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Dynamic RNA structures in the dengue virus genome

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Dengue virus is an important human pathogen that belongs to the Flaviviridae family. The viral genome is a single molecule of RNA of positive polarity that plays multiple roles during the viral life cycle. Besides encoding the viral proteins, the genome contains RNA structures that regulate different viral processes. An important feature of dengue and other flavivirus genomes is the presence of inverted complementary sequences at the ends of the molecule that mediate long-range RNA-RNA interaction and genome cyclization. Recent studies have demonstrated that alternative conformations of the genome are necessary for infectivity. In this review, we discuss the current understanding of the function of different RNA elements that modulate dengue virus replication and provide new ideas of how dynamic RNA structures participate in the viral processes.

Flexibility of Viral RNA Genomes

The genome of plus strand RNA viruses contains a wide variety of RNA signals with information to regulate fundamental processes of viral life cycles. Upon infection, the incoming genome serves as mRNA for translation, template for RNA synthesis and substrate for encapsidation. These processes must be temporally regulated to ensure efficient utilization of the genome and viral spread. RNA structures function as promoters, enhancers and repressors of translation, transcription, replication and encapsidation.[1-15] In addition, viral RNAs participate in triggering or avoiding the antiviral host response.[16,17]

Viral RNA genomes are dynamic molecules. Their secondary and tertiary structures change throughout the viral life cycle responding to alteration of the environment in the infected cell. RNA plasticity is governed by nucleotide sequences, local and long-range RNA-RNA interactions and association with viral and host proteins or ligands. Locations of enhancers, promoters or modulators of viral processes have been found in coding and uncoding regions of viral genomes.[1,18-29] Although translation initiation takes place at the 5' end and RNA replication initiates at the 3' end of the genome, cis-acting elements required for translation initiation can be found at the 3' end of the viral RNA, while elements necessary for initiation of RNA synthesis can be located at the 5' end of the viral RNA.[13,27,30-34] These observations highlight the crucial role of complex tertiary interactions that span thousands of nucleotides in viral genomes (reviewed in ref. 35–37).

It has been reported that viral RNA structures can overlap multiple functions. In addition, defined nucleotide sequences can fold into alternative RNA structures with distinct functions during viral infection.[15,38-44] The organization of regulatory RNA signals and the overlapping information in viral RNAs are possible strategies to control the utilization of the genomes for multiple functions. How, why, and when viral RNAs undergo conformational changes and how these transitions are regulated during the viral life cycles are still largely unknown.

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The Dengue Virus Life Cycle: An Overview

Dengue virus (DENV) is the single most significant arthropod-borne viral pathogen in humans. The geographical spread and incidence of DENV has increased dramatically in recent years, with an estimated 50–100 million infections annually. The lack of vaccines and specific antivirals leaves 2 billon people, mainly in poor countries, at risk and in a constant state of alarm (World Health Organization 2010).

DENV is a member of the Flavivirus genus of the Flaviviridae family.[45] The Flavivirus genus includes other important human pathogens such as yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and tick borne encephalitis virus (TBEV).[45] Flaviviruses are enveloped viruses with a single stranded, ~11 kb, positive-sense RNA genome. The genome encodes a single long open reading frame (ORF), flanked by highly structured 5' and 3' untranslated regions (UTRs).

The virus enters the host cell by receptor mediated endocytosis. Upon internalization and acidification of the endosome, fusion of viral and vesicular membranes allows release of the genomic RNA into the cytoplasm, which serves as mRNA. Translation of the single ORF at the rough ER produces a large polyprotein that is cleaved co- and posttranslationally into the mature proteins. The N-terminal of the polyprotein encodes the three structural proteins (C-prM-E), followed by seven non-structural (NS) proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) (**Fig. 1**).[46]

After translation of the RNA, virus-induced hypertrophy of intracellular membranes occurs, creating structures known as

