Chapter 3
Flaviviruses

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Abstract Flavivirus genome amplification is a complex process that involves the viral RNA, cellular and viral proteins, and a sophisticated architecture of cellular membranes induced by viral infection. The viral RNA is not just a passive template; it plays an active role acquiring dynamic tertiary structures during viral replication. RNA synthesis is regulated by *cis*-acting elements present at the 5′- and 3′-ends of the viral genome. These elements include complementary sequences that mediate genome cyclization through direct RNA–RNA interactions. Studies from many laboratories have provided compelling evidence supporting the notion that a circular conformation of the viral RNA is essential for flavivirus RNA replication. In addition, an RNA element located within the viral 5′UTR has been found to bind the viral polymerase and promote RNA synthesis. In this chapter, we describe viral proteins and RNA structures involved in flavivirus genome amplification and provide working models that explain the need of long-range RNA–RNA interactions during viral RNA synthesis.

Introduction: The Viral Life Cycle

Flaviviruses comprise one of the three genera within the Flaviviridae family; the other two are the Pestivirus and Hepacivirus. The Flavivirus genus includes, among others, the medically important mosquito borne dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and the tick-borne encephalitis virus (TBEV). Flaviviruses are enveloped viruses with a single stranded, ∼11 kb, positive-sense RNA genome with a type 1 cap (m7GpppAmp) structure at the 5′-end (Wengler and Gross 1978; Cleaves and Dubin 1979). In contrast to cellular mRNAs, flavivirus genomes are not polyadenylated (Wengler and Gross 1978). The viral RNA encodes a single long open reading frame (ORF)

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flanked by highly structured 5′ and 3′ untranslated regions (UTRs) of ∼100 and 350–700 nucleotides, respectively. As for all positive-stranded RNA viruses, the flavivirus genomic RNA is infectious and serves as mRNA. Translation of the single ORF at the rough ER produces a large polyprotein that is cleaved cotranslationally and posttranslationally into at least 10 proteins. The N-terminal of the polyprotein encodes the three structural proteins (C-prM-E), followed by seven nonstructural (NS) proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) (Rice et al. 1985) (Fig. 3.1a). The amino termini of prM, E, NS1, and NS4B are generated upon cleavage by the host signal peptidase in the ER lumen, while the processing of most of the NS proteins and the carboxyl terminus of the C protein is carried out by the viral NS3 serine protease in the cytoplasm of the infected cell (Fig. 3.1b). NS3 requires the cofactor NS2B for protease activity. In addition, NS3 comprises RNA triphosphatase and helicase activities. NS5 is the RNA-dependent RNA polymerase (RdRp), which carries a methyltransferase (MTase) domain in its NH2 terminus.

The viral replication cycle is similar for all flaviviruses (Lindenbach and Rice 2001). The virus enters a host cell via receptor-mediated endocytosis. Upon internalization and acidification of the endosome, fusion of viral and vesicular membranes

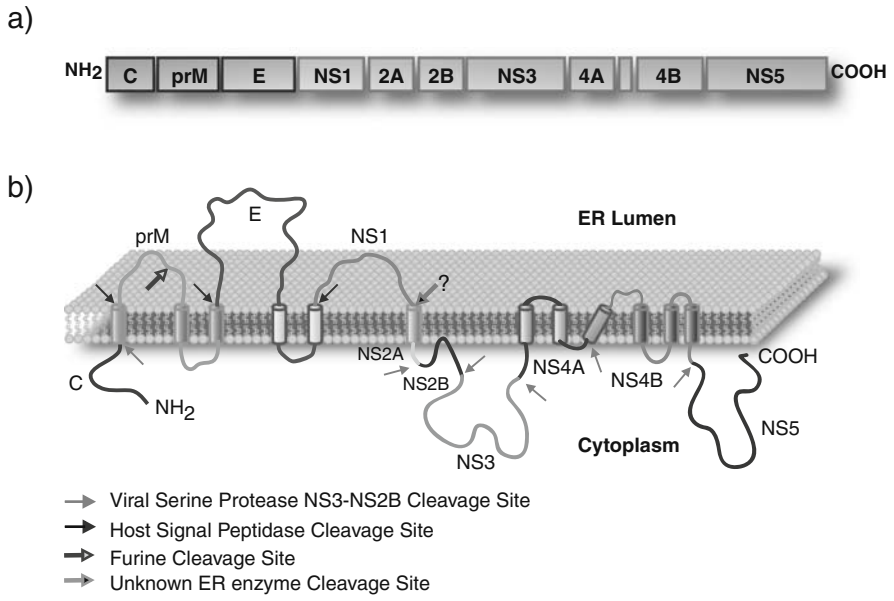


Fig. 3.1 Flavivirus proteins. (**a**) Schematic representation of the viral polyprotein. The three structural proteins C, capsid; prM precursor to membrane protein; E, envelope; and the seven non-structural (NS) proteins NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 are shown. (**b**) Membrane topology of the flavivirus polyprotein. The predicted orientation of the viral proteins across the endoplasmic reticulum (ER) membrane is shown. *Trans*-membrane domains are indicated by *cylinders* and *arrows* indicate the cleavage site of specific enzymes. The *question mark* indicates cleavage by an unknown ER enzyme.

allows entry of the nucleocapsid into the cytoplasm. After translation of the viral RNA, virus-induced hypertrophy of intracellular membranes occurs, originating membranous structures in which RNA synthesis takes place (for review see Westaway et al. 2003). Based on *trans*-complementation studies, genome packaging appears to be coupled to RNA replication (Khromykh et al. 2001b). Nascent virus particles pass through the Golgi apparatus, where prM is cleaved by furin and virion maturation occurs. Finally, the viral progeny is exocytosed via secretory vesicles.

