Insights into dengue virus genome replication

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Review
Future Virology

Since many antiviral drugs are designed to interfere with viral genome replication, understanding this step in the viral replicative cycle has gained importance in recent years. Replication for many RNA viruses occurs in cellular compartments mainly originated from the production and reorganization of virus-induced membranes. Dengue virus translates, replicates and assembles new viral particles within virus-induced membranes from endoplasmic reticulum. In these compartments, all of the components required for replication are recruited, making the process efficient. In addition, membranes protect replication complexes from RNAases and proteases, and ultimately make them less visible to cellular defense sensors. Although several aspects in dengue virus replication are known, many others are yet to be understood. This article aims to summarize the advances in the understanding of dengue virus genome replication, highlighting the *cis* as well as *trans* elements that may have key roles in this process.

In the last few years, an important number of emerging and re-emerging diseases have been associated with members of the *Flavivirus* genus and *Flaviviridae* family. Some examples are West Nile virus (WNV) in North America and dengue virus (DENV) in tropical and subtropical areas of the world. DENV transmission has been vigorously emerging in a growing number of countries over the last two decades. Many factors have contributed to the spread of the mosquito vector and the disease, among others, via the urbanization process, which has left regions of the world without sufficient running water, septic tank systems or inefficient waste management [1,2]. It is estimated that dengue annually affects 100 million people, with 2500 million people living in areas at risk of disease transmission. Dengue infection can manifest in three clinical forms of increasing severity, classical dengue fever, dengue hemorrhagic fever and dengue shock syndrome [3]. The classic dengue fever is an acute, infectious, self-limited disease characterized by high-grade fever, headache, arthralgia and myalgia. Dengue hemorrhagic fever is distinguished from classical fever by plasma leakage and thrombocytopenia. In severe cases, circulatory failure, shock (dengue shock syndrome) and death can take place [4]. Hemorrhagic fever and dengue shock syndrome are the most serious clinical manifestations of viral infection and it has been suggested that antibody-dependent enhancement and immunopathological mechanisms are implicated in such complications [5–8]. Four different serotypes of DENV have been recognized (DENV1, DENV2, DENV3 and DENV4) and, within each serotype, various genotypes are recognized [9]. Morphologically, DENV is a spherical particle of approximately 50 nm in diameter, containing a nucleocapsid of 30 nm surrounded by a lipid envelope. Two structural proteins, the envelope (E) and membrane proteins (M), are inserted in the lipid membrane [10,11]. The glycoprotein E contains most of the antigenic determinants of the virus and is essential for viral attachment and entry [12–16], while protein M, synthesized as the precursor (prM), functions as a chaperone during maturation of the viral particle [10]. The nucleocapsid is composed of the capsid protein (C), a highly basic protein with affinity to RNA, associated to the genome [17]. The viral genome is a positive polarity ssRNA of approximately 11 kb. This RNA contains a type I cap structure (m7GpppAmpN2), located at its 5′-end, and lacks the poly(A) tail at its 3′-end. As with all positive-strand viruses, the genomic RNA is infectious [18]. The unique long open reading frame of DENV genome, flanked by two untranslated regions (UTRs), which contain structural and functional elements required for viral translation and replication [19], is translated into a polyprotein that is processed co- and post-translationally, by cellular and viral proteases, to produce ten mature viral proteins. The N-terminal region encodes the structural proteins C, prM

Keywords

■ dengue virus ■ endoplasmic reticulum ■ nonstructural proteins ■ replicative complex ■ RNA–protein interactions ■ RNA–RNA interactions ■ *trans*-acting factors ■ viral proteins ■ viral replicase ■ viral RNA

and E, followed by the nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [18,20]. The N-termini of prM, E, NS1 and NS4B are cleaved by the host signal peptidase located in the lumen of the endoplasmic reticulum (ER), whereas the processing of most of the other nonstructural proteins, as well as the C-terminus of the C protein, is carried out by the viral protease NS2B-3 in the cytoplasm of infected cells [21–23]. Cleavage of the C-terminus sequence of NS1 is carried out by an unknown protease resident of the ER [24], while the furin protease, located in the Golgi apparatus, mediates cleavage of prM to M during virion maturation [25,26]. Most of the nonstructural proteins are involved in flavivirus replication, which occurs in close association with internal cellular membranes [27,28].

The first step in DENV infection is binding to the cellular receptors on the surface of the target cell [29–34]. This interaction induces the virion internalization by receptor-mediated endocytosis and subsequent fusion of the virus with the endosomal membrane, releasing the viral genome in the cell cytoplasm [35–42]. Since the viral RNA can act as mRNA, the DENV genome is associated with the rough ER where it is translated. During viral translation several changes in the host cells occur. One such change appears to be common in RNA viruses and is the induction of membrane structures that provide a membrane-bounded microenvironment required for RNA synthesis and viral morphogenesis. Replication of many positive-strand RNA viruses is intimately linked to membrane structures that enfold around the active replication complexes (RCs) [43]. Viral replication occurs in two steps, first the positive-polarity RNA is copied to an RNA of negative polarity, which, in turn, serves as a template for the synthesis of multiple strands of RNAs of positive polarity. The positive-polarity RNA can then be used for translation, for further rounds of synthesis of RNA of negative polarity, or can become associated with structural proteins C, E and M to form the viral progeny [44,45]. Finally, immature virus particles travel in vesicles to the Golgi apparatus where they undergo glycosylations to eventually travel through secretory vesicles outside the cell. During the latter process, the furin cleaves prM in M to generate mature virions, which is the last step in viral morphogenesis [43,46].

One of the key steps in the viral replicative cycle is viral RNA replication. The replication process for the DENV genome has been widely studied, and while some important features have been determined, others are not yet fully understood. Three main elements are necessary for DENV replication: *cis*-acting elements, mainly located within or in close proximity to both 5′- and 3′-UTRs; *trans*-acting factors, both of cellular and viral origin; and viral-induced membranes, which wrap RCs and compartments for viral morphogenesis.

Cis-acting elements

Cis-acting elements are proposed to function as promoters for RNA replication. The regulatory sequences in the flavivirus genomic RNA have been extensively studied and have been located at both ends of the RNA, mainly at the UTRs.

