunological defects (Chang *et al.*, 2002; Falconar, 1997). The secreted form of NS1 was originally characterized as the soluble complement-fixing antigen present in the serum and tissues of infected animals (Brandt *et al.*, 1970). Although an earlier study suggested that dimerization was necessary for NS1 secretion (Pryor and Wright, 1993), the KUN mutant with defective NS1 dimer formation was able to secrete NS1 (Hall *et al.*, 1999). Interestingly, secreted NS1 forms soluble hexameric particles of ≈11 nm (Crooks *et al.*, 1990, 1994; Flamand *et al.*, 1999). These particles appear to be three sets of dimers held together by hydrophobic interactions that could arise via sequestration of the putative membrane interaction surfaces of NS1 dimers.

It is clear that NS1 is important for RNA replication. NS1 colocalizes with VPs, the most likely sites of RNA replication (Mackenzie

et al., 1996; Westaway *et al.*, 1997b). Mutations at the first or both *N*-linked glycosylation sites led to dramatic defects in RNA replication (Muylaert *et al.*, 1996) and virus production (Muylaert *et al.*, 1996; Pryor *et al.*, 1998). Furthermore, alanine-scanning mutagenesis of YF NS1 revealed a temperature-sensitive mutation that caused a profound decrease in RNA accumulation (Muylaert *et al.*, 1997). It has been shown that KUN and YF NS1 can be supplied in *trans* (Khromykh *et al.*, 1999a, 2000; Lindenbach and Rice, 1997), and complementation studies revealed that NS1 functions at a very early stage in RNA replication. A YF mutant containing a large in-frame deletion of NS1 demonstrated undetectable levels of RNA replication with assays designed to detect the first cycle of minus-strand synthesis (Lindenbach and Rice, 1997). This mutant could not be complemented by DEN2 NS1, suggesting that DEN2 NS1 does not interact productively with the YF replicase (Lindenbach and Rice, 1999). However, this defect could be suppressed by a mutation in NS4A, indicating that an interaction involving NS1 and NS4A is required for replicase function (Lindenbach and Rice, 1999).

2. NS2A

NS2A is a relatively small (≈22 kDa), hydrophobic protein. The N terminus of NS2A is generated via the cleavage of NS1-2A by an unknown ER resident host enzyme (Falgout and Markoff, 1995), whereas the C terminus is generated by viral serine protease cleavage in the cytoplasm. Thus, this protein must be membrane spanning. In addition, a smaller (≈20 kDa), C-terminally truncated form, NS2Aα, can be made by use of an alternate, upstream serine protease cleavage site (Chambers *et al.*, 1990b; Nestorowicz *et al.*, 1994). Mutations that block either site are lethal for YF replication (Nestorowicz *et al.*, 1994). Further analyses indicated that a NS2Aα cleavage site mutant (Lys_{190}→Ser) exhibited a normal level of RNA replication but only made particles that lacked nucleocapsids (Kummerer and Rice, 2002). This defect could be complemented in *trans* by either NS2A or NS2Aα, provided that the complementing molecule contained a basic residue at position 190. Revertants and second site suppressor mutants were obtained, which either restored the basic nature around the cleavage site or changed NS3 residue Asp_{343} to a short, uncharged side chain. Although virus release was restored in these mutants, NS2Aα was not necessarily produced. Thus, the basic residue at NS2A residue 190, rather than NS2Aα cleavage per se, was an important determinant for infectious particle production. Furthermore, suppressor mutations in NS3 occurred at an acidic residue that is predicted

to be on the surface of the helicase domain of NS3 (discussed later), indicating that virion assembly also involves an interaction between NS2A and NS3 (Kümmerer and Rice, 2002). Studies with KUN replicons indicated that a deletion of NS2A was not complemented in *trans* (Khromykh *et al.*, 2000) and also suggested that NS3 was required in *cis* for virus particle formation (Liu *et al.*, 2002). NS2A was localized by cryoimmunogold staining to VPs, the presumed sites of RNA replication (Mackenzie *et al.*, 1998). Furthermore, a glutathione-*S*-transferase–KUN NS2A fusion protein was found to bind NS3, NS5, and the KUN 3′ NCR (Mackenzie *et al.*, 1998). Thus, this protein is likely to be involved in coordinating the shift between RNA packaging and RNA replication, processes that have been shown to be linked (Khromykh *et al.*, 2001b).

3. NS2B

The NS2B protein (≈14 kDa) is a small, membrane-associated protein. This protein forms a complex with NS3 and is a necessary cofactor for the serine protease in NS3 (Arias *et al.*, 1993; Chambers *et al.*, 1991, 1993; Falgout *et al.*, 1991; Jan *et al.*, 1995; Yusof *et al.*, 2000). The cofactor activity of NS2B is encoded in a conserved central hydrophilic region of 40 residues (Chambers *et al.*, 1993; Falgout *et al.*, 1993; Leung *et al.*, 2001), flanked by hydrophobic regions that mediate membrane association (Clum *et al.*, 1997). Mutations that disrupt NS2B–NS3 interaction also destroy serine protease activity (Chambers *et al.*, 1993; Clum *et al.*, 1997; Droll *et al.*, 2000; Falgout *et al.*, 1993; Jan *et al.*, 1995). JE NS2B expressed in *Escherichia coli* rendered the bacteria sensitive to the membrane-impermeable antibiotic hygromycin B, suggesting that it may also be involved in modulating membrane permeability during infection (Chang *et al.*, 1999).

4. NS3

NS3 is a large (≈70 kDa), multifunctional protein containing several enzyme activities that are involved in polyprotein processing and RNA replication. It is associated with membranes via its interaction with NS2B (Arias *et al.*, 1993; Chambers *et al.*, 1993; Clum *et al.*, 1997). Based on colocalization of NS3 and NS2B, CMs are presumed to be specialized sites of polyprotein processing (Westaway *et al.*, 1997b). NS3 has also been localized to VPs, presumed sites of RNA replication (Westaway *et al.*, 1997b). Because NS2B was not found in these latter structures, it is unclear whether NS3 involved in RNA replication is still associated with NS2B.

Based on sequence alignments, the N-terminal one-third of NS3 was predicted to be a trypsin-like serine protease (Bazan and Fletterick, 1989, 1990; Gorbalenya *et al.*, 1989a). This was confirmed by mutations in the proposed catalytic triad, which for YF NS3 are residues His_{53}, Asp_{77}, and Ser_{138} (Chambers *et al.*, 1990c; Pugachev *et al.*, 1993; Valle and Falgout, 1998; Wengler *et al.*, 1991; Zhang *et al.*, 1992). The minimal active protease domain was mapped to the N-terminal 167 amino acids of DEN NS3 (Li *et al.*, 1999). In addition to cleaving NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B, and NS4B/NS5 and generating C termini of mature capsid (Amberg *et al.*, 1994; Yamshchikov and Compans, 1994) and NS4A (Lin *et al.*, 1993a), NS2B-3 can also cleave at sites within NS2A (Nestorowicz *et al.*, 1994) and NS3 (see later). The protease preferentially cleaves after adjacent basic residues and before an amino acid containing a small, unbranched side chain (Chambers *et al.*, 1993, 1995; Lin *et al.*, 1993b; Nestorowicz *et al.*, 1994). Protease activity can be inhibited by aprotinin and substrate analogues (Leung *et al.*, 2001; Murthy *et al.*, 2000). A structural model for DEN NS3 has been proposed based on the solved structure of the hepatitis C virus serine protease (Brinkworth *et al.*, 1999). In addition, crystal structures for the DEN2 NS3 protease domain lacking the NS2B cofactor (Fig. 4), with or without a substrate inhibitor, have also been determined (Murthy *et al.*, 1999, 2000). These studies confirm the overall similarity to other members of this enzyme family, but reinforce an unusually flexible mode of substrate binding in the S1 pocket, as had been suggested from mutagenesis studies (Valle and Falgout, 1998). However, as NS2B was not included in these studies, the structure of an active flavivirus serine protease has not yet been determined. Recombinant single-chain proteases have been created by genetic fusion of the NS2B cofactor peptide and NS3 serine protease domain (Leung *et al.*, 2001; Yusof *et al.*, 2000). These molecules should be useful for improved biophysical and biochemical studies with an active protease.

The C-terminal region has been implicated in RNA replication, encoding regions of significant homology to supergroup 2 RNA helicases (Gorbalenya *et al.*, 1989b). These enzymes utilize the energy released from ATP hydrolysis to unwind RNA duplexes. Specifically, RNA-stimulated NTPase activity has been demonstrated for several full-length and N-terminally truncated NS3 proteins (Kuo *et al.*, 1996; Li *et al.*, 1999; Takegami *et al.*, 1995; Warrener *et al.*, 1993; Wengler and Wengler, 1991), and RNA unwinding activity has been demonstrated for DEN2 NS3 (Li *et al.*, 1999). Mutagenesis of the helicase active sites confirmed the essential role of this enzyme in viral replication

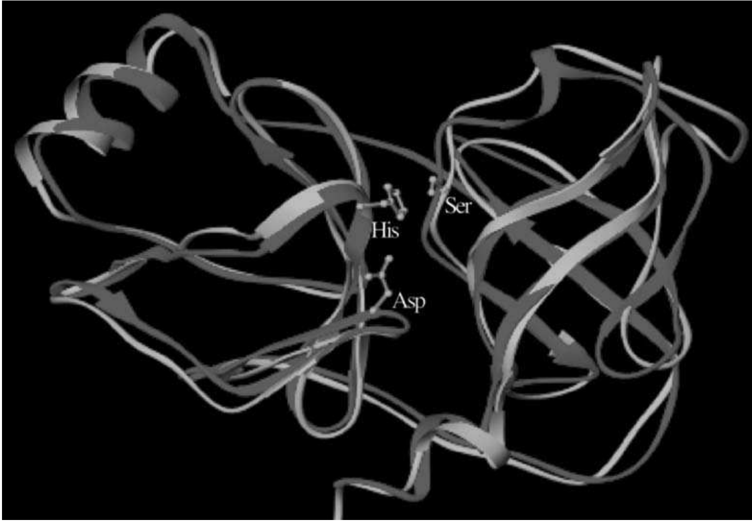


FIG 4. Structure of the viral serine protease domain of NS3. Superimposed Cα traces of DEN2 NS3 (cyan) and hepatitis C virus NS3 (magenta) serine protease domains, illustrating the typical chymotrypsin-like fold. The rms deviation between the 68 C-terminal Cα atoms of these protease structures is 0.9 Å. The catalytic triad is shown for the DEN2 protease. Figure kindly provided by Dr. H. M. Krishna Murthy. (See Color Insert.)

(Matusan *et al.*, 2001). Although helicase activities have been demonstrated or predicted for numerous positive-strand RNA viruses, their precise role in RNA replication remains unknown. Possible functions include melting regions of the RNA secondary structure involved in template recognition, increasing polymerase processivity by eliminating secondary structures, or resolving duplexes formed during the process of replication. In this regard, NS3 was shown to bind to the 3′ SL in association with NS5, and the NTPase activity of NS3 was enhanced in the presence of NS5 (Chen *et al.*, 1997; Cui *et al.*, 1998).

In addition to NTPase activity, the C-terminal region of NS3 encodes an RNA triphosphatase (RTPase) activity (Wengler and Wengler, 1993). It was originally proposed that this enzyme is involved in dephosphorylating the 5′ end of genomic RNA prior to cap addition (Wengler and Wengler, 1993), although it is presently unclear whether this activity is distinct from the NTPase activity. The RTPase activity of a purified, soluble WN NS3 C-terminal fragment was stimulated by Mg^{2+} and inhibited by poly(A), whereas NTPase activity was inhibited by Mg^{2+} and stimulated by poly(A) (Wengler and Wengler, 1993).

A recent study of the DEN2 C-terminal domain, partially purified from bacteria, demonstrated that both activities were inhibited by Mg^{2+}, high ionic strength, and a nonhydrolyzable ATP analogue, ATP-γ-S (Bartelma and Padmanabhan, 2002). Moreover, mutation of Lys_{199} in the putative NTP-binding site destroyed both activities (Bartelma and Padmanabhan, 2002; Li *et al.*, 1999). These data suggest that both activities share a common active site, although the effect of this mutation on the overall structure of the protein has not yet been addressed. It is hoped that further structural studies may bring clarity to these issues.

Truncated forms of NS3 have been observed for DEN2 and TBE, which result from an alternative serine protease cleavage site in the the helicase domain (Arias *et al.*, 1993; Pugachev *et al.*, 1993; Teo and Wright, 1997). It is unclear what the role of these cleavages are, although it is possible that the products could have a distinct function. In this regard, it has been found that KUN replicons bearing large inframe deletions in the helicase domain could be complemented in *trans*, whereas deletions in the serine protease domain could not (Khromykh *et al.*, 2000; Liu *et al.*, 2002).

5. NS4A and NS4B

NS4A and NS4B are small (16 and 27 kDa, respectively), hydrophobic proteins of unknown function. As mentioned previously, a mutation in YF NS4A allowed for the utilization of DEN2 NS1 in *trans*, suggesting a role in RNA replication (Lindenbach and Rice, 1999). This is supported by the colocalization of this protein to VPs, membrane structures presumed to be sites of RNA replication, as well as to CMs, which are thought to be involved in polyprotein processing (Mackenzie *et al.*, 1998). The C terminus of NS4A acts as a signal sequence for the translocation of NS4B into the ER lumen. However, as mentioned previously for capsid protein, processing of the NS4A–NS4B junction by signal peptidase requires NS2B–NS3 serine protease cleavage at a site just upstream of the signal peptide (Lin *et al.*, 1993a; Preugschat and Strauss, 1991). It is not yet clear why these cleavage events are coordinated in this manner. In addition, unprocessed NS3-4A and NS4A–NS4B forms have been observed (Chambers *et al.*, 1990b; Lobigs, 1992; Preugschat and Strauss, 1991). NS4B is a transmembrane protein that localizes to sites of replication and to the nucleus (Westaway *et al.*, 1997a). It is modified posttranslationally to a form that migrates faster on SDS–PAGE (Chambers *et al.*, 1990b; Preugschat and Strauss, 1991). The function of NS4B, and the nature of this modification, remain to be determined.

6. *NS5*

NS5 is a large (103 kDa), well-conserved, multifunctional protein involved in RNA replication. It contains N-terminal RNA cap-processing activity and C-terminal RdRP activity. NS5 can be phosphorylated by an associated serine/threonine serine kinase(s) (Kapoor *et al.*, 1995b; Morozova *et al.*, 1997; Reed *et al.*, 1998).

The N-terminal region (amino acids 60 to 132) of NS5 was found to contain homology with *S*-adenosyl-methionine (SAM)-dependent methyltransferases, suggesting that this protein is involved in formation of the 5′ cap (Koonin, 1993). Support for this hypothesis came from a study demonstrating that the N-terminal domain of DEN2 NS5 could transfer methyl groups from SAM to capped RNA substrates (Egloff *et al.*, 2002). Furthermore, this group determined the crystal structure of NS5 residues 7 to 267 at 2.4-Å resolution (Fig. 5), validating a structural similarity between this region and other methyltransferases (Egloff *et al.*, 2002). Interestingly, *S*-adenosyl-homocysteine copurified and cocrystallized with this

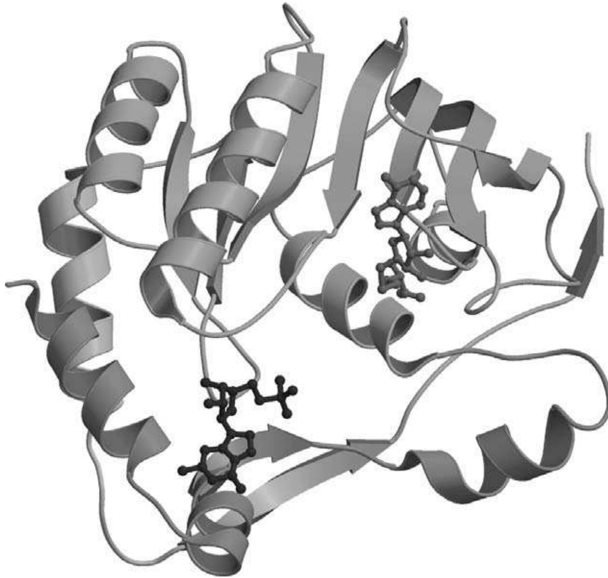


FIG 5. Structure of the NS5 methyltransferase domain. This ribbon diagram illustrates the folding of DEN2 NS5 residues 7 to 267. Individual subdomains are colored orange, cyan, and yellow, whereas bound GDPMP and *S*-adenosyl-homocysteine are colored purple and red, respectively. Figure courtesy of M. Egloff and B. Canard. (See Color Insert.)

recombinant protein, confirming that the putative SAM-binding motif actually contributes to substrate binding. A series of papers had previously demonstrated that deletions in regions that turned out to be involved in SAM binding were lethal for the replication of KUN (Khromykh *et al.*, 1998, 1999b; Liu *et al.*, 2002). Some of these deletions could be complemented in *trans*, whereas others could not (Khromykh *et al.*, 1998, 1999b; Liu *et al.*, 2002). Because the methyltransferase domain forms a compact monomeric structure, the biochemical basis for these genetic observations remains unclear. In addition, biochemical and biophysical studies revealed that GTP is bound with high specificity via an unusual set of contacts (Egloff *et al.*, 2002). Mutations that disrupted these contact sites ablated GTP binding *in vitro* (Egloff *et al.*, 2002) and were lethal in the context of a DEN2 infectious clone (Hanley *et al.*, 2002). This functionally important, unique mode of GTP binding indicates that this interaction might be an attractive drug target.

The C terminus of NS5 contains significant homology to RdRPs of other positive-strand RNA viruses (Koonin, 1993; Rice *et al.*, 1985). Polymerase activity of this protein has been confirmed with recombinant NS5 (Ackermann and Padmanabhan, 2001; Guyatt *et al.*, 2001; Tan *et al.*, 1996). The major product of these *in vitro* reactions appears to be a self-primed "copy-back" RNA (Ackermann and Padmanabhan, 2001; Guyatt *et al.*, 2001; Tan *et al.*, 1996). Based on the replication strategies of other positive-strand viruses, as well as a lack of evidence for formation of these products *in vivo*, it is expected that this mode of RNA initiation is probably not relevant for authentic RNA replication. Rather, *de novo*–initiated RNA synthesis has been demonstrated for DEN2 NS5 (Ackermann and Padmanabhan, 2001). Interestingly, self-priming vs *de novo* initiation was shown to be temperature dependent, which could reflect the temperature-induced conformational change in NS5 and/or breathing of the RNA template (Ackermann and Padmanabhan, 2001). Mutagenesis of the KUN polymerase active site motif confirmed that it is essential for virus replication and that polymerase activity could be supplied in *trans* from a KUN replicon (Khromykh *et al.*, 1998). However, *trans* complementation of this defect was inefficient in cells expressing only NS5, suggesting that cotranslational expression of additional NS proteins may be required for the RdRP to associate with other replicase components (Khromykh *et al.*, 1999a).

As NS5 has been shown to function as an RdRP, it must localize to sites of RNA replication, although this remains to be demonstrated. NS5 forms a complex with NS3 (Kapoor *et al.*, 1995b) and can stimulate NS3 NTPase activity (Cui *et al.*, 1998). Cross-linking studies

have shown that both proteins bind to the 3′ SL (Chen *et al.*, 1997). Interaction with the helicase domain of NS3 has been mapped via a yeast two-hybrid assay to a small region (DEN2 NS5 residues 320 to 368) between the methyltransferase and polymerase domains (Johansson *et al.*, 2001). Given these associations, it is surprising that NS5 can be separated physically from membrane fractions enriched for RdRP activity (Chu and Westaway, 1992; Grun and Brinton, 1987). This suggests that perhaps only a fraction of NS5 resides in active replication complexes. This would be consistent with observations from numerous other positive-stranded and negative-stranded RNA viruses, that only a limited amount of RdRP is needed for virus replication (Buck, 1996). NS5, and the phosphorylated form in particular (Kapoor *et al.*, 1995b), has been shown to localize to the nucleus (Buckley *et al.*, 1992; Kapoor *et al.*, 1995b). Interestingly, the interdomain region that directs NS5 interaction with NS3 has been shown to contain nuclear localization signals recognized by importin β1 and importin α/β (Brooks *et al.*, 2002; Johansson *et al.*, 2001).

VI. RNA Replication

The viral RNA replicase is assembled from NS proteins, together with the genomic RNA template and presumably some host factors, on cytoplasmic membranes. As discussed, localization of viral RNAs and replication proteins have indicated that VPs are the most likely sites of RNA replication (Mackenzie *et al.*, 1996, 1998; Westaway *et al.*, 1997b, 1999). It should be noted that these structures have only been seen during later times of infection, and it is presently unclear where early events in RNA replication occur. Replication begins with the synthesis of a negative-strand RNA, which serves as a template for the synthesis of additional positive-strand genomic RNA. RNA synthesis is asymmetric, leading to a 10- to 100-fold excess of positive strands over negative strands (Cleaves *et al.*, 1981; Muylaert *et al.*, 1996). Negative strands continue to accumulate throughout the infection and have been isolated exclusively in double-stranded forms (Cleaves *et al.*, 1981; Wengler *et al.*, 1978). Three species of viral RNA can be labeled metabolically *in vivo*. In pulse-chase experiments, label is first incorporated into RNA forms that sediment as a sharp peak at 20S and as a broad peak between 20S and 28S (Cleaves *et al.*, 1981). Based on its solubility in 2 *M* LiCl and resistance to single-strand specific ribonucleases, the 20S form appears to be a genome-length, double-stranded RNA, similar to the poliovirus replicative form (RF) (Baltimore, 1968; Cleaves

et al., 1981; Wengler *et al*., 1978). The heterogeneous (20S to 28S) RNA is insoluble in 2 *M* LiCl and is partially resistant to nuclease, indicating that it contains both single-stranded and double-stranded regions (Cleaves *et al*., 1981). This form, often called the replicative intermediate (RI), likely contains recently synthesized RNAs that have been displaced by nascent elongating strands (Chu and Westaway, 1985; Cleaves and Dubin, 1979). Both RF and RI forms can be chased into 40S RNA, which is insoluble in 2 *M* LiCl, fully sensitive to ribonuclease, and indistinguishable from genomic RNA (Cleaves *et al*., 1981; Wengler *et al*., 1978).

20S, 20 to 28S, and 40S RNA forms have also been described for *in vitro* RdRP reactions using infected cell extracts (Bartholomeusz and Wright, 1993; Chu and Westaway, 1985, 1987; Grun and Brinton, 1986, 1987, 1988; You and Padmanabhan, 1999). Further characterization of these extracts indicates that RdRP activity is associated with dense membrane fractions (Chu and Westaway, 1992; Grun and Brinton, 1987) that are enriched for NS2A, NS2B, NS3, and NS4A (Chu and Westaway, 1992). As discussed previously, only a fraction of NS5 cosedimented with RdRP activity (Chu and Westaway, 1992; Grun and Brinton, 1987), which could reflect regulation of the enzyme activity of NS5. Most RdRP products formed *in vitro* appear to involve the elongation of endogenous templates rather than *de novo* synthesis, although reinitiation of RNA synthesis has been described (Chu and Westaway, 1987). A system for initiation from exogenous templates has been devised (You and Padmanabhan, 1999). The major product of these reactions arises from self-primed "copy-back" synthesis and requires templates containing 5′ and 3′ cyclization sequences (You and Padmanabhan, 1999).

NS3 contains an RNA triphosphatase that presumably dephosphorylates the 5′ end of genomic RNA in preparation for 5′ cap addition (Wengler and Wengler, 1993), whereas NS5 contains a cap methyltransferase (Egloff *et al*., 2002; Koonin, 1993). However, it is not yet clear what enzyme catalyzes the guanylyl transfer reaction.

In summary, much has been learned about flavivirus RNA replication, particularly about the role of some viral proteins and RNA *cis* elements. However, we do not yet have a complete picture of this crucial process. Of particular interest will be insights into the roles of the small membrane-associated NS proteins, NS1, and host factors. In addition, fundamental issues that are not fully understood for any positive-strand RNA virus include the need for RNA replicases to associate with host membranes, as well as the regulation between replication and translation of viral genomes.

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ADVANCES IN VIRUS RESEARCH, VOL 59

FLAVIVIRUS STRUCTURE AND MEMBRANE FUSION

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Preamble
I. Introduction
II. Assembly and Maturation
III. Structure of the E Protein
IV. Architecture of Recombinant Subviral Particles and Virions
V. Fusion Characteristics
VI. Low pH-Induced Structural Changes
VII. Interactions with Membranes
VIII. Function of the prM Protein
IX. Comparative Aspects of the Flavivirus Fusion Machinery
References
Addendum
References for Addendum

Preamble

In the time since the preparation of the original version of this article in March 2002, there has been a flood of new information about flavivirus structure and membrane fusion, rendering some portions of our manuscript obsolete before going to press. In order to make this article more up-to-date, we have deleted some passages and included an addendum (page 91) summarizing the results of recent studies that we feel have made an especially important impact on the field. Several other new studies that have also made significant contributions had to be omitted because of space constraints.

I. Introduction

Flaviviruses are spherical enveloped viruses with a diameter of approximately 50 nm that contain only three structural proteins: E (envelope), prM/M (membrane), and C (capsid). The prM glycoprotein, which is present in immature virions, is cleaved late in the maturation process by a cellular protease to yield fully infectious mature virions (Figs. 1 and 2). The current view of the flavivirus life cycle,

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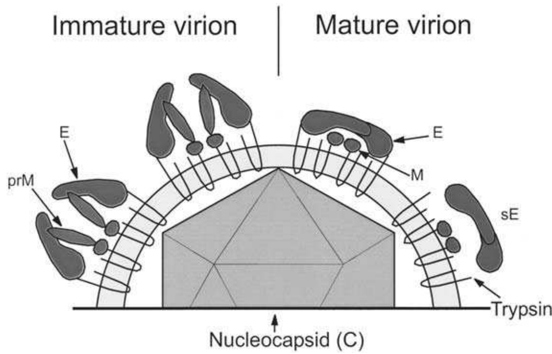
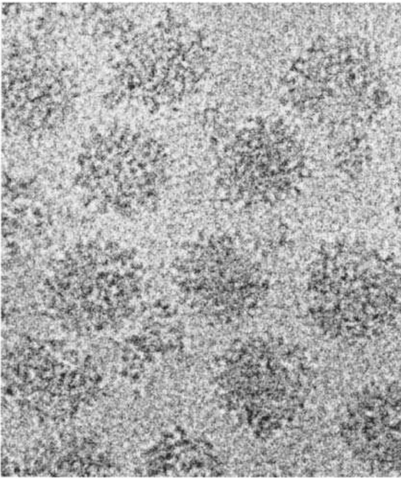


FIG 1. Schematic of a flavivirus in its immature and mature form. A soluble dimeric ectodomain fragment of the E protein (sE) isolated from mature TBE virions by limited trypsin digestion was used for structure determination by X-ray crystallography (Rey *et al.*, 1995). Adapted from Heinz and Allison (2001) with permission.

Immature TBE virus



Mature TBE virus

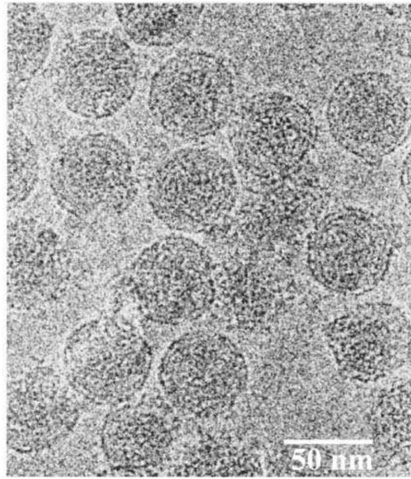


FIG 2. Cryoelectron micrographs of purified preparations of immature (left) and mature (right) TBE virions. Courtesy of S. D. Fuller, University of Oxford.

including entry, assembly, maturation, and release (Lindenbach and Rice, 2001), is shown schematically in Fig. 3. Virus entry occurs by receptor-mediated endocytosis, and the acidic pH in the endosome triggers structural alterations in the E protein that lead to the fusion of the viral membrane with the endosomal membrane and the release

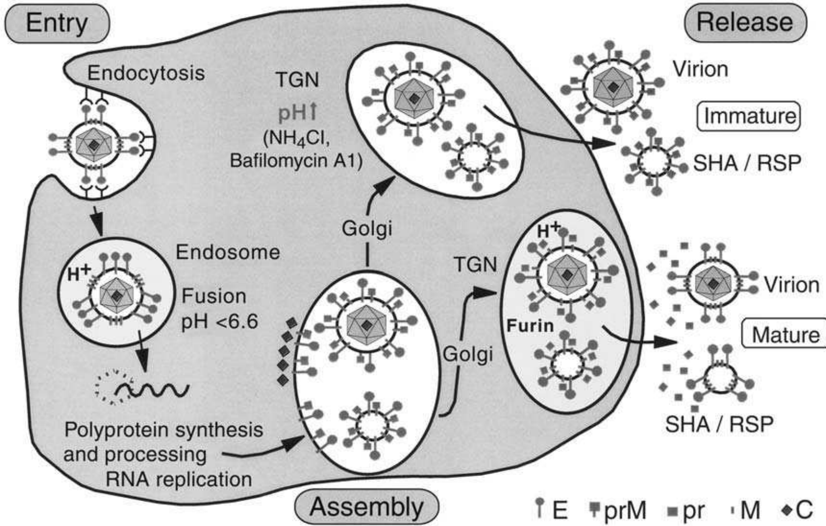


FIG 3. Schematic of the flavivirus life cycle, highlighting the processes involving the envelope glycoproteins prM and E. TGN, *trans*-Golgi network; SHA, slowly sedimenting hemagglutinin; RSP, recombinant subviral particle. Acidic compartments are depicted in yellow. Adapted from Heinz and Allison (2001) with permission.

of the nucleocapsid. During assembly, immature prM-containing virions are believed to be formed in the endoplasmic reticulum (ER) and transported through the secretory pathway of the cell. The maturation cleavage of prM is apparently mediated by furin in the *trans*-Golgi network shortly before the release of mature virions by the fusion of secretory vesicles with the plasma membrane. Capsidless subviral particles (originally designated SHA, "slowly sedimenting hemagglutinin") containing the envelope proteins but lacking the nucleocapsid are natural by-products of flavivirus assembly and are released from infected cells. Similar particles can also be generated in recombinant form (see Section II).

Significant progress has been made toward the understanding of the structure and organization of the flavivirus virion. This has included the solution of the structure of the E protein of the tick-borne encephalitis (TBE) virus at atomic resolution by X-ray crystallography (Rey *et al.*, 1995) and a 19-Å image reconstruction of a TBE virus recombinant subviral particle based on cryoelectron micrographs (Ferlenghi *et al.*, 2001). The most important structural features revealed by these studies are (1) an icosahedral arrangement of the

envelope proteins and (2) a characteristic envelope protein structure, which, together with the alphavirus E1 protein, defines a separate class of viral fusion protein. These structural data, combined with a number of functional studies, have allowed us to begin to understand the mechanisms of flavivirus assembly, maturation, and entry at the molecular level. (*For new structural information, see Addendum page 91.*)

This chapter focuses primarily on structural aspects of the envelope glycoproteins, their organization in the viral envelope, and the mechanism of virus-induced membrane fusion. Other aspects related to the virion and envelope proteins, including antigenic structure, receptor binding, the role of the E protein as a molecular determinant of virulence, and its importance as a vaccine constituent, are dealt with in separate chapters of this book.

II. Assembly and Maturation

Ultrastructural studies have revealed that flavivirus-infected cells develop an extensive proliferation of intracellular membranes and vacuoles (for review, see Lindenbach and Rice, 2001). Because virion particles first become visible in the lumen of the rough endoplasmic reticulum (RER), it is generally believed that particle formation occurs by a still undefined assembly and budding process at the RER membrane. Cryoimmunoelectron microscopy studies carried out with the Kunjin virus using drugs that inhibit intracellular protein and/or membrane transport (Mackenzie and Westaway, 2001) support a maturation model that involves virion assembly in the ER, transport of individual particles in transport vesicles to the Golgi apparatus, movement through the individual stacks into the *trans*-Golgi region, accumulation within larger vesicles, and release by exocytosis. Final proof of such a mechanism has been difficult to establish because of the inability to unambiguously demonstrate intermediate budding structures or to identify or isolate free core particles from flavivirus-infected cells. Some studies have indeed suggested that assembly can also occur at the plasma membrane (Hase *et al.*, 1987; Ng *et al.*, 1994; Rahman *et al.*, 1998), and it has been proposed that the site of budding may be virus and cell type dependent (Mackenzie and Westaway, 2001).

The apparently final maturation step for producing fully infectious virions is the proteolytic cleavage of the prM protein immediately after the sequence Arg-X-Arg/Lys-Arg, which corresponds to the consensus sequence for the cellular protease furin, an enzyme that is concentrated in the TGN but cycles between endosomes and the plasma membrane as

well (Molloy *et al.*, 1999). The efficiency of this cleavage activation, however, seems to differ among flaviviruses, and a great deal of variability in the proportion of uncleaved prM found in extracellular virus particles has been reported (Murray *et al.*, 1993; Wang *et al.*, 1999). The functional significance of this cleavage is discussed in more detail in Section VIII.

Flavivirus-infected cells have also been shown to release noninfectious subviral particles containing only the envelope proteins, and these have been designated "slowly sedimenting hemagglutinin" (Russell *et al.*, 1980; Smith *et al.*, 1970). Similar particles can also be generated in recombinant form (recombinant subviral particles, RSPs) by coexpression of the envelope proteins prM and E in the absence of other viral components. Such particles have been described for several different flaviviruses (Allison *et al.*, 1995b; Chang *et al.*, 2000; Davis *et al.*, 2001; Fonseca *et al.*, 1994; Hunt *et al.*, 2001; Konishi and Fujii, 2002; Konishi *et al.*, 1991, 1992a,b, 1994, 2001; Mason *et al.*, 1991; Pincus *et al.*, 1992; Pugachev *et al.*, 1995; Sato *et al.*, 1993; Yamshchikov and Compans, 1993), but they have been characterized most extensively in the case of TBE virus (Corver *et al.*, 2000; Ferlenghi *et al.*, 2001; Schalich *et al.*, 1996). RSPs are somewhat smaller than virions (30 vs 50 nm diameter), but like whole virions, they contain a lipid membrane, and their pathway of assembly—intracellular transport, carbohydrate processing, maturation cleavage of prM, and secretion—seems to be very similar to that of the virus. The self-assembly of RSPs suggests that specific interactions between the envelope proteins alone, in the absence of any interactions with the capsid protein, are sufficient to drive the assembly process, but it has not been ruled out that an as yet unidentified cellular cofactor interacting with either prM or E could be involved in particle formation. Further details of RSP structure and its functions are described in Section IV.

Newly synthesized prM and E proteins associate together to form heterodimers (Allison *et al.*, 1995b; Wengler, 1989), which are then incorporated into the immature particle. It has been shown that coexpression with prM is essential for proper maturation, transport, and secretion of the E protein (Allison *et al.*, 1995b; Konishi and Mason, 1993), suggesting not only that prM–E heterodimers are essential building blocks for particle formation, but also that prM might play a role in the folding of E.

The folding and oligomerization of TBE virus envelope proteins were studied in more detail by pulse-chase experiments using virus-infected cells as well as recombinant plasmid expression systems that allow the translation of prM and E together or individually (Lorenz *et al.*, 2002). By coprecipitation and cosedimentation analysis it was shown

that the two proteins rapidly formed a heterodimeric complex within a few minutes after synthesis. Analysis of disulfide bridge formation and reactivity with conformation-specific antibodies, however, revealed a significant difference between the rates of folding of prM and E. prM was shown to be a very rapidly and independently folding protein, acquiring its native folded structure very quickly ($t_{1/2} = 4$ min) in the absence of any interaction with E. In contrast, the E protein folded significantly more slowly ($t_{1/2} =$ ca. 15 min) and, although able to form disulfide bridges when expressed alone, required the coexpression of and association with prM to acquire its native conformation. This is consistent with a chaperone-like role for prM in the folding of E.

The intracellular pool of prM–E heterodimers not incorporated into particles is apparently retained in the ER, as revealed by immunolocalization studies (Mackenzie and Westaway, 2001; Mackenzie *et al.*, 1999) and the fact that both proteins remain fully sensitive to digestion with endoglycosidase H_f in cellular extracts (Courageot *et al.*, 2000; Lorenz *et al.*, 2002; Mason, 1989; Murray *et al.*, 1993). prM and E do not appear to possess typical ER retention signals (Teasdale and Jackson, 1996), which are usually dibasic motifs that reside either within the transmembrane region or in the cytoplasmic tail (which itself is almost completely lacking in both proteins), so the mechanism of ER retention is not obvious. However, using sedimentation analysis of detergent-solubilized prM–E complexes from dengue virus-infected cells, Wang *et al.* (1999) demonstrated that prM–E heterodimers gradually assemble into higher order complexes that are retained in the cell for several hours. Preliminary studies have suggested the presence of similar complexes or aggregates in TBE virus–infected cells (A. Helenius and I. Lorenz, personal communication), and in this case it has been proposed that ER retention is due to the formation of heterooligomeric clusters that are too large to be transport competent. According to this model, only proteins that separate from these clusters by budding into the ER (via virus or subviral particle formation) would be transported and secreted by the cellular exocytic pathway.

In several instances, the carbohydrate side chains in extracellular virions have been shown by endo H digestion or lectin binding (Johnson *et al.*, 1994; Schalich *et al.*, 1996; Winkler *et al.*, 1987) to be at least partially of the high mannose type, and it has been speculated that this incomplete processing is due to an overloading of the sugar-modifying enzymes by the large number of glycoproteins on the virion surface and/or their rapid transport through the secretory pathway.

III. Structure of the E Protein

Limited treatment of purified TBE virions with trypsin yields a soluble, crystallizable dimeric fragment of the E protein (SE) (Heinz *et al.*, 1991), the atomic structure of which was solved several years ago to a resolution of 2.0 Å (Rey *et al.*, 1995). (*A new high resolution structure of a recombinantly expressed dengue 2 virus dimeric E Protein fragment has been determined. See Addendum.*) The TBE virus SE fragment lacks the C-terminal membrane-spanning regions and an intervening stem structure of approximately 40 amino acids. The crystallized ectodomain (Fig. 4) is an elongated dimer with overall dimensions of 150 × 55 × 30 Å. As shown in the side view (Fig. 4), the dimer is oriented parallel to the viral membrane and has a slight curvature that corresponds to the curvature of the viral membrane. The top view (Fig. 4) shows that two monomeric subunits form a head-to-tail dimer and that each of the monomers is composed of three distinct domains, designated domains I, II, and III. The contacts between the monomers that form the dimer are not contiguous along the whole length of the molecule but are interrupted, resulting in two holes along the dimer axis. The contact at the center mostly involves hydrophilic side chains of domain II only, whereas the distal contact is largely nonpolar and is composed of residues from domains I and III on one subunit and the tip of domain II on the other.

With the exception of two short α helices in domain II, β strands are the predominating secondary structures throughout the molecule. The centrally located domain I has the structure of an eight-stranded up-and-down β barrel with its axis oriented parallel to the viral membrane. This N-terminal domain contains two disulfide bridges and on its external surface carries the single carbohydrate side chain.

Domain II has an elongated finger-like structure that is composed of two long discontinuous loops extending from domain I. One of these loops is stabilized by three disulfide bridges and forms the tip that interacts with a hydrophobic pocket provided by adjacent residues at the junction of domains I and III of the second monomer. The loop constituting this tip (cd loop; Rey *et al.*, 1995) is highly conserved among all flaviviruses and functions as an internal fusion peptide (see Section VII). Domain III contains the C terminus and has the typical fold of an immunoglobulin constant domain. It contains a single disulfide bond and is connected to domain I by a stretch of 15 amino acids. As can be seen in the side view (Fig. 4), the axis of the Ig β barrel is perpendicular to the virion surface and its tip projects higher than any other part of the protein. A significant difference between tick-borne and mosquito-borne flaviviruses can be

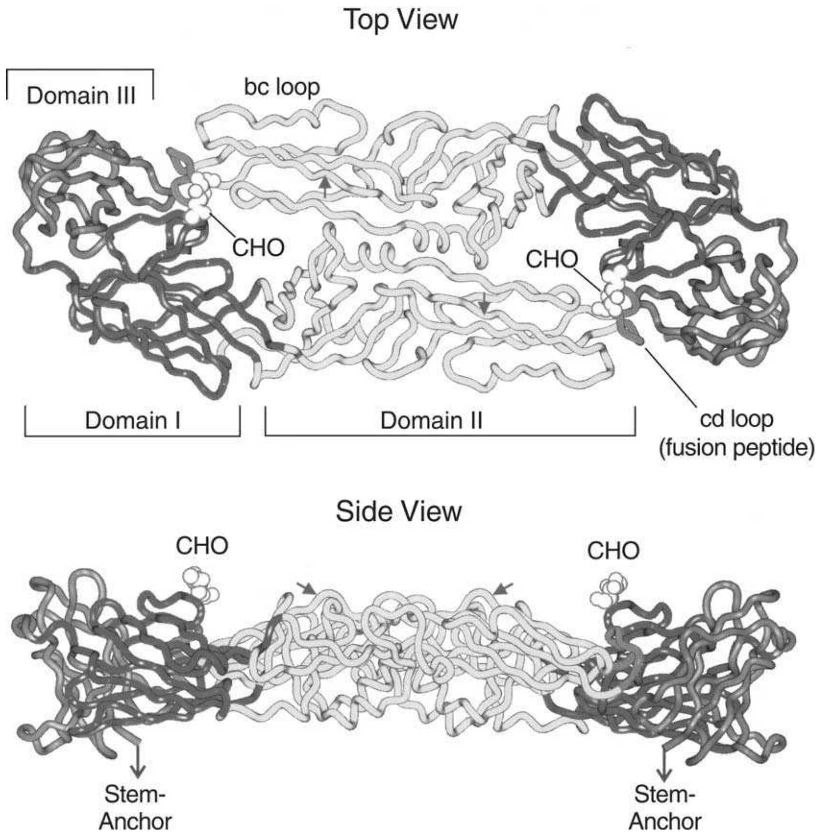


FIG 4. Top and side views of the TBE virus sE dimer, depicted as a ribbon diagram of the structure determined by X-ray crystallography (Rey *et al.*, 1995). CHO, carbohydrate attached to Asn 154. The arrow indicates the approximate position of a second glycosylation site found in certain dengue viruses (Johnson *et al.*, 1994). Adapted from Heinz and Allison (2001) with permission. (See Color Insert.)

seen on the upper lateral surface of domain III. In the latter, a tight turn between G and F sheets in the TBE E protein structure is extended by four amino acids, which in many cases include an RGD sequence reminiscent of an integrin-binding motif. Because such motifs and immunoglobulin-like domains in general are frequently involved in receptor binding, domain III has been proposed to be involved in cell surface interactions, a hypothesis that is supported, at least indirectly, by a number of studies (Bhardwaj *et al.*, 2001; Chen *et al.*, 1997; Crill and Roehrig, 2001; Hurrelbrink and McMinn, 2001; Lee and Lobigs, 2000; Mandl *et al.*, 2000; Thullier *et al.*, 2001; van der Most *et al.*, 1999). Details about

current knowledge of the interaction of flaviviruses with the cell surface are presented elsewhere in this book series.

The amino acid identity in the E protein is at least 35% between any two flaviviruses and the 12 cysteine residues are absolutely conserved. These had been assigned to six disulfide bridges in the West Nile virus E protein (Nowak and Wengler, 1987) and were found at the expected positions in the X-ray structure of the TBE virus E protein. It is therefore justified to assume that the overall structural organization and folding into three domains are similar for all flavivirus E proteins. Knowledge-based homology modeling has indeed been used to predict the three-dimensional structure of the Japanese encephalitis virus E protein (Kolaskar and Kulkarni-Kale, 1999). Structural elements that vary in size and sequence among flaviviruses are located primarily on the external surface of the molecule. The highest degree of variability is found in the loop carrying the carbohydrate side chain (E_0F_0 loop in domain I) and the fg loop in domain II (Rey *et al.*, 1995). Binding sites for antibodies (including neutralizing antibodies) have been mapped to different locations in each of the three domains, consistent with the view that the entire external surface of the E protein is antigenically active, and evidence suggests that all three domains also have the potential to be involved in cell surface recognition (Mandl *et al.*, 2001).

In the TBE virus E protein structure, the dimer contact involving the fusion peptide appears to be shielded by the single carbohydrate side chain attached to Asn 154 in domain I. Although a glycosylation site at the homologous position is found in many other flaviviruses as well, glycosylation is not absolutely conserved. The E proteins of several isolates of West Nile/Kunjin virus have been shown to be nonglycosylated, although other isolates are glycosylated at position 154 (Adams *et al.*, 1995; Chambers *et al.*, 1998; Wengler *et al.*, 1985). Similar strain-specific variations of glycosylation were also found for the St. Louis encephalitis virus (Vorndam *et al.*, 1993), the Yellow fever virus (Ballinger and Miller, 1990; Post *et al.*, 1992), and dengue viruses (Johnson *et al.*, 1994; Lee *et al.*, 1997). With dengue viruses it was shown that passaging in mosquito cells (Den 3) (Lee *et al.*, 1997) or the use of procedures that selected for an altered fusion pH (Den 2) (Johnson *et al.*, 1994) could result in the loss of the glycosylation site at Asn 153. Natural isolates of the Den 2 virus were shown to be glycosylated at position 153, whereas Den 1 virus isolates have been shown to carry an additional carbohydrate side chain at position Asn 67 (Johnson *et al.*, 1994) that resides in an exposed β sheet in domain II (Fig. 4). The precise role of glycosylation for the biosynthesis, stability,

and/or functions of the E protein remains to be determined, but it apparently varies between different flaviviruses and their strains.

The C-terminal stem-anchor region, for which detailed structural data are not yet available, consists of about 80 amino acids. (*For new structural information, see Addendum.*) Secondary structure predictions of this region indicate the presence of several distinct structural elements, which are shown schematically in Fig. 5. These include two membrane-spanning regions (TM1 and TM2) at the C terminus and two predicted α-helical regions flanking a highly conserved sequence element in the stem region, the part of the polypeptide chain that lies between the membrane anchor and domain III. Expression studies with C-terminally truncated forms of the E protein of the TBE virus (Allison *et al.*, 1999) and dengue-2 virus (P. Young, personal communication) have provided evidence that the stem probably has several functional roles, including stabilization of the prM–E complex, particle formation, and the facilitation of low pH-induced structural rearrangements (see also Section VI).

IV. Architecture of Recombinant Subviral Particles and Virions

Cryoelectron microscopy and image reconstruction studies provide evidence that flaviviruses, like alphaviruses, have an icosahedral organization of envelope proteins. The structure of a capsidless TBE

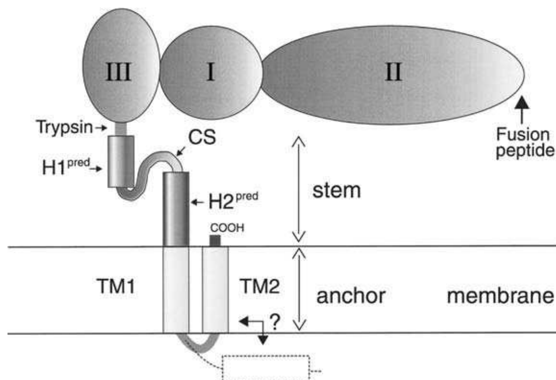


Fig 5. Schematic drawing of domains I, II, and III together with the stem-anchor region of the flavivirus E protein. $H1^{pred}$ and $H2^{pred}$, predicted α-helical regions; CS, conserved sequence element; TM1 and TM2, transmembrane segments. (*For new structure and topology data, see Addendum.*) Adapted from Allison *et al.* (1999) with permission.

virus recombinant subviral particle (RSP, see also Section II) has been determined to a resolution of 19 Å (Ferlenghi *et al.*, 2001), revealing the specific arrangement of 30 E protein dimers in an icosahedral surface lattice. Fitting of the high-resolution structure of E (Rey *et al.*, 1995) into the cryo-EM density allowed the identification of specific lateral dimer contacts between complementary surfaces that stabilize this particle (Fig. 6A). The principal contacts involve the bc loop in domain II of one subunit and a groove provided by domains I and III of the other (Fig. 6B). The position of the M protein in RSPs has not been identified unambiguously, but it could be located at local threefold symmetry axes, where some extra electron density is found that is not accounted for by the E protein. It is a characteristic structural feature of RSPs that they contain holes at the fivefold symmetry axes that are lined by the domain III of five E protein subunits (Fig. 6A). This arrangement makes the lateral surface of this domain accessible for external ligands, consistent with its putative receptor-binding function and the presence of binding sites for neutralizing antibodies (Ferlenghi *et al.*, 2001; Rey *et al.*, 1995). (*For the architecture of virions based on new structural information, see Addendum.)*

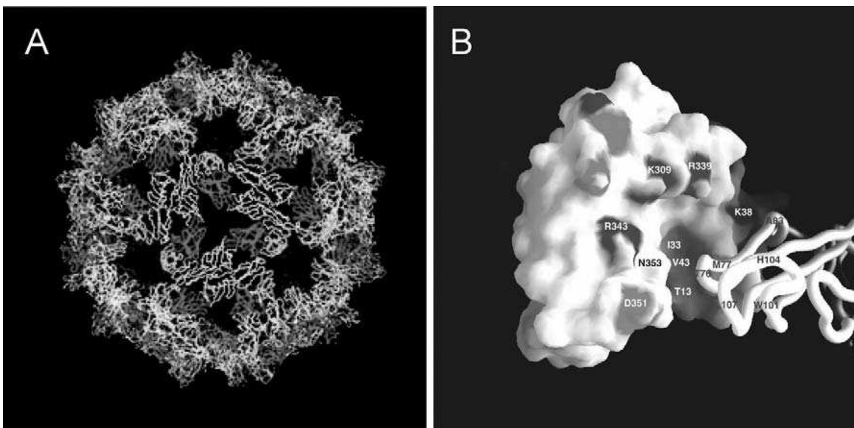


FIG 6. (A) Organization of E protein dimers in the TBE virus RSP as deduced from fitting the high-resolution sE structure into an image reconstruction of cryoelectron micrographs (Ferlenghi *et al.*, 2001). (B) The site of lateral interactions in the icosahedral lattice of E protein dimers in the TBE virus RSP (Ferlenghi *et al.*, 2001). The bc loop of one subunit (depicted as a "worm") fits into a groove in domain III of an adjacent dimer (surface representation). Electrostatic potentials are shown in red (negative) and blue (positive). Modified from Ferlenghi *et al.* (2001). (See Color Insert.)

V. Fusion Characteristics

The analysis of the fusion properties of flaviviruses in several different assay systems, including cell–cell fusion and virus–liposome fusion (Corver *et al.*, 2000; Desprès *et al.*, 1991; Gollins and Porterfield, 1986a; Guirakhoo *et al.*, 1991, 1993; Randolph and Stollar, 1990; Summers *et al.*, 1989; Ueba and Kimura, 1977; Vorovitch *et al.*, 1991), has consistently demonstrated a requirement for an acidic pH for triggering fusion activity. The pH threshold for fusion, however, is relatively high (pH 6.6 to 6.8) (Corver *et al.*, 2000; Desprès *et al.*, 1991; Gollins and Porterfield, 1986a; Guirakhoo *et al.*, 1991, 1993; Randolph and Stollar, 1990; Summers *et al.*, 1989; Vorovitch *et al.*, 1991), suggesting that fusion occurs at an early stage of the endocytic pathway, probably in early endosomes, which have a lumenal pH corresponding to the fusion threshold (Clague, 1998; Mellman, 1996).

A detailed kinetic study on the fusion properties of the TBE virus and RSPs with liposomes was conducted by Corver *et al.* (2000) using particles that were metabolically labeled in their lipid membranes with the fluorophore 1-pyrenehexadecanoic acid (C16-pyrene). A striking result of this study was that the TBE virus exhibited a fusion rate that was unprecedented by that of any other enveloped virus analyzed to date, with a fusion rate at 37°C of about 40% per second and an extent of fusion of more than 50% within the first 2 to 3 s. Also, there was no measurable lag phase, a typical kinetic feature of fusion by several other enveloped viruses (for review, see Heinz and Allison, 2000). Only by reducing the temperature to 4°C could a short lag phase of a few seconds be observed, but even at this low temperature, fusion was still quite efficient. The flavivirus fusion machinery thus seems to be the fastest and most efficient known so far, surpassing even the alphaviruses, which are known to fuse much more rapidly than orthomyxoviruses, paramyxoviruses, and HIV, for example. The possible structural basis for differences in fusion kinetics and similarities between alphavirus and flavivirus fusion machines are discussed more in Section IX.

Liposome fusion assays with the West Nile virus (Gollins and Porterfield, 1986a) and TBE virus (Corver *et al.*, 2000) have also revealed a significant influence of the lipid composition on the extent and rate of fusion. In contrast to the situation with alphaviruses (Kielian, 1995; Kielian *et al.*, 2000), both studies showed that neither cholesterol nor sphingomyelin is absolutely required for fusion, but fusion efficiency is reduced when cholesterol is omitted. *In vitro* liposome fusion studies with the TBE virus have shown that the effect of

cholesterol can at least, in part, be substituted by analogues such as coprostanol, androstanol, cholesteryl methylester, and 5-α-cholestanone (Stiasny *et al.*, in preparation). Cholesterol is thus not absolutely required in these experimental systems, but facilitates fusion in a manner that is not yet understood.

VI. Low pH-Induced Structural Changes

Exposure to an acidic pH induces dramatic structural changes in the flavivirus envelope that lead to the permanent inactivation of biological activities such as infectivity (Gollins and Porterfield, 1986b; Guirakhoo *et al.*, 1992; Heinz *et al.*, 1994), hemagglutination (Clarke and Casals, 1958; Heinz *et al.*, 1994), membrane binding (Stiasny *et al.*, 2002), and fusion (Corver *et al.*, 2000). All of the existing evidence suggests that these changes are required to drive the fusion process during virus entry. Conformational changes in the E protein have been demonstrated by examining changes in its sensitivity to protease digestion (Guirakhoo *et al.*, 1989; Kimura and Ohyama, 1988) and by the analysis of the reactivity patterns of monoclonal antibodies recognizing conformational epitopes before and after exposure to acidic pH (Guirakhoo *et al.*, 1989, 1992; Heinz *et al.*, 1994; Roehrig *et al.*, 1990). Many of these epitopes have been mapped to specific sites on the E protein, and the most significant conformational changes appear to occur in domains I and II (Heinz *et al.*, 1994). As revealed in experiments with the TBE virus, structural changes in the E protein apparently also lead to a change in the sites of contact between individual molecules, resulting in a major rearrangement of the icosahedral E protein lattice. Cross-linking, solubilization, and sedimentation analyses have shown that the E proteins are initially dimeric, but exposure of mature virions to acidic pH causes a rapid, quantitative, and irreversible conversion to a homotrimeric state (Allison *et al.*, 1995a).

In the case of the TBE virus, the low-pH–induced switch from dimers to trimers has been shown not only in the context of whole virions, but also with isolated detergent-solubilized E protein dimers (Stiasny *et al.*, 1996). It is clear that this conversion requires that the dimers first dissociate, suggesting that trimer formation is a two-step process (Fig. 7). Experimental evidence for such a two-step model has indeed been provided by the analysis of the C-terminally truncated sE dimer that was used for structure determination. In the absence of the stem-anchor region, acidic pH was shown to cause a dissociation of

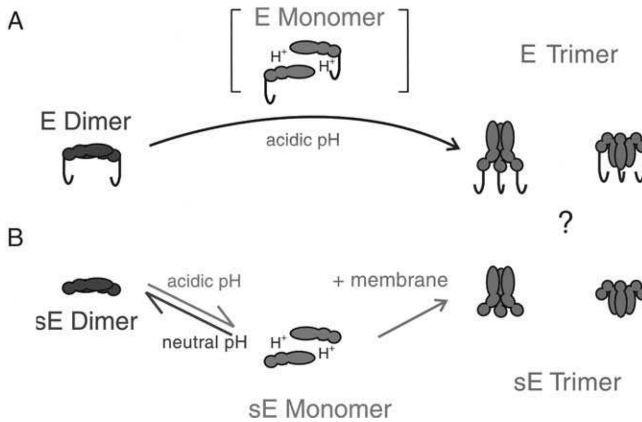


FIG 7. Proposed two-step model for low pH-induced dissociation and trimerization of (A) the E dimer (full-length) and (B) the sE dimer (lacking the stem and anchor). (A) E dimers on the virion surface or solubilized with detergent are converted irreversibly to homotrimers upon exposure to acidic pH. (B) The exposure of sE dimers to low pH results in dissociation to monomers, which is fully reversible by back-neutralization. In the presence of membranes, however, the monomeric sE proteins also irreversibly trimerize.

the dimer that was fully reversible and did not lead to trimerization. Fine mapping studies on the structural and functional role of the stem-anchor region further revealed that the predicted α helix extending from amino acids 400 to 413 ($H1^{pred}$, Fig. 5) was essential for the low-pH–induced conversion of the dimer to a trimer in solution (Allison *et al.*, 1999). Fully truncated dimers that lacked this helix, however, were able to trimerize in the presence of liposomes (Stiasny *et al.*, 2002) (see Section VII), suggesting that this structural element is not necessary for the maintenance of the trimer structure but rather acts as a facilitator of its formation. (*For new structural information on the E trimer, see Addendum.*)

The irreversibility of the formation of the low-pH, trimeric form of E suggests that—analogous to the situation with viruses that have class I viral fusion proteins—the flavivirus E protein is trapped kinetically in a metastable conformation on the surface of mature virions and is poised to undergo specific structural changes leading to an energetically more stable conformation when the appropriate trigger (in this case low pH) is applied. Consistent with this model, it was shown, using intrinsic fluorescence measurements, that the low-pH form of the TBE virus E protein is more stable to thermal denaturation than

the native, dimeric, neutral pH form (Stiasny *et al.*, 2001). However, in contrast to what has been described for certain viruses with class I fusion proteins, it was shown with the TBE virus that neither conversion from the metastable to the stable conformation nor fusion activity itself could be induced by heating or slightly denaturing conditions in the absence of the specific trigger (Stiasny *et al.*, 2001). These treatments instead only led to protein denaturation, and it has therefore been proposed that protonation of the native E dimer is indispensable for generating a monomeric intermediate structure that is required for the formation of the energetically more stable final trimeric form (Fig. 7).

VII. Interactions with Membranes

Because TBE virions that have been preexposed to an acidic pH do not exhibit any fusion activity (Corver *et al.*, 2000) and are unable to associate with liposomes *in vitro* (Stiasny *et al.*, 2002), it is clear that the fusion reaction is not induced by the final trimeric low-pH form of the E protein, but instead appears to be driven by changes in intermediate structures that are generated during the transition from the neutral form to the low-pH form. Liposome coflotation studies with soluble truncated E protein dimers treated with reversible cross-linking agents have indeed shown that the low-pH–induced dissociation of the dimer into individual monomers is essential for binding to target membranes (Stiasny *et al.*, 2002). Surprisingly, in contrast to the situation in the absence of membranes, where no trimerization of this truncated form of E occurs at low pH (see Section VI), sE monomers that associate with membranes irreversibly form trimers, as revealed by cross-linking and sedimentation analysis of liposome-bound sE proteins (Stiasny *et al.*, 2002). It can therefore be concluded that interactions in the ectodomain alone are sufficient for maintaining the trimer structure, but additional factors that act as facilitators (helix 1–H1^{pred}–in the stem; association with lipid membranes in the case of isolated sE proteins) are required for generating this structure at low pH. The precise structural role of these “cofactors” is not clear, but they both could help increase the local concentration of appropriately aligned monomers as a prerequisite for trimer formation.

Several lines of indirect evidence have suggested that amino acids 98 to 110 of domain II (Fig. 8) function as an internal fusion peptide, initiating the fusion process by inserting into the lipid bilayer of the

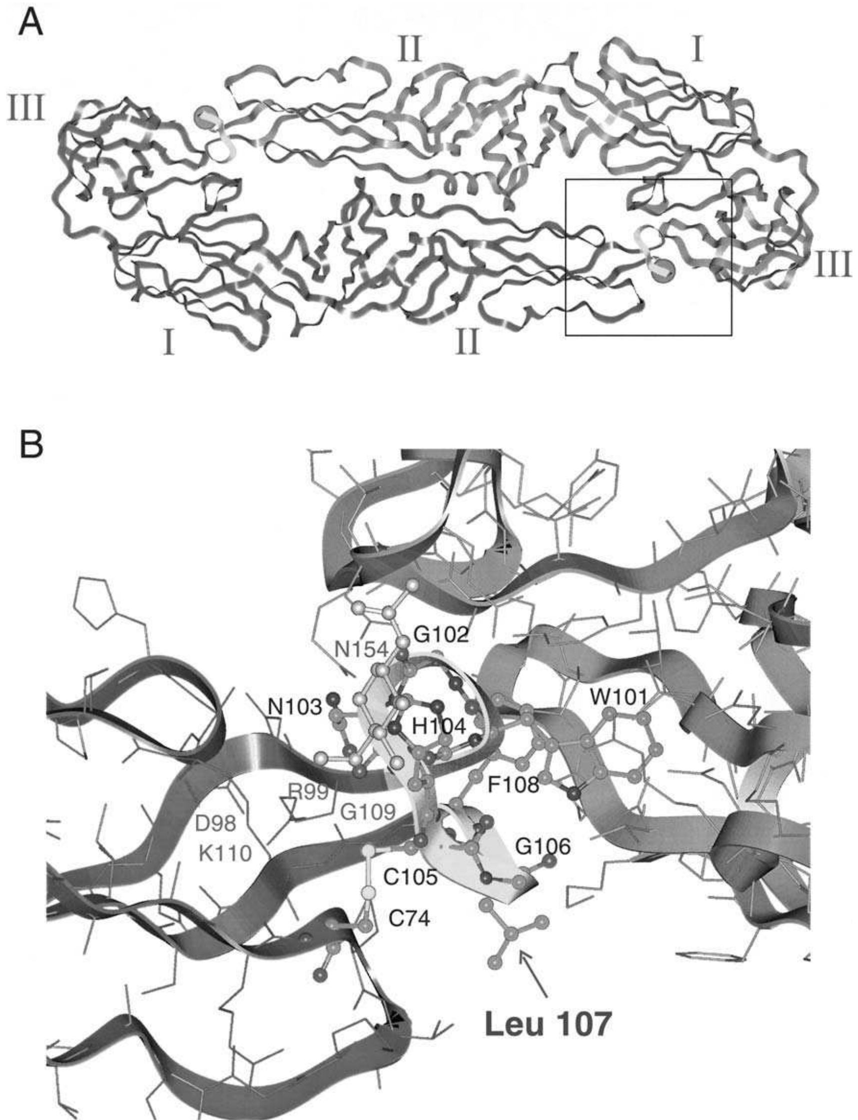


FIG 8. (A) Ribbon diagram of the TBE virus E protein (top view) with the fusion peptide highlighted in yellow and the position of Leu 107 indicated by a green ball. (B) Zoom of the boxed region in A. Adapted from Allison *et al*. (2001). (See Color Insert.)

target membrane. This region is almost absolutely conserved among flaviviruses with the exception of residue 104, which is histidine in tick-borne and glycine in mosquito-borne flaviviruses. A further variation affects amino acid 107, which is a leucine in most flaviviruses, but this position is occupied by phenylalanine in the tick-borne Powassan virus (Beasley *et al.*, 2001; Ebel *et al.*, 2001; Kuno *et al.*, 2001; Mandl *et al.*, 1993), as well as in certain strains of the JE virus (Nitayaphan *et al.*, 1990) and dengue-2 virus (Blok *et al.*, 1989). The sequence was first proposed to be a fusion peptide based on features shared with known fusion peptides of other viruses (Roehrig *et al.*, 1989), including a tetrapeptide, GLFG, that corresponds to the N terminus of the influenza virus HA2 fusion peptide and has been found in modified form in the fusion peptides of other viruses as well. Residues 100 to 108 constitute a structurally highly constrained loop (cd loop; Rey *et al.*, 1995) that is linked by a disulfide bridge to another loop (bc) of the same domain and is buried in the native dimer structure by interdigitating with a hydrophobic pocket formed by domains I and III of the other monomer (Fig. 8). The cd loop in the TBE virus E protein is further protected by the carbohydrate side chain attached to Asn 154 of the other subunit.

More direct experimental evidence for a functional role of this sequence element in fusion has been obtained by site-specific mutagenesis (Allison *et al.*, 2001) using TBE virus RSPs (see Section IV). Replacement of leucine 107 by aspartate completely abolished fusion activity, whereas fusion activity was retained, albeit at reduced levels, with a phenylalanine substitution at the same position, as expected from the natural occurrence of this amino acid in certain flavivirus strains. Although the aspartate mutant was still capable of undergoing low-pH–induced structural changes and trimer formation with the same pH dependence as the wild type (Allison *et al.*, 2001), it was unable to associate stably with lipid membranes.

It has also been shown that monoclonal antibodies that specifically recognize this region are able to inhibit fusion activity (Volkova *et al.*, 1999) and block the binding of the monomeric-truncated wild-type E protein to liposomes at low pH (Stiasny *et al.*, 2002). Furthermore, antibodies that recognize the fusion peptide region of the E protein of dengue virus type 2 bind more strongly after the virion has been exposed to acidic pH (Roehrig *et al.*, 1990), consistent with the idea that this region, upon acidification, becomes more accessible for interactions with the host membrane.

VIII. Function of the prM Protein

During biosynthesis, prM and E rapidly form a heterodimeric complex, as has been shown in pulse-chase experiments with both virus-infected cells (Courageot *et al.*, 2000; Lorenz *et al.*, 2002; Wang *et al.*, 1999) and cells transfected with constructs that express the two envelope proteins only (Lorenz *et al.*, 2002). As pointed out in Section II, prM is able to acquire its completely folded structure independently of E and appears to play a chaperone-like role for the folding of E. Sedimentation studies (Lorenz *et al.*, 2002; Wang *et al.*, 1999) have revealed that the prM–E heterodimer rapidly forms higher oligomeric assemblies that later get incorporated into particulate structures, i.e., immature virions or immature RSPs. The prM–E heterodimer thus represents the basic building block for the generation of immature viral and subviral particles, and lateral interactions between these molecules appear to drive the assembly process. The immature intracellular forms differ from mature particles by a number of criteria. Most importantly, they are structurally resistant to acidic pH and do not exhibit the low-pH–induced structural and oligomeric changes that are characteristic of their mature counterparts. Correspondingly, functions requiring these changes (fusion activity, infectivity, and HA activity) have been found to be strongly impaired or completely lacking when prM is not cleaved (Guirakhoo *et al.*, 1991, 1992; Heinz *et al.*, 1994; Randolph *et al.*, 1990; Stadler *et al.*, 1997; Wengler, 1989).

Existing evidence indicates that the cleavage of prM is mediated by furin in the TGN and requires an acidic pH. Cleavage can be prevented by raising the pH in acidic intracellular compartments using acidotropic agents such as ammonium chloride or bafilomycin A1 (Randolph *et al.*, 1990; Guirakhoo *et al.*, 1991, 1992; Heinz *et al.*, 1994) (Fig. 3) as well as specific inhibitors of furin (Stadler *et al.*, 1997). Most convincingly, only immature forms of TBE virus were shown to be secreted from a furin-deficient cell line (Stadler *et al.*, 1997). *In vitro* cleavage experiments with the TBE virus and recombinant bovine furin (Stadler *et al.*, 1997) have confirmed its dependence on a slightly acidic pH (6.7 or below) and further provided evidence that this protonation induces an irreversible conformational change that is required for cleavage to occur. Consistent with the model that prM cleavage is required for the formation of a metastable E protein arrangement as described in Section VI, *in vitro* cleavage of immature virions has been shown to activate fusion activity, HA activity, and infectivity, at least in the TBE virus system studied (Stadler *et al.*, 1997). These data are compatible with the view that prM has the function

of protecting the E protein when it passes through the TGN during exocytosis, preventing it from undergoing the irreversible conformational changes that are required for fusion in the endosome during virus entry.

The biological significance of the relatively high content of uncleaved prM found in some flaviviruses released from certain cells (Murray *et al.*, 1993; Wang *et al.*, 1999) is unclear at present, and it remains to be determined whether significant differences exist between flaviviruses with respect to the structure–function relationships of their envelope proteins. (*For new structural information on immature virions, see Addendum.*)

IX. Comparative Aspects of the Flavivirus Fusion Machinery

The determination of the structure of the flavivirus E protein (Rey *et al.*, 1995) and, more recently, of the alphavirus E1 protein (Lescar *et al.*, 2001) revealed that at least two types of structurally unrelated envelope proteins are used by viruses for mediating fusion, and these are now designated "class I" and "class II" viral fusion proteins (Gibbons *et al.*, 2000; Heinz and Allison, 2001; Lescar *et al.*, 2001). The influenza hemagglutinin is the prototype of the class I proteins, which so far have been found in orthomyxoviruses, paramyxoviruses, retroviruses, and filoviruses (Dutch *et al.*, 2000; Skehel and Wiley, 1998; Weissenhorn *et al.*, 1999). They are formed as homotrimeric complexes (see Center *et al.*, 2001, and references therein) that constitute independent spikes oriented perpendicular to the membrane, require proteolytic cleavage for activating their fusogenic potential, have an N-terminal or N-proximal fusion peptide after cleavage, and form a characteristic postfusion structure consisting of a hairpin-like arrangement that holds the N-terminal (or N-proximal) fusion peptide and the membrane anchor on the same side of this rod-like assembly (Dutch *et al.*, 2000; Skehel and Wiley, 1998; Weissenhorn *et al.*, 1999). Class II fusion proteins differ in many important aspects. They have a completely different overall fold, do not form spikes, but instead are oriented parallel to the viral membrane and arranged in an icosahedral lattice, have internal fusion peptides, and form a heterodimeric complex with a second envelope glycoprotein, the proteolytic cleavage of which activates their fusion potential. Despite these structural differences, both classes have in common that in their native form they exist in a metastable state and are converted into a more stable conformation when they encounter the appropriate trigger

(receptor binding or acidic pH). It is believed that the energy released by this conformational change is used to drive the fusion of the viral and cellular membranes. A further variation on this theme is found in rhabdoviruses (Gaudin, 2000; Gaudin *et al.*, 1999), whose fusion proteins (of still unknown structure) control fusion activity by means of reversible conformational changes rather than the irreversible changes that are characteristic of class I and II fusion proteins.

Lescar *et al*. (2001) showed that the overall fold of the flavivirus and alphavirus fusion proteins is strikingly similar, even though there is no apparent sequence homology, suggesting that these proteins have a common evolutionary origin. The similarities also include organization into three homologous domains, the location of the membrane anchor, and the position of the internal fusion peptide (Fig. 9). In addition to these features, flaviviruses and alphaviruses have a number of additional properties in common that set them apart from other enveloped viruses. They both have an icosahedral envelope symmetry, the fusion proteins are synthesized as a complex with a second protein (prM in flaviviruses and p62 in alphaviruses), and the proteolytic cleavage of this second protein generates a metastable state that is poised to undergo the irreversible conformational changes that are required for fusion upon exposure to acidic pH. In both cases, these changes lead to the formation of a stable homotrimer of the fusion protein. Both fusion proteins also appear to play an important role in virion assembly by providing lateral contacts that contribute to the icosahedral arrangement of the envelope. In the case of flaviviruses, lateral envelope protein interactions appear to be sufficient to generate capsidless subviral particles that have an icosahedral envelope arrangement (see Section IV). Although such subviral particles have not been described for alphaviruses, the importance of lateral glycoprotein contacts for maturation and budding has been suggested by work using mutants that have a defect in nucleocapsid assembly (Forsell *et al.*, 2000).

Flaviviruses and alphaviruses thus share characteristic properties not only with respect to the structure of their fusion proteins, but also with respect to the basic design of their fusion machineries. Nevertheless, there are also several notable differences. The flavivirus E protein is bifunctional, mediating both receptor-binding and fusion activities, whereas the alphavirus E1 protein has only the fusion function, and the receptor-binding activity resides in a second envelope glycoprotein, E2 (Schlesinger and Schlesinger, 2001). Alphaviruses have a diameter of 70 nm with a $T = 4$ icosahedral symmetry and bud at the plasma membrane (Schlesinger and Schlesinger, 2001), whereas

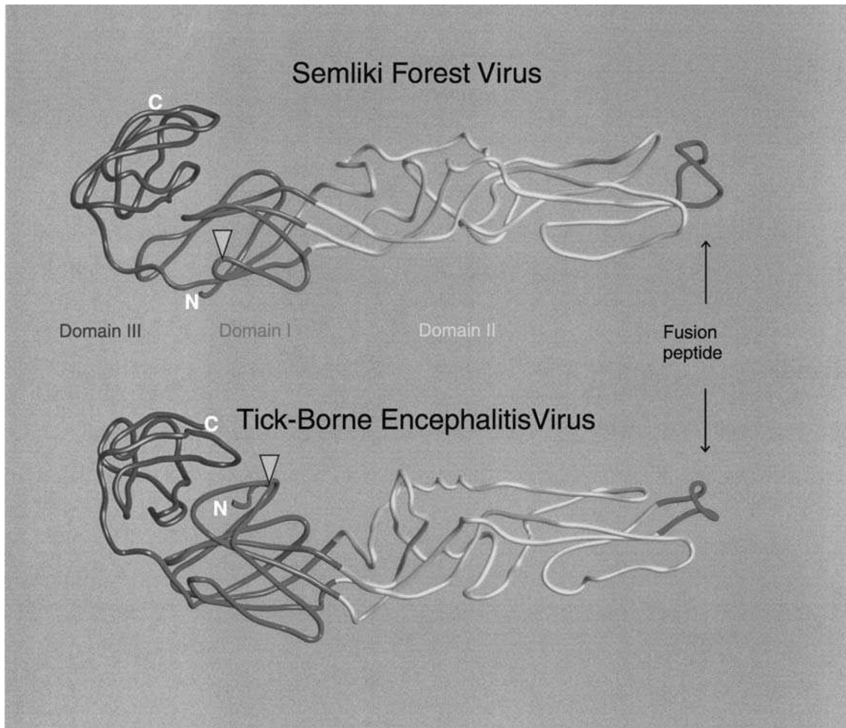


FIG 9. Comparison of the structures of the Semliki Forest virus E1 protein and the TBE virus E protein (Lescar *et al.*, 2001). Triangles mark the glycosylation sites. Reproduced from Heinz and Allison (2001) with permission. (See Color Insert.)

flaviviruses are somewhat smaller (50 nm), have a different icosahedral arrangement, and assemble intracellularly, presumably in the ER. Flavivirus envelopes are symmetrical assemblies of antiparallel, front-to-front E protein dimers in which the fusion peptide at the tip of each monomer is protected by interaction with a hydrophobic pocket provided by the second subunit. In alphaviruses, the E1 proteins are oriented back to back at twofold symmetry axes but do not form a stable homodimeric complex. They exist instead as a heterodimeric complex with E2 (the receptor-binding protein), which protects the fusion peptide and forms the outer surface of the virus particle. The initiation of fusion thus requires the low-pH–induced dissociation of the E homodimer in the case of flaviviruses and the E1–E2 heterodimer in the case of alphaviruses. The existence of this additional protein at the periphery of the virus might be responsible for the

somewhat slower kinetics of alphavirus fusion compared to that of flaviviruses because more protein movement would be required before the fusion protein itself can make an appropriate contact with the target membrane. Flavivirus fusion also does not exhibit the absolute dependence on cholesterol in the target membrane that is a characteristic feature of alphavirus fusion, but the structural basis for this difference remains to be elucidated. Both groups of viruses thus represent exciting models for studying the similarities and differences of structurally and functionally homologous, evolutionarily related proteins, and these investigations at the same time will allow new insights into how viral and cellular membrane fusion is accomplished by structurally unrelated fusion machineries. (*For new information on the post-fusion structures of flavi- and alphavirus fusion proteins, see* *References for Addendum, page 97.*)

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ADDENDUM

UPDATE ON FLAVIVIRUS VIRION AND E PROTEIN STRUCTURE (SEPTEMBER 2003)

The mature virion

Using cryo-electron microscopy and icosahedral image reconstruction, Kuhn *et al*. (2002) determined the structure of dengue virus type 2 strain S1 (a vaccine strain derived from PR-159) at a resolution of 24 Å. Computer-assisted fitting of the known atomic structure of the TBE virus E protein ectodomain into the experimental electron density revealed that the E proteins are not organized into a regular $T = 3$ pattern with quasi-equivalent contacts, as had been predicted previously. Instead, 90 E dimers are arranged into a more complex "herringbone" configuration in which 3 adjacent E monomers form the asymmetric unit. As shown in Fig. A1, the dimers on the icosahedral twofold axes do not have a quasi-3-fold relationship to the dimers at the other positions, and the structural environments of these two types of dimer are different, requiring more than one type of lateral interaction between the building blocks of the virus envelope. Because the majority of the E dimers are not involved in trimeric contacts, the quantitative conversion to the trimeric state at acidic pH would require a major rearrangement in the surface lattice.

Zhang *et al*. (2003a) refined the image reconstruction of the mature dengue virus to a resolution of about 9.5 Å. In addition to confirming the herringbone model, the improved electron density map revealed a number of other important structural details, most significantly the exact positions of the transmembrane regions of the E and M proteins and that of the E protein "stem." The transmembrane helices of both E and M were found to be antiparallel helical hairpin structures that do not extend to the interior of the viral membrane and therefore do not contact the nucleocapsid, which itself is either disordered or oriented randomly with respect to the envelope lattice. Interestingly, both of the previously predicted α-helical regions of the stem region were found to interact extensively with the outer leaflet of the viral membrane. The implications of these interactions for the membrane fusion mechanism are not yet clear, but formation of the final trimer at low pH would appear to require displacement of at least a portion of the stem from its initial position (see page 96).

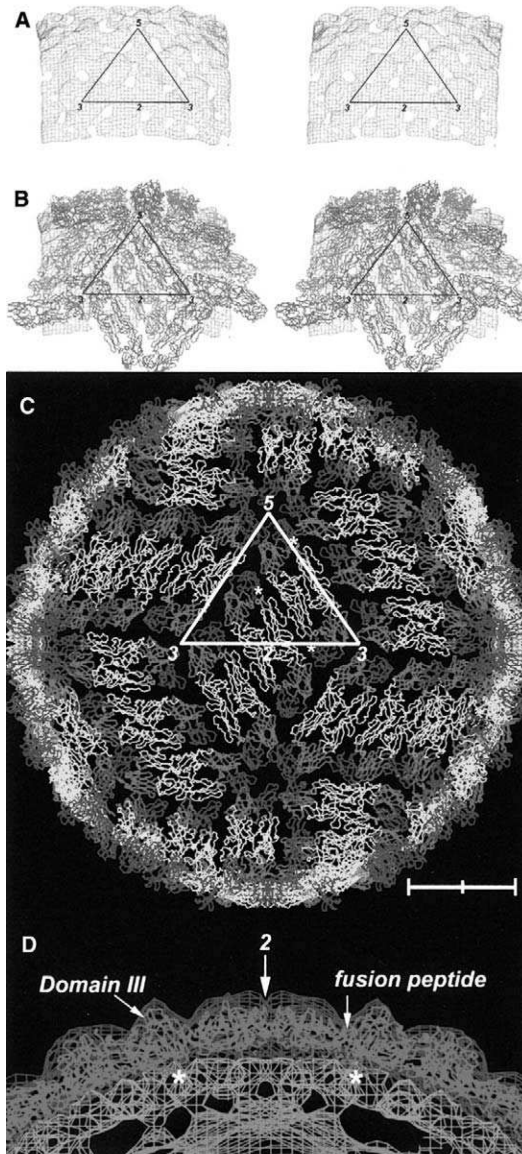

FIG A1. Envelope protein organization in mature dengue virus. (A) Stereo view of electron density from cryo-electron microscopy corresponding to the E ectodomains. (B) Fitting of the TBE virus E protein ectodomain into the experimental electron density. Green dimers are on icosahedral twofold axes, red dimers on quasi-twofold axes. (C) E protein organization of whole virus with domains I, II, and III colored red, yellow, and blue, respectively, and the fusion peptides colored green. The C-terminus of the

The immature virion

The structures of immature dengue and yellow fever virus particles have also been determined by cryo-electron microscopy to resolutions of 16 and 25 Å, respectively, using virions that were produced by treating infected cells with ammonium chloride to suppress prM cleavage (Zhang *et al.*, 2003b). In contrast to the mature form, the surface of the immature virus contains 60 icosahedrally organized trimeric spikes, each consisting of 3 prM-E heterodimers (Fig. A2). These spikes are similar in appearance to those found on alphaviruses, with the E protein monomer protruding away from the viral membrane at a 25° angle, its outer tip, including the fusion peptide, covered by the N-terminal portion of prM. In contrast to the alphaviruses, however, these spikes do not have a quasi-symmetrical distribution on the virion and are themselves asymmetrical. In spite of the fact that the geometries of both the mature and the immature virus are now known, the complex relationship between the initial spike arrangement and the final mature herringbone configuration makes it difficult to reconstruct exactly how the proteins rearrange during maturation to the metastable state, and further studies are still needed.

