



Functions of the 3' and 5' genome RNA regions of members of the genus *Flavivirus*



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ABSTRACT

The positive sense genomes of members of the genus *Flavivirus* in the family *Flaviviridae* are ~11 kb in length and have a 5' type I cap but no 3' poly-A. The 3' and 5' terminal regions contain short conserved sequences that are proposed to be repeated remnants of an ancient sequence. However, the functions of most of these conserved sequences have not yet been determined. The terminal regions of the genome also contain multiple conserved RNA structures. Functional data for many of these structures have been obtained. Three sets of complementary 3' and 5' terminal region sequences, some of which are located in conserved RNA structures, interact to form a panhandle structure that is required for initiation of minus strand RNA synthesis with the 5' terminal structure functioning as the promoter. How the switch from the terminal RNA structure base pairing to the long distance RNA–RNA interaction is triggered and regulated is not well understood but evidence suggests involvement of a cell protein binding to three sites on the 3' terminal RNA structures and a *cis*-acting metastable 3' RNA element in the 3' terminal RNA structure. Cell proteins may also be involved in facilitating exponential replication of nascent genomic RNA within replication vesicles at later times of the infection cycle. Other conserved RNA structures and/or sequences in the 3' and 5' terminal regions have been proposed to regulate genome translation. Additional functions of the 3' and 5' terminal sequences have also been reported.

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1. Introduction

Members of the genus *Flavivirus* are distantly related to those in the *Pestivirus*, *Hepacivirus* and *Pegivirus* (proposed) genera in the family *Flaviviridae* (Heinz et al., 2000; Stapleton et al., 2011; Drexler et al., 2013). All members of the family *Flaviviridae* have a single-stranded, positive-sense RNA genome that encodes a long open reading frame (ORF) of ~3400 codons. Although the genomes of the different genera share a similar gene order and some conserved nonstructural protein motifs, they diverge markedly in the *cis*-acting RNA regulatory elements located at their 3' and 5' ends. For example, polyprotein translation from pestivirus, hepacivirus and pegivirus genomes is initiated from an AUG located 3' of an internal ribosome entry site (IRES) while flavivirus polyprotein translation is dependent on a 5' type I cap.

This review is focused on the structures and functions of the 3' and 5' terminal genome regions of the members of the genus *Flavivirus* which currently contains 73 viruses classified as 53 species (Pierson, 2013) that are divided into four groups: the tick-borne

flaviviruses, the mosquito-borne flaviviruses, the no-known-vector flaviviruses and the non-classified flaviviruses (Gritsun and Gould, 2006; Pierson, 2013). A number of the arthropod-borne flaviviruses cause disease in humans. The mosquito-borne viruses are further divided into the Japanese encephalitis virus, Yellow fever virus, and Dengue virus subgroups. The majority of studies on the functions of flavivirus 3' and 5' RNA regions have been done with dengue virus (DENV) or West Nile virus (WNV) but some studies have also been done with yellow fever virus (YFV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV). The characteristics and functions of the 3' and 5' ends of the hepacivirus and pegivirus genomes have recently been reviewed by Sagan et al. (2015).

2. Conserved sequences in the terminal regions of the genome

Flavivirus genomes are ~11,000 nts in length and function both as the viral mRNA and as a template for minus strand RNA synthesis. The 5' non-coding region (NCR) is relatively short (~100 nts), while the 3' NCR is longer, ranging from ~340 to ~700 nts (Lindenbach et al., 2013). The 3' end of the genome RNA terminates with a conserved CU_{OH} instead of a poly(A) tract (Rice et al., 1985; Brinton et al., 1986; Wengler and Wengler, 1991). Short conserved

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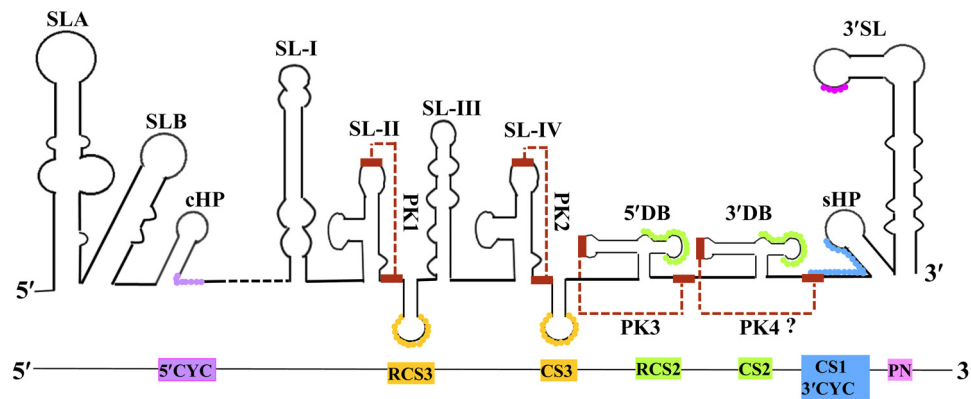


Fig. 1. Conserved 3' and 5' RNA structures and sequences in the WNV genome. The CS1 sequence includes DAR sequence at its 3' end and the 3' CYC sequence at its 5' end. The predicted PK4 interaction was not found in a SHAPE analysis of a DENV 3' RNA. SL – stem loop, cHP – capsid coding region hairpin, SHP – small hairpin, PK – pseudoknot, PN – pentanucleotide motif, CS – conserved sequence, RCS – repeated conserved sequence and CYC – cyclization sequence.

sequences located within the terminal 3' stem loop (3' SL) structure of the flavivirus genomic RNA are the terminal 5' CU 3' and a pentanucleotide motif (PN) 5' CACAG 3' in the top loop (the 5' C of this motif is base paired) (Fig. 1) (Brinton et al., 1986). The 3' CU functions as the recognition site for the viral RNA-dependent RNA polymerase (RdRp) (Khromykh et al., 2003; Nomaguchi et al., 2003). Mutational analysis of individual nts in the top loop of the 3' SL in a WNV infectious clone or in a flavivirus replicon showed that the majority were *cis* acting and that three of the nts (underlined, PN sequence in bold, 5' **cACAGUGC** 3') were essential for virus replication but did not affect translation (Khromykh et al., 2003; Elghonemy et al., 2005; Tilgner et al., 2005; Silva et al., 2007). In contrast, only the G nt in the YFV-17D PN sequence 5' CACAG 3' was found to be essential (Silva et al., 2007). Although individual substitution of the other YFV virus PN nts did not impair virus replication, the mutant viruses were outcompeted by wild type virus in co-infected cells.