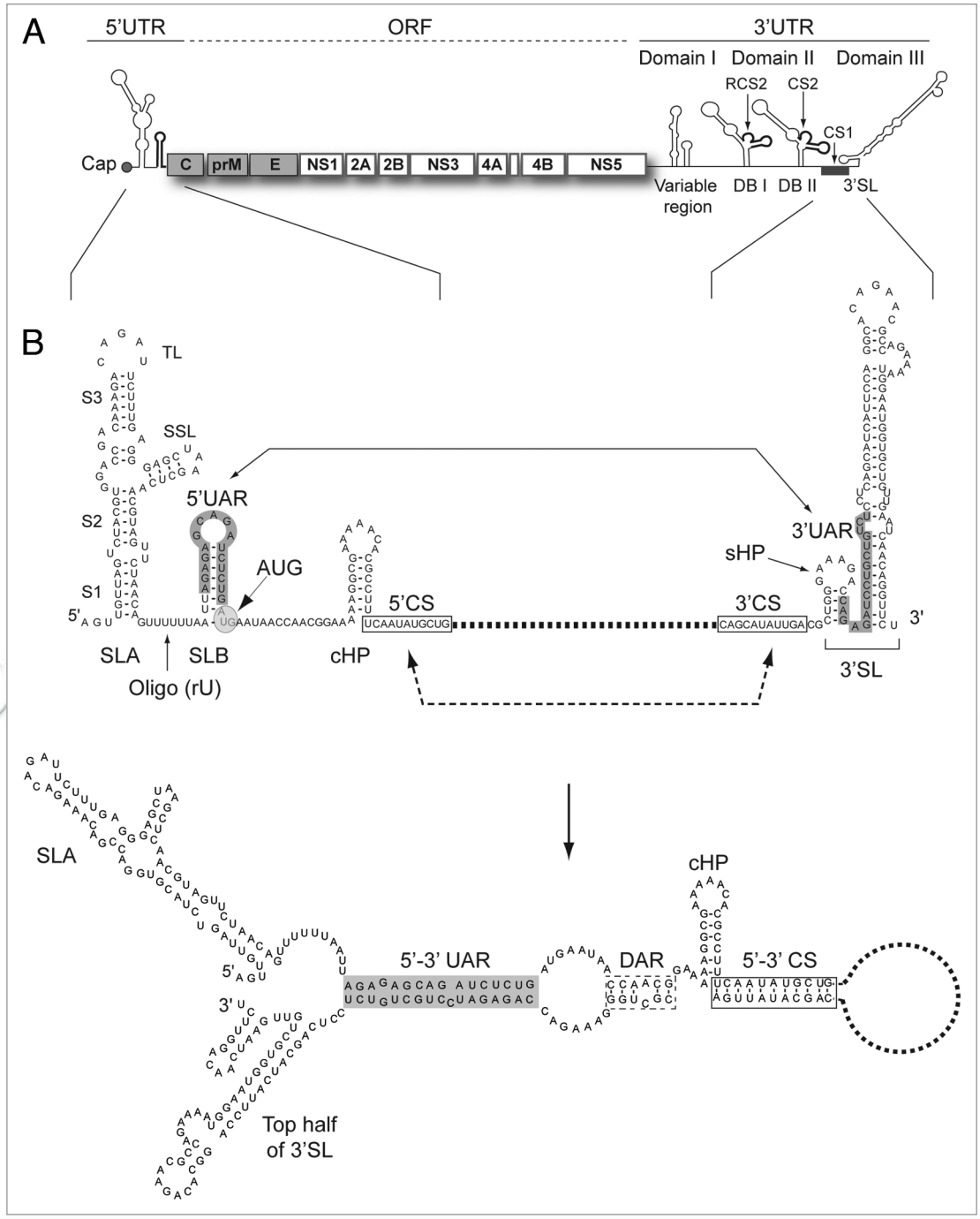

Figure 1. Schematic representation of the DENV genome. (A) Domains of the 5' and 3'UTRs, and the open reading frame indicating structural (C-prM-E) and non-structural (NS1-NS2AB-NS3-NS4AB-NS5) proteins. The predicted secondary structures of the three defined domains at the viral 3'UTR are indicated: domain I (variable region, VR), domain II (dumbbell structures, DB) and domain III (conserved sequence CS1 and 3'SL). In addition, the location of the conserved elements corresponding to RCS2 and CS2 are shown. (B) Sequence and predicted secondary structure of 5' and 3' terminal sequences of DENV are shown. On the top, the structural elements located at the 5' end: stem loop A (SLA), stem loop B (SLB), the oligo (rU) element and capsid region hairpin (cHP) are shown linked by a dash line, representing the viral genome, to the 3' stem loop (3'SL) structure. The sequence corresponding to the 5'-3' complementary regions are indicated: 5'UAR and 3'UAR are marked in grey and 5'CS and 3'CS are indicated with boxes. The bottom structure shows the cyclization of the genome mediated by hybridization of 5'-3'UAR, 5'-3'CS and adjacent DAR sequences.