The dengue virus E protein dimer

Modis *et al.* (2003) have determined the 3-dimensional structure of a soluble dimeric ectodomain fragment of the E protein of dengue virus type 2 S1 strain expressed in *Drosophila melanogaster* cells. The structures of two different crystal forms, one grown in the presence and one in the absence of the detergent β-octylglucoside (β-OG), were determined by X-ray crystallography. The dengue E protein structure was remarkably similar to that of the TBE virus E protein (37% sequence identity), with only local areas of divergence, mostly corresponding to variable loops, and there were slight differences in the orientations of the domains relative to one another. The protein was also glycosylated at two positions, Asn 67 and Asn 153, in contrast to the TBE virus E protein, in which only the latter site is used. Comparison of the forms with and without β-OG revealed a single molecule of this detergent

ectodomain fragment is indicated by an asterisk. Scale bar represents 100 Å. (D) Central cross-section showing outer protein shell (dark blue), membrane (light blue), and nucleocapsid (green). The fitted E ectodomain is shown in red. Reproduced from Kuhn *et al.* (2002) with permission. (See Color Insert.)

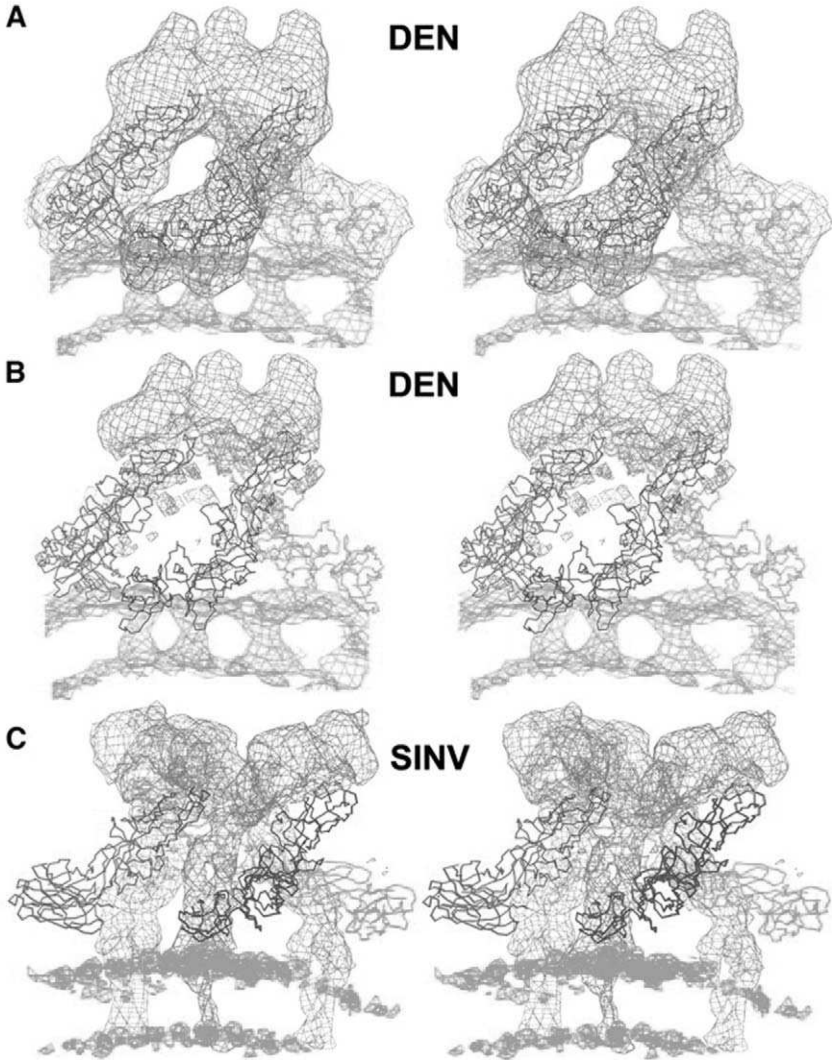

FIG A2. Stereo images of the spike structures of immature dengue virus particles (A and B) and mature Sindbis virus (C), showing the fitting of proteins E and E1, respectively, into the electron density from cryo-electron microscopy image reconstructions. In B and C, the portion of the density assigned to E and E1 were zeroed out to show the positions of the prM and E2 proteins, respectively (gray). Reproduced from Zhang *et al*. (2003b) with permission. (See Color Insert.)

bound in a pocket at the base of domain II. This pocket contains a number of residues that, when mutated, affect the pH dependence of fusion or virulence. Detergent binding resulted in a displacement of the kl loop, a small β-hairpin structure (Fig. A3). The authors propose that this flexible portion of the "hinge" between domains I and II plays an important role in the triggering of the low-pH-dependent conformational change and is a potential target for small-molecule inhibitors of fusion. In a subsequent paper, it was shown that this region indeed undergoes a conformational change at low pH (see next).

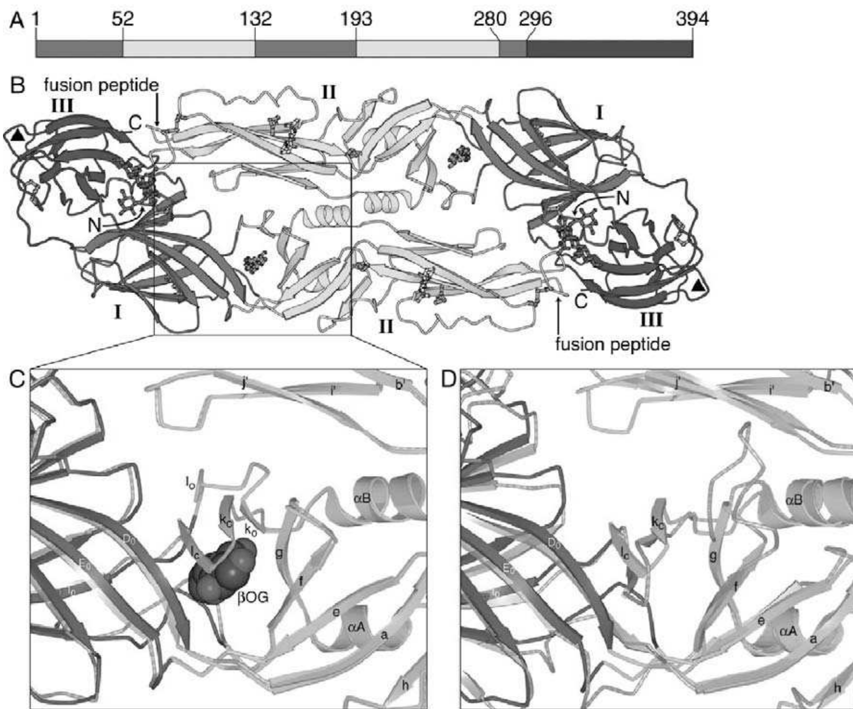

FIG A3. Dengue E protein and its ligand-binding pocket. (A) Domain definition of dengue E protein, showing domains I, II, and III in red, yellow, and blue, respectively. (B) Top view of E dimer with the domains colored as in A. Disulfide bridges are shown in orange, and the glycans on domains I and II are shown as ball-and-stick representations. A putative receptor-binding site is indicated by a triangle. (C) Enlargement of the kl hairpin region, showing the position of the bound β-OG molecule. The gray superimposed structure is the "closed" form in the absence of β-OG. (D) The same view as in C, showing the dengue virus E protein structure (closed form) superimposed onto the TBE virus E protein structure (gray). Reproduced from Modis *et al.* (2003) with permission. (See Color Insert.)

The postfusion E protein trimer

As described in Section VII, the interaction of the TBE virus sE dimer with liposomes at acidic pH leads to its irreversible conversion into a trimeric form that can be reisolated from the liposomes by detergent treatment. The application of this technology to the sE dimer isolated from TBE virus (Stiasny *et al.*, submitted) and the recombinant sE dimer from dengue 2 virus (Modis *et al.*, 2003) yielded preparations of the corresponding trimeric low-pH forms that crystallized and allowed the determination of high-resolution structures. The dengue and TBE structures were first presented at an international meeting on dengue virus in June 2003 by Stephen Harrison and Felix Rey, respectively. Since these have not yet been published, we will give only an overall description of these postfusion structures in comparison to the native dimeric structures.

In contrast to the dimers, in which the subunits exhibit a head-to-tail orientation, the subunits of the trimer are arranged in parallel. During the conversion of dimers to trimers, the domains remain intact but are reoriented relative to one another. Domain II exhibits a small rotation relative to domain I at the junction between these domains (see also the discussion of this "hinge" region in the previous section), but the most significant change in the protein is a major relocation of domain III relative to the other domains, resulting in a folded-back structure. It can also be inferred from the structure that the stem region would be displaced from its original position to pack against the trimer core. This would result in a postfusion conformation in which the fusion loop and the C-terminal membrane anchor are juxtaposed in the membrane.

The essential features of the postfusion structures of the flavivirus E proteins are very similar to those of the E1 fusion protein from the alphavirus Semliki Forest virus (F. A. Rey, personal communication), demonstrating that the formation of a folded-back structure is a characteristic of class II fusion proteins in general. This is functionally analogous to the postfusion hairpin of class I fusion proteins and suggests mechanistic similarities between these structurally unrelated classes of fusion proteins.

Acknowledgments

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ADVANCES IN VIRUS RESEARCH, VOL 59

KUNJIN RNA REPLICATION AND APPLICATIONS OF KUNJIN REPLICONS

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I. Introduction
II. Foundations for Developing a Model of KUNV RNA Synthesis
  A. Identification of Virus-Specific RNA
  B. Subcellular Location of Sites of Replication
  C. Proposed Model for Genomic RNA Synthesis
III. Extending the Scope of Replication Analyses
  A. Complexity and Motifs of Nonstructural (ns) Proteins
  B. Further Advances in Analyses of KUNV RNA Synthesis
IV. Ultrastructure of Induced Membranes, Their Association with the Replication Complex (RC), and Their Origins
  A. Comparative Electron Microscopy of KUNV-Infected Cells
  B. Cryoimmunoelectron Microscopy of ns Proteins and ds RNA in KUNV-Induced Membranes
  C. Coincidence of Labeled dsRNA and Nascent Progeny RNA in Stable Replication Complexes
  D. Biochemical Assays and Consensus Composition of the RC
  E. Putative Origins of Induced Membranes
V. Molecular Approaches to Study Formation and Operation of the RC
  A. Generation of Infectious cDNA Clones and of Subgenomic Replicons of KUNV
  B. Complementation Systems and Formation of the RC
  C. Cyclization of Genomic RNA
  D. RNA(−) Strand Synthesis on RNA(+) Strand Template
  E. RNA(+) Strand Synthesis and Recycling RF as Template
  F. Summary of the *Flavivirus* RNA Replication Model
  G. Comparisons with Similar Strategies of Other RNA Viruses
VI. Development of the Kunjin Replicon Expression System for Vaccines and Gene Therapy
  A. Design of Replicon Vectors
  B. Packaging System
  C. Transient and Stable Heterologous Gene (HG) Expression *In Vitro*
  D. HG Expression *In Vivo* and Immune Responses
VII. Concluding Remarks
References

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The Kunjin virus (KUNV) has provided a useful laboratory model for *Flavivirus* RNA replication. The synthesis of progeny RNA(+) strands occurs via asymmetric and semiconservative replication on a template of recycling double-stranded RNA (dsRNA) or replicative form (RF). Kinetics of viral RNA synthesis indicated a cycle period of about 15 min during which, on average, a single nascent RNA(+) strand displaces the preexisting RNA(+) strand in the replicative intermediate. Data on the composition of the replication complex (RC) in KUNV-infected cells were obtained from several sources, including analyses of the partially-purified still active RC, immunogold labeling of cryosections using monospecific antibodies to the nonstructural proteins and to dsRNA, radioimmunoprecipitations of cell lysates using antibodies to dsRNA and to an RC-associated cell marker, and pull-down assays of cell lysates using fusion proteins GST–NS2A and GST–NS4A. These results yielded a consensus composition of NS1, NS2A, NS3, NS4A, and NS5 strongly associated with the dsRNA template. The RC was located in induced membranes described as vesicle packets. The RNA-dependent RNA polymerase activity late in infection did not require continuing protein synthesis. Replication of genomic RNA was completely dependent on the presence of conserved complementary or cyclization sequences near the 5′ and 3′ ends. Assembly of the RC during translation in *cis* and the relationships, particularly those of NS1 and NS5 among the components, were deduced from an extensive set of complementation experiments in *trans* involving mutations/deletions in all the nonstructural proteins and use of KUN or alphavirus replicons as helpers. The KUN replicon has found useful applications also as a noncytopathic vector for the continuing expression of foreign genes, delivered either as packaged RNA or as plasmid DNA.

I. Introduction

The Kunjin virus (KUNV) is epizootic in Australia and was first isolated in 1960 from wild-caught mosquitoes together with several other new *Flavivirus* species and the Murray Valley encephalitis virus. It infects a wide range of mammalian and avian hosts via mosquito transmission and rarely causes overt disease. The genetic variation among many Australian isolates was estimated to be only about 1% (Mackenzie *et al.*, 1994), and these strains have a very close relationship to the West Nile virus New York strains. Thus the complete amino acid sequences have 98.1% identity, or 99.2% if conserved changes are

ignored, based on comparison of the coding sequences for Genbank Accession members D 00246 (KUNV) and AF 202541 (WN-NY99 virus). Among the Australian flaviviruses, KUNV is clearly the most closely related to any exotic species.

For many years, KUNV has provided a useful laboratory model for *Flavivirus* replication because of its good yields and relative ease of radiolabeling in cell cultures. Early details on the characterization of KUNV RNA and of the structural and nonstructural (ns) proteins have been reviewed elsewhere (Westaway, 1987; Westaway *et al.*, 2001). This review summarizes progress in elucidating the events of RNA replication of KUNV, develops a model for viral RNA synthesis, and describes the creation and applications of subgenomic self-amplifying KUN replicons.

II. Foundations for Developing a Model of KUNV RNA Synthesis

A. *Identification of Virus-Specific RNA*

Early studies with KUNV established by electrophoresis and RNase digestions of RNA extracted from infected cells that there was no subgenomic RNA (Boulton and Westaway, 1977). Subsequently, the intracellular RNA species in dengue-2 (DEN-2) virus–infected cells were shown to be the RF comprising double-stranded RNase-resistant RNA (dsRNA), partially RNase-sensitive RNA or replicative intermediate (RI), and genomic RNA (Cleaves *et al.*, 1981). RF and RI RNAs specified by KUNV were characterized by Chu and Westaway (1985) (see Section II,C). Subsequent nucleotide sequence analysis of the complete KUNV genome showed that it contained a single open reading frame coding for 3433 amino acids (Coia *et al.*, 1988) and that the 5′- and 3′-untranslated regions (UTRs) comprised 96 and 624 nucleotides, respectively, in a complete sequence of 11,022 nucleotides (Khromykh and Westaway, 1994).

B. *Subcellular Location of Sites of Replication*

Immunofluorescence (IF) analysis of KUNV-infected Vero cells using antibodies to envelope protein E and (synthetic) dsRNA revealed that replication appeared to be confined to foci in the perinuclear region (Ng *et al.*, 1983). These foci remained largely intact after treatment of cells with cytoskeletal-disrupting agents that caused only

relatively minor reductions in yields of infectious virus. However, IF using antiribosomal antibodies showed a progressive visible change in the rough endoplasmic reticulum from a fine to a coarse reticulum network by the end of the latent period (13 to 15 h).

C. Proposed Model for Genomic RNA Synthesis

Pulse labeling of KUNV-infected cells with [^{3}H]-uridine at 20 h post-infection (p.i.) in the presence of actinomycin D identified the three forms of intracellular RNA, namely RI, RF, and virion RNA, which were radiolabeled progressively in that order (Chu and Westaway, 1985). In pulse-chase experiments, incorporation of label in RF reached a maximum within about 20 to 30 min of commencement of a 10-min pulse, suggesting that the RF was recycling as a template. The analysis of RNA products was assisted greatly by the ability to resolve RF and RI by electrophoresis in aqueous agarose gels and in semidenaturing 7 *M* urea polyacrylamide gels and by use of LiCl precipitation for recovery of RF separated in the supernate. These observations provided the basis for the proposed model that this synthesis of viral RNA occurred in a semiconservative and asymmetric manner on a recycling RF template. The high RNase resistance of the RI (about 70%) indicated that on average only one nascent RNA strand was synthesized per cycle, based on the formula of Baltimore (1968), and it displaced a preexisting strand of the same polarity from the template. The cycle time for appearance of free newly synthesized RNA was about 15 min and included a short delay in release from the RI; as reported earlier, no subgenomic RNA was detected that might function as messenger RNA. The proposed model was tested further when an *in vitro* RNA-dependent RNA polymerase (RdRp) assay was established using lysates of KUNV-infected cells (Chu and Westaway, 1987). Thus the order of incorporation of label from ^{32}P-GTP was shown to be RI→RF→vRNA, equivalent to the *in vivo* results, and a limited reinitiation of vRNA synthesis was noted. Because cellular terminal transferases are unable to use ^{32}P-GTP as a substrate, incorporation into nascent RNA must be due to viral RdRp activity. The RdRp activity was relatively stable, was retained in the 16,000-g pellet, and was found in the supernate when membranes were dissolved by detergent treatment. Inclusion of Mg (optimum 10 m*M*) was essential for RdRp activity. Antibodies to synthetic dsRNA blocked RdRp activity in detergent-treated lysates. The specific activity in lysates was low, only about one-fiftieth of that reported for a picornavirus (encephalomyocarditis virus).

Recent pulse chase experiments with ^{32}P-GTP and Japanese encephalitis virus-infected cell lysates confirmed the above order of incorporation of label during RdRp assays and the recycling role of RF as a template in asymmetric and semi-conservative replication (Uchil and Satchidanandam, 2003a).

III. Extending the Scope of Replication Analyses

A. Complexity and Motifs of Nonstructural (ns) Proteins

Early studies indicated that as many as six unrelated ns proteins may be involved in replication (Westaway, 1973; Wright *et al.*, 1977; Wright and Westaway, 1977). The nucleotide sequence of yellow fever virus (YFV) showed that a total of seven genes in the ns region could be tentatively identified, although there was some uncertainty when assigning the gene boundaries of several due to limited data on terminal amino acid sequences (Rice *et al.*, 1985). This major advance provided a gene order and defined a single open reading frame.

The precise gene boundaries and identification of all the *Flavivirus* ns proteins were first provided by the KUNV nucleotide sequence and associated papers in 1988/1989 (Coia *et al.*, 1988; Speight *et al.*, 1988; Speight and Westaway, 1989a, 1989b). Formal confirmation of the sequential translation of each product in the polyprotein was obtained by translation mapping in KUNV-infected cells synchronized in initiation of translation (Schrader and Westaway, 1988). Complete translation of the polyprotein required about 17 min, and there was no indication of delays extending beyond about 30 min in post-translation cleavages. The gene order (the number of amino acid residues/size in kilodaltons of the KUNV polypeptides is in parentheses) is C(123/13.4)–prM(167/18.4)–E(520/53.7)–NS1(353/39.8)–NS2A(232/25.4)–NS2B(131/14.4)–NS3(619/68.9)–NS4A(149/16.1)–NS4B(255/27.5)–NS5(905/103.6). The location of signal sequences and stop transfer sequences in the KUNV structural region indicated that the amino terminus of each of prM, E, and NS1 was cleaved in the lumen of the ER by signal peptidase, as deduced by Rice *et al*. (1985) for YFV. Cleavages in the cytosol at dibasic sites defined as K/R.R↓G/S/A (Lindenbach and Rice, 2001) were confirmed by N-terminal amino acid sequencing for the KUNV NS2A–NS2B–NS3–NS4A and NS4B–NS5 junctions, and cleavage by carboxypeptidase digestion was discovered at a dibasic site in core protein C that precedes the signal sequence for prM (Speight

et al., 1988; Speight and Westaway, 1989a, 1989b). These cleavages must involve NS3 as a protease with NS2B as a cofactor (Lindenbach and Rice, 2001).

Two unexpected cleavage sites were discovered in the ns protein region of the KUNV polyprotein. These were lumenal cleavages at the junctions of NS1.NS2A and NS4A.NS4B. A signal peptide preceded the cleavage site of NS4B but not of NS2A, but both were cleaved after the conserved sequence Val-X-Ala where X is an uncharged amino acid. For the NS1.NS2A cleavage, the V-X-A sequence completed a relatively conserved octapeptide; this unusual cleavage may be mediated by a cell signal peptidase with precise specificity (Speight *et al.*, 1988). Many subsequent reports confirmed that the defined KUNV cleavage sites in the polyprotein were applicable to all *Flavivirus* polyproteins. During expression of YFV ns proteins from recombinant vaccinia virus, cleavage of the signal sequence preceding NS4B was dependent on prior cleavage by the NS2B-3 protease at the "4A/2K" site (Lin *et al.*, 1993) but this cleavage has yet to be demonstrated in wild-type *Flavivirus* infections.

Motifs indicative of roles in *Flavivirus* replication have been found only in the largest and hydrophilic proteins NS3 and NS5. NS5 is strongly conserved in size and sequence and is the putative RdRp with the essential Gly-Asp-Asp and other motifs characteristic of RdRp of other positive-strand RNA viruses (Koonin, 1991). These motifs in NS5 extend from amino acids 450 to 600. A conserved glycine-rich sequence V(V/I)DLGCGRGGW (residues 77 to 87 in KUN NS5) with homology to *S*-adenosyl methionine-utilizing (SAM) methyl transferases (Koonin, 1993) was suggested to have a role in the capping reaction for genomic RNA. Solving of the crystal structure for amino acids 1 to 268 for (NS5) of DEN-2 virus identified both the core subdomain (residues 55 to 222) of the RNA cap (nucleoside-2-0)–methyl transferase topology, and the 54 residues N-terminal to the core that provide a novel GTP-binding site (Egloff *et al.*, 2002).

Flavivirus NS3 is trifunctional, containing domains for serine protease, helicase/NTPase (RNA-stimulated), and RNA triphosphatase (RTPase) activity (Lindenbach and Rice, 2003), and can exist as a complex with NS5 (Kapoor *et al.*, 1995). The protease activity is located within the first 180 amino acids (Li *et al.*, 1999); the conserved catalytic triad His-Asp-Ser of the serine protease activity for KUN NS3 is located at amino acid positions 51, 75, and 137, respectively, with *Ser* 137 located within a strictly conserved sequence, GSTG*S*PI, and the sequence VIGLYGNG (positions 145 to 152) is the completely conserved sequence in the substrate-binding pocket (see Chang, 1997).

KUNV NS3 protease contains the conserved *Flavivirus* motifs associated with the DEAD family of RNA helicases (Gorbalenya *et al.*, 1989) including, for example, G^{198} AGKTRK (motif I), M^{283} DEAH (motif II), and S^{452} AAQRRGRXGR (motif VI). Mutation to alanine of charged residues in motifs I and VI, and in a sequence adjacent to motif IV, abolished both NS3 helicase activity and infectivity of the DEN-2 virus (Matusan *et al.*, 2001). However, the precise stage of *Flavivirus* replication requiring NS3 helicase activity remains undefined. Wengler and Wengler (1993) showed that WNV NS3 also contains the active site of a RTPase that specifically cleaves the β,γ triphosphate bond at the 5′ terminus of RNA (prior to the capping reaction). Bartelma and Padmanabhan (2002) showed that the region between amino acid residues 160 to 180 (N-terminal to helicase motif I) of DEN-2 Virus NS3 is crucial for the RNA-stimulated NTPase activity (Mg^{++} dependent) and appears to share a common active site with RTPase activity (Mg^{++} independent).

The remaining five ns proteins have no recognizable motifs that might be associated with the replication of viral RNA. NS1 is hydrophilic, contains 12 conserved cysteine residues, and is glycosylated at three conserved Asn-linked sites (Blitvich *et al.*, 2001; Pryor and Wright, 1994). KUNV NS1 is difficult to resolve by PAGE from E but was unequivocally identified by Western blotting analysis of infected cell lysates; it formed secretable homodimers in cells, and dimerization was prevented by a Pro250 to Leu mutation at a conserved site (A/G)G*P*XS, causing only a slight delay in replication (Hall *et al.*, 1999). The four small hydrophobic proteins NS2A, NS2B, NS4A, and NS4B are poorly conserved in amino acid sequence and migrate anomalously by PAGE (Coia *et al.*, 1988; Schrader and Westaway, 1988; Speight and Westaway, 1989b). Each has three or four hydrophobic sequences of 18 or more residues that are conserved in their locations within the amino acid sequences (see Chang, 1997). Characterization of the involvement in KUNV RNA replication of any of the four smallest ns proteins and NS1 required biochemical analysis and cryoimmunoelectron microscopy of infected cell ultrastructure.

B. Further Advances in Analyses of KUNV RNA Synthesis

1. Association of Induced Membranes with RNA Replication

Many electron micrographs had revealed a proliferation of cellular and induced membranes in *Flavivirus*-infected cells (Leary and Blair, 1980; Murphy, 1980), but no evidence for their involvement in virus replication or association with IF foci of viral proteins in cytoplasm.

In seminal studies to establish some relationships using the KUNV–Vero cell system, viral proteins and RNA were labeled metabolically with [^{35}S]methionine and [^{3}H]uridine media after the latent period, and membrane fractions were prepared from cytoplasmic extracts by sedimentation through sucrose density gradients (Chu and Westaway, 1992; Chu *et al.*, 1992). The fast-sedimenting or "heavy" membranes contained the virus-specified RNA and proteins, and retained virtually all of the original RdRp activity. The previously observed *Flavivirus*-induced membranes were also found by electron microscopy only in the resin-embedded thin sections of these heavy membrane fractions, together with some mitochondria and smooth and rough ER. These membranes corresponded in morphology to those described previously (Leary and Blair, 1980; Murphy, 1980) and included convoluted membranes (CM), paracrystalline arrays (PCs), and spherical smooth membrane structures (SMSs) 70 to 150 nm in diameter and often with filamentous contents. The membranes were all removed by Non-idet P40 (NP40) detergent treatment, but when the treated heavy membrane fractions were resedimented through a sucrose gradient, RdRp activity was retained in fractions sedimenting at 20 to 40S and was associated with ^{3}H-labeled RI and some RF. The structural proteins E, prM, and C were not sedimented, but ns proteins that cosedimented with RdRp activity comprised NS3, NS1, NS2A, and possibly NS4A (NS5 was degraded beyond detection of its radiolabel during the NP40 treatment). These results showed a close association of RdRp activity with induced membranes and separation of the activity from the structural proteins and NS4B. In similar studies of near complete destruction of NS3 as well as NS5 by trypsin treatment of cell lysates infected with Japanese encephalitis virus, WNV and DEN-2 virus caused no reduction in RdRp activity, indicating that only a very small proportion of NS3 and NS5 is required (Uchil and Satchidanandam 2003a, 2003b).

2. Analysis by IF of the Subcellular Location of KUNV ns Proteins

In order to assist analyses of sites of replication in KUNV-infected cells, antibodies to the (recombinant) ns proteins were prepared in rabbits and used to determine their subcellular locations at 8, 16, and 24 h. Dual labelling of infected cells with antibodies to synthetic dsRNA was also employed (Mackenzie *et al.*, 1998; Westaway *et al.*, 1997a, 1997b). At 8 h, all antibodies stained their antigenic targets in a thin perinuclear rim that extended peripherally in a diffuse pattern, except for staining of NS4B, which extended from the nucleus in a dense reticular somewhat punctate pattern. Beyond the latent period at 16 h, perinuclear staining by all the antibodies to ns proteins now

displayed a thick reticular pattern interspersed with numerous vesicular-like foci of varying sizes and distribution, except that the focal staining by NS5 antibodies was not well defined. Surprisingly, anti-NS4B antibodies also stained the nuclei, but not the nucleoli; however, no nuclear localization sequence (NLS) was identified in the NS4B amino acid sequence. Core protein C also entered the nucleus and contains a bipartite NLS preceding its carboxy-terminal hydrophobic sequence. In order to address the question of whether NS4B was assisted into the nucleus by C, cDNA of their respective genes was inserted separately into the pCINeo plasmid vector for transfection and expression in Vero cells. Cell lines were obtained using geneticin selection; C and NS4B were expressed separately and found to have the same distributions, nuclear and cytoplasmic, as in KUNV-infected cells (Westaway *et al.*, 1997a). Hence it was concluded that the migration of C and NS4B to the nucleus occurred independently of each other. IF staining of dsRNA in the cytoplasm was diffuse early (8 h), but by 16 h was coincident with some of the foci of ns proteins. By 24 h, these dual-labeled foci were more prominent but were usually engulfed in thick reticular-like staining of ns proteins. Because of the common presence of dsRNA representing the RF or putative template of RNA replication in all dual-labeled foci, they appeared to represent cytoplasmic "virus factories." Obviously the resolution of the electron microscope combined with immunogold labeling using the same suite of antibodies were required to further characterize subcellular sites of replication.

IV. Ultrastructure of Induced Membranes, Their Association with the Replication Complex (RC), and Their Origins

A. *Comparative Electron Microscopy of KUNV-Infected Cells*

Flavivirus-induced membranes CM, PC, and SMS remained associated with the purified RC during gradient sedimentation analysis (see Section III,B), but where the RdRp activity actually resided, e.g., in one or more of these induced membranes, remained unknown. Because of the intention to use cryosections in order to take advantage of mild fixation procedures to best preserve the native state of the architecture and antigenic content of induced membranes, comparisons were made of cryosections and epon-embedded sections of KUNV-infected cells at 24 h p.i. (Westaway *et al.*, 1997a, 1997b). The morphologies of CM and PC appeared similar in both types of section, but the SMS of resin sections appeared to be equivalent to the clusters

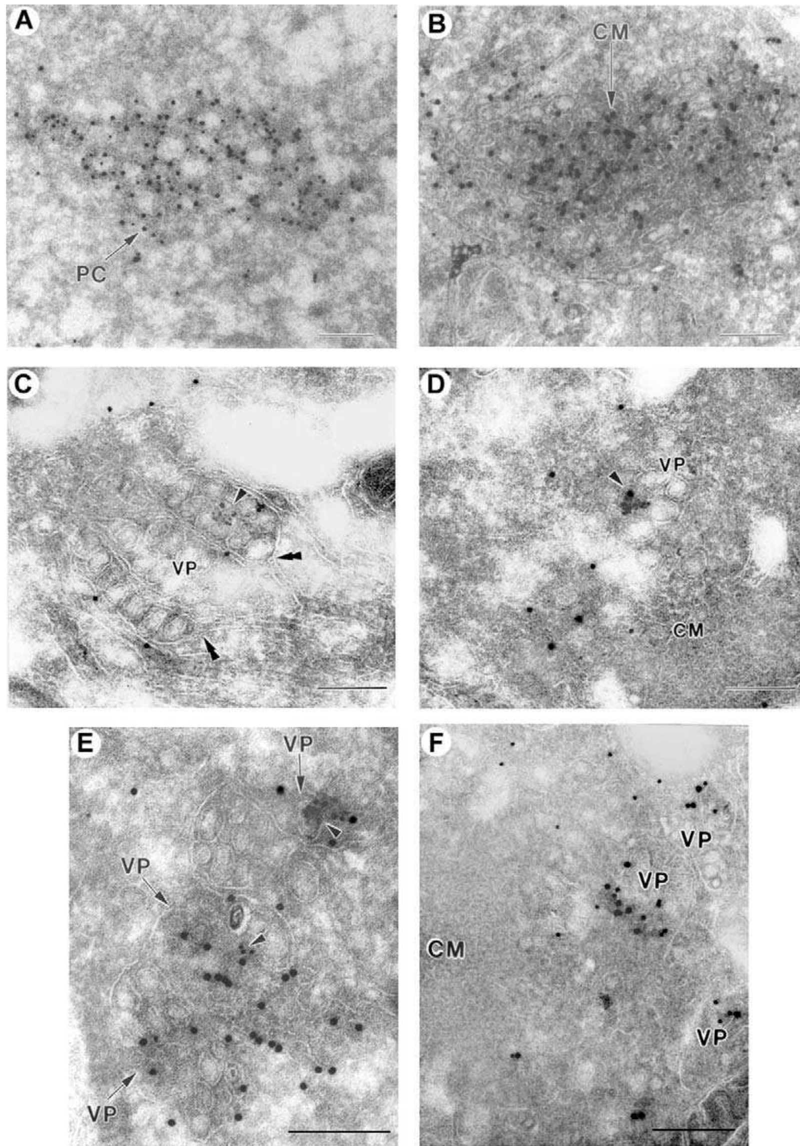


FIG 1. Immunoelectron microscopy of ultrathin cryosections of KUNV-infected cells at 24 h p.i. showing the ultrastructure of induced membranes and their apparent involvement in events of virus replication. (A and B) Components of the viral protease are immunogold labelled with antibodies to NS2B and NS3 (10- and 15-nm particles, respectively) in convoluted membranes (CM) and paracrystalline arrays (PC). (C–F) Immunogold-labeled antibodies to dsRNA (10-nm particles) are colocalized with

of 50- to 100-nm vesicles termed vesicle packets (VPs) in the cryosections. The VPs were bounded by a bilayered membrane, similar to the VPs structures described in cryosections of DEN-2 virus–infected Vero cells (Mackenzie *et al.*, 1996a, 1996b). The CM and PC were often adjacent, or continuous with connecting reticular membranes that sometimes enclosed virus particles or were extensions of the bounding membranes of the VP. Intermediate forms adjacent to CM or PC suggested that they were interconvertible.

B. Cryoimmunoelectron Microscopy of ns Proteins and ds RNA in KUNV-Induced Membranes

Cryosections of infected Vero cells were prepared at 24 h p.i. and immunolabeled using the suite of antibodies to KUNV ns proteins and dsRNA, and protein A–gold particles to visualize the label (Fig. 1). These experiments led to the demonstration of the very close association of most of the ns proteins with the dsRNA template in the induced VP, and of the apparent location of the NS2B–NS3 protease elsewhere, as detailed later (Mackenzie *et al.*, 1998; Westaway *et al.*, 1997a, 1997b).

The CM/PC in cryosections were strikingly colabeled throughout with antibodies to the viral protease NS2B-3 (Figs. 1A and 1B), and also to NS4A. This result provided the first direct evidence of an association of these induced membranes with the intracellular events and products of *Flavivirus* replication. Interestingly, these three ns proteins are cleaved sequentially during translation by NS2B-3. Also relevant are reports that cleavage in *cis* by the NS2B-3 protease of WNV NS2B/3 and of DEN-2 NS4B/5 during expression from constructs *in vitro* required or was considerably enhanced, respectively, by cotranslation with microsomal membranes (Clum *et al.*, 1997; Wengler *et al.*, 1991). The close association of KUNV NS2B and NS3 in the CM/PC was confirmed by their coprecipitation under nondenaturing conditions with their respective antibodies (Westaway *et al.*, 1997b). For the DEN-2 virus, interaction with NS3 in the protease complex

15-nm particles attached to antibodies to NS2A (C), NS4A (D), NS3 (E), and galactosyl transferase (GalT) (F), normally a marker for *trans*-Golgi. Arrowheads in C, D, and E indicate coincidence of the dual labels, and indicate the bounding membrane of the VP in F. The double arrowheads in C point to the continuity of paired membranes of the endoplasmic reticulum with the bounding membrane of the VP. Bars: 200 nm. From Westaway *et al.* (1997b, for A, B, and E), Mackenzie *et al.* (1998, for C, D), and Mackenzie *et al.* (1999, for F), with permission.

appeared to involve the central hydrophobic region of NS2B (amino acids 55 to 94) (Clum *et al.*, 1997; Falgout *et al.*, 1993), and recent modeling of the complex was based on predicted binding of an "internal core" of hydrophobic residues (amino acids 70 to 81) in NS2B to NS3pro (Brinkworth *et al.*, 1999; Matusan *et al.*, 2001; Murthy *et al.*, 1999). However, the corresponding "core" sequence in NS2B of KUNV (G^{70}SSERVDVRLDDD) and of others in the Japanese encephalitis virus subgroup is clearly hydrophilic (Chang, 1997; Coia *et al.*, 1988), and hence the interaction of NS2B in this region with NS3 of KUNV and other species probably differs from the proposed models for the DEN-2 virus. In the cryosections of KUNV-infected cells (Mackenzie *et al.*, 1998; Westaway *et al.*, 1997a, 1997b) the PC were well developed and immunolabeled with NS3 antibodies as early as 16 h. No labeling was found in CM/PC for NS1, NS2A, NS4B, or dsRNA. Some ER membranes appeared to emanate from the CM/PC and were lightly immunolabeled with antibodies to NS2B and NS3 (only). Paired smooth ER membranes connecting separate regions of CM often contained rows of virions and these were not labeled with antibodies to any of the ns proteins. However, ER (possibly rough ER) near the nucleus was strongly labeled with antibodies to NS3.

Any association of NS1 with other ns proteins in the ultrastructure was of particular interest because its involvement in replication had been proposed based on genetic analyses of YFV replication (Muylaert *et al.*, 1996, 1997) and the observation that NS1 of the DEN-2 virus was colocalized with dsRNA in VP by immunogold labeling of cryosections (Mackenzie *et al.*, 1996a). In dual-labeled cryosections of KUNV-infected cells, NS1, NS3, NS2A, and NS4A were colocated with dsRNA in VP, adjacent to CM and PC membranes in the perinuclear area (Mackenzie *et al.*, 1998; Westaway *et al.*, 1997b). The colocation was selective (Figs. 1C to 1E); within each VP, only some vesicles were labeled, others not at all. Immunogold labeling with anti-NS5 antibodies was diffuse within the cytoplasm and reticular membranes, and some co-localization of anti-NS5 andtibodies with anti-dsRNA antibodies was observed (M. T. Kenney, J. M. Mackenzie, and E. G. Westaway, submitted for publication). This clustering of ns proteins suggests that each RC moved to its location in a preformed state or complex. Gold particles appeared to be situated on the membranes of the vesicle rather than inside them.

It was concluded from ultrastructural analyses by cryoimmunoelectron microscopy that the CM/PC represented the putative site of the *Flavivirus* protease because NS2B and NS3 were colocated in abundance, and the site was adjacent to and connected to ER membranes

presumably involved in the translation of progeny viral RNA (apparently synthesized in the adjacent VP). The role, if any, of hydrophobic NS4A in the protease complex is not known; it may be merely trapped in the induced membrane during an occasional delay in cleavage from NS3. In regard to associations with the putative dsRNA template in the VP, that of NS1 was expected (see previous paragraph), and NS3 contains demonstrable helicase/NTPase activities (see Section III,A). However, the presence of NS2A and NS4A in cryosections with dsRNA was unexpected and provided the first direct evidence of their association with the apparent site of replication. The close physical connection of CM/PC, VP, ER enclosing virions, and nearby rough ER supports the interpretation that perinuclear foci observed by IF represent virus factories in which translation, RNA replication, and virus assembly occur.

Although NS4B, the largest of the hydrophobic ns proteins, did not appear to be associated with the active KUNV replicase complex (Chu and Westaway, 1992), its ultrastructural location was examined because it was labeled by IF in the nucleus as well as in the cytoplasm. The putative *Flavivirus* RNA polymerase NS5 of two other species had also been found in the nucleus of infected cells by immunofluorescence (Buckley *et al.*, 1992; Kapoor *et al.*, 1995) but not in KUNV-infected cells. By immunogold labeling of cryosections prepared at 24 h p.i., KUNV NS4B was located throughout the nucleoplasm and in perinuclear membranes, in possibly induced ER membranes and often adjacent to the CM or PC structures (Westaway *et al.*, 1997a). The association of NS4B with ER was particularly evident, in accord with its hydrophobic nature. In a cell line expressing NS4B from the pCINeo plasmid, the immunogold-labeled distribution in the ER and nucleus was equivalent to that of NS4B in KUNV-infected cells. The continued expression of NS4B in the cell line did not appear to result in the induction of specific membrane structures. In summary, the distribution of NS4B in infected cells, its lack of incorporation in the induced membranes CM/PC and VP, and its absence from partly purified replicase complexes (Chu and Westaway, 1992) have given no indication of any role for NS4B in virus replication.

C. Coincidence of Labeled dsRNA and Nascent Progeny RNA in Stable Replication Complexes

The studies reviewed involved identification in KUNV-infected cells of the ns proteins associated with RdRp activity or with putative sites of replication by radiolabeling, membrane purification, IF,

or immunogold labeling of cryosections. It was tentatively assumed that dsRNA in that context was equivalent to the RF/RI functioning as a template for viral RNA synthesis occurring in the sites so defined. However, it was possible that these assumed sites of replication represented only sites of accumulation of dsRNA and some ns proteins rather than the actual stable sites of RNA replication. Furthermore, because all the ns proteins were being synthesized continually, it was not clear whether a supply of newly synthesized proteins (viral or cellular) was required to maintain the active RC. The original model for RNA synthesis (see Section II,C) proposed that on each template only a single nascent strand was being synthesized while it displaced a preexisting strand of the same polarity. All these considerations were addressed by taking advantage of two procedures. First, it was found that bromo-substituted uridine (BrU) was taken up readily by Vero cells (similarly to tritiated uridine) and incorporated rapidly into RNA where it could be detected by immunolabeling with commercially available antibodies to BrdU. Second, all protein synthesis could be blocked by treatment of cells with cycloheximide (CHXM), which reversibly "freezes" ribosomes on messenger RNA as it is being translated.

The stability of the RC at 24 h p.i. has been examined when translation was blocked by CHXM treatment of infected cells (Westaway *et al.*, 1999). Viral RNA synthesis identified by [^{32}P]orthophosphate labeling continued with minimal if any loss throughout the CHXM block, whereas no cellular or ns proteins were found to be labeled by [^{35}S]methionine (Westaway *et al.*, 1999). This cellular translation block had no apparent effect on the location, distribution, or intensity of IF staining of dsRNA foci (Fig. 2A). These results showed unequivocally that ongoing protein synthesis was not required to maintain RdRp activity late in infection and that the RC and its apparent location shown by IF were stable *in vivo* for many hours.

In order to determine whether dsRNA foci represented the true site of viral RNA synthesis, nascent RNA was pulse labeled with BrU for 15 min in actinomycin D–treated cells at 24 h p.i. with KUNV (Fig. 2B). After the pulse, Br–RNA was shown by IF to be perfectly coincident with foci of dsRNA in dual-labeling experiments, and CHXM treatment during the pulse had no effect on the coincidental staining (Westaway *et al.*, 1999). Thus the activity of the RC in its normal location was stable in the absence of ongoing protein synthesis. Finally, infected cells were pulse labeled as before, acetone-fixed, and permeabilized by treatment with Triton X-100 to permit digestion with RNase A in high salt. If several nascent Br–RNA strands were being synthesized on each template, the expectation was that the intensity

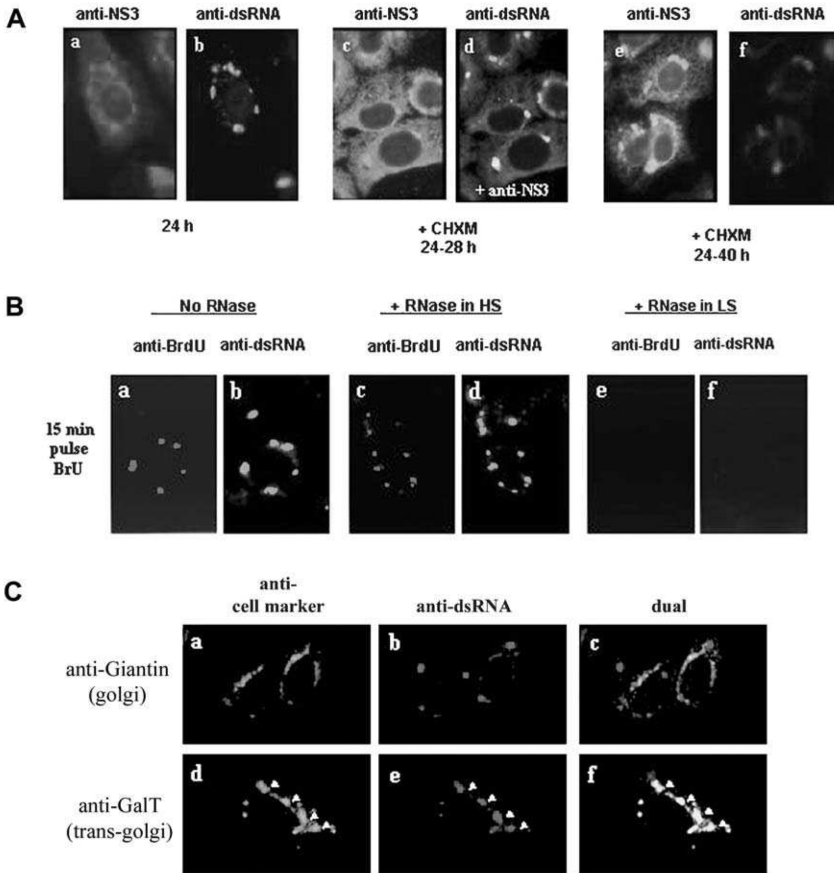


FIG 2. Immunofluorescence of KUNV-infected cells showing (A) the stability of sites of replication during CHXM treatment, (B) the coincidental location of dsRNA templates with nascent viral RNA pulse-labeled with bromo-substituted RNA (Br-RNA), and (C) the strong association of dsRNA with the *trans*-Golgi marker galactosyl transferase (GalT). (A) Cells were treated with CHXM from 24 h and dual labeled as shown; in d only, both labels are shown, producing yellow foci. (B) The pulse period of 15 min in actinomycin D–treated cells represented the time for completion of a cycle of RNA synthesis; after the pulse, cells were immediately permeabilized and treated with RNase in high salt (HS) or low salt (LS) as shown. Commercially available antibodies to BrdU are able to react with Br-RNA. (C) Note that another marker for the golgi (Giantin) is not colocated with dsRNA. From Westaway *et al*. (1999, for A, B) and Mackenzie *et al*. (1999, for C), with permission. (See Color Insert.)

of IF labeling by anti-BrdU antibodies would be reduced drastically by RNase digestion. However, if only a single nascent Br–RNA strand was being synthesized and displacing a preexisting RNA(+) strand as per the original model (see Section II,C), it would remain bound during the 15-min pulse and be protected against RNase digestion. The result showed no detectable loss in intensity of the IF label for Br–RNA after the RNase treatment and the perfect coincidence by IF with the dsRNA was preserved (Fig. 2B).

This section established that the KUN RC was stable during infection and remained active when translation was blocked, that nascent Br–RNA remained bound to the template and was located coincidentally with foci of dsRNA, and that pulse-labeled Br–RNA was resistant to RNase digestion after Triton X-100 treatment, indicating a lack of multiple nascent RNA strands on the dsRNA template. The relationship of replicating RNA to the VP membranes in lysates of WNV-infected cells was explored by Uchil and Satchidanandam (2003b) who showed that removal of the bounding membrane by Triton X-100 released the inner vesicles that still contained RNase-resistant RF. Labeled single-stranded RNAs (tails of RI and released progeny RNA) were nuclease sensitive, indicating that they were oriented outwards from the inner membranes.

D. Biochemical Assays and Consensus Composition of the RC

Because antibodies to dsRNA successfully inhibited the RdRp activity of detergent-treated KUNV-infected cell lysates (Chu and Westaway, 1985), the apparent binding properties of these antibodies were used for specific immunoprecipitation of the RC in [^{35}S]methionine-labeled lysates (Westaway *et al.*, 1997b). The precipitate (Fig. 3A) was devoid of structural proteins, but contained NS5, NS3, NS1, NS2A, and probably NS4A (not clearly resolved by PAGE from the expected migration position of NS2B). Because the *trans*-Golgi enzyme galactosyltransferase (Gal-T) was found to be associated with the apparent sites of replication by IF and by cryo-IEM (see Section IV,E), the immunoprecipitation of radiolabeled-infected cell lysates was repeated using antibodies to Gal-T (Fig. 3B). The same constellation of ns proteins was again precipitated, indicating a very close relationship of Gal-T to the RC (Mackenzie *et al.*, 1999). To confirm the RNA polymerase role of NS5, KUNV recombinant NS5 with a carboxy-terminal hexahistidine tag was produced in baculovirus-infected insect cells, purified, and presented with both specific (9-kb KUN replicon) and nonspecific (8.3-kb Semliki Forest virus replicon) templates. The polymerase

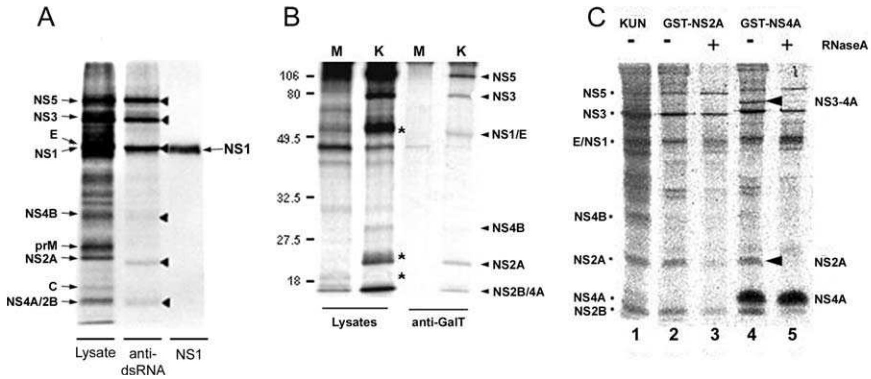


FIG 3. Affinity relationships between KUNV ns proteins and components of the replicase complex. Infected Vero cells were labeled metabolically with [^{35}S]methionine/cysteine from 22 or 24 h to 28 or 30 h, and lysates were either immunoprecipitated with antibodies to dsRNA (A) and GalT (B), or used in binding assays with GST–NS2A and GST–NS4A fusion proteins attached to glutathione Sepharose beads (C). Proteins were separated in 12.5% SDS–polyacrylamide gels for subsequent autoradiography. Arrowheads (in A and B) indicate ns proteins identified in the immunoprecipitates, including NS1, which migrated as shown in lane 3 (A) after *in vitro* translation. (B) M and K refer to mock and infected cells, respectively, and the asterisks in lane 2 indicate the structural proteins E, prM, and C found only in the untreated lysate. (C) Lanes 3 and 5 show the effect of RNase treatment on the recovery of bound proteins, and arrowheads in lane 4 indicate the locations of NS3-4A and NS2A, which were lost during RNase treatment. From Westaway *et al.* (1997b, for A), Mackenzie *et al.* (1998, for B), and Mackenzie *et al.* (1999, for C), with permission.

product obtained with either template was double stranded and migrated similarly in gels to the KUNV RF produced in Vero cells, demonstrating efficient processivity, but the RdRp activity was low and nonspecific (Guyatt *et al.*, 2001). The essential role of the RNA polymerase motif GDD in NS5 was confirmed by a mutation to GVD that caused complete loss of the RdRp activity.

By immunogold labeling of infected cells, NS2A and NS4A were colocated with dsRNA in VP (see Section IV,B), despite the lack of any known motifs associated with replication. To explore this association with the RC, both proteins were prepared as glutathione-*S*-transferase (GST) fusion proteins and used in binding assays either with the 3′UTR of KUNV RNA or with NP-40 treated KUNV-infected cell lysates labeled metabolically with [^{35}S]methionine (Mackenzie *et al.*, 1998). GST–NS2A bound specifically to the 3′UTR in pull-down assays and by Northwestern blot analysis, whereas GST–NS4A bound RNA nonspecifically. In other assays, GST–NS2A and GST–NS4A

bound putative components of the RC in lysates, particularly NS5, NS3, NS2A, and possibly NS1 (Fig. 3C). GST–NS4A also bound strongly to NS4A and uncleaved NS3–4A. After RNase treatment, the only apparent effect on GST–NS2A binding was a small reduction in the recovery of NS2A. In contrast, RNase treatment removed NS2A (and NS3–4A) from the complex bound by GST–NS4A (Fig. 3C), probably because the adjacent region of an attachment site of NS2A to (say) the 3′UTR in the replicating RNA was exposed. In addition to helping deliver the assembling RC to its membrane site, NS2A may have other roles, such as involvement in virus assembly (Kümmerer and Rice, 2002; Liu *et al.*, 2003), exposing a binding site for the RC in the RF/RI, or in the release of progeny RNA from the RC for packaging into virions or functioning as messenger RNA. A role, if any, for uncleaved NS3–4A remains uncertain. Both NS2A and NS4A fusion proteins also bound a cell protein of approximately 50 kDa that may represent the translation elongation factor 1α shown to interact with the WNV 3′UTR RNA (Blackwell and Brinton, 1997).

Data in this and previous sections provide a consensus composition of the KUNV RC, namely NS5, NS3, NS1, NS2A, NS4A, and the double-stranded RF/RI template.

E. Putative Origins of Induced Membranes

To extend studies involving the ultrastructural characterization of KUNV replication, the cellular origins of the cytoplasmic membrane structures induced during infection were investigated by both IF and cryoimmunoelectron microscopy in conjunction with a panel of antibodies raised against resident host proteins of various cellular compartments (Mackenzie *et al.*, 1999). The initial IF of KUNV-infected cells at 24 h p.i. revealed striking colocalization of dsRNA foci with a marker for the *trans*-Golgi (anti-GalT), but much less with markers for the RER [anti-PDI (protein disulfide isomerase)] and the intermediate compartment (anti-ERGIC53). Interestingly, it was apparent that some of the GalT had redistributed away from the Golgi region because the Golgi apparatus (visualized with antibodies to Giantin) appeared unaltered and some GalT also remained within the Golgi region (Fig. 2C). A further indicator of a possible role of the Golgi apparatus in the development of induced membranes was the observation that brefeldin A (a Golgi-disrupting agent) prevented induced membrane formation when added toward the end (at 12 h) of the latent period (Mackenzie *et al.*, 1999). In addition, the presence of brefeldin A from 1.5 h p.i. appeared to inhibit KUNV replication drastically

(Mackenzie and Westaway, 2001). There was no association of dsRNA foci with other cell organelles, such as endosomes and lysosomes (Mackenzie *et al.*, 1999), that are involved in the replication of togaviruses [Semliki Forest virus (Kujala *et al.*, 2001) and rubella virus (Magliano *et al.*, 1998)]. The involvement of the *trans*-Golgi marker GalT appeared to be selective, and while other markers for this region (including p230, TGN46, and possibly clathrin) showed a similar redistribution to that of GalT, some markers (such as p200 and γ-adaptin) did not. When these results were extended by EM (Mackenzie *et al.*, 1999), it was observed that the anti-GalT antibodies specifically labeled individual vesicles within the VP, and this distinctive labeling was coincident with that of anti-dsRNA in dual-labeling experiments (Fig. 1F). In contrast, anti-ERGIC53 antibodies decorated the induced CM/PC structures and some smaller vesicles within the cytoplasm near the CM/PC structures, whereas anti-PDI staining was confined to apparent ER membranes that appeared continuous with the CM/PC but was seldom observed to internally label the CM/PC. These results suggest that an intimate relationship exists between the induced membranes involved in KUNV replication, confirming that distinct functions are associated with the different membrane structures (Section IV,B). In addition, these results have also revealed possible but as yet unidentified interactions occurring between different cellular compartments that may exist in uninfected cells.

How the different cellular compartments contribute to the biogenesis and functionality of KUNV-induced membrane structure will require further investigation. Such studies may reveal possible indicators of *Flavivirus* pathogenesis and also may provide a unique approach to the isolation and purification of proteins involved in normal cellular processes.

V. Molecular Approaches to Study Formation and Operation of the RC

A. Generation of Infectious cDNA Clones and of Subgenomic Replicons of KUNV

To facilitate genetic analyses of KUNV replication, a functional full-length cDNA clone of KUNV pAKUN was assembled from cDNA clones mainly prepared by Coia *et al.* (1988). Transcripts of this cDNA template had relatively low specific infectivity ($\sim$1 plague-forming unit per 10 μg RNA; Khromykh and Westaway 1994). Subsequently,

a new full-length cDNA clone, FLSDX, was assembled from large DNA fragments obtained by reverse transcription–polymerase chain reaction (RT-PCR) of virion RNA using high-fidelity DNA polymerases (Khromykh *et al.*, 1998a). The specific infectivity of the RNA produced from the FLSDX cDNA template was $\sim 10^5$-fold higher than that produced from the pAKUN cDNA template. Sequence comparisons suggested that all but one of the several differences between wild-type viral RNA and the FLSDX cDNA clone appeared to represent errors in the original KUNV sequencing data (Liu *et al.*, 2003); this single difference was a Pro to Leu substitution of amino acid 250 in the NS1 gene, which as reported previously contributed to an initial delay in the replication of recovered virus *in vitro* and *in vivo* (Hall *et al.*, 1999). It probably also contributed to the observed lower (100- to 200-fold) specific infectivity of FLSDX RNA compared to that of purified virion RNA (Khromykh *et al.*, 1998a). Additional factors occurring as a result of preparation of RNA *in vitro* may also have contributed to the lower specific infectivity of *in vitro* RNA transcripts.

One of the most recent additions to the molecular toolbox for studying *Flavivirus* replication has been the construction of DNA-based infectious cDNA clones for WNV (Yamshchikov *et al.*, 2001) and KUNV (Khromykh *et al.*, 2001b), which allow generation of an infectious viral RNA *in vivo* by cellular RNA polymerase II from transfected plasmid DNA incorporating a cytomegalovirus (CMV) promoter. The ability to use a plasmid vector to continuously produce and accumulate genomic RNAs in cells should facilitate studies with the mutations that severely inhibit or completely abolish different events in virus replication. For example, KUNV DNA-based clones allowing the production of wild-type and replication-deficient KUN genomic RNAs were used to demonstrate for the first time the requirement for replication of *Flavivirus* RNA before its packaging into secreted virions (Khromykh *et al.*, 2001b).

In order to facilitate the study of RNA replication in isolation from virion assembly and maturation, the first *Flavivirus* subgenomic replicon cDNA constructs were prepared, from which self-replicating KUN RNAs could be transcribed (Khromykh and Westaway, 1997). These clones were constructed by deleting most of the structural region of the genome except for the 60 nucleotide sequence coding for the first 20 amino acids of the C protein and the 66 nucleotide sequence coding for the last 22 amino acids of the E protein. The retention of these two sequences was essential for RNA replication. It was also shown by mutation of the first (native) translation initiation codon that the RNA sequence *per se* in the C gene, and not the encoded amino acid sequence,

was absolutely required. Subsequently it was established that only the first 51 nucleotides in the C gene, terminating immediately downstream of a proposed cyclization sequence, were required for RNA replication (Khromykh *et al.*, 2001a; see Section V,C.) Interestingly, replication of KUN replicon RNAs in transfected BHK cells did not appear to produce the severe cytopathic effect normally characteristic of KUNV infection (Khromykh and Westaway, 1997).

B. Complementation Systems and Formation of the RC

In order to provide functional ns proteins of the RC for complementation experiments, a stable BHK cell line continuously producing replicating KUN replicon RNA (repBHK) was generated. It was achieved by the transfection of BHK cells with KUN replicon RNA containing an insertion of the cassette for the encephalomyocarditis virus internal ribosomal entry site—neomycin phosphotransferase gene (IRESNeo) downstream of the stop codon, followed by selection of replicon-expressing cells with the antibiotic G418 (Khromykh *et al.*, 1998a; Khromykh and Westaway, 1997). This repBHK cell line was then used extensively as a helper in *trans*-complementation experiments with full-length KUNV RNAs containing deletions and mutations in ns genes (Khromykh *et al.*, 1998a, 1998b, 1999b, 2000; Liu *et al.*, 2002). The replication and expression of complemented defective genomic KUNV RNAs in repBHK cells could be distinguished readily from replication of the helper replicon RNA by Northern blot analysis with a radioactive probe complementary to the sequence in the structural region and by IF analysis with anti-E antibodies. Briefly, replication of RNAs with large deletions in the genes for NS1 (84%), NS3 (71%, including the entire helicase region), and NS5 (65%, including the entire RNA polymerase domain) was complemented efficiently when these deletions were present either as a single deletion or when combined in the same defective RNA molecule (Fig. 4; Khromykh *et al.*, 2000). Additional deletions extending into the carboxy terminus of NS1 (increase to 97%) and into the amino terminus of NS5 (increase to 75%) were also complemented, but with much lower efficiencies (Fig. 4; Khromykh *et al.*, 2000). However, none of the large deletions in the four small ns genes were complemented (see open boxes I, II, and III in Fig. 4), suggesting that hydrophobic interactions of the products with membranes were critical. Interestingly, while complementation of RNAs with deletions as defined earlier in NS1 and NS5 genes permitted recovery of the secreted defective-complemented viruses, complementation of RNAs with deletions in NS3 helicase/NTPase

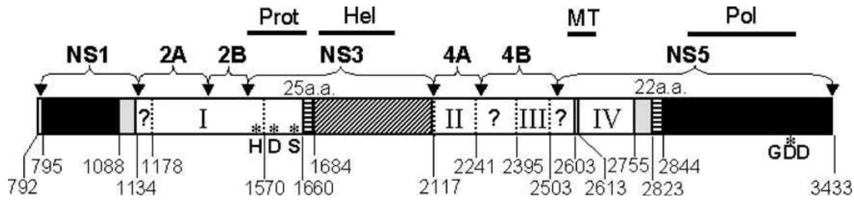


FIG 4. Map of *cis*- and *trans*-acting elements in the nonstructural region of KUN virus RNA. Numbers represent amino acid positions in the KUN polyprotein of 3433 residues and show boundaries of introduced deletions. Prot, Hel, MT, and Pol indicate corresponding functional domains of viral protease, helicase, methyl transferase, and RNA polymerase, respectively. H, D, and S with asterisks show the location of amino acids of the catalytic triad of the serine protease, and GDD with an asterisk shows the location of the characteristic RNA polymerase motif. Black boxes show *trans*-acting sequences complemented efficiently for both RNA replication and virus recovery in NS1 or in NS5. Gray boxes show *trans*-acting sequences complemented inefficiently for both RNA replication and recovery of secreted infectious virus particles in NS1, in MT of NS5, and in the carboxy-terminal region of NS5. The diagonally striped box shows the *trans*-acting sequence in NS3 complemented only for RNA replication but not for the recovery of secreted virus particles. Open boxes I to IV represent *cis*-acting sequences apparently not complemented in *trans* for any deletions. Boxes with a question mark show regions that have not been analyzed. From Khromykh *et al.* (2000), with permission.

did not produce any secreted defective virus, despite successful complementation of RNA replication (Khromykh *et al.*, 2000; Liu *et al.*, 2002). This defect in production of complemented virus demonstrated a requirement of NS3 in *cis* for virus assembly and/or secretion. Unsuccessful complementation experiments with a mutation producing a frame shift at the beginning of the NS5 gene in KUNV RNA, or with a large carboxy-terminal deletion of 760 amino acids (84% of NS5), indicated an essential role for translation of the amino-terminal region in the formation of complementable defective RC. These results led to the proposal that NS3 binds to one or more conserved regions in the amino-terminal domain of NS5 during completion of translation in *cis*, and assembly of the RC then continues on the 3'-terminal stem loop of the same RNA molecule, which becomes the template for RNA(−) strand synthesis (Khromykh *et al.*, 1999b), as discussed in Section V,D. It is possible that the remaining poorly complementable regions in NS1 and NS5, as well as some regions in two small hydrophobic ns proteins (NS2A and NS4A), may be involved in assembly of the RC (Khromykh *et al.*, 1999b, 2000). Further fine complementation mapping by introducing small deletions and point mutations into the regions required for efficient complementation, combined with

protein–protein and protein–RNA-binding assays, should assist in defining the proposed binding motifs in the components of the RC and the mechanisms of its assembly.

Complementation experiments with mutated NS1 and NS5 showed that the mechanisms for complementation of individual *Flavivirus* ns proteins may differ. For example, KUNV RNAs with lethal mutations in the NS1 gene (Khromykh *et al.*, 1999a) and YFV RNA with a large deletion in the NS1 gene (Lindenbach and Rice, 1997, 1999) could in either case be complemented by using a helper cell line stably producing NS1 alone from the noncytopathic Sindbis replicon vector. In contrast, KUNV RNA expressing a defective NS5 protein with the RNA polymerase motif GDD deleted could be complemented efficiently only in a Sindbis replicon-derived cell line expressing an NS1-to-NS5 polyprotein cassette, whereas complementation of the same RNA in a cell line expressing helper NS5 alone (and in large excess) from the Sindbis replicon was very inefficient (Khromykh *et al.*, 1999a). These contrasting results suggested that NS1 may represent a separate independent (lumenal) component of the RC and may be involved in the later stage of RC assembly by binding to the free "replicase" subunit of the RC, possibly via proposed interactions with NS4A (Lindenbach and Rice, 1999) to complete formation of the RC (see Khromykh *et al.*, 1999a). According to this model, any individually produced helper NS1 can readily substitute for the defective NS1. In contrast, NS5 is probably an integral part of the initial "replicase" subunit of the RC together with the other ns proteins (e.g., NS3, NS2A and NS4A; see Khromykh *et al.*, 1999a) and therefore cannot freely exchange with the individually produced helper NS5, possibly due to steric hindrance of any exchange within the RC.

In summary, these results provided a gradient map of complementation efficiencies among and within the ns proteins (Fig. 4) and contributed to an understanding of the assembly of the RC and the relationships of the ns proteins within the RC.

C. Cyclization of Genomic RNA

Conserved complementary cyclization sequences (CSs) in the 5′ region of the core gene and in the region preceding the conserved 3′-terminal stem loop of mosquito-borne viruses were noted by Hahn *et al.* (1987). An opportunity to explore the role of these sequences during infection became possible when the infectious cDNA clone of KUNV and the KUN replicon became available. In KUN replicon constructs, deletions in the core gene did not adversely affect *in vitro*

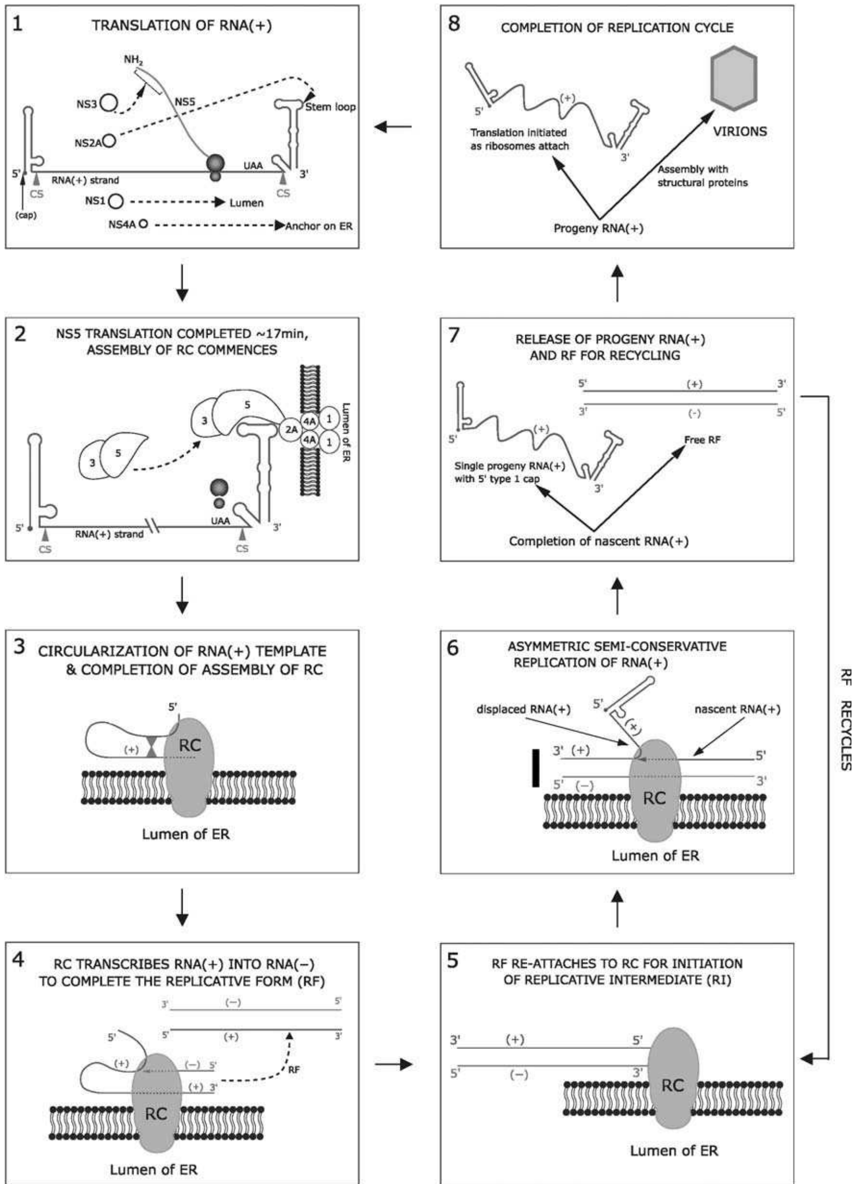
translation but caused loss of viability unless the first 20 codons were present, including the 5′ CS (5′-UCAAUAUG-3′) located at nucleotides 41 to 48 of the core gene (Khromykh and Westaway, 1997). Very large deletions of sequences in the *Flavivirus* 3′UTR beyond the stop codon were not lethal, provided that the recognizable 3′CS (Khromykh *et al.*, 2001a) were retained, e.g., for KUN replicon (Khromykh and Westaway, 1997), DEN-4 virus (Men *et al.*, 1996), and tick-borne encephalitis virus (Mandl *et al.*, 1998). Computer modeling showed that base pairing occurred between the 5′ CS (nucleotides 137 to 144) and the 3′ CS (nucleotides −97 to −104) within the covalently linked 5′ and 3′ regions of the KUNV genome. The RNA secondary structure was disrupted extensively when the 5′ CS was deleted or when noncomplementary mutations were introduced separately into either the 5′ or the 3′ CS. However, base pairing of the CS and the original secondary structure were restored when both the mutated and the complementary CSs were in the same RNA sequence. When tested in the KUN replicon, only the construct with the compensatory mutations was viable, whereas replicons with independently mutated 5′ or 3′ CS were not, and the introduced mutations in the viable construct were retained during replication (Khromykh *et al.*, 2001a). This result showed that it was the base pairing rather than the nucleotide sequence *per se* that was essential for replication to occur. Computer modeling of RNA of a range of *Flavivirus* species showed base pairing of putative CS in 5′ and 3′ locations similar to those of KUNV RNA. Results with KUN replicon RNA established the essential roles of both 5′ and 3′ CS in RNA replication *in vivo*. The CSs were also required for *in vitro* RdRp assays with DEN-2 virus–infected cell lysates as well as for the assays with purified his-tagged NS5 and terminal RNA sequences as templates (Ackermann and Padmanabhan, 2001; You and Padmanabhan, 1999). Some direct physical interaction *in vitro* involving CS in the 5′- and 3′-terminal segments of DEN-2 virus RNA was demonstrated using the psoralen/UV cross-linking method (You *et al.*, 2001). The requirement of the 5′ and 3′ CS for *in vivo* replication was readily confirmed using YF replicon RNA (Corver *et al.*, 2003) and for infections with YFV RNA transcribed from cDNA (Bredenbeek *et al.* 2003).

D. RNA(−) Strand Synthesis on RNA(+) Strand Template

Although purified NS5 of KUNV and other *Flavivirus* species has demonstrable RdRp activity *in vitro*, the activity is weak and not template specific (Guyatt *et al.*, 2001). It seems likely that additional

ns proteins are required at least for specific template recognition and copying. Cellular proteins such as EF-1α may assist by binding to the defined sequence CACA in the upper half (adjacent to the pseudoknot) of the conserved 3′-terminal stem loop, as shown for WNV RNA (Blackwell and Brinton, 1997), thus helping to promote assembly of the RC. In modeling the RC (Fig. 5), it is assumed that the consensus components NS3, NS2A, NS4A, and NS1 assemble with NS5 on or near the structurally conserved 3′-terminal stem loop as translation of the RNA(+) in *cis* is completed. A likely site for binding or commencement of assembly is at the top of the loop on the completely conserved pentanucleotide 5′CAGAC3′ (Wengler and Castle, 1986); mutations of this pentanucleotide were lethal for KUN replicon RNA synthesis (Khromykh, *et al.*, 2003). This mode of assembly is supported by data discussed in Section V,B on the proposed binding of KUNV NS3 to NS5 during translation in *cis* and on the reported interactions of the 3′UTR and/or the 3′ conserved stem loop with KUNV NS2A (Mackenzie *et al.*, 1998), KUNV NS5 (A. A. Khromykh and E. G. Westaway, unpublished results), and NS3 and NS5 of DEN-1 virus (Cui *et al.*, 1998) and Japanese encephalitis virus (Chen *et al.*, 1997). An inferred role of KUNV NS1 in complementation experiments as a separate independent (lumenal) component of the RC was also proposed (Section V,B). NS1 and NS4A of YFV have been implicated by genetic analyses involving the synthesis of minus strand RNA (Lindenbach and Rice, 1997, 1999; Muylaert *et al.*, 1996, 1997). Hydrophobic NS4A appears to provide a link between (say) hydrophobic NS2A in the assembling RC and the membrane sites of replication, where NS4A binds and can interact possibly by hydrophilic extensions between its transmembrane-spanning domains with NS1 in the lumen of the ER.

As the assembly of the RC and its attachment to the ER site of replication is completed, it is proposed that cyclization of the positive-strand RNA template occurs (see Section V,C). RNA(+) must also function as messenger RNA early and late during infection to provide the structural proteins for virus assembly as well as the ns proteins for the RC. Because the synthesis of RNA(−) occurs infrequently relative to RNA(+) synthesis, the circularization discussed in Section V,C may be a relatively rare event occurring among the total population of positive-strand RNA. However, it could be essential for appropriately presenting the template for RNA(−) synthesis. For example, if cyclization occurred concurrently with assembly of the RC on (say) the 3′-terminal stem loop, this would allow ribosomes to complete translation of RNA(+) and their subsequent release, but prevent reattachment at the 5′ end. This would preclude the risk of collision


1
TRANSLATION OF RNA(+)
NH2
NS3
NS5
Stem loop
NS2A
UAA
5'
RNA(+) strand
3'
CS
CS
NS1
Lumen
(cap)
NS4A
Anchor on ER
2
NS5 TRANSLATION COMPLETED ~17min,
ASSEMBLY OF RC COMMENCES
Lumen of ER
RNA(+) strand
UAA
3
CIRCULARIZATION OF RNA(+) TEMPLATE
& COMPLETION OF ASSEMBLY OF RC
RC
Lumen of ER
4
RC TRANSCRIBES RNA(+) INTO RNA(−)
TO COMPLETE THE REPLICATIVE FORM (RF)
RF
Lumen of ER
5
RF RE-ATTACHES TO RC FOR INITIATION
OF REPLICATIVE INTERMEDIATE (RI)
Lumen of ER
6
ASYMMETRIC SEMI-CONSERVATIVE
REPLICATION OF RNA(+)
displaced RNA(+)
nascent RNA(+)
Lumen of ER
7
RELEASE OF PROGENY RNA(+)
AND RF FOR RECYCLING
Free RF
Single progeny RNA(+)
with 5' type 1 cap
Completion of nascent RNA(+)
8
COMPLETION OF REPLICATION CYCLE
Translation initiated
as ribosomes attach
VIRIONS
Assembly with
structural proteins
Progeny RNA(+)
RF RECYCLES

between the RC and ribosomes advancing from opposite ends of the same template molecule, especially if transport of the RC to a sequestered membrane site occurs during its assembly, as proposed (Khromykh *et al.*, 1999b).

Completion of synthesis of the RNA(−) strand results in formation of the RF in which the RNA(−) strand remains base paired to the RNA(+) strand. No free RNA(−) could be detected during replication of the KUN replicon (Khromykh and Westaway, 1997). In the relevant experiment, an IRES-CAT gene cassette was inserted in the opposite orientation in the 3′ UTR. Efficient amplification of this transfected RNA occurred, but no CAT gene expression from the minus strand was detected. There was no apparent impediment to its expression because the reporter gene was translated (and CAT activity detected) from an *in vitro*–transcribed RNA(−) strand in a rabbit reticulocyte

FIG 5. Model for the RNA replication cycle of the *Flavivirus* replication complex (RC). Step 1: During translation of the ns proteins in *cis* from the genomic RNA, NS3 is assumed to bind to the terminal product NS5 at one or more conserved regions in the N-terminal domain while other polyprotein cleavage products (NS1, NS2A, and NS4A) move to the sites as indicated. Step 2: On completion of translation, assembly of the RC commences on the 3′UTR via binding of NS2A, probably at the conserved 3′-terminal stem loop at which the NS3 and NS5 components also bind. The location of the complex shown on the loop is arbitrary. The complex still attached to the RNA(+) strand is transported to the membrane site of replication by affinity of the hydrophobic regions of NS2A interacting with those of NS4A (shown as a dimer), which in turn is bound by its hydrophilic extensions into the lumen between transmembrane domains to dimeric NS1 in the lumen. Step 3: The RC is now complete and may undergo rearrangement as the RdRp domains of NS5 bind to the template, which circularizes as shown via the conserved complementary sequences (CS) located near the 5′and 3′-terminal stem loops. Step 4: The RC commences to transcribe the RNA(+) template into RNA(−), and a short dashed arrow indicates the direction of synthesis (5′ to 3′) of the nascent RNA(−) strand. The association with membranes and the consensus composition of the RC (NS1, NS3, NS5, NS2A, and NS4A) are described in the text. The RF is formed during the completion of transcription by base pairing of the newly synthesized RNA(−) strand with the RNA(+) strand. Step 5: The released RF reattaches to the RC to function as a template. Step 6: The replicative intermediate (RI) is formed as the RC commences synthesis of a RNA(+) strand by asymmetric and semiconservative replication on the RF template. The template moves through the RC while the single nascent RNA(+) strand displaces the preexisting RNA(+) strand. Step 7: The displaced RNA(+) strand is released and a type 1 ^{7}Me-guanosine cap is added by enzymic activities of NS3 (RNA triphosphatase) and NS5 (guanylyl transferase and methyl transferase), possibly while they are still associated in the RC. The RF is now free to recycle through the membrane-bound RC (late in infection, this occurs within the induced vesicle packets). Step 8: Progeny capped RNA(+) strands either assemble into virions or attach to ribosomes so the whole cycle can be repeated. Adapted from Khromykh *et al.* (1999b), with permission. (See Color Insert.)

lysate (A. A. Khromykh, unpublished result). Cleaves *et al.* (1981) noted that the rate of DEN-2 virus RNA(+) synthesis increased by at least 10-fold after the latent period relative to that of RNA(−) synthesis. In KUN replicon-transfected cells, the rate of RNA(−) synthesis appeared to no longer increase after 16 h, whereas an increase in the synthesis of positive-sense RNA did occur (Mackenzie *et al.*, 2001), hence some control mechanism must be involved. It has been shown that the KUNV core protein binds strongly and specifically to the 3′UTR of RNA(+) (Khromykh and Westaway, 1996). An accumulation of C late in the latent period may thus bind and inhibit assembly of the RC on the 3′ UTR, while initiating the process of formation of the core particle preceding virion assembly (Westaway, 1980).