Specifically, for DENV genomic RNA, the regulatory sequences are mainly formed by stem-loop and linear sequences, located at both ends of the molecule (Figure 1) [47–51]. In the 5′-UTR of the DENV genome six elements have been identified, termed stem-loop A (SLA), stem-loop B (SLB), 5′-upstream AUG region (5′-UAR), 5′-downstream AUG region (5′-DAR), 5′-cyclization sequence (5′-CS), and C-coding region hairpin (cHP). The last three elements are located within C protein-coding region [52–55].

The SLA domain is the stem loop structure located at the 5′-end of the genomic RNA (Figure 1), which is essential for viral replication. This structure has been predicted in other members of the flavivirus genus, suggesting that it is highly conserved. The Y-shaped structure predicted in the SLA domain was confirmed by enzymatic and chemical probing assays. The SLA structure has been divided into six regions: stem 1 (S1), UU bulge, stem 2 (S2), side-stem loop, stem 3 (S3) and top loop. Mutagenesis analysis of SLA structure, shows that the base pairing at the bottom of the SLA, which corresponds to S1 and S2, is necessary for viral replication regardless of the nucleotide sequence. Furthermore, while a single UU bulge is essential and sufficient for DENV replication, mutations that alter the sequence or disrupt the structure of the top loop region of the SLA impaired DENV replication. In addition, sequences downstream of the SLA modulate RNA synthesis and a minimum of ten residues between the SLA and the SLB domains are necessary for an efficient RNA synthesis. Finally, the sequence and structure adopted by the top loop region are essentials for viral replication. The use of different DENV replicon systems has demonstrated that SLA is involved in RNA replication and not in viral translation [53,56].

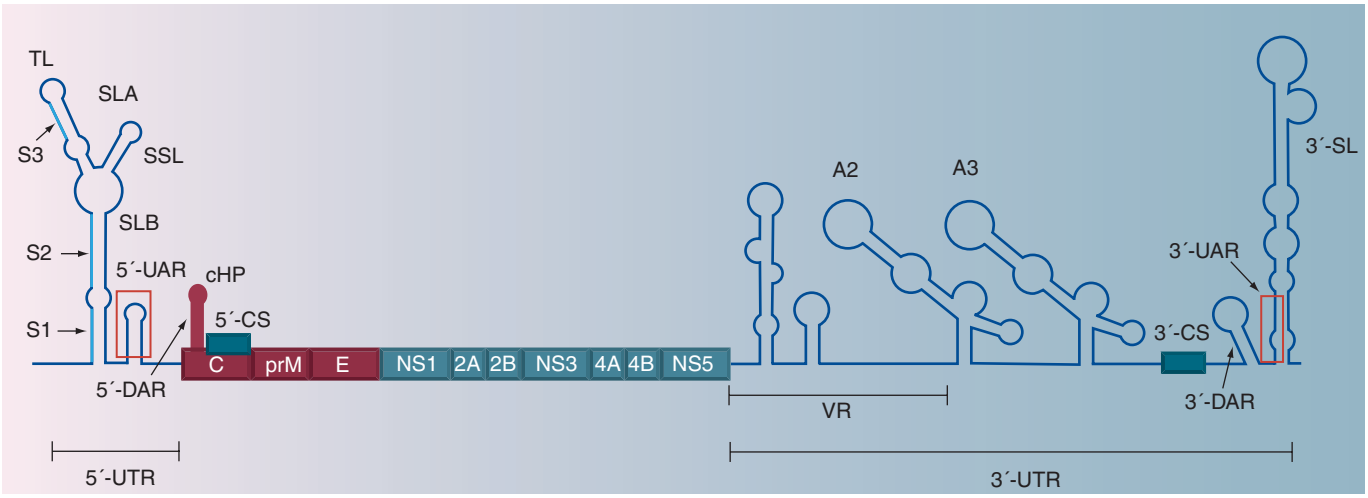

Figure 1. Dengue virus genomic RNA. *Cis*-acting elements playing essential roles in the viral replication process are emphasized. At the 5′-end is located the conserved structure known as SLA, essentially consisting of three stems, S1–3, the TL, as well as the SSL region. The next structure is SLB, which contains the 5′-UAR and, in the C-coding region, the 5′-DAR, the hairpin structure (cHP) and the 5′-CS. At the 3′-UTR the VR, the Y-shape structures, termed A2 and A3, contain conserved sequences RCS2 and CS2, respectively. The 3′-CS, the complementary sequence to the 5′-DAR, termed 3′-DAR, and the conserved 3′-SL containing the 3′-UAR are shown.
C: Capsid; CS: Cyclization sequence; DAR: Downstream AUG region; E: Envelope; prM: Precursor membrane; S: Stem; SL: Stem loop; SLA: Stem-loop A; SLB: Stem-loop B; SSL: Side-stem loop; TL: Top loop; UAR: Upstream AUG region; UTR: Untranslated region; VR: Variable region.

Another important function of the SLA sequence is the binding of NS5. The interaction between NS5 and SLA is necessary for *in vivo* and *in vitro* viral replication [19,53]. Of note is the fact that there are common structural elements at the 5′-stem loops (SLs) of the different flaviviruses [19,57–63], and they can be exchanged at least between WNV and DENV [19], suggesting that similar mechanisms for RNA synthesis are functioning in other members of the same genus.

The second element, present within the 5′-UTR, is the SLB domain (Figure 1). The presence of this element, which is also conserved among flaviviruses, contributes to viral replication; however, its structure is not essential. The importance of the SLB sequence is mainly owing to the presence of a sequence termed 5′-UAR, which is complementary to the 3′-UAR sequence present in the 3′-UTR (Figure 1). Both sequences are involved in genome cyclization [59,60,62].

Recently, an additional stretch of six nucleotides that may be involved in RNA replication and DENV circularization has been identified. This sequence, CCAACG, is located downstream of the AUG and designated 5′-DAR, is conserved in the four serotypes of DENV, and was predicted to be complementary with a sequence located in the 3′-end, termed 3′-DAR (Figure 1) [64]. DAR sequences are suggested to be important elements for the 5′–3′ genome interaction, indispensable for minus-strand RNA replication, and alternatively, DAR might play a role in the modulation of the replicative viral cycle as a potential protein binding site.