convoluted membranes and vesicle packets.[47-49] Flavivirus RNA synthesis occurs in close association with cellular membranes inside the vesicle packets in so called viral replication complexes. The process begins with the synthesis of a negative strand RNA, which serves as template for the amplification of additional positive strand genomic RNA. The enzymatic reaction is catalized by the RNA-dependent RNA polymerase (RdRp) activity of the viral NS5 protein, in association with the viral protease/helicase

NS3, other viral NS proteins and presumably host factors. The newly synthesized RNA associates with the capsid (C) protein by a still unknown mechanism. The RNA-C complex buds into the ER lumen acquiring the lipid bilayer and the viral E and prM proteins. Furin-mediated proteolysis of prM in the trans-Golgi network[50] triggers rearrangement, homodimerization of E and formation of new viral particles.[51]

Properties of the dengue virus RNA. Viral genomes of the four DENV serotypes have a type 1 cap (m7GpppAmp) structure at the 5' end and lack a poly(A) tail at the 3' end. The single ORF is flanked by UTRs that contain essential elements that modulate the function of the viral RNA. DENV 5'UTRs are between 95 to 101 nucleotides long. They contain two RNA domains with distinct functions during viral RNA synthesis. The first domain of ~70 nucleotides is predicted to fold into a large stem-loop (SLA, **Fig. 1B**). A similar structure is present at the 5'UTR of other members of the Flavivirus genus.[52-55] DENV SLA has been proposed to act as the promoter for the viral RdRp (NS5). Direct binding of NS5 to SLA was shown to be necessary for viral RNA synthesis.[13,56] The second domain of the DENV 5'UTR is predicted to form a short stem loop (SLB), which contains essential sequences for long-range RNA-RNA interaction and genome replication.[23] The two domains are separated by an oligo(U) sequence, which functions as spacer for proper function of the two stem loops.[14] The SLA is predicted to have a Y shaped structure, which was recently confirmed by enzymatic and chemical probing.[14,57] These studies indicate the presence of three helical regions (S1, S2 and S3) interrupted by bulges and highly reactive single stranded regions in agreement with the presence of a side stem loop (SSL) and a top loop (TL).[14] The conserved structural elements described within the SLA of DENV are also found at the 5' end of other members of the flavivirus genus (reviewed in ref. 58). In an initial study by Brinton and Dispoto, the 5'UTR sequences of different mosquito-borne flaviviruses were compared.[54] This study indicated that conserved secondary structures were present at the 5' end of West Nile virus (WNV), Saint Louis encephalitis virus (SLEV), DENV, yellow fever virus (YFV) and Murray Valley encephalitis virus (MVEV).[54] More recently, the predicted structures at the 5' end of the genomes of tick-borne flaviviruses and flaviviruses with no known vector were found to be similar to that observed in the mosquito-borne flavivirus.[52,55,59,60] Within the coding sequence, just downstream of the AUG translation initiation codon, a stable haipin (cHP, **Fig. 1**) was found in the DENV genome to be required for viral RNA replication.[61]

Specific structures at the 3' end of the viral genome also play crucial roles in viral RNA synthesis. The approximately 450 nucleotide long DENV 3'UTR can be divided into three domains (**Fig. 1A**). Domain I is located immediately after the stop codon,[4] and is considered the most variable region within the viral 3'UTR (VR). It exhibits extensive size variation between serotypes; it can be from more than 120 nucleotides to less than 50 nucleotides.[62-67] Domain II is of moderate conservation, comprising several hairpin motifs, including a characteristic dumbbell (DB) structure, which is duplicated in tandem (**Fig. 1**).[62-64] The DB elements contain conserved sequences named CS2 and RCS2 (repeated CS2) present in all mosquito-borne flaviviruses.[68-71] In addition, sequences within the DB elements were proposed to be involved in pseudoknot structures.[72] Although RNA elements within domains I and II are considered dispensable for flavivirus replication, these structures serve as replication enhancers.[4,73-76] Domain III is the most conserved region of the 3'UTR, bearing a conserved sequence CS1 followed by a terminal stem-loop structure (3'SL). CS1 contains a sequence involved in long-range RNA-RNA interactions between the ends of the viral genome.[68] The 3' terminal structure contains a short stem loop of 14 nucleotides (sHP) followed by a large stem loop of 79 nucleotides. The two adjacent structures involve 93 nucleotides and are referred to as 3'SL (**Fig. 1**). The presence and the essential role of the 3'SL has been supported by secondary structure predictions, covariation analysis, biochemical probing and functional studies in DENV and other members of the Flaviviridae family.[46,68,77-85]