E. RNA(+) Strand Synthesis and Recycling RF as Template

The increase in KUNV-positive strand RNA synthesis after the latent period (Mackenzie *et al.*, 2001) is accompanied by the appearance of the induced VP containing the RC (see Section IV,B). The VP appears to provide a scaffold for the RC to facilitate and maintain efficient RNA(+) synthesis. Because of the restriction to an average of only one nascent RNA(+) strand in the RI, the RF must be recycled rapidly as the template after the completion of synthesis of each strand (Chu and Westaway, 1985). During *in vitro* RdRp assays of lysates from DEN-2 virus–infected cells, release of the template from the RC and reinitiation on exogenously supplied RF templates appeared to occur (Bartholomeusz and Wright, 1993). Template exchange may also have occurred in RdRp assays when 5′- and 3′-terminal regions of DEN-2 virus RNA were supplied together in the same reaction (You and Padmanabhan, 1999).

It is proposed that an accumulation of the RC occurs in selected regions of the VP, as shown by cryoimmunoelectron microscopy (see Section IV,B). As each cycle of RNA(+) strand synthesis is completed, the RF is released and reattaches to an adjacent site comprising the membrane-bound components of the RC (Fig. 5). Note that the RF moves through the RC, which remains membrane bound. Factors controlling release and reattachment of the RF are unknown. Analyses of the stability of location and activity of the RC established that recycling of the RF is an effective strategy in maintaining RdRp activity even in the absence of ongoing protein synthesis (see Section IV,C).

An unexplored area in the *Flavivirus* RNA replication cycle is the capping reaction. A ^{7}Me-guanosine type 1 cap was shown to be present

at the 5′ end of WNV genomic RNA but not on RNA in the RF (Wengler *et al.*, 1978), and *in vitro* translation of uncapped KUNV RNA produced many products due to internal initiation apparently at several AUG codons with "Kozak" flanking sequences, whereas initiation occurred correctly at the first AUG codon of capped viral RNA (A. P. Schrader and E. G. Westaway, unpublished results). Surprisingly, in successful coinfections with poliovirus, the expected dependence of KUNV RNA translation on the cap-binding protein p220 (EIF4G, cleaved during poliovirus infection) was somehow circumvented even though translation of all cell mRNAs was blocked by p220 cleavage (Schrader and Westaway, 1990). Based on the description of the capping reaction by Bisaillon and Lemay (1997), the RTPase activity demonstrated for WNV NS3 (Wengler and Wengler, 1993) and DEN-2 NS3 (Bartelma and Padmanabhan, 2002) commences the capping reaction by cleaving the γ-phosphate from each progeny RNA(+) strand, leaving a 5′diphosphoryl-terminated RNA to which a GMP residue of GTP is linked covalently by the guanylyl transferase activity of NS5 (Egloff *et al.*, 2002), followed by methylation of the cap via the putative SAM-methyl transferase activity of NS5 (see Section III,A). It seems likely that the capping reactions occur on the 5′ terminus of each progeny RNA(+) as it commences to be displaced (5′ end first) during the initiation of synthesis of the nascent RNA(+) strand on the RF template by the RC, which could provide the required three enzymes located in NS3 and NS5. The RNA helicase activity of NS3 may also be involved in initiation of the displacement process.

F. Summary of the Flavivirus *RNA Replication Model*

The basic features of the model for *Flavivirus* replication appear to be established, although some assumptions may require confirmation. As shown in Fig. 5, genomic RNA(+) is translated, the RC commences to assemble on the 3′ UTR of the just translated RNA as it circularizes via the CS, and the complex attaches to the membrane site of replication. The template is then copied into RNA(−) by the polymerase activity of NS5 as part of the newly formed RC with the consensus composition NS5, NS3, NS1, NS2A, and NS4A. The RNA(−) strand remains bound by base pairing in the RF, and no free RNA(−) strands appear to exist in KUNV-infected cells. The RF is converted to an RI when the RC commences synthesis of a single RNA(+) strand per template by asymmetric and semiconservative replication. The completed nascent RNA(+) strand remains bound in the RF by base pairing until displaced and presumably capped during the next round of RNA(+) synthesis. Successive

rounds of RNA(+) synthesis occur on the recycling RF template as it relocates to the same or an adjacent RC in the VP. The role of the relocated *trans*-Golgi enzyme Ga1T at the site of replication remains unknown. Progeny RNA(+) strands escape from the VP site to function as viral mRNA or to form cores and commence assembly into virions.

The X-ray crystal structure of *Flavivirus* NS5 (Egloff *et al.*, 2002). may shed some light on how both copying and capping are affected by this multipurpose enzyme and may help provide information on how the components of the *Flavivirus* RC interact with each other. Also lacking is how the VP (and CM/PC) are induced and how the RC becomes located in the VP. Meanwhile the model replication system as presented invites further experimental work that should confirm, modify, or extend the salient features.

G. Comparisons with Similar Strategies of Other RNA Viruses

The strategy of *Flavivirus* replication has some features in common with that of other RNA viruses and initially warrants comparisons with the well-characterized picornaviruses, especially poliovirus. Strategies of both are based on a RNA(+) genome with a single open reading frame and gene ordering in which the structural genes are located at the 5′ end and the RNA polymerase gene is at the 3′ end of the coding region. However, the picornaviruses differ profoundly in several respects. A small protein (VPg) serves as a primer for RNA synthesis, and the RNA(+) strand is poly(A) tailed, thus there is no 3′-terminal conserved stem loop; the synthesis of RNA(+) strands is initiated rapidly on an RI consisting of one RNA(−) template and six to eight nascent RNA(+) copies; the role of the RF is not clear (Rueckert, 1996).

In contrast to picornaviruses, there are some striking parallels with *Flavivirus* RNA replication from an apparently unlikely source. The genome of the bacteriophage phi6 has three dsRNA segments (L, M, S), each of which is a template for RNA synthesis, and the largest (L; 6.6 kb) codes for the RNA polymerase (L2, theoretical size 75 kDa) and the associated components of the RC. The 2-Å resolution X-ray structure of the active *pol* subunit (phi6 protein L2 or pP2) has been presented and found to be "highly similar" to that of a flavivirus, namely hepatitis C virus NS5B; the two structures resemble each other more than either resembles any other known polymerase structure (Butcher *et al.*, 2001). In bacteria transfected with plasmids expressing cDNA copies of the phi6 genome, structures similar to procapsids are formed. These particles take up viral single-stranded RNA(+) strands and synthesize RNA(−) strands to produce dsRNA that is then used as a

template for the production of progeny RNA(+) strands (Gottlieb *et al.*, 1990). Phi6 nucleocapsids prepared from purified virions by treatment with Triton-X100 were able to synthesize progeny RNA during an *in vitro* polymerase reaction; transcription intermediates (equivalent to RI) representing the three sizes of dsRNA segments were then visualized by electron microscopy. The L transcription intermediates were always associated with only one progeny strand after a 10-min reaction, and this was attributed to very infrequent initiation followed by synthesis involving displacement of the parental strand (Usala *et al.*, 1980). The purified recombinant L2 protein efficiently utilized phi6-specific RNA(+) substrates to produce dsRNA base paired with full-length RNA(−) strands, but also accepted heterologous single-stranded RNA templates (Makeyev and Bamford, 2000). RNA transcripts of 13.5 kb produced from cDNA of fused L, M, and S dsRNA segments were infectious (Qiao *et al.*, 2000). In further analogy to *Flavivirus* RNA replication, the polymerase of phi6 and related dsRNA phages has a preference for cytidine as the penultimate nucleotide at the 3′ terminus of both RNA(+) and RNA(−) strands (Butcher *et al.*, 2001; Yang *et al.*, 2001) as occurs in the conserved termini of *Flavivirus* RNA (compare genomic RNA: 5′-AG\\CU-3′). Thus for amplification KUN replicon RNA, the penultimate cytidine in RNA(+) and RNA(−) strands was found to be essential for replication (Khromykh *et al.*, 2003) In summary, the known preferred by phi 6 RdRp for 3′ penultimate C and the use of a recycling dsRNA template for semiconservative and asymmetric synthesis of a RNA(+) strand that displaces the preexisting RNA(+) strand from the template are entirely in accord with the model for *Flavivirus* RNA replication (Fig. 5). In making comparisons of phi6 with flavivirus HCV polymerase, Butcher *et al.* (2001) commented not only on the very close similarity in crystal structure, but also on the ability of both polymerases to copy single-stranded RNA without a primer, thus indicating an unexpected evolutionary link between phi6 and the flaviviruses. This link is strengthened by the similarity to the RNA replication strategy of KUNV discussed in this review.

VI. Development of the Kunjin Replicon Expression System for Vaccines and Gene Therapy

A. Design of Replicon Vectors

The efficient C20DXrep replicon (Khromykh *et al.*, 1998a) was modified to allow inframe cloning of heterologous genes (HGs) for expression as fusion products with the amino-terminal 20 amino acids of

the C protein (C20) and the carboxy-terminal 22 amino acids of the E protein (E22; Fig. 6A). The E22 signal sequence should target the HG product to the ER membranes. To allow release of the HG product from the fused KUN peptides, sequences for the autoprotease 2A of the foot-and-mouth disease virus and/or ubiquitin were inserted at the relevant cleavage sites, and in the last construct in Fig. 6A, additional insertion of the sequence for the encephalomyocarditis virus IRES downstream of the inserted HG should result in the release of a virtually authentic HG product.

KUN replicon vectors for the stable expression of HGs were constructed by insertion of an IRESNeo cassette into the 3'UTR as indicated in Fig. 6A (or of the puromycin acetyltransferase gene between C20 and 2A) so that antibiotic-resistant cell lines expressing HGs could be selected (Fig. 6A; Varnavski *et al.*, 2000). The KUN replicon vectors were also constructed as either RNA-based or DNA-based vectors. RNA-based vectors were constructed by insertion of the bacteriophage SP6 promoter upstream of the KUN cDNA sequence to allow the production of replicon RNA *in vitro* using the SP6 RNA polymerase. In DNA-based vectors the SP6 promoter was replaced by the CMV promoter to allow the production of replicon RNA *in vivo* using cellular RNA polymerase II, and the antigenomic sequence of the hepatitis delta virus ribozyme (HDVr) was inserted downstream of the KUN sequence to ensure the formation of KUN RNA molecules with the precise 3′ termini. The last generation of RNA-based vectors also included an HDVr sequence downstream of the KUN sequence, which was shown to significantly improve their transfection/replication efficiency (Varnavski *et al.*, 2000). Some applications of these replicon vectors are discussed in Sections V,C and V,D.

B. Packaging System

The Semliki Forest virus (SFV) replicon vector pSFV1 was used for the expression of KUNV structural proteins required to package KUN replicon RNA into virus-like particles (VLPs; Fig. 6B; Khromykh *et al.*, 1998b). To avoid possible problems with KUN viral protease-mediated cleavage at the C–prM region and to eliminate any chances of recombination of the KUNV structural region with the KUN replicon sequence, prM–E genes and the core gene were cloned and expressed separately under the control of two 26S SFV subgenomic promoters in the same construct (SFV-MEC; Fig. 6B). A delay of 24 to 36 h between transfection with KUN replicon RNA and SFV-MEC replicon

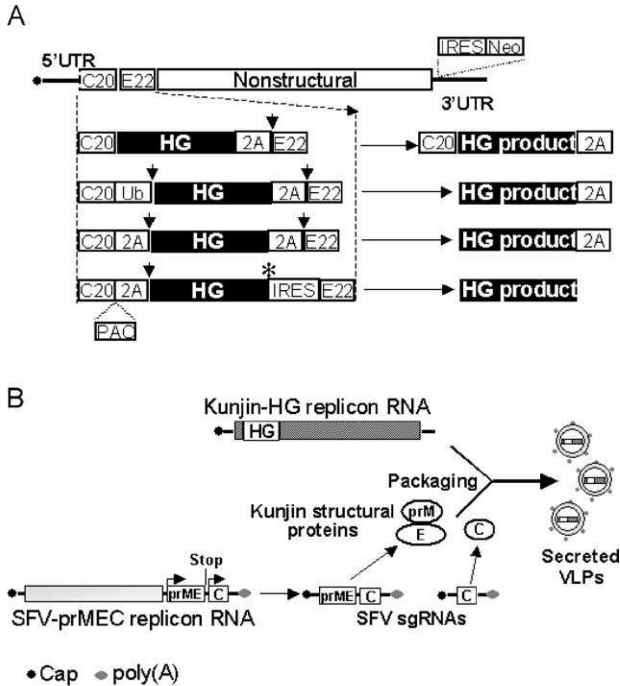


FIG 6. Kunjin replicon-based expression system. (A) HG expression strategy using Kunjin virus (KUN) replicons. C20 represents the first 60 nucleotides (20 codons) of the KUN core or C gene essential for viral RNA replication (*cis*-acting element). E22 indicates the last 66 nucleotides (22 codons) of the KUN envelope or E gene whose translation is required for the proper translocation of the following nonstructural protein (NS1) into the lumen of ER. To facilitate the release of the HG product from the adjacent E22 peptide, a sequence coding for the autoprotease 2A (2A) of foot-and-mouth disease virus (FMDV) or an IRES sequence of encephalomyocarditis virus (EMCV) was inserted between HG and E22. To facilitate the release of the HG product from the amino-terminal C20 peptide, sequences coding for the mouse ubiquitin gene (Ub) or for the FMDV 2A autoprotease were inserted between C20 and HG. Arrows show the cleavage sites for these proteases. An asterisk shows the position of the translation termination codon of the HG preceding the IRES. An IRESNeo cassette consisting of the EMCV IRES sequence, followed by the neomycin phosphotransferase gene (Neo), was inserted in the 3′UTR region of the KUN replicon vector to allow the generation of stably expressing cell lines by selection with the antibiotic neomycin. (B) KUN replicon packaging system. Packaging of KUN replicon RNA encoding a HG that after its transfection and self-amplification requires subsequent transfection with recombinant SFV replicon RNA expressing KUN structural genes. KUN structural proteins C and prM–E are produced separately from two subgenomic SFV RNAs and these then package KUN replicon RNA into VLPs that are secreted into the culture fluid. Modified from Khromykh (2000), with permission.

RNA (Khromykh *et al.*, 1998b; Varnavski and Khromykh, 1999) was required for the KUN replicon RNA to establish productive replication before the rapid shut off of cellular protein synthesis caused by the replicating SFV replicon RNA. It was shown in other studies that once established, replication of KUN viral RNA can proceed even after complete inhibition of cellular protein synthesis by treatment with cycloheximide (see Section IV,C; Westaway *et al.*, 1999). KUN VLPs were secreted and harvested at 24 to 40 h after transfection with the SFV replicon. The total amount of secreted VLPs depended on a number of factors, including the size and the nature of the gene expressed by the KUN replicon, the time between transfections with KUN and SFV-MEC RNAs, and the time of subsequent harvesting of VLPs. The maximum amount of KUN VLPs achieved so far using this packaging system was $\sim 10^7$ particles from 2×10^6 initially transfected BHK cells. We have recently developed a stable packaging cell line that produces 6×10^9 replicon VLPs from 3×10^6 transfected cells (Harvey *et al.*, submitted for publication).

C. Transient and Stable Heterologous Gene (HG) Expression In Vitro

The list of HGs transiently or stably expressed so far using KUN replicon vectors includes (i) reporter genes, i.e., green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), and β-galactosidase (β-gal); (ii) cytoplasmic viral immunogens, i.e., hepatitis C virus core and NS3 proteins (Varnavski and Khromykh, 1999), HIV Gag protein (Harvey *et al.*, 2003), human papilloma virus 16 E7 protein, respiratory syncytial virus M2 protein (D. Harrich, T. J. Harvey, A. A. Khromykh, R. Linedale, R. W. Tindle, and P. R. Young, unpublished results); and (iii) viral glycoproteins, i.e., vesicular, to stomatitis virus G protein (Varnavski and Khromykh, 1999), respiratory syncytial virus F protein, and Ebola virus glycoprotein GP (A. A. Khromykh, R. Linedale, V. V. Volchkov, and P. R. Young, unpublished results). KUN replicon vectors directed HG expression at levels similar to or slightly lower than those achievable by the cytopathic alphavirus replicon vectors in short-term (up to 3 days) transient expression studies, but significantly outperformed them as well as a conventional plasmid DNA vector when transfected cells were allowed to propagate for a prolonged (up to 5 days) period (Varnavski and Khromykh, 1999; Varnavski *et al.*, 2000). Apparently, the noncytopathic and self-amplifying nature of KUN replicons allowed transfer and efficient replication of replicon RNA in daughter cells during cell division, which in turn resulted in continuous accumulation of the HG product. In stably

expressing cell lines established by selection of a total population of antibiotic-resistant cells, the expression levels remained high and decreased only by less then 25% by passage 22. Stably expressing cultures were established for a wide range of mammalian cell lines, which so far include BHK21, Vero, 293, HEp-2, A172 (Varnavski *et al.*, 2000), SK6, L292, RK13, and 293T (A. A. Khromykh, G. Platenick, and V. V. Volchkov, unpublished results). The ability to establish persistent replication in a wide range of cell lines makes KUN replicon vectors distinct from the recently developed noncytopathic alphavirus replicon vectors with a relatively restricted host range (Agapov *et al.*, 1998; Frolov *et al.*, 1999).

D. HG Expression In Vivo *and Immune Responses*

Mice inoculated intranasally with DNA-based KUN replicon vectors encoding the reporter gene β-gal showed β-gal expression in the epithelial cells of the lung airways for up to 8 weeks after inoculation without apparent signs of inflammation (Varnavski *et al.*, 2000). This result demonstrated the potential of KUN replicon vectors for the prolonged expression of HGs *in vivo*, thus indicating their possible application in gene therapy. Intramuscular immunization with the same KUN replicon plasmid DNA resulted in the induction of antibody responses to β-gal in all mice after two immunizations, and the titers were similar to those in mice that responded to immunization with a DNA-based SFV replicon vector and higher than those induced by a conventional plasmid DNA vector (Varnavski *et al.*, 2000). Recent studies showed that KUN replicon vectors also induced protective and long-lasting CTL responses to an encoded model immunogen (I. Anraku *et al.*, 2002) and HIV-1 Gap antigen (Harvey, *et al.*, 2003). These results clearly demonstrate of all the high potential of KUN replicon vectors for developing antiviral and anticancer vaccines.

VII. Concluding Remarks

The model for *Flavivirus* RNA replication was largely based on data defining the molecular events of KUNV and replicon RNA synthesis, analyses of the ultrastructure at the sites of replication, and the biochemical and genetic studies, which together established composition of the RC. Some relevant observations with related species were also incorporated. Further research is required to flesh out the details of the model. For example, in addition to uncertainty in regard to how

the VP and CM/PC are induced, several molecular events of replication remain unexplored. These include the control mechanism(s) for the preferential synthesis of RNA(+) strands after the latent period, the 5′-capping process for released progeny RNA(+) strands, and how this RNA is transported from the sequestered VP to ribosomes for its translation or to its site of assembly with the structural proteins. Within the RC, the molecular interactions among the components and how the helicase activity of NS3 is coordinated with the RdRp activity of NS5 are also unknown. Clearly much remains to be done, and it will be of interest to compare the replication strategies of the related *Hepacivirus* and *Pestivirus* genera as they are also developed.

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ANTIGENIC STRUCTURE OF FLAVIVIRUS PROTEINS

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I. Introduction
II. Classical Flavivirus Serology
III. B-Cell Epitopes
  A. E-glycoprotein
  B. prM/M and Capsid Proteins
  C. Nonstructural Proteins
IV. T-Cell Epitopes
  A. Helper T-Cell Epitopes
  B. Cytotoxic T-Cell Epitopes
V. Conclusion
References

The increased activity of Dengue virus in the tropical regions of the world and the recent movement of West Nile virus from the eastern to the western hemisphere emphasize the fact that vector-borne flaviviruses are medically important emerging infectious diseases. These facts warrant continued efforts to decode all facets of flavivirus immunology. This chapter reviews current understanding of the antigenic fine structure of flaviviral structural and nonstructural (NS) proteins and their involvement in B- and T-cell host responses. The virion structural glycoprotein E elicits both virus-neutralizing antibodies and antiviral T_H-cell responses. Consistent with the current hypothesis of the MHC class I pathway of protein processing, immunodominant flaviviral T_C-cell epitopes mainly reside on the NS proteins. To prepare effective and inexpensive subunit vaccines, we will need to continue to better understand these structure–function relationships of flavivirus proteins.

I. Introduction

Although our understanding of the antigenic structure of flaviviruses has continued to progress, largely due to the development and application of monoclonal antibodies (Mabs) to the process, the

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pace of new discoveries in this field has slowed over the past few years. The reason for this is not certain; however, the development of promising live-attenuated virus vaccine candidates for dengue (DEN) virus and other medically important flaviviruses is certainly a contributing factor. The recent movement of West Nile (WN) virus from the eastern to the western hemisphere emphasizes the fact that vector-borne flaviviruses still "have some tricks up their sleeves," and this fact alone warrants continued efforts to better understand all facets of flavivirus immunology (Craven and Roehrig, 2001; Gubler *et al.*, 2000; Lanciotti *et al.*, 1999; Marfin *et al.*, 2001; Nash *et al.*, 2001; Petersen *et al.*, 2001). To this end, our current understanding of flavivirus B- and T-cell responses has been the subject of a number of fairly recent reviews to which the reader is also referred (Heinz, 1986; Heinz and Roehrig, 1990; Roehrig, 1997).

II. Classical Flavivirus Serology

Since the identification of flaviviruses as serologically distinct entities (first known as group B arboviruses), a number of their antigenic properties have been identified. The family *Flaviviridae*, genus *Flavivirus*, contains over 70 members, and all of these viruses have been shown to be antigenically related to some degree (Calisher, 1988; Calisher *et al.*, 1989; De Madrid and Porterfield, 1974). These antigenic relationships serve as the backbone for the taxonomy and classification of flaviviruses as discussed elsewhere in this volume. As genomic sequence information for flaviviruses has accrued, these broad, antigenically based flaviviral classification schemes have been shown to still be valid.

The pioneers in flavivirus diagnostic serology identified the basic tenents of flavivirus antigenicity. These investigators used hemagglutination inhibition (HI), complement fixation (CF), and virus neutralization (NT) to develop the first classification schemes. They determined that the HI test was quite cross-reactive among flaviviruses. They also determined that the NT assay was the most specific serological assay, often capable of differentiating very closely related flaviviruses. At that time, however, the flavivirus antigenic structure at the molecular level was not known. As the flavivirus molecular structure was deciphered, it was revealed that the envelope (E)-glycoprotein was the major protein responsible for the previously defined serological attributes of flaviviruses (Cardiff *et al.*, 1971; Eckels *et al.*, 1975; Shapiro *et al.*, 1971, 1972, 1973; Trent, 1977; Trent and Qureshi, 1971).

It has since been shown that the E-glycoprotein mediates two crucial functions in flavivirus replication: it is responsible for virus attachment to susceptible cells and for fusing virus and cell membranes, as discussed in detail elsewhere in this volume. Because of these functions, the E-glycoprotein elicits the biologically relevant virus-neutralizing, hemagglutination-inhibiting, and fusion-blocking antibodies.

III. B-Cell Epitopes

A. *E-glycoprotein*

The advent of Mab technology permitted dissection of the antigenic properties of proteins at a level never before imagined. In the 1980s, this technology blossomed as it was applied to a wide variety of flaviviruses, including WN (Besselaar and Blackburn, 1988; Peiris *et al*., 1982) tick-borne encephalitis (TBE) (Gaidamovich *et al*., 1989; Heinz *et al*., 1983b, 1984a; Stephenson *et al*., 1984), yellow fever (YF) (Barrett *et al*., 1989; Buckley and Gould, 1985; Cammack and Gould, 1986a, 1986b; Gould *et al*., 1985, 1989; Schlesinger *et al*., 1983), St. Louis encephalitis (SLE) (Mathews and Roehrig, 1984; Roehrig *et al*., 1983), Japanese encephalitis (JE) (Cecilia *et al*., 1988; Ghosh *et al*., 1989; Hasegawa, 1982; Kedarnath *et al*., 1986; Kimura-Kuroda and Yasui, 1983, 1986; Kobayashi *et al*., 1984; Srivastava *et al*., 1987), Murray Valley encephalitis (MVE) (Hall *et al*., 1988, 1990; Hawkes *et al*., 1988), and DEN viruses (Dittmar *et al*., 1980; Gentry *et al*., 1982; Halstead *et al*., 1984; Henchal *et al*., 1982, 1983, 1985; Kaufman *et al*., 1987, 1989; Monath *et al*., 1986).

These newly defined antibody reagents were used as tools to improve disease diagnosis, to evaluate antigenic drift of closely related viruses, and to better characterize the flavivirus replication cycle. The application of Mabs to dissecting the molecular antigenic structure of the flavivirus E-glycoprotein was spearheaded in a handful of laboratories. Much of what is known about the antigenic structure of the flavivirus E-glycoprotein is a direct result of these early investigations.

1. Structure of the E-glycoprotein

The molecular structure of the E-glycoprotein is reviewed in the chapter by Franz X. Heinz and Steven L. Allison; however, because many of the structure–function relationships of this protein were first identified through immunological means, a brief description of its

structure is included here. The now classic studies using Mabs to define the antigenic structure of the E-glycoprotein were first performed with TBE, SLE, and JE viruses (Guirakhoo *et al.*, 1989; Heinz, 1986; Heinz *et al.*, 1982, 1983a, 1983b; Kimura-Kuroda *et al.*, 1983, 1986; Mandl *et al.*, 1989; Roehrig *et al.*, 1983). Heinz and co-workers identified three antigenic domains (A, B, and C) on the E-glycoprotein of TBE virus. Subsequent biochemical analyses of these domains determined that the A domain, including amino acids (aa) 51 to 135 and 195 to 284, approximately, was a linearly discontinuous domain divided by the C domain (approximately aa 1 to 50 and 136 to 194). Domain B included aa 300 to 395. Based on the disulfide bridging structure of WN virus, the A domain structure was determined to be stabilized by four disulfide bridges and contained epitopes that were conformationally dependent and elicited virus-neutralizing antibody (Nowak and Wengler, 1987). The importance of conformation to virus neutralization was demonstrated elegantly in a simple study that revealed that reduced and denatured WN virus E-glycoprotein was unable to elicit antiviral neutralizing antibodies when inoculated into mice (Wengler, 1989).

Contemporary studies with SLE and JE viruses failed to identify the three biologically distinct domains (Kimura-Kuroda and Yasui, 1983; Roehrig *et al.*, 1983). However, all the epitopes identified on the SLE virus E-glycoprotein likely resided in the A domain based on their uniform sensitivity to reduction–denaturation (J. T. Roehrig, unpublished). A subsequent and more detailed evaluation of the E-glycoprotein of DEN 2 virus confirmed the antigenic characteristics and localization of the flaviviral A, B, and C domains (Roehrig *et al.*, 1998).

The B domain was shown to reside on a 9- to 10-kDa tryptic fragment of the TBE and DEN 2 virus E-glycoprotein (approximately aa 300–400) (Roehrig *et al.*, 1998; Winkler *et al.*, 1987). Expression of B domain epitopes required a native disulfide bond between Cys 11 and Cys 12 (Lin *et al.*, 1994; Mason *et al.*, 1989, 1990; Winkler *et al.*, 1987). Many of the B domain epitopes elicited virus-neutralizing antibody.

In 1995 our understanding of the structure–function relationships of the flaviviral E-glycoprotein was advanced greatly by the solving of the 2-Å molecular structure of the amino-terminal 395 amino acids of the TBE virus E-glycoprotein homodimer (Rey *et al.*, 1995). The results of this study revealed that the E-glycoprotein folded into three distinct domains (I, II, and III), which correlate directly to the previously defined antigenic domains C, A, and B (Fig. 1). From gene sequencing studies it was known that the locations of the E-glycoprotein Cys residues were conserved among all flaviviruses. It has been generally

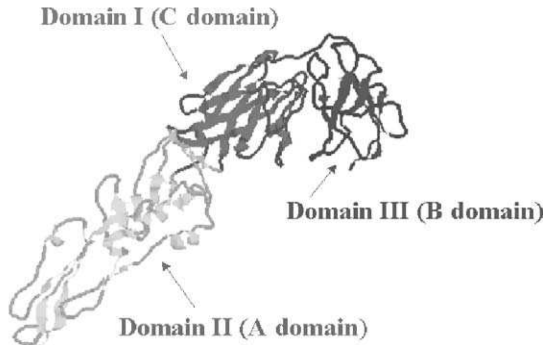

FIG 1. Crystal structure of the flavivirus E-glycoprotein monomer. Color key: red, domain I (C domain); yellow, domain II (A domain); and blue, domain III (B domain). (See Color Insert.)

assumed, therefore, that the E-glycoprotein structure is the same for all flaviviruses. The crystal structure of the DEN E-glycoprotein has been solved. Its structure is nearly identical to that of the TBE virus E-glycoprotein (Modis *et al.*, 2003).

2. Antigenic Fine Structure of the E-glycoprotein

Detailed mapping of antigenically important regions of the E-glycoprotein that readily elicit antibody has been accomplished by (1) isolating glycoprotein-reactive murine Mabs and using them to identify epitopes involved in biologically relevant virus functions; (2) preparing or expressing protein fragments and analyzing their reactivities with Mabs and polyclonal antisera; (3) preparing synthetic peptides derived from the E-glycoprotein gene sequences and analyzing the reactivities of the derived antipeptide antisera with virus and virus-specified gene products; and (4) solving the molecular structure of the E-glycoprotein and correlating this structure to the previously defined E-glycoprotein antigenic properties (Mandl *et al.*, 1989; Roehrig *et al.*, 1998).

By using many different flaviviruses to produce large panels of Mabs reactive with the E-glycoprotein, a number of general antigenic characteristics of the E-glycoprotein have become evident: (1) the E-glycoprotein is responsible for eliciting high-titered virus-neutralizing, hemagglutination-inhibiting, and cell membrane fusion-blocking antibodies; (2) authentic expression of E-glycoprotein epitopes appears to require coexpression of the prM protein; (3) the virus-neutralizing antibody can be either virus specific or virus cross-reactive; however,

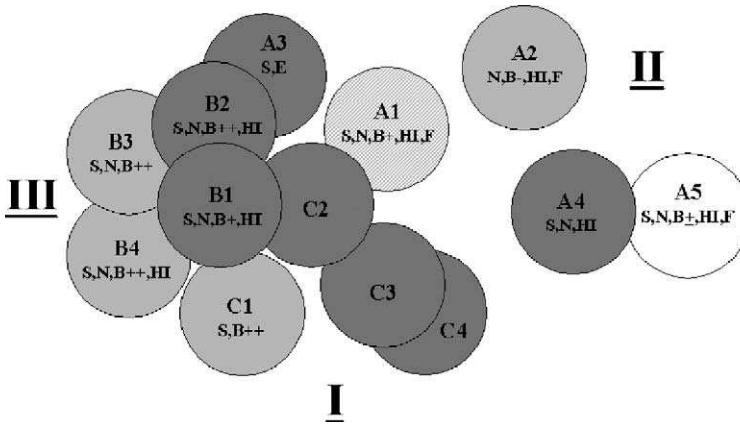

FIG 2. Biological and biochemical characteristics of DEN 2 virus E-glycoprotein epitopes mapped on a competitive binding localization assay diagram. Individual epitopes are designated by circles. Overlapping circles indicate spatially proximal epitopes. Diagrammed are A domain, A1–A5 (domain II); B domain, B1–B4 (domain III); and C domain, C1–C4 (domain I) epitopes. Biological and biochemical characteristics of these epitopes (or Mabs that bind to them) are as follows: S, surface accessible epitopes; N, elicits Mabs with virus neutralizing activity; B, elicits Mabs that block virus attachment to cells (no blocking activity to high blocking activity: B−, B±, B+, B++); E, elicits Mabs that enhances virus attachment to cells; HI, elicits Mabs that inhibit hemagglutination; and F, elicits Mabs that block virus-mediated cell membrane fusion. Virus cross-reactivity of epitope-elicited Mabs is shown by shading: type (dark gray), subcomplex (light gray), subgroup (white), and group (cross-hatched) reactive (Crill and Roehrig, 2001; Roehrig *et al.*, 1998).

virus–specific antibodies are the most potent neutralizers of virus infectivity; and (4) not all E-glycoprotein-reactive antibodies neutralize virus infectivity, suggesting that this biological activity is localized to only certain areas or epitopes in the E-glycoprotein. A summary of these biological and biochemical characteristics for each E-glycoprotein epitope of DEN 2 virus is shown in Fig. 2.

a. Localization of Epitopes by Isolating and Sequencing Mab NT Escape Variants One approach to epitope localization is to grow virus in the presence of virus-neutralizing Mab. Any surviving virus, a neutralization-escape variant, has an amino acid change that relates in some way to the binding of the selecting neutralizing Mab. By sequencing the virus genome, any amino acid changes caused by this selection process can be identified. This approach has been used successfully to identify amino acid residues in the E-glycoprotein

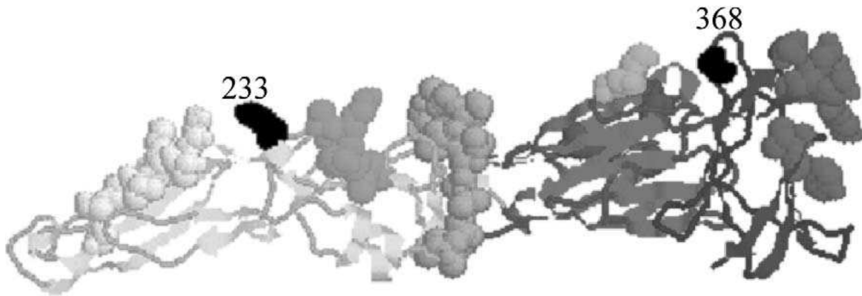

FIG 3. Localization of major neutralization regions on the E-glycoprotein identified in Mab neutralization-escape variants. A lateral view of the flavivirus monomer with domains I (red), II (yellow), and III (blue) is shown. Major neutralization regions are identified: region 1, aa 67–72 and 112 (white); region 2, aa 123–128 (green); region 3, aa 155 and 158 (cyan); region 4, aa 171, 181, and 293 (blue); region 5, aa 52, 136, and 270–279; and region 6, aa 307–311, 333, and 384–385 (purple). Also marked are two other amino acid changes associated with neutralization-escape variants (aa 233 and 368). (See Color Insert.)

associated with binding of the flaviviral-neutralizing antibody. A number of flaviviruses have been examined in this way: DEN (Beasley and Aaskov, 2001; Lin *et al.*, 1994; Lok *et al.*, 2001), JE (Cecilia and Gould, 1991; Hasegawa *et al.*, 1992; Morita *et al.*, 2001), louping ill (Gao *et al.*, 1994; Jiang *et al.*, 1993), MVE (McMinn *et al.*, 1995a), TBE (Holzmann *et al.*, 1989; Mandl *et al.*, 1989), and YF viruses (Lobigs *et al.*, 1987; Ryman *et al.*, 1997). A compiled list of amino acid changes (and their locations) identified in flavivirus neutralization-escape variants is shown in Table I. It is important to note that almost all neutralization-escape variants (31/35, 88%) have been selected using Mabs that are either type or subtype specific, indicating a high degree of antigenic drift in the identified regions. Six neutralization regions can be identified: region 1, aa 67–72 and 112; region 2, aa 123–128; region 3, aa 155 and 158; region 4, aa 171, 181, and 293, region 5, aa 52, 136, and 270–279; and region 6, aa 307–311, 333, and 384–385 (Fig. 3). Identification of these regions on the E-glycoprotein structure reveals that they are usually located on the outer, upper, or lateral surfaces of the E-glycoprotein in domains I, II, and III, suggesting easy access to the antiviral antibody. The ability of the virus-neutralizing antibody to bind to the accessible surfaces of the E-glycoprotein is consistent with the two mechanisms of flavivirus neutralization thus far identified: blocking virus attachment to susceptible cells (Crill and Roehrig, 2001) and blocking virus-mediated

TABLE I
AMINO ACID CHANGES IN THE E-GLYCOPROTEIN FOR FLAVIVIRUS MAB NT ESCAPE VARIANTS

Virus[a]	Amino acid number	Amino acid change	Mab reactivity[b]	Reference
DEN	69	T → I	Type	Lin *et al*. (1994)
	71	E → D	Type	Beasley and Aaskov (2001)
	112	S → G	Type	Lok *et al*. (2001)
	124	I → N	Type	
	279	F → S	Subcomplex	
	293	T → I	Subcomplex	
	307	K → E	Type	
	311	E → G	Type	
JE	52	Q → K (or R)	Type	Hasegawa *et al*. (1992)
	126	I → T	Type	Cecilia and Gould (1991)
	136	K → E	Type	Morita *et al*. (2001)
	270	I → S	Type	
	275	S → P	Type	
	333	G → D	Subgroup	
LI	308	D → N	Subtype	Gao *et al*. (1994)
	310	S → P	Subtype	Jiang *et al*. (1993)
	311	K → Q(or N)	Subtype	
MVE	126	A → E	Type	McMinn *et al*. (1995a)
	128	R → S	Type	
	274	F → V	Type	
	276	S → R	Type	
	277	S → I	Type	
TBE	67	D → G	Subtype	Holzmann *et al*. (1997), Mandl *et al*. (1989)
	71	A → V	Subtype	
	123	A → K	?	
	171	K → E	Complex	
	181	D → Y	?	
	233	Q → K	Subtype	
	368	G → R	?	
	384	Y → H	Subtype	
	389	S → R	Subtype	
YF	71	N → K	Type	Lobigs *et al*. (1987)
		N → Y	Type	Ryman *et al*. (1997)
		N → H	Type	
	72	D → G	Type	
	125	M → I	Type	
	155	D → G	Type	
	158	T → I	Type	

[a] DEN, dengue; JE, Japanese encephalitis; LI, louping ill; MVE, Murray Valley encephalitis; TBE, tick-borne encephalitis; YF, yellow fever.
[b] Demonstrated virus cross-reactivity of selecting Mab.

cell membrane fusion (Butrapet *et al.*, 1998; Gollins and Porterfield, 1986a; Roehrig *et al.*, 1998).

b. Epitope Mapping Utilizing Fragments of the E-glycoprotein A second approach to epitope localization is to assess the ability of E-glycoprotein fragments to bind Mabs. The fragments have been prepared in two ways. One approach is to treat virions with proteolytic enzymes (e.g., trypsin or chymotrypsin) or protein-reactive chemicals (e.g., CNBr) to produce native E-glycoprotein fragments (Guirakhoo *et al.*, 1992; Heinz *et al.*, 1984b; Roehrig *et al.*, 1998; Srivastava *et al.*, 1987; Winkler *et al.*, 1987). The Mab reactivities with reduced or nonreduced fragments can then be determined by immunoblotting. By determining the amino-terminal amino acid sequence of a Mab-reactive fragment and estimating its size on reduced polyacrylamide gels, the fragment can be localized within the E-glycoprotein amino acid backbone. A second approach is to express cloned DNA containing pieces of the E-glycoprotein gene sequence. Fragments expressed in this way can also be probed with Mabs to assess which epitopes might be contained within them. In general, this latter approach has been of limited value because of the requirements for appropriate disulfide bond formation and the coexpression of the prM and E-glycoprotein to maintain the native structure of some of the conformationally dependent E-glycoprotein epitopes (Konishi and Mason, 1993). In order to maintain any Mab reactivity, many of the expressed fragments have had to be so large that they were of limited use in precisely defining epitope location.

Nevertheless, some valuable information has been derived from E-glycoprotein fragment analysis. Mason *et al.* (1987, 1989, 1990) analyzed both the JE and the DEN 1 virus E-glycoprotein using *Escherichia coli* expression of protein fragments. Two immunogenic domains (aa 76–93 and 293–402) were identified on DEN 1 virus. The latter domain required a disulfide bridge between the last two Cys residues in the E-glycoprotein for native epitope expression. This requirement was similar to that observed previously with the TBE virus B domain and JE virus (Mason *et al.*, 1989; Winkler *et al.*, 1987). Recent expression of the domain II of DEN and Langat viruses has confirmed its role in flaviviral antigenicity and interaction with susceptible cells (Bhardwaj *et al.*, 2001; Simmons *et al.*, 1998a, 1998b, 2001a, 2001b).

Megret *et al.* (1992) completed a more extensive study with DEN 2 Jamaica virus. Sixteen *trp*E fusion proteins were prepared and analyzed with a battery of seven murine hyperimmune ascitic fluids

and 20 anti-DEN virus Mabs. Six antigenic domains were identified. One virus-specific epitope (aa 22–58) and one subcomplex-specific epitope (aa 304–332) were identified by nonneutralizing Mabs. Multiple epitopes were identified by virus-neutralizing antibodies. One region (aa 298–397) reacted with type-, subcomplex-, complex-, subgroup-, and group-reactive Mabs. Two overlapping domains (aa 60–135 and 60–205) defined group-reactive epitopes. A different study with DEN 2 virus mapped three A domain epitopes to the first 120 aa of the E-glycoprotein and four B domain epitopes to aa 300–400 (Roehrig *et al.*, 1998). A general summary of epitope locations on various defined peptide fragments of the DEN 2 virus E-glycoprotein is shown in Fig. 4. Guirakhoo *et al.* (1992) further localized the binding of domain II–reactive Mabs that are blocked when the virion contains prM to aa 200–327 in the E-glycoprotein of MVE virus.

c. Peptide Mapping of E-glycoprotein Epitopes Synthetic peptides derived from the amino acid sequence of E-glycoprotein have also been used to model epitopes. Initial studies used a variety of approaches to prepare synthetic peptides to mimic E-glycoprotein epitopes (Aaskov *et al.*, 1989; Innis *et al.*, 1989; Markoff *et al.*, 1988; Roehrig *et al.*, 1989, 1990, 1992). More recent attempts have used bacteriophage display technology to express small regions of the E-glycoprotein (Thullier *et al.*, 2001; Wu and Lin, 2001).

Results of pepscan analysis of the DEN 2 virus E-glycoprotein concluded that most of the E-glycoprotein was immunogenic (Aaskov *et al.*, 1989; Innis *et al.*, 1989). Innis *et al.* (1989) used overlapping hexapeptides and identified four putative DEN 2 virus–specific epitopes (aa 276–281, 461–467, 472–477, and 485–491) and one flavivirus group-reactive epitope (aa 205–214). Aaskov *et al.* (1989) used overlapping octapeptides and determined that the binding site of Mab 1B7 was contained in three discontinuous regions of the E-glycoprotein (aa 50–57, 127–134, and 349–356). Within the context of the E-glycoprotein molecular structure, two of these regions (aa 50–57 and 127–134) are localized to the interjacent regions between domains I and II. The third region (aa 349–356) is spatially distal to this region, residing in domain III.

Markoff *et al.* (1988) prepared 38 overlapping 15-mer peptides of the E-glycoprotein sequence of DEN 4 virus and used these reagents to screen for reactivity with a number of anti-DEN antibodies. Two of these peptides (aa 258–271 and 290–310) strongly bound mouse hyperimmune ascitic fluid. Peptides from the first 50 amino acids bound these antibodies weakly. The amino-terminal region was

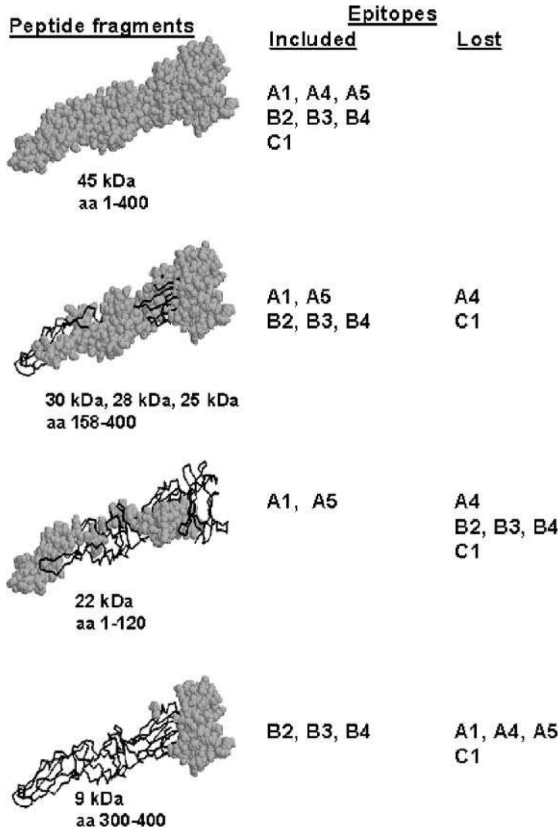

FIG 4. Localization of DEN 2 virus epitopes on a fragment of the E-glycoprotein. Various proteolytic fragments of the DEN 2 virus E-glycoprotein are mapped as a space-filled amino acid backbone. Fragment size and approximate location are listed under each model. Epitopes included or not included (lost) on each fragment are listed to the right of each model (adapted from Roehrig *et al.*, 1998).

found to be reactive with a number of immune sera from human infections. Two other peptides (aa 197–210 and 318–333) bound DEN virus–specific Mabs.

Using predictive algorithms, our laboratory prepared and analyzed synthetic peptides derived from the E-glycoprotein amino acid sequences of both MVE (Mathews *et al.*, 1991; Roehrig *et al.*, 1989) and DEN 2 (Roehrig *et al.*, 1990, 1994) viruses. These free peptides ranged in length from 9 to 39 amino acids. Comparing results between these two viruses revealed remarkable similarities. Peptides defined four

regions (aa 1–55, 79–172, 225–249, and 333–388) that could elicit an antiviral antibody. Two peptides (aa 35–55 and 352–368 for DEN 2 virus; aa 35–55 and 356–376 for MVE virus) elicited low levels of virus-neutralizing antibody in mice for both viruses. Only one DEN 2 Mab bound to a synthetic peptide (aa 333–351) (Roehrig *et al.*, 1998). A comparison of the biological and biochemical characteristics of these antipeptides is summarized in Table II.

Using a combination of both *E. coli* expression and synthetic peptides, Trirawatanapong *et al.* (1992) localized the binding site of the neutralizing MAb 3H5 to a 12–amino acid sequence (aa 386–397). This peptide also elicited a low-level virus-neutralizing antibody in rabbits. It is of interest, however, that a very similar peptide (aa 388–400) could not bind to Mab 3H5 nor elicit virus-neutralizing antibody (Roehrig *et al.*, 1990).

Two studies using phage display technology have identified three continuous polypeptides (aa 307–309, 327–333, and 386–390) capable of binding a JE virus–reactive Mab and a similar region (aa 306–314) with reactivity for a DEN virus Mab (Thullier *et al.*, 2001; Wu and Lin, 2001).

d. Immunological Approaches Used to Define the Role of the E-glycoprotein in Low pH-Catalyzed Virus-Mediated Cell Membrane Fusion Antibody blocking of low pH–catalyzed virus–mediated cell membrane fusion identified in the 1980s with WN virus was the first well-defined mechanism of flavivirus neutralization (Gollins and Porterfield, 1986a, 1986b, 1986c; Kimura *et al.*, 1986). This mechanism of neutralization has since been confirmed with other flaviviviruses (Butrapet *et al.*, 1998; Roehrig *et al.*, 1998). As E-glycoprotein amino acid sequences were compiled, it was hypothesized that the region from aa 98–110 was the actual E-glycoprotein fusion sequence. This hypothesis was based on three observations: (1) the amino acid sequence is relatively hydrophobic, a characteristic necessary for a fusion sequence; (2) the amino acid sequence has striking homologies with previously defined fusion sequences from other enveloped viruses; and (3) the amino acid sequence is highly conserved among all flaviviruses, indicating that it encoded an important viral function, and is probably not readily exposed to antiviral antibody.

This hypothesis was first examined using DEN 2 virus E-glycoprotein–derived antipeptide antibodies to probe the entire sequence of both native and low pH–treated viruses to assess the accessibility of E-glycoprotein regions at different pH values (Roehrig *et al.*, 1990). This study clearly demonstrated that certain regions of the

TABLE II

ANTIBODY RESPONSES TO FLAVIVIRUS E-GLYCOPROTEIN–DERIVED PEPTIDES AS MEASURED IN ELISA USING PEPTIDE OR PURIFIED VIRUS ANTIGENS

Amino acids		Antibody response ($\log_{10}$ GMT)[a]	
MVE	DEN2	Antipeptide	Antivirus
1–15		2.0	1.6
13–33		1.6	1.9
	1–30	3.8	3.1
35–50		3.2	3.0
35–55		3.2	ND[b]
	35–55	5.0	5.1
	49–60/121–140	2.9	1.6
	58–73/106–121	2.0	≤1.0
	72–91/93–105	2.6	1.6
77–97		2.3	2.3
	79–99	3.2	2.8
122–141		2.0	1.6
	121–140	3.2	2.2
145–169		3.1	ND
	142–172	3.5	2.8
179–197		2.4	1.6
	167–201	2.0	1.0
	208–219	≤2.0	≤1.0
230–251		3.4	3.1
	225–249	4.4	3.7
	240–262	3.2	3.1
255–266		≤2.0	ND
	255–274	≤2.0	1.0
	274–304	2.0	1.0
289–305		1.6	1.9
305–319		2.0	2.0
	302–333	3.8	1.0
336–354		2.0	2.0
	333–351	2.9	2.2
356–376		3.1	2.9
	352–368	3.5	2.2
365–376		3.1	ND
	361–388	4.1	2.8
	388–400	≤2.0	≤1.0
	398–435	2.0	1.3
	437–452	3.5	1.3
	443–493	≤2.0	≤1.0
	470–493	≤2.0	≤1.0

[a] Geometric mean titer (Mathews *et al.*, 1991; Roehrig *et al.*, 1989, 1990, 1994).
[b] Not determined.

E-glycoprotein were more accessible following a low pH–catalyzed conformational change. Not surprisingly, one of these newly exposed regions, aa 58–121, included the putative fusion domain; however, it was not colinear with a second identified region, aa 225–249. These results were confirmed in a subsequent study with TBE virus that demonstrated that three Mabs reactive with two peptides (aa 1–22 and 221–240) were more reactive with denatured virus (Holzmann *et al.*, 1993). Subsequent resolution of the E-glycoprotein crystal structure revealed that these widely spaced linear regions were either actually spatially overlapping and included in domain II or located in the pocket of domain I that interacts with the fusion sequence of the opposite monomer at neutral pH (Rey *et al.*, 1995; Roehrig *et al.*, 1998). Antigenic mapping of the DEN 2 E-glycoprotein determined that two of the three epitopes that elicited virus fusion-blocking Mabs were located on a peptide fragment composed of aa 1–120 (Fig. 4) (Roehrig *et al.*, 1998).

One of the DEN 2 virus–elicited, fusion-blocking Mabs, 6B6C-1, defined the DEN 2 epitope, A1, and was flavivirus cross-reactive, conformationally dependent, and involved in hemagglutination. This DEN 2 virus Mab was essentially identical to the TBE virus–elicited MAb that defined epitope A1 on TBE virus (Fig. 2). It has been determined subsequently that the TBE anti-A1 Mab can also block low pH–catalyzed cell membrane fusion mediated by TBE recombinant subviral particles (RSPs) (Allison *et al.*, 2001; Corver *et al.*, 2000; Stiasny *et al.*, 2002). Mutant RSPs with a Leu → Thr or Leu → Asp, but not a Leu → Phe amino acid change at Leu-107 of the E-glycoprotein have strongly impaired or completely abrogated fusion activity (Allison *et al.*, 2001). Reactivity of anti-A1 Mab with RSPs containing the Leu → Asp mutation is abolished. Figure 5 maps the important regions of the E-glycoprotein involved in low pH-catalyzed virus–mediated cell membrane fusion identified through antigenic analyses.

e. Anti–E-glycoprotein Antibodies and Their Role in Protection from Viral Infection Mab reagents used to characterize the E-glycoprotein antigenic structure have also been used to identify its protective epitopes. In these studies, susceptible animals (e.g., mice) are first passively administered known amounts of Mab and then are challenged with virus. The relative ability of a Mab to protect the animal from virus challenge, when compared to other similarly tested Mabs, serves as a direct measurement of the protective efficacy of the eliciting

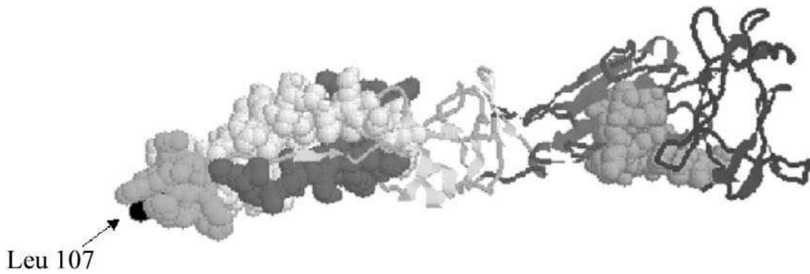

FIG 5. Important regions of the E-glycoprotein involved in low pH–catalyzed virus-mediated cell membrane fusion identified through antigenic analyses. A lateral view of the flavivirus monomer with domains I (red), II (yellow), and III (blue) is shown. Highlighted regions are identified as space-filled amino acids. Regions exposed when virus is treated at low pH: aa 1–22 (TBE, orange), aa 58–121 (DEN 2, white), and aa 225–249 (DEN 2, purple). This last region is nearly identical, with a similar region (aa 221–240) in TBE virus. The proposed flavivirus fusion sequence (aa 98–110) is shown in cyan. The aa Leu 107, associated with TBE virus fusion, is marked in black. (See Color Insert.)

epitope. In this way, epitopes eliciting virus-neutralizing vs nonneutralizing, type-specific vs cross-reactive, and complement-fixing vs nonfixing antibodies can be compared directly.

The first such study used SLE virus (Mathews and Roehrig, 1984). Subsequent investigations with JE (Butrapet *et al.*, 1998; Kimura-Kuroda and Yasui, 1988; Roehrig *et al.*, 2001; Zhang *et al.*, 1989), MVE (Hawkes *et al.*, 1988), YF (Brandriss *et al.*, 1986), and DEN (Kaufman *et al.*, 1987) viruses have generally confirmed the results from the SLE virus investigation. A correlation of virus-neutralizing activity with protection has been observed for all flaviviruses (for review, see Heinz and Roehrig, 1990). Epitopes that elicit antiviral antibodies that do not possess virus-neutralizing activity are poor protectors. The ability of cross-reactive antibodies to cross-protect from challenge with related viruses was investigated using JE, WN, and MVE viruses (Roehrig *et al.*, 2001). This study revealed that while cross-protection could be observed, this protection was not as potent as protection mediated by type-specific, virus-neutralizing Mab. Nevertheless, cross-neutralizing antibodies demonstrated some level of cross-protection against heterologous viruses, and some nonneutralizing antibodies also afforded measurable levels of protection. A study with YF virus suggested that protection mediated by virus-neutralizing antibodies required an intact Fc region (Schlesinger and Chapman, 1995).

TABLE III

ANTI–E-GLYCOPROTEIN MABS USEFUL IN FLAVIVIRUS SEROLOGY AND IDENTIFICATION

Virus Mab	Virus specificity	ELISA IgM	ELISA IgG	ELISA Ag capture[a]	IFA	Reference
SLE						
6B5A-2	SLE	–	–	D	+	Roehrig *et al.* (1983)
4A4C-4	SLE	–	–	C	+	Hunt *et al.* (2002); Roehrig *et al.* (1983); Tsai *et al.* (1987, 1988)
6B6C-1	All flavis	+	–	D	+	Han *et al.* (1988); Hunt *et al.* (2002); Johnson *et al.* (2000); Martin *et al.* (2000, 2002); Monath *et al.* (1984); Palmer *et al.* (1999); Roehrig *et al.* (1983)
JE						
JE314H52	JE	–	–	D	+	Unpublished
6B4A-10	JE complex	–	–	C	+	Guirakhoo *et al.* (1992); Roehrig *et al.* (2001)
6A4D-1	JE/MVE	–	–	D	+	Guirakhoo *et al.* (1992); Roehrig *et al.* (2001)
MVE						
4B6C-2	MVE	–	–	D	+	Hawkes *et al.* (1988, 1990)
WN/KUN						
3.91D	WN/KUN	–	–	C	+	Adams *et al.* (1995); Hall *et al.* (1987); Hunt *et al.* (2002)
H5.46	WN	–	–	–	+	Gould *et al.* (1990); Lanciotti *et al.* (2000)
10A1	KUN	–	–	–	+	Adams *et al.* (1995); Hall *et al.* (1987)
2B2	WN/KUN	–	–	–	+	Adams *et al.* (1995); Hall *et al.* (1987)
YF						
5E3	YF	–	–	C,D	+	Monath and Nystrom (1984); Schlesinger *et al.* (1983)
2D12	YF	–	–	D	+	Schlesinger *et al.* (1983)
864	Vaccine YF	–	–	D	+	Gould *et al.* (1985)
117	wt YF	–	–	D	+	Gould *et al.* (1989)
DEN						
D2-1F1-3	DEN1	–	–	D	+	Unpublished
3H5-1-21	DEN2	–	–	C,D	+	Henchal *et al.* (1985); Kuno *et al.* (1985)
D6-8A1-12	DEN3	–	–	D	+	Unpublished
1H10-6-7	DEN4	–	–	C,D	+	Henchal *et al.* (1982); Kuno *et al.* (1985)
4G2	All flavis	–	+	C	+	Hall *et al.* (1987); Henchal *et al.* (1982); Johnson *et al.* (2000)

[a] Used as capture (C) or detector (D) antibodies.

f. E-glycoprotein–Specific Mabs Useful in Flaviviral Disease Diagnosis and Virus Identification A valuable "spin-off" of E-glycoprotein epitope mapping studies has been the application of E-glycoprotein–specific Mabs in various flavivirus diagnostic assays. These Mabs have been useful in virus identification (as virus-specific or cross-reactive reagents) in indirect immunofluorescence assay, as antigen capture and detector antibodies in antigen detection assays, and as antigen capture or detector antibodies in serological assays to detect antiviral IgM and IgG. A referenced list of Mabs (arranged by virus) and their application in diagnostic flavivirology is shown in Table III.

B. prM/M and Capsid Proteins

Little specific information is available on the antigenic structure of prM/M proteins. The low virus-neutralizing activity of anti-prM antibody was initially identified with JE virus and has been confirmed with Langat virus (Holbrook *et al.*, 2001; Takegami *et al.*, 1982). This result may be consistent with the previous observation that an anti-prM Mab prepared against Langat virus was protective from virus challenge (Iacono-Connors *et al.*, 1996). This Mab, however, did not neutralize Langat virus infectivity. Kaufman *et al.* (1989) prepared five DEN prM/M-reactive Mabs and identified both DEN virus complex and subcomplex reactivity. Two of these Mabs (2H2 and 5C9) also had low-level virus-neutralizing activity (1:10); however, other investigators were unable to reproduce the virus-neutralizing activity of the 2H2 Mab (Aaskov *et al.*, 1988). McMinn *et al.* (1995b) associated a valine to alanine change at aa 76 of the prM with escape from neutralization by MVE virus. This was the only report in which escape from antibody neutralization was associated with an amino acid change in the prM protein.

Even less is known about the B-cell response to C protein. Six Mabs were derived following immunization with the purified nucleocapsid protein of DEN 2 virus (Bulich and Aaskov, 1992). These Mabs were used to corroborate localization of the flavivirus C protein in the nucleus of the virus-infected cell (Tadano *et al.*, 1989; Gould *et al.*, 1983). Pepscan analysis identified the binding site for these Mabs to be a 9–19, a site adjacent to the possible nuclear localization sequence. Another anti–DEN 2 virus C-protein Mab has also been identified, but its epitope correlate was not identifed (Roehrig *et al.*, 1998).

C. Nonstructural Proteins

Even though the flavivirus NS1 protein can elicit high-titered antibody in flavivirus-infected or -immunized animals, its function in flavivirus replication remains an enigma (Falkler *et al.*, 1973). Because of this, precise epitope mapping and subsequent linkage to structural and functional activities of NS1 have not been done. Interest in the antigenic structure of NS1 peaked after it was determined that NS1 immunization or passive transfer of anti-NS1 Mabs could protect animals from viral challenge (Gould *et al.*, 1986; Henchal *et al.*, 1988; Iacono-Connors *et al.*, 1996; Schlesinger *et al.*, 1985, 1986, 1987).

The general approach to mapping important B-cell epitopes on NS1 is similar to that used for the E-glycoprotein. Because the NS1 is not incorporated into the virion and does not elicit virus-neutralizing antibodies, NS1-related neutralization-escape variants cannot be prepared. Epitope maps have been developed for the NS1 proteins of DEN 2 and MVE viruses (Hall *et al.*, 1990; Henchal *et al.*, 1987). In the DEN 2 virus study, a competitive binding assay identified six epitopes arranged in a spatial continuum. All but one of these anti-NS1 Mabs were DEN virus serotype specific in indirect immunofluorescence assays. This apparent serotype specificity corroborated previous studies that used polyclonal antisera and has served as the foundation for studies using NS1 as a virus-specific serological antigen (Huang *et al.*, 1999; Russell *et al.*, 1970; Wu *et al.*, 2001). Two of these Mabs had complement-fixing activity, similar to other Mabs specific for YF virus NS1 protein (Schlesinger *et al.*, 1985). In the MVE virus NS1 study, six unlinked epitopes were identified by six Mabs (Hall *et al.*, 1990). Four of these Mabs were MVE virus specific and two cross-reacted with only the closely related Alfuy virus.

Immunoblots that identified three different NS1 protein specificity patterns were observed with Mabs derived from immunization of mice with immunoaffinity-purified NS1 from DEN 2 virus, PR159 strain (Falconar and Young, 1991). This study identified conformationally dependent NS1 dimer epitopes, conformationally dependent NS1 epitopes conserved on both the dimer and the monomer, and conformationally stable NS1 monomer epitopes. Although some of these Mabs were DEN 2 virus serotype specific, more broadly cross-reactive epitopes were also identified.

More precise NS1 epitope localization studies have been performed using NS1 protein–derived fragments or NS1 gene sequence–deduced synthetic peptides. Putnak and co-workers (1988) investigated the binding of polyclonal antibodies and Mabs to *E. coli*–expressed

fragments of DEN 2 NS1. This study demonstrated that the polyclonal murine anti-NS1 antibody reacted best with the NS1 amino terminus, whereas the polyclonal rabbit anti-NS1 antibody was most reactive with the carboxy-terminal region. The previously described murine Mabs reacted most reproducibly with a fragment composed of aa 273–346 (Henchal *et al.*, 1987; Putnak *et al.*, 1988). An analysis using *E. coli trp*E-expressed DEN 1 NS1 gene fragments identified a Mab-reactive region near the amino terminus (aa 57–126) (Mason *et al.*, 1990). Four linear NS1 epitopes (aa 25–33, 61–69, 111–119, or 113–121 and aa 299–307 or 301–309) could be identified using pepscan analysis with 174 overlapping nonameric peptides of the DEN 2 NS1 protein (Falconar *et al.*, 1994). The location of these linear epitopes may be consistent with the previously identified antigenicity of the NS1 amino and carboxy terminus. One subsequent study measured ELISA reactivity of human DEN patient sera on synthetic peptides derived from NS1 and NS3 (Garcia *et al.*, 1997). Five peptides, two from NS1 and one from NS3, appeared to be recognized by DEN infection–immune sera; however, differences between this reactivity and the reactivity of serum samples from nonimmune control subjects were small.

There is very little detailed knowledge about the B-cell reactive antigenic structure of the other flaviviral NS proteins (NS2a, NS2b, NS3, NS4a, NS4b, and NS5). A handful of anti–NS3-reactive Mabs have been prepared for DEN 1 virus; however, they were not used to map epitopes (Tan *et al.*, 1990). Eight anti–YF virus NS5 protein-specific Mabs were used to identify a nuclear association of this protein in virus-infected cells (Buckley *et al.*, 1992). Studies using purified NS5 from SLE virus-infected cells showed that unlike the E-glycoprotein, the NS5 was serotype specific when tested by complement fixation (Qureshi *et al.*, 1973a, 1973b, 1973c). This observation was used to develop a solid-phase radioimmunoassay that was specific for SLE virus (Trent *et al.*, 1976). The delineation of the antibody responses to the NS5 protein and the ability of this protein to serve as a type-specific antigen await further study.

IV. T-Cell Epitopes

A. *Helper T-Cell Epitopes*

Studies in mouse models have determined that flavivirus structural proteins are important in eliciting an antiviral helper T-cell (T_H) response (Chaturvedi *et al.*, 1987; Kulkarni *et al.*, 1992). The virus

specificity of this T_H-cell response can be serotype specific or virus cross-reactive (Rothman *et al.*, 1989; Uren *et al.*, 1987). An anti–DEN virus T_H-cell response can be detected as early as 2 weeks after immunization, can be stable for at least 12 weeks, and is still measurable at 1 year after immunization. When DEN virus–primed lymphocytes are restimulated *in vitro* using a cell lysate of recombinant baculovirus-infected cells, only recombinants containing either a combination of antigens (C-prM-E-NS1-NS2a) or the E-glycoprotein alone resulted in cellular proliferation, suggesting that the E-glycoprotein alone can be responsible for this proliferative response.

Predictive algorithms (amphipathicity, Rothbard motif, and α-helix formation) have been used to identify and synthesize T_H-cell epitopes. In one such study, three epitopes on the E-glycoprotein of JE, West Nile, and DEN viruses were identified: aa 436–445, JE virus; aa 430–439, DEN 4 virus; and aa 9–19, DEN 2 virus (Kutubuddin *et al.*, 1993). *In vitro* blastogenesis assays determined that two of these peptides (JE virus aa 436–445 and DEN 4 aa 430–439) elicited a virus cross-reactive response. The third peptide, DEN 2 aa 9–19, elicited a serotype-specific response that was also mouse haplotype dependent.

A second study utilized the previously described *trp*E fusion proteins of DEN 2 virus and identified three large regions (aa 22–205, 267–354, and 366–424) that stimulated an *in vitro* blastogenic response of virus-primed lymphocytes (Leclerc *et al.*, 1993; Megret *et al.*, 1992). Four peptides (aa 135–157, 270–298, 295–307, and 337–359) capable of eliciting an *in vitro* blastogenic response of virus-primed lymphocytes were subsequently identified.

In a comprehensive analysis of the murine flavivirus T_H-cell response to the E-glycoprotein, previous results with MVE virus were used to guide an analysis of the T_H-cell epitopes of DEN 2 virus (Mathews *et al.*, 1991, 1992; Roehrig *et al.*, 1994). Twenty-five synthetic peptides identified many regions capable of priming mice *in vitro* for both an antipeptide and an antivirus lymphocyte blastogenic response. For the first time, it was determined that while some peptides failed to prime mice for an *in vitro* antivirus blastogenic response, they could still prime animals *in vivo* for an enhanced antibody response. By comparing results between MVE and DEN viruses, it was shown that the location of important T_H-cell epitopes might be conserved between flaviviruses (Table IV). One T_H-cell epitope identified on one MVE virus peptide (aa 230–251) was subsequently mapped to aa 239–251 (Mathews *et al.*, 1992). By covalently cross-linking another of these T_H-cell epitopes (aa 352–368) to a normally nonimmunogenic peptide, a significant B-cell response to the nonimmunogenic

TABLE IV

T_H-CELL PRIMING ACTIVITY OF DEN 2 VIRUS E-GLYCOPROTEIN–DERIVED PEPTIDES

Peptide (amino acids)		Priming activity[a]		
MVE	DEN 2	P/P	P/V	*In vivo*
1–15		–	–	ND[b]
13–33		–	–	ND
	1–30	+	+	–
35–50		+	–	ND
35–55		+	–	ND
	35–55	+	+	–
	49–60/121–140	–	–	–
	58–73/106–121	+	–	+
	72–91/93–105	+	–	+
77–97		–	–	
	79–99	+	+	+
122–141		–	–	ND
	121–140	–	–	ND
145–169		+	–	ND
	142–172	+	+	+
179–197		+	–	ND
	167–201	+	–	ND
207–230		–	–	ND
	208–219	+	+	–
230–251		+	+	+
	225–249	+	+	+
	240–262	+	–	ND
255–266		–	–	ND
	255–274	+	–	ND
289–305		+	–	ND
	274–304	+	–	+
305–319		–	–	ND
	302–333	+	–	+
336–354		–	–	ND
	333–351	+	+	+
356–376		+	–	ND
	352–368	+	+	+
365–376		+	–	ND
	361–388	+	–	+
	388–400	–	+	–
	398–435	–	–	+

[a] Priming activity as measured in peptide/peptide (P/P, in one or more haplotypes of mice) or peptide/virus (P/V) *in vitro* lymphocyte blastogenesis tests or by *in vivo* priming by peptide for an enhanced antiviral antibody response (Mathews *et al*., 1991; Roehrig *et al*., 1994).

[b] Not defined.

peptide could be elicited (Roehrig *et al.*, 1992). Because of the extensive cross-reactivity that occurs among $CD4^+$ T-cell responses elicited by flaviviruses and the unusual propensity for these viruses to induce cytotoxic effector activity in this compartment, a more definitive characterization of the range and dominance of T_H epitopes and their relationship to protective immunity is needed. Such information may be of benefit for the design of future flavivirus vaccines.

B. Cytotoxic T-Cell Epitopes

Because the subject of flavivirus cytotoxic T-cell (T_C) epitopes is dealt with elsewhere (Rothman, 2003), only a brief summary of this topic is included here. Consistent with the current hypothesis of the MHC class I pathway for protein processing and generation of T_C-cell–reactive peptides, the immunodominant flaviviral T_C-cell epitopes reside on the flavivirus nonstructural proteins.

Studies have identified NS3 as an immunodominant target for DEN, MVE, KUN, and JE virus–reactive, human and murine T_C-cells, and T_C-cell clones (Aihara *et al.*, 1998; Bukowski *et al.*, 1989; Dharakul *et al.*, 1994; Gagnon *et al.*, 1996; Green *et al.*, 1993, 1997; Hill *et al.*, 1992; Kulkarni *et al.*, 1992; Kurane *et al.*, 1989, 1991, 1993, 1998; Livingston *et al.*, 1994, 1995; Lobigs *et al.*, 1994; Mathew *et al.*, 1996; Parrish *et al.*, 1991; Regner *et al.*, 2001a, 2001b; Rothman *et al.*, 1993; Spaulding *et al.*, 1999; Zeng *et al.*, 1996; Zivny *et al.*, 1999). NS3-specific T_C-cell clones can demonstrate a variety of virus reactivities ranging from virus-specific to flavivirus complex cross-reactive. T_C-cell epitopes have usually been defined by presenting them to virus-primed T cells in the form of vaccinia virus constructs or synthetic peptides. Depending on the immunizing virus and the subset of lymphocyte cloned, NS3 T_C-cell epitopes have been identified at aa 71–79 and 235–243 (Zivny *et al.*, 1999); aa 202–211, 241–249, and 351–361 (Zeng *et al.*, 1996); aa 224–234 (Kurane *et al.*, 1998; Mathew *et al.*, 1998); aa 296–310 (Rothman *et al.*, 1996; Spaulding *et al.*, 1999); and aa 500–508 (Livingston *et al.*, 1994). T_C-cell epitopes have also been identified on NS1/NS2a and NS4a/NS4b proteins of DEN and KUN viruses (Green *et al.*, 1997; Hill *et al.*, 1992; Rothman *et al.*, 1993) and on the flavivirus structural proteins, E (aa 331–339) (Aihara *et al.*, 1998; Livingston *et al.*, 1994; Rothman *et al.*, 1996) and C (aa 47–55) (Gagnon *et al.*, 1996). The $CD8^+$ T-cell response to flavivirus infection has unusual features, as discussed in detail elsewhere (Müllbacher *et al.*, 2003). It is clear that this response exhibits substantial cross-reactivity, resulting from the conserved epitopes within the NS3

protein; however, CTL effector responses at the epitope-specific level can vary. A more thorough investigation is needed of the factors that govern the $CD8^+$ T-cell repertoire against flaviviruses, the functional activities associated with dominant epitopes, and the relationship of these responses to protective immunity. These studies are likely to have great impact on strategies for designing and improving vaccines for dengue and other flaviviruses.

V. Conclusion

The current popular approach to flavivirus vaccine development is to construct full-length infectious cDNA virus clones and use them as substrates to produce chimeric flaviviruses or fully attenuated and stable viruses capable of eliciting a complete and long-lasting immunity. It is likely, however, that these vaccines will be expensive to produce, difficult to maintain because of requirements for a cold chain, and costly for the consumer. Many countries that would be targeted for DEN, JE, and YF virus vaccines are poor, so the development of economical and efficient subunit vaccines may still be necessary.

To prepare effective and inexpensive subunit vaccines, we will need to continue to make progress in understanding the structure–function relationships of the flavivirus proteins. Past attempts to develop DEN virus subunit vaccines taught us valuable lessons that should not be forgotten. These studies produced many recombinant antigens of the E-glycoprotein, most of which elicited only weak virus-neutralizing antibodies, most likely due to the requirement for coexpression of the E-glycoprotein and the prM protein for the preservation of native epitopes. Therefore, it will be important to gain a better understanding of the antigenic structure of the prM protein and its function in the virus. An accurate assessment of the role of disulfide bonding in the expression of E-glycoprotein epitopes would also be useful. Before the NS1 protein can be employed as a viable vaccine candidate, its function in virus replication and its interaction, if any, with the virion envelope proteins must be determined. The full contribution of NS3 and NS5 to flavivirus antigenicity and protective immunity needs to be fully examined. By making continued progress in understanding these and other aspects of flavivirus immunity, successful development and implementation of a variety of live-attenuated or subunit flavivirus vaccines should be available soon.

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ADVANCES IN VIRUS RESEARCH, VOL 59

5′- AND 3′-NONCODING REGIONS IN FLAVIVIRUS RNA

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I. Introduction
II. Conserved Linear Sequence Features of NCRs in Flavivirus RNA
 A. Complementary Nucleotides at the 5′ and 3′ Termini of NCRs
 B. Cyclization Sequences
 C. Additional Conserved Linear Sequences in 3′ NCRs of Mosquito-Borne Flaviviruses
 D. Conserved Linear Sequence Features of 3′ NCRs of the TBE Subgroup of Tick-Borne Flaviviruses
III. Secondary Structure of NCRs
 A. Secondary Structure of the 5′ NCR
 B. The 3′ SL
IV. Mutational Changes in the 5′ or 3′ NCR and the 3′ SL May Result in Attenuation of Virus Virulence
V. Hypothesis for Hydrogen Bonding between 5′ and 3′ NCRs
VI. Additional Secondary Structure Predictions in the 3′ NCR
VII. Summary and Future Directions
 References

I. Introduction

The major human pathogens among the flaviviruses are yellow fever (YF) virus, the four serotypes of dengue (DEN) viruses, Japanese encephalitis (JE) virus, and the tick-borne encephalitis (TBE) viruses. Flaviviruses contain a positive-stranded RNA genome that is approximately 10,500 nucleotides in length. The RNA contains a type 1 cap at its 5′ terminus (Cleaves and Dubin, 1979) but is not polyadenylated (Wengler and Wengler, 1981), and it encodes a single, long open reading frame (ORF) (reviewed in Chambers *et al.*, 1990). All evidence indicates that virus-specific proteins are derived by co- or posttranslational cleavage of the polyprotein encoded by the ORF. These include three structural proteins [capsid (C), premembrane (preM), and envelope (E)] and at least seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, in that gene order). Most cleavages of the polyprotein are mediated by the host cell enzyme signal peptidase or by a viral protease complex requiring a central hydrophilic

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domain in NS2B and the amino terminus of NS3 (Chambers *et al*., 1993; Falgout *et al*., 1991). Genomic RNA contains noncoding regions (NCR) 5′ and 3′ to the ORF. The 5′-NCR is about 100 nucleotides in length, and the 3′-NCR is 400 to nearly 800 nucleotides in length (Table I). The conserved structural and nucleotide sequence elements of these NCRs and their function in RNA replication and translation are the subjects of this review.

TABLE I
COMPARISON OF THE LENGTH IN NUCLEOTIDES OF 5′ AND 3′ NCRS IN GENOMES OF SELECTED FLAVIVIRUSES

Virus genome[a]	5′ NCR[b]	3′ NCR	Total genome length	Method[c]	Reference[d]
DEN1, strain WP74	97	466	10,736	RNA	NC 001477
DEN2, strain NGC	89	451	10,723	DNA	NC 001474
DEN3, strain H87	93	432	10,696	DNA	NC 001475
DEN4, strain 814669	101	384	10,649	RNA	NC 002640
JE, strain JaOAR5982	95	585	10,976	DNA, RNA	NC 001437
WN, strain NY99	96	631	11,029	DNA, RNA	AF 196835
MVE	95	614	11,014	DNA, RNA	NC 000943
YF, strain 17D	118	511	10,862	DNA	Rice *et al*. (1985)
TBE, strain Sofjin	*131*	*518*	*10,894*	*DNA, RNA*	*AB 062064*
TBE, strain Neudorfl	*132*	*764*	*10,469*	*DNA, RNA*	*NC 001672*
POW	*111*	*480*	*10,839*	*DNA, RNA*	Mandl *et al*. (1993)
CFA[e]	113	559	10,695	DNA, RNA	NC 001564

[a] Names of viruses are abbreviated as follows: DEN, dengue (numbers indicate dengue serotypes 1 to 4); JE, Japanese encephalitis; WN, West Nile; YF, yellow fever; MVE, Murray Valley encephalitis; TBE, tick-borne encephalitis; POW, Powassan; and CFA, cell-fusing agent. Strain names are abbreviated as follows: WP74, Western Pacific, 1974; NGC, New Guinea C; NY99, New York, 1999; and 17D, attenuated YF vaccine strain. Data for mosquito-borne viruses are indicated in plain type. Data for tick-borne viruses are indicated in italics.

[b] Number of nucleotides assigned by sequence analysis to the 5′ NCR and 3′ NCR for each virus genome is indicated where noted, and the total genome size in nucleotides is also indicated where noted.

[c] DNA sequence data were obtained by sequencing a series of overlapping cloned DNA fragments spanning the genome. RNA sequence data were obtained by direct sequencing of RNA or by sequencing of uncloned DNA fragments generated from RNA.

[d] Either a published manuscript listed in the references or a Genbank accession number is given.

[e] Has no known vector.

The NCRs in genomic RNA must be involved in the initiation of negative-strand synthesis and in any processes related to switching from negative-strand synthesis to the production of progeny virion RNAs. NCRs may also play a role in the packaging of nascent virions and contain nucleotide sequences required to initiate translation. Of the viral proteins, NS1, NS2A, NS3, NS4A, and NS5 have been implicated directly or indirectly in RNA replication. The role of NS1 in this process is not defined, but mutations in NS1 have been shown to affect the initiation of minus-strand synthesis (Lindenbach and Rice, 1997, 1999; Muylaert *et al.*, 1997). NS2A is a small hydrophobic protein localized to the replication complex that may target it to membrane organelles (Mackenzie *et al.*, 1998). NS3 contains an RNA helicase and nucleotide triphosphatase activities in addition to its protease activity (Utama *et al.*, 2000; Warrener *et al.*, 1993; Wengler and Wengler, 1991), and NS4A may form a required association with NS1 during replication (Lindenbach and Rice, 1999). NS5 functions as an RNA-dependent RNA polymerase (Ranjith-Kumar *et al.*, 2001; Tan *et al.*, 1996) and also contains a potential methyl-transferase activity, possibly required for the capping of progeny viral genomes (Koonin, 1993).

The currently accepted model for flavivirus RNA replication is a semiconservative one. Input genome RNA must first be copied to form full-length minus strands in order to generate stable double-stranded replicative forms (RF). Free minus-strand genomic RNA is not detected (Chu and Westaway, 1985; Khromykh and Westaway, 1997), and the plus and minus RNA strands in the RF appear to be exact copies of each other, such that there are no overhanging unpaired nucleotides at the 3′ termini of either strand (Wengler and Wengler, 1981). Plus-strand RNA synthesis is thought to proceed in replicative intermediates, using RFs as a template. No subgenomic virus-specific RNAs have been detected in flavivirus-infected cells, in support of the concept that the genomic plus-strand RNA is the only virus-specific mRNA. The RNA replication process appears to be localized to perinuclear membrane organelles, probably in complexes containing nascent RNA and NS proteins in association with cellular proteins (Chu and Westaway, 1985, 1987; Ng *et al.*, 1989; Westaway *et al.*, 1999).