A conserved hairpin structure in mosquito- and tick-borne flavivirus is located 12–16 nt downstream the AUG start codon within the C-coding region, termed cHP (Figure 1). This structure is 14-nt long and has been proposed to have a dual function in the DENV replicative cycle. During translation, this element appears to be responsible for an efficient translation initiation from the C-start codon. Mosquito-borne flaviviruses have poor translation context sequences; thus, it has been proposed that the cHP element compensates for this deficiency, making the scanning translation machinery stall in the first AUG [52]. Furthermore, it has been demonstrated that this element is also important for RNA synthesis, but its exact function during replication remains to be determined [65]. These two distinct functions of the cHP are sequence independent, and structure and position dependent.

However, the main sequence involved in genome cyclization is a 10-nt sequence, highly conserved among flaviviruses, located within the C protein-coding region. This element, known as the CS, located at the 5′-end, together with its complementary sequence present at the 3′-UTR (3′-CS) (Figure 1), favored close RNA–RNA interaction between the 5′- and 3′-ends of the viral genome [66,67]. In the case of DENV, these

sequences are CAAUAUGCUG in the 5′-CS, and CAGCAUAUUG in the 3′-CS [59]. The interaction between both CS sequences is required to allow stable association of both UAR sequences, as have been demonstrated by the solution structure of the 5′–3′ interaction, as well as by chemical and enzymatic analysis of the complex [56].

In summary, direct RNA–RNA interactions take place between the 5′- and 3′-ends of the DENV genome mediated by three pairs of inverted complementary sequences, namely CS and UAR and, most likely, DAR sequences [48,55,57,59,60,64].

The RNA–RNA interaction was demonstrated by visualization of individual circular RNA molecules by atomic force microscopy [59]. Moreover, the importance of the presence of the cyclization sequences at the 5′- and 3′-ends of the viral genome was initially demonstrated by *in vitro* replication assays using recombinant DENV NS5 polymerase [66,67], and later by the use of DENV replicons, showing that the cyclization process is essential for DENV RNA synthesis but it is not required for viral translation [60].

On the other hand, the 3′-end of the flavivirus genome also contains important *cis*-acting elements for viral replication. Downstream of the viral polyprotein stop codon there is a variable region (VR) followed by two structural elements, termed A2 and A3, which have been predicted using folding algorithms to form identical dumbbell structures (Figure 1). Finally, at the 3′-end, there is a highly conserved stem loop structure, known as 3′-SL.

The importance of the VR in dengue replication was demonstrated by introducing mutations in recombinant cDNA clones or in a replicon context in mammalian cells. In both cases, it was observed that VR modulates viral growth and RNA synthesis in mammalian but not in mosquito cells [60,68]. The basis of the differential behavior of a sequence in two different cell types could be explained by the presence of a different set of host factors acting to modulate the RNA synthesis in each host. However, further studies are required to determine the precise role of this sequence.

In addition, a clinical study using isolates from patients from Thailand demonstrates that the VR sequence contains insertions or deletions. Interestingly, in this study, the viruses with insertions showed higher levels of replication with respect to virus without insertions. However, no correlation between severity of the disease and specific sequences or structures within the UTRs was observed [69].

Downstream of the VR there are two elements known as A2 and A3 domains, which have similar secondary structures and act as modulator elements for RNA synthesis. Both domains contain highly conserved structures named RCS2 and CS2, respectively (Figure 1). Deletion mutants within RCS2 and CS2 of the DENV4 cDNA clone demonstrated that these elements were not essential for DENV replication [48]. However, a 30-nt deletion in the A3 domain induced an attenuated phenotype, and experimental evidence indicates that this deletion does not alter the translation process but severely impairs RNA synthesis [70]. The 3′-CS, which is responsible for genome circularization is located downstream of the A3 domain.

At the end of the 3′, there is a conserved 3′-SL structure (Figure 1), which has been demonstrated to be essential for viral replication by several experimental approaches. It was observed that in the infectious clone of DENV2, an 11-nt element located at the bottom of the SL was indispensable for viral replication and the structure at the top of the SL was critical for viral growth in mammalian cells [71]. Moreover, the sequence and structure present in the pentanucleotide (CACAG) located at the top of the 3′-SL of WNV is important for an efficient viral RNA synthesis [72]. The 3′-UAR sequence, which is complementary to 5′-UAR, is located within the 3′-SL domain.

Finally, the dinucleotide terminal CU_{OH} of the flavivirus genome has been demonstrated to play a critical role in the replication of Kunjin virus, WNV and DENV [72–74]. The functional involvement of that sequence in replication could be related to the assembly of the RC.

The actual model for DENV minus-strand RNA synthesis proposes that after viral translation and accumulation of viral proteins, the *cis*-elements CS, UAR and possibly DAR induce circularization of the viral genome. In this conformation, the NS5 polymerase binds to the SLA, which in a circularized molecule is close to the 3′-end, the site where minus-strand RNA synthesis initiates.

Importantly, mutations in CS or UAR sequences, which are known to prevent cyclization, inhibit viral replication but not translation, indicating that cyclization is only required for replication. This fact would support the notion that once the viral RNA is in the cytoplasm of the infected cells, after decapsidation it adopts a linear conformation favoring viral translation, and later, when viral proteins are synthesized, viral RNA cyclization occurs to allow viral replication initiation. Although

this model was correct, the question remains as to which elements, molecules or conditions are involved in switching translation to replication or in altering the conformation of the viral RNA. The conformation of viral RNA, and its association with cellular or viral proteins in different compartments of the ER, are important aspects that need to be analyzed in order to fully understand regulation of DENV translation and replication.

To this respect, in recent years, some interesting features related with DENV replication have been described. The first is a repair mechanism of 3′-end deletions. The proposed repair mechanism involves two steps: first, a nontemplate-based nucleotide addition takes place to restore the structure of the 3′-SL and is carried out by the terminal nucleotidyl transferase activity of the NS5; and the second step involves the evolutionary selection of the 3′-end sequences, based on RNA structures with the highest fitness that can support viral replication [75]. Given the importance of the sequences present in the 3′-end of the viral RNA, it is likely that this repair mechanism plays an important role in viral infection and merits more detailed analysis.

The other feature described in flavivirus replication is the accumulation of a small viral subgenomic noncoding RNA species. These shorter RNAs, observed in DENV infection with the four serotypes [76], have been characterized in detail for WNV [77,78]. This subgenomic RNA presumably derives from the incomplete degradation of the genomic viral RNA carried out by the 5′–3′ XRN1 exoribonuclease in P bodies [78]. The length of this RNA is approximately 0.4 kb for DENV (corresponding to the length of the DENV 3′-UTR), and is given by the presence of a stable RNA stem loop structure in the beginning of the 3′-UTR, where the XRN1 is stalled [76,78]. It has been proven that this subgenomic RNA is essential for viral-induced cytopathocity and pathogenicity; however, the precise molecular mechanism of action of these molecules is still unknown [78].