A conserved feature of DENV and other flavivirus genomes is the presence of inverted complementary sequences at the ends of the RNA that mediate long-range RNA-RNA interactions.[23,52,53,68,86-88] The significance of genome cyclization during viral replication is now beginning to be uncovered. At least two pairs of complementary regions and adjacent nucleotides are necessary for DENV genome cyclization (**Fig. 1B**) (reviewed in ref. 37). These regions are known as 5'-3'CS and 5'-3'UAR, of 11 and 16 nucleotide long, respectively, and adjacent sequences named DAR.[89] The 5'CS is located inside the ORF, encoding the N-terminus of the capsid protein, and the 3'CS is located upstream of the highly conserved 3'SL (**Fig. 1B**). The 5'UAR is in the 5'UTR, just upstream of the translation initiator AUG, and the 3'UAR is located within the 3'SL overlapping the sHP and the bottom half of the large stem. Visualization of the DENV genome using atomic force microscopy (AFM) confirmed cyclization of individual molecules by long-range RNA-RNA interactions involving these sequences.[23]

Studies from many different laboratories using infectious clones and replicon systems provided compelling evidence for the essential role of genome cyclization during flavivirus replication.[23,76,86,90-93] Mismatches within complementary regions 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' base pairing rescued RNA synthesis indicating a role of RNA-RNA complementarity rather than the nucleotide sequence per se for viral replication.[23,91,94]

Alternative RNA Structures are Necessary for Dengue Virus Replication

The sHP structure formed within the 3'SL at the viral 3'UTR overlaps with the 3'UAR sequence (**Fig. 2**). Multiple sequence alignment with the RNAz software was used to analyze the formation of the sHP structure in different mosquito-borne flavivirus genomes. Although low nucleotide conservation was observed, the sHP structure was predicted with high probability as a functional RNA element. The consensus sHP structure showed co-variation in three position of the stem. In addition, in