Table I shows a list of the lengths of the respective 5′ and 3′ NCRs in representative flavivirus genomes. The total number of nucleotides in each genome is also shown for comparison. Flaviviruses have been speciated, and species have been subgrouped, by results of virus cross-neutralization assays (Calisher *et al.*, 1989). The results of such assays depend on antigenic differences among the respective envelope glycoproteins of the different viruses. Seven subgroups were thus defined,

but at least 15 viruses remained ungrouped, including YF virus. For example, the four serotypes of dengue viruses form one subgroup or "complex" among the mosquito-borne flaviviruses. Similarly, the Japanese encephalitis virus complex includes JE, West Nile (WN), and Murray Valley encephalitis (MVE) viruses. The tick-borne flaviviruses (e.g., TBE virus strains Sofjin and Neudorfl and Powassan virus, see Table I) form a complex that is serologically distinct from the mosquito-borne flaviviruses. Cell-Fusing Agent virus (CFA) was isolated from cultured mosquito cells and appears to have no serological relationship to other flaviviruses in the cross-neutralization assay, has no vector in nature, and causes no illness in humans or animals (Cammisa-Parks *et al*., 1992).

Flaviviruses have also been grouped by phylogenetic analysis, based on sequence data obtained for a 1000-nucleotide cDNA generated from the 3′ termini of the NS5 genes of several dozen isolates (Kuno *et al*., 1998). The hierarchical levels of relatedness defined by this technique were organized in descending order as follows: "cluster," "clade," and "species," where a clade was defined as a group of viruses that share 69% or higher pairwise nucleotide sequence identity among the members. Results of the analysis for grouping flaviviruses in clades were quite comparable to those obtained by the cross-neutralization assay as it was used to define antigenic complexes or subgroups, suggesting a parallel evolution of genome sequences and antigenic character. These findings will be seen to be relevant as we discuss differences in 5′ and 3′ NCR nucleotide sequences among viruses of different antigenic subgroups or clades.

Data for viruses listed in Table I show that the 5′ NCRs of flavivirus RNAs are relatively short compared to the 5′ NCRs of other positive-strand RNA viruses. Lengths of the 5′ NCR sequences are between 89 nucleotides, for dengue serotype 2 (DEN2) strain New Guinea C (NGC), and 132 nucleotides, for TBE strain Sofjin. Tick-borne flaviviruses (strain Sofjin, strain Neudorfl, and Powassan) tend to have slightly longer 5′ NCRs than mosquito-borne viruses. For comparison, the 5′ NCR of a typical isolate of the hepacivirus, hepatitis C virus, is 341 nucleotides in length (Fukushi *et al*., 1994), whereas that of a picornavirus, poliovirus type 1, is 742 nucleotides in length (Rezapkin *et al*., 1998). This discrepancy in length of the 5′ NCRs of these otherwise related positive-strand RNA virus genomes is due primarily to the fact that the hepatitis C and poliovirus 5′ NCRs each contain an internal ribosome entry site (IRES) required for translation initiation (Bergamini *et al*., 2000; Brown *et al*., 1992; Poyry *et al*., 2001; Wang *et al*., 1995). IRESs are fairly large tRNA-like structures composed of

hundreds of nucleotides. In contrast, translation of flavivirus RNAs is thought to initiate by ribosome scanning from the 5′ cap structure (Ruiz-Linares, 1989) so possibly there is an adaptive advantage related to having a short 5′ NCR.

DEN virus genomes had the shortest 3′ NCRs among the selection of flavivirus genome sequences surveyed, ranging from 384 nucleotides for DEN4, strain 814669, to 466 nucleotides, for DEN1, strain WP74. The 3′ NCRs in viruses of the JE group shown in Table I ranged from 573 to 631 nucleotides in length. The length of the YF vaccine strain 17D 3′ NCR was in between that of the DEN and JE complex viruses at 511 nucleotides. The 3′ NCRs of the TBE viruses, other than strains Neudorfl and Sofjin, vary greatly in length due to the presence of long poly(A) tracts in some genomes that are not present in others and due to deletions of sequences flanking the poly(A) tract within the variable region (see later for details). Many dozens of complete genome sequences of flavivirus have been entered in Genbank. In no case do the lengths of the 5′ and 3′ NCRs vary significantly from those noted for viruses listed in Table I.

II. Conserved Linear Sequence Features of NCRs in Flavivirus RNA

A. Complementary Nucleotides at the 5′ and 3′ Termini of NCRs

The first complete flavivirus genome sequence to be published was that of the YF virus, strain 17D (Rice *et al.*, 1985). The authors noted that the extreme 5′- and 3′-terminal sequences of the YF genome were partially homologous to those of the WN virus, which had been determined separately (Wengler and Wengler, 1981), and that for both genomes (i) the 5′-terminal two nucleotides of the plus-strand sequence (5′-AG) were complementary to the 3′-terminal two nucleotides (UC-3′) and (ii) there was a short (five-nucleotide) region of identity between a segment near the 3′ termini of plus-strand RNAs and the predicted 3′ termini of minus-strand RNAs for each of the two virus genomes due to the presence of a complementary sequence in the 5′ NCR of the plus strand (the sequence 5′-UGUGU in the 5′ NCR and the sequence 5′-ACACA in the 3′ NCR, as shown in Fig. 1). For the YF genome, the sequence ACACA initiated at the 13th nucleotide from the 3′ end of the RNA and spanned nucleotides –13 through –9 (reading in the 3′ to 5′ direction is indicated by a minus sign), and for the WN genome the ACACA sequence spanned nucleotides –11 through –6 (Fig. 1).

YFV	(+) 5'	AGUAAAUCCUGUGUGC . . . A**ACACA**AAACCACU 3'
	(−) 3'	UCAUUUAGG**ACACA**CG . . . UUGUGUUUUGGUGA 5'
WNV	(+) 5'	AGUAGUUCGCCUGUGUG . . . A**ACACA**GGAUCU 3'
	(−) 3'	UCAUCAAGCGG**ACACA**C . . . UUGUGUCCUAGA 5'
MVE	(+) 5'	AGACGUUCAUCUGCGUGAGC . . . GAGAAGACCACAGGAUCU 3'
	(−) 3'	UCUGCAAGUAGACGCACUCG . . . CUCUUCUGGUGUCCUAGA 5'
DEN3	(+) 5'	AGUUGUUAGUCUACGUG . . . AUCAACAGGUUCU 3'
	(−) 3'	UCAACAAUCAGAUGCAC . . . UAGUUGUCCAAGA 5'
KUN	(+) 5'	AGUAGUUCGCCUGUGUGAGCU . . . GGUGCGAGA**ACACA**GGAUCU 3'
	(−) 3'	UCAUCAAGCGG**ACACA**CUCGA . . . CCACGCUCUUGUGUCCUAGA 5'
JE	(+) 5'	AGAAGUUUAUCUGUGUGAAC . . . GAGGAAGA**ACACA**GGAUCU 3'
	(−) 3'	UCUUCAAAUAG**ACACA**CUUG . . . CUCCUUCUUGUGUCCUAGA 3'

FIG 1. Nucleotide sequences of 5′ and 3′ termini of a selection of mosquito-borne flavivirus positive-strand (+) genome RNAs are shown aligned with the nucleotide sequences of 3′ and 5′ termini of the respective minus (−) strands, predicted to result when the positive strand is used as a template to form a replicative intermediate during virus replication. Dotted lines indicate the intervening nucleotides of the respective full-length genomes. Flavivirus conserved 5′- and 3′-terminal dinucleotides and their complementary nucleotides are boxed. The semiconserved pentanucleotide sequence ACACA is shown in bold in the 3′ termini of positive- and negative-strand RNAs, respectively, where present. Genome sequences are as follows: YF, yellow fever (Rice *et al.*, 1985); WNV, West Nile (Wengler and Wengler, 1981); MVE, Murray Valley encephalitis (Genbank number NC00943); DEN3, dengue serotype 3 (Genbank number NC001475); KUN, Kunjin (Genbank numbers L24511 and L24512); and JE, Japanese encephalitis (Genbank number NC001437).

The identical five-nucleotide sequences near the 3′ termini of both plus- and minus-strand RNAs were posited as recognition sites for the viral replicase that could be used in common during either positive- or negative-strand RNA synthesis (Rice *et al.*, 1985).

An examination of a selection of genome sequences for other flaviviruses reveals that the complementary pairs at the ends of the RNA (5′-AG/UC-3′) are completely conserved among mosquito- and tick-borne viruses. The CFA virus genome is unique among the flaviviruses in that it terminates with GC-3′ and its 5′-terminal two nucleotides (5′-GU) are not complementary. The survey also revealed that the sequence ACACA and its complement are not well conserved in analogous positions in 3′ and 5′ NCRs, respectively, of flavivirus genomes (Fig. 1). For example, JE and Kunjin virus genomes do contain such sequences in the predicted locations, whereas the related MVE virus genome does not, and viruses of the DEN subgroup do not. Therefore, the significance of the “ACACA-type” sequences near the 3′ termini of

plus and minus strands of the YF, WN, JE, KUN, and perhaps other flavivirus genomes must be regarded as uncertain, especially since the functionality of other better conserved segments of the NCRs in RNA replication has been demonstrated.

B. Cyclization Sequences

The most conserved linear sequence feature of the 5′ termini of flavivirus genomes is the cyclization sequence (5′CS in Fig. 2; Hahn *et al.*, 1987; Khromykh *et al.*, 2001). The motif is so named because of the existence of a complementary sequence in the 3′ NCR. Base pairing between 5′ and 3′ end sequences would in theory permit the formation of cyclized plus strands, perhaps during the initial phases of RNA replication. This conformation was initially predicted to permit the viral replicase to bind to both ends of the template RNA simultaneously in order to assure the generation of full-length copies (Hahn *et al.*, 1987). Similar "panhandle" structures are thought to form, for example, during the replication of alphavirus (Hsu et al, 1973) and bunyavirus (Hewlett *et al.*, 1977) RNAs and of phage QB RNA (Blumenthal and Carmichael, 1979).

For mosquito-borne virus genomes, the 5′ cyclization sequence is known as the "5′CS" [indicating the 8 nucleotide sequence UCAAUAUG, spanning nucleotides +137 through +144 of the Kunjin virus (KUN) genome, for example, where the plus sign indicates nucleotides are numbered from the 5′ terminus]. The 5′CS actually lies within the ORF, about 34 to 40 nucleotides downstream from the start codon (Fig. 2). For tick-borne virus genomes, two potential 5′ cyclization sequences have been identified (Khromykh *et al.*, 2001; Mandl *et al.*, 1993), here designated "C1" and "C2" (Fig. 3). C1 and C2 differ markedly in linear sequence from each other and from that of the 5′CS in mosquito-borne virus genomes (Table II). Tick-borne virus 5′-NCRs contain a very short ORF that is terminated with a stop codon within the NCR (Fig. 3). The most 5′-terminal potential cyclization sequence (C1; the 11 nucleotide sequence GGAGAACAAGA, spanning nucleotides +115 through +125 for TBE viruses) lies downstream from this mini-ORF and wholly within the 5′ NCR (Mandl *et al.*, 1993). C2 (the 11 nucleotide sequence GGGGCGGUCCC, spanning nucleotides +164 through +174 for TBE) lies downstream from C1, within the true ORF, in a position analogous to that of the 5′CS in mosquito-borne virus genomes (Khromykh *et al.*, 2001).

The 3′ extreme of the 8 nucleotide 3′ cyclization sequence (CYC; Fig. 2) in mosquito-borne virus RNAs is variably located 99 to 112

FIG 2. Some nucleotide sequence features of 5′ and 3′ NCRs of mosquito-borne flavivirus genomes are represented. Horizontal lines represent nucleotide sequences of DEN2, DEN4, JE, MVE, WN, and YF genome 5′ NCRs (aligned in vertical order on the left) and 3′ NCRs (aligned in vertical order on the right). Upward vertical ticks in the 5′ NCR indicate the location of the 5′-most start codon for translation of the single ORF in the flavivirus genome. Downward vertical ticks indicate the location(s) of translational stop codons at the 5′ ends of 3′ NCRs. The flavivirus-conserved stem and loop secondary structure predicted to form at the 3′ end of all genomes is indicated by a loop at the end of the linear sequence (SL). The relative locations of the conserved eight nucleotide cyclization sequences, 5′CS and CYC, respectively, at the 5′ and 3′ ends of the genome are indicated by open boxes with cross-hatching. The relative location of the conserved sequence, CS2, in all genomes is indicated by a stippled box, and the location of the CS2 tandem repeat sequence, RCS2, in all but the YF genome is indicated by a second stippled box upstream from CS2. Similarly, the relative locations of the conserved tandem repeat sequences, CS3 and RCS3, are indicated by black, cross-hatched boxes in genomes of viruses of the JE subgroup. The locations of three tandem repeat sequences in the upstream portion of the YF genome 3′ NCR are indicated by open rectangles.

nucleotides from the 3′ terminus of the genome (Table II). This locus is just upstream from the conserved 3′ terminal stem and loop structure common to all flavivirus genomes (see later). The CYC sequence is included within the upstream portion of a longer sequence element, "CS1" (Figs. 2 and 4) that is also semiconserved among mosquito-borne virus genomes (Fig. 5). For tick-borne viruses, the 11 nucleotide 3′

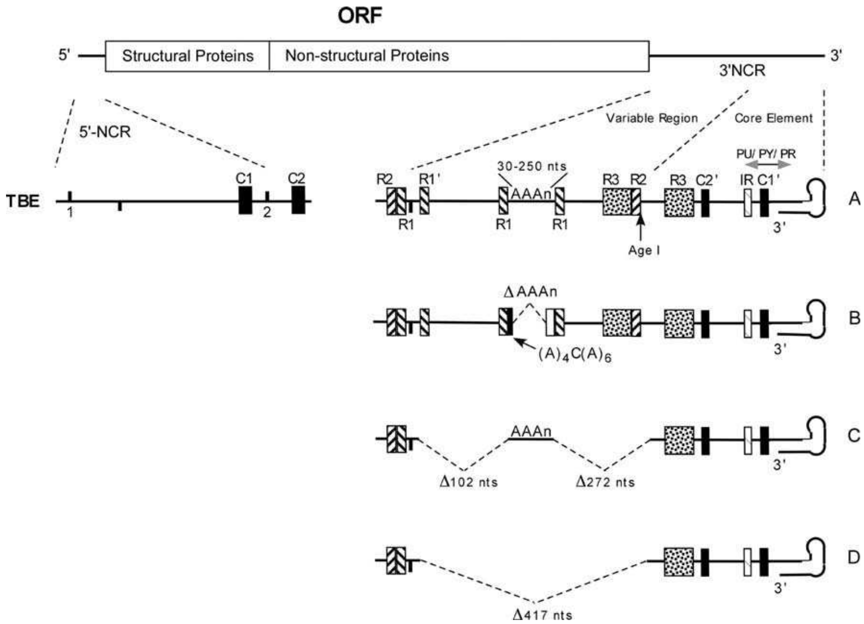

FIG 3. Some nucleotide sequence features of 5′ and 3′ NCRs of TBE virus strain genomes are represented. The 5′ NCR of TBE virus is represented on the left as a horizontal line. The start codon for translation of a very short ORF in the 5′ NCR is represented by a single upward vertical tick, numbered one (1); the start codon for translation of the long ORF in TBE genomes is represented by an upward tick, numbered two (2). Stop codons for the short ORF in the 5′ NCR and for the long ORF at the 5′ end of the 3′ NCR are indicated by a downward vertical tick. Four of six different possible nucleotide sequence motifs for 3′ NCRs of TBE viruses are indicated on the right as horizontal lines (adapted from Wallner *et al.*, 1995). Motif A, strains Neudorfl and 263; B, strain Ljubljana I; C, strain 132; and D, strain RK1424. The flavivirus-conserved 3′ stem and loop structure is indicated by a loop in the horizontal line and is labeled "SL." The location of the restriction endonuclease site *Age*I in TBE genomic DNA is indicated by an arrow defining the boundary between the variable region and the core element in the strain Neudorfl 3′ NCR. Black boxes indicate the relative locations of cyclization sequences C1 and C2 in the 5′ NCR and C1′ and C2′ in the 3′ NCR. Relative locations of TBE virus–conserved repeat sequences R1, R1′, R2, and R3 in the 3′ NCR are indicated by cross-hatched or stippled boxes. IR, conserved inverted repeat. PU/PY/PR, conserved homopurine, pyrimidine, and purine-rich segments in 3′ NCR RNA, respectively. Dotted lines indicate the locations of deletions in the genomes of some TBE strains relative to the motif found in strain Neudorfl RNA. The notation "AAAn" indicates the location of a poly(A)tract in the strains Neudorfl, 263, and 132 genomes. The location of a severely truncated poly(A)tract in the strain Llubljana 1 genome is specifically indicated by an arrow.

TABLE II

RELATIVE LOCATIONS OF PROPOSED CYCLIZATION SEQUENCES (CS) IN GENOMIC RNA OF *FLAVIVIRUS* SPECIES

Virus	Translation initiation[a]	Proposed CS and locations[b]	Genbank accession No.	Reference for CS
Mosquito borne		5′-**U**CAAUAUG 3′-AGUUAUAC		Hahn *et al*. (1987); 5′CS/CYC in Fig. 2
Kunjin	97	5′ (**137**) (**-104**) 3′	D00246, L24511, L24512	Khromykh *et al*. (2001)
JE	96	5′ (**136**) (**-111**) 3′	M10370	Fig. 2
MVE	96	5′ (**136**) (**-111**) 3′	NC000943	Hahn *et al*. (1987)
WN	97	5′ (**137**) (**-105**) 3′	M12294	Hahn *et al*. (1987)
DEN1	81	5′ (**118**) (**-103**) 3′	M87512	Fig. 2
DEN2	97	5′ (**134**) (**-103**) 3′	M20558	Fig. 2
DEN3	94	5′ (**132**) (**-103**) 3′	M93130	Fig. 2
DEN4	102	5′ (**136**) (**-99**) 3′	M14931	Fig. 2
YF	119	5′ **(156)** (-**112**) 3′	NC002031	Fig. 2
Cell-fusing agent	114	5′ (**169**) **C**CCCGUUCUGG 3′ GGGGCA-GGUC (**-134**)	M91671	Cammisa-Parks *et al*. (1992)
	114	5′ (**149**) **G**CCAGGG 3′ CGGUCCC (**-471**)	M91671	CS "D"/CS"C" in Khromykh *et al*. (2001)

Tick borne				Khromykh *et al.* (2001)
TBE	133	5′ (**164**) **G**GGGC^{GG}UCCC	U27495	C2/C2′ in Fig. 3
		3′ CCCCG$_{G}$AGGG**G** (**-193**)		
Powassan	112	5′ (**136**) **G**GGGGC^{GG}UCC	L06436	C2/C2′ in Fig. 3
		3′ CCCCCG–AG**G** (**-189**)		
Louping ill	130	5′ (**160**) **G**GGGGC^{GG}UCC	Y07863	C2/C2′ in Fig. 3
		3′ CCCCCG$_{G}$ AG**G** (**-193**)		
TBE	133	5′ (**115**) **G**GAGAACAAGA	U27495	Mandl *et al.* (1993) C1/C1′ in Fig. 3
		3′ CCUCUUGUUC**U** (–**81**)		
Powassan	112	5′ (**88**) **G**GAGAACAAGA	L06436	Mandl *et al.* (1993)
		3′ CCUCUUGUUC**U** (–**81**)		
Louping ill	130	5′ (**112**) **G**GAGAACAAGA	Y07863	Gritsun *et al.* (1997)
		3′ CCUCUUGUUC**U** (–**81**)		

[a] Numbered position of first nucleotide in the AUG codon.

[b] The 5′ and 3′ CS (cyclization sequence) shown are base paired. The first nucleotide of each CS is shown in bold. Additional but nonconserved 5′/3′ *trans* complementary basepairs flanking the CSs are not shown. Positions of the first nucleotide of each CS in relation to the 5′ or the 3′ terminus (indicated by a minus sign) of each genome are shown in bold within parentheses. Reference is made to Figs. 2 and 3 for mapping of the CSs in mosquito- and tick-borne virus genomes.

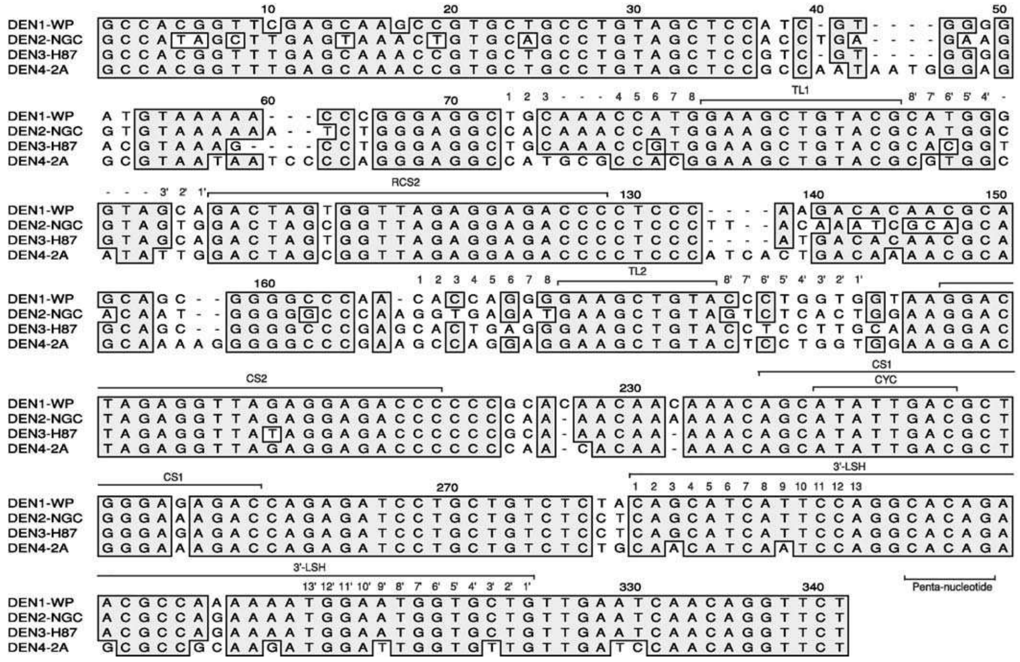

FIG 4. Nucleotide sequences of 3′ NCRs of dengue virus genomes representing each of the four serotypes are shown. Shaded areas indicate sequence homology. Dashes indicate gaps introduced into a sequence in order to maximize its alignment with the other sequences. Nucleotides that form the long stem and loop within the flavivirus-conserved 3′-SL (see text) are indicated by a horizontal line above sequence data, labeled 3′-LSH. The location of the flavivirus-conserved pentanucleotide sequence, 5′-CACAG-3′, within the 3′-SL, is indicated by a line below sequence data. The conserved sequences, CS1, the cyclization sequence, CS2, and RCS2 are indicated by horizontal lines above relevant sequence data. The conserved

CS1:

AGC**AUAUUGAC**ACCUGGGAAAAGAC **MVE**
AGC**AUAUUGAC**ACCUGGGAU.AGAC **WN**
AGC**AUAUUGAC**ACCUGGGAAUAGAC **JE**
AGC**AUAUUGAC**GC.UGGGAG.AGAC **DEN1**
AGC**AUAUUGAC**GC.UGGGAG.AGAC **DEN3**
ACC**AUAUUGAC**GCCAGGGAA.AGAC **YF**

CS2:

GGACUAGAGGUUAGAGGAGACCC **MVE, WN, JE**
GGACUAGAGGUUAGAGGAGACCC **DEN1**
GGACUAGAGGUUAUAGGAGACCC **DEN3**
GGUCUAGAGGUUAGAGGAGACCU **YF**

CS3 (1) and RCS3 (2) in MVE (JE subgroup) 3'NCR:

1 CCCCAGGAGGACUGGGUUACCAAAGCUG
2 CCCCAGGAGGACUGGGUAAACAAAGCCG

Upstream Tandem Repeats in the YF 17D 3' NCR:

1 ***UAA**CCGGGAUACAAACCACGGGUGGAGAACCGGACUCCCCACA
2 GAAACCGGGAUAUAAACCACGGCUGGAGAACCGGGCUCCGCACU
3 GAAACCGGGAUAAAAACUACGGAUGGAGAACCGGACUCCACACA

FIG 5. Nucleotide sequences of some conserved motifs in 3′ NCRs of mosquito-borne flavivirus genomes. Deletions in CS1 with respect to the sequence for MVE (Hahn *et al.*, 1987) are indicated by a period. Nucleotide substitutions with respect to the sequences of CS1 and CS2 in the MVE genome are indicated by underlining the substituted nucleotides. Nucleotides comprising the 3′ cyclization sequence (CYC) in CS1 are shown in bold print. The stop codon that comprises the first three nucleotides of the most upstream tandem repeat in the YF genome 3′ NCR (Rice *et al.*, 1985) is also shown in bold and indicated by an asterisk. Nucleotide sequence data are from Genbank (Table I) or from the aforementioned references.

sequence complementary to C1 [C1′, the sequence CCUCUUGUUCU (Mandl *et al.*, 1993)] is localized to the most upstream portion of nucleotides required to form the conserved stem–loop, spanning nucleotides –81 to –71 (Table II; Fig. 3). The 10 nucleotide 3′ segment complementary to C2 [C2′, the sequence CCCCGGAGGG (Khromykh *et al.*, 2001)] is located further upstream from the 3′ terminus of the genome, spanning nucleotides –184 to –193, for example, in the TBE genomes.

Hahn and colleagues (1987) further predicted that the double-stranded panhandle structure potentially resulting from base pairing of the 5′ and 3′ cyclization sequences in the mosquito-borne virus genomes that were under analysis could be further stabilized by the presence of additional nonconserved complementary nucleotides

small stem and loop structures, TL1 and TL2 (Proutski *et al.*, 1997b), are also so indicated. Nucleotides presumed to base pair in the formation of the long stem in the 3′-SL and in the formation of the short TL1 and TL2 stems are numbered.

upstream from the 5′CS at the 5′ end of the genome in a pyrimidine-rich segment of the genome and downstream from the 3′ CYC sequence at the 3′ end of the genome in a purine-rich region. Thus there were a total of 11 or 12 contiguous base pairings possible for cyclization of YF, MVE, WN, and DEN2 RNAs, resulting in a predicted thermal stability of from −9.1 kcal (for MVE RNA) to −12.3 kcal (for YF RNA). This was deemed to be sufficient free energy to cyclize flavivirus RNAs based on the previous observation that alphavirus RNAs could cyclize under physiological conditions (Hsu *et al.*, 1973) and that the free energy of cyclization of alphavirus RNA measured thermodynamically was determined to be −13.5 kcal at 25°C (Frey *et al.*, 1979). Additional but noncontiguous complementarity further upstream from the 5′CS and downstream from the 3′ CYC sequence was predicted to stabilize the panhandle further for some of the RNAs, resulting in an increase of potential thermal stability to −20.5, −25.5, and −33.7 kcal, respectively, for the panhandle form of the WN, MVE, and YF genomes (Hahn *et al.*, 1987). The predicted thermal stability of the double-stranded regions potentially formed by base pairing of C1 with C1′ and of C2 with C2′ in the TBE virus genome was −19.8 and −30.9 kcal, respectively (Khromykh *et al.*, 2001). Potential cyclization sequences have also been identified in the CFA genome (Cammisa-Parks *et al.*, 1992; Table II).

Several bits of evidence demonstrate that base pairing of the cyclization sequences is required for RNA replication. First, in a study of the requirements for nucleotide sequences of the DEN4 3′ NCR for virus replication, Men and co-workers (1996) created a series of mutant DEN genomic RNAs by the transcription of DEN4 infectious DNAs containing internal deletions centered about a unique *Apa*I restriction endonuclease site located between the conserved tandem repeat sequences CS2 and RCS2 (see later) in wild-type DEN4 DNA (Figs. 2 and 4). Eight mutant RNAs containing deletions ranging in size from 30 to 262 nucleotides, in which all or portions of CS2, RCS2, and CS1 were deleted, were evaluated for their infectivity. Although all the RNAs gave rise to viruses that were reduced in replication competence compared to wild type, only one RNA (mutant 3′d172–83) had a lethal phenotype. The deletion in this RNA was unique in that it included all of the 23 nucleotide CS1 segment in DEN4 RNA, whereas the maximum downstream extent of any of the other seven deletions spared the entirety of CS1. These results demonstrated that CS1, including the 8-nucleotide cyclization sequence, was indispensible for virus replication.

Second, Khromykh and Westaway (1997) evaluated the replication competence of several in-frame internal deletion mutant RNAs derived from a full-length infectious DNA copy of the Kunjin virus genome

("replicons"). They demonstrated that an RNA with a deletion starting at codon 20 of the capsid gene and extending to include downstream sequences encoding the premembrane protein and the entire ectodomain of the envelope glycoprotein (mutant C20rep) replicated efficiently in hamster kidney (BHK) cells, whereas an otherwise identical deletion mutation, in which the 5′ end of the deletion extended upstream to codon 2 of the capsid gene (mutant C2rep), was lethal for RNA replication. Similarly, another mutant RNA in which the capsid start codon and sequences downstream encoding the remainder of capsid, all of the premembrane protein, and the amino-terminal majority of the envelope protein (mutant ΔCME) was also replication defective. C20rep RNA contained the 5′CS for the Kunjin genome, whereas the mutants C2rep and ΔCME RNAs did not. This suggested that either the RNA sequence (including the 5′CS) within the ORF encoding the amino terminus of capsid or translation of the amino terminus of capsid was necessary for RNA replication. The results of follow-up experiments favored the former hypothesis, that nucleotide sequences comprising the 5′ terminus of the ORF, including the 5′CS, were required for RNA replication.

Subsequent studies specifically addressed the requirement for both the 5′CS and the 3′CYC sequence for RNA replication. You and Padmanabhan (1999) reported on an *in vitro* assay for RNA-dependent RNA polymerase (RdRP) activity in DEN2-infected mosquito cell extracts using exogenous subgenomic RNAs containing the 5′-terminal 230 nucleotides and/or the 3′-terminal 373 nucleotides of the DEN2 genome as templates. 5′-terminal DEN2 sequences containing the 5′CS sequence, either linked covalently to the 3′-terminal DEN2 RNA sequence or supplied in *trans*, were required for replication to initiate on the 3′-terminal RNA segment. Mutagenesis of either the 5′ or the 3′ cyclization sequence such that base pairing between them was abrogated resulted in total loss of *in vitro* RdRP activity, but when both sequences were mutagenized such that the mutant cyclization sequences were complementary, RdRP activity was detected. This demonstrated that cyclization sequences were required for synthesis of a covalently closed double-stranded hairpin molecule *in vitro*. Free progeny RNA molecules were not detected in this system. However, Khromykh and colleagues (2001) addressed the relevant question *in vivo* using the KUN replicon system. They initially created a 5′CS deletion mutation in KUN replicon RNA and demonstrated that the resulting RNA was no longer competent for replication. Next, they created substitution mutations in either the 5′CS or the 3′CYC sequence in order to disrupt base pairing between them. As seen in the *in vitro* study, abrogation of

their complementarity by mutagenesis of either cyclization sequence was also lethal for RNA replication. However, when both cyclization sequences were mutagenized with respect to the wild-type sequence such that their capacity to base pair was maintained, RNA replication, including *de novo* minus and plus strand synthesis, was restored.

These results clearly demonstrated that complementarity, but not the respective wild-type nucleotides, of the 5′CS and 3′CYC sequences was necessary for flavivirus RNA replication to proceed. Future studies will likely be designed to prove that the input plus strand RNA actually does cyclize *in vivo* prior to or as a requirement for initiation of RNA replication and to determine how localization of the cyclization sequences, at nearly equal distances from the 5′ and 3′ termini of the genome, is related to their function in mediating the initiation of RNA synthesis. Moreover, it will be of interest to learn whether both pairs of complementary cyclization sequences present in the genomes of tick-borne viruses are required for the replication of tick-borne virus RNA or whether only one complementary pair is sufficient. Cellular and viral proteins that participate in the cyclization process are also yet to be identified. For further discussion of a model for panhandle formation related to additional complementarity of nonconserved sequences in the 5′ and 3′ NCRs (Khromykh *et al.*, 2001), see later.

C. Additional Conserved Linear Sequences in 3′ NCRs of Mosquito-Borne Flaviviruses

1. The Pentanucleotide

The 3′ NCRs of mosquito-borne flaviviruses contain a number of conserved sequences that are localized primarily to the 3′-terminal portion of this region of the genome. The most 3′-terminal of these sequences is the pentanucleotide 5′-CACAG, which forms part of an unpaired region forming a closed loop within the conserved stem and loop structure formed by the 3′-terminal ~95 nucleotides of all flavivirus genomes (Hahn *et al.*, 1987). For example, in DEN virus genomes, the pentanucleotide sequence occurs between nucleotides –43 and –47 (Figs. 4 and 6). This sequence and its location in the genome are completely conserved among mosquito- and tick-borne flaviviruses for which sequence data are available. It is also incompletely conserved in the 3′ stem and loop structure predicted to be present in CFA genome RNA; the fourth nucleotide is C instead of A (Cammisa-Parks *et al.*, 1992). The functional requirement for the conserved pentanucleotide sequence in the virus life cycle is not known, but it has been suggested that the pentanucleotide may play an indispensible role in the binding of cellular or

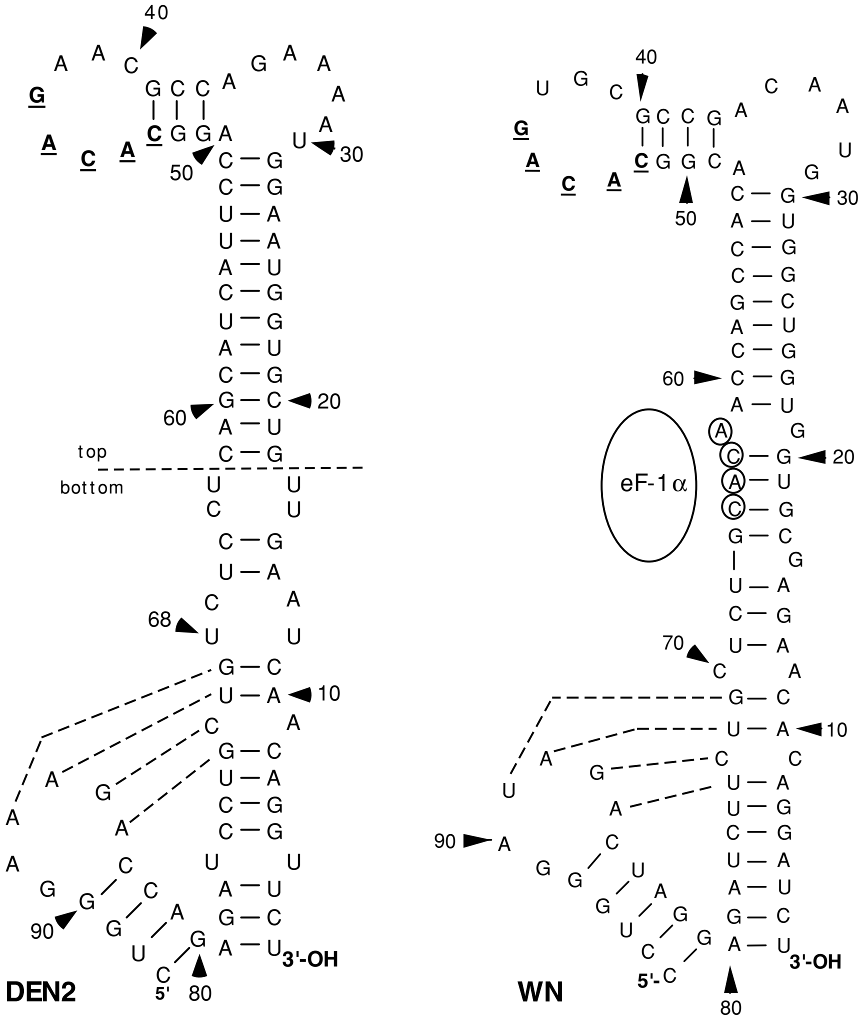

FIG 6. Nucleotide sequences of 3′-SLs in WN strain E101 and DEN2 strain NGC genomes (Zeng *et al.*, 1998) are shown in the expected conformation and numbered from the 3′ end of each genome. The flavivirus-conserved pentanucleotide sequence 5′-CACAG-3′ is indicated in bold underlined type. The "top" and "bottom" portion of the DEN2 3′-SL as designated in a previous study with respect to the WN 3′-SL is shown by a labeled horizontal dashed line (Zeng *et al.*, 1998). Essential nucleotides of the putative binding site for the translation elongation factor, eF-1α, to the WN 3′-SL are circled (Blackwell and Brinton, 1997). Nucleotides potentially involved in pseudoknot formation in both DEN2 and WN 3′-SLs are linked by dashed lines (Shi *et al.*, 1996a).

viral proteins to the conserved 3′ stem and loop structure (see later) during RNA replication (Khromykh *et al.*, 2001).

2. CS1

The next upstream conserved sequence in mosquito-borne flavivirus RNA is CS1, which has already been mentioned earlier in describing localization of the CYC sequence (Figs. 2, 4, and 5). The CS1 sequence is not present in 3′ NCRs of tick-borne flavivirus genomes, although, as will be shown, the characteristic 3′ cyclization sequence(s) in those genomes also occurs in the context of a larger conserved sequence motif (Wallner *et al.*, 1995). CS1, as originally described by Hahn and colleagues (1987), consisted of a 25 nucleotide segment in the MVE 3′ NCR that was nearly but not totally conserved among viruses of the same subgroup and between subgroups. For example, CS1 for the MVE genome differs from that of the WN genome by one nucleotide substitution and the deletion of a single nucleotide, whereas CS1 in the JE virus genome differs from that of MVE by only a single nucleotide substitution. The sequence in the MVE genome differs from that found in the DEN1 and DEN3 genomes in length in that the DEN sequences appear to contain two discrete single nucleotide deletions and two discrete single nucleotide A to G substitution mutations with respect to the reference. DEN2 and DEN4 genomes contain the identical deletion mutations but only one of the two substitution mutations with respect to the MVE sequence. The CS1 sequence in the YF virus genome contains three substitution mutations and one deletion with respect to the MVE sequence. The 8-nucleotide CYC sequence is contained in the upstream half of CS1, between nucleotides –95 and –102 in DEN genomes. The CYC sequence itself is completely conserved among these and other mosquito-borne virus genomes.

3. CS2 and RCS2 and Analogous Upstream Tandem Repeat Sequences

The conserved tandem repeat sequences CS2 and RCS2 were also initially described by Hahn and co-workers (1987). CS2 and RCS2 are not found in 3′ NCRs of tick-borne flavivirus genomes. For mosquito-borne viruses, CS2 is located closest to the 3′ terminus of the genome, 12 to 22 nucleotides upstream from CS1 in the DEN, YF, and JE subgroup genomes (Figs. 2, 4, and 5). For the Genbank genome sequences of individual strains surveyed, the 20 nucleotide sequence of CS2 is completely conserved for viruses in the JE subgroup (MVE, WN, and KUN) and among all four serotypes of DEN viruses, except for a single internal nucleotide substitution (G to U) found in the DEN3 genome. The CS2 sequence in the YF 17D genome (Rice *et al.*, 1985) deviated from that

seen in genomes of viruses of the JE subgroup by two nucleotide substitutions, resulting in A to U and C to U changes in the sequence.

In the MVE genome, and similarly in genomes of the other JE subgroup viruses, RCS2 is 51 nucleotides upstream from the 5′ terminus of CS2 and represents an exact duplication of the 20-nucleotide sequence of CS2. In the DEN genome sequences, RCS2 is located 62 (for DEN1) to 68 nucleotides (for DEN4) upstream from CS2 (see Fig. 4). CS2 is not repeated upstream in the YF genome. Instead, the YF genome contains three unique tandem repeats at the 5′ terminus of its 3′ NCR. The first of these repeats is 40 nucleotides long in the YF strain 17D genome, and it initiates downstream from the third of three in-frame stop codons found at the 3′ terminus of the YF 17D ORF (Figs. 2 and 5). The second and third iterations of the repeat are 44 nucleotides in length. Save for the fact that the second repeat is 4 nucleotides longer than the first, there are only 5 nucleotides different between the two segments. Similarly, the third repeat differs from the first by only 4 nucleotides in 40. The second repeat initiates 4 nucleotides downstream from the 3′ terminus of the first one, and the third repeat in turn initiates 6 nucleotides downstream from the 3′ terminus of the second.

Not all YF virus genomes contain an analogous set of three tandem repeat sequences, as found in vaccine strain 17D. A study of partial nucleotide sequence data for genomes of 13 YF field isolates (Wang *et al.*, 1996) revealed that only the West African strain genomes contained a 511-nucleotide 3′ NCR, including three tandem repeat sequences. Genomes of strains from central and east Africa and from South America had shorter 3′ NCRs (443 to 469 nucleotides) due to the lower number of YF-specific repeat sequences. Central and east African strains had only two YF-specific repeats, and those from South America had only one copy of the “repeat” sequence. On the basis of overall nucleotide sequence relatedness, it was speculated that duplication of the repeated sequence took place in west Africa after introduction of the YF virus into South America. The resulting variation in lengths of 3′ NCRs of the genomes of YF virus isolates, due to duplication and/or deletion of redundant nucleotide sequences within the 5′ proximal portion of the 3′ NCR, is reminiscent of that observed among the genomes of different strains of TBE virus (see later).

The JE subgroup viruses have an additional pair of repeats upstream from CS2 and RCS2 in the 3′NCR (CS3 and RCS3 in Figs. 2 and 4). For the MVE genome (Hahn *et al.*, 1987), these sequences are each 28 nucleotides in length and are situated 120 nucleotides apart in the MVE 3′NCR. The most upstream of these repeat segments has its 5′ terminus 153 nucleotides downstream from the stop codon in the

MVE ORF. There are only three nucleotides different between the sequences of CS3 and RCS3 in the MVE 3′ NCR.

4. Function of Conserved Tandem Repeat Sequences in Mosquito-Borne Flavivirus Replication

Relatively little is known regarding this subject. Perhaps more work will be done in this area in the future, as the use of full-length infectious DNAs to effect site-directed mutagenesis of the flavivirus genome has become more common. However, it is clear that deletion of conserved repeat sequences alters virus growth properties. As mentioned previously, Men and co-workers (1996) created a series of eight different mutant dengue genomic RNAs derived from DEN4-infectious DNAs containing internal deletions centered about a unique *Apa*I restriction endonuclease site located at nucleotide –171 in the DEN4 genome, about midway between CS2 and RCS2 (Figs. 2 and 4). Thus, all or portions of CS2 and/or RCS2 were deleted in six of the resulting seven viable deletion mutant viruses. (The eighth mutant RNA contained a deletion of CS1, which proved to be lethal, as discussed previously.) The maximum 3′ boundary of any deletion that gave rise to virus was 113 nucleotides from the 3′ end of the genome, preserving CS1 and the 3′ stem and loop (SL). One DEN4 mutant containing a 121-nucleotide deletion that included all of RCS2 and most of the upstream portion of the 3′ NCR (mutant 3′d303-183) was only slightly impaired for replication in cultured monkey kidney cells compared to wild-type virus. In contrast, a mutant containing a 60-nucleotide deletion that included almost all of CS2 (mutant 3′d172-113) was reduced in peak titer by about 10-fold with respect to wild type in both experiments. This suggested that CS2 may be more critical for viability than RCS2. Interestingly, a mutant that contained a deletion of only 30 nucleotides (3′d172-143) between CS2 and RCS2, but sparing both conserved sequences, was more impaired for replication than either mutant 3′d172-113 or 3′d303-183 in that its peak titers in the two assays were reduced compared to wild type by about 100-fold. This indicates that nonconserved nucleotides between CS2 and RCS2 are important for virus viability, possibly because of a requirement for spacing between the two conserved sequences or possibly because this deletion removed a DEN and JE subgroup conserved SL structure (TL2) predicted by Proutski and colleagues (1997b). Mutant virus 3′d172-143 is currently under study as a potential DEN vaccine candidate (Durbin *et al.*, 2001).

The DEN4 genome could tolerate deletion of more than half its 3′ NCR, provided the deleted sequences were upstream from nucleotide

–113, without complete loss of viability. A deletion of 202 nucleotides, extending from the stop codon (at nucleotide –384) to nucleotide –183 (3′d384-183) produced a viable virus that formed plaques in monkey cells and was only a little more reduced in peak titer compared to the vaccine candidate, mutant 3′d172-143, in the two growth assays, and a 262-nucleotide deletion of all but 10 nucleotides between the stop codon and nucleotide –113 produced viable virus after transfection of mosquito cells. However, this virus did not form plaques in monkey cells.

Khromykh and Westaway (1997) addressed the requirement for CS3 and RCS3, found in the JE subgroup virus genomes, using the KUN replicon system. They started with replicon ΔME, which was stable and replicated efficiently *in vivo*. A 76-nucleotide deletion (KUN nucleotides 10,422 to 10,499) was created in ΔME DNA between the stop codon and the upstream boundary of RCS3 (Fig. 2) to create mutant ΔME/76 RNA. A second mutant RNA derived from ΔME DNA, ΔME/352, contained a 352-nucleotide deletion of KUN nucleotides 10,422 to 10,775, which included all of RCS3 and CS3, nonconserved sequences between these two boxes, and additional nucleotides extending upstream to overlap the upstream boundary of mutant ΔME/76 RNA. Whereas ΔME/76 RNA replicated efficiently in BHK cells, comparable in level to that of the "wild-type" ΔME replicon RNA, only very low-level replication of ΔME/352 RNA was detected above the threshold of sensitivity of the assay. Unfortunately, data are lacking relative to the phenotype of either deletion mutation in the context of a replication-competent KUN virus genome. Proutski and colleagues (1997b) offered the hypothesis that the phenotype of mutant ΔME/352 RNA was related to the deletion of nonconserved nucleotides that form a conserved secondary structure in all JE subgroup virus genomes.

D. Conserved Linear Sequence Features of 3′ NCRs of the TBE Subgroup of Tick-Borne Flaviviruses

1. The Pentanucleotide

The pentanucleotide 5′-CACAG-3′ is the only linear nucleotide sequence that is conserved in both mosquito- and tick-borne flavivirus genomes (see earlier discussion).

2. Features Unique to TBE Viruses

The absolute lengths of the 3′ NCRs in TBE virus genomes vary much more than those of mosquito-borne viruses and even compared to those of other tick-borne viruses, such as Powassan virus

(Table I). For example, the 3′ NCR in TBE strain Neudorfl virus is 764 nucleotides in length (Table I), whereas that of TBE strain RK 1424 virus is only 350 nucleotides in length (Fig. 3; Wallner *et al.*, 1995). Accumulated nucleotide sequence data also show that this variability is due to differences in length of the upstream portion of the 3′ NCR; the most 3′-terminal 325 nucleotides are conserved in all strains and share a high degree of sequence identity. Wallner and co-workers (1995) referred to the conserved 3′-terminal segment as the "core element" of the 3′ NCR and designated any upstream sequences as part of a "variable region." Their findings were remarkable in that the viruses under study are all strain variants of a single serotype. In contrast, the 3′ NCRs of the four serotypes of DEN viruses, for example, are nevertheless highly conserved with respect to both length and sequence content, perhaps because the DEN 3′ NCR is minimal in the functional sequence content required for a viable flavivirus (Fig. 2). Thus the DEN 3′ NCR may be analogous to the core element of the 3′ NCR in TBE virus genomes. A comparison of the conserved sequences in 3′ NCRs of TBE virus genomes to those of the Powassan virus genome (Mandl *et al.*, 1993) revealed that the latter genome has conserved features similar to TBE strain Neudorfl genome (to be described), except that the poly(A) tract found in strain Neudorfl and other TBE genomes is not present in the Powassan virus genome.

The core element of the TBE 3′ NCR appears to be sufficient for a viable TBE virus, as the 3′ NCR of strain RK1424 virus is virtually devoid of a variable region (Fig. 3). Essential elements of the core are as follows. (1) The approximately 100 3′-terminal nucleotides of the core region are predicted to form the flavivirus-conserved SL structure, which has been mentioned previously and which is discussed in greater detail later. Among the TBE virus genomes, the nucleotide sequence within the 3′ stem and loop varies only in segments that are not required for base pairing to form the stem region of the secondary structure, with the exception of the conserved CACAG pentanucleotide box. (2) There is a short inverted repeat sequence (IR in Fig. 3) about 150 nucleotides upstream from the 3′ terminus. The sequence has a very high GC content and a correspondingly high predicted thermal stability of ~19 kcal. Therefore, Wallner and coworkers (1995) suggested that there is a high probability that the IR actually forms a hairpin structure *in vivo*. (3) Adjacent to the IR sequence and overlapping it by three nucleotides is a 20-nucleotide stretch of purine residues, extending toward the 3′ terminus of the RNA. This is the longest homopurine (PU) sequence within the entire genome of the TBE strain Neudorfl virus, apart from the poly(A) tract

in the variable region of the 3′ NCR. (4) A few nucleotides further downstream from the PU sequence is a 14-nucleotide homopyrimidine (PY) sequence. (5) Thirty nucleotides still further downstream from the PY box exists a second conserved 14-nucleotide pyrmidine-rich (PR) sequence (consisting of 13 pyrimidine residues and one purine residue). The 3′ cyclization sequence, C1′, lies within the PR box. The PU, PY, and PR boxes and of course the sequence of C1′, among other features of the TBE 3′ NCR, are semiconserved in the Powassan virus genome (Mandl *et al*., 1993), suggesting their fundamental importance in replication. Homopurine and -pyrimidine stretches have been found to function in processes related to the regulation of translation (Avni *et al*., 1994; Behe, 1995), stabilization of RNA (Czyzyk-Krzeska *et al*., 1994), or in (retrovirus) RNA replication (Hungnes *et al*., 1992). It is perhaps noteworthy that the PU, PY, and PR boxes are found at loci in the genome analogous to those occupied by CS2 and CS1 in the mosquito-borne virus genomes, although there is no significant sequence homology between TBE or Powassan virus-specific domains and those of mosquito-borne viruses.

The remainder of the core region in TBE virus 3′ NCRs contains one copy of sequence R3. This is a 76-nucleotide segment. The 3′ terminus of R3 occurs 45 nucleotides upstream from IR. R3 is a tandem repeat sequence in the genomes of virus strains Neudorfl, 263, Ljubljana I, and Aina, where the repeat is found upstream from the core element in the variable region of the respective 3′ NCRs. However, R3 is not repeated in the variable regions of the genomes of virus strains Hypr, Crimea, and RK 1424 (Fig. 3).

The variable region contains additional tandem repeat sequences. R1 is a 26-nucleotide segment that constitutes the 3′ terminus of the ORF, including the stop codon. Well-conserved R1 repeat sequences flank the poly(A) tracts in the 3′ NCRs of strains Neudorfl, 263, and Ljubljana I genomes. R1′ is a 26-nucleotide semiconserved repeat of the R1 sequence that is found 14 nucleotides downstream from the stop codon at the 3′ terminus of the ORF. R1′ contains an additional UAA stop codon that is in-frame with the one designated to be functional at the 3′ terminus of the most upstream R1 segment. R1′ was identified in the genomes of virus strains Neudorfl, 263, Ljubljana, I, Aina, Hypr, and Crimea but not in those of strains 132 and RK 1424. The designation “R2” identifies a conserved 26-nucleotide sequence upstream from R1 in the 3′ terminus of the ORF. R2 is repeated once in the variable region of the 3′ NCR, just downstream from R3 in the genomes of virus strains Neudorfl, 263, Ljubljana I, and Aina. An R2 repeat sequence was also identified in an analogous location in the 3′ NCRs of virus

strains Hypr and Crimea, despite the lack of an R3 repeat sequence in the variable region of the 3′ NCRs in those genomes.

The poly(A) tract in the variable region of the 3′ NCR in the TBE virus genome was first described for strain Neudorfl virus (Mandl *et al.*, 1991) and was believed to constitute the 3′ terminus of the entire genome. It was later shown to be an internal part of an otherwise more conventional 3′ NCR sequence in the strain Neudorfl genome, and similar tracts were also identified in the genomes of strains 263 and 132 (Wallner *et al.*, 1995). The strain Ljubljana I genome contains an oligo(A) tract at the analogous locus in its 3′ NCR, the sequence $(A)_4C(A)_6$ (Fig. 3). The poly(A) tracts in the strain Neudorfl genome appeared to be heterogeneous in length, ranging from 30 to 250 nucleotides, but the degree of heterogeneity was difficult to assess due to the possibility that some of it was created artifactually either by stuttering of reverse transcriptase and/or *Taq* polymerase used in generating sequence data. A full-length cDNA copy of the strain Neudorfl genome containing a poly(A) tract only 49 nucleotides in length produced infectious RNA *in vitro* (Mandl *et al.*, 1997).

Wallner and co-workers (1995) made an effort to define a mechanism for the evolution of the diverse lengths of the 3′ NCRs in these closely related TBE virus strains. They attempted to relate their findings (1) to other strain-specific parameters, such as the year, geographic origin, or source of the virus isolate, (2) to a dendrogram showing evolutionary relationships among the strains, derived by comparing sequence data for a 375-nucleotide segment of the NS5 gene, and (3) to a second dendrogram derived by comparing sequence data for the core elements of the various 3′ NCRs. These analyses failed to yield any workable hypothesis to explain their observations. However, the close agreement between the dendrogram obtained by comparing the sequences of the NS5 coding regions to that obtained by comparing those of the core elements suggested that the sequences evolved in parallel. This implied that the heterogeneity in length of the variable regions did not evolve by intragenic recombination. Additional consideration of the details of the results of the sequence analysis led to the conclusion that the observed diversity in lengths of the 3′ NCRs in these genomes was most likely due to deletion events and that therefore the viruses were likely to have evolved from a common ancestor with a long 3′ NCR, such as those found in the genomes of strains Neudorfl and 263. Furthermore, these “long” 3′ NCRs were thought to have arisen from an ancestral virus with a “short” 3′ NCR by duplication events, as the variable region consists in a large part of direct repeats of sequence elements found in the core element and in the ORF.

Other unique sequences in the variable region may have arisen by intragenic recombination over short segments within subdomains.

Size variation of the 3′ NCRs among closely related strains of other positive-strand RNA viruses has also been observed. (1) Among alphaviruses the 3′ NCR in the genome of an avirulent variant of Semliki Forest virus was 334 nucleotides longer than that of the wild-type variant. The additional sequences consisted largely of tandem repeats (Santagati *et al.*, 1994). (2) The 3′ NCRs of pestiviruses are also known to contain conserved and variable regions. One strain of bovine viral diarrhea virus was found to have a 41-nucleotide deletion in the variable region of its 3′ NCR as compared to two other strains of this virus (Deng and Brock, 1993). There was at least a loose inverse correlation of the variation in length of the 3′ NCRs in TBE strains to their virulence; strains Neudorfl and 263 were less virulent in mice than strain Hypr (which had a relatively short 3′ NCR). However, strain Ljubljana caused severe disease in humans, despite having a genome with a "long" 3′ NCR (Fig. 3).

Subsequently, Mandl and co-workers (1998) investigated the spontaneous occurrence of deletions in the 3′ NCR of strain Neudorfl viruses that had been passaged many times in cultured hamster kidney (BHK) cells or of infectious DNA-derived strain Neudorfl non–3′ NCR mutant viruses that had been passaged in both BHK and suckling or adult mouse brain. They determined the 3′ NCR nucleotide sequence for 14 mutant viruses thus derived. The frequency with which 3′ NCR deletions arose appeared to correlate best with the initial number of passages of virus in BHK cells. The spontaneous internal deletions that occurred in the 3′ NCR had the following characteristics. (1) All deletions affected the variable region exclusively. (2) The deletions affected any and all sequence elements of the variable region [from nucleotide 10,394 to nucleotide 10,811, which is 15 nucleotides downstream from the *Age*I restriction endonuclease site (at nucleotide 10,796) that defines the boundary between the core element and the variable region (Fig. 3)]. (3) The poly(A) tract was removed in all deletion events, although short homoadenosine sequences were often retained. (4) There was some tendency for the deletions to cluster around certain nucleotides. The three largest deletions detected in this study were all of more than 300 nucleotides, and all of these resulted in the removal of the R2 repeat adjacent to the *Age*I site, the upstream R3 repeat sequence, the intervening heterogeneous sequence between R3 and the nearest R1 repeat, all of the poly(A) tract, and all or most of the R1 repeat that flanks the poly(A) tract at its upstream boundary.

These same investigators (Mandl *et al*., 1998) also created six 3′ NCR deletion mutants in the context of the strain Neudorfl infectious DNA. The smallest of these mutations deleted all of the variable region, from the *Age*I site to the stop codon in the ORF (Fig. 3). The remaining five larger deletions also initiated on their upstream ends at the stop codon and extended to varying lengths on the downstream end into the core element. The longest deletion included all of the 3′ NCR from the stop codon to the PU tract. All of these deletion mutant DNAs gave rise to viable mutant viruses, except for the longest of the deletions, just described (which included in its extent both potential 3′ cyclization sequences, C1′ and C2′, and the conserved IR sequence). Among the viable mutant viruses, only the one with the largest deletion, extending from the stop codon to the downstream end of R3 in the core element, was reduced in peak titer compared to all other wild-type and mutant viruses by about 100-fold in a growth curve. This result was in contrast to that observed for internal deletion mutations of the 3′ NCR in the DEN4 virus genome, where all deletion mutants had, to varying degrees, a reduced capacity for replication in tissue culture. However, it could be argued that the short DEN genome 3′ NCRs are already functionally similar to the TBE core element, such that deletion mutations invariably remove sequences required for fully efficient replication.

The viable mutant TBE viruses were all comparable to wild type in lethality after intracerebral infection of suckling mice, although there was a correlation between the sizes of the deletions and the survival times after infection. However, there were marked differences in lethality of the viruses for 5-week-old mice after subcutaneous inoculation. This difference in virulence was directly correlated with the size of the deletion in the 3′ NCR, such that the mutant virus with the largest deletion was more than 10,000-fold less virulent than wild-type strain Neudorf1. In contrast, the virus containing a deletion limited only to the entire variable region of the wild-type strain Neudorfl 3′ NCR was indistinguishable from its parent in each of these assays. Thus the attenuation phenotype could be attributed to deletions of portions of the core element only. The variable region did not appear to have any relevant function for the growth of TBE virus in cell culture or in mice, as demonstrated by the occurrence of spontaneous deletions that affected almost all parts of this segment of the 3′ NCR. Additional support for this conclusion arose from the finding that the engineered strain Neudorfl deletion mutant virus that contained a deletion of the entire variable region exhibited no change in its biology compared to the wild-type strain Neudorfl virus. These results also raised the

possibility that TBE strains with "short" 3′ NCRs had undergone spontaneous deletion mutations during the processes of virus isolation and normal additional passaging in tissue culture.

It remains unclear why the variable region sequences exist and persist in naturally occurring TBE isolates. There may be some adaptive advantage associated with retention of the variable region, related perhaps to replication in ticks. Mandl and co-workers (1998) noted that the core element of the TBE strain Neudorfl 3′ NCR contains 10 predicted SL secondary structures (Gritsun *et al.*, 1997; Rauscher *et al.*, 1997; see later), including the IR sequence and the 3′-SL. They suggested that differences in virulence among mutant viruses might be related to the effects of deletions in altering or abrogating the more upstream of these conserved secondary structures.

Similar observations regarding the requirement for the core element in tick-borne virus genomes were made by Pletnev (2001). Four internal deletions were created in the 3′ NCR of an infectious DNA for an egg passage-attenuated Langat virus, a tick-borne virus highly antigenically related to TBE viruses. These all had their upstream boundaries at the stop codon for the ORF and extended downstream 320, 374, 449, and 471 nucleotides, respectively. All of these deletions eliminated short upstream portions of the core element, and only the 320 nucleotide deletion yielded viable virus when deletion mutant RNA was transfected into susceptible cells. Thus deletion of as little as 54 additional nucleotides of the core element (the difference between the 320-nucleotide deletion and the 374-nucleotide deletion) was lethal, even though the 3′ SL, C1′, C2′, and other more downstream features of the TBE 3′ NCR (Fig. 3) were intact in the 374-nucleotide deletion mutant. The viable 3′ NCR deletion mutant virus was more attenuated in mice than its already attenuated parent virus.

III. Secondary Structure of NCRs

A. *Secondary Structure of the 5′ NCR*

Brinton and Dispoto (1988) determined the nucleotide sequences of the 5′ NCRs of WN virus and of seven different isolates of the mosquito-borne flavivirus, St. Louis encephalitis (SLE) virus. They then compared the sequences to those of YF, MVE, and DEN viruses. While only short regions within the 5′ NCRs were conserved among different subgroups, significant sequence homology was observed among viruses of the same subgroup, and an almost complete conservation of nucleotide sequence was observed among different strains of the same

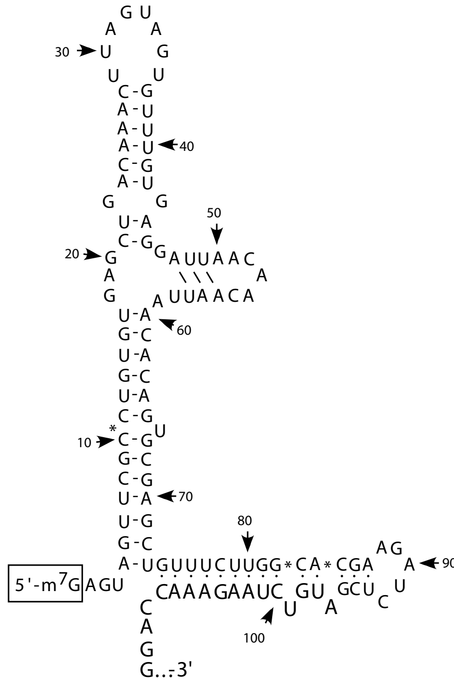

Fig 7. Proposed secondary structure of the WN 5′ NCR (adapted from Brinton and Dispoto, 1988). Nucleotides are numbered from the 5′ terminus, excluding the cap, which is shown boxed. Nucleotides of the ORF, starting with nucleotide number 96, are shown in large, bold type (adapted from Brinton and Dispoto, 1988).

virus, i.e., the seven isolates of SLE virus and two different strains of WN virus. For each 5′ NCR sequence, secondary structures of similar size, shape, and predicted thermodynamic stability could be formed. In each case, the structures consisted of a stem with a small top loop and a larger side loop (Fig. 7). In most cases, a second short stem could be formed by 3′-terminal sequences of the 5′ NCR, including the predicted start codon for the long ORF and downstream nucleotides in the ORF. The predicted thermal stability of the long stem structures ranged from −17.3 kcal (for the SLE sequence) to −27.8 kcal (for the WN strain E101 sequence, as shown). The conservation of this secondary structure in unrelated flavivirus genomes was taken as evidence of its possible importance in replication.

The relative importance of the predicted double-stranded regions in the 5′ NCR was studied by deletion mutagenesis of an infectious DNA

copy of the DEN4 strain 814669 genome (Cahour *et al.*, 1995). The linear sequence of the 5′ NCR is partially conserved between DEN and WN species (Fig. 7). For example, nucleotides 1–7, 11–16, 21–25, 55–60, and 69–77, numbering from the 5′ terminus of the WN strain E101 sequence, are present in the 5′ NCRs of both the DEN4 strain 814669 and the WN strain E101 genomes. Conservation of sequence and predicted secondary structure are much higher when any two DEN 5′ NCRs are compared. Nine distinct deletions of from 5 to 12 nucleotides each were created between nucleotides 50 and 87 of the DEN4 sequence in the context of a DEN4 infectious DNA. In addition, two deletions of 5 and 26 nucleotides, respectively, were created downstream (involving nucleotides 94 to 98; d94-98) and upstream (d18-43) from the central targeted domain. Nucleotide 98 is four nucleotides upstream from the start codon of the DEN4 ORF. Mutants d55-60, d62-68, d69-75, and d82-87 contained deletions of nucleotides that were base paired in the long stem region of the 5′ NCR, whereas mutants d50-54, d58-64, d76-81, d73-77, d76-87, and d94-98 contained deletions in the shorter stem or non–base-paired regions within the long stem. Mutant d18-43 contained a deletion of the upper loop and the adjacent upper portion of the long stem, as shown for the WN 5′ NCR in Fig. 7. Five mutations were deemed lethal, in that infectious virus could not be recovered after transfection of cultured monkey kidney cells with RNA derived *in vitro* by the transcription of mutagenized DNAs. These were mutants d55-60, d58-64, d62-68, d69-75, and d94-98. Nucleotides 55 to 75 in the DEN4 genome are analogous to nucleotides 60 to 80 in the WN 5′ NCR secondary structure shown in Fig. 7. In general, failure of the four contiguous deletion mutations (spanning DEN4 nucleotides 55 to 75) to yield viable viruses was ascribed to a requirement for those nucleotides for formation of the base pairs that constitute the bottommost portion of the long stem and the short stem, which also requires nucleotides in the ORF for its formation.

The viable mutant viruses derived in this study were distinguishable from each other by plaque size in cultured mosquito and monkey cells, growth curves on these two cell types, relative levels of RNA in infected cells as assessed by dot-blot hybridization, and translation efficiency of the ORF in an *in vitro* system. Mutant d82-87 had a host range-restricted phenotype in that it uniquely failed to replicate in mosquito cells and in adult mosquitoes, apparently due to a defect in RNA synthesis. Translation of a portion of the DEN4 ORF containing the d82-87 5′ NCR was also least efficient among all the mutants tested. However, the cell-free translation assay was not predictive of the viability of mutant viruses; RNAs containing some of the

lethal deletion mutations, e.g., d55-60, exhibited enhanced activity in translation compared to wild-type control RNA. Therefore, the dominant effect of deletion mutations on the viability of mutant viruses appeared to be at the level of RNA synthesis. The host range-restricted phenotype of mutant d82-87 also suggested that binding to RNA of host-specific factors, presumably cellular proteins, was a secondary determinant of the viability of mutant viruses.

Shi and co-workers (1996a) performed a computer-based analysis of the 3′ negative-sense (−) RNA predicted to be synthesized in the process of replication of the 5′ NCR of WN strain E101 virus. The presence of an SL secondary structure in 3′ (−) NCR RNA, formed by the sequence complementary to the SL predicted to occur in the 5′ NCR, was subsequently confirmed by spectroscopy, analysis of thermal melting curves, and selective RNase digestion of an *in vitro*-transcribed 75-nucleotide RNA representing the SL within the 96 nucleotide 3′ NCR of the WN-negative strand. The 75-nucleotide transcript was then shown to bind specifically to four discrete proteins in three RNA–protein complexes in lysates of BHK cells and in lysates of embryonic fibroblasts derived from C3H/RV and C3H/He mice. C3H/RV mice are homozygous for the flavivirus resistance gene *Flv*, whereas C3H/He mice are susceptible to flavivirus infection (Groschel and Koprowski, 1965). Resistance of mice to the pathologic effects of encephalitic flavivirus infection had been shown previously to relate to reduced production of progeny plus strand RNAs at the cellular level. Because RNA–protein complexes formed in lysates of C3H/RV cells were indistinguishable in size from those formed in C3H/He cells (and in BHK cells), an investigation was conducted to determine whether the complexes between C3H/RV proteins and WN 3′ (−) SL RNA were different in stability from those formed between C3H/He proteins and WN 3′ (−) SL RNA. Complexes 1 and 3 formed in lysates of C3H/RV cells were shown to have a significantly longer half-life than the analogous complexes formed in C3H/He cell lysates.

B. The 3′ SL

Analysis of the YF genome sequence originally revealed the presence of a potential SL structure formed by the 3′-terminal 87 nucleotides (Rice *et al.*, 1985). Similar stem–loop secondary structures with similar predicted thermal stability have been subsequently proposed for the 3′ termini of all flavivirus genomes for which nucleotide sequence data are available (see, e.g., Brinton *et al.*, 1986; Hahn *et al.*, 1987; Irie *et al.*, 1989; Wengler and Castle, 1986). In fact, similar structures are

predicted to form at the 3′ termini of the genomes of the other Flaviviridae, the hepaci- and pestiviruses (Blight and Rice, 1997; Deng and Brock, 1993). The stem consists of about 30 hydrogen-bonded base pairs and is variably interrupted by bulges due to the predicted apposition of nucleotides that are unable to hydrogen bond. Sequence analysis also predicted a smaller adjacent stem–loop structure that is a consistent feature of the flavivirus 3′ NCR, upstream from the long stem–loop. Together the two adjacent structures involve 90 to 100 nucleotides at the 3′ end of the genome and are referred to collectively, for purposes of this discussion, as the 3′ SL (Fig. 6). The 3′ SL has a predicted thermal stability of −40 to −45.2 kcal, largely due to the free energy state of the long stem region.

The nucleotide sequence of the 3′ SL is only partially conserved among unrelated flavivirus species (e.g., Fig. 6), although conserved segments do exist. For example, as already noted, the distal loop region of the long stem contains the flavivirus-conserved CACAG box, and in general there is a similarity among the nucleotide sequences of the predicted loop regions, both the double loop that terminates the long stem and in the single loop atop the adjacent short stem (the sequence 5′ GANAGA-3′) (Shi *et al.*, 1996b), nucleotides –83 to –89 in the DEN2 3′ SL and nucleotides –85 to –91 in the WN 3′ SL. In addition, there is similarity among flavivirus genomes at nucleotides –1 to –6, which includes the 3′ terminal 3′ UC dinucleotide that is conserved among all known flavivirus genome sequences except that of the CFA virus. For example, Fig. 6 shows the similarity in sequence between 3′ termini of the DEN genomes (the sequence 3′-UCUUGG-5′, conserved in all four DEN serotypes) compared to the 3′ terminus of the WN genome (the sequence 3′-UCUAGG-5′, conserved in KUN, JE, and MVE genomes). Upstream from these six nucleotides, forming one side of the lower half of the long stem, one finds the semiconserved pentanucleotide ACACA, which has been discussed previously. The 3′ SL nucleotide sequence is best conserved among the closely related members of the same subgroup or clade, for example, among the four DEN serotypes and among species assigned to the JE subgroup.

Physical and biochemical evidence for the existence of the 3′-terminal long stem and loop in WN virus RNA was provided by Brinton and co-workers (1986). In their efforts to determine the nucleotide sequence of the genome at its 3′ end, they subjected virus RNA to digestion with five different ribonucleases. Comparison of the gel mobility of the end products of these reactions to that of a sample of RNA that was sequenced by selective chemical degradation revealed that most of the

3′-terminal 84 nucleotides were relatively resistant to one or more of the RNases. This suggested that the nucleotides in question were involved in a secondary partially double-stranded structure that conferred the observed resistance to degradation. Drawing on the previous prediction of a 3′-terminal SL structure in the YF virus genome (Rice *et al.*, 1985), these workers hypothesized that the last 84 nucleotides of the WN genome were involved in a similar motif. WN virus RNA did not exhibit significant resistance to RNase digestion upstream from nucleotide –84.

Further analysis of interactions among nucleotides of the 3′ SL suggested the existence of a pseudoknot tertiary structure (Shi *et al.*, 1996a), potentially formed by hydrogen bonding between nucleotides comprising one strand of the long stem (nucleotides –71 to –74 in the WN 3′-SL; Fig. 6) and nucleotides contained in the loop structure atop the adjacent short stem (nucleotides -86 to -89 in the WN 3′ SL). In order for the pseudoknot to form in the 3′ SL for most flavivirus species, one must posit the occurrence of G-A and G-U base pairs in some genomes (see DEN2 3′-SL; Fig. 6). Such atypical hydrogen bonding has been described for other RNA species (Heus and Pardi, 1991; Wimberly *et al.*, 1993), and pseudoknots have been described at the 3′-ends of other viral genomes (Dreher and Hall, 1988; Jacobson *et al.*, 1993; Pleij *et al.*, 1985). Replication of phage QB RNA was shown to be dependent on interaction of the replication complex with a pseudoknot formed by internal base pairing of the 3′ terminus of the genome to a single-strand RNA segment 1200 nucleotides upstream (Klovins and van Duin., 1999).

WN and DEN3 3′ model RNAs that included the lower half of the long stem and the upstream adjacent small stem and loop (nucleotides –1 to –11 linked covalently via four U residues to nucleotides –71 to –96, for the WN sequence; Fig. 6) were synthesized *in vitro* (Shi *et al.*, 1996a). Results from RNase probing, circular dichroism spectral analysis, UV-melting experiments, mutagenesis of the RNA sequence, and molecular modeling were consistent with the existence of the predicted pseudoknot in each of the two RNAs that were studied. A consequence of this prediction was that the RNA in a pseudoknot was predicted to form a cleft that could be functional in protein or metal binding. A potential weakness of this work, borne of necessity, was the fact that it was conducted using *in vitro*–transcribed RNA representing a small, discontinuous segment of the 3′ NCR. Thus, possible interactions between and among the segments that were studied and more upstream sequences in the genome could not be assessed.

1. Binding of Cellular Proteins to the 3′ SL

Because it likely contains RdRP promoter elements, the 3′ SL is likely to interact with both cellular and viral proteins required for viral RNA replication, which must initiate with the synthesis of full-length minus-strand RNAs. The host range of most flaviviruses, both in nature and in the laboratory in tissue culture, is very broad. For example, flaviviruses can replicate efficiently in mammalian, avian, insect, reptile, and amphibian hosts, as well as in cell cultures from these hosts (Brinton, 1986). Therfore, one might expect that host cell proteins required for flavivirus replication are highly conserved or that domains required for this function may be conserved within a variety of multifunctional proteins of disparate size in different cell types. Cell proteins that bind 5′ and/or 3′ NCRs of genomes of several different RNA viruses have been described, including those of mouse hepatitis virus (Furuya and Lai, 1993), bovine coronavirus (Spagnolo and Hogue, 2000), measles virus (Leopardi *et al*., 1993), Rubella virus (Nakhasi *et al*., 1990, 1991), Sindbis virus (Pardigon *et al*., 1993), hepatitis A virus (Schultz *et al*., 1996; Yi *et al*., 2000), and hepatitis C virus (Luo, 1999; Spangberg and Schwartz, 1999). In many cases, the cited reports refer to binding of cellular translation factors to IRES elements found in the 5′ NCRs of hepaciviruses and picornaviruses, among others, but not present in the flavivirus 5′ NCR. For further information, the reader is referred to reviews of this subject with emphasis on positive-strand RNA viruses in this volume (Brinton, 2001; Strauss and Strauss, 2002).

In general, the literature on binding of proteins to 3′ NCRs of positive-strand RNA virus genomes does not shed light on the functional requirements associated with protein binding. However, two reports [for mouse hepatitis virus RNA (Huang and Lai, 2001) and poliovirus RNA (Herold and Andino, 2001) suggest that cellular proteins may be involved in linkage of 5′ and 3′ termini of replicating RNA virus genomes through the formation of ribonucleoprotein (RNP) complexes. For poliovirus, formation of this RNP was required for the initiation of negative-strand RNA synthesis.

Cell proteins required for flavivirus replication have not yet been identified with certainty. However, the specific interaction of a radiolabeled RNA representing the nucleotide sequence of the WN 3′ SL with proteins in extracts of uninfected BHK cells was reported by Blackwell and Brinton (1995). (BHK cells are a common laboratory cell substrate for growth of the WN virus to very high titers.) Gel mobility shift assays, UV cross-linking, Northwestern blotting, and ion-exchange chromatography were initially used to identify 56-, 84-, and 105-kDa

proteins that bound to a truncated *in vitro*–synthesized fragment of the WN and DEN3 3′ SLs. The unidentified 105-kDa protein species also had significant affinity for an RNA representing the sequence of the 3′ (negative-sense) SL (see earlier discussion). The hypothesis was advanced that this protein may play a role in the initiation of both plus- and minus-strand RNA synthesis. Similar specificity has been reported for other viral systems. For example, one of the cellular proteins that binds rubella virus RNA appears to have specific affinity for both minus- and plus-sense SL structures at the 3′-termini of the respective RNAs (Nakhasi *et al*., 1991).

Blackwell and Brinton (1997) subsequently identified the 56-kDa protein that bound the WN 3′ SL in BHK cell lysates as the translation elongation factor, eF-1α. The site for eF1-α binding was localized to nucleotides –62 to –65, the sequence 3′ACAC-5′ (Fig. 6). Li and Brinton (2001) made the possibly relevant observation that an *in vitro*–synthesized RNA containing the nucleotide sequence of the WN 3′ SL inhibited *in vitro* translation of WN RNAs containing 5′-terminal portions of the WN ORF and of foreign mRNAs. In contrast, an RNA containing nucleotides representing the 3′-terminal secondary structure of rubella virus RNA enhanced *in vitro* translation. These contrasting results may indicate that the affinity of the WN 3′ SL for translation elongation factors is significant for upregulating WN viral translation or downregulating translation of cellular mRNAs *in vivo*. Plant and animal elongation factors eF1-α, β, and γ are recognized components of replicases in other systems. Das and coworkers (1998) demonstrated that the polymerase of the negative-strand virus, vesicular stomatitis virus (VSV), which replicates in both insects and mammals, binds eF1-α tightly, and that the resulting complex binds eF1-β and γ. All three elongation factors are required for VSV replicase activity in an *in vitro* system. In earlier work, Blumenthal *et al*. (1976) demonstrated that RNA bacteriophage Qβ uses three cellular proteins as components of its replicase, the bacterial translation elongation factors, Ts and Tu, and ribosomal protein S1. Further work is required to establish with certainty the physiological significance of the observation that the WN 3′ SL RNA binds translation elongation factors.

In a more recent study, an *in vitro*–synthesized, 83-nucleotide RNA representing the JE virus 3′ SL nucleotide sequence was evaluated for binding of specific proteins in lysates of neonatal mouse brain (Ta and Vrati, 2000). Three RNP complexes thus derived were stable in the presence of high-salt buffer. UV cross-linking and Northwestern blotting analysis identified three proteins involved in RNPs, with

apparent molecular masses of 32, 35, and 50 kDa. Gene products derived from a neonatal mouse brain cDNA library were screened for binding to the JE 3′ SL, and a 36-kDa protein, MOV34, was thus identified. Subsequent experiments showed that the MOV34 protein could also bind to the 3′ SL in native full-length JE virus genomic RNA. MOV34 is a 26S proteasome subunit protein that belongs to a family also thought to be involved in RNA transcription and translation. A computer-based comparison of the amino acid sequences of translation elongation factor eF1-α and MOV34 revealed no detectable sequence similarity between them, even when constraints for detection were set very low (L. Markoff, unpublished data). However, both of these proteins contain a high proportion of positively and negatively charged amino acids, which could effect an association with RNA and/or cell membrane organelles. The translation elongation factor sequence is 463 amino acids in length and includes 23 Asp residues, 31 Glu residues, 17 Arg residues, and 47 Lys residues for a net positive charge of +10. MOV34 is 309 amino acids in length and contains 18 Asp residues, 23 Glu residues, 11 Arg residues, and 23 Lys residues for a net negative charge of –8.

2. Binding of Viral Proteins to the 3′ SL and the Upstream Proximal Portion of the 3′ NCR

As mentioned previously, the viral nonstructural proteins NS3 and NS5 contain domains with helicase and polymerase activities, respectively, required for flavivirus RNA synthesis. It is logical to expect that the viral helicase and RdRP both bind the 3′ termini of plus-strand RNA in order to initiate negative-strand synthesis. Chen and coworkers (1997) made lysates of BHK cells infected with JE virus and first demonstrated that these lysates retained viral RNA synthetic activity. Next, they probed active lysates for proteins that could bind an exogenous 585-nucleotide *in vitro*–synthesized RNA representing the JE 3′ NCR by UV cross-linking. They focused on proteins that specifically formed in lysates of infected cells, as compared to uninfected cells. Two proteins, p71 and p110, were thus identified. Mapping experiments revealed that protein p110 required only the 3′-terminal 80 nucleotides of the JE genome for binding, whereas protein p71 appeared to have a more diffuse binding site on the 585-nucleotide probe. The two proteins were subsequently identified as NS5 and NS3, respectively. The results of immune precipitation assays suggested further that NS5 and NS3 formed a complex with each other in association with the 3′ NCR.