A single copy of a sequence, designated conserved sequence 1 (CS1), is located just 5' of the 3' terminal SL structure. The 5' end of CS1 contains a highly conserved 8 nt sequence, designated the 3' cyclization sequence (3' CYC). An exact complement of the 8 nt 3' CYC sequence is located in the capsid coding region near the 5' end of the genome (Fig. 1) (Hahn et al., 1987). Although the results of an initial study suggested that the 3' and 5' CYC sequences were not important as long as they were complementary (Khromykh et al., 2001), a subsequent study showed that substitution of some 3'–5' CYC base pairs with alternative base pairs reduced virus replication efficiency (Basu and Brinton, 2011). Analysis of additional mutant WNV infectious clones indicated that disruption of five adjacent CYC base pairs was lethal while two or three CYC mismatches reduced but did not abolish virus replication efficiency and revertants with increased replication efficiency were rapidly generated (Basu and Brinton, 2011). Several different mutant genomes with three adjacent mismatching CYC substitutions were rescued by the same spontaneous single nt second site mutation that created an additional base pair on the internal genomic side of the 3'–5' CYC structure. Sequences adjacent to the CYC sequence that extend the long distance 3' RNA–5' RNA interaction include the 5' upstream AUG region (UAR) that interacts with a complementary 3' UAR sequence located in SLB downstream of the 3' CYC (Fig. 2A) (Alvarez et al., 2005b; Zhang et al., 2008) and either one (DENV) or two (WNV) 5' downstream of the AUG codon region (DAR) sequences that interact with complementary 3' DAR sequence(s) located in the capsid protein coding region between the 3' UAR and 3' CYC sequences (Fig. 2A) (Dong et al., 2008; Friebe and Harris, 2010). The UAR and DAR sequences are not well conserved among flaviviruses but sufficient complementarity between the 3' and 5' UAR and also

between the 3' and 5' DAR sequences is maintained within individual flavivirus genomes (Fig. 2B) (Gritsun and Gould, 2007a,b; Alvarez et al., 2008).

3. Long distance 3'–5' RNA–RNA interactions are required for minus strand synthesis

Interaction between 3' and 5' terminal flavivirus genome sequences has been demonstrated by atomic force microscopy (Alvarez et al., 2005b), structure probing (Dong et al., 2008; Polacek et al., 2009a), and electrophoretic mobility shift assays (Alvarez et al., 2005b; Zhang et al., 2008). Terminal region RNA fragments with mutations that disrupted 3'–5' CYC base pairing were not able to form either the long distance CYC or UAR interactions. In contrast, mutations that disrupted 3'–5' UAR base pairing disrupted the long distance 3'–5' UAR interaction but not the 3'–5' CYC interaction (Polacek et al., 2009a). The data suggest that the flavivirus genomic 3'–5' RNA–RNA interaction initiates with the interaction of the 3' and 5' CYC sequences, followed by 3'–5' DAR sequence pairing, then pairing of the 5'–3' UAR nts (Friebe et al., 2011). Data from several studies indicated that additional sequences located 3' of the 5' CYC are also involved in genome cyclization (Basu and Brinton, 2011; Friebe et al., 2012; Liu et al., 2013).

Genome cyclization due to the 3'–5' long distance RNA–RNA interaction is required for the initiation of minus strand RNA synthesis but not for translation (Khromykh et al., 2001; Corver et al., 2003; Lo et al., 2003; Alvarez et al., 2005a). The methyl transferase (MTase) region of the viral NS5 protein interacts with sites on the 5' terminal stem loop structure (SLA) bringing the RdRp region of NS5 in close proximity to the 3' end of the genome in the context of the 3'–5' long distance RNA–RNA interaction and viral minus strand RNA synthesis is initiated *de novo* (Fig. 3B) (Filomatori et al., 2006; Dong et al., 2008; Lodeiro et al., 2009).

It was recently hypothesized that 3'–5' base pairing might occur between ends of separate genome RNAs forming noncovalent concatemers (Lott and Doran, 2013). High concentrations of genome RNA present in infected cells at later times of infections were postulated to enhance concatemer formation. An advantage of this type of end pairing would be that individual genomes would not need to switch between the linear (translation) and cyclized (minus strand synthesis) forms. However, such a model would result in increased efficiency of minus strand synthesis which is not consistent with the limited amount of minus strand synthesized or with the decrease in minus strand levels at later times in the infection cycle when genome levels are high. Also, WNV mutants that produced increased amounts of minus strand RNA produced reduced levels of genome RNA and virus (see Section 6) (Davis