Trans-acting factors

RNA synthesis is a highly regulated process, which depends on *cis*- and *trans*-acting factors. *Cis*-acting elements, as already described, play different roles during viral replication, and some of them are independent of the presence of proteins, while some others involve the interaction with *trans*-acting factors. Both *trans*-acting factors and *cis*-acting elements must act coordinately to produce adequate levels of viral RNA at the right sites. The specificity and affinity of the viral RNA for certain proteins suggests that they are important elements in the viral replicative cycle. Two types of *trans*-acting factors have been involved in viral replication: viral and cellular factors.

Viral *trans*-acting factors

It has been previously described that DENV RNA replication takes place in close association with viral-induced membranes. Partial fractionation of cytoplasmic extract from infected cells and coimmunolocalization assays show that the membrane fractions enriched in RNA-dependent RNA polymerase activity contain the viral proteins NS1, NS2A, NS3, NS4A, NS4B and NS5 [79–82].

Immunolocalization experiments performed with different cell types indicate that proteins NS1, NS4A and NS4B colocalized with the RNA replication sites [80,81]. Although the specific role of these proteins in viral replication is not completely understood, it is possible that NS1 and the hydrophobic protein NS4A act as structural components of the replication complex, anchoring the complexes to the viral-induced membranes. The genetic interaction between NS1 and NS4B has been observed by *trans*-complementarity assays [83]. In addition, it has been demonstrated that NS4B interacts with NS3, and this association modulates the NS3 activity. The interaction of NS4B with NS3 causes NS3 dissociation from ssRNA and promotes dsRNA unwinding activity [84].

The multifunctional and multidomain proteins NS3 and NS5 are the only proteins with catalytic activities encoded by DENV. The NS3 protein is a viral protease (NS3pro) that requires association with the cofactor NS2B to form a heterodimeric complex, and it also has a RNA triphosphatase as well as RNA helicase activities [85–88].

Besides host-encoded proteases, the NS3pro is important to produce individual and functional proteins from the 375-kDa viral polyprotein. It mediates the cleavage of the peptide bonds between C–prM, NS2A–NS2B, NS2B–NS3, NS3–NS4A and NS4A–NS5 [20]. In addition, the role of the RNA triphosphatase and helicase activities of NS3 has been demonstrated to be essential for viral replication [88].

Since RNA replication takes place in the ER membranes [89], the cap type I structure has to be added by a protein located in this specific compartment; for DENV, the cap is added by the viral protein NS5. The NS5 consists of a N-terminal

guanylyltransferases and methyltransferase [90–93] and a C-terminal RNA-dependent RNA polymerase domain [94]. For flavivirus, both guanine-N7 methyltransferase (N7-MTase) and nucleoside-2′-*O*-methyltransferase activities have been identified, but only the N7-Mtase activity has been shown to be essential for RNA replication [95,96]. It is likely that flavivirus RNA capping and methylation are coupled to RNA synthesis (for review see [96]). However, further experiments are required to elucidate the specific steps and exact mechanism for viral RNA capping.

In addition to the methyltransferase activity, NS5 also has RNA-dependent RNA polymerase activity (RDRP). The C-terminal polymerase domain of NS5 is essential for positive- and negative-strand RNA synthesis. Using recombinant DENV NS5 (RDRP), it has been demonstrated that *in vitro* RNA synthesis requires sequences present at the 5′- and 3′-end of the genome as previously described [66, 67]. Moreover, it has been shown, by atomic-force microscopy, that the NS5 RDRP domain of DENV2 binds to the circularized DENV2 genome [53]. The presence of a promoter element in the SLA enhances *de novo* negative-strand RNA synthesis and template discrimination [53].

Since the main functions known for NS5 are methyltransferase and the RDRP, it would be expected that this protein is mainly located within the ER; however, most of the protein is located in the nucleus. The nuclear localization of NS5 can be explained by the presence of a bipartite nuclear localization sequence (aNLS and bNLS) in the region between the N-terminal methyltransferase and C-terminal RDRP domains of NS5. The aNLS, recognized by the importin a/b, is considered to be responsible for the nuclear localization of NS5; however, bNLS is also recognized by importin b1 nuclear transporters. Moreover, it has been established that NS5 is able to be exported from the nucleus to the cytoplasm by the exportin CRM1 and, hence, can shuttle between the nucleus and the cytoplasm [97]. Interestingly, the DENV2 NS5 protein has been found to accumulate in the nucleus late in infection as a hyperphosphorylated form that is unable to bind NS3, whereas the hypophosphorylated form of NS5 is cytoplasmic and complexed to NS3. Thus, it is also possible that phosphorylation and the interaction with NS3 may also be modulating the compartamentalization and function of the viral RNA replicase [98]. To this respect, it has been demonstrated *in vitro* that the interaction between both proteins enhances the NTPase activity of NS3 [99]. This activity is essential for the conversion of the replicative form of RNA to the replicative intermediate [100,101].

It has been suggested that the presence of NS5 into the nucleus can antagonize the antiviral response by modulation of IL-8 production in infected cells [102]. IL-8 has been speculated to enhance viral production by antagonizing the effects of interferons [103], as has been described for other viruses, including picornaviruses, encephalomyocarditis virus, HCV, citomegalovirus and HIV [103–107]. However, a more direct role for IL-8 in inhibiting virus replication has recently been suggested [107], implying that inhibition of IL-8 production may serve to assist virus production. Thus, it has been suggested that after initial induction of IL-8 transcription by NS5 alone or in cooperation with host cell factors [102], the production of IL-8 could be regulated by the concentration of nuclear/cytoplasmic NS5 and its interaction with cellular and/or viral factors [108]. These studies imply that, in addition to being a key enzyme in viral RNA replication, NS5 may have a role in viral pathogenesis related to its localization in the nucleus. Further studies directed towards determining the levels of NS5 in the nucleus and the cytoplasm, the level of phosphorylated forms of this protein, as well as its interaction with other cellular or viral factors into the nucleus are required in order to extend our knowledge about the function of NS5 in viral replication and pathogenesis.