3. Nucleotide Sequence Specificity of the 3′ SL

As mentioned previously, the nucleotide sequence of the 3′ SL in the genomes of different species of flaviviruses is only partially conserved. Zeng and colleagues (1998) asked whether nucleotide sequences of the WN 3′ SL could replace those of the DEN2 3′ SL, in the context of the DEN2 genome. This was done by site-directed substitution mutagenesis of sequences encoding the 3′ SL in a DEN2-infectious DNA (Polo *et al*., 1997). RNA derived from "D2/WN-SL" DNA was infectious in that DEN2-specific immune fluorescence was observed in transfected cells, but the resulting virus was highly impaired for replication; it never achieved a titer greater than 100 pfu/ml in monkey cells. In contrast, DEN2 wild-type virus derived from RNA transcribed *in vitro* typically grew to titers >10E6 pfu/ml. This demonstrated that the nucleotide sequence, not merely the secondary structure of the 3′ SL, was a determinant of replication efficiency.

An attempt was then made to define which DEN2 3′ SL nucleotides were required for efficient virus replication by restoring DEN2 3′ SL–specific sequence elements in the context of the D2/WN-SL mutant DNA. "Top" and "bottom" portions of the DEN2 (and WN) 3′ SLs were defined arbitrarily according to previous work by Blackwell and Brinton (1995) (Fig. 6). Six additional DEN2 3′ SL mutant DNAs were created, and the growth phenotypes of the resulting viruses in LLCMK2 cells are summarized in relation to their 3′ SL structure in Fig. 8. The mutations were "lethal," meaning no positive immunofluorescence could be detected in transfected cells; "sublethal," meaning the viruses replicated about as well as the parent mutant, D2/WN-SL; or "viable," meaning peak titers in LLCMK2 cells were equal to or no worse than 10- or 100-fold reduced compared to the wild-type DEN2 virus. Results identified an 11-bp segment, comprising the uppermost portion of the bottom of the wild-type DEN2 3′ SL sequence, as being essential in viable virus mutants (DEN2 nucleotides 7–17 and 63–73 in Fig. 6). In addition, the study demonstrated that the DEN2-specific nucleotide sequence of the "top" portion of the DEN2 3′ SL was not required for efficient replication, but that its base-paired structure was essential. A mutant in which the entire top portion of the WN 3′ SL was substituted for that of the DEN2 3′ SL grew as well as the wild-type virus, but a second mutant, in which base pairing in the top portion of the long stem was abrogated by mutagenesis of the DEN2 nucleotide sequence, had a lethal phenotype. Defects in replication of all mutant viruses appeared to be at the level of RNA replication, suggesting a critical role of the 3′ SL for initiation of negative-strand RNA synthesis.

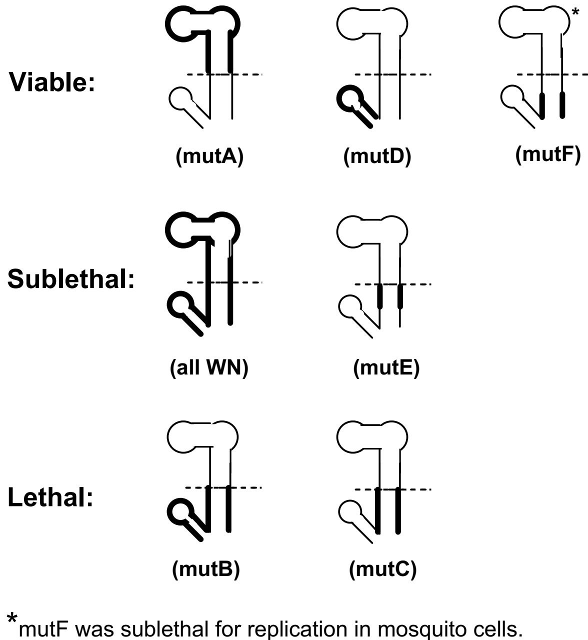

FIG 8. Chimeric DEN2/WN 3′ SL nucleotide sequences in DEN2 genomic DNA. The 3′ SL secondary structure is depicted as a line drawing. DEN2 nucleotide sequences are depicted as a thin line, and WN nucleotide sequences are depicted as a thick line. "Top" and "bottom" portions of the 3′ SL are indicated by horizontal dashed lines bisecting the long stem in the 3′ SL. The chimeric nucleotide sequences indicated were substituted for the wild-type nucleotide sequence of the 3′ SL in DEN2 infectious DNA, and the resulting mutant DNAs were used to derive RNAs that yielded DEN2 viruses with the indicated phenotypes after transfection of cultured monkey kidney cells (see text). Viable viruses all retained the DEN2-specific 11-bp segment comprising the top portion of the bottom section of the long stem in the 3′ SL. The DEN2mutF virus (asterisk) was notable for its host range-restricted phenotype in mosquito cells, where its replication was 100,000-fold reduced, compared to the wild-type DEN2 virus (Zeng *et al.*, 1998).

Mutant D2/WN-SL(mutF) or "mutant F" virus (Fig. 8) was of special interest in that it displayed a viable phenotype in LLCMK2 cells but was severely impaired for replication in cultured mosquito cells. Thus, site-directed mutagenesis of either the 5′ NCR (Cahour *et al.*, 1995) or the 3′ SL (Zeng *et al.*, 1998) may result in the genesis of host range-restricted mutant DEN viruses. These findings support the hypothesis that host cell proteins play an important role in virus replication. Zeng and co-workers (1998) also demonstrated that DEN2 mutant F virus was defective in RNA synthesis at early times after infection of

mosquito cells using an assay that did not distinguish between plus- and minus-strand synthesis. However, a more specific mechanism for the host range-restricted phenotype of DEN2 mutant F virus has not been elucidated thus far. For example, using an *in vitro* assay for RdRP activity (described previously), You and co-workers (2001) demonstrated that an RNA containing the DEN2 mutant F 5′ and 3′ NCR nucleotide sequence was not defective for replication in a mosquito cell extract compared to its activity in an extract of monkey kidney cells. Because this assay detected only negative-strand synthesis, perhaps the results indicate that DEN2 mutant F replication is defective at the level of progeny positive-strand RNA synthesis. Other efforts to detect differential binding of mosquito cell proteins to the DEN2 mutant F 3′ SL, using techniques pioneered by Blackwell and Brinton (1995), have thus been unsuccessful (Yu and Markoff, unpublished results).

Introduction of mutant F mutational changes (Markoff *et al*., 2002) into infectious DNAs for human virulent DEN1 and DEN4 viruses has resulted in the production of mutant viruses that have a host cell-restricted phenotype similar to that of the DEN2 mutant F virus. The DEN1 mutant F virus was subsequently shown to be attenuated and highly immunogenic in a monkey model for DEN virus infection of humans and is currently a candidate for DEN vaccine development.

IV. Mutational Changes in the 5′ or 3′ NCR and the 3′ SL May Result in Attenuation of Virus Virulence

Mutational changes, either in the ORF or in the 5′ and/or 3′ NCRs, could be expected to alter virus growth properties and therefore virulence. For example, Chiou and Chen (2001) reported on the properties and genome sequences of two naturally occurring isolates of JE virus found in an area of Taiwan where local residents have high levels of JE antibodies. One of these isolates, strain T1P1, exhibited 10,000-fold less neurovirulence in mice compared to the other strain, CH1932. A comparison of the complete genome sequences of these two strains revealed that they differed by seven nucleotides. Two of these sequence differences resulted in amino acid changes in NS3, and there were two others in the 3′ NCR, upstream from the 3′ SL. The other three nucleotide sequence differences between the two strains were "silent" ones in the M and NS5 genes. The inference was made that the nucleotide sequence differences noted in NS3 and in the 3′ NCR were responsible for the marked differences in neurovirulence between strain T1P1 and

strain CH1932. No attempt was made to determine whether the 3′ SL mutational difference between strains T1P1 and CH1932 resulted in altered binding of virus- or cell-specific proteins to the 3′ NCR. See also discussions of the work of Cahour (1995), Durbin *et al*. (2001), Mandl *et al*. (1998), Men *et al*. (1996), Pletnev (2001), and Zeng *et al*. (1998) in conjunction with Markoff *et al*. (2002) for other examples of attenuation of flavivirus virulence resulting from mutagenesis of the 5′ or 3′ NCR.

V. Hypothesis for Hydrogen Bonding between 5′ and 3′ NCRs

The existence *in vivo* of the the previously predicted secondary structures formed by *cis* interactions of nucleotides within the 5′ NCR (Brinton and Dispoto, 1988) and within the 3′ NCR (Brinton *et al*., 1986; Hahn *et al*., 1987; Rice *et al*., 1985) must be reconsidered in light of a more recent disclosure of a possible interaction between nucleotides of the 5′ and 3′ NCRs, including the cyclization sequences, in *trans* to form double-stranded panhandle-like structures (Khromykh *et al*., 2001). Sequences representing the 5′-terminal 150 to 170 nucleotides, an intervening poly(A)tract, and the 3′ terminal 110 to 120 nucleotides of the KUN and other mosquito-borne virus genomes, the TBE genome, and the CFA genome were folded by computer using the "MFOLD" program (Mathews *et al*., 1999) and were based on the assumption of hydrogen bonding between the respective 5′ and 3′ cyclization sequences. Results of this analysis revealed that the 5′ and 3′ NCRs in all flavivirus genomes contained *trans* complementary sequences upstream and downstream from the conserved cyclization sequences, such that secondary structures of very similar shape and very high thermal stability could be formed between them.

Khromykh *et al*. predict an extensive *trans* interaction between the KUN genome 5′ and 3′ NCRs. Very similar structures were predicted for the JE, YF, DEN, TBE, and CFA genomes. The model predicts for the KUN genome that (1) 3′ nucleotides –1 to –8 hydrogen bond to 5′ nucleotides +45 to +52 and (2) 3′ nucleotides –11 to –14 and –68 to –106 hydrogen bond with 5′ nucleotides +77 to +150. As a result, (1) 3′ NCR nucleotides –15 to –67 remain available to form a 3′ *cis* stem and loop structure, a truncated 3′ SL composed of nucleotides previously comprising the top portion of the long stem and including the distal loop regions (Fig. 6), and (2) 5′ NCR nucleotides +1 to +44 and +53 to +76 are available in *cis* to form short stem–loop structures,

conserving part of the 5′ SL predicted by Brinton and Dispoto (1988) (Fig. 7), including its distal loop. Therefore, the distal loop in the 5′ NCR could still form. The pseudoknot predicted by Shi *et al.* (1996a) and involving a portion of the 3′ SL conserved in the 5′/3′ panhandle structure could also still form, as the semiconserved nucleotides that form the loop atop the short stem in the conventional model of the 3′ SL secondary structure (5′-CANAG-3′; Fig. 6) are available for hydrogen bonding with the conserved remnant of the long SL in the model proposed by Khromykh and co-workers (2001). It is worthy to note that the predicted thermal stability of the panhandle exceeds those of the earlier predicted 5′ SL and 3′ SL by a wide margin. In addition, the predicted thermal stability of mutant KUN RNA 5′/3′ hydrogen-bonded complexes that lacked complementary cyclization sequences was very little different from that of the wild-type complex. This suggests that the requirement for the cyclization sequences relates to their specific functional necessity, e.g., in initiation of RNA synthesis or translation or packaging, rather than to their additive effect on thermal stability of the panhandle. One must view all of these hypotheses for base pairing with an open mind for two reasons: (1) Computer power to predict folding and base pairing in nucleic acids is increasing rapidly and therefore any of these hypotheses are subject to evolution based on the state of the art in computer programs and computer power and (2) the "wild card" in any computer-based prediction is the unknown but certain effect of cellular and possibly viral proteins on the secondary and tertiary structure of the RNA.

VI. Additional Secondary Structure Predictions in the 3′ NCR

Proutski and colleagues (1997b) analyzed the distal 330 to 400 nucleotides of published flavivirus 3′ NCR sequences for predictable secondary structure using a genetic algorithm that purportedly simulates the natural folding pathway that takes place during RNA elongation and allows prediction of tertiary interactions as well as secondary structure. They then used a second program to detect compensatory mutations (meaning mutations in both strands of a base-paired region that conserve hydrogen bonding) in the 3′ NCRs of related viruses. The presence of compensatory mutations was taken as an indication that the base pairing in that region was likely to occur *in vivo*, as random mutational events conserved the double strandedness.

As a result of this analysis, three regions of secondary structure could be defined within what could be described as the core element

(Wallner *et al.*, 1995) of 3′ NCRs in mosquito-borne as well as tick-borne viruses (Figs. 2 and 3). Region I consisted of nucleotides upstream from CS1, CS2, and RCS2 in mosquito-borne virus genomes or the 5′-most extreme of the core element in tick-borne virus genomes. This is a region of high variability in linear nucleotide sequences. However, all flavivirus sequences in region I formed very similar long hairpin structures with a branching stem–loop side structure or, in some cases, a bulge–loop on the 5′ side of the main hairpin. Downstream from this long hairpin in region I was a conserved shorter hairpin of similar configuration. Nucleotides in region II included those assigned to the CS2 and RCS2 boxes plus intervening sequences. Unlike region I, where a single consensus secondary structure was predicted, region II nucleotides exhibited differences in folding patterns that separated the viruses into three groups: (1) DEN and JE subgroup viruses, (2) tick-borne viruses, and (3) YF virus. Region II in genomes of viruses in the DEN and JE subgroups was shown to contain tandem repeat sequences (TL1 and TL2; Fig. 4) that potentially form conserved short SL structures. TL1 is located upstream from RCS2, and TL2 is located between RCS2 and CS2. The secondary structures in region II for tick-borne viruses were so different from those predicted for mosquito-borne viruses that no one element was conserved between the two groups. Region III consisted of nucleotides traditionally believed to constitute CS-1 and the 3′ SL. A 3′-terminal SL structure was predicted by this analysis, just as it was predicted by the MFOLD program used by Khromykh *et al.* (2001). As in the Khromykh model for panhandle formation, the length of the long stem at the genome 3′ terminus in the Proutski model (Proutski *et al.*, 1997b) is shorter than that predicted by older methods due to the alternative hydrogen bonding of nucleotides that form the lower portion of the long stem shown in Fig. 6. The Khromykh model (Khromykh *et al.*, 2001) and predictions for the secondary structure within the 3′ NCR put forward by Proutski and colleagues (1997b) are not incompatible if one posits that sequences not included in the 3′ SL in the Proutski model are base paired with nucleotides of the 5′ NCR.

The model for the secondary structure of the 3′-terminal 380 nucleotides of the YF genome was subsequently applied to the sequences of several different YF isolates and to the sequences of vaccine strains 17D-204 and 17DD (Proutski *et al.*, 1997a). An association between secondary structure of the 3′ NCR and virus virulence was claimed in that all wild strain genomes were likely to fold similarly and in a significantly different way from the attenuated vaccine strains. In addition, strain 17DD had an intermediate folding pattern that was less

different from that of wild strain genomes than was the strain 17D-204 3′ NCR. The authors noted that strain 17DD is more virulent in mice than strain 17D-204 and suggested that mutagenesis of the primary nucleotide sequence of the 3′ NCR to alter the secondary structure could be a useful strategy for vaccine development. The point may be well taken with respect to mice, but after more than 60 years of experience, there is no indication that the strain 17DD vaccine is any less attenuated in humans than is the strain 17D-204 vaccine (Monath, 1999).

The same group (Proutski *et al.*, 1999) applied similar reasoning to an analysis of the results of Men *et al.* (1996) and Khromykh and Westaway (1997), which were described earlier, in which each of these groups of investigators created internal deletions in the 3′ NCRs of DEN4 and KUN virus genomes, respectively. Consideration of the resulting alterations of the 3′ NCR secondary structure resulting from deletions in the DEN4 genome, in particular, could help explain the observed discordance between the size of the deletion and the growth phenotype of the resulting mutant DEN4 viruses.

Olsthoorn and Bol (2001) also sought to detect a secondary and tertiary structure in the 3′ NCR, and they performed a computer analysis of 191 flavivirus genomic sequences representing four serogroups (DEN, JE, YF, and TBE), also using the MFOLD program. This program yielded a set of suboptimal structure predictions, which were then checked manually for the presence of pseudoknots and for phylogenetic consistency. The analyses confirmed the presence of conserved serogroup-specific secondary structures for each of the four serogroups in the 3′ NCR upstream from the 3′ SL. As in the work of Proutski and colleagues (1997b), the existence of conserved SL structures that involved CS2 and RCS2 was confirmed for viruses of the DEN and JE subgroups. These were paired "dumbbell"-shaped structures that included the conserved stem and the respective TL1 and TL2 loop sequences identified previously. Olsthoorn and Bol (2001) proposed that TL1 and TL2 loops could each form pseudoknots by hydrogen bonding with conserved tetranucleotide sequences pk1 and pk2 present in predicted linear regions of the DEN and JE subgroup 3′ NCRs. For example, in the DEN serogroup, nucleotides in the TL1 sequence (5′-GAA**GCUGU**A-3′; Fig. 4), which forms a loop within a secondary structure that includes CS2, could form hydrogen bonds (bold letters in the TL1 sequence) with conserved sequence pk1 (5′-ACAGC-3′), which lies downstream from CS2. Similarly, in the upstream dumbbell-shaped secondary structure that includes RCS2, the nucleotides 5′-GCUGC-3′ in TL2 could hydrogen bond with linear

sequence pk2 (5′-GCAGC-3′), which also lies between RCS2 and CS2. For JE serogroup viruses, pk1 and pk2 were both located downstream from CS2. In the YF genomes, which lack RCS2 (Fig. 2), three pseudoknots were predicted, one of which was similar in its genesis to the more downstream pseudoknot in DEN and JE serogroup genomes. As in the analyses of Proutski *et al*. (1997a), the TBE genomes were folded very differently from those of the mosquito-borne viruses, resulting in the formation of a single predicted pseudoknot within the core element. The internally consistent results of this study and the compatibility of the results with the work of others (Proutski *et al*., 1997b; Rauscher *et al*., 1997) constitute additional evidence that pseudoknot formation may be required for proper function of the 3′ NCR in flavivirus RNA replication.

VII. Summary and Future Directions

The flavivirus genome is a capped, positive-sense RNA approximately 10.5 kb in length. It contains a single long ORF, flanked by a 5′ NCR, which is about 100 nucleotides in length, and a 3′ NCR ranging in size from about 400 to 800 nucleotides in length. Replication is thought to occur by a semiconservative mechanism requiring double-stranded replicative form RNA to generate replicative intermediate RNA containing nascent single strands. The 5′ and 3′ NCRs must play a role in the initiation of negative-strand synthesis on virus RNA released from entering virions, switching from negative-strand synthesis to synthesis of progeny plus strand RNA at late times after infection, and possibly in the initiation of translation and in the packaging of virus plus strand RNA into particles. However, none of these mechanisms is as yet well defined. The presence of conserved and nonconserved complementary nucleotide sequences near the 5′ and 3′ termini of flavivirus genomes suggests that “panhandle” or circular RNA structures are formed transiently by hydrogen bonding at some stage during RNA replication.

Genomes of flaviviruses that differ according to vector (tick-borne viruses vs mosquito-borne viruses) and by antigenic subgroup may also contain unique or characteristic nucleotide sequence features in their noncoding regions. However, a few such features are conserved in all flavivirus genomes. These are as follows: (1) All vector-borne flavivirus genomes begin with the dinucleotide sequence 5′-AG and end with the complementary nucleotide sequence UC-3′. The functional significance of these sequences is not known but may play a role in

cyclization of the genome during or as a prerequisite for replication. (2) While the nucleotide sequence of the 5′NCR is not well conserved among flavivirus species, the 5′NCRs of all flavivirus genomes do contain a predicted stable stem and loop structure. (3) All flavivirus genomes contain a 3′-terminal approximately 100-nucleotide sequence that is predicted to form a highly stable stem and loop structure, the 3′ SL. The actual nucleotide sequence of this domain is well conserved among viruses of the same subgroup but is much less well conserved across subgroups. The 3′ SL is known to bind the virus-coded RNA-dependent RNA polymerase protein NS5 and to be required for binding of a virus-coded helicase, NS3, to the 3′NCR. In addition, the 3′ SL apparently binds several cellular proteins required for virus replication. One of these proteins has been identified as the translation elongation factor eF1-α. Mutations of the 3′ SL can be shown to affect the host range of the virus, suggesting that binding of host cell proteins is critically important for replication. (4) Within the nucleotide sequence forming the distal loop of the 3′ SL in all vector-borne flavivirus genomes, there is a conserved nucleotide sequence, 5′-CACAG. The function of this conserved pentanucleotide sequence in replication is unknown.

Functionally analogous group- and subgroup-specific nucleotide sequence elements have been identified in the 5′ and 3′ NCRs of flavivirus genomes. For example, mosquito-borne flavivirus genomes contain conserved complementary 8-nucleotide sequences. One is located in the 3′ NCR, referred to as the cyclization sequence (CYC). The other is located in the 5′ terminus of the long ORF, just downstream from the start codon that initiates translation of the capsid protein gene segment, referred to as the “5′CS.” The CYC sequence and the 5′CS are thought to hydrogen bond, helping to form a panhandle structure during RNA replication. Tick-borne flavivirus genomes contain two analogous pairs of 5′/3′ complementary sequences, each 11 nucleotides in length. The 3′-terminal motifs in tick-borne virus genomes are herein referred to as C1′ and C2′, and the 5′-terminal motifs are referred to as C1 and C2. The CYC sequence in mosquito-borne virus genomes and C1′ are analogous in their position in the 3′ NCR, just upstream from nucleotides comprising the 3′ SL. However, the complement of C1′, C1, lies wholly within the 5′ NCR in tick-borne virus genomes, not in the ORF. C2′ lies upstream from C1′, and its complement at the 5′ end of the genome, C2, lies within the ORF, in a location analogous to that of the 5′CS in mosquito-borne virus genomes. The 5′ and 3′ complementary sequences are necessary for viral RNA replication, but the specific nucleotide sequence of,

for example, the CYC sequence and the 5′CS in mosquito-borne virus genomes can be substituted by a random pair of complementary sequences 8 nucleotides in length without much loss of replication efficiency.

Mosquito-borne viruses of the dengue subgroup contain the shortest 3′ NCRs in this group (∼400 nucleotides), with a minimum of conserved features. CS1 is a 20-nucleotide segment just upstream from the 3′ SL. The 8-nucleotide CYC sequence comprises the upstreammost part of CS1. Deletion of CS1 nucleotide sequences was specifically lethal for DEN4 virus replication, but the functional requirement for the 12 nucleotides of CS1 that do not compose the CYC sequence is not known. CS2 is a 23-nucleotide conserved element that lies 12 to 22 nucleotides upstream from CS1. CS2 is one of a pair of repeated sequence elements in the 3′ NCR; the repeat sequence RCS2 lies 51 to 62 nucleotides upstream from CS2 in the genome of mosquito-borne viruses. All or parts of CS2 and RCS2 were dispensible for DEN-4 virus replication, but the respective mutant viruses were mildly to markedly reduced in replication efficiency.

3′ NCRs of viruses in the JE subgroup are larger in size than those of the DEN subgroup (∼500 to ∼600 nucleotides) and contain additional conserved repeat sequence motifs, in addition to all the conserved motifs described for the 3′ NCR in DEN virus genomes. These additional repeat sequences are referred to as CS3 and RCS3, respectively, in relation to their increasing distance from the 3′ terminus of the genome compared to CS2 and RCS2. CS3 and RCS3 are each 28 nucleotides in length and are separated by 120 nucleotides in the 3′ NCRs of JE subgroup viral genomes.

The YF virus genome is unique in several ways from that of the other mosquito-borne flaviviruses, including its organization of the 3′ NCR, which contains CS1 and CS2 but lacks the repeat segment RCS2 found in the other genomes of the mosquito-borne virus group. The YF strain 17D vaccine virus 3′ NCR also contains a series of three unique 40-nucleotide tandem repeats at the 5′ end of its 3′ NCR. Also, unlike other flavivirus genomes, the yellow fever 3′ NCR contains three in-frame stop codons for translation of the ORF, the last of which lies within the upstream boundary of the first of the tandem repeats.

For tick-borne flaviviruses, the 3′ NCRs of tick-borne encephalitis virus strains have been analyzed extensively. The genome of TBE strain Neudorfl virus genome contains the longest 3′ NCR in this group, at 764 nucleotides. This segment has been divided, by comparative analysis of the nucleotide sequences of strain Neudorfl and other TBE viruses and the results of mutagenesis experiments, into a “core

element" and a "variable region." The core element is directly analogous to the entirety of the DEN virus 3′ NCR and includes the most distal portion of the 3′ NCR, containing the 3′ SL. The cyclization sequence, C1′, lies within a mixed purine/pyrimidine-rich tract upstream from the 3′ SL, which is analogous in position and sequence to CS1 in mosquito-borne flavivirus genomes. Upstream from this PR segment exists consecutive pyrimidine- and purine-rich segments, each about 14 nucleotides in length. These could be said to be analogous to CS2 found in mosquito-borne virus genomes. The second cyclization sequence motif, C2′, lies upstream from C1′, also within the core element. The core element also includes one of a pair of long tandem repeats in the archetypal strain Neudorfl 3′ NCR, R3, which is 76 nucleotides in length.

The structure of the variable region, which comprises the upstream portion of the 3′ NCR in strain Neudorfl and related TBE virus genomes, is complex. The variable region contains a second iteration of the R3 sequence found in the core element. It also contains additional repeated sequences, R1 and R2. R1 is repeated three times in the strain Neudorfl genome. One of these repeats lies within the 3′ terminus of the ORF. The single repeat of R2 also lies within this same distal segment of the ORF. The most striking feature of the variable region in TBE genomes is the presence of a poly(A) segment that can be as long as 250 nucleotides. The function of the variable region remains a puzzle because there exist naturally occurring highly related TBE virus variants with much shorter 3′ NCRs compared to that of strain Neudorfl, completely lacking a variable region. In addition, the variable region nucleotide sequence can be completely removed from the strain Neudrofl genome by site-directed mutagenesis or by spontaneous mutation that occurs during repeated *in vitro* tissue culture passage, with no effect on virus replication in tissue culture or on mouse neurovirulence.

In recent years, computer-based methods have been used to predict the existence of additional secondary and tertiary structures within the 3′ NCR and to predict additional interactions between 5′ and 3′ NCRs based on the potential for hydrogen bonding of complementary segments in these two regions of the genome, in addition to that afforded by the known cyclization sequences. The significance of this work remains to be seen and requires better knowledge and understanding of the possible effects of viral and cellular proteins on folding of the RNA, which can only be gained by further *in vitro* studies with engineered RNAs and virus infectious clones.

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ADVANCES IN VIRUS RESEARCH, VOL 59

MANIPULATION OF CELL SURFACE MACROMOLECULES BY FLAVIVIRUSES

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I. Introduction
II. The Flavivirus Receptor Binding Protein
III. Cell Targets for Flaviviruses
  A. Dendritic Cells
  B. Monocytes and Macrophages
  C. Endothelial Cells
  D. Lymphocytes
  E. Neural Cells
  F. Basophils/Mast Cells
  G. Platelets
  H. Cell Targets: An Overview
IV. Cell Surface Macromolecules Involved in Flavivirus Attachment
  A. Glycosaminoglycans
  B. CD14
  C. Fc Receptors
  D. Complement Receptors
  E. Virus Binding Proteins Identified on Cells
V. Cell Surface Macromolecules Modulated by Flavivirus Infection
  A. MHC Class I
  B. MHC Class II
  C. Adhesion Molecules
VI. Other Cell Surface Macromolecular Modifications Triggered by Flavivirus Infection
  A. Complement Deposition
VII. Conclusions
References

Cell surface macromolecules play a crucial role in the biology and pathobiology of flaviviruses, both as receptors for virus entry and as signaling molecules for cell–cell interactions in the processes of vascular permeability and inflammation. This review examines the cell tropism and pathogenesis of flaviviruses from the standpoint of cell surface molecules, which have been implicated as receptors in both virus–cell as well as cell–cell interactions. The emerging picture is one that encompasses extensive regulation and interplay among the invading virus, viral immune complexes, Fc receptors, major histocompatibility complex antigens, and adhesion molecules.

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I. Introduction

Flaviviruses comprise a rich and diverse family of agents that infect a variety of hosts and cause a wide spectrum of disease. Three disease types are recognized for flaviviruses, namely encephalitis, hemorrhagic fever, and fever–arthralgia–rash. Disease distinctions are not absolute, and overlapping pathologies among various flavivirus members are often observed. The ability of flaviviruses to cause such divergent clinical syndromes, associated with virus replication in a number of different organs, has profound implications for the types of cell surface molecules the virus recognizes as receptors. Mutational analyses of the flaviviral E protein have demonstrated a striking ability of flaviviruses to adapt to different cells and receptors. Given the considerable homologies among them, flaviviruses show a remarkable capacity to cause vastly different diseases with a minimum of alterations in the E protein.

The cell surface molecules, which act as receptors for flaviviruses, are only starting to be identified. In addition to providing the molecules involved in virus attachment and penetration, the host cell erects a battery of surface structures that mediate communication with other cells and trigger host defense and pathological processes. Many of these are modulated by flavivirus infection and contribute to the overall picture of pathogenesis.

II. The Flavivirus Receptor Binding Protein

The flavivirus E protein is a multifunctional protein involved in cell receptor binding (Anderson *et al.*, 1992; Chen *et al.*, 1996; He *et al.*, 1995) and virus entry via fusion with a host cell membrane (Rice, 1996). Some of the functional activities of the E protein, notably membrane fusion, are regulated by interaction with a second viral protein, prM. It is believed that the association of prM with E stabilizes certain pH-sensitive epitopes on the E protein, thereby preventing the conformational changes that normally occur at acidic pH and activate the fusogenic activity of the E protein (Allison *et al.*, 1995; Guirakhoo *et al.*, 1992; Heinz *et al.*, 1994). In addition to its normal role in flavivirus assembly, the prM protein has also been included in novel recombinant formulations in which it is generally coexpressed with the E protein; the resultant E/prM complexes have been shown to be immunogenic and protective as vaccines against challenge with several flaviviruses, including Japanese encephalitis virus (Mason *et al.*,

1991), yellow fever virus (Pincus *et al.*, 1992), dengue virus (Fonseca *et al.*, 1994), and tick-borne encephalitis (TBE) virus (Heinz *et al.*, 1995).

In TBE virus, the majority of extracellular virus is largely free of prM protein due to a late intracellular processing event that generates a carboxy-terminal fragment designated M and which together with the E and C proteins are believed to constitute the protein components of the mature virus particle (Heinz *et al.*, 1994). Cleavage of prM to M enhances low pH–dependent virus–cell fusion (Guirakhoo *et al.*, 1991) and infectivity (Guirakhoo *et al.*, 1992; Heinz *et al.*, 1994; Randolph *et al.*, 1990; Shapiro *et al.*, 1972; Wengler, 1989). Dengue virions containing prM are still infectious (Randolph *et al.*, 1990) and bind to permissive cells in a manner that can be blocked using E-specific antibodies (He *et al.*, 1995; Wang *et al.*, 1999). Virus particles containing mainly E and prM also show antibody-enhanced binding to Fc receptor-bearing K562 cells as well as to platelets (Wang *et al.*, 1995). Thus, in addition to being requisite precursors to mature virus particles, virus particles containing prM possess many properties associated with mature virus particles.

Flaviviruses appear to gain entry to the cell by the endocytic pathway (Rice, 1996). At low pH, the E protein undergoes a conformational change (Allison *et al.*, 1995) involving dissociation of the E dimer (Stiasny *et al.*, 1996), thereby exposing a hidden fusion peptide, followed by reorganization of E into a trimer (Allison *et al.*, 1995), in which the fusion peptide is brought close to the membrane-anchoring carboxy terminus (Ferlenghi *et al.*, 2001). Remarkably similar structural features and conformational rearrangements have been noted between the flavivirus E protein and the alphavirus E1 (Heinz and Allison, 2001; Lescar *et al.*, 2001; Pletnev *et al.*, 2001; Strauss and Strauss, 2001), suggesting a common evolutionary origin for these two virion surface proteins.

Considerable homology exists among flaviviral E proteins, raising the possibility that different flaviviruses may have similar receptor-binding motifs. For example, many mosquito-borne flaviviruses contain an RGD sequence (e.g., residues 388–390 of the Murray Valley encephalitis virus E protein), which has been implicated in virulence (Lobigs *et al.*, 1990) and receptor binding by analogy with integrin-binding motifs (Rey *et al.*, 1995). Mutagenesis studies of the yellow fever virus (Van der Most *et al.*, 1999) and Murray Valley encephalitis virus (Hurrelbrink and McMinn, 2001) RGD motifs, however, have cast doubt on the role of integrins in flavivirus attachment or entry.

Studies with TBE virus have identified important determinants for pathogenicity within the suspected receptor-binding site on the upper-lateral surface of domain III (Mandl *et al.*, 2000). Acquisition of heparan sulfate-binding mutations by passaging TBE in cell culture has also implicated amino acids in this region in receptor binding (Mandl *et al.*, 2001). The selection of virus mutants on the basis of weak binding to brain membranes has been used with several neurotropic flaviviruses (Holbrook *et al.*, 2001; Ni and Barrett, 1998; Ni *et al.*, 2000) and has identified a variety of mutations within domain III as well as other regions of E. For dengue virus, blocking of virus cell binding correlates more closely to virus neutralization for mAb 3H5 than for mAb 1B7 (Wang *et al.*, 1999). This may suggest that mAb 3H5 neutralizes dengue virus predominantly by blocking virus–cell attachment, whereas mAb 1B7 neutralizes dengue virus largely by a postattachment mechanism. The mAb 3H5-binding site on the dengue viral E protein has been partly characterized (Hiramatsu *et al.*, 1996; Megret *et al.*, 1992; Trirawatanapong *et al.*, 1992) and probably encompasses, at a minimum, residues 383–385 (Hiramatsu *et al.*, 1996) within domain III. More recent data involving a larger number of monoclonal antibodies indicate that mAbs that interact with domain III are in fact the most effective blockers of virus–cell attachment (Crill and Roehrig, 2001). A putative heparan sulfate-binding site on the dengue-2 E protein is also located within this region (Chen *et al.*, 1997), and comparative sequencing of dengue type 2 genomes has implicated amino acid 390 of the E protein as a major determinant of pathogenicity (Leitmeyer *et al.*, 1999). The pH-dependent conformational "hinge" region (between domains I and II) of the E protein has also been implicated in virulence, receptor interaction, and/or membrane fusion (Hurrelbrink and McMinn, 2001; Lee *et al.*, 1997; Monath *et al.*, 2002). Further mutagenesis studies will undoubtedly help define the sites of the E protein involved in flavivirus–cell macromolecule recognition.

III. Cell Targets for Flaviviruses

A. Dendritic Cells

Transmission of flaviviruses to humans generally occurs via the bite of an infected mosquito or tick. In the case of dengue, inoculated virus is thought to first replicate in skin Langerhans (dendritic) cells (Palucka, 2000; Taweechaisupapong *et al.*, 1996a, 1996b; Wu *et al.*,

2000). Dendritic cells have also been shown to be involved in the transport of intradermally inoculated West Nile virus to local draining lymph nodes, with a subsequent accumulation of leukocytes (Johnston *et al.*, 2000). It is likely that dendritic cells will prove to be efficient carriers of a wide number of flaviviruses from their cutaneous site of infection to lymphoid and possibly other tissues.

Given the importance of dendritic cells in initiating immune responses (Banchereau *et al.*, 2000), they probably play a pivotal role in stimulating host defense against invading flaviviruses. Dengue virus infection of immature myeloid dendritic cells has been shown to induce their maturation accompanied by the expression of major histocompatibility complex (MHC) class I and II antigens; the costimulatory molecules CD40, CD80, and CD86; and the dendritic cell marker CD83 (Libraty *et al.*, 2001). Such changes were seen in both dengue-infected and bystander cells, indicating that upregulation of cell surface molecules could be a consequence of virus infection as well as virus-induced cytokine expression. Similarly, Langerhans cells infected with West Nile virus, as well as an alphavirus, Semliki Forest virus, express increased cell surface MHC class II and appear to undergo maturation to a cell type similar to lymphoid dendritic cells (Johnston *et al.*, 1996). The efficient presentation of both MHC class I– and II–associated viral peptides on the surface of dendritic cells permits the generation of potent cytotoxic and helper T cell responses (see also Section V,A).

B. Monocytes and Macrophages

Monocytes and macrophages have long been recognized as major targets of flavivirus replication in the human host (Halstead, 1989; Halstead *et al.*, 1977; Scott *et al.*, 1980). They are also important host cells for the antibody-enhanced replication of certain flaviviruses (see Section IV,C). Because of their presence in the circulation, blood monocytes may be particularly important to the pathogenesis of hemorrhagic viruses, such as dengue. Because most of the pathological changes associated with dengue virus are hemostatic in nature, it is suspected that blood cells, particularly virus-infected blood monocytes, orchestrate many of these effects.

Dengue virus–infected human monocytes have been shown to be potent sources of vasoactive cytokines such as tumor necrosis factor (TNF)-α (Anderson *et al.*, 1997) and interleukin (IL)-1β (Chang and Shaio, 1994). Monocytes are also known producers of several other vasoactive mediators, including IL-6, platelet-activating factor (PAF), prostaglandins, thromboxanes, leukotrienes, and nitric oxide

(Bulger and Maier, 2000; Funk, 2001; Lefer, 1989; Maruo *et al.*, 1992; Montrucchio *et al.*, 2000; Szabo and Billiar, 1999), any of which could have powerful effects on endothelial cell physiology. A crucial aspect in understanding dengue pathogenesis will be the identification of additional vasoactive mediators, which trigger the key dysfunctional events in vascular integrity.

Various tissue macrophages are undoubtedly important in the pathogenesis of flaviviral diseases but have, to date, not received much attention. Skin mononuclear cells, pulmonary, splenic, and thymic macrophages and liver Kupffer cells have been recognized carriers of viral antigen (Halstead, 1989). In the liver, virus or viral antigen has been found in Kupffer cells and hepatocytes in infections with yellow fever (Monath *et al.*, 1989) and dengue (Bhamarapravati *et al.*, 1967; Hall *et al.*, 1991; Halstead, 1989; Rosen and Khin, 1989). Destruction of Kupffer cells, possibly by apoptosis, has been reported in the liver of some patients with fatal dengue (Huerre *et al.*, 2001). Primary cultures of Kupffer cells apparently undergo an abortive infection with dengue virus in which viral antigen but no progeny virus is produced (Marianneau *et al.*, 1999).

C. Endothelial Cells

Many flaviviruses invade either visceral or central nervous system tissues following initial replication in dendritic cells, monocytes, or macrophages. Often this necessitates a transfer of virus across blood vessel endothelial layers.

For neurotropic flaviviruses, endothelial cells of the cerebral microvasculature constitute a barrier that must be overcome in order to gain access to the central nervous system. How this occurs remains uncertain. Transendothelial passage of virus may direct infection of cerebral microvascular endothelial cells, may transport across the endothelial layer, or both (Dropulic and Masters, 1990). Japanese encephalitis virus has been observed electron microscopically to traverse mouse cerebral endothelial cells by transcytosis (Liou and Hsu, 1998). Alternatively, virus may spread from blood vessels to the olfactory neuroepithelium and from there to olfactory neurons (McMinn *et al.*, 1996; Monath *et al.*, 1983).

Even normally nonneurotropic flaviviruses may occasionally invade the central nervous system under certain conditions. Modulation of the blood–brain barrier by anesthetics (Ben-Nathan *et al.*, 2000) or lipopolysaccharide (Lustig *et al.*, 1992) has been reported to facilitate neuroinvasion by a normally noninvasive strain of West Nile virus.

Flaviviruses may also trigger the production of soluble factors that perturb the integrity of the blood–brain barrier, leading to increased leakage of proteins and cells into the central nervous system (Chaturvedi *et al.*, 1991). These studies indicate that even nonneurotropic flaviviruses may infect tissues of the central nervous system or otherwise affect the integrity of the blood–brain barrier under special circumstances.

Transendothelial migration of individual leukocytes (e.g., lymphocytes, monocytes, neutrophils, eosinophils) is regulated in a highly specific manner by the differential expression of selected adhesion molecules on endothelial cells (reviewed in Crockett, 1998; Lowell and Berton, 1999). Flaviviruses, including dengue (Anderson *et al.*, 1997) and West Nile (Shen *et al.*, 1997) viruses, activate endothelial cell adhesion molecule expression by either direct (virus-mediated) or indirect (cytokine-mediated) mechanisms (see Section V,C). In the presence of leukocyte-attracting chemokines, such virus-triggered activation of the vascular endothelium may contribute toward the migration of leukocytes into extravascular tissues. In addition to being a mechanism for virus dissemination, this process may also be a factor in phenomena such as leukopenia and particularly neutropenia (loss of circulating leukocytes, neutrophils) often observed in flavivirus, particularly dengue, infection (reviewed in Halstead, 1989). Due to the lack of suitable animal models for severe dengue disease, i.e., dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), there are difficulties in assessing the roles of such events, particularly the identification of adhesion molecules mediating the transendothelial migration of neutrophils using blocking antibodies against specific integrins, as has been performed for other disease states (Doerschuk *et al.*, 1990; Gao *et al.*, 1994; Issekutz and Issekutz, 1993; Laberge *et al.*, 1995; Springer, 1995).

The hallmark feature of increased vascular permeability in hemorrhagic flavivirus (e.g., dengue) infection suggests that vascular endothelial cells may mediate the fluid leakage and hemorrhaging that occur in DHF/DSS. Endothelial cells line the inner surface of blood vessels and play essential roles in maintaining an antithrombogenic surface and regulating vascular permeability. Increased vascular permeability can arise from a variety of mediators associated with acute inflammation and shock (Bulger and Maier, 2000; Funk, 2001; Lefer, 1989; Michel, 1988; Montrucchio *et al.*, 2000; Schnittler *et al.*, 1990). It is thought that vascular permeability is largely controlled by changes in endothelial cell–cell contact, which result in gap formation, thus allowing for fluid exchange between blood and interstitial tissue

fluid (Michel, 1988). An electron microscopic study of endothelium from DHF biopsy samples revealed the occasional presence of gaps (Sahaphong *et al.*, 1980), thus providing evidence that endothelial cell features may indeed be perturbed during DHF/DSS.

Although dengue virus infects endothelial cells *in vitro* (Andrews *et al.*, 1978; Avirutnan *et al.*, 1998; Killen and O'Sullivan, 1993), there is no evidence that endothelial cell infection occurs clinically, as neither virus particles nor viral antigen has been detected in the endothelium of tissue specimens (Halstead, 1988, 1989; Sahaphong *et al.*, 1980), in contrast to that seen in cases of ebola (Zaki *et al.*, 1999) or hantaan hemorrhagic fever (Gavrilovskaya *et al.*, 1999; Wang *et al.*, 1997). It is likely that dengue virus mediates endothelial cell activation via an indirect route, involving blood monocytes, which are a major cell target for dengue virus infection (Halstead *et al.*, 1977b; Scott *et al.*, 1980). A major candidate event in such a route is the activation of endothelial cell adhesion molecules by a factor(s) (particularly TNF-α) produced by dengue virus–infected blood monocytes (Anderson *et al.*, 1997).

TNF is a key cytokine in a variety of normal and pathological immune responses, including immunoregulation, regulation of cell proliferation, cytotoxicity, and in the mediation of endotoxic shock (Fiers, 1991; Tartaglia and Goeddel, 1992; Tracey and Cerami, 1993; Vassalli, 1992). Monocyte-derived TNF-α appears to play a pivotal role in dengue-associated endothelial cell activation (Anderson *et al.*, 1997) and may be an important effector in the manifestation of DHF/DSS. Support for the clinical significance of this observation comes from observations of elevated TNF levels in the sera of patients with severe dengue disease (Green *et al.*, 1999b; Hober *et al.*, 1993; Vitarana *et al.*, 1991; Yadav *et al.*, 1991). Taken together, current evidence indicates that dengue virus represents a rather unique group of viruses that target monocytes, thereby triggering the production of factors such as TNF-α, which in turn affect other cell targets, including endothelial cells. While the overall picture of endothelial cell dysfunction in DHF/DSS is obviously more complex than can be explained by any single factor, the role of TNF in dengue pathogenesis would seem to merit particular attention.

Current knowledge of endothelial cell responses observed in endotoxic shock may be instructive for the understanding of vascular leakage in DHF/DSS. Plasma leakage induced by endotoxin (lipopolysaccharide, LPS) from gram-negative bacteria encompasses a complex cascade of processes, including activation and functional alteration of endothelial cells. Major mediators of endothelial cell perturbation

in endotoxic shock are LPS itself, as well as cytokines such as TNF-α and IL-1β (Bevilacqua, 1993). These factors can modulate endothelial cell function to varying degrees by activating cytokine and vasoactive factor release (Rink and Kirchner, 1996; Shanley *et al.*, 1995), upregulating adhesion molecule expression (Bevilacqua, 1993; Luscinskas *et al.*, 1991; Moser *et al.*, 1989; Smith *et al.*, 1989), and mediating transendothelial migration of specific leukocytes (Issekutz *et al.*, 1995; Luscinskas *et al.*, 1991; Morzycki *et al.*, 1990; Moser *et al.*, 1989; Smith *et al.*, 1989). Additional factors, particularly lipid mediators such as PAF, leukotrienes, thromboxanes, and prostaglandins, may contribute to further endothelial cell dysfunction, including vascular leakage (Bulger and Maier, 2000; Funk, 2001; Lefer, 1989; Montrucchio *et al.*, 2000). While the involvement of these vasoactive mediators is recognized in endotoxic shock, more needs to be learned of their role in the vascular dysfunction that occurs in severe dengue disease.

D. Lymphocytes

Although lymphocytes are potently involved in the host response and immunopathology of flavivirus (especially dengue) diseases, their role as virus-permissive host cells is unclear. Dengue virus has been identified in circulating B cells from acutely ill dengue patients by immunocytochemistry and by recovery of infectious virus after passage in mosquitoes (King *et al.*, 1999). *In vitro* studies showed that cells and cultured cell lines of both B and T cell derivation could be infected with dengue virus (Bielefeldt-Ohmann *et al.*, 2001; Kurane *et al.*, 1990; Marchette and Halstead, 1978; Mentor and Kurane, 1997; Sung *et al.*, 1975; Takasaki *et al.*, 2001; Theofilopoulos *et al.*, 1976). Continued passage of dengue virus in lymphoblastoid (Raji) cells can give rise to dengue virus variants capable of replication in human lymphocytes (Brandt *et al.*, 1979). Interestingly, lymphocytes do not appear to undergo antibody-enhanced dengue virus infection (Brandt *et al.*, 1979; Kurane *et al.*, 1990), even though B cells do have Fc receptors (Dijstelbloem *et al.*, 2001; see Section IV,C).

E. Neural Cells

The initial stages of pathogenesis for neurotropic flaviviruses appear to be common for flaviviruses in general in that the virus progresses from the subcutaneous site of inoculation to lymph nodes, followed by viremia and replication in extraneural tissues. Invasion into the

central nervous system is marked by high virus titers in the brain and detectable virus or viral antigen in neurons (Albrecht, 1968). Cell destruction in tick-borne encephalitis may be less extensive than that seen in herpes simplex type 1 encephalitis (Studahl *et al.*, 2000), although this is variable and may involve considerable inflammation (Chu *et al.*, 1999; Matthews *et al.*, 2000; Suzuki *et al.*, 2000). Susceptible cell types include both neurons and glial cells (Chu *et al.*, 1999; Ramos *et al.*, 1998; Steele *et al.*, 2000).

F. Basophils/Mast Cells

As notorious producers of vasoactive mediators, mast cells have been a source of controversial speculation for years in dengue pathogenesis. Cells resembling degranulated mast cells have been reported in skin perivascular infiltrates from DHF/DSS cases (Bhamarapravati *et al.*, 1967). Dengue patients showed elevated levels of urinary histamine (a major granule product of mast cells), which correlated with disease severity (Tuchinda *et al.*, 1977), suggesting that mast cells may have a contributory role in the pathogenesis of dengue. Although antihistamine treatment does not resolve shock in severely dengue-diseased patients (Halstead, 1989), histamine is only one of several potent vasoactive factors produced by mast cells (Benyon *et al.*, 1991; Bradding *et al.*, 1993; Galli *et al.*, 1984; Grabbe *et al.*, 1994; Marshall and Bienenstock, 1994; Moller *et al.*, 1991, 1993, 1998; Nilsson *et al.*, 1995; Schwartz and Austen, 1984), some of which could cause vascular dysfunction in dengue infection. DHF/DSS patients have been reported to have elevated serum levels of IgE (Pavri *et al.*, 1979), which has been speculated to relate to IgE-triggered histamine release in the manifestation of shock (Pavri and Prasad, 1980).

Mast cells reside mainly in the tissues, often closely associated with blood vessels (Alving, 1991; Anton *et al.*, 1998; Pesci *et al.*, 1996; Pulimood *et al.*, 1998; Selye, 1966; Selye *et al.*, 1968). They are present in large numbers in the skin (Marshall *et al.*, 1987), where transmission of insect-borne flaviviruses occurs. Basophils, however, comprise about 1% of total circulating cells and would be accessible to virus in the blood. Dengue virus infects basophil/mast cell–like KU812 cells in an antibody-enhanced manner, coupled with the release of vasoactive cytokines, IL-1β and IL-6 (King *et al.*, 2000, 2002). This cell line, which can be differentiated easily toward either a basophil or mast cell phenotype (Saito *et al.*, 1995), may provide further insights into potential roles for basophils and mast cells in dengue disease.

Dengue patients show increased serum levels of anaphylatoxins C3a and C5a (Malasit, 1987), which can attract (Nilsson *et al.*, 1996) and activate (Kownatzki, 1982) mast cells. Among the expected mast cell secretion products would be vasoactive factors, including histamine, which has been detected in elevated amounts in the urine of dengue patients (Tuchinda *et al.*, 1977).

G. Platelets

Evidence for platelet involvement in dengue pathogenesis comes from at least two (probably related) sources. First, thrombocytopenia (loss of circulating platelets) is one of the most consistent clinical features of severe dengue infection (Halstead, 1989). Second, viral immune complexes have been detected on platelets from dengue patients (Boonpucknavig *et al.*, 1979; Phanichyakarn *et al.*, 1977a). Functional studies on platelets in dengue-diseased individuals have been sparse, but include a markedly reduced half-life (Mitrakul *et al.*, 1977), deficient ADP release (Mitrakul *et al.*, 1977), increased adhesiveness (Doury *et al.*, 1976), increased tagging by complement fragments (Malasit, 1987), and increased release of β-thromboglobulin and platelet factor 4 (Srichaikul *et al.*, 1989). There is also evidence for platelet activation in dengue patients (Doury *et al.*, 1976; Krishnamurti *et al.*, 2001; Srichaikul *et al.*, 1989). Although these results relate to a variety of platelet functions, they do indicate a general alteration in platelet physiology, which is consistent with platelet involvement and triggering of thrombocytopenia in dengue disease.

Dengue virus has been recovered from washed patient platelets (Scott *et al.*, 1978), and virus has been reported to bind to platelets in the absence of antibody as assayed using immunofluorescence and immunoperoxidase techniques (Funahara *et al.*, 1987). However, the levels of antibody-independent bound virus are very low compared to the levels of virus bound in the presence of dengue-specific antibodies (Wang *et al.*, 1995). As noted earlier, dengue immune complexes have been demonstrated on platelets from dengue patients (Boonpucknavig *et al.*, 1979; Phanichyakarn *et al.*, 1977a). Weiss and Halstead (1965) originally proposed the possibility that dengue virus interactions with platelets might be involved in the thrombocytopenia observed in severe dengue disease. The finding that dengue virus binding to platelets is dependent on a virus-specific antibody is consistent with epidemiological and experimental data linking preexisting host antibodies to an increased risk of DHF/DSS (reviewed in Halstead, 1990).

Several other viruses have been shown to bind directly to platelets (Bik *et al.*, 1982; Danon *et al.*, 1959; Forghani and Schmidt, 1983; Larke and Wheelock, 1970; Lee *et al.*, 1993; Zucker-Franklin *et al.*, 1990). Platelet association may stabilize or protect blood-borne viruses (Larke and Wheelock, 1970) and may function as a mechanism of hematogenous dissemination (Forghani and Schmidt, 1983). Virus binding to platelets has been suggested to be a contributing mechanism to thromobocytopenia arising from infections with vaccinia (Bik *et al.*, 1982), chikungunya (Larke and Wheelock, 1970), and rubella (Bayer *et al.*, 1965). Thrombocytopenia in these virus infections is generally much milder than that observed in severe dengue disease.

Levels of dengue virus in the blood can exceed 10^7 infectious units/ml (Gubler, 1988; Monath, 1994). Such high viremic titers are likely necessary to ensure infection and transmission of the obligate mosquito intermediary host (Monath, 1994). Assuming a reasonable particle:infectivity ratio of 100:1, virus particle titers in blood may rival normal platelet counts (3×10^8/ml). Such parity between numbers of virus particles and platelets suggests that antibody-enhanced binding of virus to platelets may have a profound effect on platelets. Circulating virus-immune complexes are detected in DHF/DSS, and levels of immune complexes have been correlated with severity of disease (Ruangjirachuporn *et al.*, 1979) and some of these are platelet associated (Boonpucknavig *et al.*, 1979; Phanichyakarn *et al.*, 1977a). These observations suggest that sufficient binding of virus immune complexes to platelets may occur to tag the majority of circulating platelets. Such an event could lead to immune clearance by the reticuloendothelial system, thereby precipitating the thrombocytopenia frequently associated with severe dengue disease.

It is likely that molecules other than Fc receptors on the platelet surface may mediate antibody-enhanced binding of dengue virus (Wang *et al.*, 1995). Drug-induced thrombocytopenias provide interesting examples in this regard. It is known that given the appropriate accessory ligand (i.e., drug), IgG can bind to platelets through either the Fc receptor or other surface proteins. A variety of clinical thrombocytopenias are known that involve an immune component in pathogenesis. Many of these reflect activities of host antibodies, which react with proteins on the surface of platelets. These antibodies may be autoimmune in nature (i.e., antibodies that bind to platelet surface molecules) or dependent on a third party ligand (drug or protein), which then induces binding of the antibody–ligand complex to either the platelet Fcγ receptor or to another surface protein. For example, a number of individuals are susceptible to drug-dependent

thrombocytopenia when administered drugs such as heparin or quinine/quinidine (Aster, 1989; Hackett *et al.*, 1982). While heparin-dependent antibodies bind to the platelet Fcγ receptor (Adelman *et al.*, 1989; Chong *et al.*, 1989a, 1989b; Kelton *et al.*, 1988), quinine/quinidine-dependent antibodies bind to platelet protein heterodimers GPIIb/IIIa and GPIa/IX (Berndt *et al.*, 1985; Chong *et al.*, 1983; Christie *et al.*, 1987; Devine and Rosse, 1995). This latter category of immune-mediated thrombocytopenia may be relevant to the understanding of dengue-associated thrombocytopenia, as patient antibodies mediate dengue virus binding to platelets via a platelet surface protein other than the Fcγ receptor (Wang *et al.*, 1995).

Communication between platelets and endothelial cells is a frequent intermediate step in certain events such as platelet adhesion, aggregation, and regulation of vascular permeability. How this occurs in dengue infection and what the effects are on endothelial cell function are unknown. Binding of viruses to platelets can have potentially profound immunological effects [e.g., the stimulation of TGF-β release by platelets bound by Epstein–Barr virus (Ahmad and Menezes, 1997)]. In light of reports of altered platelet function in dengue patients, discussed earlier, there is a tantalizing need to determine the immunological consequences of antibody-enhanced dengue virus binding to platelets in terms of platelet as well as endothelial cell physiological responses.

Many products of complement activation can also be deposited on platelets (Devine, 1992). In view of evidence for complement activation in severe dengue disease (Halstead, 1989; Malasit, 1987), binding of complement products might play a role in the immune destruction of platelets leading to thrombocytopenia. Platelets display surface receptors, e.g., C1q receptor (Peerschke and Ghebrehiwet, 1987, 1998), membrane cofactor protein (Seya *et al.*, 1986), and decay-accelerating factor (Devine *et al.*, 1987), for specific components of complement activation. In addition, the platelet surface can act as a substrate for the deposition of C3dg and C5b-9 (Devine, 1992). Fragments of C3 have been detected on the platelets of DHF/DSS patients (Malasit, 1987).

In addition to immune complex deposition on platelets, thrombocytopenia associated with DHF/DSS might also arise by the immune destruction of platelets through antiplatelet autoantibodies. Antiplatelet autoantibodies have been reported in the sera of dengue patients (Lin *et al.*, 2001), although they have also been detected in patients recovering from a variety of viral infections (Imbach, 1994). Antiplatelet antibodies are strongly linked to the pathogenesis of immune-mediated thrombocytopenias, such as idiopathic thrombocytopenic purpura (Winkelstein and Kiss, 1997).

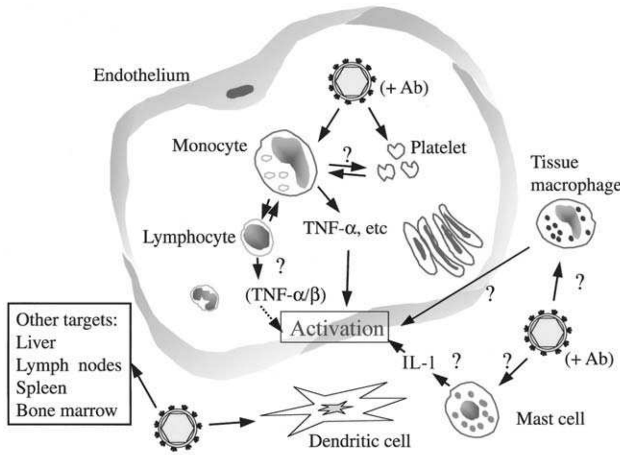

FIG 1. Model showing surface interactions of hemorrhagic flavivirus (dengue) with extra- and intravascular cell targets. Intravascularly, the presence of subneutralizing levels of antivirus antibodies stimulates virus attachment to platelets and infection of monocytes. This results in immune complex deposition on platelets and secretion of vasoactive factors from virus-infected monocytes. Among such vasoactive factors are cytokines, particularly TNF-α, which activates increased surface expression of adhesion molecules on endothelial cells. Extravascularly, virus infection of tissue macrophages, mast cells, and dendritic cells may result in the release of additional factors, which contribute to endothelial cell perturbation. (See Color Insert.)

H. Cell Targets: An Overview

While this brief discussion of cell targets for flaviviruses is by no means complete, it highlights some of the major interactions as they relate to pathogenesis. Because pathogenesis is probably best understood for dengue, Fig. 1 illustrates the interactions of hemorrhagic flavivirus (e.g., dengue) with cell targets both within and outside the vascular system.

IV. CELL SURFACE MACROMOLECULES INVOLVED IN FLAVIVIRUS ATTACHMENT

A. Glycosaminoglycans

Glycosaminoglycans and proteoglycans (i.e., proteins bearing glycosaminoglycans) are important cell surface molecules involved in a variety of ligand recognition and cell signaling processes (Gallo, 2000). Because glycosaminoglycans are widely distributed on cells,

they are attractive candidates as virus receptors. Some degree of specificity (i.e., virus tropism) may arise from the compositional heterogeneity of glycosaminoglycans, as well as quantitative differences in the degree of expression on various cell types.

Flaviviruses seem to share, with a large number of virus families, the ability to bind glycosaminoglycans (Birkmann *et al.*, 2001; Dechecchi *et al.*, 2000, 2001; Duisit *et al.*, 1999; Feldman *et al.*, 1999, 2000; Giroglou *et al.*, 2001; Goodfellow *et al.*, 2001; Heil *et al.*, 2001; Hsiao *et al.*, 1999; Hulst *et al.*, 2000, 2001; Lin *et al.*, 2000; Liu and Thorp, 2002; Patel *et al.*, 1993; Rue and Ryan, 2002; Shukla *et al.*, 1999; Shukla and Spear, 2001). Glycosaminoglycans such as heparin and its structural analogues have been investigated for their ability to bind dengue virus and thereby to gain insights as to the structural requirements for dengue receptors. Potential glycosaminoglycan-binding motifs have been identified on the dengue viral E protein at two sites, the best characterized of which appears to be composed of amino acids 188, 284–295, and 305–310 and which may also play a role in virus–cell attachment (Chen *et al.*, 1997). Heparin (minimum of 10 carbohydrates) and an uncharacterized highly sulfated heparin sulfate isolated from bovine liver were found to show the best binding to dengue E protein (Chen *et al.*, 1997). Attachment of dengue virus to human hepatoma cells has also been reported to be inhibited by heparin (Hilgard and Stockert, 2000). A further study involving a panel of natural and synthetic polyanionic, sulfated compounds suggested that binding of the dengue E protein required a highly sulfated (and highly charged) oligosaccharide with a minimum size of 39Å and a high degree of structural flexibility (Marks *et al.*, 2001).

The role of glycosaminoglycans in natural (i.e., nontissue culture-adapted) strains of flaviviruses needs to be studied further. It has long been recognized that dengue virus passaged in various host cell types can give rise to virus variants with altered cell specificity (Brandt *et al.*, 1979; Halstead *et al.*, 1984a, 1984b, 1984c). Passage-dependent mutations of the dengue virus E protein at a number of different amino acid residues have been documented (Lee *et al.*, 1997). Following passage of TBE virus in cultured BHK-21 cells, virus mutants were selected that contained more positively charged amino acids in the putative receptor-binding region of the E protein, resulting in dependence on cell surface heparan sulfate (Mandl *et al.*, 2001). Such mutants were diminished in their neurovirulence in mice as well as in their replication in primary chicken cells and plaque formation in porcine kidney cells (Mandl *et al.*, 2001). A large number of other viruses have also been shown to undergo loss of virulence upon adaptation to cell culture

associated with heparan sulfate utilization (Bernard *et al.*, 2000; Byrnes and Griffin, 2000; Klimstra *et al.*, 1998, 1999; Lee and Lobigs, 2000; Neff *et al.*, 1998; Sa-Carvalho *et al.*, 1997).

B. CD14

CD14 and the Toll-like receptor (TLR) pattern recognition receptors are involved in the innate response to lipopolysaccharide and other microbial products (Diamond *et al.*, 2000; Imler and Hoffmann, 2000). A role for CD14 and TLR4 has been found for respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000), suggesting that these receptors may have a broader involvement in host response than previously thought. A possible role for CD14 in dengue infection has been postulated on the basis of inhibition of dengue virus infection of human monocytes with bacterial lipopolysaccharide (Chen *et al.*, 1999). However, this has been disputed (Bielefeldt-Ohmann *et al.*, 2001) and requires further investigation.

C. Fc Receptors

As indicated earlier, flaviviruses are capable of initiating infection of appropriate host cells through as yet largely unidentified primary receptors. In addition, a number of flaviviruses are capable of using subneutralizing levels of virus-specific antibodies to attach to and gain entry to cells bearing Fc and/or complement receptors (Cardosa *et al.*, 1983; Halstead, 1982; Halstead and O'Rourke, 1977a; Schlesinger and Brandriss, 1981a) by a process known as antibody-dependent enhancement (ADE) of infection (Table I). ADE has been documented for dengue (Halstead *et al.*, 1980), West Nile (Peiris and Porterfield, 1979), yellow fever (Schlesinger and Brandriss, 1981b), tick-borne encephalitis (Phillpotts *et al.*, 1985) and Japanese encephalitis (Cecilia and Ghosh, 1988) viruses. Early work with dengue virus and monocytes differentiated between trypsin-sensitive and trypsin-resistant cell surface molecules as the putative receptors for antibody-independent and antibody-dependent infection, respectively (Daughaday *et al.*, 1981).

To date, dengue virus appears to be the only flavivirus in which strong evidence exists for antibody-dependent enhancement as a major contributing factor to severe disease (Halstead, 1980; Thein *et al.*, 1997). Severe dengue disease, encompasing conditions known as dengue hemorrhagic fever/dengue shock syndrome, involves several well-defined hemostatic abnormalities, including the leakage of

TABLE I
FcRs for Antibody-Enhanced Infection of Dengue Virus

Cell	FcγR[a]	Dengue virus replication		FcγR for ADE
		Ab independent	Ab enhanced	
Monocyte	I,II,III	Yes[b]	Yes[b]	I, II[f]
Dendritic cells	II	Yes[c]	No[c]	None
Mast cell/basophil	I,II	No[d]	Yes[d]	Unknown
Kupffer cell	I,II,III	No[e]	Unknown	Unknown

[a] Compiled from van de Winkel and Anderson (1991), Dijstelbloem *et al.* (2001), Okayama *et al.* (2000), Anselmino *et al.* (1989), and Tuijnman *et al.* (1993).
[b] From Halstead and O'Rourke (1977).
[c] From Wu *et al.* (2000) and Libraty *et al.* (2001).
[d] From King *et al.* (2000).
[e] Abortive infection, but expressing viral antigen (Marianneau *et al.*, 1999).
[f] From Littaua *et al.* (1990) and Kontny *et al.* (1988).

plasma into interstitial spaces, as well as thrombocytopenia and bleeding (Halstead, 1990; Kurane *et al.*, 1994). The potential to cause severe hemorrhagic disease is a general property of dengue viruses and is not limited to any one viral serotype (Gubler, 1998; Rigau-Perez *et al.*, 1998). Although different strains of dengue may influence the severity of hemorrhagic symptoms (Leitmeyer *et al.*, 1999; Rico-Hesse *et al.*, 1997), it is also generally accepted that pathogenesis depends on immunopathological processes (Rothman and Ennis, 1999). Thus the roles of prior immunity, antibody-enhanced virus infection, and immune-mediated pathologic effects on the vascular system are key points in understanding the pathogenesis of dengue hemorrhagic disease.

While the pathogenesis of severe dengue disease is not completely understood, it is clear from laboratory and epidemiological studies that a considerable risk factor is prior immunity. Severe dengue disease, DHF/DSS, rarely occurs in seronegative individuals suffering their first dengue infection, but instead occurs in individuals who have preexisting dengue viral antibodies, either from a previous infection or from passive antibody transfer, e.g., following maternal transmission of antibodies to the fetus (Kliks *et al.*, 1988, 1989). Estimates suggest that 99% of children suffering from DHF/DSS have preexisting immunity from a prior dengue virus infection (Halstead, 1988). Consequently, from this and other studies, it has been calculated that prior exposure to dengue increases the risk for hemorrhagic disease in a

second dengue infection by at least 15-fold (Halstead, 1980; Thein *et al.*, 1997). Preexisting serum antibodies can potentiate virus infection by the mechanism of antibody-dependent enhancement, giving rise to amplified virus replication and to an increased potential for the development of hemorrhagic symptoms (Halstead, 1989). Viremic titers are higher in secondary dengue infections in both humans (Gubler *et al.*, 1979) and experimental monkeys (Halstead *et al.*, 1973). Antibody-enhanced dengue virus infection of human blood monocytes is necessary for the production of endothelial cell activators (Anderson *et al.*, 1997), thereby providing a link between antibody-dependent enhancement and alteration of endothelial cell properties, which might contribute to vascular permeability in dengue infection.

For certain other viruses, e.g., influenza (Tamura *et al.*, 1993) and HIV (Takeda *et al.*, 1990, 1992), distinct "neutralizing" and "antibody-enhancing" epitopes have been identified on the respective viral attachment proteins. Surprisingly, no systematic approach has yet been undertaken to identify regions on the E protein that are essential for ADE, even though this issue was raised as a challenge to research on dengue many years ago (Halstead, 1988).

Human Fcγ receptors are currently categorized into three classes: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). While FcγRI shows high affinity for monomeric IgG, FcγRII and FcγRIII bind monomeric IgG poorly and are more likely involved in binding immune complexes (Dijstelbloem *et al.*, 2001). FcγRII is the most widely distributed, being expressed on most circulating leukocytes (van de Winkel and Anderson, 1991). Monocytes express all three FcγRs to varying degrees (van de Winkel and Anderson, 1991), although FcγRI and FcγRII predominate, whereas FcγRIII appears to be limited to a subpopulation ($\sim$10%) of monocytes (Anderson *et al.*, 1990; Passlick *et al.*, 1989). FcγRIII constitutes the major FcγR on macrophages (Fanger *et al.*, 1989), although FcγRI and FcγRII are also present (Tuijnman *et al.*, 1993; van de Winkel and Anderson, 1991). It is also important to recognize that FcR expression on cells, including macrophages, can vary depending on the microenvironment (Tomita *et al.*, 1994).

Although strong evidence exists for FcγR involvement in ADE of dengue virus, the participating FcγRs *in vivo* have not yet been identified rigorously. In cultured cell lines (monocytic U937 or erythroleukemic K562 cells), FcγRI (Kontny *et al.*, 1988) and FcγRII (Littaua *et al.*, 1990) have been shown to mediate ADE of dengue virus infection. That FcγRI has the ability to mediate ADE of dengue has been demonstrated using COS cells transfected with FcγRI (Schlesinger and Chapman, 1999).

Dengue and DHF patients show elevated serum levels of interferon (IFN)-γ (Kurane *et al.*, 1991). Because IFN-γ can upregulate both MHC class I and II molecules as well as FcγR (particularly FcγRI) expression in monocytes (Erbe *et al.*, 1990; Perussia *et al.*, 1983), the chances for ADE may be increased, thereby creating a vicious cycle involving positive cytokine feedback and virus amplification (Kurane and Ennis, 1992). IFN-γ has been shown to enhance ADE of dengue virus infection of human monocytic U937 cells (Kontny *et al.*, 1988), although any enhancing effect on dengue infection of peripheral blood monocytes may be negated by the antiviral properties of IFN-γ (Sittisombut *et al.*, 1995).

Mast cells and basophils express mainly FcγRII (Anselmino *et al.*, 1989; Okayama *et al.*, 2001a; Wedi *et al.*, 1996) and some (IFN-γ–inducible) FcγRI (Okayama *et al.*, 2000, 2001b) as well as the high-affinity FcεRI for IgE (Guo *et al.*, 1992; Sperr *et al.*, 1994). As noted previously, the basophil/mast cell KU812 cell line exhibits antibody-enhanced dengue virus infection and produces vasoactive cytokines (King *et al.*, 2000).

Although FcγR-mediated ADE of flaviviruses has been examined extensively as a mechanism for virus amplification, the biological consequences for the participating host cell are not well understood. Because FcγR-mediated cell signaling is complex, the functional effects of virus–antibody interactions with cell surface FcγRs need to be investigated. Monocytes infected with dengue virus in the presence of antibody release cytokines such as TNF-α (Anderson *et al.*, 1997). Induction of TNF-α requires infectious virus (Anderson *et al.*, 1997), suggesting that virus replication (or perhaps expression of one or more crucial viral genes) is responsible for the stimulation of TNF-α release. Therefore, in this case, the FcγR is likely facilitating antibody-enhanced virus replication rather than providing a signal triggered by virus binding to the FcγR. Similarly, antibody-enhanced dengue virus infection of KU812 basophil/mast cells produces IL-1β, IL-6 (King *et al.*, 2000, 2002), and selected chemokines (King *et al.*, 2002). Suppressive effects of antibody-enhanced flavivirus or alphavirus infection on monocyte cytokine secretion have also been reported (Lidbury and Mahalingam, 2000; Yang *et al.*, 2001).

Both activating (FcγRI, FcγRIIa, and FcγRIIIa) and inhibitory (FcγRIIb) forms of FcγRs exist, which mediate signal transduction via a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) or inhibitory (ITIM) motif, respectively (Dijstelbloem *et al.*, 2001). The ITAM and associated molecules are necessary for the endocytosis of FcR-bound immune complexes (Amigorena and Bonnerot, 1999) and

therefore play a likely role in the initiating events of antibody-enhanced flavivirus infection. Although not necessary for FcγRII, an accessory subunit (homo- or heterodimeric γ or ζ chains) is required for signaling through FcγRI and FcγRIIIa (Ravetch, 1994). A further FcγR (FcγRIIIb) lacks transmembrane and cytoplasmic domains and is instead anchored to the cell surface membrane via a glycosylphosphatidylinositol (GPI) linkage (Selvaraj *et al.*, 1988; Simmons and Seed, 1988). It apparently does not participate in signal transduction and has been speculated to sequester and accumulate immune complexes at specific sites on the cell surface (Huizinga *et al.*, 1988; Selvaraj *et al.*, 1988).

The roles of activating and inhibitory FcRs in viral ADE have not yet been ascertained. Activating FcRs are expressed on monocytes, macrophages, granulocytes, natural killer (NK) cells, and platelets but not on most lymphocytes (Dijstelbloem *et al.*, 2001). Inhibitory FcRs, however, are found on B cells, dendritic cells, and macrophages (Dijstelbloem *et al.*, 2001). Interestingly, ADE of dengue virus is best documented for monocytes/macrophages and related cell lines (Halstead, 1989). In contrast, lymphocytic cells (Brandt *et al.*, 1979; Kurane *et al.*, 1990) and dendritic cells (Wu *et al.*, 2000) do not appear to support antibody-enhanced dengue virus infection. Whether this is due to differential expression of activating versus inhibitory FcRs remains to be investigated.

FcRs for IgE (primarily the high-affinity FcεRI) are expressed on cells such as monocytes, macrophages, mast cells, basophils, and dendritic cells and are structurally related to FcγRs (Ravetch, 1994). Their role in binding IgE and/or immune-complexed flaviviruses, such as dengue, remains unexplored. Similarly unexplored is the potential role of the neonatal Fc IgG receptor (FcRn), structurally related to MHC class I and involved in IgG transport across cells (Ghetie and Ward, 2000). In addition to being expressed on certain epithelial and endothelial cells, FcRn is also expressed functionally on monocytes, macrophages, and dendritic cells (Zhu *et al.*, 2001).

D. Complement Receptors

In addition to the FcγR, the antibody-complexed flavivirus has been shown to be taken up by a macrophage cell line using the complement receptor-3 (Cardosa *et al.*, 1983). In the case studied—West Nile virus infection of mouse P388D1 macrophages—ADE was mediated by the presence of antiviral IgM and was inhibited with a CR3-blocking antibody. This mode of ADE was, however, found to be quantitatively less

productive than the more commonly studied route of ADE, i.e., involving FcγR-mediated uptake route of IgG–virus complexes (Cardosa *et al.*, 1983).

E. Virus Binding Proteins Identified on Cells

The recent demonstration of DC-SIGN as a functional dengue virus receptor on human dendritic cells represents an important advance in the definitive identification of flavivirus receptors (Navarro-Sanchez *et al.*, 2003; Tassaneetrithep *et al.*, 2003). Several studies have identified cell surface proteins that bind flaviviruses, generally assayed by virus overlay blots of SDS–PAGE-resolved cell proteins (Table II). Further work is required to confirm the involvement of these and other proteins as receptors in flavivirus infection.

V. Cell Surface Macromolecules Modulated by Flavivirus Infection

A number of flaviviruses are able to stimulate the expression of cell surface molecules. Notable among these are adhesion molecules and major histocompatibility antigens. Multiple mechanisms appear to be involved, including virus- and cytokine-dependent pathways.

A. MHC Class I

Flavivirus infection of a number of cell types causes an increase in cell surface MHC class I expression (King and Kesson, 1988; King *et al.*, 1989; Libraty *et al.*, 2001; Liu *et al.*, 1989; Lobigs *et al.*, 1996; Shen *et al.*, 1995a, 1997). Evidence for both virus-dependent (Lobigs *et al.*, 1996) and cytokine-dependent (Libraty *et al.*, 2001; Shen *et al.*, 1997) mechanisms has been reported. One process appears to be driven by the amount of flaviviral peptides generated by proteolysis and imported into the transporter associated with antigen processing (TAP), which results in increased cell surface expression of peptide-loaded MHC class I (Momburg *et al.*, 2001). The upregulation of MHC class I molecules by flaviviruses is perhaps reminiscent of that observed in infections by coronaviruses (Suzumura *et al.*, 1986) but stands in contrast to the virus-manipulated downregulation of MHC class I by viruses such as herpesviruses (Jennings *et al.*, 1985; Ploegh, 1998), adenoviruses (Sparer and Gooding, 1998), poxviruses (Boshkov *et al.*, 1992), and HIV (Scheppler *et al.*, 1989). Although enhanced

TABLE II
Flavivirus Binding Proteins on Cells

Cell	Virus	Binding protein(s)	Reference
Human erythroleukemic K562 cells	Dengue-2	100 kDa	Rothwell *et al.* (1996)
Human and mouse neuroblastoma cells	Dengue-2	65 kDa	Ramos-Castaneda *et al.* (1997)
Human monocytic, B and T cell lines	Dengue-2	32, 45, 72 kDa	Bielefeldt-Ohmann (1998); Bieldfeldt-Ohmann *et al.* (2001)
Monkey kidney Vero cells	Dengue-4	44, 74 kDa	Martinez-Barragan and del Angel (2001)
Mosquito C6/36 cells	Dengue-4	40, 45 kDa	Salas-Benito and del Angel (1997)
Mosquito C6/36 cells	Dengue-2	65, 80 kDa	Munoz *et al.* (1998)
Human hepatoma HuH-7 cells	Dengue-1	33- and 37-kDa proteoglycans	Hilgard and Stockert (2000)
Pig kidney PS cells	TBE	35 kDa	Kopecky *et al.* (1999)
Human dendritic cells	Dengue	DC-SIGN	Navarro-Sanchez *et al.* (2003) Tassaneetrithep *et al.* (2003)
Vero cells; mouse neuroblastoma cells	West Nile	105 kDa	Chu and Ng (2003)

MHC class I expression would be expected to lead to greater cytotoxic T (Tc) cell–mediated cytolysis, it would render cells less susceptible to recognition by NK cells. Evidence has been presented that flavivirus-infected cells in fact show reduced susceptibility to NK cells at the cost of enhanced Tc cell–mediated lysis (Lobigs *et al.*, 1996). It has been suggested that such a response may permit flaviviruses to evade an early NK cell response and thereby allow for substantial amplification of virus during the viremic phase of infection (Momburg *et al.*, 2001). Nevertheless, evidence shows that NK cells are activated during dengue infection (Green *et al.*, 1999a), and NK cell–mediated cytotoxicity has been reported to correlate with the severity of disease (Homchampa *et al.*, 1988).

Dendritic cells also undergo upregulation of MHC class I molecules following infection with dengue virus (Libraty *et al.*, 2001). Compared to other antigen-presenting cells, dendritic cells have superior T cell–stimulating activities (McKinney and Streilein, 1989; Timares *et al.*, 1998). Because antigen presentation via dendritic cell MHC class I can provoke exceptionally strong proliferation in CD8-bearing T cells (Bhardwaj *et al.*, 1994; Elbe *et al.*, 1994; McKinney and Streilein, 1989), much of the overall cytotoxic T cell response arising in flavivirus infection may be dictated at the level of the dendritic cell.

B. MHC Class II

West Nile virus infection induces MHC class II expression in mouse macrophages (Shen *et al.*, 1995a), mouse astrocytes (Liu *et al.*, 1989), rat Schwann cells (Argall *et al.*, 1991), and human myoblasts (Bao *et al.*, 1992). Upregulation of dendritic cell MHC class II occurs in response to dengue (Libraty *et al.*, 2001) and West Nile (Johnston *et al.*, 1996) virus infection. Given the potent ability of dendritic cells to activate T cells (Banchereau *et al.*, 2000), the communication between dendritic cell MHC class II–peptide complexes and recognition molecules on CD4-expressing T cells should provide insights into some of the molecular processes underlying T cell activation.

C. Adhesion Molecules

Adhesion molecules are expressed on a variety of cells and mediate a spectrum of processes (Ley, 2001; Roebuck and Finnegan, 1999; Springer, 1995). From the standpoint of flaviviruses, the most significant processes likely concern adhesion molecules on vascular endothelial cells, as these cells regulate permeability as well as

transendothelial migration of leukocytes (Springer, 1995). Of particular importance are intercellular adhesion molecule 1 (ICAM-1; CD54), vascular cell adhesion molecule-1 (VCAM-1; CD106), and E-selectin (CD 62E), which are upregulated on the surface of the endothelium by inflammatory cytokines, cellular stress, and virus infection (Roebuck and Finnegan, 1999).

In the case of dengue, activation of endothelial cells occurs *in vitro* via TNF-α released from antibody-enhanced dengue virus infection of monocytes (Anderson *et al.*, 1997). Such activation involves upregulation of adhesion molecules E-selectin, ICAM-1, and VCAM-1. Evidence that similar activation processes occur *in vivo* comes from clinical studies showing elevated serum levels of TNF-α (Green *et al.*, 1999b; Hober *et al.*, 1993; Vitarana *et al.*, 1991; Yadav *et al.*, 1991) and soluble VCAM-1 (Murgue *et al.*, 2001) in dengue- and DHF/DSS-infected patients. Surprisingly, serum levels of soluble ICAM-1 were actually found to be lower than those of control subjects, although this may reflect plasma protein loss through leakage (Bethell *et al.*, 1998). Moreover, the function of soluble forms of ICAM-1 remains unclear, and their expression appeazrs to be regulated differently from that of membrane-bound ICAM-1 (Komatsu *et al.*, 1997; van Den Engel *et al.*, 2000).