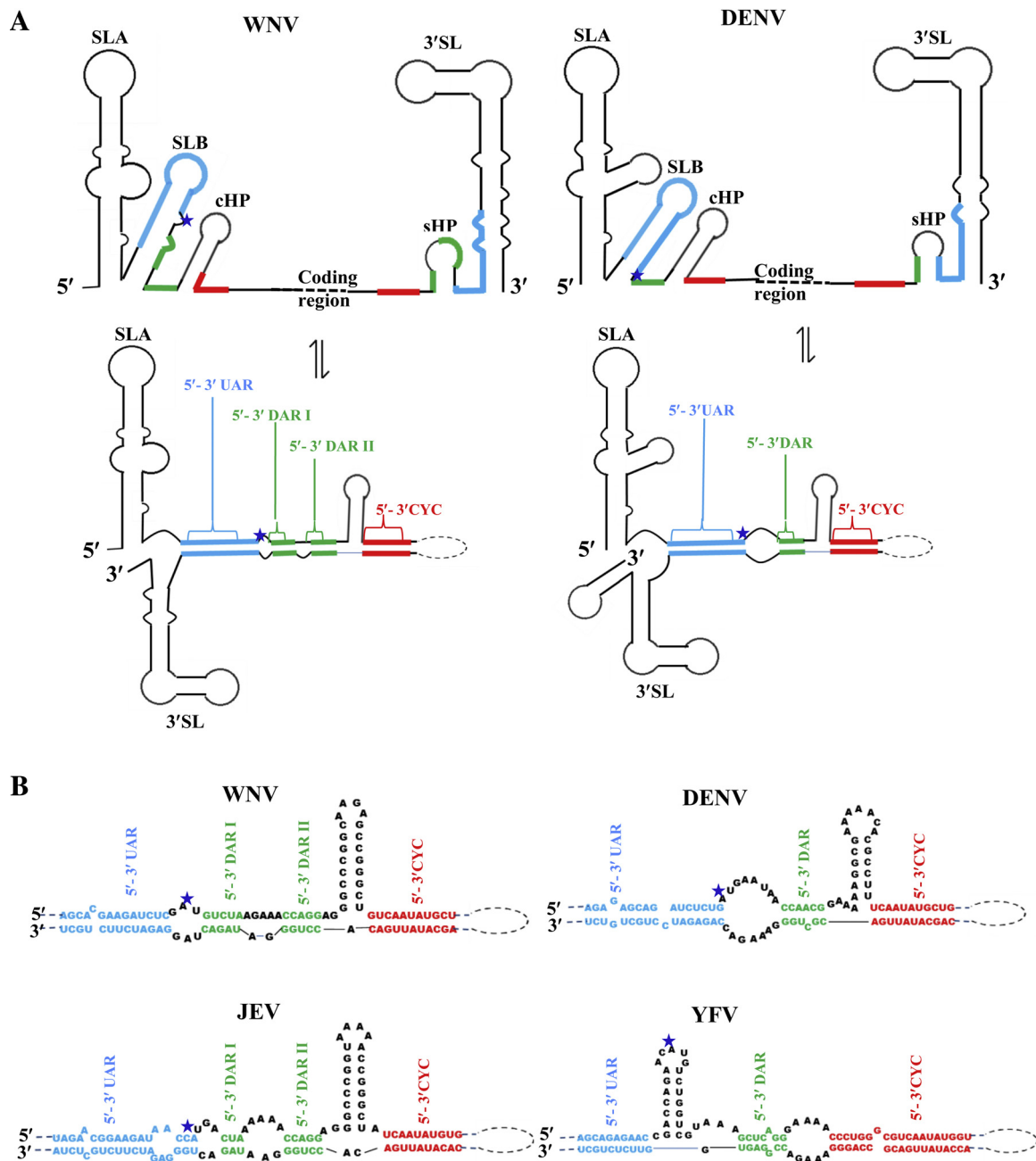


Fig. 2. The flavivirus genome 3'–5' long distance interaction. (A) The structures of the “linear” and “cyclized” genomes of WNV and DENV. (B) Examples of 3' and 5' UAR, DAR and CYC sequence pairing for WNV, JEV, DENV, and YFV.

et al., 2007). The complex multiple step process required to initiate the synthesis of minus strand RNA appears to be important for preferentially limiting the levels of minus strand RNA and also for keeping the level of total intracellular viral RNA low during the initial stages of the virus life cycle when sufficient viral protein to counteract the cell interferon response has not yet been translated.

4. Additional conserved 3' NCR sequences

The remainder of the mosquito-borne flavivirus 3' NCR contains several conserved direct repeat sequences including conserved sequence 2 (CS2) and repeated conserved sequence 2 (RCS2) located 5' of CS1 as well as CS3 that is followed by RCS3 (Fig. 1). Sequence alignments suggest the flavivirus 3' NCR evolved by

multiple duplications of an ancient RNA motif homologous to a long repeat sequence identified in tick-borne flaviviruses and that the short direct repeat sequences remaining in the 3' NCRs of current mosquito-borne flaviviruses are evolutionary remnants of this ancient long repeat sequence (Olsthoff and Bol, 2001; Gritsun and Gould, 2007a,b; Gritsun et al., 2014). The number of remnant repeat sequences in the 3' NCR varies in different flavivirus genomes.

The 5' end of the 3' NCR is known as the variable region (VR) (Beasley et al., 2001). The length and sequence of this region varies significantly between different arthropod-borne flaviviruses (Kato et al., 2012). One to three copies of a repeated conserved sequence (RYF) are present in the VRs of different natural isolates of YFV and in some non-vector flaviviruses (Gritsun and Gould, 2007a,b). Spontaneous deletions in this region occur during passage of flaviviruses