Flavivirus Replication Complexes

Flavivirus RNA replication occurs in close association with cellular membranes in so-called viral replication complexes (RCs). Replication begins with the synthesis of a negative-strand RNA, which serves as a template for the synthesis of additional positive-strand genomic RNA. The enzymatic reaction is catalyzed by the RdRp activity of the viral NS5 protein, in association with the viral protease/helicase NS3, other viral NS proteins, and presumably host factors. RNA synthesis is asymmetric, leading to a 10- to 100-fold excess of positive over negative strands (Cleaves et al. 1981; Uchil and Satchidanandam 2003b). Negative strands continue to accumulate throughout the infection and have been isolated exclusively in double-stranded forms. Three species of viral RNA can be metabolically labeled: a ribonuclease-resistant double-stranded RNA (dsRNA) called replicative form (RF); a form partially resistant to ribonucleases, likely composed by RNAs with complementary nascent elongating strands, known as replicative intermediates (RI); and the genomic vRNA that is fully sensitive to ribonucleases. The three RNA forms have also been described for in vitro RNA polymerase reactions using infected cell extracts (Grun and Brinton 1986; Chu and Westaway 1987; You and Padmanabhan 1999; Uchil and Satchidanandam 2003b).

Different lines of evidence revealed that RNA replication appears to be confined to discrete foci, mainly in the perinuclear region (Ng et al. 1983; Westaway et al. 1997; Mackenzie et al. 1999). Data on the composition of the RCs in flavivirus-infected cells were obtained by confocal and electron microscopy together with co-immunoprecipitations using specific antibodies to different NS proteins and to dsRNA. The results indicated that proteins NS1, NS2A, NS3, NS4A, NS5, and for some viruses NS4B co-localize with dsRNA (Mackenzie et al. 1996; Westaway et al. 1997; Mackenzie et al. 1998; Miller et al. 2006). Interestingly, the DENV and YFV NS5 RNA polymerases were found predominantly in the nucleus, showing weak staining in the perinuclear region that co-localized with the RCs (Buckley et al. 1992; Kapoor et al. 1995; Miller 2006; Uchil et al. 2006). This observation is in agreement with the finding that a small amount of NS5 is involved in active RNA replication (Grun and Brinton 1987; Chu and Westaway 1992; Uchil and Satchidanandam 2003a). Furthermore, active RCs were also found in the nucleus of infected cells (Uchil et al. 2006).

Membranes have been suggested to play a structural and organizational role in flavivirus replication, possibly offering a suitable microenvironment for viral

RNA synthesis and viral morphogenesis. The membranous structures found in flavivirus-infected cells seem to originate from different cellular organelles (for review see Mackenzie 2005). Convoluted membranes (CM) and paracrystalline structures (PC) are the putative sites of viral polyprotein processing, whereas proliferating ER and vesicles of 70–100 nm in diameter, found as vesicle packets (VP) enclosed by an outer membrane, may represent the sites of viral RNA replication. The association of the replicative forms of the viral RNA with the VP has been shown by electron microscopy and confirmed by biochemical analysis (Mackenzie et al. 1996; Grief et al. 1997; Westaway et al. 1997; Uchil and Satchidanandam 2003a). Biochemical studies with flavivirus-infected cells and cell extracts active for RNA synthesis were used to probe the architecture of the RC (Uchil and Satchidanandam 2003a). Treatment of the extracts with nucleases in the presence or absence of different detergents suggested that the three viral RNA species (RF, RI, and vRNA) reside in a membrane enclosed nuclease-resistant compartment. It was proposed that the RF resides within the inner membrane of a double membranous structure, whereas the nascent genomic RNA was extruded from the vesicles but retained inside the outer bounding membrane of the VP (Uchil and Satchidanandam 2003a).

It has been shown that protein NS4A is required for induction of the membranous structures CM/PC by different flaviviruses. Cleavage of a 2 K domain at the C-terminus of NS4A by the viral serine protease leads to a large accumulation of intracellular membranes in DENV-infected cells (Miller et al. 2007) and relocalization of matured NS4A to Golgi membranes in WNV-infected cells (Roosendaal et al. 2006). These results suggest that proteolytic processing of NS4A could regulate the membrane rearrangements observed upon infection. A role for NS1 during RNA replication was proposed. Mutation of the N-glycosylation sites of NS1 led to a dramatic defect in RNA replication (Muylaert et al. 1996). In addition, deletions within YFV NS1 resulted in viruses with defects at early stages of RNA replication, presumably during minus-strand synthesis (Lindenbach and Rice 1997). Interestingly, this defect was suppressed by a mutation in NS4A, providing genetic evidence for NS1–NS4A interaction in RNA replication (Lindenbach and Rice 1999). Although NS1, NS2A, and NS4B of different flaviviruses have been implicated in RNA synthesis, their precise roles in this process remain unclear.

