

MOLECULAR BIOLOGY OF FLAVIVIRUSES

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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

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 (≈ 50 nm), spherical particles containing an electron-dense core of ≈ 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/cm³, 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

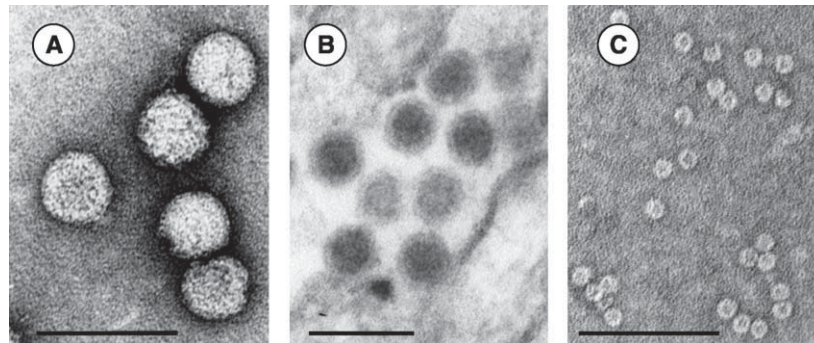


FIG 1. Electron micrographs of flavivirus particles. Bars represent 100 nm. (A) Negatively stained DEN2 virus particles isolated from an infected mouse brain homogenate via rate zonal sedimentation through a sucrose gradient. (B) St. Louis encephalitis virus particles can be seen in this thin section 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 non-ionic detergents reveals discrete nucleocapsids (120 to 140S; 1.30 to 1.31 g/cm³), 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 (≈ 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/cm³), and can undergo acid-catalyzed fusion similar to virions (Schalich *et al.*, 1996).

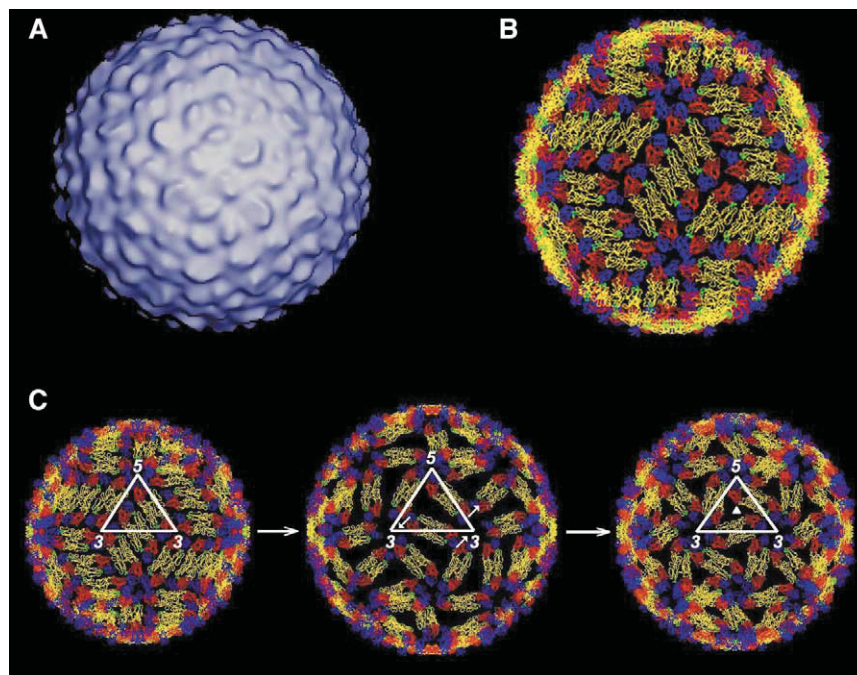


FIG 2. DEN2 virion structure determined by cryoelectron microscopy. (A) The relatively smooth surface of the DEN2 virion is visible in this model based on image reconstruction. (B) The unusual “herringbone” arrangement of E protein dimers fitted into the electron density map. In this image, E protein 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.

Cryoimage reconstruction of TBE RSPs suggests a T=1 icosahedral arrangement of 30 E protein dimers (Furlonghi *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, m⁷GpppAmpN₂ (Cleaves and Dubin, 1979; Wengler *et al.*, 1978). Additional methylation of the N₂ 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

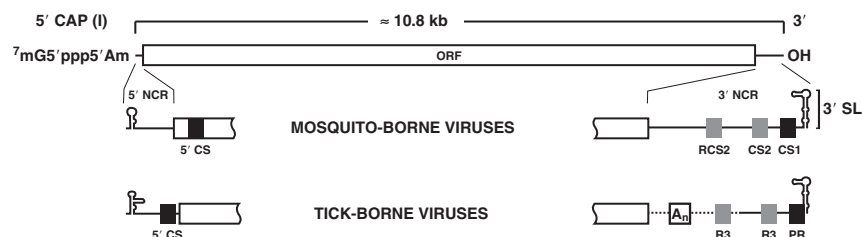
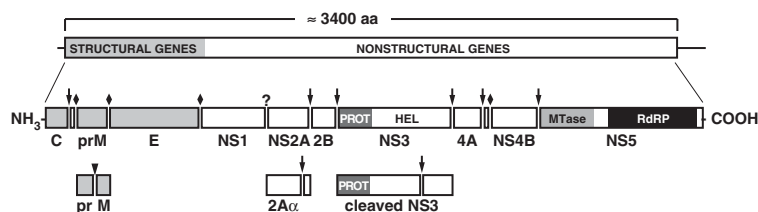
A. Flavivirus genome: RNA elements**B. Flavivirus genome: protein expression**