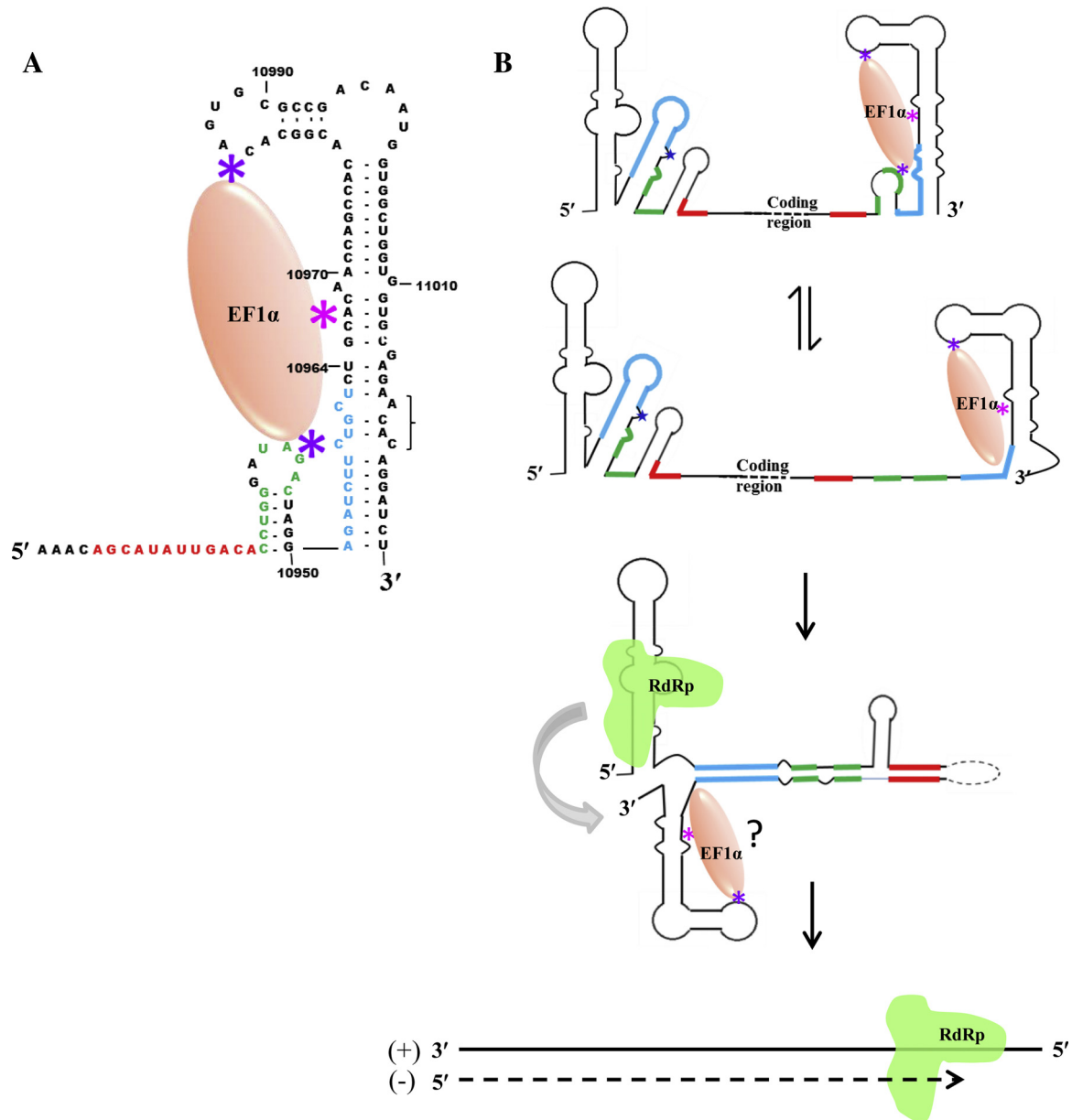


Fig. 3. The cell protein eEF1 α interacts with three sites on the 3' terminal genome RNA structures and facilitates minus strand synthesis. (A) eEF1 α interacting with the WNV 3' SL and sHP. The eEF1 α interaction regions on the viral RNA are indicated by asterisks. A metastable cis-acting element in the 3' SL is indicated by a bracket. The sequences involved in 3'–5' long distance RNA pairing are indicated by colors: UAR (blue), DAR (green), and CYC (red). (B) The binding of eEF1 α to the 3' terminal region may further destabilize the metastable element facilitating the opening of the bottom of the 3' SL and leading to the initiation of 3'–5' base pairing. The 5' SLA functions as the promoter for the initiation of the synthesis of a single minus strand RNA.

in the laboratory (Gritsun et al., 1997; Mandl et al., 1998; Mutebi et al., 2004). The longer VRs of natural isolates suggest increased length may have a selective advantage during infections in vector or host species. However, a shorter VR in a tick borne encephalitis genome was associated with higher virulence in a mouse model (Sakai et al., 2014). The VR of mosquito-borne flaviviruses contains an A-U rich region that is thought to have evolved due to the RdRp stuttering on the UAA stop codon. The length of the A-U rich region varies among arthropod-borne flaviviruses with the WNV and Murray Valley encephalitis virus genomes having the longest A-U rich regions (Gritsun and Gould, 2006). The functional roles of the VR region or the A-U sequence in the flavivirus lifecycle are not currently known. A possible role is that this relatively flexible and unstructured region facilitates the folding of downstream 3' NCR conserved RNA structures.

5. Conserved RNA structures in the terminal regions of the genome

The sequences at both the 3' and 5' ends of flavivirus genomes form conserved RNA secondary structures (Fig. 1). The 5' terminal nts form SLA (Brinton and Dispoto, 1988; Dong et al., 2008; Lodeiro et al., 2009; Polacek et al., 2009a). Additional conserved 5' RNA structures include SLB and the capsid coding region hairpin (cHP) (Dong et al., 2008; Polacek et al., 2009a). A conserved capsid-coding region 1 (CCR1) RNA structure was reported to be located on the 5' side of the 5' CYC and may facilitate virion assembly (not shown in Fig. 1) (Groat-Carmona et al., 2012). The genome has a terminal 3' SL but not a 3' terminal poly(A) tract. One function of the terminal 3' SL is protection of the RNA from 3' exonuclease digestion. The predicted secondary structures of the flavivirus 3' SL and the adjacent

small hairpin (sHP) (Fig. 1) have been confirmed by structure probing (Brinton et al., 1986), NMR spectrometry (Davis et al., 2013) and selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) (Sztuba-Solinska et al., 2013). Deletion of either the 3' SL or the 5' SLA is lethal for flavivirus infectious clones (Lai et al., 1992; Cahour et al., 1995; Yu and Markoff, 2005). The 3' UAR sequence overlaps the 3' SL and both the 3' UAR and 3' DAR sequences overlap the 3' sHP (Fig. 2A). The 5' UAR overlaps SLB while the 5' DAR(s) is located either within SLB and/or 5' of it (Fig. 2A). A conserved U rich linker is located between SLA and the 5' UAR sequence and is thought to provide flexibility that facilitates the structural changes occurring during switches between linear and cyclized forms of the genome. The start codon for the polyprotein is located on the 3' side of SLB. The DENV 5' CHP was reported to be involved in the selection of the start codon used (Clyde and Harris, 2006).

A low-temperature transition observed with a truncated WNV 3' model RNA consisting of the lower part of the 3' SL and the sHP was initially attributed to the melting of a putative pseudoknot between nts in the sHP loop and ones on the 5' side of the 3' SL (Shi et al., 1996a). However, in a subsequent study, a low-temperature transition was also observed with longer 3' SL RNAs containing mutations that abolished the predicted pseudoknot interaction. A region near the bottom of the 3' SL consisting of two conserved, *cis*-acting base pairs flanked below by a C/C bulge and above by a C/A bulge in the WNV 3' SL were shown to be responsible for the low temperature transition (Fig. 3A) (Davis et al., 2013). Substitutions that maintained the predicted secondary structure in this region but changed the primary sequence or orientation of either the U-A or G-C base pairs located between the bulges negatively affected virus replication. An exception was the substitution of the G-C pair with a G-U pair which had little effect on virus replication. Neither of the U-A base pairs located above and below this region was detected by the NMR analysis indicating that these interactions are weak. Mutation of the C/A bulge or adjacent nts was also detrimental to virus replication. These findings support the hypothesis that this region is metastable and imparts functionally important conformational flexibility to the lower part of the 3'-terminal SL. Adjacent, *cis*-acting U-A and G-C base pairs flanked below and above by bulges located near the bottom of the 3' SL appears to be a common feature of flavivirus genomes (Silva et al., 2007; Iglesias and Gamarnik, 2011). The lower of the two bulges consistently begins 7 bp from the bottom of the 3' SL and structure probing of both the WNV and DENV 3'-5' RNA-RNA long-distance interactions (Alvarez et al., 2005b; Dong et al., 2008) indicated that the 3' end of the 3' UAR sequence is located one nt above the upper bulge of the metastable region. Base pairing in the 3' SL above the metastable region does not change during formation of the 3'-5' RNA-RNA interaction (Figs. 2A and 3A). The conservation of both the position and characteristics of the metastable 3' SL structural feature consisting of small, symmetrical bulges flanking two conserved base pairs in divergent flavivirus RNAs and the location of this feature at the upper end of the region of the 3' SL that must unpair to facilitate formation of the long distance 3'-5' RNA-RNA interaction strongly suggest that this metastable feature is involved in regulating the switch from the 3' SL and CHP to the 3'-5' RNA-RNA interaction. Mutations that disrupted base pairing at the bottom of the 3' SL were lethal suggesting that the opening of the base pairs at the bottom of the 3' SL must occur as part of a coordinated mechanism during the formation of the 3'-5' RNA-RNA interaction (Davis et al., 2013).