Multifunctional Viral Proteins Involved in Flavivirus RNA Replication

NS5 is the largest and the most conserved of the flavivirus proteins. It contains an N-terminal S-adenosyl-methionine (SAM)-dependent methyl transferase domain (MTase) and a C-terminal RdRp domain. Recently, the structure of the NS5 C-terminal domain of WNV and DENV revealed a classical RdRp-fold bearing palm, thumb, and fingers motifs (Malet et al. 2007; Yap et al. 2007). The presence of a priming loop found in these structures is consistent with a primer-independent

(de novo) mechanism of initiation of RNA synthesis proposed for flaviviruses. The NS5 from WNV, DENV, and YFV were shown to possess guanine N7 and ribose 2′-O MTase activities involved in formation of the 5′ cap (Egloff et al. 2002; Ray et al. 2006; Dong et al. 2007; Zhou et al. 2007). The crystal structures of WNV and DENV MTase domain showed a single binding site for the methyl donor SAM (Egloff et al. 2002; Zhou et al. 2007). In addition, a positively charged surface adjacent to the SAM binding site was proposed to be the recognition site for the capped-RNA substrate. Deletions or mutations within the MTase domain were shown to be lethal for the replication of WNV and DENV (Khromykh et al. 1998, 1999b; Ray et al. 2006). In addition, mutagenesis of the Kunjin polymerase active site motif confirmed that it is essential for viral replication and that polymerase activity could be supplied in *trans* from a Kunjin replicon. However, *trans*-complementation in cells expressing only NS5 was found to be inefficient, suggesting that co-translational expression of additional NS proteins may be required for the RdRp to associate with other replicase components (Khromykh et al. 1999a).

The multifunctional NS3 protein bears a protease, helicase, nucleotide triphosphatase (NTPase), and 5′RNA triphosphatase (RTPase) activities. Its N-terminal 180 amino acids comprise the serine protease, and its C-terminal region has conserved domains found in the DEXH family of NTPase/RNA helicases. RNA-stimulated NTPase and RNA unwinding activities have been characterized in DENV, WNV, YFV, and JEV (Warrener et al. 1993; Li et al. 1999; Utama et al. 2000; Borowski et al. 2001; Benarroch et al. 2004; Yon et al. 2005). Crystal structures have been recently reported for the helicase of DENV and YFV (Wu et al. 2005; Xu et al. 2005). A study with a DENV infectious clone showed that an active helicase was essential for virus viability (Matusan et al. 2001). Although helicases have been implicated in the replication of flaviviruses genomes, their precise role in RNA synthesis remains unknown. Possible functions include unwinding dsRNA intermediates that arise during RNA amplification, destabilizing secondary structures of the RNA to increase polymerase processivity, or participating in RNA recruitment at specific subcellular locations.

Two-hybrid systems and co-immunoprecipitation studies using infected cells and recombinant proteins demonstrated that the helicase domain of NS3 of DENV binds NS5 (Kapoor et al. 1995; Johansson et al. 2001). This interaction occurred in the absence of other viral proteins but was dependent on the NS5 phosphorylation state (Kapoor et al. 1995). NS5 has been detected in both the cytoplasm and the nucleus. Only a hyperphosphorylated form of NS5, which was unable to interact with NS3, has been detected in the nucleus of DENV-infected cells (Kapoor et al. 1995; Brooks et al. 2002). NS5 contains two functional nuclear localization sequences (NLSs) and binding to importin β1 was demonstrated (Johansson et al. 2001; Brooks et al. 2002). Whether the nuclear localization of NS5 plays a role in the viral replication cycle or is part of a mechanism used by the virus to alter a cell function is currently unknown. The N-terminal region of NS5 that was shown to be required to bind NS3 was also found to bind importin β1, suggesting a competition between NS3 and importin β1 that may play a role in controlling the subcellular localization of NS5.

According to the crystal structure of NS5, the putative binding site for NS3 would be near the entrance of the RNA template tunnel, consistent with the proposed role of its activity in RNA unwinding during RNA synthesis (Malet et al. 2007). In addition, NS5 was reported to stimulate the NTPase and RTPase activities of NS3 (Yon et al. 2005), suggesting a functional interaction between these two viral proteins.

5′UTR Elements and Promoter Signals for RNA Synthesis

The 5′UTRs of flavivirus RNAs are relatively short and almost complete sequence conservation was observed among different strains of the same virus. In contrast, sequence comparisons between different flaviviruses, such as DENV, WNV, YFV, and SLEV, showed little conservation (Brinton and Dispoto 1988). Interestingly, the predicted secondary structure of 5′UTRs of different flaviviruses is very similar. These structures consist of a large stem-loop with a side stem-loop (named Stem-Loop A, SLA, Fig. 3.2). The conservation of this secondary structure in unrelated flaviviruses was taken as evidence of its possible importance in viral replication. In most cases, a second short stem-loop named SLB, which includes or ends at the translation initiator codon, could be formed downstream of SLA. The structure of SLA and the sequence upstream of the translation initiator AUG were found to be essential *cis*-acting elements for viral RNA synthesis (Cahour et al. 1995; Filomatori et al. 2006; Kofler et al. 2006). In addition, a conserved stem-loop structure present just downstream of the initiator AUG of DENV type 2 (DENV2) RNA was reported to act as a regulatory element important for start codon selection in translation initiation (Clyde and Harris 2006).

Cahuer and co-workers reported the first evidence of functional 5′UTR elements in flavivirus replication in vivo (Cahour et al. 1995). In this study, deletions from 5 to 25 nucleotides were incorporated throughout the 5′UTR by mutagenesis of an infectious DNA copy of DENV4. The dominant effect of the deletions appeared to be at the level of RNA synthesis and many of the mutations were found to be lethal. More recently, a similar approach was used to study the role of SLA structure during DENV2 replication (Filomatori et al. 2006). In this case, site-directed mutagenesis was done to (a) open the bottom of the stem of SLA structure, (b) reconstitute the stem with sequences that differed from the wild type, and (c) substitute nucleotides at the top and side loops of SLA. Alteration of the stem of SLA was found to be lethal, while reconstitution of the stem yielded an infectious RNA with a phenotype similar to the parental virus. In addition, infectious viruses could not be recovered after transfection of RNAs carrying substitutions at the top loop of SLA. In this case, revertant viruses with single substitutions, partially recovering the wild type sequence, were rescued in cell culture. In the same study, using a DENV2 replicon system that allows discrimination between translation and RNA synthesis, it was shown that the SLA structure was essential for RNA amplification, whereas no crucial role of SLA was connected with translation of the input RNA.