Cellular *trans*-acting factors

It is well established that cellular factors are important components of the RCs of several viruses. The initial approach to find cellular proteins that could be involved in viral replication is to determine which factors are able to interact with the *cis*-acting elements. This approach has been used successfully for several viral systems to identify host factors involved in viral replication. Using mobility-shift assays, UV-induced crosslinking and RNA-affinity chromatography, several proteins have been found to bind to the *cis*-acting elements of flavivirus RNAs. These factors are elongation factor 1a (EF1a), polypyrimidine tract binding protein (PTB), La, T-cell intracellular antigen-1 (TIA-1), the related protein (TIAR), Y Box binding protein-1 (YB-1), calreticulin, PDI and hnRNP A1, A2/B1 and Q [109–114].

Elongation factor 1a

Although the regular function of the translation EF1a is to catalyze the elongation step in mRNA translation, it has also been implicated in

cytoskeleton organization, ubiquitin-mediated protein degradation, cell proliferation and senescence (for review see [115]). The use of different methodologies, such as electrophoretic mobility shift assay (EMSA), mobility supershift assays, UV-crosslinking and coimmunoprecipitation assays demonstrate that EF1a binds to WNV and the DENV 3′-CS–SL region [109,116]. Moreover, using footprinting assays, deletion/mutation analyses and RNA binding assays, the sequence CAC, located at the main stem of the 3′-SL, was found to be the main binding site of EF1a to WNV RNA [109]. This protein has been found to be involved in WNV genome replication; however, its function during DENV replication is unknown. One possible function for EF1a could be targeting the RNA to the compartment of viral replication, since it has been found associated with the ER membranous fraction. Besides its interaction with flavivirus genomic RNA, EF1a is implicated in binding and activation of the RNA polymerase RNA-dependent complex of other RNA viruses, such as vesicular stomatitis virus [117].

Polypyrimidine tract binding protein

The PTB protein is a widely expressed protein containing four RNA recognition motifs involved in RNA interaction. It shuttles between the nucleus and cytoplasm and has multiple roles in mRNA metabolism, including localization, polyadenylation, regulation of alternative splicing and alternative translation initiation, among others [118–121]. The preferred RNA-binding site of PTB is a U/C tract whose interaction could cause a conformational change in the RNA [120].

The PTB protein also has the ability to bind to the 5′- and 3′-UTRs of several viral genomic RNAs favoring viral translation and replication [122–125]. In this way, the role of PTB has been extensively studied in the replicative cycle of picornaviruses and HCV [126–128]. In both cases, PTB binds to specific sequences within the 5′-UTR, promoting translation initiation, dependent on an internal ribosomal entry site. PTB also binds to the 3′-UTR of several flaviviruses, such as DENV, Japanese encephalitis virus and HCV [110,129,130]. The specific binding of PTB to the 3′-SL of DENV was initially demonstrated *in vitro* through EMSA and UV-crosslinking assays [110].

The participation of PTB in the HCV and DENV replicative cycles was evaluated after silencing and/or overexpression of PTB. Increased PTB expression levels induced augmentation in viral RNA levels determined by real-time reverse transcriptase PCR, as well as in the levels of the reporter protein encoded by viral replicons, while the opposite was observed after silencing the protein expression [131–134]. These results suggest that PTB is a positive regulator of translation and replication for HCV and DENV.

Since most of the functions of PTB are carried out in the nucleus, the major location of this protein is in the nuclear compartment; however, it also shuttles between the nucleus and the cytoplasm [120]. PTB translocates from the nucleus to the cytoplasm during DENV infection in Vero and in Huh-7 cells. The observed translocation was more prominent in Vero than Huh-7 cells, suggesting that intrinsic differences between these two cell types could be related to the observed difference in translocation efficiencies [131]. Colocalization studies with NS1, NS3 and NS5 in the ER and immunoprecipitation with NS4A has implicated PTB in the formation of the replication complex [131–133], but the precise role of PTB during DENV infection is still unknown.

The translocation of PTB from the nucleus to the cytoplasm has been documented during infection with several other viruses, such as poliovirus, rhinovirus, feline calicivirus, HCV and DENV [131,132,134–136]. PTB has been involved in the regulation of viral internal ribosome entry segment-dependent translation, as well as in the replication of mouse hepatitis virus and HCV [131,136]. In feline calicivirus replication, PTB has been proposed to play a role in the switch from translation to replication, since it downregulates viral translation and stimulates viral RNA replication [135]. In other viruses, such as coxsackievirus B3, PTB has been implicated in the circularization of the genome, favoring the interaction between the 5′- and 3′-UTRs and permitting an efficient viral translation [137].

TIA-1 & TIAR

The TIA-1 and the related protein, TIAR, are broadly expressed host factors carrying out multiple functions mainly related to mRNA modification pathways and apoptosis [138–140]. TIA-1 and TIAR shuttle between the nucleus and the cytoplasm, where they bind to the 3′-UTR of several cellular mRNAs causing translational inhibition. In addition, both proteins have been involved in the cytoplasmic storage or degradation of mRNA in structures known as stress granules [141,142]. These host factors interact with the 3′-UTR of the negative strand of WNV, specifically with two short AU sequences [111]. The ability of TIA-1 and TIAR to bind those regions

was associated with a higher viral replication efficiency determined by plaque size, virus yield and genomic RNA levels [111]. Both TIA-1 and TIAR have been found to colocalize with WNV and DENV nonstructural proteins and with dsRNA in the perinuclear region of infected cells, thus suggesting a role for these proteins in viral replication [143]. However, the ability of TIA-1 and TIAR proteins to bind to the positive or negative RNA strands of DENV is unknown. Taking into consideration the cellular function of both proteins, it has been proposed that they could act as stabilizers of the 3′-SL(-) region, inducing conformational changes that prepare the RNA to interact with the replication complex to initiate the positive-strand RNA synthesis.

Y-Box binding protein-1

The YB-1 protein is a cold shock factor involved in several cellular functions, such as DNA repair of growth-related genes and stress response to extracellular signals. In the cytoplasmic compartment it has been described as an mRNA chaperone and translational modulator [144].