The 3' sHP and the 5' CHP are present only in the non-cyclized "linear" form of the flavivirus genome because the nts forming the stems of these structures are involved in alternative long distance base pairs in the cyclized genome RNA (Fig. 2). Deletion of the whole sHP or mutation of a particular sHP loop nt in a WNV infectious clone or introduction of mutations disrupting the sHP stem

in a dengue infectious clone are lethal (Davis et al., 2013; Villordo and Gamarnik, 2013). However, mutation of sHP stem nts that disrupted base pairing of only the sHP stem or of only the 3'-5' DAR interaction negatively affected, but did not abolish, virus replication and spontaneous revertants with restored pairing in both contexts were generated (Clyde et al., 2008; Villordo et al., 2010; Iglesias and Gamarnik, 2011). These findings suggest that the correct balance between the "linear" and circular forms of the genome RNA is required for efficient viral replication. Some nt substitutions in the loop and stem of a DENV sHP had a greater negative effect on virus replication in C6/36 mosquito cells than in mammalian cells (Villordo and Gamarnik, 2013) suggesting that this region of the RNA may be involved in differential protein interactions in the cells of mosquito and mammalian hosts (Villordo and Gamarnik, 2013).

6. Additional conserved RNA structures in the 3' NCR

One or two conserved "dumbbell-like" (DB) or "Y-shaped" secondary structures are located in the 3' NCR upstream of the CYC sequence (Gritsun et al., 2014). Mosquito-borne flaviviruses have two copies, a 5' DB and a 3' DB (Fig. 1). The conserved RCS2 and CS2 sequences overlap one of the loops of the 5' DB and 3' DB, respectively. Although formation of pseudoknots between 5 nts in the top loop of each dumbbell structure and a complementary 3' sequence was predicted (PK3 and PK4) (Olsthoorn and Bol, 2001) and initially analyzed by structure probing using a DENV4 3' NCR RNA (Romero et al., 2006), a recent SHAPE analysis of a DENV2 3' NCR RNA provided support for the existence of only the PK3 interaction of the 5' DB (Sztuba-Solinska et al., 2013). Deletion of an entire DB region had a greater negative effect on virus replication than deletion of CS2 or RCS2 (Alvarez et al., 2005a). The DB structures and pseudoknot sequences are not essential for replicon replication but function as replication enhancers (Funk et al., 2010; Manzano et al., 2011). Deletion of the top SL of the 3' DB ($\Delta 30$) in a DENV4 infectious clone generated a virus that was attenuated in humans but still highly immunogenic and showed restricted replication in mosquitoes (Durbin et al., 2001; Troyer et al., 2001). Although deletion of the same nts in DENV1 produced an immunogenic, attenuated virus, deletion of the same region did not significantly attenuate either DENV2 or DENV3 (Blaney et al., 2004a,b; Durbin et al., 2006; Blaney et al., 2008).

Mutation of nts in the top loop of the 5' DB that disrupted the predicted PK3 interaction did not affect replicon translation and similar mutations in the 3' DB terminal loop (PK4) had only a slight negative effect. However, mutation of both of these sequences reduced translation by 60% (Manzano et al., 2011). Additional data suggested that non-canonical translation of viral RNA occurring in the presence of an inhibitor of translation initiation factor 4E (eIF4E) was facilitated by the presence of both DB pseudoknot interactions. Another study reported that mutation of the CS2 and RCS2 sequences negatively affected translation (Wei et al., 2009). The variable findings of experiments testing the functions of the DB structures, pseudoknots and CS2/RCS2 sequences could be due to differential effects of the mutations or deletions tested on genome folding in other regions of the genome.

The numbers and types of RNA structures and conserved sequences in the variable region located 5' of the dumbbell structures vary among different subgroups of flaviviruses (Olsthoorn and Bol, 2001; Gritsun and Gould, 2007a,b; Gritsun et al., 2014; Selisko et al., 2014). In WNV genomes, four additional conserved SLs (SL-I, SL-II, SL-III, and SL-IV) are located 5' of the dumbbell structures (Fig. 1). The top loop sequences of SL-II and SL-IV are involved in predicted conserved pseudoknot interactions (PK1 and PK2, respectively). The conserved sequences CS3 and RCS3 are located in the lower loop of SL-IV and SL-II, respectively. In WNV

genomes, SL-II stalls the cellular 5'–3' exoribonuclease XRN1 as it digests the genome RNA from the 5' end, generating the 3' subgenomic flavivirus RNA (sfRNA) (Funk et al., 2010; Silva et al., 2010). When present, SL-IV serves as a backup “stop” for XNR1 when SL-I is absent (Funk et al., 2010; Chapman et al., 2014). The functions of sfRNA have been recently reviewed by Clarke et al. (2015).

7. 3'-Terminal minus strand RNA structure

The 3' terminal nts of the minus strand RNA form a conserved SL structure (Brinton et al., 1986; Shi et al., 1996b). Although the nts at the 3' end of the minus strand and the 5' end of the genome are complementary, the SL structures formed by these sequences differ due to the formation of G–U base pairs (Figs. 1 and 4). Because minus strand RNA is found only in replication intermediates either base paired with the genome template or associated with multiple nascent elongating genomes in infected cells, the 5' sequence of the minus strand is not thought to be free to fold into RNA structures. However, a 5' (–)RNA probe was reported to bind to the cell protein hnRNP A2 (Katoh et al., 2011).