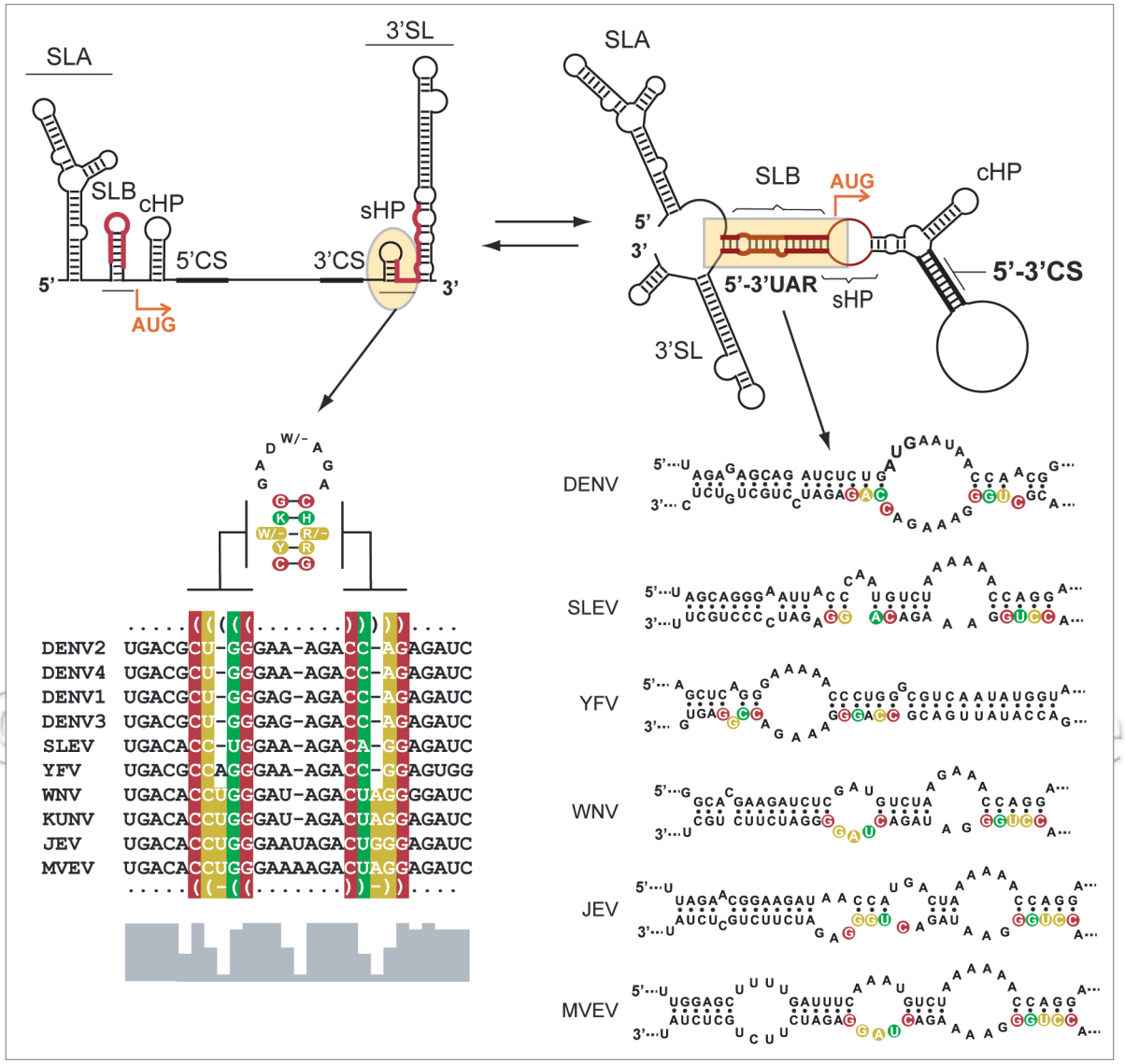

Figure 2. Schematic representation of linear and circular conformations of the DENV genome indicating the formation of mutually exclusive structures. The local sHP structure present in the linear conformatin is shown on the left and the extended duplex including the same nucleotides in the circular conformation is shown on the right. Underneath of the linear representation of the genome, multiple alignment of the sHP sequence of mosquito-borne flaviviruses is schematically shown. The nucleotides of the stem of the sHP are colored: the conserved positions are shown in red; yellow and green indicate the co-variation positions with two or three different base pairs, respectively. On the top of the alignment, the consensus sHP secondary structure is shown. Nucleotides are represented with IUPAC codes: W (A/U), K (G/U), H (not G), D (not C), R (A/G), Y (C/U) or—(deleted). The bottom histogram shows the nucleotide conservation of the indicated region. On the right, underneath of the circular representation of the genome, the predicted changes in the sHP structure of different flavivirus genomes upon 5'-3' end hybridization is shown. The nucleotides corresponding to the stems of the sHP are indicated with the same color code indicated on the left.

all sequences analyzed, the sHP region overlapped with nucleotides predicted to be involved in long-range RNA-RNA interactions (**Fig. 2**).

Recently, the functional role of the sHP structure during DENV replication was demonstrated using mutated infectious clones and reporter viruses.[15] RNA genomes carrying substitutions in the stem of the sHP including 1, 2 or 3 mismatches were impaired for replication. Translation of the input RNA was unaffected while genome amplification was greatly compromised. Selection of replicating viruses in cell culture showed spontaneous revertions within the sHP restoring the structure.[15] Mutations in the loop of the sHP were better tolerated and viral stocks, retaining the original mutations, were obtained. In addition, viruses carrying nucleotide substitutions in the stem of the sHP that retain the structure were viable. These studies indicated an absolute requirement of the sHP structure for viral infectivity. Because the two mutually exclusive structures, the extended duplex and the sHP, are essential for viral RNA replication, both conformations of the genome should exist in the infected cell. Therefore, it has been proposed that transitions between circular and linear forms of the RNA must occur during DENV RNA synthesis.