This protein was identified as one of the proteins eluted from an affinity chromatography column prepared with the 3′-UTR of genomic DENV RNA. Several other RNA binding proteins were eluted with YB-1, such as hnRNPQ, A1, A2/B1 and Q [114,144]. The interaction of YB-1 with the viral RNA was further confirmed by EMSA and footprinting assays. In addition, *in silico* studies predicted several potential YB-1 binding sites along the DENV 5′-UTR and the nonstructural protein-coding region, but these predictions have not been proven. The significance of the interaction of YB-1 with DENV genome is unknown. However, the silencing of YB-1 in DENV-infected cells suggests that this protein may have a role as an antiviral factor, or it could be participating in the switching from viral translation to replication. In addition, it might be implicated in antiviral early immune response as part of the interferon signaling pathway, as has been described for adenovirus infection [114,145].

La

The La protein is an RNA binding protein, which plays several roles in the cell. The best-established roles for La protein are to provide protection against 3′-exonucleolytic RNA degradation and to bind and splice the RNA pol III primary transcripts [146].

The interaction of La protein with viral RNAs in other systems, including vesicular stomatitis virus, human parainfluenza virus, Rinderpest virus, rabies, HIV, HBV, HCV and several picornaviruses, has been reported [147–153]. In those systems, translocation of the La protein from the nucleus to the cytoplasm have been observed, and it has been suggested that La may function in RNA stabilization, as well as in viral translation and/or replication.

Furthermore, the La protein has been recently involved in the inhibition of interferon activation through its interaction, in early times postinfection, with the negative-strand RNA of respiratory syncytial virus and with the Sendai viral genome. This interaction avoids the recognition and signaling by an intracellular sensor of infectious RNA, RIG-I, contributing to the viral evasion of the host immune system [154].

For flavivirus, it has been reported that La protein binds to a loop of the 3′-UTR and has a functional role in the replication of Japanese encephalitis virus [155]. For DENV, the interaction of La protein with the 5′- and 3′-UTR (3′-SL–3′-CS) of the genomic RNA, as well as with the 3′-UTR of the negative-strand RNA, was demonstrated by EMSA, UV-crosslinking and immunoprecipitation assays [110,112,113]. Furthermore, in DENV-infected cells, the La protein is redistributed from the nucleus to the cytoplasm [156].

Since La protein interacts *in vitro* with both ends of the DENV genome, it has been suggested that it could play a role in the stabilization of the replication complex or in switching translation to replication. To this respect, the addition of recombinant La protein to an *in vitro* replication system inhibits RNA synthesis in a dose-dependent manner, suggesting that La protein downregulates the DENV replicative cycle [156]. Of note is the fact that La protein was immunoprecipitated with NS3 and NS5, further suggesting a role for La protein in the DENV replicative cycle [113].

Replication complexes

The replication process for several RNA viruses requires the induction of membrane structures that provide a membrane-bounded microenvironment. This environment separates viral RNAs and proteins from the cellular components allowing for efficient RNA synthesis and viral morphogenesis. Although all groups of positive-strand RNA viruses use membrane structures, their architecture and origin differs among the various groups (reviewed in [157]). DENV-induced membrane structures show positive immunolabeling with calnexin, PDI and CLIMP63, indicating that membranes are

derived from the ER [158]. Moreover, positive immunolabeling with syntaxin 17 indicates that virus-induced membranes may also be derived from the smooth ER [159]. For Kunjin virus and other flavivirus-infected cells, the *trans*-Golgi marker β-1-4-galactosyltransferase, as well as other *trans*-Golgi markers, such as p230 and TGN46, also localized within the vesicles, suggesting that *trans*-Golgi membranes are also involved in the formation of flavivirus RCs [160].

Apparently, upon DENV infection, genomic RNA associates with ribosomes at the ER during viral translation. Viral proteins are processed co- and post-translation forming the RC. Assembly of the RC initially requires the proliferation and formation of invaginations of the ER membranes. Presumably, this process is induced by NS4A and NS3, in conjunction with cellular and other viral proteins [161–163]. It is thought that NS4A oligomers, intercalated into the luminal leaflet of the ER membrane, through the peripheral membrane domain, would dilate the luminal leaflet forming the invaginations (Figure 2). These invaginations give rise to membranous vesicles or vesicle packets that have been described as the site for viral replication, since NS proteins and dsRNA have been located into these membranous structures. The interior of the vesicles are connected with the surrounding cytosol via a pore (Figure 2). Although the composition of the pore and its biogenesis is unknown, it is quite likely that it regulates the import of molecules or factors required for viral replication and the export of newly formed genomes for translation or assembly. However, the proteins or factors involved in the regulation or 'gating' process are not known. In this regard, Welsch *et al.* calculated that even though the size and volume of the vesicle may harbor up to 50 ssRNA molecules, the electron lucent appearance of the vesicles suggests that they contain only a few RNA molecules [164]. It is thought that following replication within the virus-induced vesicles, the RNA is exported to the convoluted membranes (CMs), which are located in close proximity with the vesicles (Figure 2). For Kunjin virus, the immunolabeling of the CM with antibodies anti-NS3 and NS2B suggests that CMs are the sites for polyprotein processing [165,166]. However, for DENV, this aspect is unclear. The presence of syntaxin 17 and the absence of ribosomes in the CM induced by DENVs suggest that the CMs are derived from smooth ER membranes. The close proximity between the CM and virus-induced vesicles and the presence of NS3 in the CM allowed Wesch *et al.* to speculate that CM may represent a storage site for proteins and lipids required for DENV replication [164]. Since it is known that translation and replication are coupled processes and that replication of the nascent RNA molecule is required for packing [57], it could be expected that nucleocapsid formation and virus budding into the ER lumen occur in close proximity of the vesicles and the CM, where viral RNA is released. To this respect, the first step in viral morphogenesis is the association between the RNA and the C protein to generate nucleocapsids. Recently, it has