8. Cell protein involvement in unpairing of the stem at the bottom of the 3' SL

Initiation of minus strand RNA synthesis occurs after the bottom portion of the 3' SL stem and the 3' sHP stem have opened followed by long distance pairing of 3' and 5' sequences (Fig. 3). As discussed in Section 3, a conserved metastable region in the 3' SL is involved in regulating the opening of the lower part of the stem (Davis et al., 2013). Cell proteins binding to the genome 3' NCR and/or to a viral replication complex protein may function to facilitate the initiation of minus strand viral RNA synthesis. A number of different cell proteins have been reported to bind to a 3' NCR flavivirus probe including La, PTB, PABP, NF-9 FBP1, DDX5, eEF1A, TIAR/TIA-1 (De Nova-Ocampo et al., 2002; Polacek et al., 2009b; Chien et al., 2011; Gomila et al., 2011; Li et al., 2013; Albornoz et al., 2014) but the region of the 3' NCR to which these proteins bound was not mapped and some of these proteins also bound to additional flavivirus RNA terminal sequences (De Nova-Ocampo et al., 2002; Yocupicio-Monroy et al., 2007; Li et al., 2014) as well as to some of the viral proteins (Li et al., 2013, 2014). Additional studies identified cell proteins binding to particular regions within the flavivirus 3' NCR. La, MOV34, YB-1, P100 (NF-κB2), PTB, and eEF1a (Blackwell and Brinton, 1997; Ta and Vratil, 2000; Kim and Jeong, 2006; Davis et al., 2007; Paranjape and Harris, 2007; Lei et al., 2011; Vashist et al., 2011) were reported to bind to a flavivirus 3' SL, DDX6 was reported to bind to the 5' DB and 3' DB (Ward et al., 2011) and Caprin 1, G3BP1/2 and USP10 were reported to bind to the VR of the 3' NCR (Ward et al., 2011).

A number of technical issues complicate studies attempting to demonstrate a direct functional contribution of a viral RNA–cell protein interaction to viral replication. In some cases, protein binding sites are located in hairpin loops but in other cases, secondary and sometimes also tertiary RNA structures are critical features in protein binding sites on viral RNA. Protein–RNA interactions typically occur by an induced fit mechanism that can alter either or both the protein and RNA structures (Williamson, 2000). Although a higher affinity of a cell protein for a viral RNA may be needed for effective competition of the viral RNA with the host RNA binding partner, RNA–protein interactions that are involved in viral RNA synthesis initiation cannot be too strong since they must be functionally dynamic. Attachment of an RNA probe to an affinity column only at one end is preferable to attaching probes with biotin incorporated along their length for preserving the RNA structure. Also, mutation, deletion or truncation can alter RNA structures in probes

or genomes used to map the location of interacting sites. Because it is possible that cell proteins identified from pull down assays may not bind directly or specifically to the viral RNA, when possible, follow up *in vitro* binding analyses are needed to test the specificity of the viral RNA–cell protein interaction and to map the binding sites so that the effect of mutating these sites on virus replication can subsequently be tested. However, low concentrations of protein and probe need to be used to prevent non-specific interactions in the *in vitro* assays. Also, a mammalian protein expressed in bacteria may not behave as the protein does in mammalian cells due to the lack of post-translational modifications. Expressed recombinant RNA-binding proteins may already be bound to RNA. Testing the effect on virus yields of knockdown of a candidate viral RNA-binding cell protein indicates whether the cell protein is important for efficient virus production but does not distinguish between effects due to a direct viral RNA–cell protein interaction and secondary effects due to reducing cell functions of the protein that are needed for efficient virus replication.

Three cell proteins, 52, 84 and 105 kDa, present in BHK cytoplasmic extracts were shown to bind specifically to a probe consisting of the 3' SL and the sHP of WNV in gel shift and UV-crosslinking assays done in the presence of competitor RNAs on fractions obtained by sequential chromatography of cytoplasmic extracts (Blackwell and Brinton, 1995). For only one of these cell proteins have extensive follow up studies been done. The 52 kDa protein was purified to near homogeneity by ammonium sulfate precipitation and liquid chromatography and identified as eukaryotic elongation factor 1 alpha (eEF1A) by MassSpec sequencing. The eEF1A–WNV 3' SL interaction was confirmed by Western blotting and supershift assays. In mammalian cells, eEF1A, the second most abundant protein after actin, carries aminoacylated tRNAs into translating ribosomes (Riis et al., 1990). RNA footprinting and filter binding assays identified one major (60% of the binding activity) and two minor binding sites (each ~20% of the binding activity) for eEF1A on the WNV genome 3' RNA (indicated by asterisks in Fig. 3A) (Blackwell and Brinton, 1997). The similar binding activity of eEF1A for WNV, YFV, DENV2 and TBEV 3' terminal RNA probes indicated that this interaction is conserved among the arthropod-borne flaviviruses and may also be a common characteristic of the genomes of other members of the genus *Flavivirus* (Davis et al., 2013). Nts in the WNV 3' SL protected by eEF1A in a footprinting assay identified a 5'-CACAA-3' sequence located on the 5' side of the upper stem as the major contact region for eEF1A on the 3' SL (Blackwell and Brinton, 1997). A subsequent mutagenesis study of this region of the 3' SL in a WNV infectious clone showed that a single nt bulge plus a C located one or three nts below the bulge were essential for efficient eEF1A binding in this region and that this interaction was required for efficient virus replication (Davis et al., 2007).

Substitutions in nts on the 3' side of the 3' SL that disrupted base pairing in the major eEF1A binding site increased *in vitro* eEF1A binding activity to the 3' SL RNA and also increased minus strand RNA levels in cells transfected with an infectious clone with these mutations (Blackwell and Brinton, 1997; Davis et al., 2007). Conversely, mutations in this region that decreased *in vitro* eEF1A binding to the 3' SL RNA also decreased minus strand levels in transfected cells (Davis et al., 2007). However, increased synthesis of minus strand viral RNA reduced rather than increased the amount of intracellular genome RNA and progeny virus produced. This is likely due to stronger activation of the innate immune response by the higher than normal levels of viral RNA produced at early times after infection (Scherbik et al., 2013a).