FIG 3. Flavivirus genome organization. (A) RNA elements of the 5'-capped, ≈ 10.8 -kb flavivirus genome are illustrated. Similar structural 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 frame (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 intriguing, 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 Q β 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 Vрати, 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 (Olsthorn 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 (≈ 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 Cys₄–Cys₁₅, Cys₅₅–Cys₁₄₃, and Cys₁₇₉–Cys₂₂₃ 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, Pro₂₅₀ 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 [³H]ethanolamine or [³H]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 [^3H]inositol, and labeling with [^3H]ethanolamine or [^3H]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, NS2A α , 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 NS2A α cleavage site mutant (Lys₁₉₀→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 NS2A α , 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 Asp₃₄₃ to a short, uncharged side chain. Although virus release was restored in these mutants, NS2A α was not necessarily produced. Thus, the basic residue at NS2A residue 190, rather than NS2A α 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 His₅₃, Asp₇₇, and Ser₁₃₈ (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; Warrenner *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

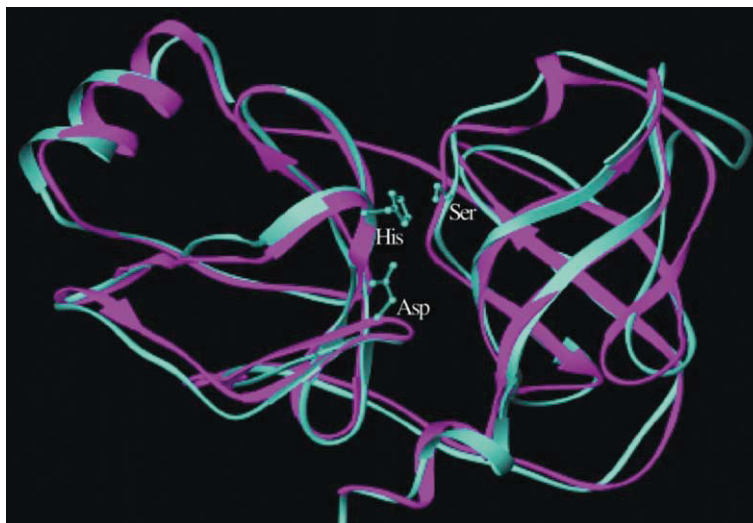


FIG 4. Structure of the viral serine protease domain of NS3. Superimposed C α traces of DEN2 NS3 (cyan) and hepatitis C virus NS3 (magenta) serine protease domains, illustrating the typical chymotrypsin-like fold. The rms deviation between the 68 C-terminal C α atoms of these protease structures is 0.9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

(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 Mg²⁺ and inhibited by poly(A), whereas NTPase activity was inhibited by Mg²⁺ 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 Lys₁₉₉ 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 in-frame 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

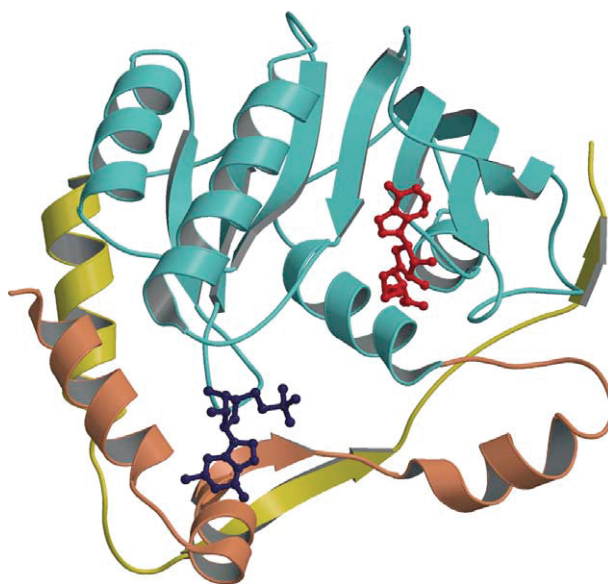


FIG 5. Structure of the NS5 methyltransferase domain. This ribbon diagram illustrates the folding of DEN2 NS5 residues 7 to 267. Individual subdomains are colored orange, cyan, and yellow, whereas bound GDPMP and *S*-adenosyl-homocysteine are colored purple and red, respectively. Figure courtesy of M.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

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 β 1 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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