A Balance between Different Forms of the Dengue Virus Genome is Necessary for Infectivity

Different lines of evidence indicate that the DENV genome exist in at least two alternative conformation in the infected cell.[13,15,23,91] However, little is known about mechanisms modulating the switch from one to the other conformation. It is likely that host or viral proteins that interact with the RNA could participate in this process. Several host proteins have been reported to bind the viral RNA,[95-103] however, the role of these proteins in viral replication remains elusive. Useful information was obtained using infectious DENV clones to test the effect of mutations that displace the equilibrium towards the circular or the lineal conformation of the RNA on viral replication.[15] In this study, substitutions within UAR to increase the stability of the long-range RNA-RNA interaction were analyzed. The mutant tested contained 4 additional GC base pairs (Mutant Cyc$^+$). A second mutant was designed to stabilize the competing sHP structure. In this case, two additional GC base pairs were introduced in the stem of the sHP (Mutant sHP$^+$). When the full length viral RNAs were transfected into cells both mutations (Cyc$^+$ and sHP$^+$) impair viral replication. Using reporter DENV carrying a luciferase gene, it was shown that viral translation was efficient while RNA synthesis was almost undetectable. While the wild type virus takes between 2 to 3 days to infect a complete monolayer, the mutants took between 9 to 12 days. Analysis of the replicating viruses revealed a wide variety of spontaneous mutations at both ends of the genome. To obtain information of nucleotide changes in individual genomes, the ends of the isolated RNA were ligated, and both ends were sequenced in independent clones. In all the replicating genomes, the nucleotide changes tended to restore the wild type equilibrium between the two competing structures. Two types of spontaneous revertions were observed. In the first case, the stability of the structure altered by the mutation was restored. In the second type, spontaneous mutations were rescued by stabilizing the competing structure. For instance, transfection of the mutant sHP$^+$ yielded viruses with mutations that stabilized the 5'-3'UAR interaction. This observation highlighted the importance of the relative stability between the two competing conformations rather than the absolute stability of each structure. In a current model, it has been proposed that a balance between at least two conformations of the DENV genome is necessary for RNA replication and viral infectivity.[15]

Unlike well studied riboswitches in cellular RNAs, the importance of conformational changes in viral RNAs during infection is a new area of investigation. How and why the viral RNA switches convert from one to another structure is still unclear. Overlapping structures could have evolved in viral genomes to provide a way to control the switch between different RNA conformations. In this regard, mutant DENV in which the sHP was uncoupled from the 3'UAR sequence did not replicate, and revertant viruses were not rescued, suggesting a role for the overlapping nature of these RNA elements.

Promoter Signals for Dengue Virus RNA Synthesis and the Role of Genome Cyclization

How viral RdRps discriminate viral from cellular RNAs and how viral polymerases specifically initiate RNA synthesis at the 3' end of the genome are questions that remain largely unanswered. In the case of DENV, the SLA structure present at the 5' end of the genome specifically binds the viral polymerase NS5 and promotes RNA synthesis. RNA molecules carrying the SLA serve as templates for de novo RNA synthesis in vitro, while heteropolymeric viral or cellular RNAs, lacking the SLA, are not copied by the viral enzyme.[13]

Footprinting analysis, RNA binding assays and functional studies identified structural elements and sequences within the SLA that are essential for in vitro RNA synthesis and viral RNA replication in infected cells.[14,104] Substitution of nucleotides at the top loop of the promoter SLA impairs RNA synthesis in vitro and viral replication in cells. Evolution and selection of viruses in cell culture that partially restore the top loop sequence also reverted the viral phenotype, and the ability of the SLA to promote RNA synthesis in vitro.[13] In addition, it was found that, although efficient binding of the polymerase to the SLA is necessary for activity, binding was not sufficient to promote RNA synthesis.[104] In this regard, SLA mutants were found that uncoupled the RNA binding requirements from those needed for RNA synthesis. These observations support the idea that specific SLA-NS5 interactions are necessary for the enzyme to acquire a catalytically active state.