Two phases of ICAM-1 upregulation have been noted in West Nile and Kunjin virus infection of human embryonic fibroblasts, namely an early (~2 h postinfection) virus-dependent process and a later (~24 h postinfection) event that is mediated by type 1 interferon (Shen *et al.*, 1995b).

For neurotropic flaviviruses, such as West Nile virus in the mouse, the development of encephalitis has been correlated with viremia (Weiner *et al.*, 1970), suggesting virus penetration of the blood–brain barrier. The endothelium of the brain microvasculature normally represents a block between circulating virus and the central nervous system. Expression of endothelial cell adhesion molecules, thereby facilitating leukocyte adherence and diapedesis through the endothelium, may be an important mode of dissemination of virus-infected monocytes or other leukocytes into the brain. West Nile virus infection of human endothelial cells causes the upregulation of E-selectin, ICAM-1, and VCAM-1 (Shen *et al.*, 1997), which could mediate the transendothelial migration of leukocytes. Upregulation of these adhesion molecules was observed to occur early (2–4 h) in infection and appeared to be triggered by the virus rather than by cytokines (Shen *et al.*, 1997).

Further studies are required to clarify the role of endothelial cell adhesion molecule expression in the neuroinvasion of certain flaviviruses. Assuming such a role is confirmed, it will be incumbent to identify the mechanisms by which either free or cell-borne flaviviruses

are stimulated to cross the vascular endothelial layer. For virus-infected leukocytes, such stimulation likely arises, at least in part, from chemokines produced by cells of the central nervous system. Astrocytes infected with JE virus have been reported to release chemokines (RANTES and MCP-1), which may play a role in the transendothelial migration of leukocytes (including those possibly carrying virus) across the blood–brain barrier (Chen *et al.*, 2000). Thus, once neural infection is initiated, the process could be amplified by the production of leukocyte-attracting chemokines at the site of infection.

VI. Other Cell Surface Macromolecular Modifications Triggered by Flavivirus Infection

A. Complement Deposition

Complement activation is well documented in dengue disease (Nishioka, 1974; Phanichyakarn *et al.*, 1977b; Russell *et al.*, 1969), with peak activation and the production of C3a and C5a occurring at the time of vascular leakage and/or shock (Malasit, 1987). Complement activation is likely to be largely mediated by immune complexes consisting of IgG and virus (Bokisch *et al.*, 1973a, 1973b; Shaio *et al.*, 1992; Sobel *et al.*, 1975), although the low levels of circulating immune complexes detected in patients have stimulated thought as to other possible mechanisms (Malasit, 1987). Receptors for C3a and C5a are found on a wide variety of cells, including many human peripheral blood leukocytes (Chenoweth and Hugli, 1978; Fureder *et al.*, 1995; Kretzschmar *et al.*, 1993; Nilsson *et al.*, 1996; van Epps and Chenoweth, 1984). C5a receptors have been reported on endothelial cells, although at lower levels than myeloid cells (Zwirner *et al.*, 1999).

Although endothelial cells do not appear to be major targets for dengue virus *in vivo* (Halstead, 1988, 1989; Sahaphong *et al.*, 1980), endothelial cells infected with dengue virus *in vitro* can become a substrate for deposition of C3dg and C5b-9, provided the dengue antibody is present (Avirutnan *et al.*, 1998). The presence of complement activation products on the endothelial cell surface could be a contributing factor to vascular permeability (Saadi *et al.*, 1995). Furthermore, anaphylotoxins and/or deposition of sublytic C5b-9 on the endothelial cell surface has the potential to activate the expression of adhesion molecules (Foreman *et al.*, 1994), cytokines (Saadi *et al.*, 2000), chemokines (Selvan *et al.*, 1998), cyclooxygenase-2

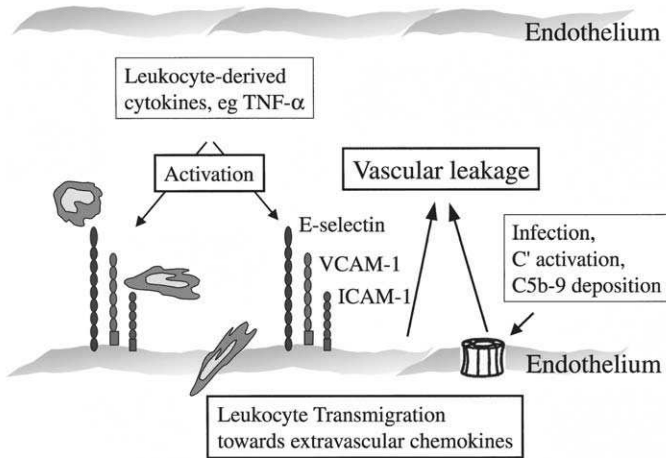

FIG 2. Model depicting possible events in endothelial cell surface perturbation during hemorrhagic flavivirus (dengue) infection. Endothelial cell activation, leading to upregulation of adhesion molecules (E-selectin, VCAM-1, ICAM-1), can be triggered by monocyte-derived cytokines (Anderson *et al.*, 1997) or by deposition of C5b-9 and other products of complement activation (Avirutnan *et al.*, 1998). C5b-9 is represented as a membrane attack complex pore structure, although the deposition of C5b-9 on dengue-infected cells appears associated with sublytic, rather than lytic, responses (Avirutnan *et al.*, 1998). Increased adhesion molecule expression, along with uncharacterized vasoactive factors, can lead to endothelial leakage and can mediate rolling, adhesion, and transendothelial migration of leukocytes into extravascular tissues. Similar processes may also contribute to the invasion of cell-borne neurotropic flaviviruses through the endothelial blood–brain barrier. (See Color Insert.)

(Bustos *et al.*, 1997), tissue factor (Saadi and Platt 1995), heparan sulfate proteoglycan proteinases (Ihrcke and Platt, 1996), and even functional or morphological changes such as permeability loss and gap formation (Saadi *et al.*, 1995).

Thus, in addition to being activated by leukocyte-derived cytokines (Anderson *et al.*, 1997), endothelial cells may also be coaxed toward a more permeability-enhancing state by virus infection and virus-mediated complement deposition. At present, the lack of evidence for *in vivo* infection of endothelial cells by dengue virus would suggest that the cytokine-mediated pathway is dominant. Figure 2 shows a model illustrating the potential role of endothelial cell perturbation by monocyte-derived cytokines and complement activation products in initiating vascular permeability and leukocyte extravasation in severe hemorrhagic flavivirus disease.

VII. Conclusions

Much remains to be learned about the primary receptors for flaviviruses, though much knowledge has been gained about the initial interactions of flaviviruses with cell surface structures. The ability of flaviviruses to affect cell entry through heparan sulfate–type proteoglycans, as well as their dexterity to adjust mutationally to different receptors, depending on host cell type, illustrates the plasticity of the viral E protein to adapt to changing conditions and to ensure successful virus replication. Beyond this, certain flaviviruses, notably dengue virus, are masters at exploiting host antibody and Fc receptor–bearing cells to dramatically amplify viral replication. Flavivirus replication is coupled to altered cellular expression of cytokines, chemokines, and cell surface molecules, which shape the host response and immunopathogenesis associated with flavivirus infections. Ongoing and future characterization of the cell surface structures that mediate these events will be helpful in understanding the mechanisms of flavivirus-induced disease and in developing therapeutic and/or preventive strategies.

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FLAVIVIRUS EVOLUTION

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ORIGINS, EVOLUTION, AND VECTOR/HOST COADAPTATIONS WITHIN THE GENUS *FLAVIVIRUS*

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I. Introduction
II. Phylogenetic Analysis of the Genus *Flavivirus*
III. Impact of Host/Virus Coadaptation on Flavivirus Evolution
  A. Nonvectored Viruses
  B. Tick-Borne Flaviviruses
  C. Mosquito-Borne Flaviviruses
IV. What Are the Origins of Flaviviruses?
V. Does Recombination between Flaviviruses Occur and, if so, What Is the Likely Impact?
References

Although viruses in the genus *Flavivirus* share complex antigenic interrelationships, they can be divided into four phylogenetic/ecological groups: two mosquito-borne groups, a tick-borne group, and nonvectored viruses. These divisions largely reflect the selective constraints imposed on the viruses by the vertebrate hosts, the invertebrate vectors, and the associated ecologies. Phylogenetic trees based on the flavivirus genetic sequence show characteristic branching patterns that reflect these groupings. This review describes the evolution and possible origins of individual flaviviruses, correlating ecological and epidemiological characteristics with their phylogenies and geographic dispersal. It will also become apparent that many of the phylogenetic lineages that define species diverged relatively recently, and the

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subsequent dispersal and epidemiology of these viruses have therefore been significantly influenced by increasing human population densities and activities such as recreation, urbanization, land reclamation, transportation, and deforestation. This review also considers some of the likely implications of persistent/chronic infections in relation to virus dispersal and recombination between related flaviviruses on phylogenetic analysis and vaccine development strategies.

I. Introduction

Viruses in the genus *Flavivirus* differ from other members of the family *Flaviviridae* in their antigenic, ecological, and epidemiological characteristics. Many of the flaviviruses have been shown to infect both vertebrate and invertebrate species, a feature not common to viruses in other genera of the family, i.e., *Pestivirus* and *Hepacivirus* or indeed cell-fusing agent (CFA) and Tamana bat virus (TBV), both of which are tentative species in the genus *Flavivirus*. Currently, 73 flaviviruses have been assigned individual identities either as species, of which there are 50, or subtypes, of which there are 23. These viruses have been classified into 12 groups largely on the basis of phylogenetic analysis of sequences representing the nonstructural NS5 gene (Heinz *et al.*, 2000). Their approximate geographic distribution is shown in Fig. 1. Publications show that the phylogenetic trees are very similar when constructed using sequence data derived from genes encoding the NS5 protein, the NS3 protein, the envelope (E) protein, or the entire genome (Billoir *et al.*, 2000; Charrel *et al.*, 2001; Gaunt *et al.*, 2001; Kuno *et al.*, 1998; Marin *et al.*, 1995; Zanotto *et al.*, 1995). Subdivision of the viruses within the 12 main groups also takes into account their known antigenic relationships (Calisher, 1988; Porterfield, 1980), the type of disease they cause, the type of vector and host with which they are known to be associated, their ecological characteristics, and their geographic distribution (Karabatsos, 1985; Theiler and Downs, 1973). Undoubtedly, as more isolates are identified and more sequence data become available, there will be additional viruses and changes of the positions of some viruses in the current ICTV scheme of classification (Heinz *et al.*, 2000). For example, on the basis of recently published phylogenetic trees (Gaunt *et al.*, 2001) and also shown in Fig. 2, the Kadam virus (KADV), which is currently grouped with mammalian tick-borne viruses, might be placed more appropriately in the seabird tick-associated virus group, i.e., together with the Tyuleniy virus (TYUV), Saumarez Reef virus (SREV), and Meaban virus (MEAV), in

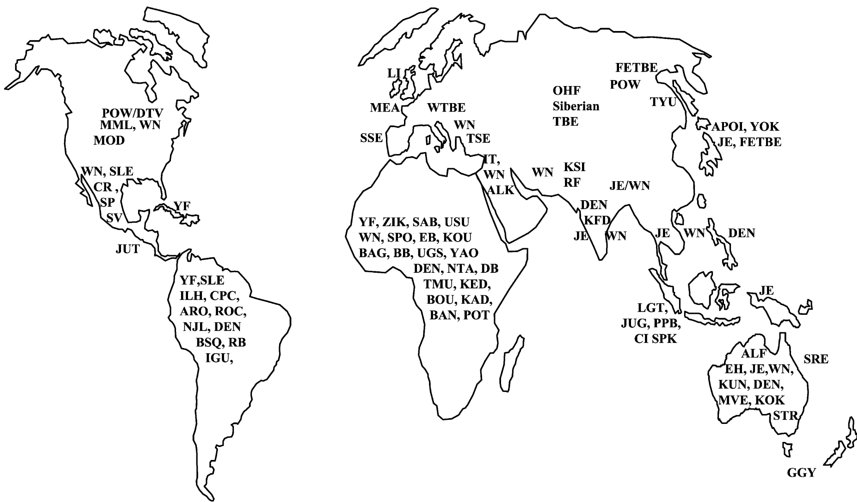

FIG 1. World map illustrating the approximate geographic location, by land mass, of the recognized flaviviruses. Some viruses are shown more than once to emphasize their wide geographic distribution.

a strongly supported phylogenetic group. Moreover, in the NS3 gene, KADV has the seabird amino acid signature in the DEAH box. However, independent analysis of genetic distances in the E, NS3, or NS5 genes of a representative group of flaviviruses infers that KADV constitutes a third group in addition to the mammalian and seabird groups (X. de Lamballerie, unpublished observation). Clearly more sequence data will be required to resolve this. Other apparent discrepancies in the current scheme of classification are (1) the positioning of St. Louis encephalitis virus (SLEV) in the Japanese encephalitis virus (JEV) group and (2) the positioning of Kedougou virus (KEDV), Spondweni virus (SPONV), and Zika virus (ZIKV), each of which will need to be reconsidered now that more data are available. In some cases, these discrepancies reflect the antigenic relationships of the viruses in plaque reduction neutralization and hemagglutination-inhibition tests rather than phylogenetic relationships, around which this review will focus. Nevertheless, such apparent deficiencies in the classification scheme are minor and the phylogenetic tree presented in this review provides sufficient robust information for us to be able to interpret the evolutionary characteristics of the flaviviruses. We can also predict, with reasonable certainty, the geographic origins and subsequent dispersal patterns of most of the recognized viruses in the genus, taking into account their associated vector–host coadaptations.

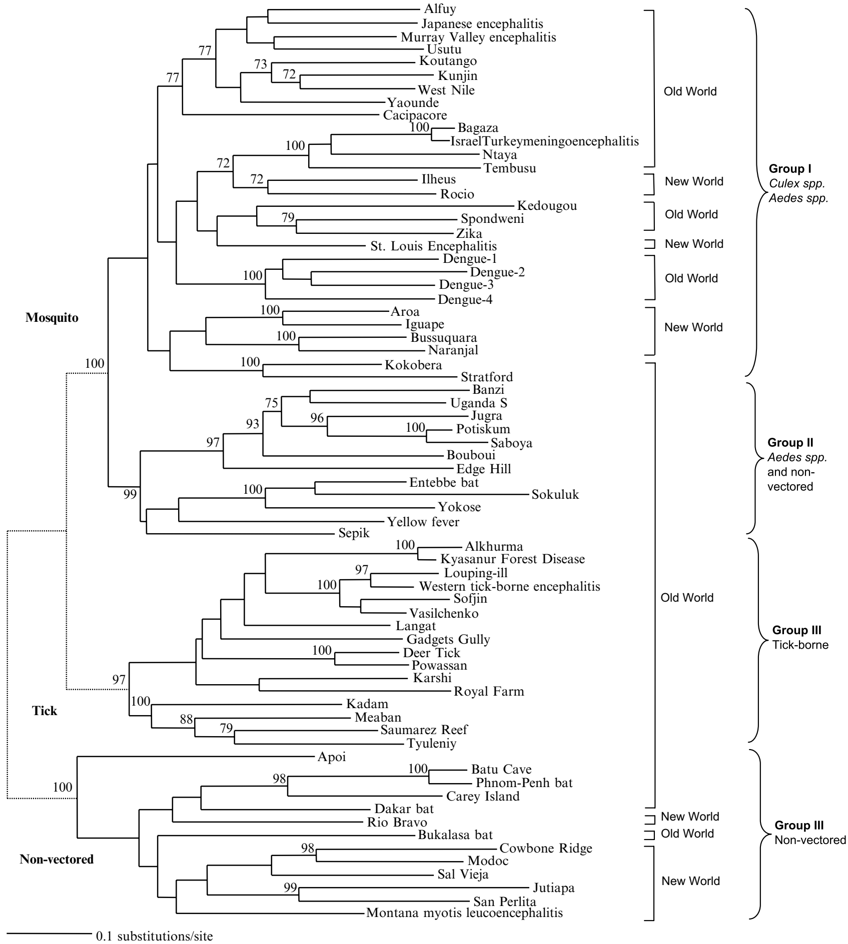

FIG 2. Maximum likelihood (ML) phylogenetic tree of NS5 nucleotide sequences (990 bp) from 70 flaviviruses. Because there are major differences in base composition among the three major groups of viruses (tick-borne, mosquito-borne, no-known vector; Jenkins *et al.*, 2001) and because this affects phylogenetic accuracy, trees inferred for these groups were estimated separately and then joined in a final phylogeny in which all branch lengths were reoptimized. A highly variable region of 18 amino acids where the alignment was uncertain was also removed prior to analysis. All trees were constructed using the general time-reversible (GTR) model of nucleotide substitution, allowing each codon position to have a different rate of change. Bootstrap values (from 1000 replicate neighbor-joining trees estimated under the ML substitution model) are shown where >70%. All analyses were undertaken using the PAUP* package (Swofford, 2002). The tree is midpoint rooted for purposes of clarity only and all horizontal branch lengths are drawn to scale.

II. Phylogenetic Analysis of the Genus *Flavivirus*

Flaviviruses are known to occur on every continent other than Antarctica, where they may well have been introduced many times but have either remained undetected or failed to become established due to the unfavorable environmental conditions. Individual species have distinct geographic distributions that reflect very closely (1) the different vertebrate and/or invertebrate hosts they infect, (2) the ecology of the natural habitat in which they are found, (3) the climatic conditions that pertain to their distribution and, in more recent times, (4) the impact of human urbanization and transportation on their dispersal. Data describing the geographic locations where each virus was first isolated, their antigenic characteristics, the vertebrate and invertebrate hosts with which they are associated, their pathogenicity, and their taxonomic status have been published previously (Heinz *et al.*, 2000; Karabatsos, 1985; Theiler and Downs, 1973).

Early investigations of the biological and antigenic characterization, as well as the nucleotide sequence of the flaviviruses, enabled the following conclusions to be drawn.

- The antigenic and phylogenetic relationships between flaviviruses correlate quite closely (Mandl *et al.*, 1988).
- Viruses within the mammalian tick-borne virus group (in old terminology, referred to as the TBE complex viruses) shared greater sequence identity than that measured between mosquito-borne flaviviruses, leading to the proposition that tick- and mosquito-borne flaviviruses had been subjected to different selective constraints during their evolution (Shiu *et al.*, 1991).
- The genus *Flavivirus* was monophyletic (Venugopal *et al.*, 1994).
- Tick- and mosquito-borne viruses represented separate phylogenetic lineages (Shiu *et al.*, 1991; Venugopal *et al.*, 1994).
- The close antigenic and phylogenetic relationships and the characteristics of their geographic dispersal led to the proposal that TBE complex viruses had evolved in a continuous or gradual manner across Asia and Europe (Gao *et al.*, 1993), apparently contrasting with the mosquito-borne viruses, which appeared to have evolved in a more discontinuous fashion.
- Phylogenies relating to dengue viruses, constructed on the basis of different genes within the genome were congruent (Blok *et al.*, 1992), implying that recombination has not played a significant part in the evolution of flaviviruses. This interpretation is reconsidered in this review, as it has now been demonstrated

convincingly that recombination does occur among closely related dengue viruses, strains of JEV, and strains of SLEV (AbuBakar *et al.*, 2002; Holmes *et al.*, 1999; Tolou *et al.*, 2001; Twiddy and Holmes, 2003; Uzcategui *et al.*, 2001; Worobey *et al.*, 1999).

Prior to 1995 there had been only limited phylogenetic analyses of viruses in the genus *Flavivirus*, but from the details provided earlier, it was already clear that tick- and mosquito-borne viruses had diverged relatively early in their evolutionary history; subsequently, more detailed phylogenies (Marin *et al.*, 1995; Zanotto *et al.*, 1995, 1996b) confirmed that they had distinctly different evolutionary characteristics. Viruses in the mammalian tick-borne virus group were shown to have evolved in a progressive fashion across the northern hemisphere, which was described as "clinal" evolution because there was a direct correlation between genetic and geographic distance between the viruses (Zanotto *et al.*, 1995).

Phylogenetic trees that compared mosquito- and tick-borne flaviviruses showed this as an asymmetric (i.e., ladder-like) topology in which tick-borne viruses had apparently branched continuously through time. This contrasted sharply with the more balanced (pectinate) branching structure of mosquito-borne flaviviruses in which there were relatively long periods of time (and presumably loss of lineages) between the intense cladogenesis, thereby supporting a "boom and bust" model of evolutionary change (Zanotto *et al.*, 1996b). Estimation of the relative degree of amino acid divergence between tick- and mosquito-borne flaviviruses led to the additional conclusion that mosquito-borne viruses had evolved approximately 2.5 times faster than tick-borne viruses (Zanotto *et al.*, 1995). This can be explained largely by the fact that ticks known to be capable of transmitting flaviviruses have a relatively long life cycle, lasting from 2 to 5 years during which the tick takes only three blood meals. These blood meals precede the change from larvae to nymphs, from nymphs to adults, and the period when the adult lays her eggs. If a larva becomes infected, it remains infected throughout the complete life cycle and may transmit the virus through the egg to the next life cycle. The efficiency of transovarial transmission appears to be very low (significantly less than 1%). Nevertheless, because adult ticks lay thousands of eggs, this is probably a significant mechanism for the long-term survival of tick-borne flaviviruses. Because the level of virus infectivity in the tick remains relatively low, the overall effect of this protracted life cycle will be to constrain virus turnover. Moreover, transmission of tick-borne flaviviruses between ticks occurs when they cofeed on vertebrate hosts that

may not even develop a viremia. Thus, while the vertebrate host is important for virus transmission between ticks, it probably plays a small role in total virus turnover and generating genetic variation. In contrast, mosquitoes feed several times during their relatively short life cycle and virus titers increase rapidly to high levels in the mosquito and vertebrate host before they transmit the virus to the next mosquito through the viremic host. Thus, for mosquito-borne flaviviruses, the entire process is far more dynamic (i.e., a greater number of replication cycles) and presents a greater opportunity for genetic variation in the vertebrate host. No-known-vector (NKV) viruses have not been studied in as much detail, but because they do not appear to produce high levels of viremia in their natural hosts, it seems likely that their rate of turnover would be relatively low compared to mosquito-borne viruses. The phylogenetic trees seem to support this concept.

As more sequence data became available (Kuno *et al.*, 1998), it became clear that most of the NKV viruses had diverged distinctly from tick- and mosquito-borne viruses, although three bat-associated NKV viruses—Yokose virus (YOKV), Entebbe bat virus (EBV), and Sokoluk virus (SOKV)—were grouped within the mosquito-borne viruses, indicating that they have lost vector transmission secondarily.

However, it was still not clear from the phylogenetic trees which of the three distinct groups (tick-borne, mosquito-borne, or NKV) was the most divergent. Although midpoint-rooted trees (which assume a molecular clock) depict the NKV as the most divergent, this branching order cannot be resolved without the use of adequate outgroups.

Figure 2 depicts a maximum likelihood phylogenetic analysis of most recognized flaviviruses based on partial NS5 gene sequence data, allowing a different rate of change for each codon position. The Alkhurma virus (ALKV), which was isolated in Saudia Arabia (Charrel *et al.*, 2001), is not yet officially classified, but is genetically most closely related to the Kyasanur Forest disease virus (KFDV) and will probably be classified as a subtype of KFDV. A partial envelope gene sequence of a second unclassified virus, Wesselsbron virus (WSLV), which is found in Africa and Thailand, is now available (unpublished observations) and its genetic similarity with Yellow fever virus (YFV) suggests that it will be included in the clade that contains YFV. Moreover, the complete sequence of TBV has been determined (de Lamballerie *et al.*, 2002), and phylogenetic data imply that TBV is a distantly related nonvectored virus. This supports evidence that TBV does not cross-react antigenically with any of the recognized flaviviruses but shares several similar characteristics with them (Price, 1978).

Several features of the maximum-likelihood tree (Fig. 2) merit highlighting.

- NKV and tick- and mosquito-borne viruses diverged early in the evolution of the genus *Flavivirus* and possibly in that order of divergence. This observation is based on the rooting of the tree by adding CFAV and TBV as outgroups for the entire genus (data not shown). Independently, the same root position was found by midpoint rooting (data not shown) in the absence of the two outgroups. In this case the root was placed at the middle of the longest branch of the tree, which also indicated that the NKV group radiated before the split of the mosquito and tick virus groups. Moreover, this root position supports our classification into four monophyletic groups (groups I to IV), as it does not fall within one of the ingroups in a branch leading to a terminal taxon (e.g., in the branch leading to APOIV within group IV).
- Some NKV viruses (SOKV, YOKV, and EBV) diverged with mosquito-borne viruses, i.e., diverging distinctly from the remaining NKV viruses and these viruses then diverged from mosquito-borne viruses. According to Fig. 2, YFV occupies a basal lineage to these three NKV; however, there is no bootstrap support for this position. Previous NS5 trees and other trees based on the E gene or the NS3 gene place these NKV viruses basal to the YFV lineage. It is difficult to decide which of these opinions represents the most robust interpretation, but more sequence data and future analyses should resolve this problem.
- Both of the rooting methods referred to earlier (i.e., the use of outgroups and midpoint rooting) suggest that the most divergent NKV virus is Apoi virus (APOIV), which was isolated from rodents in Hokkaido. Two distinct lineages then diverged to form a rodent NKV clade, all the viruses of which occur only in the New World, and a bat NKV clade, which contains viruses in both the New and the Old World. The precise position of Bukalasa bat virus (BBV) and Montana myotis leucoencephalitis virus (MMLV) remains to be determined, as the bootstrap figures were too low to assign them with any certainty. Previous trees assigned them to the bat clades.
- Tick-borne viruses diverged to two distinct evolutionary lineages, one of which contained the seabird tick-borne viruses that are normally associated with *Ornithodorus* spp., but the most divergent virus in this clade is KADV, which is found in Africa and is normally associated with herded livestock and *Rhipicephalus appendiculatus*. The position of KADV, which is ecologically distinct

from the other seabird-associated viruses, implies that there may be more viruses to be identified in this part of the tree. The other clade contains the mammalian tick-borne virus group, many of which are normally associated with rodents and *Ixodes* spp. in forest environments.

- The most divergent mosquito-borne viruses are all found in the Old World and are designated group II viruses. They are associated primarily with *Aedes* spp. and some of them produce hemorrhagic disease in primates.
- A subsequent divergence split the viruses into other Old World clades containing *Aedes* spp.–associated viruses, including viruses known to cause hemorrhagic disease and clades of viruses that are associated primarily with *Culex* spp. and encephalitic disease. These *Culex*-associated viruses, most of which infect birds, form related groups in either the Old World or the New World.
- Phylogenetic data imply that *Aedes* and *Culex* spp. viruses in group I emerged after *Aedes* spp. viruses in group II and therefore are rooted in the Old World.
- Some viruses show unusually long branch lengths compared with the related viruses. Assuming there are no sequence errors, this suggests that flaviviruses show some variation in their rates of evolutionary change. For example, in many published trees, the dengue type 2 (DENV-2) virus has a longer branch length than the other dengue serotype viruses, which could be explained by the fact that DENV-2 viruses have evolved more than the other dengue serotypes because they have dispersed more widely and caused more major epidemics, i.e., their turnover has been more extensive. This is not as clear in Fig. 2 as it is in some previously published trees, which also show a different order of evolution for the four dengue serotypes (Billoir *et al.*, 2000; Gaunt *et al.*, 2001; Gould *et al.*, 2001).

III. Impact of Host/Virus Coadaptation on Flavivirus Evolution

Phylogenetic data clearly illustrate how the adaptation of each virus to specific vertebrate and invertebrate hosts influences virus evolution, dispersal, epidemiology, and possibly even pathogenesis of the flaviviruses. While similar conditions may prevail with other animal, plant, insect, or bacterial viruses, we know of no other genus where correlations between so many parameters have appeared so distinct.

A. Nonvectored Viruses

Relatively little is known about the NKV viruses compared with arthropod-transmitted flaviviruses. Nevertheless, there are clear indications from the phylogenetic tree that their emergence and dispersal have been dictated largely by the rodents or bats with which they are associated. It is likely that the success of the NKV viruses in nature reflects the fact that rodents and bats aggregate in large numbers, providing excellent conditions for transmission between these hosts. While it has to be admitted that the number of isolates of individual NKV viruses is limited, current evidence suggests that many of these viruses occupy distinct geographic areas, presumably being largely dependent on the particular rodent or bat species with which they are associated. As shown later, this contrasts with mosquito-borne viruses, many of which have wide geographic distributions. Despite this lack of detailed studies, evidence supports the notion that at least some of the bat-associated NKV viruses may effectively remain dormant in bats for long periods of time (Sulkin, 1962; Sulkin *et al.*, 1964). Moreover, rodents or bats from which NKV viruses are isolated appear healthy at the time of virus isolation, suggesting that the virus infects the host persistently. If this is the case, the turnover of these viruses would be expected to be relatively low, as persistent infections are generally characterized by low-level virus replication. One might even speculate that if any flaviviruses coevolve with their hosts, the NKV viruses are the most likely candidates, although this is not supported by the time scale of flavivirus evolution.

Apart from APOIV, the other branch of this basal node gave rise to two clades: one occupied exclusively by rodent-associated viruses (and one bat-associated virus) in the New World and the other occupied exclusively by bat-associated viruses in either the New or the Old World, but so far not one of them is found in both the New World and the Old World. It is interesting that the three NKV viruses that diverged with mosquito-borne viruses are all Old World viruses associated with bats, i.e., only rodent-borne NKV viruses are exclusively New World viruses.

B. Tick-Borne Flaviviruses

Although tick-borne viruses have a common ancestor, they diverged early to form two distinct clades, determined by the combination of arthropod and vertebrate host with which they are most often associated. Most of the tick-borne flaviviruses can infect and be transmitted by several different tick species, even though they may be associated

most commonly with one species. For example, far eastern Asian strains of TBEV have been isolated from 18 different tick species (Smorodintsev and Dubov, 1986; Zlobin and Gorin, 1996). This explains, at least in part, how such viruses are able to occupy successfully such wide geographic regions of the northern hemisphere. It is also worth noting that the Gadgets Gully virus (GGYV), which is a member of the mammalian tick-borne virus group, was isolated from *Ixodes uriae*, a tick species associated with seabirds, suggesting that it has retained the ancestral condition.

The Powassan virus (POWV) has been isolated from several different types of tick, including *Ixodid* spp., and also mosquitoes, including *Culex* spp. (Professor Lvov, Moscow, personal communication). The association of POWV with this wide range of arthropods could partly explain the relatively wider geographic dispersal (New World and Old World) of this virus compared with most of the other tick-borne flaviviruses (Old World only). It is interesting that POWV is found in the same region of Russia, the Primorsk region, as the far eastern subtype of TBEV; they overlap ecologically in Russia. It is estimated to be responsible for between 1% and 4% of all TBE cases in the Primorsk region (Leonova *et al.*, 1991). However, in Canada, the United States, and Central and South America, only the POWV lineage is recognized. Perhaps the most logical explanation for this is that POWV was introduced into the New World from the Old World relatively recently, i.e., after the TBEV group emerged in the Old World.

Seabird-associated tick-borne flaviviruses, i.e., SREV, MEAV, and TYUV, are found commonly in ornithophilic ticks under small rocks, boulders, and stones on islands frequented by migratory seabirds and penguins. It is presumed that they have been introduced to these often uninhabited and remote islands by the seabirds, some of which fly enormous distances annually from the northern to the southern hemisphere. It seems most likely that related viruses will eventually be shown to be present on many equivalent islands on the routes flown by the migratory birds. It is important to note that the three Arch Rock virus, which was isolated from *I. uriae* off the coast of Oregon in the United States, is shown in Fig. 2 as TYUV. These viruses are known to be antigenically very similar and, until a Russian strain of TYUV has been sequenced, are assumed to be strains of the same virus. The Tyuleniy virus was isolated from Tyuleniy island, off the coast of north eastern Russia, but antigenically closely related viruses have also been found on other islands in the same region of Russia.

Viruses in the mammalian tick-borne virus group comprise a continuous evolutionary lineage or cline (Zanotto *et al.*, 1995, 1996a). On

the basis of its antigenic properties, POWV is found in far eastern Russia and North America, including both Canada and the United States. The strain for which sequence data are available (Mandl *et al.*, 1993) was isolated in Ontario, Canada, from a child with encephalitis, and although clinical cases are not too common, occasional outbreaks occur among individuals exposed to questing forest ticks in endemic regions. A genetically closely related subtype of POWV virus, provisionally designated deer tick virus (DTV), was isolated from ticks collected in coastal New England (Telford *et al.*, 1997). In the New World, POWV and DTV appear to have established in different ecological niches (Ebel *et al.*, 2001). Whereas sequences for the lineages recognized as POWV were all obtained from viruses isolated in New York or Canada and were associated with a groundhog *I. cookei* cycle, lineages corresponding to the strains of DTV were all obtained from viruses isolated either on the Atlantic Coast of the United States or from Wisconsin and were primarily associated with deer ticks and the white-footed mice upon which they feed most frequently. In Russia, POWV has been isolated from *Haemaphysalis neumanii*, *Anopheles hyrcanus*, *Aedes togoi*, humans, and small rodent species. The phylogenetic tree (Fig. 2) shows that POWV is genetically relatively closely related to GGYV virus, which was isolated from *I. uriae* found in the penguin rookeries under rocks or in tussock grass (*Poa foliosa*) on Macquerie Island, in the southern Pacific Ocean south of Tasmania. GGYV has never been associated with clinical disease in humans, possibly because few humans have ever been exposed to the virus. Based on the fact that the Storm Petrel (*Oceanites oceanicus*) breeds in the antarctic and migrates to the arctic, whereas the migratory pattern of the arctic tern (*Sterna paradisaea*) is the reciprocal of this, it was suggested that GGYV could represent a natural link between subarctic and subantarctic tick-borne flaviviruses (St. George *et al.*, 1985).

The Kyasanur Forest disease virus, one of the next viruses in the mammalian tick-borne virus lineage, is found almost exclusively in or at the edge of the Kyasanur Forest in the Shimoga district, Karnataka (then Mysore) state of India (Work *et al.*, 1959; Work and Trapido, 1957). The virus was discovered in 1957 when an unusual number of monkeys were found dying with a disease that resembled hemorrhagic fever in humans. Some of the investigators, exposed to these dead and dying monkeys, also developed fatal hemorrhagic disease. The outbreak in monkeys and humans may have arisen as the result of deforestation, intended to extend the building and grazing land for villagers and local farmers. It has been suggested that monkeys, which normally spent most of their time in the forest trees, increasingly came

down to the ground and roamed the cleared areas, foraging for food scraps left by humans. This exposed the monkeys to KFDV-infected ticks in the undergrowth (Venugopal *et al.*, 1994). The most abundant tick species in this region is *Haemaphysalis spinigera*, but the virus has also been isolated from seven other different species of *Haemaphysalis*, *Dermacentor*, and *Ixodes* ticks. It seems reasonable to assume that KFDV has circulated among the indigenous forest tick and rodent species for at least a few centuries and causes disease in humans only rarely. Estimates of the time of divergence between ALKV and KFDV based on previous publications (Zanotto *et al.*, 1996b), and assuming a molecular clock, would be about 500 to 600 years ago. Presumably, KFDV and other strains/subtypes of this virus exist in other parts of India but are not recognized either because they do not cause clinical disease in humans or because the level of exposure to humans is low. Indeed, this seems quite likely, because a genetically closely related virus, Alkhurma virus (ALKV), was isolated from the blood of several humans with severe hemorrhagic fever in Saudi Arabia in 1995 (Zaki, 1997). Since that time, 16 cases have been confirmed, of which 4 have died. Complete genome sequence data and phylogenetic analysis of the human isolate (Charrel *et al.*, 2001) have now confirmed that KFDV and ALKV should be classified as genetic subtypes of the same virus species.

Further support for the idea that there may be many more as yet unrecognized but closely related tick-borne viruses in this region of the world comes from the isolation and identification of Karshi virus (KSIV) from Uzbekistan and Royal Farm virus (RFV), a subtype of KSIV, from Argasid ticks collected near Kabul, Afghanistan. Despite their phylogenetic placement within the mammalian tick-borne virus group, these viruses have not been associated with human disease. However, it is quite likely that this merely indicates a lack of appropriate diagnostic facilities in this region of the world.

The Langat virus (LGTV) makes the genetic link among the African, the southern Ocean, the south Asian, and Middle East viruses with those originally referred to as tick-borne encephalitis complex viruses, which are found in Asia, including Japan, central Europe, Scandinavia, western Europe, and the British Isles. LGTV infects humans but is generally regarded as less virulent than TBEV in Europe and Russia. Laboratory-attenuated strains of LGTV have been tested as live-attenuated vaccines in humans. In the natural environment, LGTV infects rodents, particularly the ground rat (*Rattus muelleri validus*) and the long-tailed rat (*Rattus sabanus vociferans*). The virus is found in *Ixodes* and *Haemaphysalis* ticks in Malaysia and neighboring Thailand and shares with TBEV and LIV the ability to be transmitted

between ticks cofeeding (see later) on rodent hosts (Labuda, personal communication).

Omsk hemorrhagic fever virus (OHFV), which is found in Siberia, diverges distinctly (not included in Fig. 2) from the Asian and European viruses (Marin *et al.*, 1995; Zanotto *et al.*, 1995) that typically produce encephalitis. Although this virus is antigenically closely related to other TBE complex viruses, OHFV is not a typical TBE complex virus. In terms of the hemorrhagic disease it produces in humans, OHFV resembles KFDV and ALKV, possibly reflecting an evolutionary connection between these viruses. OHFV is associated most frequently with the lowland forest–steppe and steppe areas of western Siberia, which characteristically feature marshy land, lakes, and shallow rivers, i.e., land containing much moisture. Common shrews and root voles are most numerous on the lake banks and in dry habitats, and muskrats, water voles, and root voles are found in floating vegetation. The muskrats originated in North America and were first released in the lakes of northern Kalunda and southern Baraba in 1936–1937. Almost all the muskrats and some other introduced species died in an intense epizootic of unknown etiology at the time. During the early studies, local investigators named OHF "muskrat disease" because they associated human infections with the infected muskrats. The virus was first isolated in 1954 from the brains of muskrat corpses. Importantly, the virus was subsequently isolated from the brains and other organs of healthy muskrats (Kharitonova and Leonov, 1985), and it is commonly believed that OHFV produces persistent infections in many of the animals that survive the initial infection. However, no natural virus infection was detected in any wild or domesticated animals inhabiting areas where OHFV was known to be present in muskrats, although antibody was detected in rodents, indicating that these animals were in contact with the virus. This difference in susceptibility to OHFV was confirmed by comparing experimentally infected muskrats and water voles. There were no signs of disease in the water voles (Kharitonova and Leonov, 1985). The fact that OHFV is highly virulent for the North American muskrat but not virulent for the indigenous rodents in Siberia supports the concept of an Old World origin for these viruses. This is additional support for the idea that POWV, a reasonably close relative of OHFV, was introduced to the New World from far east Asia.

The clinal biogeographic distribution of the TBE complex viruses and the underlying factors that led to the cline have been described in detail previously (Gould *et al.*, 2001; Zanotto *et al.*, 1995, 1996b). If we consider the genetic stability and dispersal of these viruses from the point of view of their associated hosts and ecology, it is remarkable

how precisely these viruses have established their individual niches. Despite the fact that small genetic differences between individual strains have been identified (Hayasaka *et al.*, 2001), the tick-borne viruses in far eastern Russia and Japan, i.e., a massive area of territory, are extremely closely related and are phylogenetically distinct from the other TBE-related viruses. In Siberia and central Europe, the TBE complex viruses are typified by the Siberian subtype, Vasilchenko (Vs) strain (Gritsun *et al.*, 1993), and in western Europe by the European subtype. All these viruses can be found in ticks and associated rodents in forest environments. The clinal pattern of evolution of the mammalian tick-borne virus group rules out the likelihood that migratory birds contribute significantly to the geographical dispersal of these viruses, even though strains of TBEV have been isolated from birds (Ernek, 1960). For a long time it has been believed that the virulence for humans of the far eastern strains of TBEV is higher than that of the central European strains, which seems to be supported by evidence that these viruses target different cells in the brains of monkeys when compared experimentally (Votiakov *et al.*, 1978).

For many years it was assumed that transmission of tick-borne encephalitic viruses occurred when a noninfected tick took a blood meal from an infected and viremic mammalian host. The tick became infected by the virus in the ingested blood and, after moulting to the next stage of its life cycle, transmitted the virus to a noninfected, susceptible mammalian host, which developed a viremia, enabling continuation of the cycle of transmission. Viremic transmission does undoubtedly occur and has been demonstrated experimentally many times (Reid, 1984). However, transmission via this mechanism does not adequately explain how tick-borne flaviviruses are maintained in the natural environment, as many species of rodents do not appear to develop viremia when exposed to the virus, even though they are considered the most important hosts for virus transmission. An alternative method of virus transmission was provided by the discovery of nonviremic transmission of tick-borne bunyaviruses and, subsequently, flaviviruses by ticks cofeeding on vertebrate hosts (Jones *et al.*, 1997). Nonviremic transmission is the process by which virus passes from infected to noninfected ticks feeding in close proximity to each other on vertebrate hosts. It is not necessary for the vertebrate host either to be susceptible to virus infection or for it to develop a viremia. The implications of this virus transmission process are that a much wider range of natural hosts than was previously recognized may contribute to the transmission of TBE. During the feeding process, an infected tick transfers virus from its salivary gland to the

dendritic cells that move freely under the superficial layers of skin. The cofeeding, noninfected tick becomes infected when the infected dendritic cells are transferred to its salivary gland during the feeding process. Nonviremic transmission was shown to occur in the natural environment (Jones *et al.*, 1997) between Louping ill virus (LIV)-infected and noninfected *Ixodes* spp. cofeeding on blue mountain hares (*Lepus timidus*). The discovery of virus transmission by cofeeding ticks on nonviremic animals is fundamental to our understanding of the process of tick-borne virus evolution and dispersal.

It has been known for many years that environmental and climatic conditions determine the geographic range of ticks, but it is now believed that the focal distribution of European subtype TBEV may be determined by the geographically variable degree of synchrony in the seasonal questing activity of larval and nymphal *Ixodes ricinus* (Randolph *et al.*, 1999). The most important criteria for successful transmission and maintenance of the virus in the forest environment can be summarized as follows.

- Cofeeding on suitable mammalian hosts must occur between noninfected larvae and infected nymphs. This ensures continuation of the transmission cycle through the generations of ticks. Experimental evidence suggests that cofeeding transmission can occur even on immune hosts (Jones *et al.*, 1997; Labuda *et al.*, 1997).
- To increase the possibility of long-term virus survival, there must be a high ratio of larvae to cofeeding-infected nymphs.
- There must be high humidity to ensure a high ratio of cofeeding larvae.
- Larval–nymphal synchrony requires a rapid fall in ground-level temperatures from August to October in the previous year, which causes large numbers of unfed larvae to pass the winter in quiescence from which they emerge synchronously with nymphs in the spring.

It follows that such criteria will only be met within relatively narrow geographic zones in Eurasia. This explains to a large extent the very highly defined areas in which the TBEVs occur at significant levels. It also helps explain the annual variability in the incidence of tick-borne encephalitis in humans. Presumably, when all the criteria defined earlier are satisfied, the relative proportion of virus-infected ticks will be high and the number of infected humans will reflect this. In years when relatively low numbers of ticks and therefore humans become infected, the virus might have to survive long term in larvae and nymphs that do not find suitable conditions for cofeeding until

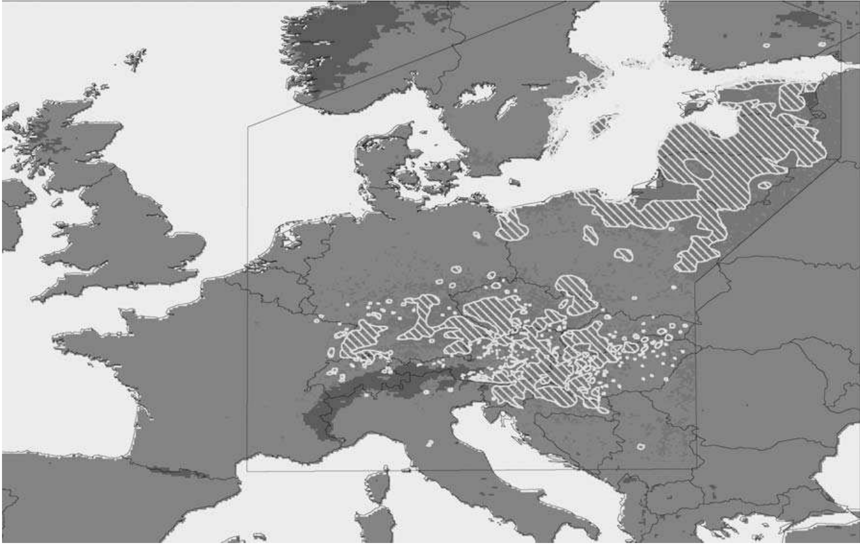

FIG 3. Predicted (red) and observed (yellow hatched) pan-European distributions of foci of TBEV based on analysis of remotely sensed environmental values and elevation within the outlined area. The virus occurs extensively to the east of this area, but is not yet mapped in any detail. Frequent cloud contamination in high mountain areas (darker green) prevents analysis there (Randolph, 2000). (See Color Insert.)

the following year. Geographic information systems (GIS) are now being used to predict the distribution of disease by detecting and precisely mapping elevation, temperature, and moisture conditions (among other parameters) of the vegetation, which are the most significant determinants of arthropod distribution. By augmenting ground-collected climatic information and vector habitat distribution with remotely sensed data, risk maps have been produced that predict the pan-European distribution of TBEV using GIS satellite data (Fig. 3). The prediction maps fit well with the recorded disease distribution (Randolph, 2000).

In addition to the tick-borne flaviviruses described earlier for which the natural habitat is the Eurasian forest floor, another distinct subgroup of tick-borne flaviviruses has evolved in its own characteristic habitat. These viruses infect and cause encephalomyelitis in sheep and goats. They are referred to collectively as LIV and include subtypes isolated from Turkey (TSEV), Greece (GGEV), Spain (SSEV), and the British Isles, including Ireland and Norway. Phylogenetic trees constructed from either complete or partial envelope gene

sequences of tick-borne flaviviruses (Charrel *et al.*, 2001; McGuire *et al.*, 1998) imply that their lineages emerged more recently than that of the far eastern TBEV subtype. Current evidence supports the idea that TSEV/GGEV emerged earliest of the sheep encephalomyelitic viruses and was probably transported with sheep/goats to France and Spain (SSEV). The British mainland strains of LIV represent the least divergent lineages of the tick-borne flaviviruses. They were almost certainly introduced to the British Isles following commercial exchanges of sheep and cattle between western Europe and Ireland during the past few centuries (McGuire *et al.*, 1998).

The evolution of these viruses represents an example of how human agricultural practices may have an impact on processes in the natural environment. Presumably, the seasonal introduction of nonimmune sheep and goats onto grazing land that skirted the forests of Eurasia exposed them to ticks infected with TBEV. While their high susceptibility to the virus ensured its amplification by viremic transmission, their mere presence also provided an excellent source of blood for ticks, which increased significantly in numbers and raised the probability of cofeeding transmission between ticks on other indigenous but insusceptible species such as the blue mountain hare (Jones *et al.*, 1997). Hence the virus became established in the area and was no longer entirely dependent on sheep for its perpetuation.

C. Mosquito-Borne Flaviviruses

While it is recognized that some mosquito-borne flaviviruses have been isolated from ticks and some tick-borne flaviviruses from mosquitoes, it is clear from all recently published phylogenetic trees that once the recognized tick- and mosquito-borne viruses diverged, they then remained separated ecologically. The phylogenetic tree presented in this review (Fig. 2) shows that mosquito-borne viruses diverged to form two distinct groups (group I and group II). Group II viruses contain those associated primarily with *Aedes* spp. (SEPV, YFV, etc.), which appear to have diverged first, and three viruses that have no known vector (SOKV, YOKV, and EBV). In previous trees, these three NKV appeared more divergent than SEPV and YFV (Gould *et al.*, 2001; Kuno *et al.*, 1998). This tree therefore infers early emergence of SEPV and YFV in the evolution of the genus *Flavivirus*. Further evidence in the form of sequence data will be required to resolve this anomaly. The other clade in group II viruses contains mosquito-borne viruses mostly associated with *Aedes* spp. These viruses were isolated in Africa (BOUV, SABV, UGSV, BANV), Asia (JUGV), and

Australia (EHV), i.e., they are all Old World viruses. Because JUGV and EHV are both close relatives of African viruses, they could represent the dispersed species of these African viruses. The other mosquito-borne viruses form group I, comprising Old World *Aedes* spp.-associated viruses and Old or New World *Culex* spp.-associated viruses. Gaunt *et al*. (2001) noted that all mosquito-borne viruses associated with hemorrhagic disease include *Aedes* spp. vectors in their life cycle, whereas all encephalitic mosquito-borne viruses include *Culex* spp. vectors in their cycle. At this stage of research, it has not proved possible to identify specific sequences within the viral genomes that determine these different disease characteristics, but this may be only a matter of time.

It is also important to emphasize that these divisions and interpretations are not absolute. For example, KOUV and YAOV, which diverge with the known *Culex* spp.–associated viruses, are also associated with *Aedes* spp. Moreover, other viruses are found in the *Aedes* spp. group when analyzed in the NS5 gene but in the *Culex* spp. group when analyzed in the E gene or the NS3 gene. Such examples either represent survival of the genetic elements required for *Aedes* spp. specificity within the *Culex* spp. lineages and vice versa or may infer recombination in these viruses.

YFV is the most well studied of the group II viruses, and its ecology and epidemiology have been described in detail (Monath, 1991; Strode, 1951; Theiler and Downs, 1973). This virus is confined endemically to Africa and South America within the tropics of Cancer and Capricorn only very occasionally making epizootic excursions outside these boundaries. In the Old World, it is found predominantly in central and west Africa, although strains have been isolated from east Africa. It is transmitted year-round among simian species and their associated *Aedes* spp. mosquitoes. *A. africanus* is the principal vector, feeding on monkeys living in the canopy of the humid equatorial African forests. In the nearby savannah regions, where there are dense populations of monkeys, *A. furcifer*, *A. africanus*, and *A. luteocephalus* are the principal sylvatic vectors, and any of these mosquitoes may also transmit the virus to humans during epidemics. Depending on their distribution, *A. vitattus*, *A. metallicus*, *A. opok*, *A. neoafricanus*, and *A. keniensis* are also associated with virus transmission. In areas where there are long periods of dry weather and also in urban areas along the west coast of Africa, epidemic outbreaks occur intermittently and are associated most frequently with *A. aegypti*, which breeds in and around urban dwellings. In Africa, the virus is not noticeably virulent for simian species, implying a long-term relationship between

YFV and these vertebrate hosts. Moreover, Africans appear more resistant to YFV than Caucasians (Strode, 1951).

In South America, YFV is also transmitted year-round in the humid equatorial forests and surrounding grasslands among simian species and mosquitoes (genus *Haemagogus*). However, in contrast with Africa, the virus often causes fatal infections in the South American monkeys, which is interpreted as indicating that YFV was introduced from the Old World into the New World relatively recently. It is considered likely that *A. aegypti* originated in Africa (Strode, 1951; Tabachnick, 1991) and that YFV was transported to the New World in the infected mosquitoes, humans, and animals that traveled on the ships that crossed the Atlantic Ocean from the 15th century onward. Prior to the eradication of *A. aegypti* from the Amazon basin in the 20th century, urban epidemics of yellow fever were common in the Americas and even in Europe (Burke and Monath, 2001; Strode, 1951); indeed there are detailed accounts of yellow fever in humans in the Mississippi Valley in 1878 (Bloom, 1993). However, it must be emphasized that outbreaks of yellow fever in North American and in European cities almost certainly resulted from multiple introductions of the virus off the ships that traded with Africa. In other words, this virus was never endemic, it was "pseudoepidemic" in these cities. Presumably, this was also true in South America with the first introductions of YFV, but the indigenous mosquitoes and other wildlife species provided suitable hosts for the virus to become established in the South American forests and surrounding savannah regions.

Three of the other group II viruses, JUGV, SEPV (Asia), and EHV (Australia), have been found only outside Africa. Because the remaining mosquito-borne viruses in group II are found only in Africa, it is reasonable to assume that JUGV, SEPV, and EHV or more divergent viruses representing their lineages were exported through human commercial activity from Africa to Asia and Australia where they adapted to the local ecology. Clearly, the relatively limited dispersal of these viruses reflects to some extent the sampling successes of scientists and the association of these viruses with mosquitoes that are not particularly anthropophilic or ornithophilic. Many more sylvatic and emerging flaviviruses are likely to be discovered in the future. However, without human commercial and agricultural activities, particularly the large-scale movement of humans, animals, and mosquitoes, YFV would almost certainly have been confined to Africa.

There have been many discussions as to why YFV has failed to become established in Asia (Gould *et al.*, 2001; Strode, 1951), despite the fact that the climate, the ecology, and the vector species appear

to be capable of sustaining YFV. These previous discussions will not be elaborated here. However, some additional thoughts are presented for consideration. Latin America contrasts with Africa and Asia and may provide part of the answer to this question. Throughout the 20th century, YFV has caused major human epidemics in Africa wherever *A. aegypti* has been prevalent. This virus also caused major epidemics in the New World, prior to the eradication of *A. aegypti* at the beginning of the 20th century. However, *A. aegypti* has returned to and is now widespread across Latin America, but in countries like Cuba, which still see epidemic outbreaks of dengue fever almost annually, yellow fever has never reappeared.

However, at the time yellow fever was causing epidemics in Cuba and South America, i.e., during the late part of the 19th century, dengue fever was present but was a much less significant problem. It is easy to provide a rational explanation for why yellow fever has not reappeared in Cuba. First, the eradication of *A. aegypti* totally removed the virus from the island because it was not established in sylvatic reservoirs. Second, since abolition of the slave trade, Africa is no longer a major source, into Latin America, of introduced yellow fever. However, in mainland South American jungles where the population density of *A. aegypti* has increased significantly in the past decades, sylvatic YFV has survived and is now potentially a serious epidemic threat to the human population. This threat is potentially even more serious than has been realized locally in Brazil because it has been demonstrated that Brazilian strains of YFV differ in their capacity to be neutralized by yellow fever–specific neutralizing antibodies (Buckley and Gould, 1985). Indeed, if a nonneutralizable strain of YFV becomes the predominant endemic/epidemic virus in a Latin American country, it is possible that the 17DD live-attenuated vaccine would fail to protect vaccinees against this virus.

Today, DENV is both endemic and epidemic in humans. Asia, rather than Africa, is a rich source for global dispersal of this virus. All four DENV serotypes have been introduced into Latin America and also to the Pacific Islands from Asia (Gubler, 1997), where there is no yellow fever. Because these viruses have evolved to be transmitted efficiently by *A. aegypti* and *A. albopictus*, they are now the cause of dengue fever (DF) and, in many cases, dengue hemorrhagic fever (DHF) in the Caribbean, in mainland South America, and in the Pacific Islands.

Presumably, multiple introductions of YFV into Asia, on the scale of its introduction into Latin America during the past 400 to 500 years, could have led to its establishment in Asia and subsequent global

spread. However, YFV is less predominant on the east coast of Africa and compared with the west coast of Africa, there is a significantly lower level of immunity to YFV, inferring that the disease is infrequent in the population. Moreover, far fewer movements of slaves and the associated infected mosquitoes took place eastward out of Africa during the past 500 years. In further support of these arguments is the fact that yellow fever has never been observed in the Pacific Islands where the climate is suitable and the mosquitoes are present. This is clearly because the virus was never introduced either from Africa or from Latin America at a sufficiently frequent number of intervals or at a sufficiently high level to be able to become endemic. However, human transport and commercial activity between Asia and the Pacific Islands has been very intense during the past 100 years, thus accounting for the presence of DF and DHF. Moreover, American genotype DENV-2 viruses are now known to have emerged more recently than many Asian viruses, as illustrated in trees that compare global collections of DENV-2 virus (Twiddy *et al.*, 2002; Uzcategui *et al.*, 2001). Thus, the DENV-2 viruses that were introduced in the late 19th or early 20th century either from Africa, with YFV, or from Asia did not become established in the sylvatic environment in the Americas and were eradicated effectively when *A. aegypti* was eliminated. This explains why today all the viruses found in Latin America are the least divergent viruses from Asia.

In the context of YFV and its failure to spread to Asia, there is an apparent paradox because Sepik virus (SEPV) and Wesselsbron virus (WESSV), the two most closely related viruses to YFV (Gaunt *et al.*, 2001; Kuno *et al.*, 1998) (unpublished observations), are found both in Africa and Asia; in contrast to YFV, they have dispersed successfully out of Africa to Asia. This can be explained most adequately by the fact that while these viruses are not as well studied as YFV, they have evolved in different habitats from YFV and have presumably been transported many times in mosquitoes associated with animals that are moved commercially between Africa and Asia. Similar dispersal patterns out of Africa are seen with alphaviruses and bunyaviruses.

It is also possible that the high virulence of YFV for humans has contributed to its failure to be introduced into Asia. In urban areas of Africa, where yellow fever produces only spasmodic outbreaks, the level of immunity is low. When introduced into these areas, yellow fever produces a severely incapacitating, clinically very recognizable, and often fatal disease. Few humans sick with yellow fever are capable of traveling significant distances. In contrast, DENV, SEPV, and WESSV produce less incapacitating infections in most infected

humans or animals. Therefore, movement and spread of these viruses by infected humans or animals are more likely to occur.

Group I viruses contain both *Aedes* and *Culex* spp.–associated virus clades. ZIKV and SPOV share strong sequence similarity in the NS5 and E genes (Gaunt *et al.*, 2001; Kuno *et al.*, 1998). ZIKV has been isolated in various parts of central and west Africa, and an antigenically indistinguishable virus has been isolated from regions of Malaysia (Marchette *et al.*, 1969). In Africa, ZIKV shares the ecological habitat with YFV but produces a much milder disease in humans than YFV. SPOV was first isolated in South Africa from the mosquito *Mansonia uniformis* but has since been isolated from several different mosquito species, the most common of which is *Aedes circumluteolous*. KEDV, another African virus, occupies a distinct lineage from ZIKV, SPOV, and the four dengue serotype viruses, reflecting its different ecological history. Nevertheless, like the other group I *Aedes* spp.–associated viruses, KEDV does infect humans.

Among all arboviruses, epidemic DENVs (group I) are the only ones to have apparently "escaped" the ties of a sylvatic existence and evolved the capacity to circulate in all tropical regions of the world among the human population in the urban environment. In this sense, humans are not "dead-end" hosts for dengue infections. This is largely due to the close association of the DENVs with *A. aegypti*, which lives and breeds successfully in the urban environment, transmitting the DENVs among humans. Admittedly, YFV achieves this position effectively but only temporarily during urban epidemics in Africa and very occasionally in South America. However, yellow fever epidemics invariably remain localized and die out relatively quickly. New epidemics then arise when sylvatic YFV is reintroduced into rural or urban areas. As far as is known, sylvatic strains of DENV are not associated with major human epidemic outbreaks (Gubler, 1997). Presumably, the localized distribution and feeding preferences of the mosquitoes in the more remote and forested areas ensure that very few humans are exposed to the sylvatic strains associated with these mosquitoes and the sylvatic strains may be less well adapted to the new mosquito and/or human hosts (Wang *et al.*, 2000). In further support of this latter observation, experimental infections of *A. aegypti* and *A. albopictus* indicate that these urban vectors are more susceptible to urban than to sylvatic strains of DENV-2, consistent with the hypothesis that urban dengue emerged via adaptation to peridomestic mosquito vectors (M. Diallo and A. Sall, personal communication; A. C. Moncayo and S. C. Weaver, personal communication).

Why have the DENVs been so much more successful in spreading globally than YFV? In addition to the points discussed earlier, the DENVs also infect and are transmitted efficiently to humans by *A. albopictus*. This Asian species has now spread globally through the tropical and subtropical regions. Originally a forest mosquito, *A. albopictus* has adapted to the human environment and is found in rural areas as well as periurban localities. *A. aegypti* is better adapted to the urban ecosystem of cities. DENVs effectively therefore have the best of both worlds, i.e., in the city environment they can be transmitted to humans by *A. aegypti*, but in the rural environment, *A. albopictus* provides an excellent vector for their continued transmission through human populations. The phylogenetic trees show that the DENV-2 were introduced from Asia into the New World where they then spread between different South American countries, evolving *in situ* (Uzcategui *et al.*, 2001). These viruses have adapted to the urban environment, being more readily transmissible by a wider variety of *Aedes* spp. (and other species?) than YFV. In other words, they have evolved more effective between-epidemic “out of the wild” survival strategies than YFV in the urban environment. Whether the DENVs have developed sylvatic cycles in Latin America remains to be seen.

The *Culex* spp.–associated flaviviruses in group I consist of a widely dispersed variety of viruses. Nevertheless, with the exception of West Nile virus (WNV), these viruses occur either in the New World or in the Old World, never in both. This implies that even though they infect birds, they are not dispersed across the two major oceans by birds. In terms of its epidemiology, the most well-known New World virus in this group is SLEV, which is associated with migratory birds that move the virus between South America and North America. The other recognized New World viruses, ROCV, ILHV, NJLV, BUSV, IGUV, and AROAV, are not as well studied, but with the possible exception of ROCV and ILHV appear to be less widely distributed. This may reflect the local animal species with which they are associated and the time in the past when they emerged. On the basis of a recent phylogenetic analysis (Kramer and Chandler, 2001), the most divergent lineages of SLEV isolates were found in Argentina and the least divergent were found in North America, inferring that SLEV was first successfully introduced into and became established in South America and then evolved and dispersed northward during the past centuries. Further support for this concept comes from the fact that all of the New World *Culex* spp.–associated viruses are found in South America but few of these are found in North America. Another interesting observation is

that each of the major clades in group I contains viruses from both the Old World and the New World. It therefore seems most likely that several different viruses have been introduced into Latin America, from the Old World, during the past centuries rather than a single virus being introduced with subsequent divergence to generate the different species found in the Americas.

Of the Old World *Culex* spp.–associated flaviviruses, WNV has clearly been the most successful in terms of its global distribution. This virus infects and is transmitted by a wide range of *Culex* spp., other mosquito species, and even ticks (Monath and Heinz, 1996). Its close association with migratory birds is well documented (Malkinson *et al.*, 2002; Rappole *et al.*, 2000). Phylogenetic trees constructed using the sequences of a wide geographic range of WNV isolates show that African strains are among the most divergent (Briese *et al.*, 2002; Lanciotti *et al.*, 1999, Platonov *et al.*, 2001; Scherret *et al.*, 2001), inferring that WNV may have radiated from Africa being vectored by *Culex* spp. and using migratory birds as its primary mode of geographic dispersal. The phylogenies suggest that WNV dispersed out of Africa to the Mediterranean countries, northern Europe, eastern Europe, southern Asia, including India, Malaysia, and Australasia. Confirmation of this predicted pattern of emergence awaits more sequence data and more detailed phylogenetic analysis. Interestingly, KUNV, the closely related subtype of WNV, is found only in or very close to Australia but as far as is known, WNV has not been found in Australia. Phylogenetic analyses of strains of WNV and KUNV show that KUNV appeared relatively recently (Platonov *et al.*, 2001, Scherret *et al.*, 2001). It therefore seems likely that WNV dispersed eastward across Asia into Australasia, and KUNV emerged either immediately before or soon after being introduced into Australia.

Until 1999, when WNV was first identified in New York (Briese *et al.*, 1999), there were no extant Old World *Culex* spp.–associated viruses in the New World. The method by which WNV was introduced into North America is not known, but it was almost certainly the direct result of human transportation. It is extremely unlikely that the virus was introduced by an infected bird or mosquito flying in from the Old World, and if this were the method then it should have occurred many times in the past and presumably, other African *Culex* spp.–associated viruses, such as USUV and YAOV, would also have been introduced into the New World. One acceptable explanation is that an infected bird(s) and/or mosquito(es) was inadvertently transported to New York, from the Middle East, on a commercial airliner. Irrespective of the method by which WNV gained access to the United States, it

dispersed north and southward along the east coast of America on the recognized bird migratory routes and westward, reaching the Midwest by September 2002. By mid-2003, the virus had been identified in both the Carribean and South America.

JEV is found in many parts of Asia, including Japan, mainland China, Taiwan, India, Malaysia, Indonesia, and Australasia. It is interesting that although WNV and JEV appear to share similar vectors, i.e., *Culex* spp., and vertebrate hosts, i.e., birds, horses, and pigs, JEV is not as widely distributed across the world as WNV. One possible explanation for this is that WNV emerged in Africa or nearby before dispersing northward and eastward, whereas JEV emerged after the parent lineage had dispersed out of Africa into Asia. This hypothesis offers a rational explanation for why JEV has not become established on the bird migratory routes that connect Africa and the more westerly parts of Europe and Asia. The phylogenetic tree (Fig. 2) also supports this suggestion. The closest relatives of JEV are Usutu virus (USUV), an African virus, Murray Valley encephalitis virus (MVEV), and Alfuy (ALFV), which are Australian viruses. In evolutionary terms, these lineages diverged relatively recently and have either African or Asian roots. Therefore, because JEV is not found in Africa and is closely related to the Australian viruses, it could be argued that this virus emerged relatively recently in Asia. Perhaps the most interesting recent observation was the discovery of USUV in Vienna, Austria (Weissenbock *et al.*, 2002). The fact that USUV and JEV are closely related and that USUV, until its discovery in Vienna, was found only in Africa infer that USUV could be the African equivalent of JEV in the same way that KUNV is the Australian equivalent of WNV! Because MVE and ALFV are close relatives of JEV, they almost certainly emerged at or around the same time as JEV in or nearby Australia.

During the past two centuries, JEV has caused many human epidemics in Asia. One of the many factors that undoubtedly contribute to this disease pattern is the very extensive pig and poultry farming industries that have developed in Asia. In addition to being hosts for the virus, these animals and birds are excellent amplification hosts for the *Culex* spp. that transmit JEV. Moreover, they are usually situated near major human populations. This farming industry has almost certainly also contributed to the successful dispersal of JEV across Asia. Although many phylogenetic trees based on JEV sequence data have been published (Chen *et al.*, 1990, 1992; Paranjpe and Banerjee, 1996; Uchil and Satchidanandam, 2001), there is no clear pattern of dispersal other than that of JEV eastward to Australia (Hanna *et al.*,

1996), the multiple introductions of JEV into the Indian subcontinent (Uchil and Satchidanandam, 2001), and the suggested differentiation of JEV strains into northern and southern Asian genotypes (Chen and Beaty, 1982; Chen *et al.*, 1992). However, following rigorous analysis of 107 envelope gene sequences (Uchil and Satchidanandam, 2001), it is now clear that different clusters of JEV strains from the same country are widely divergent. Therefore, one can speculate that an ancestral virus was introduced into southeast Asia during the past few centuries and as JEV emerged, the descendant strains radiated throughout Asia aided and abetted by human commercial activities. As the quantity of sequence data increase and the quality of phylogenetic analysis improves, more definite patterns of emergence and dispersal should become evident.

What is the likelihood that JEV or other *Culex* spp.–associated viruses will be introduced into the Americas? The short answer to this question is "very likely." Usutu virus has appeared in Vienna (Weissenbock *et al.*, 2002) apparently for the first time, presumably introduced by birds migrating from Africa. JEV was first detected in northeast Australia in 1995 (Hanna *et al.*, 1996). WNV is now known to be quite widespread in Europe, the Middle East, and Asia (Burke and Monath, 2001) and was recently introduced into North America (Briese *et al.*, 1999). These viruses are clearly dispersing ever more widely. Why does this appear to be happening now rather than hundreds of years ago? Because these viruses have emerged relatively recently, they therefore have had insufficient time to become widely dispersed. Additionally, the commercial movement of birds and the inadvertent movement of mosquitoes through increasing transportation have increased the likelihood of their dispersal across the major oceans. Nevertheless, it must be borne in mind that the successful establishment of any of these *Culex* spp.–associated viruses into a new country is still a relatively low-risk event, otherwise why have SLEV and WNV apparently been introduced only once into the Americas? Could JEV spread more westerly and become established in the New World? Because the degree of transportation and commercial activity between the Old and New World is continuing to increase, and we have recently witnessed the appearance of USUV in Austria (Weissenbock *et al.*, 2002), we have to assume the answer is yes. However, it may not be that simple. With SLEV and WNV already established in the New World, it might be difficult for JEV and other introduced viruses from group I to compete and become established.

Several *Culex* spp.–associated flaviviruses, KUNV, ALFV, MVEV, KOKV, and STRV, are virtually exclusive to Australia. Although

occasional isolates have been found in southeast Asia, these viruses do not appear to have spread widely out of Australia in a westerly direction. From this point of view alone, therefore, it is likely that they emerged in Australia or very nearby. Why have these viruses not radiated out of Australia and spread westerly? First, more divergent but related viruses are already established in Asia, providing a form of natural immunity in nearby countries. Second, because the Australian viruses diverged recently, they have not yet had sufficient time to disperse widely. Third, the disease incidence of any flaviviruses in Australia is generally low compared with many Asian countries, and Australian control measures across its borders are well organized, thus restricting the movement of these viruses both in and out of the country.

IV. What Are the Origins of Flaviviruses?

In attempting to answer this question, we need to consider (1) their relationships with other genera in the family *Flaviviridae*, (2) the land mass in which the genus *Flavivirus* probably originated, (3) how they were dispersed, and (4) the likely time period during which the genus, as we recognize it today, emerged and evolved.

The genus *Flavivirus* was originally placed in the family *Flaviviridae* because viruses in other genera, i.e., *Hepacivirus* and *Pestivirus*, share similar genome strategies. Nevertheless, the three genera are antigenically distinct and share less than 20% overall sequence identity across the other genera. Within the family *Flaviviridae*, only the genus *Flavivirus* contains arboviruses, but it may be significant that many of the most divergent viruses in this genus are nonvectored; this suggests that the ancestral condition within the *Flaviviridae* is that of nonvector transmission and that vector-borne transmission was a relatively recent innovation. If so, it supports the notion that the NKV viruses were the first to diverge within the genus *Flavivirus*. At least some viruses in each of the genera infect wildlife species, but there are no known human pestiviruses. CFA was isolated from an *A. aegypti* cell line, and is considered originally to have been an African mosquito (Rodhain and Rosen, 1997; Strode, 1951; Tabachnick, 1991). The genome strategy of CFAV is very similar to that of viruses in the genus *Flavivirus*, but they are antigenically distinct, and CFAV shares no more than about 20% nucleotide identity with flaviviruses and slightly less than this when compared with hepaciviruses and pestiviruses. Nevertheless, it was proposed that CFAV should be a virus species in

the genus *Flavivirus* (Cammisa Parks *et al.*, 1992); it has been used successfully as an outgroup for the genus in phylogenetic analysis (Marin *et al.*, 1995; Zanotto *et al.*, 1995, 1996b).

TBV, which was isolated in 1973 in Trinidad from the salivary glands, saliva, and spleen of the insectivorous bat *Pteronotus parnellii* (Price, 1978), has the typical morphological and physical features of a flavivirus (Kuno *et al.*, 1998), but has not been shown to cross-react antigenically with any of the flaviviruses. Moreover, the genome sequence of TBV shares less than 20% identity with viruses in the genus *Flavivirus*, and phylogenetic analysis has thus far failed to resolve its position with respect to other members of the family Flaviviridae. It is important to remember that even the most genetically distant of the recognized flaviviruses in the phylogenetic tree shares antigenic similarity with all of the other viruses in the genus *Flavivirus*. Therefore, until more viruses, more sequence data, and new methods of analysis become available, we can only suggest that CFAV and TBV represent the closest viruses to the flaviviruses and may provide a genetic link between pestiviruses and flaviviruses.

The tentative conclusion at this stage would be that the genus *Flavivirus* originated from nonvectored mammalian viruses. Whether the most divergent viruses originated in the Old or the New World is not entirely clear, as TBV and CFAV were discovered, respectively, in Trinidadian bats and African mosquitoes. Nevertheless, the most divergent viruses in each of the three major branches at the deepest nodes of the flavivirus tree are Old World viruses. NKV viruses are found in either the New World or the Old World, implying that they were dispersed widely following emergence, possibly by bats or rodents transported over the oceans. All the early tick-borne and mosquito-borne lineages define Old World viruses in Africa or Asia, and because all the major clades contain African viruses, this presently seems the most likely origin of the arthropod-transmitted flaviviruses.

The methods of dispersal of mosquito- and tick-borne viruses are much more easy to understand, and the underlying reasons for these dispersal patterns have been defined in detail in this review and elsewhere (Gould *et al.*, 2001; Gubler, 1997; Strode, 1951; Theiler and Downs, 1973). The influence of human commercial and military transportation has been tremendous. There are no better examples of this than YFV from Africa to the Americas via the slave trade and the dengue viruses that are transported from Asia to the Americas, as discussed earlier. It is difficult to identify the precise geographic point of origin of the dengue viruses, but all trees infer that DENV-4 was the first serotype to emerge. Moreover, there is strong evidence based on

phylogenetic evidence and ecological data that the DENVs originated in monkeys, i.e., they were sylvatic and cross-species transfer to humans subsequently occurred independently in all four serotypes (Holmes and Twiddy, 2003; Rodhain, 1991; Rudnick, 1978, 1984; Wang *et al.*, 2000; Wolfe *et al.*, 2001). Whether the DENVs first emerged in Africa and dispersed into Asia or vice versa is debatable. It is believed that *A. aegypti* originated in Africa, and it would therefore seem logical that the DENVs also originated in Africa. Moreover, dengue fever first appeared in the Americas at the same time as YFV was being introduced, implying that the DENVs were being introduced to the Americas from Africa on the slave ships. However, sylvatic dengue is recognized with all four serotypes in Asia. This is not the case in Africa, although this could simply reflect the small number of African samples. What is now becoming clear is that the evolutionary history of dengue virus is very recent, with the sylvatic DENVs emerging during the past 1000 years and endemic/epidemic transmission in human populations becoming established as recently as the past few hundred years (Holmes and Twiddy, 2003; Twiddy *et al.*, 2003; Wang *et al.*, 2000).

Overall, it has been estimated that the recognized flaviviruses diverged from an ancestral virus during the past 5000 to 10,000 years (Zanotto *et al.*, 1996b). While this estimate may have originally been considered by some to be in error by orders of magnitude, it is now recognized that the evolution of this genus is likely to have followed the ending of the ice age and to have accompanied the development of human civilization and farming practices during the past 10,000 years.

V. Does Recombination between Flaviviruses Occur and, if so, What Is the Likely Impact?

Until relatively recently, the only published data (Blok *et al.*, 1992) implied that recombination did not occur among flaviviruses, and it was assumed that flavivirus evolution occurred clonally with genetic variation arising through the accumulation of mutations. However, there is now convincing evidence to support recombination among closely related strains of DENVs, i.e., at the intraserotypic level (AbuBakar *et al.*, 2002; Holmes *et al.*, 1999; Tolou *et al.*, 2001; Twiddy and Holmes, 2003; Uzcategui *et al.*, 2001; Worobey *et al.*, 1999). Recombination at this level has implications for our understanding of flavivirus evolution because trees constructed without taking recombination into account may not reveal true genetic relationships. In this

context, it was noted that SLEV appears in different relative positions with respect to other flaviviruses when analyzed using sequence data derived from either the E or the NS5 gene, implying recombination within SLEV (Gaunt *et al*., 2001), and we have also referred to other flaviviruses that occupy different positions in trees constructed using sequences from different genes. A second implication of recombination is the possibility that virus phenotype may alter as the result of recombination. This would be particularly important if recombination among flaviviruses led to increased virulence for mammalian hosts or to altered arthropod specificity.

Recombination is not a new phenomenon in virology, but to occur and then to be recognized requires dual or multiple infections of a single host cell by distinguishable viruses and the appropriate computer software with which to compare the sequences. New and improved software is now available, and many more cases of recombination are being recognized. The likelihood of mixed flavivirus infections occurring is increasing almost exponentially, largely as the result of major increases in global trading and transportation, urbanization and population density, modern animal farming practices, and arthropod densities, all of which, as we have indicated, are undoubtedly contributing significantly to the increasing global dispersal of the flaviviruses. Indeed, many of these viruses now overlap geographically and ecologically in different regions of the world. Arthropods are excellent reservoirs for viruses, as flavivirus-infected ticks and mosquitoes continue to feed and reproduce apparently as normal, thus increasing the possibility of exposure to a second virus and therefore increasing the risk of recombination. Humans infected chronically with TBEV (Gritsun *et al*., 2003) and healthy birds carrying SLEV or WNV may also serve as potential reservoirs for mixed infections. If recombination occurred among flavivirus species such as TBEV and OHFV or between tick-borne and mosquito-borne flaviviruses, the consequences for disease epidemiology could be profound. It is pertinent to reflect on the fact that in the genus *Alphavirus*, recombination between Eastern equine encephalitis virus and a virus related to Sindbis virus resulted in the production of Western equine encephalitis virus. The circumstances under which this occurred can only be guessed at. However, to put it in perspective, individual flavivirus trees constructed using genes from any part of the genome or the entire genome are relatively congruent, implying that most recombination in flaviviruses occurs between closely related viruses. Nevertheless, infectious hybrids of flaviviruses, based on a backbone of live-attenuated yellow fever vaccine, can now be generated in the

laboratory (Chambers *et al.*, 1999; Monath *et al.*, 1999, 2000) and are under development as chimeric live-attenuated vaccines for inoculation into humans. Additionally, specific mutations that attenuate flaviviruses are being introduced into live-attenuated hybrid vaccines (Blaney *et al.*, 2002). If such vaccines recombine with wild strains of virus, there is a risk that the attenuating mutations/genes will be replaced with nonattenuating mutations/genes. It will be important to establish whether such chimeric viruses present an acceptable risk when they are administered to large numbers of humans as live-attenuated vaccines.

At this time there is no direct evidence to show that recombination between very closely related flaviviruses has had a significant impact on viral pathogenesis. However, in Latin America, both Asian and Latin American strains of DENV are known to cocirculate (Uzcategui *et al.*, 2001) and until now, only Asian strains of DENV have been shown to cause the more severe form of disease, i.e., dengue hemorrhagic fever (Leitmeyer *et al.*, 1999). It follows that if there are viral genetic determinants of hemorrhagic fever, recombination between viruses with these determinants and viruses without these determinants could lead to an increased likelihood of more severe epidemics in Latin America and elsewhere.

In summary, flaviviruses represent a relatively large and mixed group of viruses, comprising those that have remained almost untouched by human influence and those whose epidemiology and evolution have been significantly influenced by humans and the consequences of their commercial and recreational activities. This human influence will undoubtedly continue, and it is therefore likely that flaviviruses will assume a larger importance in disease emergence in the future. Viruses such as the DENVs, WNV, and USUV are testimony to this.

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ADVANCES IN VIRUS RESEARCH, VOL 59

MICROEVOLUTION AND VIRULENCE OF DENGUE VIRUSES

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I. Introduction
II. Genetic Variation within Dengue Serotypes
III. Phylogenetics of Dengue Viruses
  A. Phylogeny of Dengue Serotype 1
  B. Phylogeny of Dengue Serotype 2
  C. Phylogeny of Dengue Serotype 3
  D. Phylogeny of Dengue Serotype 4
IV. Viral Determinants of Disease
V. Viral Determinants of Transmission
VI. Viral Displacement and Epidemiology
References

The evolution of dengue viruses has had a major impact on their virulence for humans and on the epidemiology of dengue disease around the world. Although antigenic and genetic differences in virus strains had become evident, it is mainly due to the lack of animal models of disease that has made it difficult to detect differences in virulence of dengue viruses. However, phylogenetic studies of many different dengue virus samples have led to the association between specific genotypes (within serotypes) and the presentation of more or less severe disease. Currently, dengue viruses can be classified as being of epidemiologically low, medium, or high impact; i.e., some viruses may remain in sylvatic cycles of little or low transmissibility to humans, others produce dengue fever (DF) only, and some genotypes have been associated with the potential to cause the more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) in addition to DF. Although the factors that contribute to dengue virus epidemiology are complex, studies have suggested that specific viral structures may contribute to increased replication in human target cells and to increased transmission by the mosquito vector; however, the immune status and possibly the genetic background of the host are also determinants of virulence or disease presentation. As to the question of whether dengue viruses are evolving toward virulence as they continue to spread throughout the world, phylogenetic and

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epidemiological analyses suggest that the more virulent genotypes are now displacing those that have lower epidemiological impact; there is no evidence for the transmission of antigenically aberrant, new strains.