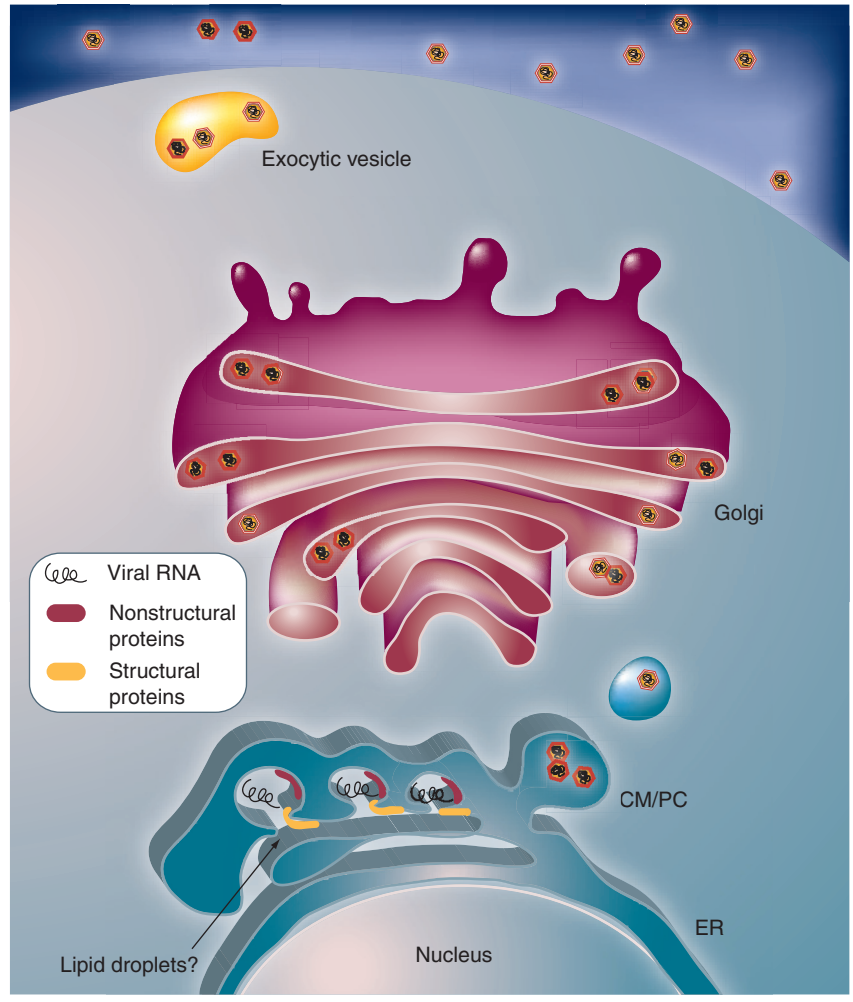

Figure 2. Coupling between dengue virus translation, replication and morphogenesis in viral-induced membranes. Upon infection, the viral genome associates with membranes from the ER. Ribosomes translate viral RNA in structural and nonstructural proteins. Both types of proteins are arranged in different sides of the ER membranes. Nonstructural proteins, such as NS3 and NS4A, and probably other cellular and viral proteins, induce invaginations in the ER. Inside these invaginations, RNA replication takes place. The interior of the vesicles is connected with the surrounding cytosol via a pore. Through this pore, the RNA is exported to the CM, which are located in close proximity to the vesicles. The first step in viral morphogenesis is the association of viral RNA with capsid protein, which has been found to be associated with lipid droplets. Virus budding through the ER occurs in close proximity to or opposite the vesicles. Finally, virions travel to distal ER and later to Golgi in secretory vesicles where virion maturation takes place.
CM: Convoluted membrane; ER: Endoplasmic reticulum; PC: Paracrystalline arrays.
Adapted with permission from [164].

been described that the C protein accumulates around the lipid droplets (LDs) and that this association is crucial for infectious particle formation (Figure 2). LDs are ER-derived organelles that contain a core of neutral lipids enclosed by a monolayer of phospholipids. Although the place and mechanism by which the C protein recruits the viral RNA to form the nucleocapsid are still uncertain, Samsa *et al.* have suggested that either the C protein associates with LDs early in the infection and then is mobilized to the ER during viral morphogenesis, or the genomic RNA interacts with the C protein on the surface of LDs to form the nucleocapsids, which could be transferred to the ER membranes for the assembly of new viral particles [167]. To date, ultrastructural studies of RCs have not described the presence of LDs within viral-induced membranes. However, the fixation method used during sample preparation may have avoided its observation [167]. Further studies are required to elucidate the precise location of LDs and the dynamics of nucleocapsid formation within virus-induced membrane structures.

After nucleocapsid formation, the viral envelope has to be acquired. One important aspect suggested for DENV and other flaviviruses is that the viral envelope is obtained by budding into the ER lumen. Using electron tomography, Welsch *et al.* observed that budding of viral particles occurs in a close proximity to vesicles and CMs [164]. Immature viral particles accumulate in the lumen of dilated ER cisternae and are afterward transported to the *cis*-Golgi for further maturation. Viral particle accumulation occurs in specific membrane structures, which have been described as paracrystalline arrays (Figure 2).

Despite the importance of membrane composition of DENV RCs, little is known about this aspect. For other members of the *Flaviviridae* family, such as HCV, it has been described that the RCs are present in cholesterol-rich membranes; moreover, the presence of cholesterol is important for an efficient viral replication [168,169]. For DENV, it has been described that cholesterol depletion reduces viral yield, as well as viral RNA synthesis, suggesting that cholesterol is necessary for viral entry and postentry processes [33,170–172]. Since viral replication and the morphogenesis take place in the virus-induced membranes, it is likely that both process are affected by cholesterol-disrupting drugs. Given that the exact composition and cholesterol content of the virus-induced membranes are unknown, more studies directed to understanding this aspect are necessary. Moreover, it will be interesting to know if the amount of cholesterol and phospholipids composition are the same in the different membrane structures, vesicles, CM and paracrystalline arrays, and which participate in viral replication.

It is clear that the subversion of cellular machinery and pathways is crucial for virus propagation and survival. In particular, induction of membrane proliferation and reorganization seem to be central to flavivirus replication. Thus, the study of the different cellular and viral components involved in flavivirus replication and in inducing membrane proliferation is necessary for a comprehensive understanding of the viral replicative cycle, and to pave the way for the identification of specific antiviral targets.