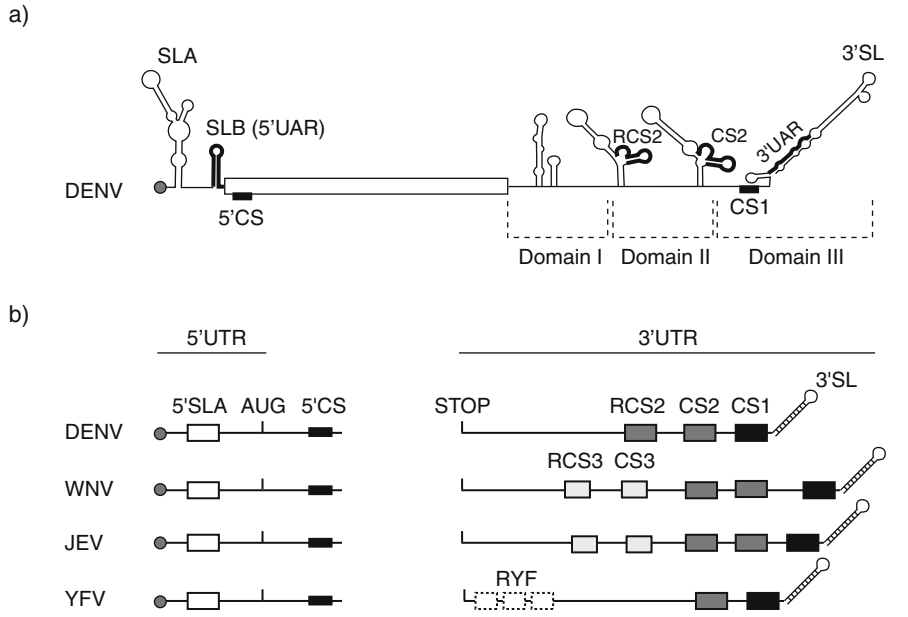
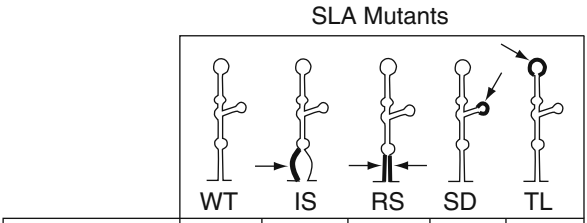


Fig. 3.2 Conserved 5′ and 3′UTR RNA structures of mosquito-borne flaviviruses. (**a**) Schematic representation of RNA elements found at the 5′ and 3′UTRs of DENV genome. The predicted secondary structures of defined domains are indicated: at the 5′-end, stem-loop A (SLA), stem-loop B (SLB, containing 5′UAR), and 5′CS are indicated; at the 3′-end, domains I, II, and III are shown. In addition, location of the 3′ stem-loop (3′SL) and the conserved sequence RCS2, CS2, and CS1 are indicated. (**b**) RNA elements of 5′ and 3′UTRs of dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) are schematically shown. At the 5′-end the 5′SLA, the translation initiator codon AUG, and the cyclization sequence 5′CS are indicated. The presence and relative location of the different conserved RNA elements at the 3′UTRs, CS1, CS2, RCS2, CS3, RCS3, and the yellow fever tandem repeats (RYF) are indicated by *boxes* in different *colors*.

RNA–protein interaction studies have been used to demonstrate direct binding of DENV polymerase to RNA molecules carrying the SLA structure. Moreover, using an in vitro assay to measure RdRp activity of DENV2 NS5, it was shown that SLA was a critical determinant for template specificity (Filomatori et al. 2006). The polymerase was able to initiate de novo and copy RNA templates bearing the SLA, while it was very inefficient in copying viral or non-viral RNA templates lacking this structure. A remarkable correlation between the requirement of DENV SLA sequence/structure for viral replication in transfected cells and the need of this element for in vitro polymerase activity was observed (Fig. 3.3). Based on in vivo and in vitro results, it was proposed that the SLA functions as the promoter for DENV negative-strand RNA synthesis. Further studies are necessary to extrapolate these observations to other flaviviruses.



	WT	IS	RS	SD	TL
Viral replication in cell culture	+++++	–	+++	+++++	–
RdRp activity in vitro	100%	20%	80%	100%	5%

Fig. 3.3 Structure–function correlation of DENV stem-loop A (SLA) mutants. On the *top*, schematic representation of RNA mutants: interrupted stem (IS), reconstituted stem (RS), side loop mutant (SD), and top loop mutant (TL). The level of DENV replication in BHK cells transfected with full-length viral RNAs carrying the respective mutation is compared with the viral RdRp activity observed in vitro using RNA templates of 160 nucleotides carrying the same mutations (Filomatori et al. 2006).