RNA molecules carrying the SLA structure fused to non-viral sequences were efficient templates for RNA synthesis provided that the length of the RNA was less than 500 nucleotides. In contrast, DENV minigenomes of 2,000 bases containing the viral inverted complementary sequences at the ends of the RNA, with the ability of 5'-3' hybridization, were active templates for polymerase activity.[13] This observation indicated that polymerase activity was independent of template length only when the RNA was able to circularize. Because the viral genome is about 11 kb, RNA cyclization would play a role placing the 3' end of the molecule near the 5' end promoter site. Previous studies also provided evidence of the requirement of 5'-3' RNA-RNA interactions for polymerase activity. Using DENV infected cell extracts with exogenous RNAs, Padmanabhan and collaborators showed that viral RNAs of 770 nucleotides were copied by the RdRp only in the presence of competent cyclization sequences.[87] The current model for DENV RNA synthesis proposes that the viral polymerase binds to the core promoter SLA at the 5' end of the genome. This interaction yields an active polymerase, which is repositioned near the 3' end initiation site by genome cyclization (**Fig. 3**).

Experimental evidence supports the idea that two conformations of the DENV genome are necessary during the process of viral RNA synthesis. The mechanism for minus strand RNA synthesis involves cyclization of the genome. However, it is still unknown the role of the lineal form of the RNA during this process. In addition, it is unclear which conformation of the RNA is required for plus strand RNA synthesis. It is possible that switching from circular to linear conformations inhibit minus

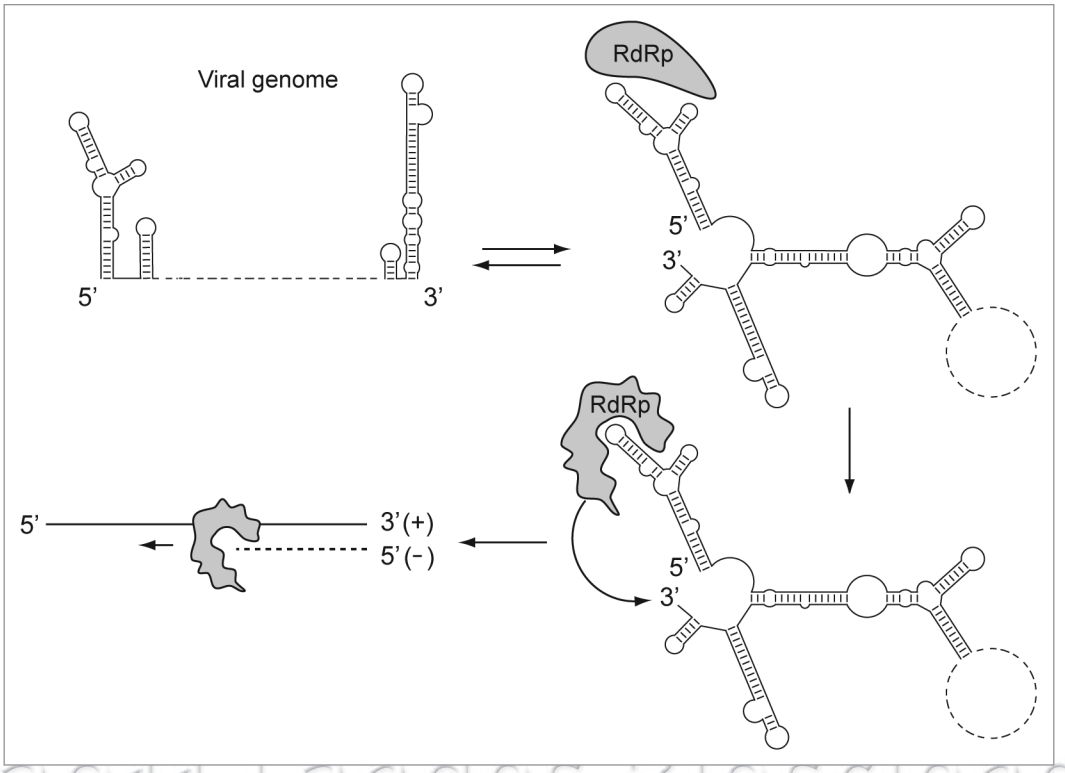

Figure 3. Model proposed for DENV minus strand RNA synthesis. The viral genome acquires two conformations and the balance between these two forms of the RNA is critical for RNA synthesis. The viral RNA-dependent RNA polymerase (RdRp) binds to the promoter SLA at the 5' end of the genome. Binding of the RdRp to the promoter element induces activation of the protein. The long range RNA-RNA interaction facilitates relocation of the RdRp at the 3' end of the genome and induces conformational changes within the 3'SL, which allows polymerase initiation of minus strand RNA synthesis.