Conclusion

Dengue virus, similar to many positive-strand RNA viruses, requires *cis*-acting elements, mainly located within the 5′- and 3′-UTRs, *trans*-acting factors from cellular and viral origin and viral-induced membranes located within the ER, for viral replication. However, DENV and other flaviviruses differ from other positive-strand RNA viruses due to the fact that cyclization of the viral genome occurs through RNA–RNA interactions and does not require the presence of viral or cellular proteins. It is not known which elements trigger viral cyclization, but the conformation of the viral RNA (linear or cyclizated form) may be an important regulator element of viral translation and replication. All nonstructural proteins from DENV are important *trans*-acting factors for viral replication. While some proteins are important components for anchoring the RC to the ER membranes, others, such as NS3 and NS5, carried out the main catalitic acivities: NS3 as the helicase, nucleoside thriphosphatase and protease; and NS5 as RNRP and methyltransferase. Among cellular *trans*-acting factors, the nuclear proteins with affinity to RNA, such as PTB, La and YB-1, most likely play a role during viral replication. Finally, DENV infection induces synthesis and rearrangement of membranes from the ER and *trans*-Golgi. Within the ER, vesicles, CMs and paracrystalline arrays are generated. DENV uses these membranes to translate, replicate and produce new viral particles.

Future perspective

Although the cells have developed different mechanisms to detect the presence of viral infection, viruses have also developed strategies to be less visible to the defense sensors. One of these strategies is to induce synthesis

and rearrangement of internal membranes. Specifically, DENV uses these membranes to translate, replicate and produce new viral particles. In this compartment within the ER, all components required for replication are recruited, making the process more efficient. In addition, membranes protect RC from RNAases and proteases that could interfere with the viral replicative cycle. Thus, the intervention of viral infection requires the consideration of these aspects.

Given the importance of DENV infection in world health, different approaches have been developed to block or inhibit viral infection. Although different steps in the viral replicative cycle can be interfered with, such as binding, entry, replication or morphogenesis, and viral release, in the last years, many antiviral drugs have been designed to interfere with viral genome replication. Following this, the need to understand this step has gained importance. Two important viral components of the RC can be considered as important targets for antiviral drug design: NS5 and NS3. The presence of the RDRP and methyltransferase activities in NS5 and the importance of both activities for DENV replication make NS5 an excellent target to interfere with viral infection. In this respect, it is

Executive summary

Dengue virus epidemiology

- Dengue virus (DENV) transmission has been vigorously emerging in a growing number of countries during the last two decades.

DENV structure & genomic organization

- DENV genome is a single-stranded positive-polarity RNA that encodes for three structural and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

DENV replication

- Three main elements are necessary for DENV replication: *cis*-acting elements, mainly located within or in close proximity to both 5′- and 3′-untranslated regions (UTR), *trans*-acting factors, both of cellular and viral origin, and viral-induced membranes, which wrap replication complexes and provide compartments for viral morphogenesis.

Cis-acting elements

- The *cis*-acting elements required for DENV replication are mainly located at both ends of viral genome in the 5′- and 3′-UTR.
- The cyclization sequences, as well as the upstream UAG region located within both ends of the viral genome, and maybe the downstream AUG region, induce circularization of viral genome.
- The secondary structure of the stem loop located at the 3′-end (3′-SL), as well as the secondary structure of SL structures within the 5′-UTR, are required for an efficient negative-strand RNA synthesis.
- The RNA dependent RNA-polymerase NS5 binds to the 5′-UTR to initiate viral replication.

Trans-acting factors

Viral *trans*-acting factors:

- The multifunctional and multidomain proteins NS3 and NS5 are the only proteins with catalytic activities encoded by DENV.
- NS5 has two main activities: RNA-dependent RNA-polymerase and methyltransferase.
- NS3 has activities of protease, helicase and nucleoside triphosphatase. The function of NS3 can be regulated by its association with other viral proteins.
- NS1 and the small nonstructural proteins may be required for anchoring of the viral replication complex to membranes of the endoplasmic reticulum (ER).

Cellular *trans*-acting factors:

- Several cellular proteins, such as EF1a, polypyrimidine tract binding protein (PTB), La, YB-1, calreticulin, PDI and the heterogenous nuclear factors A1, A2/B1 and Q, have been found bound to the 5′- or 3′-UTR of DENV.
- PTB and La translocate from the nucleus to the cytoplasm during DENV infection and act as positive and negative regulators of viral replication.
- The YB-1 protein may have a role as an antiviral factor or it could be participating in the switching from viral translation to replication.

Replication complex

- Formation of the replication complex initially requires the proliferation and the formation of invaginations of the ER membranes, presumably induced by NS4A and NS3 in conjunction with cellular and other viral proteins.
- Invaginations have been described as the site for viral replication.
- The viral RNA is exported to the convoluted membranes, which may represent a storage site for proteins and lipids required for DENV replication.
- The first step in viral morphogenesis is the association between the RNA and the C protein to generate nucleocapsids. The C protein accumulates around lipid droplets in the ER.
- Immature viral particles accumulate in the lumen of dilated ER cisternae and afterward are transported to the *cis*-Golgi for further maturation.

necesary to further study the methyltransferase activity and the importance of cap addition, not only in viral translation but also in viral replication. Conversely, NS3 can also be an excellent target for drug design, since the three activities present in this protein are indispensable for viral replication.

Other viral proteins, such as NS1 or the modulation in cholesterol levels in the host cells, may also be targeted to interfere with DENV replication.

Although several aspects in DENV replication are understood, others need further analysis. One important aspect to highlight is the need for the study of the replication process in mammalian and in mosquito cells. It is not clear if the same cellular structures that are induced in mammalian cells are also present in mosquito cells. Furthermore, it needs to be established if the cellular proteins that bind to the 3′- and 5′-UTR of DENV using mammalian cell extracts will be the same when mosquito cell extracts are used. Conversely, even though genome cyclization occurs in the absence of viral and cellular proteins *in vitro*, it is not known whether cellular or viral proteins are required *in vivo* to stabilize or induces RNA–RNA contacts. Moreover, it is not known if there is a switch that induces translation termination and favors replication initiation or if viral cyclization is also required for the positive-strand RNA synthesis. All these aspects need to be solved in order to understand the viral replicative cycle in hosts, mosquito and mammalian cells.

One important aspect that has to be evaluated in the coming years is the role of the presence of NS5 within the nucleus of infected cells. It is known that NS5 modulates the expression of IL-8; however, it is relevant to determine if this protein is playing an active role in the regulation of the expression of additional genes, as well as its role in the relocation of nuclear proteins to the cytoplasm during viral infection.

Finally, the isolation of RC from infected cells will allow the more precise determination of the role of cholesterol in viral replication, as well as the distribution of RC in lipid microdomains. This aspect will be important in antiviral drug design. Certainly, the understanding of DENV replication will be expanded in the coming years and, hopefully, provide important clues to reduce the burden of this important infection in areas of the world suffering epidemics of DENV.

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