Cis-Acting Elements at the 3′UTRs

The 3′UTR sequences exhibit great variability among different flaviviruses; however, several conserved features and conserved secondary structures have been elucidated (Wallner et al. 1995; Proutski et al. 1997; Rauscher et al. 1997; Shurtleff et al. 2001; Thurner et al. 2004; Gritsun and Gould 2007). The 3′UTRs are between 350 and 700 nucleotides long and can be divided into three domains based on sequence/structure conservation (Fig. 3.2a). Domain I is the region immediately following the stop codon that is hypervariable and contains deletions and insertions in most flaviviruses. YF contains unique tandem repeats within domain I, known as RYF (Bryant et al. 2005) (Fig. 3.2b). Domain II is a region of moderate conservation comprising several hairpin motifs, including a characteristic dumbell (DB) structure with a conserved sequence named CS2 motif, present in all mosquito-borne flaviviruses (Olsthoorn and Bol 2001; Gritsun and Gould 2006; Romero et al. 2006). This DB structure is repeated in tandem in members of the DEN and JE subgroups, containing a repeated conserved sequence (RCS2) motif (Fig. 3.2b). Domain III is the most conserved region of flavivirus 3′UTRs, bearing a terminal stable stem-loop structure (3′SL). The presence of the 3′SL has been supported by secondary structure predictions, co-variation analysis, and biochemical probing. An essential role of the 3′SL in flavivirus replication has been extensively documented (for review see Markoff 2003). Upstream of the 3′SL there is a highly conserved sequence named CS1 motif (Hahn et al. 1987).

Work from many laboratories allowed to define several essential elements within domain III of the 3′UTR: (i) a pentanucleotide sequence CAGAC mostly present in a loop of the 3′SL (Wengler and Castle 1986; Khromykh et al. 2003; Tilgner and

Shi 2004; Elghonemy et al. 2005); (ii) a region within CS1 that contains a complementary sequence to a region present within the coding sequence of the capsid protein (Hahn et al. 1987; Men et al. 1996; Corver et al. 2003); (iii) the 3′ terminal nucleotides of the 3′SL, including the last CU_{OH} conserved in all mosquito and tick-borne flaviviruses (Khromykh et al. 2003; Tilgner and Shi 2004); (iv) a region within the stem of the 3′SL that contains a complementary sequence to a region present upstream of the translation initiator AUG at the viral 5′UTR (named UAR in DENV and CSA in TBEV) (Alvarez et al. 2005b; Kofler et al. 2006); and (v) specific bulges within the 3′SL structure (Yu and Markoff 2005). Deletions or mutations within any of these *cis*-acting RNA elements abolish viral replication. In contrast, RNA structures within domains I and II are considered dispensable for flavivirus replication. However, these structures are believed to serve as replication enhancers because deletion mutants within domains I and II exhibit decreased viral RNA synthesis and attenuation (Men et al. 1996; Mandl et al. 1998; Bredenbeek et al. 2003; Lo et al. 2003; Alvarez et al. 2005a).

Inverted Complementary Sequences in Flavivirus RNAs

Flavivirus genomes possess inverted complementary sequences at the ends of the RNA (Fig. 3.4), similar to those observed in the negative-strand RNA bunya-, arena-, and orthomyxoviruses. These inverted complementary sequences have been suggested to allow the ends of the genome to associate through base pairing, leading to circular conformations of the RNA (panhandle-like structures).

Two pairs of inverted complementary sequences can be found at the ends of mosquito- and tick-borne flavivirus genomes (Fig. 3.4). In all mosquito-borne flaviviruses (MBF), there is a region within CS1 that complements perfectly with a sequence located in the coding region of the C protein. This pair of complementary sequences is known as cyclization sequence 5′–3′CS (Hahn et al. 1987) (Fig. 3.4). The second pair of inverted complementary sequences was first noticed using folding prediction algorithms on flavivirus RNAs (Hahn et al. 1987; Khromykh et al. 2001a; Thurner et al. 2004). A sequence located just upstream of the translation initiator AUG at the 5′UTR was found to be complementary to a region present within the stem of the 3′SL. This pair of complementary sequences is known as cyclization sequence 5′–3′UAR (the name stands for *u*pstream *A*UG *r*egion) (Alvarez et al. 2005b) (Fig. 3.4). Alignment of MBF sequences indicates high conservation of 5′–3′CS, whereas less sequence conservation was observed within 5′–3′UAR (Fig. 3.4). Two pairs of complementary sequences, CSA and CSB, were proposed as possible cyclization elements in the case of tick-borne flaviviruses (TBF) (Mandl et al. 1993; Khromykh et al. 2001a). The 5′CSA is located upstream of the initiator AUG and is complementary to the 3′CSA located within the stem of the 3′SL, which is reminiscent of the location of the MBF 5′–3′UAR. The 5′–3′CSB sequences are found in similar locations as the MBF 5′–3′CS.

Requirement of 5′–3′CS base pairing was first analyzed in RdRp reactions using DENV-infected cell extracts and exogenous RNA templates including the 5′- and