The two minor eEF1A binding sites, one in the top loop of the 3' SL that contains the PN sequence and one in the sHP loop (Fig. 3A), each account for ~20% of *in vitro* eEF1A binding activity to a wild type 3' SL probe (Blackwell and Brinton, 1997). However, mutation of some of the nts in the top loop as well as deletion of the entire

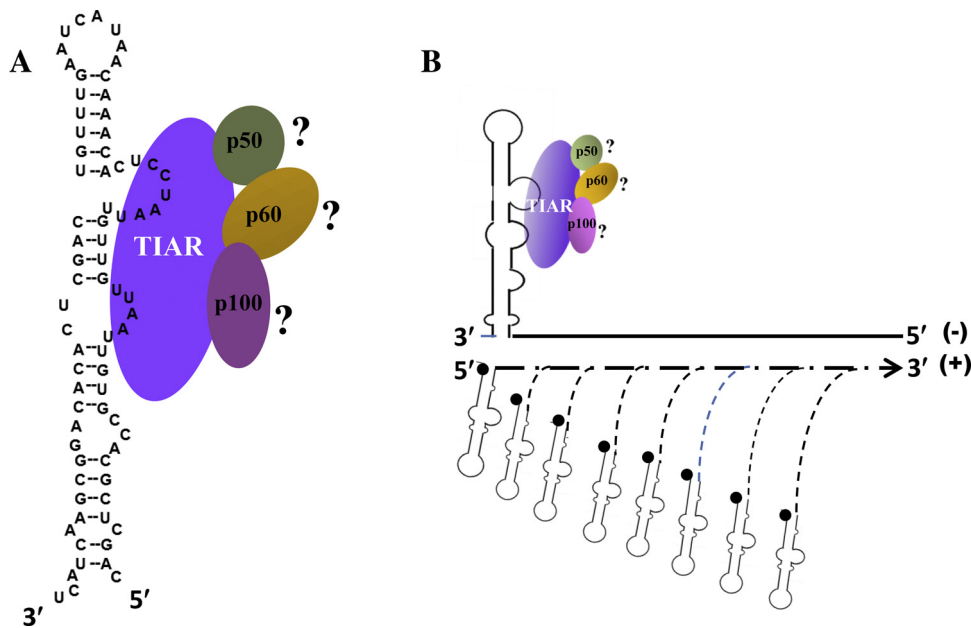


Fig. 4. Efficient genome RNA synthesis occurs within replication vesicles from a minus strand template. (A) Four cell proteins including TIAR bind to the 3' SL of a WNV minus strand RNA. (B) The 5' SLA of nascent genome RNAs may form as part of the capping process. The released 3' end of the minus strand template would then fold into the terminal RNA structure. The binding of a complex of cell proteins including TIAR to the 3' SL of the minus strand template may enhance recruitment and possibly also, the positioning of NS5 for efficient reinitiation of genome RNA synthesis. The 5' cap of nascent genomes is indicated by a black circle.

SHP or of G87 (counting from the 3' end of the genome) in the loop of the SHP were lethal indicating that both of the minor binding sites are functionally important (Davis et al., 2013). A G87C substitution in an infectious clone was also lethal and decreased *in vitro* eEF1A binding activity to the 3' SL RNA by 53%. Substitution of G87 with an A or U reduced eEF1A binding by 42% and 34%, respectively. Genomes with either of these two substitutions replicated poorly but were able to carry out a sufficient amount of viral RNA synthesis to generate revertants with the parental G. These data support the involvement of the interaction of eEF1a at several sites on the flavivirus 3' SL in facilitating minus strand RNA synthesis. Interaction of eEF1A with the genome 3' SL may occur as an occasional event when an eEF1A is released from a ribosome involved in translating the genome ORF as it nears the stop codon. Whether or not eEF1A remains associated with the genome 3' SL after the bottom of the stem has opened and the 3'–5' RNA–RNA interaction has formed is not known. Co-immunoprecipitation data suggested the possibility that eEF1A may be able to interact with NS3 and maybe also with NS5 (Davis et al., 2007). If eEF1A does remain attached to the top part of the 3' SL in the cyclized genome, it might help to recruit NS5 to the 5' SLA after the 3'–5' RNA–RNA interaction forms. The 3'–5' RNA–RNA interaction inhibits new translation initiation. eEF1a is not expected to be required for initiation of genome synthesis from the minus strand RNA template and minus strand RNA amplification decreases as exponential genome RNA synthesis begins inside the replication vesicles.

9. Cell protein involvement in facilitating the 3'–5' RNA–RNA interaction

It has been hypothesized that the 3' and 5' ends are far apart in the context of a “linear” flavivirus genome. This may or may not be true. Whole genome RNA fold data suggest that internal base pairing may bring the two ends relatively close together in the “linear” form in the absence of the 3'–5' UAR–DAR–CYC RNA–RNA interactions. The involvement of viral or cell proteins in facilitating the 3'–5' RNA–RNA interaction has been suggested. Binding of the promiscuous RNA-binding cell protein La (Vashist et al., 2011), the

viral C protein (Ivanyi-Nagy and Darlix, 2012) and the viral NS3 protein (Gebhard et al., 2012) to both the 3' and 5' RNA ends of flavivirus genomes has been reported. However, unless the proteins binding to each end of the RNA can interact with each other or one protein can bind simultaneously to both the 3' and 5' RNAs this does not seem to be a viable mechanism.

10. Cell protein involvement in exponential production of genome RNA

A single minus strand is copied from a genome template generating a dsRNA (Fig. 3B) (Chu and Westaway, 1987). As viral proteins facilitate ER membrane invagination, viral dsRNA is recruited into the forming replication vesicles. Initiation of genome synthesis from the minus strand template in the dsRNA complex is inefficient when it is located outside the replication vesicles but becomes very efficient inside these vesicles with rapid reinitiation of the minus strand template resulting in exponential amplification of genome RNA (Fig. 4B) (Chu and Westaway, 1987). How this rapid reinitiation is regulated is not known.

The first step likely involves freeing the 3' terminal sequence of the minus strand so that it can form the terminal SL structure that is needed for interaction with viral and cell proteins (Fig. 4). How the release of the 3' end of a minus strand template from the dsRNA is accomplished is not known. One possibility is that the binding of an NS5 MTase to the 5' terminal nts of nascent plus strands as part of the capping process may result in the formation of SLA at the 5' end of each nascent genome and this would release the 3' end of the minus strand template. The rapid reinitiation of genome RNA from the minus strand template inside the replication vesicles may be facilitated by cell proteins present in the vesicles that bind to the 3' SL of the minus strand. TIAR/TIA-1 and GAPDH have been identified as cell proteins that bind preferentially to the 3' SL of the flavivirus minus strand RNA (Li et al., 2002; Yang et al., 2009).