strand RNA amplification, providing a mechanism to control the 100:1 ratio of plus versus minus strand RNA found in infected cells. The same genomic RNA must also function as template for translation and encapsidation. Interaction of the 5'UTR with translation initiation factors could modulate hybridization of the ends of the genome. Encapsidation is one of the most obscure steps of the DENV replication cycle.[105] It is still unclear how and where the capsid protein recruits the viral genome for assembly. It is possible that a certain conformation of the RNA is also necessary for viral capsid recognition; as it was previously demonstrated for retroviruses.[38,39]

Future studies should investigate the role of dynamic structures in the RNA during each step of viral replication and determine the participation of proteins in modulating these conformational transitions.

Dual Functions of 5'-3'UAR Hybridization in Dengue Virus Replication

The DENV polymerase is able to recognize and copy an RNA molecule that corresponds to the viral 5' terminal region but not the one corresponding to the viral 3'UTR.[13,87] However, when both molecules are present in the same reaction mix, they form an RNA-RNA complex in which the 5'RNA molecule provides the SLA promoter in trans to copy the molecule carrying the 3' UTR sequences.[13,14,87,91] Based on this observation, a trans-initiation assay that recapitulates RNA synthesis at the 3' end of the viral RNA was developed.[13,14,87,91,104] In this assay, polymerase activity depends on both the SLA structure and RNA-RNA interactions between two separate molecules resembling the ends of the genome. The two RNA molecules are copied, resulting in two different products. It has been shown that this trans-initiation activity not only depends on 5'-3' complementarity to bring the two molecules into proximity, but also requires the location of 3'UAR within the 3'SL.[104]

The nucleotide sequences of 5' and 3' UAR fold locally into conserved RNA structures, the SLB and the 3'SL, which are predicted to change upon 5'-3' hybridization (**Fig. 2**). The stem of SLB and the bottom half stem of the 3'SL open. As a consequence of this, the last nucleotides of the genome become less structured. Probing studies using West Nile virus RNA, which contains analogous sequences to those present in the DENV genome, confirmed these predicted conformational changes.[106] Additional evidence indicated that the viral polymerase is unable to use templates with structured 3' ends.[104] It has been shown that the presence of the 3'SL structure represses RNA synthesis when fused to the SLA promoter.[104] Information obtained from the 3D structure of the viral RdRp provides an explanation for this observation. It has been reported that the DENV protein has a narrow template channel, which would only accommodate a 3'

end of an RNA in a single stranded form.[107] Therefore, it is likely that the 3'SL unwinds before entering the template channel of the enzyme. Structural changes around the 3' terminal nucleotides were previously reported to be essential for other plus strand RNA viruses. In these cases, melting 3' end RNA structures was a prerequisite for polymerase initiation of RNA synthesis.[8,43,108-110]

Because UAR hybridization induces a conformational change at the 3' end of the genome, it has been proposed a dual function for the long-range RNA-RNA interaction during RNA synthesis: (i) bringing the promoter SLA near the 3' end initiation site and (ii) releasing the inhibitory effect of the 3'SL structure, facilitating access of the polymerase to the 3' end of the genome.

Perspectives

A great deal has been learned in the last few years about the role of RNA structures of the DENV genome that modulate viral replication. However, many questions still remain. How does the SLA interact with the polymerase to promote RNA synthesis on the authentic 3' end of the genome? How does the 3'SL change its conformation during the initiation process in the infected cell? Why does the virus need a balance between different conformations of the genome? What is the role of helicases and RNA chaperones in modulating the architecture of viral genomes? Another important question is whether the functional structures identified as regulators of RNA synthesis are necessary for both minus and plus strand RNA amplification. Tools to discriminate the requirements for these two processes are still lacking, which represent an important challenge to establish the similarities and differences between these two steps of DENV RNA replication. While it is not surprising to find a core promoter for RNA synthesis at the 3' end of a viral genome, it is still intriguing why certain RNA viruses would have promoters or enhancer elements for RNA replication at the 5' end of the RNA. 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 different processes. It is evident that this is the beginning of an exciting new area of investigation. Further analysis of functional dynamic RNA structures will help to clarify the molecular mechanisms by which RNA viruses regulate the multiple roles of their genomes during the viral life cycle.

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