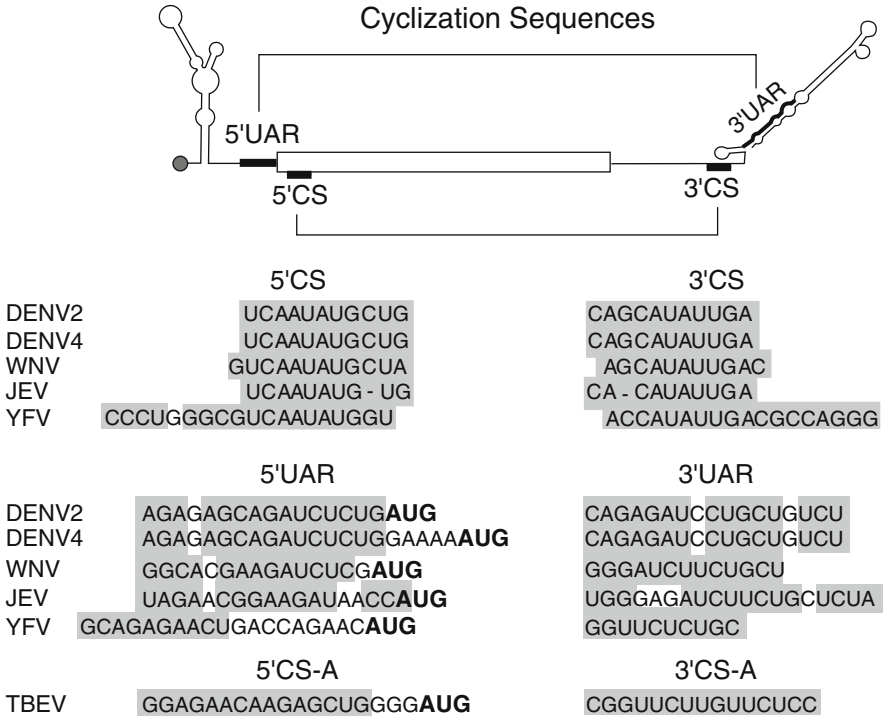


Fig. 3.4 Sequence and location of flavivirus cyclization sequences. On the *top*, a schematic representation shows the location of 5′–3′UAR and 5′–3′CS regions of mosquito-borne flaviviruses. The *bottom* panels show the nucleotide sequences of the complementary regions 5′–3′CS and 5′–3′UAR of dengue virus type 2 and 4 (DENV2, Genebank number U87412 and DENV4 Genebank number M14931), West Nile virus (WNV, Genebank number M12294), Japanese encephalitis virus (JEV, Genebank number NC001437), and yellow fever virus (YFV, Genebank number NC002031); and the 5′–3′CSA of tick-borne encephalitis virus (TBEV, Genebank number U27495). The *gray boxes* denote the inverted complementary sequences. In *boldface* the translation initiator AUG is indicated.

3′-end viral sequences. In this study, it was shown that 5′–3′CS complementarity was necessary for polymerase activity (You and Padmanabhan 1999). Subsequent studies specifically addressed the requirement of sequence complementarity in vivo using a Kunjin virus replicon system (Khromykh et al. 2001a). In agreement with the in vitro study, specific mutations in the 5′CS or 3′CS abolished RNA amplification while reconstitution of potential base pairings with foreign sequences restored replicon replication. More recent studies confirmed the requirement of CS complementarity for RNA amplification of DEN and WN viruses (Lo et al. 2003; Alvarez et al. 2005a).

The specific role of 5′–3′UAR complementarity was addressed using infectious DENV2 and DENV replicons. These studies indicated that mismatches within 5′–3′UAR did not alter translation of the viral RNA but greatly decreased RNA

synthesis, leading in some cases to undetectable levels of viral replication. Compensatory mutations that restored 5′–3′UAR base pairing rescued RNA synthesis. In addition, the mutants with the compensatory changes within UAR were shown to replicate less efficiently than the parental virus, suggesting that 5′ and 3′UAR sequences could play additional roles during viral replication (Alvarez et al. 2005b; Alvarez et al. 2006).

The requirement of the putative cyclization sequences CSA and CSB in TBEV was recently investigated using a replicon system (Kofler et al. 2006). This work provided clear evidence that complementarity of 5′–3′CSA, which is analogous in location to the 5′–3′UAR cyclization elements in DENV, was essential for TBEV replication. Interestingly, no crucial function was connected with the CSB elements, suggesting that only one pair of the two putative complementary sequences would be required to mediate 5′–3′ interactions in TBF genomes. In summary, there is compelling evidence indicating that sequence complementarity between the ends of flavivirus genomes is essential for viral RNA synthesis.

Cyclization of the Viral Genome

Direct interaction between two RNA molecules carrying the 5′ and 3′ terminal sequences of a flavivirus genome was first observed using psoralen/UV cross-linking, and a role of 5′–3′CS complementarity for the interaction was proposed (You et al. 2001). More recently, an electrophoretic mobility shift assay was employed to study the formation of RNA–RNA complexes with molecules carrying the terminal DENV sequences (Alvarez et al. 2005b). In this work, the specific contribution of both cyclization elements, 5′–3′CS and 5′–3′UAR, was demonstrated by mutagenesis analysis. Single mismatches within the complementary sequences were shown to increase the apparent dissociation constants of specific RNA–RNA complexes.

The first direct evidence of long-range RNA–RNA interactions between the ends of a flavivirus RNA was obtained by visualization of individual molecules using atomic force microscopy (AFM) (Alvarez et al. 2005b). Because single-stranded RNA molecules acquire compact tertiary structures that preclude visualization of intramolecular contacts, the RNAs used in that analysis were hybridized with antisense RNA molecules to generate elongated double-stranded segments (Fig. 3.5). This strategy allowed visualization of long-range RNA–RNA contacts at single-stranded regions. Using AFM in air, a model RNA molecule of 2 kb carrying the 5′ and 3′ terminal sequences of DENV2 as well as single molecules of the full-length genomic RNA were visualized in linear and circular conformations, whereas control molecules with deletions of 3′CS and 3′UAR were only observed in linear forms. Cyclization of DENV RNA was observed in the absence of proteins (Fig. 3.5). However, it is possible that binding of cellular or viral proteins to the ends of the RNA enhances or disrupts genome cyclization.