I. Introduction

Understanding dengue virus variation is especially important because we still know little about the disease; what has become evident over the many years of dengue research is that dengue epidemiology is determined by many factors, including those in the host, vector, and environment. Dengue virus evolution is also determined by many complex interactions, from the cellular to the populational level, in humans and mosquitoes. Some of the genetic changes that occur during the natural transmission cycles of dengue viruses have ultimately affected virulence or the potential to cause disease in humans, and it would benefit us greatly if we could narrow these down for inclusion in the development of control measures. The understanding of these virus-specified determinants of virulence has been difficult to achieve because of the lack of *in vivo* and *in vitro* markers to correlate with disease severity in humans. Therefore, what little we understand today has been acquired indirectly by the association between evolutionary groupings of virus strains and their epidemiological and/or disease clinical presentations. These associations are still being revealed, as in the case of dengue viruses of serotypes 1 and 4, about which we have little information, and as more dengue virus samples are being analyzed. It is also evident that we have a limited window in time from which to derive our information—virus fossils do not exist, and virus strains have only recently begun to be acquired and stored properly, with documentation of the associated epidemiological and clinical associations. Therefore, it behooves us to understand the mechanisms of dengue virus evolution, but we are only at the beginning stages of being able to associate specific viral sequences or structures with virulence for humans. The study of laboratory-prepared, attenuated dengue viruses is the subject of other reviews in this volume, and virulence in that context refers to the propensity of candidate vaccine viruses to cause any disease at all. This review attempts to summarize our current interpretations of dengue virus sequence analyses and how we can use this information to follow, understand, and possibly predict the evolution of dengue viruses around the globe.

II. Genetic Variation within Dengue Serotypes

As with other viruses, the evidence for strain differences among dengue viruses was first detected serologically using antibodies made by inoculating laboratory animals (Sabin, 1952). However, none of these animals showed signs of disease comparable to humans. Nonhuman primates develop transient viremias, but even the apes, our most closely related primates, do not develop the rash and hemorrhagic symptoms consistent with DF or DHF. Some investigators have resorted to using mouse neurovirulence (after intracranial inoculation) as a surrogate model of virulence for humans. The first observations concerning differences in strain virulence came from clinical and epidemiological associations, where more severe disease was associated with specific outbreaks and the virus strains were isolated from patients with hemorrhagic disease (Barnes and Rosen, 1974; Gubler *et al.*, 1978; Rosen, 1977, 1986). Meanwhile, others observed an increase in disease severity in patients who had been infected by more than one dengue virus serotype; this became known as the immune enhancement phenomenon (Halstead, 1970, 1988). Thus, two opposing theories of dengue pathogenesis came into existence, with one describing virulence as a viral characteristic and the other relating the severity of disease to the immune status of the host. Controversies surrounding both of these theories still exist, but it is most probable that both the virus and the host immune system play a role in dengue pathogenesis.

The first genetic evidence for differences between dengue viruses of the same serotype came from RNA fingerprinting studies (Repik *et al.*, 1983; Vezza *et al.*, 1980). This method uses an enzyme to digest viral RNA into strands, with the number and size of strands varying according to the entire viral sequence. This is a relatively crude surveying technique that does not give results that are directly comparable across strains. The resulting groupings of viral samples were called "topotypes," reflecting the two-dimensional topology of the RNA strand electrophoresis patterns. Another method, using a similar approach of digesting a cDNA copy of the viral RNA template with endonucleases, gave somewhat more resolution, but it also failed to identify the regions of the genome in which the sequences differed among viruses (Kerschner *et al.*, 1986). It was not until the mid-1980s that direct sequencing of specific genome regions became amenable to the study of many different viruses. Because primer-extension sequencing off of the viral RNA required very large quantities of relatively pure virus preparations to obtain clear sequence information,

the first analyses of dengue virus variation used relatively short sequences for comparison or used very few strains (Blok *et al.*, 1989; Chu *et al.*, 1989; Rico-Hesse, 1990). What has become clear is that with more sequence information, i.e., either full genomic sequences for one virus or entire genes for many different viruses, our understanding of dengue evolution and virulence has increased dramatically. Current controversies center on how to interpret this information rather than whether dengue viruses differ in their potential to cause disease. The classification of viruses into genetic groups ("genotypes") within serotypes is constantly changing, as methods for sequencing and evolutionary analysis improve and the available database expands.

Current methods for obtaining dengue virus sequences no longer require a viable virus isolate; in fact, the entire genome sequence can be obtained by enzymatic amplification of the viral RNA template in the patient's blood sample (Leitmeyer *et al.*, 1999). Thus, most of the sequences available from the 1990s to date were generated with the reverse transcriptase–polymerase chain reaction (RT-PCR), which has substantially improved the quality or fidelity of the sequences available from many laboratories around the world. Unfortunately, there is no uniform approach to determining which sequences will be compared among strains; this has led to a vast quantity of information available on the sequence database (GenBank) for which there has been no systematic interpretation. In addition, there are numerous sequences that contain errors (sequencing or editing artifacts), which have led to serious mistakes in interpretation, especially regarding the possibility of intramolecular recombination (see later). Therefore, it is wise to obtain virus isolates for further genetic characterization if in fact the initial sequence information obtained proves to be unusual.

III. Phylogenetics of Dengue Viruses

As sequencing methods have become more accessible technically and financially, there has been a concomitant increase in the number of dengue virus strains that have been analyzed. This has led to a refinement in the analyses of dengue evolution (thus we now speak of "microevolution" of dengue viruses), but this advance has also been dependent on the development of statistical methods for determining sequence relationships, known as "phylogenetics." In the last decade, the computer algorithms used in the generation of phylogenetic or evolutionary trees of virus relationships have increased in number

and complexity; this is a field of research that is evolving on its own, and most virologists tend to apply the algorithms that are handy, compatible with their computer, or are used by nearby colleagues. Therefore, it is difficult to say which phylogenetic method is best unless one does several comparisons under specific dataset assumptions (number of taxa, character weighing, rooting options, etc.). Currently, the consensus among "phylogeneticists" is that a large number of taxa (virus strains), with long sequence strings (i.e., one gene or more) analyzed by the maximum likelihood (ML) method, with its incorporated transition/transversion rate calculation, and with bootstrap (statistical) support for the branching patterns, are probably sufficient to yield an accurate phylogenetic tree for viral genotype classification (Hillis, 1998; Lemmon and Milinkovitch, 2002). Calculations of molecular clocks (rates of evolution over time), theoretical ancestors, and selective pressure estimations require many assumptions for which we have no hard evidence; these interpretations may be misleading when based solely on laboratory or *in vitro* observations. It is clear that we also suffer from a taxa inclusion bias because we obtain samples from humans who are ill (and historically analyze those from patients with more severe disease) and do not have enough samples from vertebrates involved in sylvatic cycles or from mosquitoes. Thus, quantitations of natural dengue virus diversity (Holmes, 1998; Zanotto *et al.*, 1996) are premature. Currently, the accuracy of phylogenetic trees is usually limited by the number and type of taxa analyzed and the investigator's access to high-speed computing resources.

The selection of domains or genome regions for sequence comparison is very important with regard to analysis outcome and limits of interpretation. When using the maximum parsimony method of phylogenetic analysis, it is common to state how many parsimony informative sites were included in the dataset; however, this approach is not available in ML and we are left with bootstrap values to support the monophyletic or genotype groupings after estimating numerous trees. It has become clear that different areas of the dengue virus genome evolve or fix mutations at different rates and will sometimes exhibit "hot spots" of higher mutation rates within a region (e.g., the E gene or the 3′-untranslated region). In general, when long enough sequences are used, the trees generated from different genome regions usually correspond or overlap, and discrepancies occur only when trying to interpret the minor branches of the trees (i.e., genotype groupings usually remain the same). It is this characteristic of varying mutation rates across the genome that prevents us from establishing a uniform cutoff rate of divergence for different genotypic groups; thus,

it is important to state that arbitrary cutoffs for genotypes apply only to the region or sequences being compared (Rico-Hesse, 1990). Until more complete genome sequences of dengue viruses are available, we will not be able to establish the natural, full range of nucleotide or amino acid variability within serotypes and genotypes; in addition, viruses or templates should be of low passage from their original sources to avoid artificial selection of mutants (Lee *et al.*, 1997). This is a difficult but not impossible task given the large number of mutations that can potentially occur in all areas of the dengue genome. At this point in time the actual mechanisms governing the natural selection of dengue mutants have not been determined, and we are limited to statistical inferences of positive or negative selection on specific genome regions or amino acids (Twiddy *et al.*, 2002a, 2002b). Suffice it to say that these mechanisms appear to influence codon usage (Jenkins *et al.*, 2001), RNA folding (Brinton and Dispoto, 1988; Leitmeyer *et al.*, 1999; Shi *et al.*, 1996), or protein structures of dengue and other flaviviruses; i.e., there are structural and functional limitations to the plasticity of dengue viruses in addition to the dogma of immune selection by host antibodies.

The possibility of intramolecular recombination among dengue viruses has received considerable attention (Gould *et al.*, 2001; Holmes and Burch, 2000; Holmes *et al.*, 1999; Uzcategui *et al.*, 2001; Worobey *et al.*, 1999); however, no virus isolates meeting stringent criteria for recombination have yet been described. In one instance, most of the purported recombinant sequences acquired from the GenBank database were shown to contain sequencing artifacts, which made them behave as recombinants in statistical analyses (Worobey *et al.*, 1999). In other examples, the virus isolate can no longer be obtained for independent verification (Holmes *et al.*, 1999) or the investigators were not able to meet virological criteria for recombinant classification (Tolou *et al.*, 2001; Uzcategui *et al.*, 2001). Such criteria include obtaining a virus isolate whose purity is confirmed by plaquing, probe hybridization, and/or neutralization with serotype-specific antisera, ruling out mixed templates, and direct sequencing of multiple amplicons from several RNA template preparations, as has been done with polioviruses (Cuervo *et al.*, 2001; Liu *et al.*, 2000). Although the possibility of recombination among dengue viruses may not be remote because dual (serotype) infection of humans has been demonstrated (Gubler *et al.*, 1985; Laille *et al.*, 1991; Lorono-Pino *et al.*, 1999), the probability of simultaneous infection of cells in human or vector hosts may be low because of replication interference (Dittmar *et al.*, 1982). However, this phenomenon has not kept other single-stranded,

nonsegmented RNA viruses, such as poliovirus, from often producing inter- and intraserotype recombinants, which has occurred in vaccine (live-attenuated, trivalent Sabin strain) recipients. It remains to be determined whether and at what frequency dengue viruses do undergo recombination in nature, where the main concern would be the creation of interserotype hybrids, which might be capable of escaping immunity to the four known serotypes. So far, the aforementioned phylogenetic or statistical methods for detecting recombinants have possibly demonstrated evidence for dengue virus hybrids within but not across serotypes.

The ultimate application of understanding virus evolution is to derive information that could be helpful in disease control. Thus, it is important that all of the information used for phylogenetic tree interpretation meet certain standards so that our conclusions are not erroneous. Both the clinical and epidemiological information we use to relate samples or genotypes to disease potential should therefore clearly meet dengue case definitions for disease classification (i.e., DF or DHF/DSS). Countries reporting outbreaks or epidemics should use the resources of reference laboratories to confirm their findings. In fact, the best samples for full genome sequencing of dengue viruses have come from prospective studies, where patients are enrolled and sampled when febrile and their clinical progress documented along with other immunological and epidemiological parameters (Rico-Hesse *et al.*, 1998; Vaughn *et al.*, 2000). Dengue virus phylogenies cannot be interpreted without clear information about their phenotypic or biological properties, and the trees will only be as accurate as the tests used to address our hypotheses. Therefore, different trees can be generated with differing sequences or discrete genome regions, depending on the question posed. For most dengue serotypes, we are still attempting to determine which genotypes are associated with higher virulence, severe disease, or larger epidemics. However, for dengue serotype 2 and 3 viruses, we appear to have identified genotypes that have undergone greater spread than the other genotypes and have the potential to cause DHF. The transmission of these genotypes is being monitored in several countries and the ministries of health have understood the urgency to reduce transmission of these strains, albeit by vector reduction.

In an attempt to clarify or unify the current classification of dengue genotypes within serotypes, phylogenies of all four serotypes are described using nucleotides from the entire E gene region. All sequences were aligned with representatives of the other serotypes (using the Clustal W program) (Aiyar, 2000; Higgins *et al.*, 1996) and were

compared by the computing-intensive ML method, with inherent estimations of transitions/transversions, and with bootstrap support for branching patterns (Swofford, 2002). Only the latest versions of sequences deposited in the GenBank database were used for comparisons to avoid the inclusion of laboratory artifacts or errors. These results and interpretations should not be construed to represent actuality but rather the best approximations of dengue virus evolutionary relationships we have at this time.

A. Phylogeny of Dengue Serotype 1

The first genetic comparison of dengue type 1 strains was reported in 1983 using RNA fingerprinting (Repik *et al.*, 1983); these investigators were able to distinguish three geographic groupings (Caribbean, Pacific/Southeast Asian, and African) among 12 different strains isolated at different times. A review published in 1990 mentioned the possibility of up to eight topotypes of serotype 1 viruses, but the original data were not published (Trent *et al.*, 1990). Subsequent E gene sequencing studies confirmed the original groupings and also noted a failure to detect specific virulence markers (Chu *et al.*, 1989); that is, there was no correlation of specific amino acid sequences in this gene with dengue disease of greater or lesser clinical severity. Another sequencing study, using only 240 nucleotides from the E/NS1 gene junction, showed that 40 serotype-1 strains could be classified into five genotypic groups when using an arbitrary cutoff point of 6% divergence for groupings, but because more strains were analyzed, the global routes of transmission of these viruses could be followed (Rico-Hesse, 1990). A large number of strains were analyzed in another report (Chungue *et al.*, 1995), but this study used a different region of the genome (180 nucleotides from E gene) for comparisons and the results were not directly comparable; however, these investigators used the 6% divergence cutoff mentioned earlier to distinguish three genotypes. Only recently has a more complete study of serotype 1 strains been reported (Goncalvez *et al.*, 2002), where the full E gene sequences were determined, allowing for the correction of some of those reported earlier. The trees reported in that study differ from the analysis shown here only in that representatives of the other serotypes were included as an outgroup in the current study. Also, genotypes are denoted here by their apparent geographical origins (and not necessarily to where they have since spread) and not by numbers (Roman numerals).

The phylogeny of 36 dengue serotype 1 viruses is shown in Fig. 1. The statistical analyses of validity of branching patterns (bootstrap) continue to support the classification of these viruses into five genotypes: sylvatic/Malaysia, Americas/Africa, South Pacific, Asia, and Thailand. The tree also supports the previous hypothesis (Wang *et al.*, 2000) that sylvatic strains, one from Malaysia in this case, evolved earlier from a hypothetical ancestor shared by all dengue viruses, which is why most sylvatic viruses are basal (branch off first) in all ML-estimated trees. However, only one dengue type 1 virus has been isolated under these ecological conditions (from a sentinel monkey) (Rudnick, 1965); it is also unclear if this virus cycle still exists, as field work has ceased and other isolates from Malaysia are now from urban epidemics and involve other genotypes (Chow *et al.*, 1994). It is clear, however, that this sylvatic genotype, albeit represented by one isolate, is of low epidemiological importance to humans, as it is not causing detectable disease in humans and there is no evidence that reintroduction from a sylvatic cycle is required for persistence of dengue transmission year-round. Some of the other four genotypes may no longer exist either, as one of them is represented only by older Thai strains (from 1954 to 1964) and the Japan/Hawaii strains that are basal to the Asian genotype (including a Thai 1980 strain) have no other closely related neighbors. Only further sampling will allow clarification of these observations.

Because the clinical classifications of the patients from which some of these older viruses were obtained are unclear and because sequences for serotype 1 viruses, which have caused more severe dengue, are lacking in most analyses (e.g., recent Thai viruses), it is not possible to find an association between some of these genotypes and increased virulence. Only from the most recent study do we know that the American/African genotype has the potential to cause DHF because of the inclusion of two samples from patients in Venezuela (Goncalvez *et al.*, 2002). In addition, this genotype had been spreading to many other countries or geographic regions during the past decade (see Brazil, 1997; Colombia, 1996; Peru, 1991; and Venezuela, 1997). Therefore, this genotype would be considered of higher epidemiological impact than the sylvatic genotype. It is evident from these results that a more complete and systematic survey of serotype 1 samples is necessary before a link can be established between specific genotypes and virulence of these viruses.

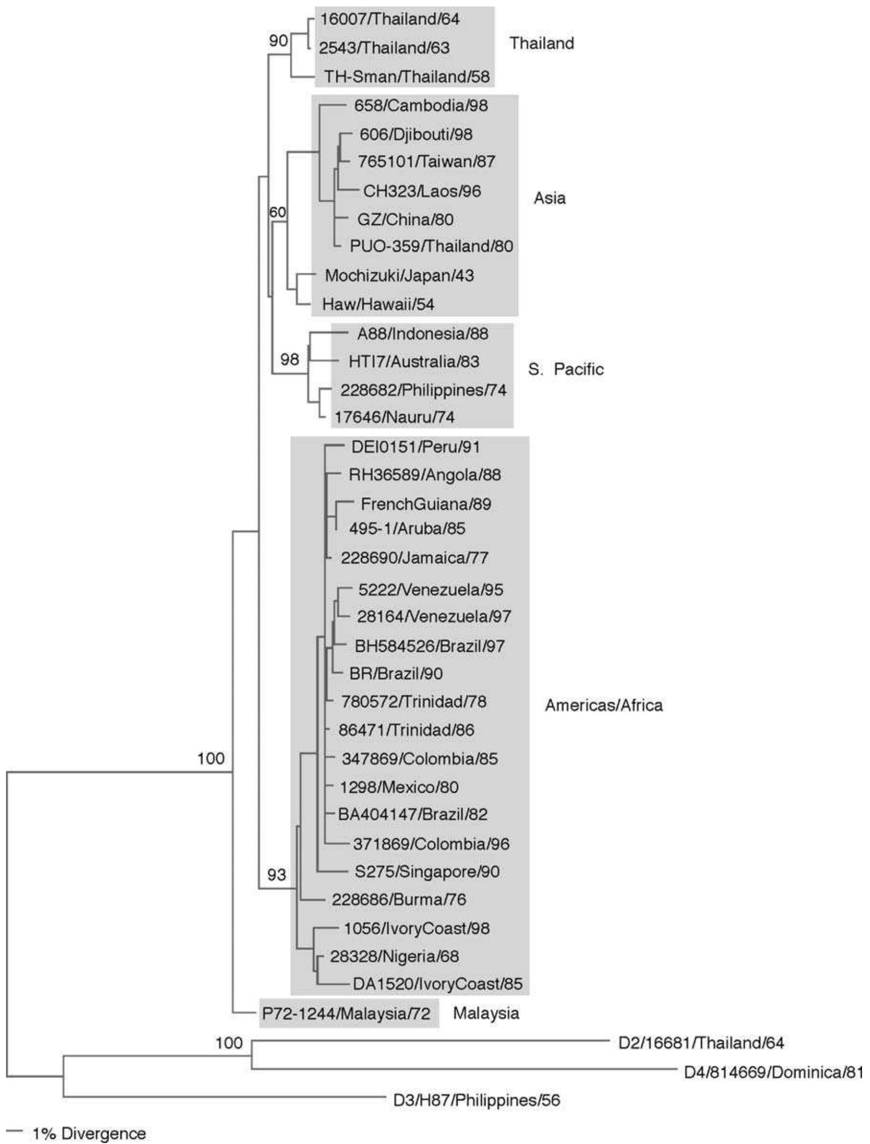

FIG 1. Phylogeny of 36 serotype 1 dengue viruses using 1485 nucleotides from the E gene. Representatives of the other serotypes were used as an outgroup. Strains are denoted by number, country of isolation, and year of isolation; genotypes are shaded and names given to the right. Bootstrap values of statistical support for major branches are shown as percentage equivalents.

B. Phylogeny of Dengue Serotype 2

Dengue viruses of serotype 2 have traditionally been studied in more detail than those belonging to the other serotypes because of their association with more frequent and severe epidemics. In southeast Asia, the first detailed descriptions of disease and epidemics were those caused by viruses belonging to serotype 2 (Burke *et al.*, 1988; Sangkawibha *et al.*, 1984); in the Americas, the appearance of serotype 2 virus was associated with the first epidemics of DHF in this region (Kouri *et al.*, 1983). It was with viruses of this serotype that numerous methods for detecting genetic differences among strains were first attempted: antigen signature analysis compared specific epitopes using monoclonal antibodies to the E glycoprotein (Monath *et al.*, 1986), restriction enzyme mapping of cDNA and probing detected some nucleotide differences (Kerschner *et al.*, 1986), and RNA fingerprinting of many different patient samples from the same location gave an estimate of the large number of variants circulating during 1 year in Thailand (Walker *et al.*, 1988). One large fingerprinting study concluded that numerous variants circulated in southeast Asia over a 25-year period (Trent *et al.*, 1989), and in one summary, up to 10 distinct topotypes of serotype 2 viruses could be identified (Trent *et al.*, 1990). It was not until the first sequencing studies that an attempt was made to identify the genetic differences between strains from DF patients and viruses isolated from DHF patients. Comparisons of E gene sequences from 12 serotype 2 viruses showed no correlation between disease severity and specific nucleotides or amino acids (Blok *et al.*, 1989), and a similar conclusion was drawn after comparison of the NS1 gene from eight virus strains (Blok *et al.*, 1991). Subsequent full genome analyses of viruses from patients in southeast Asia also failed to identify specific sites that might determine virulence (Mangada and Igarashi, 1998; Pandey and Igarashi, 2000).

Sequence analysis of many more strains of serotype 2 first gave rise to the idea that specific genotypes could show differences in virulence potential, resulting in epidemics of DHF. A study of 40 strains from different areas of the tropical world, collected over a 45-year period, showed that dengue serotype 2 viruses could be classified into five genotypes by comparison of the E/NS1 gene junction sequences (240 nucleotides) (Rico-Hesse, 1990). Subsequent comparisons of E gene sequences from 16 strains (Lewis *et al.*, 1993), then more sylvatic strains (Wang et al., 2000), and, more recently, the comparison of only the 3′-untranslated region of serotype 2 strains continued to support these groupings (Shurtleff *et al.*, 2001). Subsequently, after the

analysis of many other samples, especially those from southeast Asia, a region with a long history of DHF epidemics and numerous cocirculating virus variants, the groupings were broken down into four genotypes (Rico-Hesse *et al.*, 1998): sylvatic/west Africa, Americas, southeast Asia, and Malaysia/Indian subcontinent (Fig. 2). It became clear that the introduction of the southeast Asian genotype into the Americas in 1981, specifically into Cuba, and its subsequent spread to other parts of the Caribbean were directly associated with the appearance of DHF in the western hemisphere (Rico-Hesse, 1990). Prior to 1981, there had been sporadic reports of cases of dengue with hemorrhagic manifestations, but it was unclear if the DHF/DSS case definitions had been met. It took a much more detailed analysis of the viruses circulating in the Americas to directly link more severe dengue disease with the southeast Asian genotype virus. The availability of samples from patients with well-documented clinical records from three countries with DHF cases (Brazil, Mexico, and Venezuela) helped prove this point (Rico-Hesse *et al.*, 1997). Others have continued to corroborate this hypothesis (Gubler, 1998) and, to this date, all dengue serotype 2 viruses from DHF/DSS patients have been shown to belong to the southeast Asian genotype. The American and Malaysia/Indian subcontinent genotypes have so far been isolated from patients with DF only and are therefore considered to be of intermediate epidemiological impact.

An anomaly that currently has no explanation is the repeated, independent isolation of viruses genetically similar to the original New Guinea C (NGC) prototype (isolated in 1944) virus by several laboratories around the world: Cuba (Guzman *et al.*, 1995), Venezuela (Rico-Hesse *et al.*, 1997), Mexico (P. A. Armstrong, unpublished), China (GenBank, AF204177), and Vietnam (Twiddy *et al.*, 2002a). Because these contemporary, independently isolated and sequenced samples vary (in general, <2%) from the old, high-passage NGC prototype virus, it is possible that these isolates represent contamination with the NGC laboratory strain, which had been passaged to fix some mutations. The NGC virus has been used for many decades in most national laboratories as the reference strain for serotype 2. It could have potentially been maintained under different *in vitro* conditions, which could have made the resulting sequences different for these countries. The only other explanation seems to be that NGC is, in fact, being transmitted in these countries. However, there is very little evidence to support this, as these isolations are very rare. The disease associated with these samples is not unusual in that there are a mixture of DF and DHF cases from which these were purportedly isolated; this is the only type of isolate that remains from the Cuban 1981 epidemic (Guzman *et al.*, 1995).

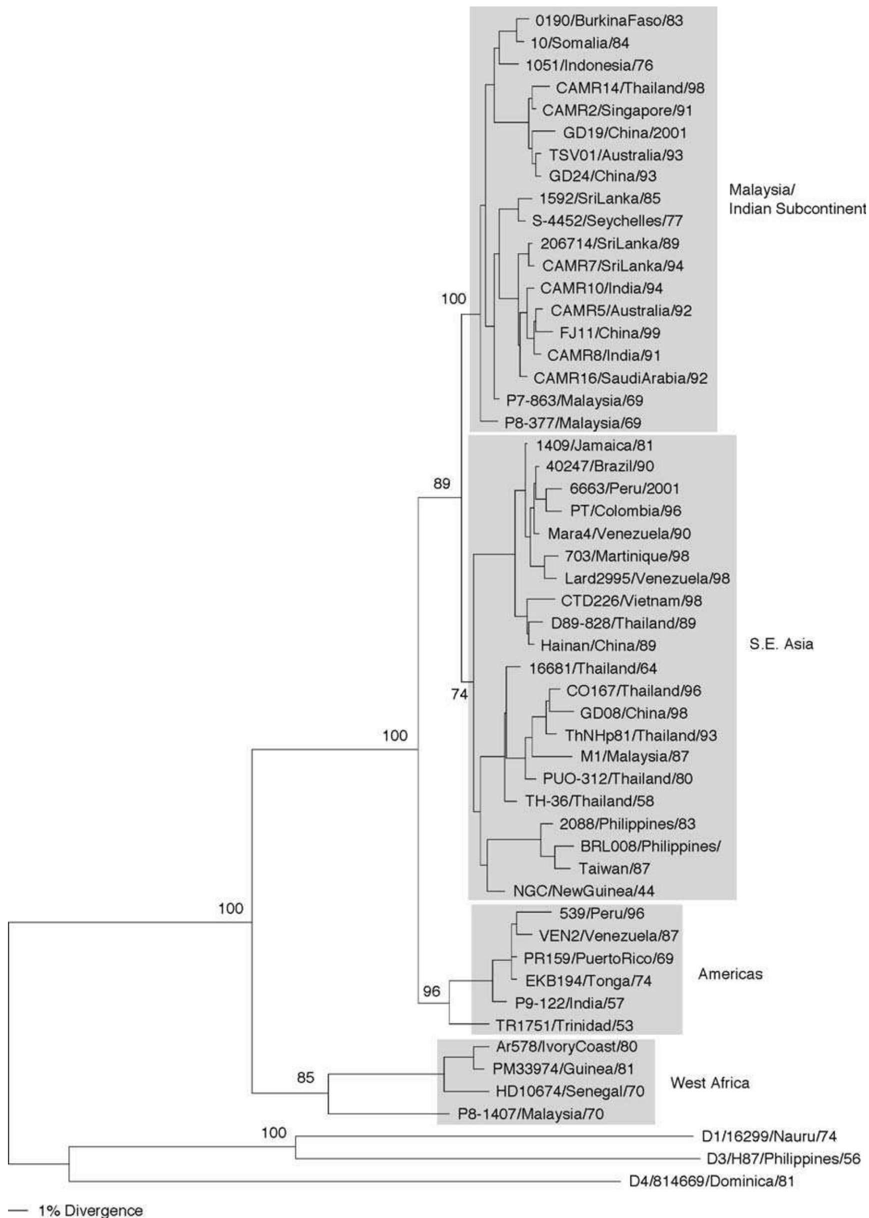

FIG 2. Phylogeny of 50 serotype 2 dengue viruses using 1485 nucleotides from the E gene. Representatives of the other serotypes were used as an outgroup. Strains are denoted by number, country of isolation, and year of isolation; genotypes are shaded and names given to the right. Bootstrap values of statistical support for major branches are shown as percentage equivalents.

As for serotype 1 viruses, serotype 2 sylvatic viruses do not seem to spread to other urban areas even within west Africa (the Malaysia/Indian subcontinent genotype has been imported) and could be classified as being of low epidemiological impact. Furthermore, because of their basal or ancestral position on the phylogenetic tree (Fig. 2), it seems likely that they were the first to evolve from the progenitor shared by all four serotypes. Because of their great genetic distance from the other genotypes, it is hard to determine if they are the origin of all other serotype 2 viruses. Further research is required to explain the low transmissibility or virulence of these viruses to humans and whether this is a consequence of their adaptation to canopy-dwelling mosquito vectors, which do not bite or infect large numbers of humans.

Serotype 2 viruses have also shown the potential to establish transmission in very distant areas of the world. It has been documented previously that the southeast Asian and the Malaysia/Indian subcontinent genotypes have spread from one continent to another in a matter of years (Rico-Hesse, 1990). Classical epidemiological investigations have helped trace the pathways of these introductions and the establishment of endemic or hyperendemic cycles of transmission in some countries (e.g., Venezuela, Brazil). Currently, the main concern is that the southeast Asian genotype, of high epidemiological impact, continues to spread through several countries, including Peru and Mexico. In the case of Peru, good epidemiological evidence shows that the native, American genotype did not cause DHF in patients, even upon secondary infection (Watts *et al.*, 1999). It remains to be seen if and how severe the epidemics will become upon the establishment of transmission of the southeast Asian genotype (Rocha *et al.*, 2002). In Mexico, the introduction of the southeast Asian genotype has been gradual, with the first documented viruses from cases in south and central Mexico in 1995 (Rico-Hesse *et al.*, 1997), but with some areas of northern Mexico (close to the Texas border) still occupied by the American genotype (P. A. Armstrong and R. Rico-Hesse, personal communication). The availability of a very large database of serotype 2 sequences (several hundred strains) will facilitate the documentation of movement of dengue 2 around the world.

C. Phylogeny of Dengue Serotype 3

The first published report of the analysis of genetic variation in serotype 3 viruses occurred in 1972 (Russell and McCown, 1972), but used serologic tests to show this difference; the geographical distinction of strains (Puerto Rico and Tahiti, separate from Asian strains)

still holds true. Fingerprinting studies identified five topotypes for this serotype (versus 8 and 10 for dengues 1 and 2, respectively) (Trent *et al.*, 1990); this lower number of distinct groups is evident in serotype 4 viruses also. Thus, the interpretation of the phylogeny of serotype 3 viruses is somewhat more complex because the rates of fixation seem to be lower, i.e., there is less distance or divergence between the genotypic groups.

The first phylogenetic analysis of these viruses required the comparison of the entire E gene sequences (Lanciotti *et al.*, 1994), and four genotypes were distinguished. This classification has remained unchanged with the study and inclusion of new isolates (Fig. 3), although the lines demarcating genotypic groups are somewhat blurred mainly by older strains (see Philippines 1956 and Thailand 1962). Again, most of the genotypes can be identified by geographical origin, but there is no evidence for a sylvatic group: Americas (most basal), Indian subcontinent, Thailand, southeast Asia/South Pacific.

In this case, the genotype with low epidemiological potential seems to be the one that circulated in the Americas prior to 1989; isolates of this type came from patients with DF only. In the 1980s through the 1990s, in both the Americas and the South Pacific, there were two independent introductions of new genotypes: the southeast Asian genotype was associated with large epidemics with DHF in Tahiti and Fiji (Chungue *et al.*, 1993) and the Indian subcontinent genotype was introduced to Central America in the mid-1990s (Balmaseda *et al.*, 1999; Harris *et al.*, 1998, 2000; Usuku *et al.*, 2001), displacing the American genotype in both areas.

Evidence shows that the Indian subcontinent genotype has evolved, since 1989, to produce more DHF in Sri Lanka (Lanciotti *et al.*, 1994), a country that maintains good epidemiological and virological records. Current studies are focused on documenting the mutations responsible for this phenotypic variation, as was done for viruses of serotype 2 (Messer *et al.*, 2002). In prospective studies in Thailand, the direct correlation between viremia caused by dengue serotype 3 viruses and the severity of disease (DHF) has also been shown (Libraty *et al.*, 2002). Thus, it is important to determine if some of these genotypes also replicate to higher levels in human cells, which are targets involved in the pathogenesis of dengue disease. The more virulent Indian subcontinent genotype has also been associated with epidemics currently occurring in Mexico and Central and South America, and viruses from these outbreaks need to be studied more closely, especially with the new assay systems described later. It remains to be seen if viruses of serotype 3 have consistent genetic differences

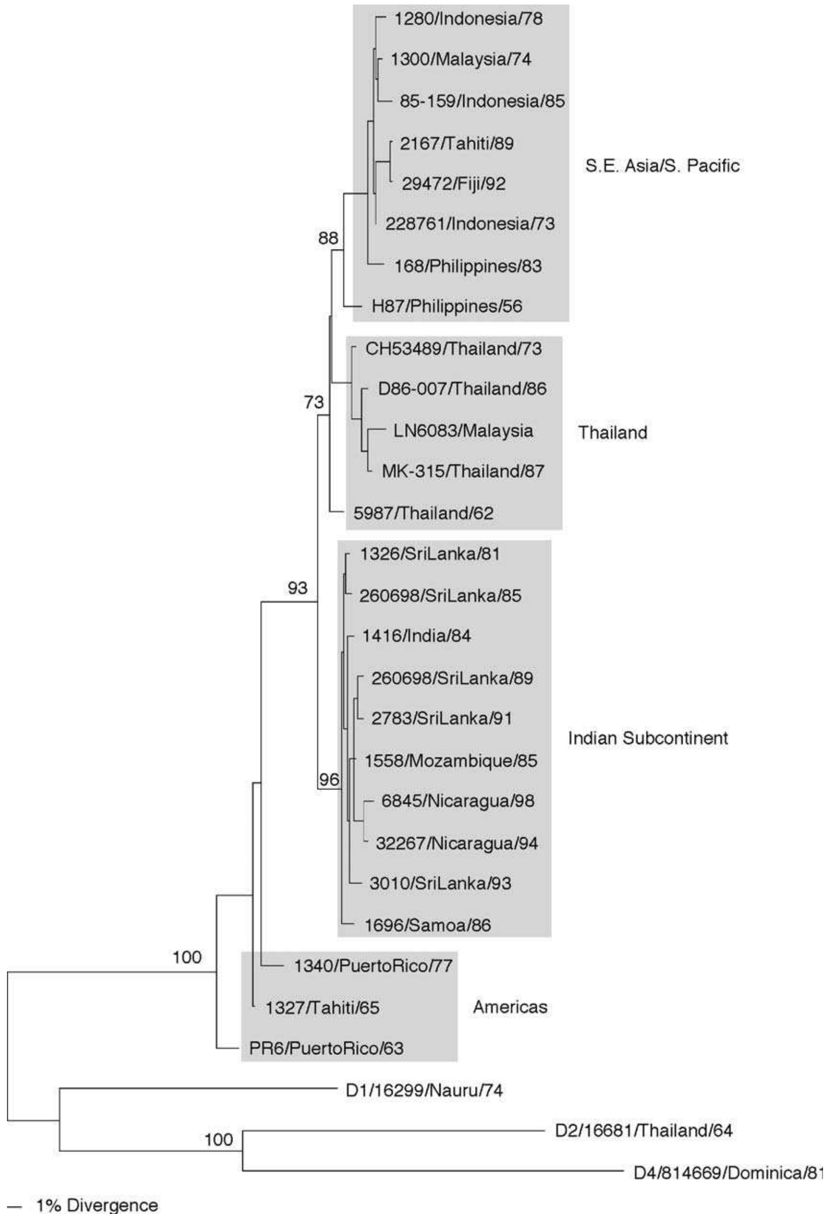

FIG 3. Phylogeny of 26 serotype 3 dengue viruses using 1479 nucleotides from the E gene. Representatives of the other serotypes were used as an outgroup. Strains are denoted by number, country of isolation, and year of isolation; genotypes are shaded and names given to the right. Bootstrap values of statistical support for major branches are given as percentage equivalents.

in regions of the viral genome that encode determinants of more severe disease.

D. Phylogeny of Dengue Serotype 4

Dengue viruses of serotype 4 were shown to differ antigenically and genetically in 1986 (Henchal *et al.*, 1986), and five topotypes were identified in 1990 (Trent *et al.*, 1990). However, comparison of short (180-nucleotide) sequences from the E gene failed to classify 28 isolates into genotypic groups (Chungue *et al.*, 1995). Only after the comparison of full E gene sequences was it possible to distinguish two genotypes (Lanciotti *et al.*, 1997) and the rates of variation among 20 strains were shown to reach a maximum of 6%. The inclusion of longer nucleotide sequences (preM-E genes) has failed to add more resolution to the phylogenetic trees, and only two genotypes could be resolved (Fig. 4); the inclusion of only E gene sequences from one sylvatic virus (Wang *et al.*, 2000) adds one extra group, as for serotype 1 viruses. Thus, there are currently the Malaysia, southeast Asia, and Indonesia genotypes. As with the other serotypes, it is not known if the sylvatic cycle in Malaysia still exists, as that country had imported the urban cycle virus, apparently from southeast Asia (see Malaysia 1969). At this point in time, it seems that the genotype that includes viruses from Indonesia, the South Pacific, and the Americas is the one of greater epidemiological impact, if only because it continues to spread to other countries, mainly in the Americas (Lanciotti *et al.*, 1997). It is obvious that we know much less about the genetic variation of viruses of this serotype other than that their rates of mutation in nature are lower and that there is less transmission of this virus, as evidenced by the lack of epidemics associated strictly with these viruses.

IV. Viral Determinants of Disease

As mentioned earlier, dengue virus virulence has been difficult to measure because of the lack of *in vivo* and *in vitro* models of disease. One approach to determining the viral sequences or structures involved in virulence has been to compare the entire genome amplified directly from samples from patients with clearly differing disease presentations, after having the virus isolates identified as to serotype and genotype, usually from the same blood sample. This requires samples of serum or plasma containing high titers of virus from patients in the acute phase of disease (before the onset of severe symptoms, if they

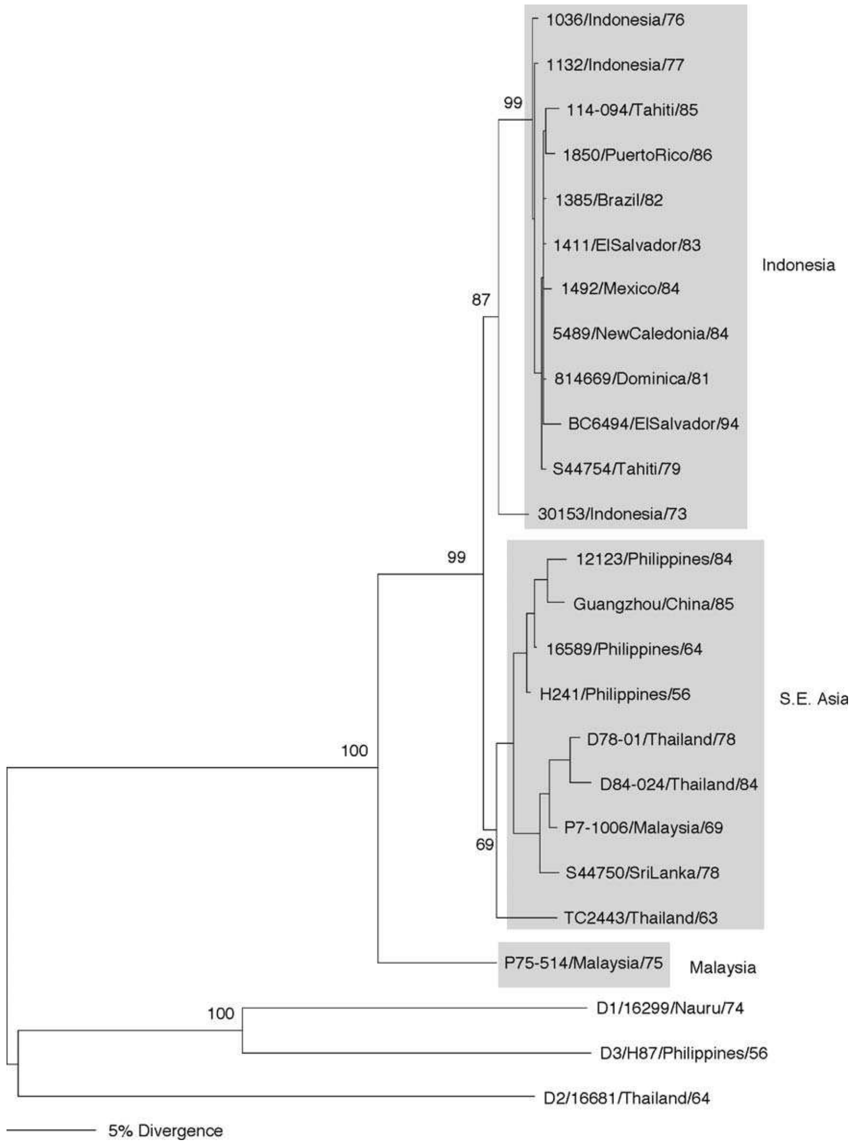

FIG 4. Phylogeny of 22 serotype 4 dengue viruses using 1635 nucleotides from preM–E genes, except for two strains from Malaysia, for which only E gene sequences were available (1485 nucleotides). Representatives of the other serotypes were used as an outgroup. Strains are denoted by number, country of isolation, and year of isolation; genotypes are shaded and names given to the right. Bootstrap values of statistical support for major branches are shown as percentage equivalents.

develop) and ultralow temperature storage (−70 °C) until processing and sequencing with RT-PCR methods. So far, these conditions have been met only for dengue viruses belonging to serotype 2, with a detailed comparison between sequences belonging to the American (low virulence) and southeast Asian (high virulence) genotypes (Leitmeyer *et al.*, 1999). The results pointed to consistent differences between these genotypes in certain nucleotides and folding patterns of the 5′- and 3′-untranslated regions of the viral genome, along with amino acid charge differences in the prM, E, NS4b, and NS5 genes. Others had shown that mutations in the untranslated regions of dengue serotype 4 infectious clones yielded viruses with altered mouse neurovirulence (Blaney *et al.*, 2001) and that differences in amino acid position 390 of the E glycoprotein of serotype 2 viruses also have such an effect (Sanchez and Ruiz, 1996). The working hypothesis is that substitutions at these positions affect RNA structures and properties of the E protein and contribute in some way to alter the pathogenesis of DHF. Support for this hypothesis has come from complex, prospective studies showing that severe disease correlates with higher titers of virus in the bloodstream when associated with southeast Asian genotype virus infection (Vaughn *et al.*, 2000).

Current studies of viral determinants of disease involve the modification of infectious clones (Kinney *et al.*, 1997) and the measurement of the effects of mutations in new, target cell assay systems. The discovery of dendritic cells as primary targets for dengue virus replication (Wu *et al.*, 2000; in contrast to many earlier studies focusing on monocytes as target cells) has led to attempts to use this characteristic as a correlate of differences in virulence. For instance, it has been shown (Cologna and Rico-Hesse, 2003) that the replacement of southeast Asian genotype 5′- and 3′-untranslated regions and one amino acid (E-390) with American genotype structures significantly reduce the level of replication of infectious clone-derived viruses in human, primary dendritic cell cultures. The virus yield (measured by quantitative RT-PCR) of the triple mutant (three regions substituted) was identical to that of a wild-type American genotype virus in the same assays, whereas that of the unmodified southeast Asian virus was 10-fold higher. Others have shown that the modification of amino acid residue 390 alone of the E protein reduces the rate of replication of dengue virus infectious clones in human monocytes (Pryor *et al.*, 2001). It is anticipated that dendritic cell assay systems may serve as an *in vitro* surrogate for dengue virus virulence, but many more detailed investigations are necessary to determine the molecular mechanisms involved in the differences in virus yield. However, a comparison of 12

different wild-type, low-passage, dengue serotype 2 viruses belonging to the southeast Asian and American genotypes almost uniformly showed lower virus output by the latter in primary human dendritic cells. It remains to be seen whether these dengue viruses behave similarly in other human target cells, but it is tempting to speculate that the differences in the behavior of these viruses could explain the increase in cytokine release (i.e., as a result of higher virus burdens in infected monocytes and dendritic cells) and the subsequent plasma leakage by endothelial cells seen in DHF (Bosch *et al.*, 2002). Another assay system under development involves the use of dengue virus–infected, primary lymphocyte cell cultures in chambers below human, primary endothelial cell cultures to measure the effects of cytokine secretion by lymphocytes on the latter cells as a surrogate for plasma leakage *in vivo* (C. Kubelka, R. Cologna, and R. Rico-Hesse, personal communication). Thus, the availability of *in vitro* systems to measure the effect of virus infection on isolated human target cells might also help us understand the mechanisms behind immune enhancement of disease. Other desirable models of disease might also include immunodeficient mice that have been reconstituted with human cells or factors that may be involved in the pathogenic cascade that leads to more severe dengue disease. However, no one has yet reported successful engraftment of dendritic cells or the reproduction of severe dengue symptoms in such an animal model (An *et al.*, 1999; Lin *et al.*, 1998; Wu *et al.*, 1995).

V. Viral Determinants of Transmission

Another way of measuring dengue virus epidemic potential comes from studies of the ability of different viruses to infect and be transmitted to other humans by the mosquito vector, *Aedes aegypti* (Gubler *et al.*, 1979). It follows that if some dengue viruses are capable of producing higher and longer viremias in their human hosts, these viruses could possibly infect more vectors and thus have a higher chance for transmission. This hypothesis was tested by infecting low-passage (F2–F4 generation) *A. aegypti* mosquitoes collected in McAllen, Texas, and Iquitos, Peru, with the same 12 wild-type dengue virus preparations used for dendritic cell infections described earlier and measuring time for virus dissemination to the salivary glands as a surrogate for transmission potential (Armstrong and Rico-Hesse, 2001). These mosquitoes were infected *per os* with the same amount of virus as measured by quantitative RT-PCR and mosquito infectious dose.

Results suggested that the transmission of southeast Asian genotype viruses is more robust, with more efficient infection and dissemination in mosquitoes from different geographical areas. However, others have shown that mosquito populations also vary in their ability to become infected by, and to disseminate, a single dengue virus, albeit a very high passage strain (Bosio *et al.*, 1998, 2000). It is clear that mosquitoes vary in their ability to get infected and reproduce enough virus to become infectious to humans by injection of their saliva. Laboratory-maintained strains of *A. aegypti* seem to have lost their potential to replicate dengue viruses differentially (southeast Asian versus American genotype), and there is also some variability of this transmission efficiency within field-derived populations (Armstrong and Rico-Hesse, 2001). Additional studies are needed to identify the factors that influence mosquito vectorial capacity, including the genetics of high and low transmitters (Miller and Mitchell, 1991).

VI. Viral Displacement and Epidemiology

Although the efficiency of dengue virus transmission is a complex phenomenon, the displacement of one genotype by another more "virulent" type has been documented in the past and is currently occurring in some countries. To understand this process, it is important to have good continuous epidemiological surveillance systems in place to detect the spread of different dengue virus genotypes. Such systems have allowed for the detection of the displacement of the American genotype of dengue serotype 2 by the southeast Asian genotype in the Americas in at least four countries in the past (Brazil, Colombia, Mexico, and Venezuela) (Rico-Hesse *et al.*, 1997), and presently in one country (Peru) (Rocha *et al.*, 2002). The failure to detect DHF in Peru has been associated with the lack of transmission of the southeast Asian genotype, although the American genotype virus was being transmitted at high rates (Watts *et al.*, 1999). Unfortunately for the people of Peru, the southeast Asian genotype virus has been detected in mosquitoes in Iquitos, and this regional population has therefore become a natural test bed for the virus virulence hypothesis.

The same phenomenon of displacement seems to be occurring for dengue serotype 3, where the more virulent Indian subcontinent genotype had appeared after a nearly 20-year hiatus by the native American genotype in Nicaragua and Guatemala (Balmaseda *et al.*, 1999; Usuku *et al.*, 2001). The transmission of this virus has been associated with massive outbreaks of DF and DHF in Brazil, Ecuador, el Salvador,

and Honduras (World Health Organization, 2002). These introductions and/or displacements have been clearly correlated with the appearance of DHF. Thus, by monitoring the transmission of specific dengue genotypes, we can predict the appearance of more severe epidemics. This phenomenon seems to occur uniformly because virus transmission is so high that invariably those with the propensity to present with severe disease become infected. However, more studies are needed to identify the viral determinants that impart to the virus an increased potential to replicate in certain hosts and vectors and a subsequent role in the epidemiology and pathogenesis of dengue disease.

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INDEX

A

A172 cells, 133
ACACA-type sequences, 182–183
Acidic pH
  catalyzed conformational change, 154
  E-glycoprotein fusion and, 154–155
  E protein and, 231
  fusion activity and, 77
  induced dissociation, 75–76
Acidotropic agents, 80
Active protease domain, 40
ADE. *See* Antibody-dependent enhancement
S-adenosyl methionine-utilizing methyl transferases
Adenoviruses, 249
Adhesion molecule(s), 229, 235
  cell surface macromolecules and, 249
  expression, 237, 253
  flaviviruses and, 251–253
  functions of, 251
ADP release, 239
Aedes, 285
  viral associations of, 295
Aedes aegypti, 295
  DEN virus transmission by, 334
  Dengue virus and, 299
  eradication of, 297
  in New World, 295
  origin of, 305
Aedes africanus, YFV and, 295
Aedes albopictus, Dengue virus and, 300
Aedes furcifer, 295
Aedes keniensis, 295
Aedes luteocephalus, 295
Aedes metallicus, 295
Aedes neoafricanus, 295
Aedes opok, 295
Aedes togoi, 288
Aedes vitattus, 295
Affinity relationships, between ns proteins and RC, 115
Afghanistan, 289
Africa, 306
*Age*I, 202
Agricultural practices, 294
Alanine-scanning mutagenesis, 38
Alfuy virus (ALFV), 302
Alkhumra virus (ALKV), 283
Alphavirus, 4
  recombination between, 307
  Western equine encephalitis virus and, 6
Alphavirus(es)
  3′ NCR in, 201
  cyclization, 190
  E1-E2 heterodimer of, 83
  E1 protein, 66, 81, 82–83
  fusion rate of, 74
  replicon vectors, 132
  replicons, 96
  vs. flaviviruses, 4, 82–83
Amino acid
  changes in E-glycoproteins, 148
  divergence, 282
Ammonium chloride, 80
Anaphylatoxins, 253
  C3a, 239
  C5a, 239
Androstanol, 75
Anopheles hyrcanus, 288
Antarctica, 281
Anti-dsRNA, 117
Anti-E-glycoprotein
  antibodies, 154–155
  Mabs, 156
Anti-ERGIC53, 117
Antibodies. *See also* Monoclonal antibodies
  anti-BrdU, 114
  anti-NS1, 37, 159

Antibodies. *See also* Monoclonal antibodies (*cont.*)
- anti-NS4B, 106
- anti-prM, 157
- antiribosomal, 102
- to dsRNA, 112–114
- flaviviral-neutralizing, 147
- fusion blocking, 145
- neutralizing, 2, 73
- responses to synthetic peptides, 153

Antibody-dependent enhancement (ADE), 244
- antiviral IgM and, 248
- CR3 blocking antibody, 248
- FcRs in, 248
- IFN-γ and, 247

Antigenic analysis, 5
- of DEN 2 E-glycoprotein, 154
- *vs.* phylogenetic analysis, 281

Antigenic cross-reactivity, 2
Antigenic groups, 3
Antigenic structure, 66
Antihistamine treatment, 330
*Apa*I, restriction endonuclease site, 196
Apoi virus (APOIV), 284, 286
Apoptosis, of Kupffer cells, 234
Aprotinin, 40
Aqueous agarose gels, 102
Arbovirus(es)
- classification of, 3–4
- group A, 3
- group B, 4
- HI and, 2–3
- history of, 1–2
- isolation, 2
- morphology of, 4

Argasid ticks, 299
Arthropods, 1–2
- viral recombination and, 307

Asia, YFV in, 297–298
Asp_{77}, 40
Asp_{343}, 38
Assembly, of flaviviruses, 64, 66–68
Astrocytes, 253
ATP hydrolysis, 40
Australia, flaviviruses in, 303
Autoprotease 2A, 130

B

B cells
- epitopes, 143–159
- inhibiting FcRs on, 248
- response to C protein, 157

Bacteriophage
- display technology, 150
- Qβ, 150

Bafilomycin A1, 80
Base pairing, long-distance, 32
Basophils
- Fc receptors in, 247
- flaviviruses and, 238–239

BBV. *See* Bukalasa bat virus
BHK cells
- HG expression in, 132
- KUN replicon RNAs in, 119
- lysates, 211
- mutant viruses in, 201
- replication in, 191
- replicon RNA in, 197

Binding proteins, 230
Binding sites, for neutralizing antibodies, 73
Biochemical assays, of RC, 114–116
Biosynthesis, prM formation in, 80
Bisaillon, M., 127
Blackwell, J. L., 209–210
Blood-brain barrier, 235
- chemokines and, 253

Blood vessel endothelial layers, 234
Bol, J., 218–219
Bovine coronavirus, 209
Bovine viral diarrhea virus, 201
Br-RNA strands, 112
Brefeldin A, 116
Brinton, M. A., 203, 207, 209–210
Bromo-substituted uridine (BrU), 112
Brown, L. V., 2
Budding, 24–25
Buescher, Edward, 2–3
Bukalasa bat virus (BBV), 284
Bunyaviridae, 4
Butcher, S. J., 129

C

C20, 129–130. *See also* C (capsid) protein(s)
C (capsid) protein(s), 26, 33–34
  accumulation of, 126
  amino-terminal 20 amino acids of, 129–130
  antigenic structure of, 157
  B-cell response to, 157
  C-terminal hydrophobic domain of, 34
  cleavage among, 33
  composition of, 7
  of flavivirus, 63
  migration, 106–107
  in RNA replication, 191
  structure of, 27
  TBE, 33
C-terminal
  of E proteins, 69
  hydrophobic anchor, 33
  of NS5, 43
  NS1 and, 36
  of NS2A, 38
  peptide sequencing, 37
  RNA replication and, 40
  stem-anchor region, 72
C3a receptors, 253
C5a receptors, 253
Calisher, C. H., 4
Cap-binding protein, p220, 127
Capping
  reaction, 127
  type 1, 177
  of viral genomes, 179
Capsid gene, CS1 and, 31
Capsidless subviral particles (SHA), 65
Carbohydrate side chains
  in extracellular virions, 68
  in JE E protein, 71
Carboxy-terminal hexahistidine tag, 114
Carboxyl-terminal hydrophobic sequence, 107
Carboxypeptidase digestion, 103
Carey Island virus, 5
Casals, Jordi, 2
CAT. *See* Chloramphenicol acetyltransferase
C5b9, 241
CD14, 244
C3dg, 241
cDNA(s), 107
  clone of KUNV, 121
  cloned flavivirus, 28
  clones, 117–119
  epitope localization and, 149
  FLSDX, 118
  library, 211
  virus clones, 163
C20DXrep replicon, 129
CEEV. *See* Encephalitis virus
Cell-cell interactions, 229
Cell-fusing agent (CFA) virus(es), 180, 278
  divergence of, 304
  genome, 182
Cell proliferation, 236
Cell receptor binding, 230
Cell surface macromolecules, 229
  in flavivirus attachment, 242–249
  modulation of, 249–253
Cell surface proteins, 24
Cell tropism, 229
Cellular protein binding, to 3′-SL, 209–214
Cellular receptors, 23
Cellular terminal transferases, 102
Central European encephalitis virus (CEEV), 5, 16
Central nervous system
  chemokines from, 253
  flaviviruses in, 234
  neurotropic flaviviruses in, 237
Cerebral microvasculature, 234
CF. *See* Complement-fixation technique
CFA. *See* Cell-fusing agent virus
Chemokines
  of central nervous system, 253
  expression, 253
  leukocyte-attracting, 235
Chen, C.-J., 211
Chen, W.-J., 214
Chikungunya, 240
Chiou, S.-S., 214
Chloramphenicol acetyltransferase (CAT), 132
  gene expression, 125
5-α-cholestanone, cholesterol and, 75
Cholesterol, 74–75, 84
Cholesteryl methylester, 75
CHXM. *See* Cycloheximide
Circular dichroism analysis, 208

cis
acting elements, 120
cleavage in, 109
NS3 and, 39
secondary structures and, 215
translation in, 100
Clathrin-coated pits, 24
Cleavage(s)
anchC-prm, 34
by carboxypeptidase digestion, 103
of class I proteins, 81
in cytosol, 103
of NS1-2A, 36
polyprotein, 177
of prM protein, 66, 80
protonation and, 80
signalase, 34
in vitro experiments, 80
Clustal W program, 322
CM/PC structures
analysis, 109–111
anti-ERGIC53 and, 117
induction, 133–134
CMs. *See* Convoluted membranes
Codon usage, 320
Colocation, in VPs, 110
Comparative electron microscopy, of KUNV-infected cells, 107–109
Complement activation, 253
Complement fixation (CF) assays, 142
Complement-fixation (CF) technique, 2
human poliomyelitis virus and, 3
Complement receptors, 248–249
Complementation experiments
of KUNV NS1, 123
with NS1 and NS5, 121
Consensus composition, of RC, 114–116
Conserved linear sequence
cyclization sequences and, 183
of NCRs, 179–203
Conserved linear sequence features, of 3′NCRs of TBE, 197–203
Conserved linear sequences, in 3′ NCRs, 192–197
Convoluted membranes (CMs), 24
induced membranes and, 106
morphologies of, 107
polyprotein processing and, 42
Coprostanol, 75
Coronaviruses, 249
Co-translation, with microsomal membranes, 109
Coxsackievirus, 3
C1q receptor, 241
CR3, 248
Cross-reactions, antigenic, 5
Cryoelectron micrographs, 26
DEN2, 25, 65
of flaviviruses, 72
of TBE virions, 64
Cryoimage reconstruction, 27
Cryoimmunoelectron microscopy
of dsRNA, 109–111
with Kunjin virus, 66
NS1 and, 105
of ns proteins, 109–111
Cryosectioning, 24
CS1, 31–33
CYC sequence in, 194
in mosquito-borne virus genomes, 217
structure of, 194
CS2, 30, 194–196
in mosquito-borne virus genomes, 217
vs. RCS2, 196
CS. *See* Cyclization
CS3, *vs*. RCS3, 196
5′CS
PR and, 32
RNA replication and, 192
Csyteines, conserved, 36
CTL effector responses, 162–163
Cuba
DEN2 and, 326
YFV in, 297
Culex, 285, 300
New World, 5
viral associations of, 295
CYC. *See* Cyclization
3′CYC sequence, RNA replication and, 192
Cyclization (CYC)
conserved complementary, 121
in CS1, 194
energy for, 190
Flavivirus, 186–187
of flaviviruses, 220
of genomic RNA, 121
mutagenized, 192
sequences (CS), 31, 100, 183–192, 219
3′ cyclization sequences (CS), 183–184
5′ cyclization sequences (CS), 183

Cycloheximide (CHXM), 112, 132
Cyclooxygenase-2 expression, 253
Cysteine residues, 34
  in E proteins, 71
Cytokines
  dengue virus and, 334
  expression, 253
  leukocyte-derived, 254
Cytopathic effect, of KUNV infection, 119
Cytoplasmic
  membranes, 24
  viral immunogens, 132
Cytosol, 103
Cytotoxic effector activity, 162
Cytotoxic T-cell (T_c)
  epitopes, 162–163
  MHC class I and, 249
Cytotoxicity, TNF and, 236

D

DAF. *See* Decay accelerating factor
Dalldorf, Gilbert, 3
Dawson, J. R.
de Madrid, A. T., 4
DEAD family, 104
DEAH box, KADV and, 279
Dear tick virus (DTV), 288
Decay accelerating factor (DAF), 241
  human, 37
Deforestation, 278
Deletion(s)
  mutations, 205–206
  spontaneous, 201
DEN1 virus
  Americas/Africa, 324
  Asia, 324
  assembly in, 123
  E-glycoprotein analysis, 149
  mutant F and, 214
  phylogeny of, 322–323, 324
  South Pacific, 324
  strain WP74, 181
  sylvatic/Malaysia, 324
  Thailand, 324
  vs. DEN2 virus, 71
DEN2 virus, 285
  3′-SL replacement, 212
  3′ stem-loop, 29
  Americas, 326
  cryoelectron micrograph of, 64
  CS1 in, 31
  cyclization, 190
  E-glycoprotein analysis of, 149–150
  E-glycoprotein epitopes of, 146
  E/NS1 gene junction, 325
  epidemiological impact of, 320, 328
  epitopes, 151
  fusion in, 152
  genomic sequencing, 333
  genotypes, 326
  glycosylation in, 71
  infected Vero cells, 109
  Mab, 154
  Malaysian/Indian subcontinent, 326
  NS1, 36, 37, 110
  NS3, 42, 109–110
  phenylalanine in, 79
  phylogeny of, 325–328, 327
  RF RNA in, 101
  3′SLs in, 193
  southeast Asia, 326
  strain New Guinea C (NGC), 180
  structure of, 26, 27
  sylvatic/west Africa, 326, 328
  synthetic peptide analysis, 151
  transmission of, 321
  vs. DEN1 virus, 71
DEN3 virus
  Americas, 329
  CS1 and, 31
  displacement, 335–336
  epidemiologic potential, 329
  genotypes of, 329
  Indian subcontinent, 329
  phylogeny of, 328–331
  southeast Asia/South Pacific, 329
  Thailand, 329
  transmission of, 321
DEN4 virus
  3′ model RNAs, 208
  3′ NCR and, 32
  classification of, 331
  CS1 and, 31
  3′CS and, 122
  deletion in, 196–197
  deletion mutagenesis in, 202, 205
  E-glycoprotein sequencing, 150
  emergence of, 305

DEN4 virus (*cont.*)
  Indonesia, 331
  Malaysia, 331
  mutant F and, 214
  ORF, 205
  phylogeny of, 331, 332
  replication, 29
  southeast Asia, 331
  strain 814669, 205
  topotypes of, 331
Denaturation, thermal, 76–77
Dendritic cells
  flaviviruses and, 332–333
  inhibiting FcRs on, 248
  MHC class I and, 231
  T-cell stimulation by, 231
Dendrogram, 200
Dengue (DEN) virus(es), 177, 297
  3′ NCR nucleotide sequences of, 181, 188
  ADE of, 244–245
  in Americas, 306
  antigenic differences of, 315
  antihistamine treatment of, 330
  B cells and, 237
  combinations of, 7
  CS1 and, 194
  domain II expression in, 149
  E/prM complexes and, 231
  ELISA reactivity of, 159
  Endothelial cells and, 254
  epidemiology, 316, 335
  epitope localization in, 147
  evolution, 315
  genetic differences of, 315
  genomes, 181
  genomic sequencing, 318, 320, 321
  glycosylation in, 71
  hemagglutinins of, 2
  hemorrhagic disease and, 245
  in humans, 232–233
  increased activity of, 141
  interferon and, 235
  intramolecular recombination among, 320
  isolates, 320
  Mab reagents and, 155
  mature, 92
  microevolution of, 318
  mutagenic analysis of, 333
  mutant genomic RNAs, 196
  mutations, 320
  3′NCRs of, 221
  nucleotide sequence of, 203
  origin of, 306
  pathogenesis, 33
  phylogenetics, 315, 318–331
  phylogenies related to, 281–282
  platelets and, 239
  recombination between, 6, 306
  subunit vaccines, 163
  surface interactions on, 242
  thrombocytopenia and, 239
  transmission, 334–335
  vaccine candidates for, 141
  vectors, 299
  viral determinants of, 331–334
  viral RNA template of, 318
  virulence of, 315
  vs. YFV, 300
Dengue fever (DF), 297, 315
  ape *vs.* human, 317
  in Latin America, 335–336
  in New World, 297
Dengue hemorrhagic fever (DHF), 6, 235, 297
  American/African strain and, 323
  in Americas, 317, 320
  ape *vs.* human, 317
  interferon and, 235
  in Latin America, 335–336
  plasma leakage and, 334
Dengue infections, viremic titers in, 246
Dengue serotypes, genetic variation within, 317–318
Dengue shock syndrome (DSS), 235
DHF. *See* Dengue hemorrhagic fever
DHF/DSS, 245, 315
  case definitions, 326
  fluid leakage in, 235
  ICAM-1 and, 252
  IgE levels and, 238
  thrombocytopenia and, 240–241
  TNF and, 236
Dimerization
  of NS1, 36
  NS1 secretion and, 37
Disease associations, of flaviviruses, 8–12

Dispersal
of flaviviruses, 279
virus, 277–78
Dispoto, J. H., 203
Dissociation, pH induced, 83
Disulfide bridges, 35
E-glycoprotein epitopes and, 163
E-glycoprotein structure and, 144
in E proteins, 68
in NS1, 36
Double-stranded RNA (dsRNA), 100. *See also* Replicative form (RF)
analysis, 112
antibodies to, 112–114
in DEN2, 101
foci, 112
foci colocalization, 116
in KUNV-induced membranes, 109–111
nascent progeny RNA and, 111–114
of phi6, 128
synthesized, 106
template, 111
DSS. *See* Dengue shock syndrome
DTV. *See* Dear tick virus
Dual-labeled cryosections, of KUNV-infected cells, 110
Dual-labeled foci, 106–107

E

E22. *See also* E (envelope) protein(s)
signal sequence, 130
E1-E2 heterodimer, 83
E (envelope) dimers, 26, 64, 69, 73, 77, 78
of DEN2 virus, 73
dissociation of, 231
protonation of, 77
of TBE, 78
E (envelope) protein(s), 25–26, 34–35
amino acid identity in, 71
antibodies against, 5
C terminus of, 69
carboxy-terminal 22 amino acids of, 130
cleavage among, 33
conformational changes in, 75, 231
crystallized ectodomain of, 69
dimer, 93, 95
elasticity of, 255
epitopes and, 75
flavivirus, 63, 81
folding of, 67, 80
functions of, 230
Heinz, 35
homology among, 231
icosahedral organization of, 72
metastable arrangement of, 80
mutational analysis of, 230
N-glycosylated, 35
N-terminal domain of, 69
NS1 and, 35–36
oligomerization of, 67
prM protein and, 34, 68, 228
schematic of, 72
sequence data, 279
structural alterations of, 64
structural comparison of, 83
structure of, 35, 69–72, 91–97
of TBE virus, 26
thermal denaturation of, 76–77
TM1 of, 72
TM2 of, 72
trimer, 96
vs. alphavirus E1 protein, 231
E gene sequencing analysis
of DEN1, 322
of DEN2, 325
of DEN3, 329
E1 glycoprotein, 35
E-glycoprotein(s)
A domain of, 144
amino acid changes in, 148
antibody responses to, 153
antigenic characteristics of, 145–146
antigenic differences among, 179
antigenic domains of, 144
antigenic fine structure of, 145–157
B domain of, 144
C domain of, 144
conformational change of, 154
crystal structure, 145, 154
of flaviviruses, 142–143
fragment analysis, 149
fusion and, 152–154
fusion sequence, 152
mapping of, 145
neutralization regions of, 147
pepscan analysis of, 150
peptide mapping of, 150
protective epitopes of, 154

E-glycoprotein(s) (*cont.*)
specific Mabs, 157
structure of, 143
synthetic peptides of, 150
E/NS1 gene junction, 322
E/prM complexes, 230
E1 proteins
alphavirus, 82–84
structural comparison of, 83
E2 proteins, E1 protein and, 84
E-selectin, 252
Eastern equine encephalitis virus (EEEV), 2, 6
Ebola virus, glycoprotein GP, 132
Edge Hill virus (EHV), 295
divergence of, 296
EEEV. *See* Encephalitis virus
EF-1α, 123
EF-Tu, 31
EF1A. *See* Translation elongation factor 1A
EHV. *See* Edge Hill virus
Electron densities, 26
Electron microscopy, 4
of endothelium, 236
of flavivirus particles, 25
Electrophoresis, of intracellular RNA, 102
ELISA reactivity, of human DEN, 159
Elongation factors, 210
translation, 211
Encephalitis virus, 1, 6, 230. *See also* specific strains
classification of, 3–4
human, 2
neutralization tests on, 5
viremia and, 252
Encephalomyocarditis virus
internal ribosomal entry site, 119
IRES, 130
Endocytic pathway
flaviviruses and, 227
fusion and, 74
Endocytosis, receptor-mediated, 24, 64
Endoglycosidase H_f digestion, 68
Endothelial cell physiology, 233–234
Endoplasmic reticulum (ER), 24
assembly and, 65
colocalization, 116
lumen, 42
retention signals, 68
rough (RER), 66
translocation, 33
in virion assembly, 66
Endosomes, 74
Endothelial cells, 234–237
activation, 246, 252
C5a receptors in, 253
cerebral microvascular, 234
DHF/DSS and, 236
function of, 237
plasma leakage by, 334
surface perturbation, 254
Endotoxic shock, 236
Endotoxin, 236
Enzymatic amplification, 318
Epidemiology
of Dengue virus, 335–336
virus, 277–279
Epitope(s)
B-cell, 143–159
conformational, 75
cytotoxic T-cell, 162–163
DEN 2 virus, 151
disulfide bridges and, 144
helper T-Cell, 159
localization of, 146
mapping, 149–150
on NS1, 158
T-Cell, 159–163
ER. *See* Endoplasmic reticulum
Escherichia coli
E-glycoprotein analysis in, 149
expression, 152
JE NS2B and, 39
[^{3}H]ethanolamine, 36
YF NS1 labeling, 37
Evolution, recombination and, 6
Exocytosis, in maturation, 66

F

Fc receptors, 229
antibody enhancement for, 240–241
in B cells, 237
in cell types, 248
flaviviruses and, 244–248
on platelets, 240

Fcγ
  RIIs, 242–243
  Rs, 247
Fcγ receptors, 240
  ADE and, 246
  classes of, 246
Ferlenghi, I., 73
Fever-arthralgia-rash, 230
Filoviruses, 81
Finlay, Carlos, 1
Flaviviridae, 4, 304
  3′ termini of, 207
  genera of, 7
  serology of, 142
  taxonomy of, 5–7, 13–15, 16
Flavivirus, 4
  antigenic interrelationships of, 277
  cDNA clones for, 118
  CS, 186–187
  evolution, 284
  ns proteins, 103
  pathogenesis, 117
  phylogenetic analysis of, 281–285
  phylogenetic groups, 277
  protease, 110
  replication, 104
  replication model, 127–128
  replication strategies, 128
  RNA replication, 100
  serology of, 142
  taxonomy of, 13–15, 16
  virion, 91–97
  vs. phi6, 128–129
  vs. Picornaviruses, 128–129
Flavivirus(es)
  3′-SL in, 209
  adhesion molecules and, 251–253
  amino acid divergence between, 282
  antigenic structure of, 141–142
  antigenicity, 142
  assembly of, 66–68
  basophils and, 238–241
  binding proteins, 250
  cell interactions of, 23–24
  cell surface macromolecules, 229, 242–249
  cell targets for, 232–242
  cell tropism of, 229
  chimeric, 163
  classification, 8–12
  cleavage activation in, 67
  conserved linear sequences of, 192–197
  CS2 location in, 194
  Culex-associated, 300
  dendritic cells and, 232–233
  diagnostic serology, 142
  disease types, 232
  dispersal patterns of, 279
  divergence in, 304
  E protein structure in, 81
  endocytic pathway and, 231
  endothelial cells and, 234–237
  envelope changes in, 75–77
  envelopes, 83
  Fc receptors and, 240–248
  FcγR-mediate ADE of, 247
  fusion, 84
  fusion machinery, 81–84
  fusion properties, 74–75
  genome features, 220
  genome organization, 30
  genomes, 28, 29, 219
  genomic RNA, 28
  genomic sequence information, 142
  geographic distribution of, 278, 280
  geographic origins of, 279
  glycosaminoglycans and, 243
  hemorrhagic, 242
  infectious synthesized, 307–308
  lifecycle, 63–64
  lymphocytes and, 237
  macromolecular modifications by, 253–254
  mast cells and, 238–239
  maturation of, 66–68
  MHC class I and, 249
  molecular clones, 28
  mosquito-borne, 4, 32, 203
  mosquito-borne *vs.* tick-borne, 31
  mutagenesis of, 196
  3′ NCR, 178
  5′ NCR, 178
  NCR nucleotide sequences of, 182
  NCRs of, 184
  neural cells and, 237–38
  neutralization, 147, 152
  neutralization-escape variants, 147, 148
  nonarthropod-borne, 4
  nonneurotropic, 230–235
  nonvectored, 277

Flavivirus(es) (*cont.*)
  origins of, 5, 304–306
  particles, 25–27
  pathogenesis of, 229
  phylogenetic analysis of, 180
  phylogenetic tree of, 280
  platelets and, 239–241
  receptor binding protein, 230–232
  recombination between, 306–308
  replication, 209
  reverse genetics on, 28
  RNA genome of, 177
  RNA replication, 192
  schematic of, 64
  serological comparisons of, 4
  structure of, 63–64
  subgroups of, 179–180
  T-cells and, 162–163
  taxonomic placement of, 7–16
  tick-borne, 4, 32, 180
  transendothelial passage of, 234
  translation initiation in, 7–16
  transmission of, 232
  vector-borne, 141
  vs. alphaviruses, 4, 82–83
Fluorophore 1-pyrenehexadeconoic acid, 74
Folding
  of E proteins, 80
  predicting, 216
  prM *vs.* E proteins, 68
Foot-and-mouth disease virus, 130
Freund, Jules, 2
Furin, 34, 66
  during maturation, 65
  prM cleavage and, 80
  recombinant bovine, 80
Fusion
  acidic pH and, 77
  activation, 81
  cell-cell, 74
  characteristics, 74–75
  E-glycoprotein and, 143
  E protein and, 230
  flavivirus, 84
  initiation, 77–78
  lipid composition and, 74
  low pH and, 75
  machinery, 81–84
  monoclonal antibodies and, 79
  pH and, 74
  of viral and cellular membranes, 82
  virus-liposome, 74
Fusion proteins
  alphavirus *vs.* flavivirus, 82–83
  class I, 77
  *trp*E, 160
Fusogenic trimeric complexes, 26

G

Gadgets Gully virus (GGYV), 287
Gal-T. *See* Galactosyltransferase
β-galactosidase (β-gal), 132
  expression in mice, 133
Galactosyltransferase (Gal-T), 114, 128
  replication and, 116–117
1,4-galactosyltransferase, 24
Gel mobility shift assays, 29–30
  of 3′-SL, 209–210
GenBank, 318
  Accession, 101
  dengue sequencing artifacts in, 320
  flavivirus genomes in, 181
Geneticin selection, 107
Genomic
  DEN sequencing, 320
  DEN2 sequencing, 333
  replication, 29
  RNA, NCRs in, 1173
  structure, 28
  translation *vs.* replication, 32
Geographic distribution, of flaviviruses, 8–12
Geographic information systems (GIS), 293
GFP. *See* Green fluorescence protein
GGYV. *See* Gadgets Gully virus (GGYV)
GIS. *See* Geographic information systems
Glial cells, 238
Glutathione-*S*-transferase (GST), 115
Glutathione-*S*-transferase-KUN NS2A fusion protein, 39
Glycoproteins
  TBE structural, 34
  viral, 132
  viral surface, 23
Glycosaminoglycans, 242–244

Glycosylation sites
N-linked, 34, 36
of TBE E protein, 71
Glycosylphosphatidylinositol (GPI)
linkage, 248
NS1 and, 36
Golgi apparatus, in maturation, 66
GPI. *See* Glycosylphosphatidylinositol
Granulocytes, activating FcRs on, 248
Green fluorescene protein (GFP), 132
GST. *See* Glutathione-*S*-transferase
GST-NS2A, 100
GST-NS4A, 100
Guanylyl transferase, 127
Guatemala, 335
Guirakhoo, F., 150

H

HA activity, 80
HA2 virus fusion peptide, 79
Haemaphysalis neumanii, 288
Haemaphysalis spinigera, 289
Hamster cell proteins, 29
WN RNA and, 30
Helicase domain, of NS3, 39
Helicases
mutagenesis of, 40
NS3 and, 111
in ns proteins, 211
role of, 41
supergroup 2 RNA, 40
Helper T-Cell (T_h)
anti-DEN virus and, 160
epitopes, 159–162
murine flavivirus and, 160
priming activity, 160
Hemagglutination
inhibiting, 145
inhibition (HI), 2
inhibition (HI) assays, 142
low pH and, 75
technique, 2
Hemagglutinin
influenza, 81
slowly sedimenting, 67
Hemorrhagic disease, 288
Hemorrhagic fever, 230
HEp-2 cells, 133
Hepacivirus, 7, 134, 279
taxonomy of, 15
Hepaciviruses, 7
3′ termini of, 207
ribosomal entry sites of, 16
Heparan sulfate, 243
binding mutations, 232
expression, 253
proteoglycans, 255
Heparin
dengue binding properties of, 243
thrombocytopenia and, 241
Hepatitis A virus, 209
Hepatitis C virus
5′ NCR, 170
core, 132
NCR binding and, 209
vs. Flavivirus, 129
Hepatitis dela virus, ribozyme (HDVr), 130
Herpes simplex type 1 encephalitis, 238
Herpesviruses, 249
Heterologous genes (HGs), 129–130
expression, 132–133
HI. *See* Hemagglutination
High-titer, 3
Hirst, George, 2
His_{53}, 40
Histocompatability complex antigens, 229
HIV
epitopes, 246
Gag protein, 132
MHC class I and, 249
Homopurine sequence (PU), 198–199
Homopyrimidine sequence (PY), 199
Homotrimeric complexes, 81
Homotrimeric state, of E proteins, 75
Host cell proteins, 213
Host species, of flaviviruses, 8–12
Host/virus coadaptation, impact of, 285–304
Human papilloma virus, 132
Human population densities, 277
Humoral responses, against NS1, 37
HVC polymerase, 129
Hydrogen bonding, between NCRs, 209–211
Hydrophobic residues, binding of, 110
Hygromycin B, 39

I

ICAM-1, 251
Icosahedral symmetry, 26
ICTV classification, 279
Idiopathic thrombocytopenic purpura, 241
IF. *See* Immunofluorescence analysis
IFN. *See* Interferon
IFN-γ, 247
IgE, 238
IgG
  binding to platelets, 240
  complement activation and, 253
IgM, antiviral, 248
IL. *See* Interleukin
IL-6, 238
IL-1β, 238
Image reconstruction studies, of flaviviruses, 72
Immune enhancement phenomenon, 317
Immune responses
  dendritic cells and, 233
  HG expression and, 133
Immunization
  intramuscular, 133
  with KUN replicon, 133
Immunoblotting
  Mab reactivities and, 149
  NS1 specificity patterns and, 158
Immunocytochemistry, B cells and, 237
Immunoelectron microscopy, of KUNV-infected cells, 108
Immunofluorescence (IF) analysis, 102
  indirect, 157
  of KUNV-infected cells, 116–117
  of KUNV-infected Vero cells, 101
  of KUNV ns proteins, 106–107
Immunoglobins
  Fc receptors, 24
  opsonization with, 24
Immunogold labeling, ns proteins and, 115
Immunoreceptor motifs
  ITAM, 247
  ITIM, 247
Immunoregulation, 236
India, 288
Induced membranes
  flavivirus, 107
  Flavivirus replication and, 109
  putative origins of, 116–117
  with RNA replication, 105–106
  ultrastructure of, 107–117
Infectivity
  humoral responses and, 37
  low pH and, 75
  ticks and, 282
  in virions, 80
Inflammation, 229
Influenza
  epitopes, 246
  hemagglutinin, 81
Initiation, self-priming *vs. de novo,* 46
Inoculation, intraperitoneal, 1
[^{3}H]inositol, 36
  labeling, 37
Interferon (IFN), 247
Interleukin (IL), 233
Internal fusion peptides, 77–79
  similarities among, 82
Internal ribosome entry site (IRES), 180–181
  in 5′ NCRs, 209
  encephalomyocarditis virus, 119
International Catalogue of Arborviruses, 5
International Committee on Taxonomy of Viruses, 7
  Flaviviridae and, 16
International Union of Microbiology Societies, 7
Inverted repeat sequence (IR)
  structure of, 198
  in TBE 3′NCR, 198
Ion-exchange chromatography, of 3′-SL, 209–210
IRES. *See* Internal ribosome entry site
IRES-CAT gene cassette, 125
IRESNeo, 130
ITAM. *See* Immunoreceptor motifs
ITIM. *See* Immunoreceptor motifs
Ixodes cookei, 288
Ixodes ricinus, 292
Ixodes uriae, 287, 288