Interaction of the viral protein NS5 with capped- and uncapped-RNA molecules corresponding to 5′-end ∼200 nucleotides of WNV and DENV genomes was

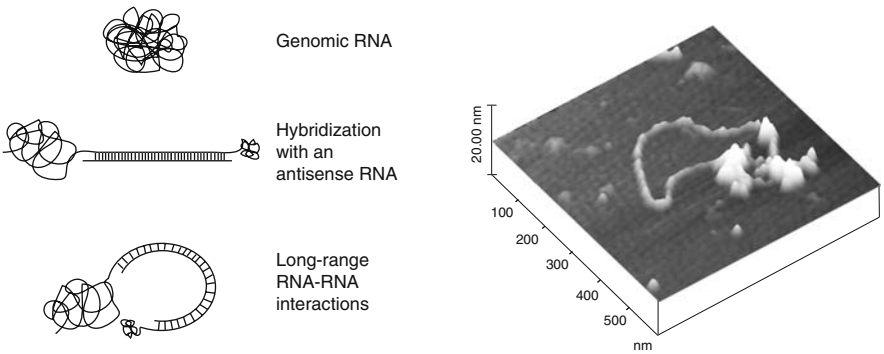


Fig. 3.5 Cyclization of DENV genomic RNA. On the *left*, a schematic representation of the viral genome in a compact conformation and the strategy used to visualize RNA–RNA contacts hybridizing the central part of the molecule with an antisense RNA molecule. On the *right*, a representative image of a single molecule of DENV genomic RNA obtained by tapping mode atomic force microscopy in circular conformation is shown. The 10.7 kb RNA molecule was hybridized with an antisense RNA of 3.3 kb resulting in a linear double-stranded region with single-stranded overhangs of 6970 and 451 nucleotides at the 5′- and 3′-ends, respectively (Alvarez et al. 2005b).

recently demonstrated (Filomatori et al. 2006; Dong et al. 2007). Deletion analysis of 5′-end sequences of DENV RNA indicated that the SLA structure was essential while 5′CS and 5′UAR sequences were dispensable for NS5 binding. Interestingly, interaction of DENV NS5 with an RNA–RNA complex formed between the 5′- and 3′- ends of the viral genome was observed, suggesting that the viral polymerase recognizes SLA even in the context of interacting 5′- and 3′-end viral sequences. Moreover, binding of NS2A, NS3, and NS5 to the 3′UTR of different flaviviruses has been also reported (Chen et al. 1997; Mackenzie et al. 1998). Whether binding of these viral proteins or other *trans*-acting factors modulates long-range RNA–RNA interactions in the context of the viral genome remains to be defined.

Proteins Interacting with the Viral RNA

Several reports have shown that defined RNA elements present at the 3′UTRs of flavivirus genomes differentially enhanced viral replication in distinct host cells. For instance, specific nucleotides of the bottom long stem of WNV and DENV 3′SL greatly enhanced replication competence in mosquito cells but had no effect on replication in mammalian cells (Zeng et al. 1998; Yu and Markoff 2005). In addition, it was found that deletion of the variable region encompassing domain I of DENV 3′UTR, reduced viral replication in mammalian cells without altering replication in mosquito cells (Alvarez et al. 2005a). These and other observations suggest that host cell specific factors bind the viral 3′UTRs. Significant effort has been made to identify host proteins that interact with the viral RNA. The eukaryotic elongation factor 1 alpha (EF-1 α) was identified to bind the 3′SL of WNV and

DENV (Blackwell and Brinton 1997; De Nova-Ocampo et al. 2002). Binding of EF-1 α to the viral RNA was mapped by footprinting analysis. This study defined a main binding site in the middle of the 3′SL (Blackwell and Brinton 1997). In addition, the human La autoantigen and the human PTB were found to interact with the 3′UTR of genomic DENV4 (De Nova-Ocampo et al. 2002; Garcia-Montalvo et al. 2004). In the case of JEV, a 36 kDa protein MOV34 was found to bind the 3′SL (Ta and Vrati 2000).

Search for proteins that bind the negative-strand RNA of flaviviruses has also been pursued. Four host proteins that bind specifically to the 3′ terminal sequences of the negative strand of WNV RNA were detected using BHK cell extracts (Shi et al. 1996). Purification of one of these proteins revealed to be TIAR, an RNA-binding protein containing three RNA recognition motifs (Li et al. 2002). Furthermore, a related protein TIA-1 was also shown to bind the same RNA. Interestingly, the growth of WNV was inhibited in a TIAR knockout cell line, indicating the functional importance of this protein. In addition, the human La autoantigen, calreticulin, and the protein disulfide isomerase were shown to interact in vitro with the 3′-end of the negative strand of DENV4 (Yocupicio-Monroy et al. 2003). Although several host proteins have been identified that bind the viral RNA and a functional role was proposed for some of them, the participation of these proteins during flavivirus RNA replication remains to be defined.

A Model for Minus-Strand RNA Synthesis

Filomatori and co-workers demonstrated that RNA molecules of ∼160 nucleotides carrying the DENV SLA structure were efficient templates for in vitro RNA polymerase activity, whereas longer RNA molecules of ∼2000 nucleotides, carrying the SLA in the same location were inefficient templates (Filomatori et al. 2006). In contrast, when the two pairs of cyclization sequences (5′–3′CS and 5′–3′UAR) were introduced at the ends of the RNA, polymerase activity became independent on the length of the template. Thus, it was hypothesized that the distance between the promoter SLA and the 3′-end of the template was critical for in vitro polymerase activity and that long-range RNA–RNA interactions would bring the 3′-end of the molecule near the SLA. This idea was consistent with the previous work of Padmanabhan and collaborators (Ackermann and Padmanabhan 2001; You et al. 2001).

A model for DENV minus-strand RNA synthesis was proposed (Fig. 3.6). In this model, the viral NS5 protein binds the promoter SLA at the 5′-end of the RNA, ∼11 kb away from the initiation site. Cyclization of the viral genome through long-range RNA–RNA interactions could place the 3′-end of the RNA near the polymerase-SLA complex, allowing initiation of RNA synthesis. Therefore, only molecules in circular conformation would be competent templates for minus-strand RNA synthesis. In addition, it is possible that interaction of the polymerase with specific nucleotides within the SLA could induce conformational changes in the protein, facilitating the recognition of the 3′-end of the template. According to the

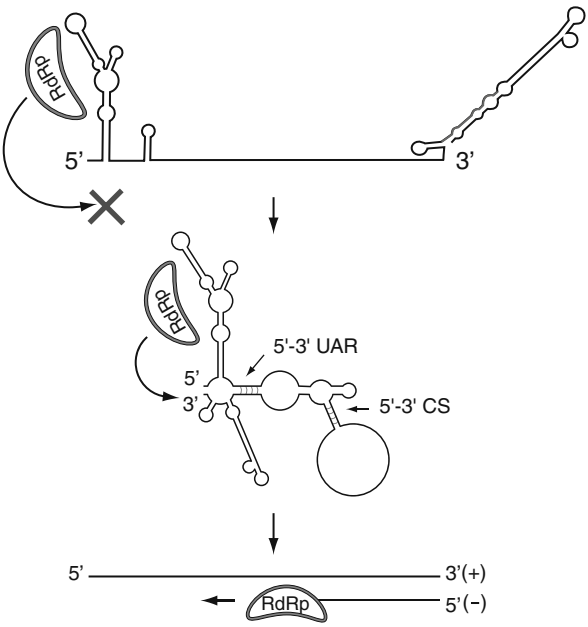


Fig. 3.6 Model for DENV minus-strand RNA synthesis. The viral RNA-dependent RNA polymerase (RdRp) binds the promoter SLA at the 5′-end of the RNA. The genome in a linear conformation does not permit the RdRp to initiate minus-strand RNA synthesis, however, cyclization of the RNA through 5′–3′CS and 5′–3′UAR contacts allows the RdRp to reach the 3′ initiation site.

crystal structure of DENV RdRp the template channel has dimensions that would only permit access to a ssRNA chain (Yap et al. 2007). Therefore, it is likely that the 3′SL structure unwinds before entering the template channel of the enzyme during initiation of minus-strand RNA synthesis. This process could be aided by the helicase activity of NS3 or by cellular *trans*-acting factors interacting with the viral 3′SL. Alternatively, base pairings between 5′ and 3′UAR of MBF or between 5′ and 3′CSA of TBF genomes, which were predicted to open the bottom half stem of the 3′SL, could release the 3′-end nucleotides, rendering the structural changes around the 3′SL presumably necessary for the initiation process.

In summary, the model proposes a core promoter at the 5′-end of the genome and long-range RNA–RNA interactions as essential elements for initiation of RNA synthesis. Although the 3′SL structure has been shown to be essential for flavivirus RNA synthesis, the molecular details by which this element participates during the process remain unclear. While it is not surprising to find a core promoter for RNA synthesis at the 3′-end of a viral genome, it is intriguing why certain plus-strand RNA viruses would have promoters or enhancer elements for RNA replication at the 5′-end of the RNA. In this case, the requirement of genome cyclization may provide advantages for viral replication such as control mechanisms to amplify only full-length templates or coordination of translation, RNA synthesis, and RNA packaging by overlapping signals involved in these processes. Further analysis of the RNA conformations required in each viral process will help to clarify the molecular details by which flaviviruses replicate their genomes.

Perspectives

Much has been learned in the last years about flavivirus RNA replication. A model for minus-strand RNA synthesis has been proposed that explains the need of genome cyclization, and roles of viral proteins and RNA *cis*-acting elements have been uncovered. An important aspect in flavivirus RNA replication that remains undefined is the mechanism of positive-strand RNA amplification. It has been proposed that the RF form is the template for genome amplification. We can speculate that the SLA could also serve as the promoter for positive-strand RNA synthesis. In this case, the SLA would have to work in *trans*, transferring the polymerase to the 3′-end of the negative strand, to initiate positive-strand RNA synthesis. A similar strategy has been previously proposed for the cloverleaf structure present at the 5′-end of poliovirus RNA (Andino et al. 1990). For DENV, a *trans*-initiation activity of SLA in vitro has been observed (Ackermann and Padmanabhan 2001; You et al. 2001; Filomatori et al. 2006), but not experimental evidence in vivo has yet been provided. Otherwise, the negative-strand RNA could carry its own promoter element either at the 5′- or the 3′-end of the molecule to facilitate positive-strand RNA amplification. In vivo experiments that allow discrimination between negative- and positive-strand RNA synthesis will be necessary to identify *cis*- and *trans*-acting factors specifically involved in each of these processes.

Formation of the cap at the 5′-end of the viral RNA requires the MTase activity of NS5. Because the cap structure precedes the SLA, it is possible that binding of NS5 to the promoter element is also involved in cap methylation. Consistent with this idea, recent studies using a recombinant WNV MTase have reported a requirement of SLA sequences for in vitro cap RNA methylation. The challenge will be to define how both enzymatic activities of NS5 are coordinated during flavivirus RNA synthesis.

Another intriguing question is: How is the vRNA released from the VP to the cytosol for translation and RNA packaging? The close association between the VP and the CM/PC reveals a level of organization that might allow the vRNA to be transported to the sites of protein synthesis or RNA packaging. Additional studies are needed to understand how the viral RNA is recruited to specific places of the infected cell for each step of the viral life cycle.

Finally, different RNA viruses use long-range RNA–RNA interactions as a strategy to allow *cis*-acting regulatory elements such as enhancers, promoters, and silencers to act from long distances (Pogany et al. 2003; Ray and White 2003). In addition, the dynamics of RNA tertiary structures allow modulation of specific functions of RNA–RNA contacts by *trans*-acting factors. In the future, we will have to uncover the functional significance and the underlying connections of these common viral strategies.

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