J

Japanese encephalitis (JE) virus(es), 2, 4, 5, 31, 177
  3′-SL binding, 211
  ACACA-type sequence and, 183

anti-prM antibody and, 157
assembly in, 123
chemokines and, 253
complex, 180
dispersal, 302–303
E-glycoprotein analysis, 149
E-glycoprotein structure and, 144
E/prM complexes and, 230
E protein of, 71
epidemiology of, 302
epitope localization in, 147
3′NCRs of, 221
phenylalanine in, 79
SLEV and, 279
strain CH1932, 214–215
strain T1P1, 214–215
subgroup genomes, 197
subgroups, 195
virus-reactive Mab, 152
Jugra virus (JUGV), 295
divergence of, 296

K

K562 cells, TBE infected, 231
Kadam virus (KADV), 279
divergence of, 279
Karnataka, 288
Karshi virus (KSIV), 289
neutralization tests on, 5
Kedougou virus (KEDV), 279, 299
KFDV. *See* Kyasanur Forest disease virus
Khromyke, A. A., 190, 191, 197, 218
Koutango virus (KOUV), 295
Kozak flanking sequences, 127
KSIV. *See* Karshi virus
KU812 cells, 238
dengue infected, 247
Kuhn, R, J, 91
KUN replicon, 121
expression system, 131
immunization with, 133
packaging system, 130–132
RNA transfection, 130–132
system, 197
vectors, 129–130, 132
Kunjin (KUN) virus(es), 100
3′ NCR, 39
ACACA-type sequence and, 183
base pairing in *trans* of, 210
cDNA clones for, 118, 121
cytopathic effect of, 119
dual-labeled cryosections of, 110
E protein of, 71
gene order, 103
ICAM-1 and, 252
immunofluorescence of, 113
infected cells, 107–109
isolation of, 100
maturation of, 66
mutant RNA, 215
ns proteins, 106–107
nucleotide sequence, 103
polymerase activity, 44
polyprotein, 103–104
radiolabeling of, 101
replication, 117–118
replicon expression system, 129–133
replicon RNA, 119, 129
replicons, 39, 100, 117–119, 190–191
RNA, 120, 122
RNA synthesis analyses, 105–107
trans and, 38
viral protease-mediated cleavage, 130
viral RNA, 132
VLPs, 132
KUNV-Vero cell system, 105
Kyansanur Forest disease virus (KFDV), 283, 288
ALKV and, 289
hemorrhagic disease and, 288
neutralization tests on, 5

L

L292 cells, HG expression in, 133
La autoantigen, 31
Land reclamation, 277
Langat virus(es) (LGTV), 289
anti-prM Mab for, 157
deletion mutations in, 203
domain II expression in, 149
neutralization tests on, 5
Latin America, 297
Lectin binding, of cellular extracts, 68
Lemay, G., 127
Lepus timidus, 292
Lescar, J., 82

Leukocytes, transendothelial migration of, 235, 251
Leukotrienes, 233, 237
LGTV. *See* Langat virus
LiCl, 45–46
  precipitation, 102
Lipid composition, 74
Lipid envelope removal, 26
Lipid membrane labeling, 74
Lipopolysaccharide (LPS), 236–237, 244
Liposome fusion assays
  of TBE, 74
  of West Nile virus, 74
Liposomes
  in acidic pH, 77
  coflotation studies, 77
Live-attenuated hybrid vaccines, 308
Liver
  flavivirus infection and, 234
  Kupffer cells, 234
Localization, of DEN 2 virus epitopes, 151
Louping ill virus (LIV), 2
  classification of, 4
  distribution of, 293
  epitope localization in, 147
  neutralization tests on, 5
  nonviremic transmission and, 292
Low pH. *See* Acidic pH
LPS. *See* Lipopolysaccharide
Lymph nodes, 234
Lymphocyte blastogenic response, 237
  antipeptide, 160
  antivirus, 160
  to *trp*E fusion proteins, 160
Lymphocytes
  activating FcRs on, 248
  flaviviruses and, 237
Lysates, of JE-infected BHK, 211

M

M (membrane) protein(s), 25–26, 34
  antigenic structure of, 157
  E dimes and, 26
  of flavivirus, 63
  glycoprotein precursor of, 34
  in RSPs, 73
  structure of, 27
  virus-encoded, 7
Mabs. *See* Monoclonal antibodies
Macrophages
  activating FcRs on, 248
  flavivirus replication in, 233–234
  inhibiting FcRs on, 248
  pulmonary, 234
  splenic, 234
  thymic, 234
Major histocompatibility complex (MHC) antigens
  cell surface macromolecules and, 249
  class I, 233, 249–251
  class I pathway, 162
  class II, 233, 251
Malaysia, 289
Mammalian cells, NS1 and, 35
Mandl, C., 201
Mansonia uniformis, 299
Markoff, L., 150
Mast cells
  Fc receptors in, 247
  flaviviruses and, 238–239
Materies morbi, 1
Maturation
  of flavivirus, 63
  of flaviviruses, 66–68
Maximum likelihood (ML) method, 313
  features of, 284
  of NS5 nucleotide sequences, 280
Maximum parsimony method, 319
^{7}Me-guanosine type 1 cap, 126
Meaban virus (MEAV), 279, 287
Measles virus, 209
Melnick, Joseph, 3
Membrane-anchoring carboxy terminus, 231
Membrane anchor(s), 82
Membrane binding, low pH and, 75
Membrane cofactor protein, 241
Membrane formation, 116
Membrane fusion, 230. *See also* Fusion
Membrane interaction, 77–79
Men, R., 190, 218
[^{35}S]methionine labeling, 105, 114
Methylation, of N_2 residue, 28
Methyltransferases, 179
  SAM-dependent, 43
Mexico, 328
MFOLD program, 215
  3′ NCR and, 218

Mg^{2+}
  NS3 and, 41
  RdRP activity and, 102
MHC. *See* Major histocompatibility complex antigens
Minus strand synthesis, 179
Mississippi River, 1
MMLV. *See* Montana myotis leucoencephalitis virus
Modis, Y., 93
Molecular clocks, 319
Monkeys
  Kyasanur Forest disease virus and, 288–289
  YFV and, 295–296
Monoclonal antibodies (MAbs), 141
  advent of, 143
  anti-A1
  anti-DEN virus, 150
  anti-E-glycoprotein, 156
  anti-prM, 157
  binding with E-glycoprotein fragments, 149
  C protein, 157
  DEN 2, 152
  domain II-reactive, 150
  E-glycoprotein-specific, 157
  E-glycoprotein structure and, 154–155
  against envelope proteins, 5
  fusion inhibition and, 79, 154
  glycoprotein-reactive murine, 145
  3H5, 152
  JE virus-reactive, 152
  reactivity patterns of, 75
  virus-neutralizing, 146
Monocytes
  activating FcRs on, 248
  blood, 233
  Dengue infected, 233
  flavivirus replication in, 233–240
  TNF-α and, 247
Montana myotis leucoencephalitis virus (MMLV), 284
Mosquito, 1
  DEN virus transmission by, 334–335
Mosquito-borne flaviviruses, 294–304
  CYC sequence in, 221
  group II, 303
  TBE virus and, 281
Mosquito life cycle, 283
Motifs, of nonstructural proteins, 103
Mouse, 1
  astrocytes, 251
  brain, neonatal, 211
  coxsackievirus and, 3
  hepatitis virus, 209
  hyperimmune ascitic fluid, 150
  macrophages, 251
  Schwann cells, 251
  virus isolation in, 2
MOV34 protein, 211
  mouse, 31
mRNAs, 7
  vs. flavivirus genomes, 28
Murine hyperimmune ascitic fluids, 149–150
Murphy, Frederick, 4
Murray Valley encephalitis virus(es) (MVEV), 2, 4, 31, 36
  CS1 and, 194
  cyclization, 190
  genome, 195
  isolation of, 100
  JEV and, 302
  Mab reagents and, 155
  NS1 study of, 158
  nucleotide sequence of, 203
  synthetic peptide analysis, 151
Muskrats, 290
Mutagenic analysis
  of 3′ model RNAs, 208
  of 5′ NCR, 213
  of 3′-SL, 213
  of dengue viruses, 333
  of flavivirus genome, 196
  site-specific, 79
  of TBE, 232
Mutant D3/WN-SL(mutF), 213
Mutant F virus, of DEN2, 214
Mutations
  compensatory, 217
  deletion, 201–03
MVEV. *See* Murray Valley encephalitis virus(es)
Myeloid cells, 253
Myoblasts, human, 251

N

N-terminal domain
  of E proteins, 69
  of NS3, 40
  of NS5, 43
  of NS2A, 38
Natural killer (NK) cells, 248
NCR. *See* Noncoding regions
3′ NCR. *See* 3′-noncoding region
5′ NCR. *See* 5′-noncoding region
Negative-strand intermediate, 24
Neomycin phosphotransferase gene, 119
Neural cells, flaviviruses and, 237–238
Neurons, 238
Neurovirulence, 214
Neutralization, flavivirus, 152
Neutralization tests, 5
  limitations of, 5–6
New Guinea C (NGC) prototype, 326
New York, 301
NGC. *See* DEN2 virus
Nicaragua, 335
Nitric oxide, 233
NK cells, 251
NLS. *See* Nuclear localization sequence
No known vector (NKV) viruses, 283
  divergence of, 283, 284, 304
  success of, 286
Nobel prize, 1
Non-ionic detergents, 26
3′-noncoding regions (NCR), 29–32
  binding of viral proteins to, 211
  cis interaction with, 215
  conserved linear sequences in, 192–197
  conserved motifs in, 189
  deletion mutations, 195–196, 201–202
  in DEN, 198
  of flavivirus RNAs, 180
  hydrogen bonding and, 215–216
  internal deletions in, 218
  lengths, 181, 197–198
  mutational changes in, 214
  nucleotide sequences features of, 184, 185, 188
  pentanucleotide of, 192–194, 197
  poly(A) tract in, 200
  RdRP bonding of, 220
  RNA(−), 206
  secondary structures, 203, 216–219
  size variation of, 201
  structure of, 217
  in TBE, 197–103
  termini, 181–183
  tertiary structures in, 218–219
5′-noncoding regions (NCR), 28–29, 31
  cis interaction with, 215
  cyclization sequences in, 186
  deletion mutagenesis for, 204
  distal loop of, 216
  of flavivirus RNAs, 180
  hydrogen bonding and, 215–216
  mutagenesis of, 213
  mutational changes in, 214–215
  nucleotide sequences features of, 184, 185
  secondary structure of, 203–206, 204
  termini, 181–183
  in tick-borne virus genomes, 221
  tick-borne *vs.* mosquito-borne, 180
  translation for, 205
  of YF virus, 181
Noncoding regions (NCRs), 178
  conserved linear sequence features of, 181–203
  DEN2 mutant F, 214
  secondary structures of, 203–214
Noncytopathic vector, KUN replicon as, 100
Noninfectious particles, 26
Nonstructural (ns) proteins, 33, 35–45, 103–105, 158–159
  complementation among, 120–121
  flaviviral, 141
  in KUNV-induced membranes, 109–111
  RC and, 114, 119
  recombinant, 106
  in RNA replication, 179
  subcellular location of, 106–107
Nonvectored viruses, 286
North America, 296
Northwestern blotting, 209–210
Nott, Josiah Clark, 1
ns. *See* Nonstructural proteins
NS1 protein(s), 35–38
  antigenic structure of, 170
  carboxy terminus of, 119
  cleavage among, 33
  DEN2, 36, 38
  dimerization and, 37

epitopes, 158
gene deletion, 119
high-titered antibody response and, 158
homodimers of, 36
humoral responses against, 37
KUNV, 36, 105
in minus strand synthesis, 179
MVEV, 36
NS4A and, 38, 42
RC and, 121
RNA replication and, 37–38
SDS-PAGE of, 36
specificity patterns, 158
translocation of, 35
Triton X-114 and, 36
vs. NS5, 121
YFV, 37, 38
NS3 proteins, 29, 39–42, 132
3′ NCR and, 211
binding properties of, 120
cis and, 39
cleavage of, 33, 40
cytotoxic T-cells and, 162
DEN2, 38, 40, 109–110
displacement and, 127
Flavivirus, 104
flavivirus antigenicity and, 163
gene deletion, 119
helicase domain of, 39
helicase/NTPase, 111, 119
as immunodominant target, 162
KADV, 279
KUNV, 109–110
N-terminal of, 40
NS5 and, 41
NS2B and, 39
nucleotide triphosphatase in, 179
as protease, 103
and RdRP activity, 134
residue, 38
RNA helicase of, 179
sequence data, 279
TBE, 42
viral serine protease domain of, 41
NS5 proteins, 29, 43–45, 104
3′ NCR and, 211
amino terminus of, 120
antigenic structure of, 159
binding of, 39, 120
cleavage among, 33
flavivirus antigenicity and, 163
gene deletion, 119
guanylyl transferase activity of, 127
in KUNV-infected cells, 111
methyltranferase domain, 43
NS3 and, 41
polymerase activity of, 127
RdRP activity of, 125, 179
RdRP and, 45–46
RNA polymerase and, 114–115
sequence data, 279
vs. NS1, 120
X-ray crystal structure of, 128
NS4A, 42
cleavage among, 33
GST and, 116
hydrophobic, 111
immunogold labeling and, 116
NS1 and, 38, 42
NS2A proteins, 38–39
C terminus of, 38
cleavage of, 33
GST and, 116
immunogold labeling and, 116
N terminus of, 38
structure of, 179
NS2Aα proteins, 38
NS2B proteins, 39
cleavage among, 33
as cofactor, 103
cofactor activity of, 39
JE, 39
KUNV, 109–110
NS3 and, 39
NS4B proteins, 42
in KUNV-infected cells, 111
migration, 106–107
Ntaya virus, 5
NTPase, 41
NS3, 41, 44, 111
Nuclear localization sequence (NLS), 107
Nucleocapsids, 26
replication and, 38
uncoating, 24
virus, 24
Nucleotide sequence(s) ,5
3′-SL, 207, 212
of flavivirus genomes, 219
KUNV, 103
repeats, 195

Nucleotide sequence(s) (*cont.*)
  specificity, 212–214
Nucleotide triphosphatase, 179

O

Oceanites oceanicus, 288
OHFV. *See* Omsk hemorrhagic fever virus
Olfactory neuroepithelium, 234
Oligomerization, of E proteins, 67
Olsthoorn, R., 218–219
Omsk hemorrhagic fever virus (OHFV), 289
Open reading frame (ORF), 177
  DEN4, 205
  mutational changes in, 214
Opsonization, 24
ORF. *See* Open reading frame
Ornithodorus, 284
Orthomyxoviruses, 81

P

[^{32}P]orthophosphate labeling, 114
Padmanabhan, R., 191
PAF. *See* Platelet-activating factor
pAKUN, 117, 118
[^{3}H]palmitate, YF NS1 labeling, 37
Paracrystalline arrays (PC)
  induced membranes and, 106
  morphology of, 107
Paramyxoviruses, class I proteins in, 81
Pathogenesis
  dengue, 238
  Flavivirus, 117
  of flaviviruses, 229
  initial stages of, 237
  platelets and, 239
Pathogens, 177
Pattern recognition receptors, 244. *See also* CD14
PC. *See* Paracrystalline arrays
pCINeo plasmid vector, 107
Pentanucleotide, 192–194
  of 3′NCRs of TBE, 197
Pepscan analysis
  of C protein Mab, 157
  of DEN2 virus E-glycoprotein, 150
Peptidase, host signal, 33, 35–36
Peptide sequencing
  C-terminal, 37
  E-glycoprotein, 150
Perinuclear staining, 106
Peru
  DEN2 in, 328
  DHF in, 335
Pestivirus, 7, 134, 278
  taxonomy of, 15
Pestiviruses, 7
  3′ NCRs of, 201
  3′ termini of, 207
  ribosomal entry sites of, 16
Petidase host signal, 34
Phage display technology, 152
Phi6, 128–129
  RNA synthesis of, 129
Phnom Penh bat virus, 5
Phosphatidylinositol-specific phosphatidyl lipase C (PI-PLC), 37
Phylogenetic analysis, 5
  of dengue viruses, 315, 318–331
  of *Flavivirus*, 281–285
  of flaviviruses, 180
  maximum parsimony method of, 319
  recombination and, 6
  vs. antigenic analysis, 281
Phylogenetic trees
  building, 6
  comparative, 282
  computer generation of, 318–319
  interpretation, 321
  of mosquito-borne viruses, 294
  of NS5 nucleotide sequences, 280
Phylogeny
  of DEN1, 322–323, 324
  of DEN2, 325–328, 327
  of DEN3, 328–331
  of DEN4, 331, 332
PI-PLC. *See* Phosphatidylinositol-specific phosphatidyl lipase C
Picornaviruses, 102
  replication strategies of, 128
  vs. Flavivirus, 128–129
Plasmid DNA vectors, 132
Platelet-activating factor (PAF), 233, 237
Platelets
  activating FcRs on, 248
  factor 4, 239

flaviviruses and, 239–241
virus binding to, 239–240
Pletnev, A., 203
pol, subunit of phi6, 128
Poliomyelitis virus, 2
in mice, 3
Poliovirus
5′ NCR, 170
replication strategies of, 128
RNP formation in, 209
Poly(A), 41
Polyadenylate sequence, 32
Polyclonal antibodies, 158
Polyclonal antisera, 145
Polymerase
in ns proteins, 211
processivity, 41
Polyprotein
CMs and, 42
of KUNV, 103–104
N-terminal of, 33
NS3 and, 39
processing, 32–33
Porterfield, J. S., 4
Powassan virus (POWV)
associations of, 287
clinical cases of, 288
genome, 198
neutralization tests on, 5
phenylalanine in, 79
Poxviruses, 249
PR. *See* Pyrimidine-rich sequence
Prelysosomal endocytic compartment, 24
Primorsk, 287
prM-E heterodimers, 68
pulse-chase experiments and, 80
sedimentation analysis of, 80
prM protein(s), 26, 34
antigenic structure of, 157
cleavage, 80
cleavage 4, 33
E protein and, 34, 35, 68, 230
of flavivirus, 65
folding of, 80
function of, 80–81
maturation cleavage of, 65
in mature virus particles, 231
pr segment of, 34
proteolytic cleavage of, 66
translocation of, 33
Prostaglandins, 233, 237
Protease(s)
digestion, 75
inhibiting, 40
in maturation, 66–67
recombinant single
chain, 40
viral, 171
Proteasome subunit protein, 211
Protein A-gold particles, 109
Protein-reactive chemicals, 149
Proteins
class I, 81
class II, 35
class II fusion, 81–82
Protelycan proteinases, 254
Proteoglycans, 242–244
Proteolysis, 249
Proteolytic processing
epitope localization and, 149
at NS1/2A junction, 36
Protonation
cleavage and, 80
of E dimer, 77
Proutski, B., 196, 197, 216
TBE genome folding and, 219
Pseudoknots, 32, 208
in TL loops, 219
Pteronotus parnellii, 305
PU. *See* Homopurine sequence
Pull-down assays, of cell lysates, 100
Pulse-chase analysis
on E proteins, 67
prM-E heterodimers and, 80
RF RNA and, 102
of TBE, 34
Pulse labeling
CHXM treatment and, 112
of KUNV-infected cells, 102
Putative fusion domain, 35
of E-glycoprotein, 154
Putative receptor-binding function, of E proteins, 73
Pyrmidine-rich sequence (PR), 32, 199

Q

Qβ replicase, 31
Quinine/quinidine, 241

R

R3, 32
Rabies, 2
Radioimmunopreciptations, 100
Rats, LGTV and, 289
RCS2, 30, 194–196
  in mosquito-borne virus genomes, 217
  in MVE genome, 195
  vs. CS2, 196
RCS3, *vs.* CS3, 196
RdRP. *See* RNA-dependent RNA polymerase
Receptor-binding, 66
  motifs, 231
Recognition motifs, 29
Recombinant subviral particles (RSPs), 26, 67
  architecture of, 72–73
  fusion properties of, 74
  production, 33
  TBE, 25, 72–73, 79
Recombination
  dengue viruses, 6
  between flaviviruses, 306–308
  interserotype, 321
  intramolecular, 320
  intraserotype, 321
Release, of flavivirus, 64
repBHK, 119
Replicase, YF, 38
Replication
  3′-SL and, 209
  analyses, 103–107
  asymmetric, 127
  brefeldin A and, 116
  complexes, 111–114
  defects, 212
  flavivirus, 219
  Flavivirus, 104
  Flavivirus RC, 125
  host cell proteins and, 213
  ns proteins in, 103
  RNA, 25
  semiconservative, 127
  tandem repeat sequences in, 196–197
Replication complex (RC), 100
  accumulation, 126
  assembly, 100, 127
  biochemical assays of, 114–116
  complementation systems of, 119–121
  consensus composition of, 114–116
  formation of, 119–121
  induced membranes and, 107–117
  KUNV ns proteins and, 115
  NS1 and, 121
  ns proteins of, 119
  operation of, 117–129
  RNA replication cycle of, 124–125
  stability of, 114
Replications proteins, 24
Replicative form (RF), 100, 101
  conversion to RI, 127
  minus RNA strands in, 179
  poliovirus, 45
Replicative form RNA (RI RNA), RNase resistance of, 102
Replicons
  KUNV, 117–118, 190–191, 197
  mutated KUN, 122
  vectors, 129–130
Reporter genes, 132
RER. *See* Endoplasmic reticulum
Respiratory syncytial virus (RSV), 130–132, 244
Restriction endonucleus site, *Apa*I, 190, 196
Retroviruses, 81
Reverse genetics, 28
Reverse transcriptase-polymerase chain reaction (RT-PCR), 318
  of Dengue, 333
Rey, F. A., 96
RFV. *See* Royal Farm virus
RGD motifs, 231
Rhabdoviruses, 82
Rhipicephalus appendiculatus, 284
RI RNA. *See* Replicative form RNA
Ribonucleases, 207
Ribosomal entry sites, 16
Ribosomes, CHXM and, 112
Ribonucleoprotein (RNP) complexes, 209
  proteins involved in, 210–211
RK13 cells, 133
RNA
  20S–28S, 46
  binding protein, 29
  copy-back, 42
  C2rep, 191

C20rep, 191
5′diphosphoryl-terminated, 127
elongation, 217
flavivirus genomic, 28
folding, 320
genomic, 7, 26, 121–122
helicase, 7, 104
heterogeneous, 46
intracellular, 102
nascent progeny, 111–114
packaging, 39
progeny virion, 179
pseudoknots, 208
recognition motifs, 29
replicase, viral, 45–46
replication, 24, 25, 29, 31, 37–38, 39, 117– 118
replicon, 132
20S, 46
40S, 46
secondary structure, 41
triphosphatase, 41, 46, 104
triphosphate, 127
virus-specific, 101
WNV 3′UTR, 116
RNA(+), 24
control mechanisms for, 134
cyclization of, 123
synthesis, 100, 126, 127
translation, 127
RNA(−)
analysis of, 206
3′NCR, 206
polymerase of, 210
strand synthesis, 123–126
strands, 179
synthesis initiation, 212
RNA-dependent RNA polymerase (RdRP), 7, 31, 100, 102
bonding with 3′NCR, 220
in DEN2, 191
in induced membranes, 107
NS3 and, 134
NS5 and, 44, 45–46, 125
promoter elements, 209
RNA fingerprinting, 317
DEN2 and, 325
DEN3 and, 329
RNA polymerase
II, 130
NS5 and, 114–115
RNA replication, 45–46
C protein and, 191
C-terminal region and, 40
cycle, 124–125
cyclization sequences for, 190
flavivirus, 100, 179
induced membranes with, 105–106
NS3 and, 39
NS5 and, 43
ns proteins in, 179
of phage QB, 208
virus genomes, 209
RNA synthesis, 182
3′-SLs and, 210
genomic, 102
KUNV, 95–96, 105–107
model for, 112
[^{32}P]orthophosphate labeling and, 112
plus-strand, 179
RNase
digestion, 112
probing, 208
RNP. *See* Ribonucleoprotein complexes
Rockefeller Institute, 2
Rooting methods, 284
Royal Farm virus (RFV), 289
neutralization tests on, 5
RSPs. *See* Recombinant subviral particles
RSV. *See* Respiratory syncytial virus
RT-PCR. *See* Reverse transcriptase-polymerase chain reaction
RTPase, 41. *See also* RNA, triphosphatase
Rubella virus
NCR binding and, 209
thrombocytopenia and, 240
Russia, 287
Russian spring-summer encephalitis virus (RSSEV), 2, 4, 5, 16

S

S-adenosyl-homocysteine, 43
S-adenosyl-methionine (SAM), 43
S1 pocket, 40
Sabin, Albert, 2
Saumarez Reef virus (SREV), 279, 287
SDS-PAGE analysis

SDS-PAGE analysis (*cont.*)
  epitope localization and, 149
  of KUNV NS1, 105
  of NS1, 36
  NS4B and, 42
  virus binding proteins and, 249
sE monomers, 77
sE proteins, liposome-bound, 77
Secondary structure, prediction, 216–219
Sedimentation analysis
  gradient, 107
  of prM-E complexes, 68
  of prM-E heterodimers, 80
Sedimenting (70S) hemagglutinin (SHA), 26
Selective pressure estimations, 319
Semliki Forest virus (SFV), 35, 96
  3′ NCR of, 201
  E1 protein structure, 83
  replicon vector pSFV1, 130
Sentinal monkey, 323
Sepik virus (SEPV), 294
  divergence of, 296
  YFV and, 298
Ser_{138}, 40
Serine kinase(s), serine/threonine, 43
Serine protease, 7
  in NS3, 39
  structure of, 41
  trypsin-like, 40
  virus-encoded, 33
Serine protease cleavage
  NS2B-BS3, 42
  NS2B-NS3, 42
Serologic testing, 2–3
Serology, flavivirus diagnostic, 142
SFV. *See* Semliki Forest virus
SFV-MEC replicon, 132
SHA. *See* Sedimenting (70S) hemagglutinin
Shi, P., 206
Shope, Robert, 3
Siberia
  OHFV in, 290
  TBE complex viruses in, 291
Signal peptidase, 177
Signal peptide, GPI-anchoring, 36
Signalase cleavage, 34
Sindbis-related virus, 6
Sindbis replicon vector, 121
Sindbis virus, 209
SK6 cells, 133
Skin Langerhans. *See* Dendritic cells
Skin mononuclear cells, 234
Skin perivascular infiltrates, 238
SL. *See* Stem and loop, 3′
SLEV. *See* St. Louis encephalitis virus
3′SLs, 193
Smooth membrane vesicles, 24
SMS. *See* Spherical smooth membrane structures
South America, YFV in, 296, 297
Spectral analysis, 208
Spherical smooth membrane structures (SMS), 106
Sphingomyelin, 74
Spondweni virus (SPONV), 279
  ZIKV and, 299
SREV. *See* Saumarez Reef virus
ssRNA, 7
St. Louis encephalitis virus (SLEV), 2, 4, 5, 7
  E-glycoprotein structure and, 144
  epidemiology of, 300
  glycosylation in, 71
  JEV group and, 279
  Mab reagents and, 155
  migratory birds and, 300
  nucleotide sequence of, 203
  recombination between, 307
3′ Stem and loop (SL), 29, 206–208
  binding of viral proteins to, 211
  DEN4, 31
  DEN2/WN nucleotide sequence, 213
  mutagenesis of, 213
  NS3 and, 211
  NS5 and, 211
  nucleotide sequence of, 201, 212–214
  protein binding to, 209–214
  RdRP promoter elements in, 209
  RNA(−) synthesis initiation and, 212
  structure, 207
  WN, 31
Sterna paradisaea, 288
Stop codon, 202
Storm Petrel, 288
Structural proteins, 33–35
  flaviviral, 141
Study formation, of RC, 117–129
Substrate analogues, 40

Substrate bonding, in S1 pocket, 40
Subviral particles. *See also* Recombinant subviral particles
  of alphaviruses, 82
  noninfectious, 67
  structure of, 67
Sucrose density gradients, 105
Synthetic peptides
  antibody responses to, 153
  binding site localization with, 152
  of E-glycoprotein, 150

T

T-cells. *See also* Helper T-cell
  CD4-expressing, 251
  CD4+ responses, 162
  dendritic cells and, 251
  epitopes, 159–163
  response, 162
293T cells, 133
Tamana bat virus (TBV), 278
Tandem repeat sequences
  in flavivirus replication, 196–197
  R1, 199
  R2, 199–200
  R3, 199
  upstream, 194–196
TAP, 249
Taxa inclusion bias, 319
Taxonomy
  flaviviral, 5–7
  of *Flaviviridae,* 13–15
  genome based, 16
TBE. *See* Tick-borne encephalitis virus(es)
TBV. *See* Tamana bat virus
T_c. *See* Cytotoxic T-cell
Template
  exchange, 126
  recognition, 41
3′-terminal motifs, 220–221
5′-terminal motifs, 220–221
3′-terminal poly(A) tract, 7
5′-terminal type I cap, 7
T_h. *See* Helper T-Cell
Thailand, 289
Theiler, Max, 1
  HI and, 3
Theoretical ancestors, 319
Thermal stability, of 5′/3′ NCR panhandle, 216
Thrombocytopenia, 239
β-thromboglobulin, 239
Thromboxanes, 237
TIA-1, 29
TIAR, 29
  knockout cell line, 29
Tick
  life cycle, 282
  viruses in, 2
Tick-borne encephalitis (TBE) virus(es), 5, 16, 26, 32, 42, 177, 171, 280–288, 286–294,
  3′ NCRs of, 197–203
  clades of, 286–287
  cleavage in, 80
  components of, 231
  conserved linear sequence features of, 197–203
  3′CS and, 122
  deletion mutations, 201–202
  dendrogram, 200
  E dimer, 78
  E-glycoprotein homodimer, 144
  E-glycoprotein structure and, 144
  E/prM complexes and, 231
  E protein of, 67, 71
  E protein structure, 83
  environmental conditions and, 292
  epitope localization in, 147
  evolutionary relationships among, 200
  folding, 219
  fusion properties of, 74
  genome, 222
  genomic structure, 189
  GIS data and, 293
  heparan sulfate and, 243
  isolation of, 287
  Mabs, 154
  mammalian, 287
  mosquito-borne flaviviruses and, 281
  mutational analysis of, 232
  NCRs of, 185
  3′NCRs of, 222
  neural cell destruction of, 238
  OHFV and, 290
  origins, 294
  pan-European distributions of, 303

Tick-borne encephalitis (TBE) virus(es), (*cont.*)
 pathogenicity of, 232
 recombination between, 307
 RSPs, 73, 79
 sE dimer of, 70
 seabird-associated, 287
 strain 267, 199
 strain Aina, 199
 strain Ljubljana I, 199
 strain Neudorfl, 196, 198, 199, 200, 202
 strain RK 1424, 198
 strain Sofjin, 180
 tandem repeats in, 199
 transmission criteria for, 292
 transmission of, 291
 Vasilchenko (Vs) strain, 291
 virions, 64, 69
 vs. MBE, 70–71
Tick-borne viruses
 CS of, 183
 isolation of, 305
Tissue factor expression, 253
TL1 loops, 219
TL2 loops, 219
TLR. *See* Toll-like receptor
TM. *See* Transmembrane, segments
TNF. *See* Tumor necrosis factor
TNF-α, 233
 endothelial cells and, 252
 endotoxic shock and, 237
Tobacco mosaic virus, 31
Togaviridae, 4
Toll-like receptor (TLR), 244
Topotypes, 317
trans, 38
 acting elements, 120
 base pairing in, 210
 complementation experiments in, 100
 DEN2 NS1 in, 42
 interactions with NCRs, 215
 KUN replicon and, 44
trans-complementation experiments, 25
 repBHK and, 119
trans-Golgi region, 24, 65
 colocalization, 116
 in maturation, 66
Transcytosis, 234
Transendothelial migration, of leukocytes, 235, 237, 251
Transendothelial passage, 234
Transfection/replication efficiency, 130
Transition/transversion rate calculation, 319
Translation
 in *cis*, 100
 of DEN4 ORF, 205
 initiation, 7–16, 180
 initiation codon, 118
 mapping, 103
 MOV34, 211
 processing, 32–33
 of viral genome, 24
Translation elongation factor 1A (EF1A), 30–31
Translocation, of NS1, 35
Transmembrane
 proteins, 42
 region, 35
 segments, of flavivirus E protein, 72
Transmission
 agriculture and, 294
 criteria for, 292
 DEN virus, 334–335
 monitoring, 335–336
 nonviremic, 292
 of TBEV, 291
Transportation, 278
 virus dispersal and, 305
Trimerization
 E, 26
 two-step model for, 75–76
Triphosphate, 5′-terminal, 105
Trirawatanapong, T., 152
Triton X-100
 CHXM treatment and, 112
Triton X-114, NS1 and, 36
tRNAs, EF1A and, 30–31
*trp*E fusion proteins, 149–150
Trypsin
 ADE and, 244
 treatment, of TBE virions, 69
Tryptic digest analysis, of NS1, 36
Tuberculosis, 2
Tumor necrosis factor (TNF), 233
 immune response and, 236
 monocyte-derived, 236
Turnip yellow mosaic virus, 31
293 cells, 133
Tyrosine phosphorylation, 37

Tyuleniy island, 287
Tyuleniy virus (TYUV), 279, 287

U

Ultrastructural studies, 66
Untranslated regions (UTRs), 101
Urbanization, 277
Urea polyacrylamide gels, semidenaturing 7 *M*, 102
[^{3}H]uridine, 102
  labeling, 105
Urinary histamine, 328
Usutu virus (USUV), 302
  in Austria, 303
3′ UTR, 127
UTRs. *See* Untranslated regions
UV cross-linking, 29–30
  of 3′-SL, 209–210
UV-melting experiments, on 3′ model RNAs, 208
Uzbekistan, 289

V

Vaccines
  live-attenuated, 28
  live-attenuated virus, 142
  production, 163
  strategies, 28
Vaccinia, 240
  virus enzyme, 105
Vascular
  leakage, 236–237
  permeability, 229
Vasoactive
  cytokines, 233
  factor release, 237
  mediators, 233–234
VCAM-1, 252
Vector-host coadaptations, 279
Vector(s)
  differences, 220
  KUN replicon, 129–130
  replicon, 129–130
  species, 8–12
Vero cells
  BrU in, 112
  DEN2 virus infected, 109
  HG expression in, 133
Vesicle packets (VP), 24, 109
  colocation in, 110
  induction, 134
  NS1 and, 37–38
Vesicular stomatitis virus G protein, 132
Vesicular stomatitis virus (VSV), 210
Viral determinants
  of dengue, 331–334
  of DENV transmission, 334–335
Viral displacement, 335
Viral fusion proteins, 66
Viral genome, 24
  translation, 24
Viral immune complexes, 229
  on platelets, 239
Viral life cycle, 23–25
Viral replicase proteins, 29
Viral RNAs, 24
Viral serine protease cleavage, NS2A and, 38
Viral surface glycoprotein, 23
Viremia, 237
  encephalitis and, 252
Viremic titers, 240
  in dengue infections, 246
Virion(s), 7, 24
  architecture of, 72–73
  assembly, 82
  extracellular, 68
  formation, 66
  fusion activation in, 80
  fusion activity of, 77
  immature virion, 93, 94
  mature, 91
  prM-containing, 65
  RNA, 102
  TBE, 64
Virulence
  dengue, 331
  determinants of, 316
  E protein and, 66
Virus
  proteins, 32–45
  receptors, 243
Virus-neutralizing
  antibody, 152
  assays, 142
  high-titered, 145

Virus particles
  assembly of, 24
  mature, 231
Virus(es)
  attachment, 143
  binding proteins, 249
  classification, discrepancies in, 279
  cross-neutralizing assays, 179
  cross-reaction of, 3
  entry, 229
  evolution, 321
  genome sequencing, 146
  identification, 157
  isolation, 2
  mutant, 205–206
  origins, 200
  positive-strand, 44
  production, 24
  replication, 32
  revertant, 34
  RNA, 128–129
  titers, 238
VLPs, KUN, 132
Voles, 290
VP. *See* Vesicle packets
VSV. *See* Vesicular stomatitis virus

W

Wallner, G., 198–200
Webster, L. T., 2
Wengler, G., 12
Wesselsbron virus (WESSV), 283
  YFV and, 298
West Nile (WN) virus(es), 6–7, 29
  3′ model RNAs, 208
  3′-SL, 31, 193, 207, 209–210
  3′-SL replacement, 212
  ACACA-type sequence and, 183
  cDNA clones for, 117–118
  classification of, 4
  CS1 in, 31
  Culex and, 301
  cyclization, 190
  dendritic cells and, 233
  E protein of, 71
  epidemiology of, 300
  ICAM-1 and, 252
  inhibition, 29
  Mab reagents and, 155
  MCH Class II and, 251
  movement, 141, 142
  in New York, 100, 301
  nucleotide sequence of, 203
  RNA, 30
  success of, 301
  3′UTR RNA, 116
Westaway, E. G., 190, 197, 218
Western blotting analysis, 105
Western equine encephalitis virus, 2
  Alphavirus and, 6
White-footed mice, 288
Whitman, Loring, 3
WN. *See* West Nile virus

X

X-ray crystallography, 26
  of TBE, 65

Y

Yaounde virus (YAOV), 295
Yellow fever virus(es) (YFV), 1, 177, 294
  3′-SL, 208
  ACACA-type sequence and, 183
  in Americas, 306
  classification of, 4
  CS1 in, 31
  CS1 sequence of, 194
  cyclization, 190
  E/prM complexes and, 231
  epidemiology of, 295
  epitope localization in, 147
  genome sequence, 206
  genomes, 195
  glycosylation in, 71
  hemagglutinins of, 2
  Mab reagents and, 155
  3′NCRs of, 221–222
  NS1, 37
  ns protein genes of, 103
  nucleotide sequence of, 203
  replication, 38, 117
  secondary structures of, 218

in South America, 296
strain 17D, 181
strain 17D-204, 218
strain 17DD, 218
virulence of, 298–299
vs. dengue virus, 300
You, S., 191
DEN genomic RNAs and, 196

Z

Zeng, L., 212
Zhang, W., 93
Zhang, Y., 91
Zika virus (ZIKV), 279
SPONV and, 299

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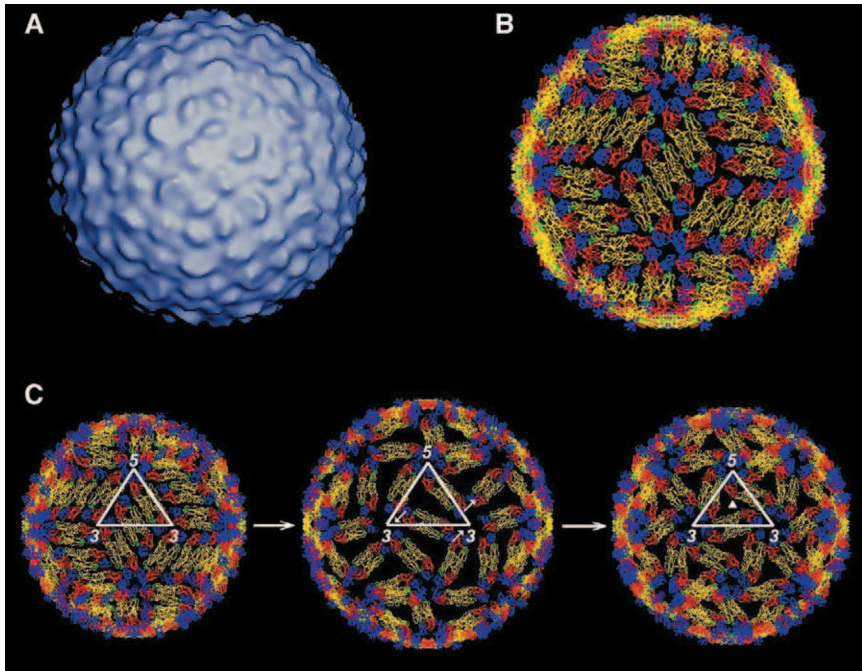
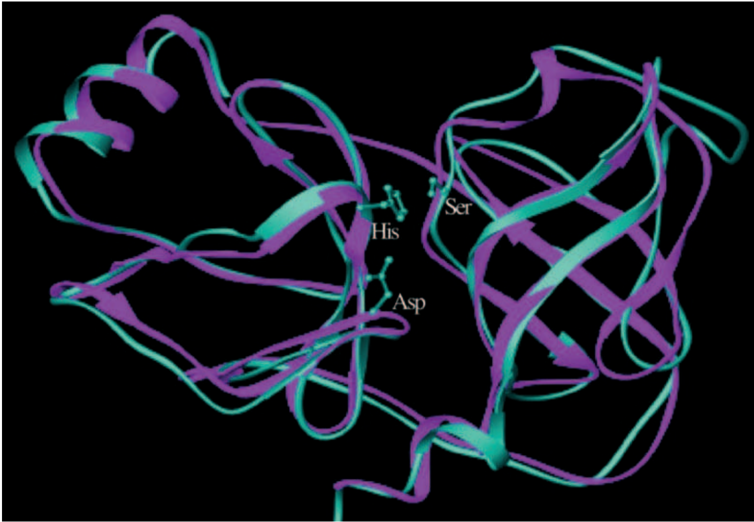
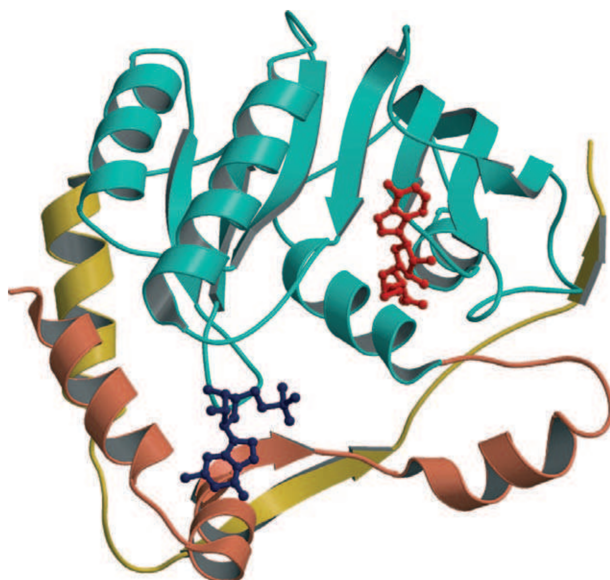


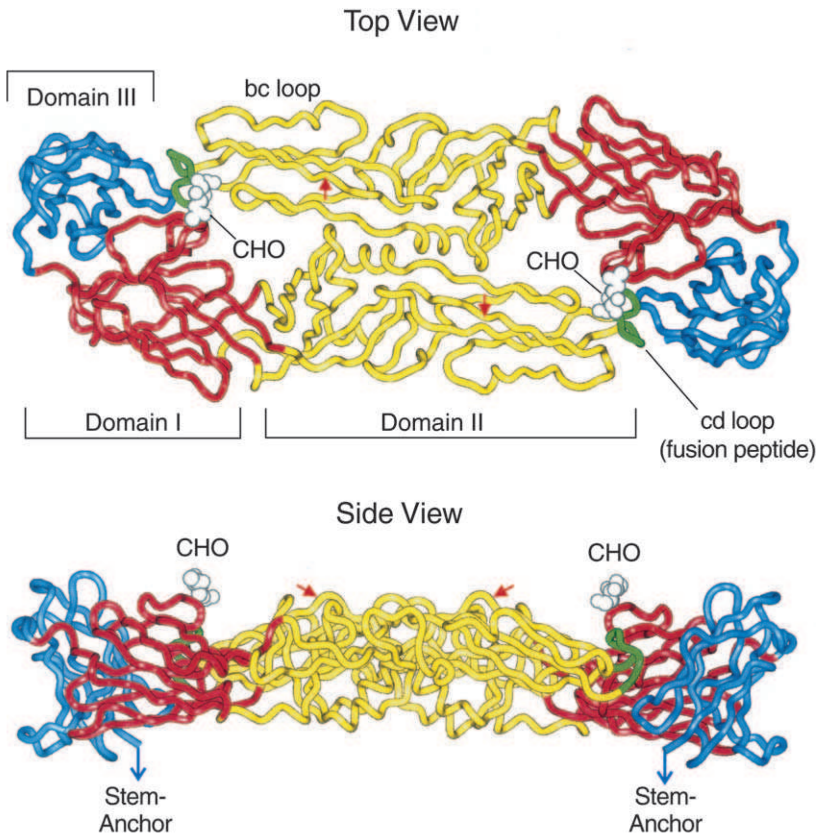
LINDENBACH AND RICE, FIG 2. DEN2 virion structure determined by cryoelectron microscopy. (A) The relatively smooth surface of the DEN2 virion is visible in this model based on image reconstruction. (B) The unusual "herringbone" arrangement of E protein dimers fitted into the electron density map. In this image, E protein domains I, II, III, and the fusion peptide are colored red, yellow, blue, and green, respectively. (C) Proposed acid-catalyzed rearrangement of E protein dimers into the fusogenic state. An icosahedral asymmetric unit is represented by the triangle, and three- and fivefold axes of symmetry are illustrated. Small arrows indicate the proposed rotation of the E protein. Images adapted from Kuhn *et al*. (2002) and used with permission. Composite image kindly provided by Dr. R. J. Kuhn.



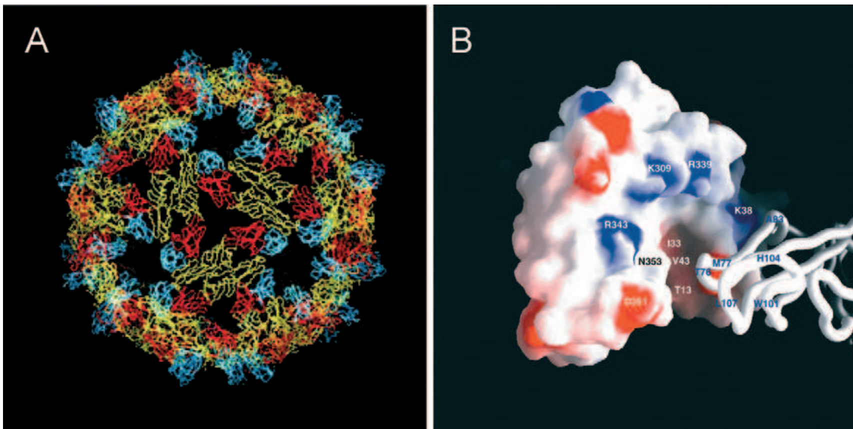
LINDENBACH AND RICE, FIG 4. Structure of the viral serine protease domain of NS3. Superimposed Cα traces of DEN2 NS3 (cyan) and hepatitis C virus NS3 (magenta) serine protease domains, illustrating the typical chymotrypsin-like fold. The rms deviation between the 68 C-terminal Cα atoms of these protease structures is 0.9 Å. The catalytic triad is shown for the DEN2 protease. Figure kindly provided by Dr. H. M. Krishna Murthy.



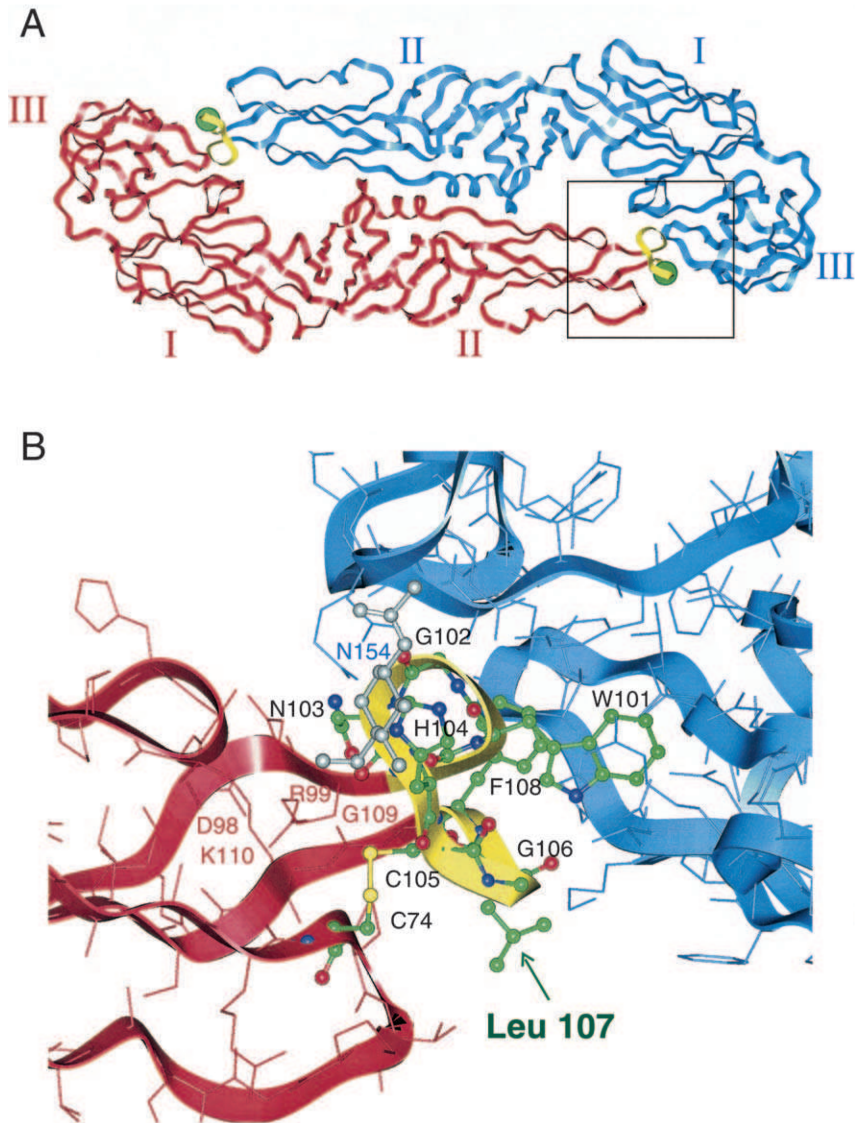
LINDENBACH AND RICE, FIG 5. Structure of the NS5 methyltransferase domain. This ribbon diagram illustrates the folding of DEN2 NS5 residues 7–267. Individual subdomains are colored orange, cyan, and yellow, whereas bound GDPMP and *S*-adenosylhomocysteine are colored purple and red, respectively. Figure courtesy of M. Egloff and B. Canard.



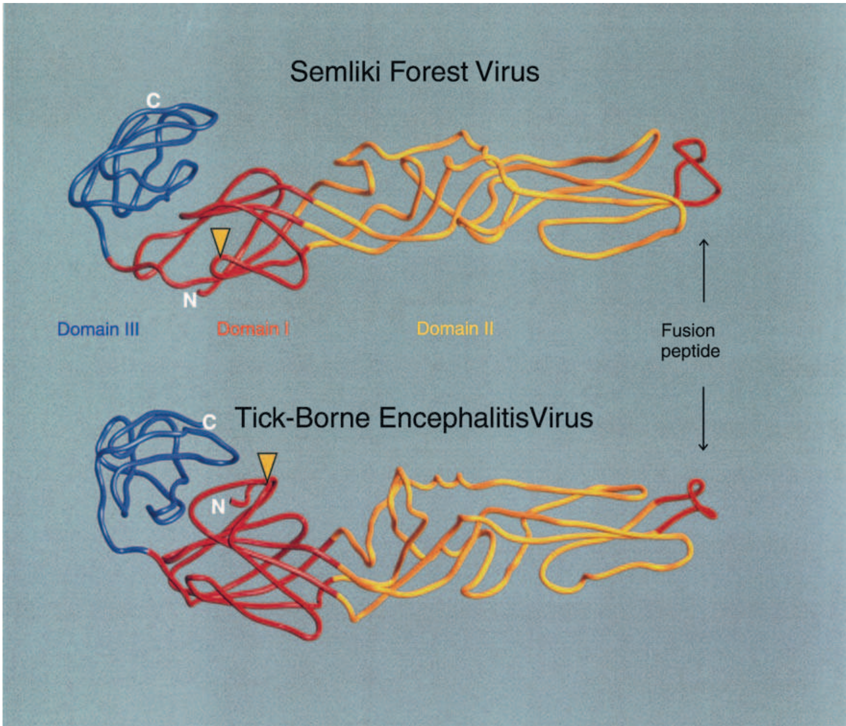
HEINZ AND ALLISON, FIG 4. Top and side views of the TBE virus sE dimer, depicted as a ribbon diagram of the structure determined by X-ray crystallography (Rey *et al*., 1995). CHO, carbohydrate attached to Asn 154. The arrow indicates the approximate position of a second glycosylation site found in certain dengue viruses (Johnson *et al*., 1994). Adapted from Heinz and Allison (2001) with permission.



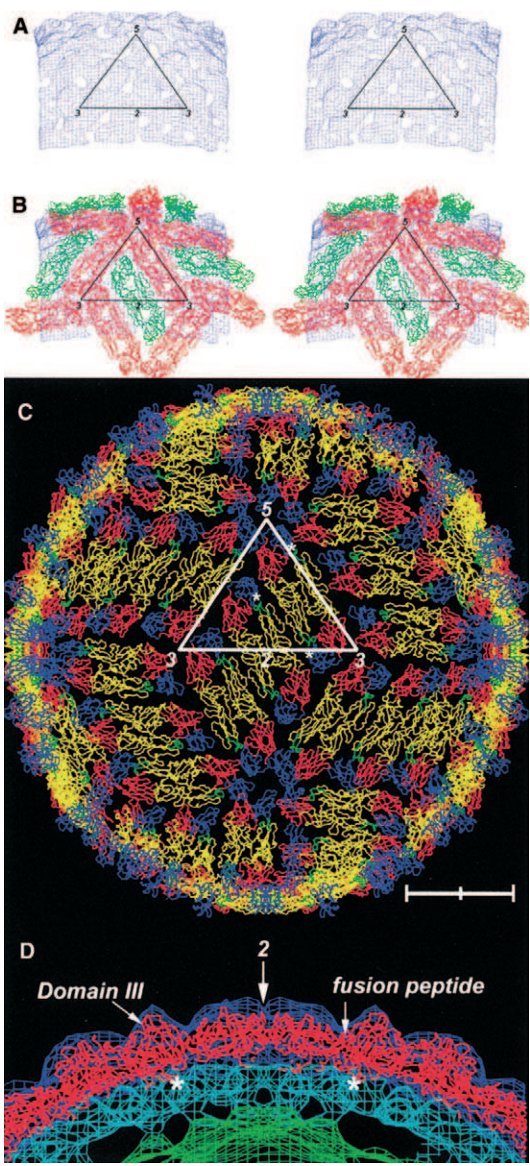
HEINZ AND ALLISON, FIG 6. (A) Organization of E protein dimers in the TBE virus RSP as deduced from fitting the high-resolution sE structure into an image reconstruction of cryoelectron micrographs (Ferlenghi *et al*., 2001). (B) The site of lateral interactions in the icosahedral lattice of E protein dimers in the TBE virus RSP (Ferlenghi *et al*., 2001). The bc loop of one subunit (depicted as a "worm") fits into a groove in domain III of an adjacent dimer (surface representation). Electrostatic potentials are shown in red (negative) and blue (positive). Modified from Ferlenghi *et al*. (2001).



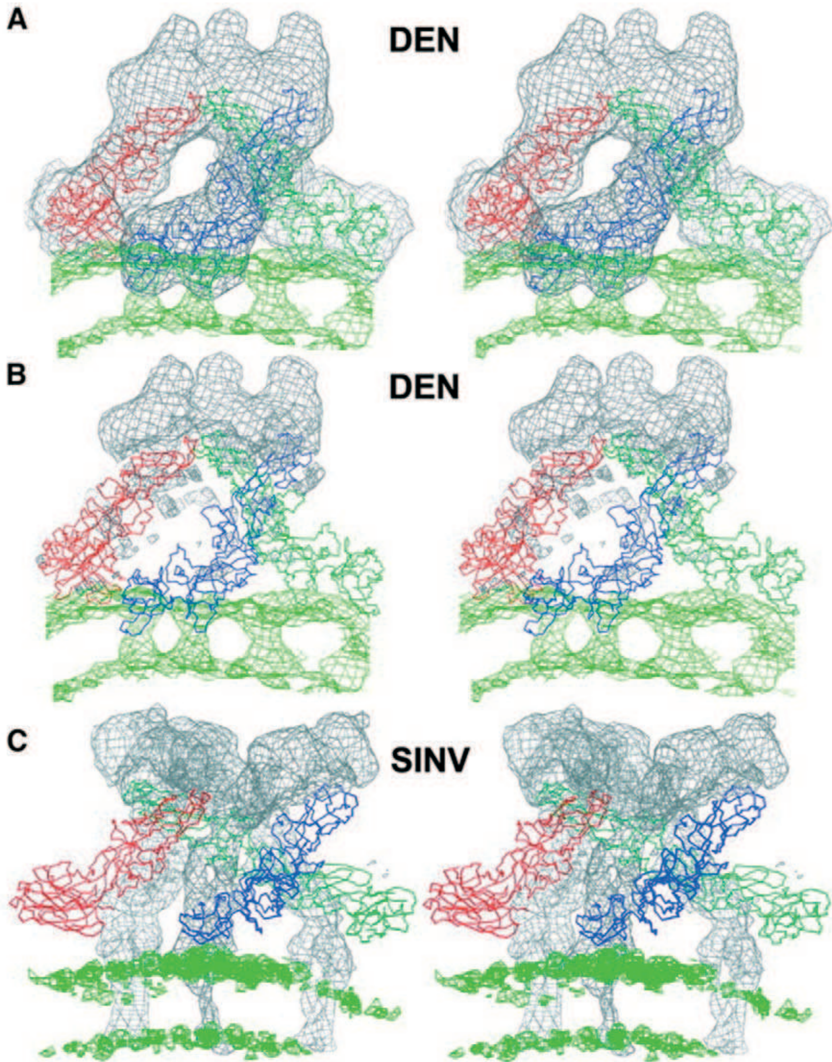
HEINZ AND ALLISON, FIG 8. (A) Ribbon diagram of the TBE virus E protein (top view) with the fusion peptide highlighted in yellow and the position of Leu 107 indicated by a green ball. (B) Zoom of the boxed region in A. Adapted from Allison *et al*. (2001).



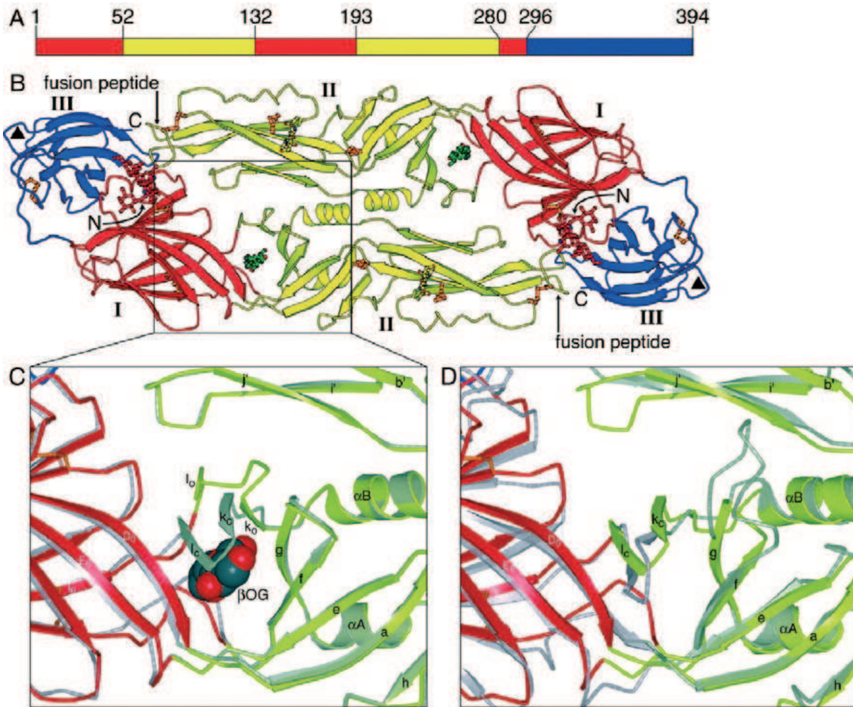
Heinz and Allison, Fig 9. Comparison of the structures of the Semliki Forest virus E1 protein and the TBE virus E protein (Lescar *et al*., 2001). Triangles mark the glycosylation sites. Reproduced from Heinz and Allison (2001) with permission.


A
B
C
D
2
Domain III
fusion peptide

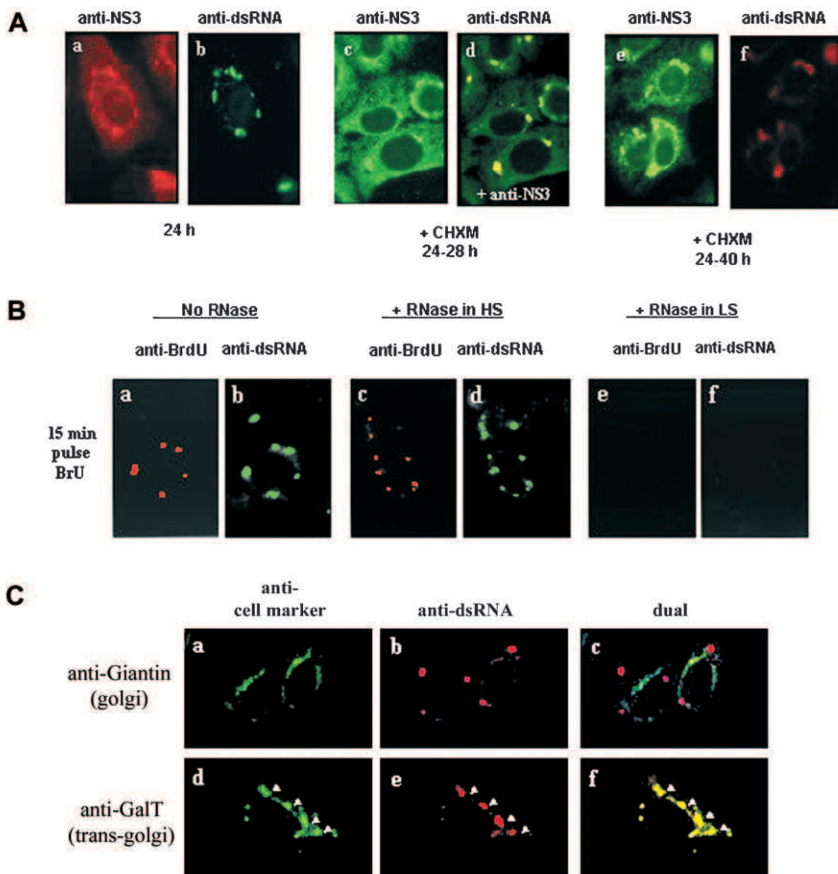
HEINZ AND ALLISON, FIG A1. Envelope protein organization in mature dengue virus. (A) Stereo view of electron density from cryo-electron microscopy corresponding to the E ectodomains. (B) Fitting of the TBE virus E protein ectodomain into the experimental electron density. Green dimers are on icosahedral twofold axes, red dimers on quasi-twofold axes. (C) E protein organization of whole virus with domains I, II, and III colored red, yellow, and blue, respectively, and the fusion peptides colored green. The C-terminus of the ectodomain fragment is indicated by an asterisk. Scale bar represents 100 Å. (D) Central cross-section showing outer protein shell (dark blue), membrane (light blue), and nucleocapsid (green). The fitted E ectodomain is shown in red. Reproduced from Kuhn *et al*. (2002) with permission.



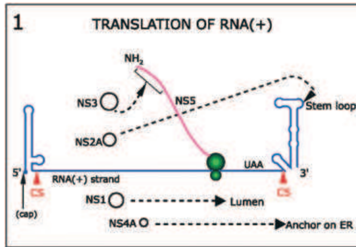
HEINZ AND ALLISON, FIG A2. Stereo images of the spike structures of immature dengue virus particles (A and B) and mature Sindbis virus (C), showing the fitting of proteins E and E1, respectively, into the electron density from cryo-electron microscopy image reconstructions. In B and C, the portion of the density assigned to E and E1 were zeroed out to show the positions of the prM and E2 proteins, respectively (gray). Reproduced from Zhang *et al*. (2003b) with permission.

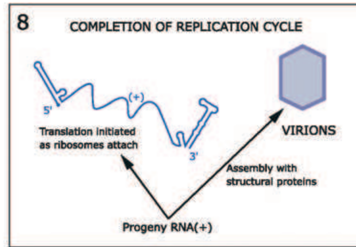


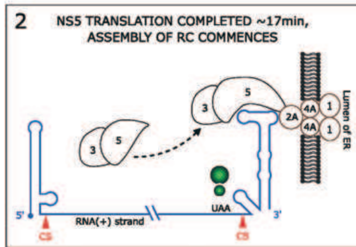
HEINZ AND ALLISON, FIG A3. Dengue E protein and its ligand-binding pocket. (A) Domain definition of dengue E protein, showing domains I, II, and III in red, yellow, and blue, respectively. (B) Top view of E dimer with the domains colored as in A. Disulfide bridges are shown in orange, and the glycans on domains I and II are shown as ball-and-stick representations. A putative receptor-binding site is indicated by a triangle. (C) Enlargement of the kl hairpin region, showing the position of the bound β-OG molecule. The gray superimposed structure is the "closed" form in the absence of β-OG. (D) The same view as in C, showing the dengue virus E protein structure (closed form) superimposed onto the TBE virus E protein structure (gray). Reproduced from Modis *et al.* (2003) with permission.

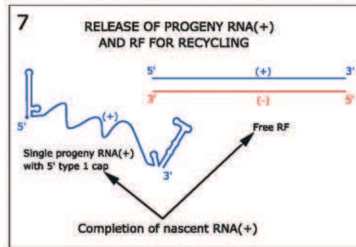


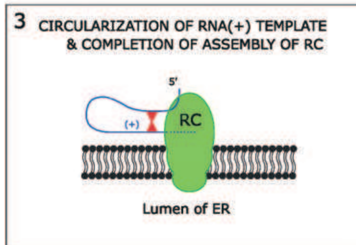
WESTAWAY *ET AL.*, FIG. 2. Immunofluorescence of KUNV-infected cells showing (A) the stability of sites of replication during CHXM treatment, (B) the coincidental location of dsRNA templates with nascent viral RNA pulse-labelled with bromo-substituted RNA (Br-RNA), and (C) the strong association of dsRNA with the *trans*-Golgi marker galactosyl transferase (GalT). (A) Cells were treated with CHXM from 24 h and dual labeled as shown; in d only, both labels are shown, producing yellow foci. (B) The pulse period of 15 min in actinomycin D-treated cells represented the time for completion of a cycle of RNA synthesis; after the pulse, cells were immediately permeabilized and treated with RNaseA in high salt (HS) or low salt (LS) as shown. Commercially available antibodies to BrdU are able to react with Br-RNA. (C) Note that another marker for the golgi (Giantin) is not colocated with dsRNA. From Westaway *et al*. (1999, for A, B) and Mackenzie *et al*. (1999, for C), with permission.

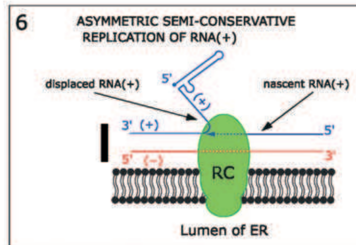


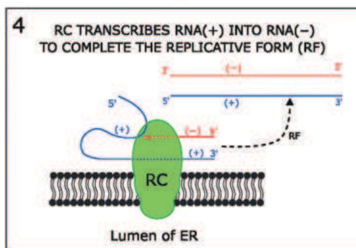


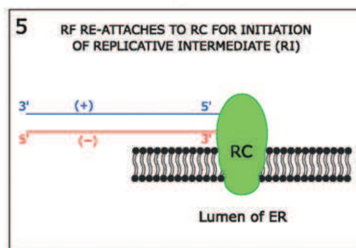






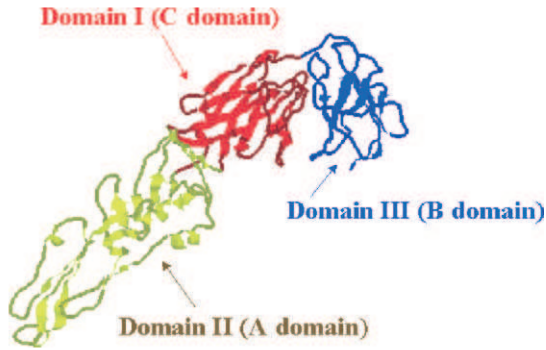




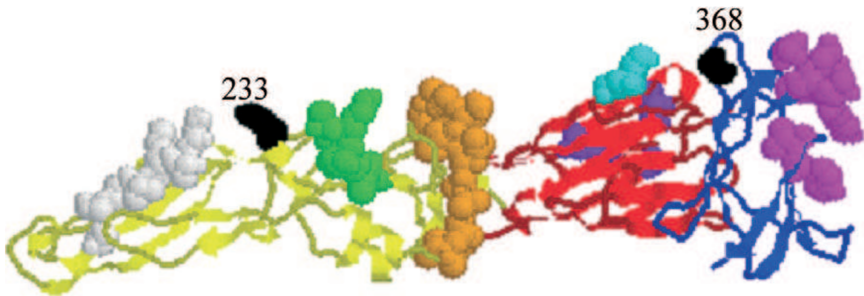


RF RECYCLES

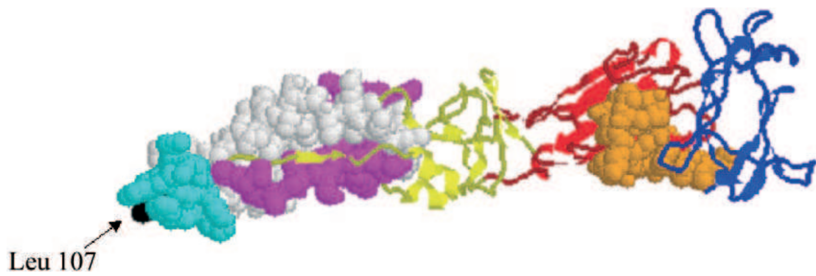
WESTAWAY *ET AL.*, FIG. 5. Model for the RNA replication cycle of the *Flavivirus* replication complex (RC). **Step 1:** During translation of the ns proteins in *cis* from the genomic RNA, NS3 is assumed to bind to the terminal product NS5 at one or more conserved regions in the N-terminal domain while other polyprotein cleavage products (NS1, NS2A, and NS4A) move to the sites as indicated. **Step 2:** On completion of translation, assembly of the RC commences on the 3′UTR via binding of NS2A, probably at the conserved 3′-terminal stem loop at which the NS3 and NS5 components also bind. The location of the complex shown on the loop is arbitrary. The complex still attached to the RNA(+) strand is transported to the membrane site of replication by affinity of the hydrophobic regions of NS2A interacting with those of NS4A (shown as a dimer), which in turn is bound by its hydrophilic extensions into the lumen between transmembrane domains to dimeric NS1 in the lumen. **Step 3:** The RC is now complete and may undergo rearrangement as the RdRp domains of NS5 bind to the template, which circularizes as shown via the conserved complementary sequences (CS) located near the 5′and 3′-terminal stem loops. **Step 4:** The RC commences to transcribe the RNA(+) template into RNA(−), and a short dashed arrow indicates the direction of synthesis (5′ to 3′) of the nascent RNA(−) strand. The association with membranes and the consensus composition of the RC (NS1, NS3, NS5, NS2A, and NS4A) are described in the text. The RF is formed during the completion of transcription by base pairing of the newly synthesized RNA(−) strand with the RNA(+) strand. **Step 5:** The released RF reattaches to the RC to function as a template. **Step 6:** The replicative intermediate (RI) is formed as the RC commences synthesis of a RNA(+) strand by asymmetric and semiconservative replication on the RF template. The template moves through the RC while the single nascent RNA(+) strand displaces the preexisting RNA(+) strand. **Step 7:** The displaced RNA(+) strand is released and a type 1 ^{7}Me-guanosine cap is added by enzymic activities of NS3 (RNA triphosphatase) and NS5 (guanylyl transferase and methyl transferase), possibly while they are still associated in the RC. The RF is now free to recycle through the membrane-bound RC (late in infection, this occurs within the induced vesicle packets). **Step 8:** Progeny-capped RNA(+) strands either assemble into virions or attach to ribosomes so the whole cycle can be repeated. Adapted from Khromykh *et al.*, (1999b), with permission.



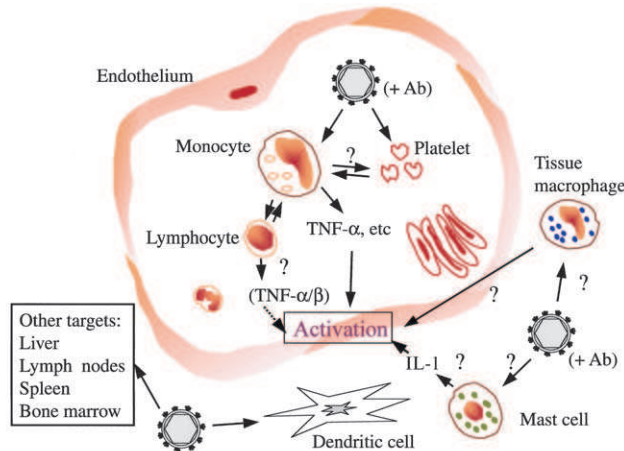
ROEHRIG, FIG 1. Crystal structure of the flavivirus E-glycoprotein monomer. Color key: red, domain I (C domain); yellow, domain II (A domain); and blue, domain III (B domain).



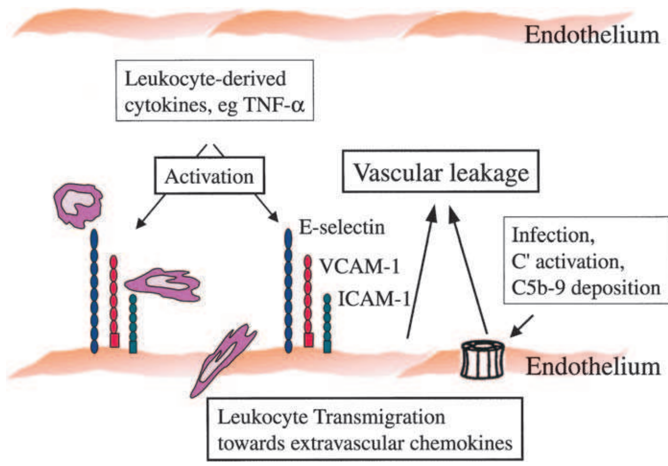
ROEHRIG, FIG 3. Localization of major neutralization regions on the E-glycoprotein identified in Mab neutralization-escape variants. A lateral view of the flavivirus monomer with domains I (red), II (yellow), and III (blue) is shown. Major neutralization regions are identified: region 1, aa 67–72 and 112 (white); region 2, aa 123–128 (green); region 3, aa 155 and 158 (cyan); region 4, aa 171, 181, and 293 (blue); region 5, aa 52, 136, and 270–279; and region 6, aa 307–311, 333, and 384–385 (purple). Also marked are two other amino acid changes associated with neutralization-escape variants (aa 233 and 368).



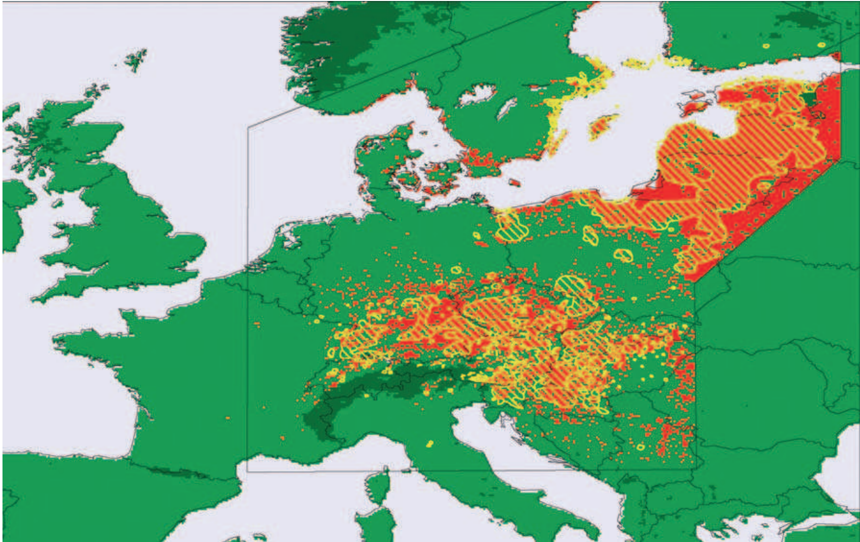
ROEHRIG, FIG 5. Important regions of the E-glycoprotein involved in low pH-catalyzed virus-mediated cell membrane fusion identified through antigenic analyses. A lateral view of the flavivirus monomer with domains I (red), II (yellow), and III (blue) is shown. Highlighted regions are identified as space-filled amino acids. Regions exposed when virus is treated at low pH: aa 1–22 (TBE, orange), aa 58–121 (DEN 2, white), and aa 225–249 (DEN 2, purple). This last region is nearly identical with a similar region (aa 221–240) in TBE virus. The proposed flavivirus fusion sequence (aa 98–110) is shown in cyan. The aa Leu 107, associated with TBE virus fusion, is marked in black.



ANDERSON, FIG. 1. Model showing surface interactions of hemorrhagic flavivirus (dengue) with extra- and intravascular cell targets. Intravascularly, the presence of subneutralizing levels of antivirus antibodies stimulates virus attachment to platelets and infection of monocytes. This results in immune complex deposition on platelets and secretion of vasoactive factors from virus-infected monocytes. Among such vasoactive factors are cytokines, particularly TNF-α, which activates increased surface expression of adhesion molecules on endothelial cells. Extravascularly, virus infection of tissue macrophages, mast cells, and dendritic cells may result in the release of additional factors, which contribute to endothelial cell perturbation.



ANDERSON, FIG. 2. Model depicting possible events in endothelial cell surface perturbation during hemorrhagic flavivirus (dengue) infection. Endothelial cell activation, leading to upregulation of adhesion molecules (E-selectin, VCAM-1, ICAM-1), can be triggered by monocyte-derived cytokines (Anderson *et al.*, 1997) or by deposition of C5b-9 and other products of complement activation (Avirutnan *et al.*, 1998). C5b-9 is represented as a membrane attack complex pore structure, although the deposition of C5b-9 on dengue-infected cells appears associated with sublytic, rather than lytic, responses (Avirutnan *et al.*, 1998). Increased adhesion molecule expression, along with uncharacterized vasoactive factors, can lead to endothelial leakage and can mediate rolling, adhesion, and transendothelial migration of leukocytes into extravascular tissues. Similar processes may also contribute to the invasion of cell-borne neurotropic flaviviruses through the endothelial blood–brain barrier.



GOULD *ET AL*., FIG 3. Predicted (red) and observed (yellow hatched) pan-European distributions of foci of tick-borne encephalitis virus based on analysis of remotely sensed environmental values and elevation within the outlined area. The virus occurs extensively to the east of this area, but is not yet mapped in any detail. Frequent cloud contamination in high mountain areas (darker green) prevents analysis there (Randolph, 2000).