Four cell proteins, p108, p60, p50, and p42, were reported to bind specifically to the 3' terminal SL of the WNV minus strand RNA (Shi et al., 1996b) and the 42 kDa protein was identified as TIAR by MassSpec sequencing after purification on an adipic acid

RNA affinity column (Li et al., 2002). The closely related protein, TIA-1, was also shown to bind to the WNV minus strand 3' SL. These proteins bind to AU-rich sequences in the 3' NRCs of some cell mRNAs, are expressed in most tissues and shuttle between the nucleus and cytoplasm of infected cells (Taupin et al., 1995; Dember et al., 1996; Dixon et al., 2003; Cok et al., 2004). The binding sites for these proteins on the WNV minus strand 3' SL were mapped to two 5 nt AU sequences in the two side loops (Fig. 4A) (Emara and Brinton, 2008). Deletion or C substitution of the AU-rich sequences in either loop in a WNV infectious clone was lethal and partial deletion or substitution of these sequences reduced the efficiency of virus replication. Decreased protein binding efficiency to mutant 3'(-)SL RNAs *in vitro* consistently correlated with decreased plus strand RNA synthesis and virus production in cells transfected with infectious clones containing the same mutations but neither minus strand RNA levels nor viral protein levels were decreased. The data strongly suggest that the interaction between TIA-1/TIAR and the 3' terminal SL of the WNV minus strand RNA facilitates exponential genome RNA synthesis from the minus-strand template.

11. Cell protein involvement in viral RNA translation

After release of an incoming flavivirus genome into the cytoplasm, the genome is translated to produce a polyprotein that is processed by cell and viral proteases into the mature viral proteins. The viral nonstructural proteins must be synthesized before viral RNA replication can begin. During the early phase of the virus life cycle, the few genome RNAs present alternatively function as mRNA for translation and as templates for minus strand RNA synthesis. The "linear" form of the genome is used for translation while the "cyclized" form with the 3'–5' long distance RNA–RNA interaction is required for minus strand RNA initiation (Khromykh et al., 2001; Corver et al., 2003; Lo et al., 2003; Alvarez et al., 2005a). Genome translation predominates at this stage of the viral replication cycle so that sufficient amounts of viral proteins accumulate to counteract the cell innate responses and to remodel ER membranes to form replication vesicles. The low levels of membrane-associated viral RNA present early in the replication cycle reduce detection by the cytoplasmic RNA sensors (Courtney et al., 2012; Scherbik et al., 2013a). The cell protein far upstream element (FUSE) binding protein 1 was reported to bind to both the JEV 3' and 5' NCRs and to function as a suppressor of viral translation (Chien et al., 2011) while the cell protein DDX3 was reported to bind to NS3 and NS5 as well as to both the JEV 3' and 5' NCRs and to positively regulate viral translation.

A type 1 cap structure (m⁷GpppAmp) is added to the 5' end of nascent genomes by NS5 which has guanylyltransferase as well as N7 and 2'-O-S-adenosyl methionine MTase activities in its N-terminal domain (Ray et al., 2006; Zhou et al., 2007; Issur et al., 2009). The NS5 MTase interacts with a set of mapped contact sites on the 5' SLA RNA to carry out N7 methylation and then repositions on the 5' RNA to complete the 2'-O-methylation step (Egloff et al., 2002; Dong et al., 2007; Egloff et al., 2007; Dong et al., 2008). The 2'-O methylation modification of the flavivirus cap protects genomes from restriction by innate immune response IFIT family members (Daffis et al., 2010) and N7 methylation functions to enhance viral translation (Dong et al., 2014).

Most flavivirus infections do not induce stress granules which would inhibit cap-dependent translation (Courtney et al., 2012; Tu et al., 2012). The cell eIF2 α kinase PKR is not activated by viral RNA in infected cells which is likely due to the low levels of membrane-associated viral RNA present during initial stages and sequestering of the viral RNA replication complexes in ER membrane vesicles

at later stages (Elbahesh et al., 2011). Flavivirus NS2A was also reported to be able to block PKR activation (Tu et al., 2012) and the JEV capsid protein was reported to inhibit stress granule formation through interaction with the cell protein Caprin-1 (Katoh et al., 2013). At later times, a large pool of structural proteins must be produced to assemble nascent virions. It is not known how flavivirus genome RNA translation efficiently competes with cell mRNA translation. Dephosphorylation of p70S6K and 4E-BP1 in infected cells was detected starting at 24 h after infection (Villas-Boas et al., 2009) and cap-independent translation of genome RNA was observed when eIF4E levels were low (Edgil et al., 2006) **suggesting that flavivirus RNA translation may switch to an as yet unknown cap-independent mechanism at later times of infection.** Both the 3' and 5' NCRs of the flavivirus genome were shown to be required for cap-independent translation. Mutagenesis/deletion studies have implicated the conserved CS2 and RCS2 and/or the DB structures in the regulation of flavivirus translation (Wei et al., 2009; Manzano et al., 2011). Cell proteins that bind near the 3' end of the genome and modulate translation efficiency have been reported. The cell protein YB-1 binds to the DENV 3' SL and functions as a negative regulator of genome translation (Paranjape and Harris, 2007). Binding of TIA-1 to the 3' NCR of tick borne encephalitis virus was reported to negatively affect genome translation (Albornoz et al., 2014). DDX5 and DDX3 have been reported to bind to both the 3' NCR and the 5' NCR and to positively regulate genome translation (Li et al., 2014). Poly(A) binding protein (PABP) binds to an unmapped region of the 3' NCR located 5' of the dumbbell RNA structures and facilitates *in vitro* translation (Polacek et al., 2009b). However, deletion of the entire variable region of the tick borne encephalitis virus 3' NCR, including the AU-rich region, had no effect on translation (Hoenninger et al., 2008).

12. Other functions of flavivirus 3' and 5' NCRs

It has not been possible to maintain many of the full length flavivirus infectious clones in bacteria. A cryptic bacterial promoter was identified in the 5' NCR region of a full length DENV infectious clone (Li et al., 2011). The N-terminally truncated polyprotein translated in bacteria from mRNA transcribed from this promoter was initiated at an in-frame AUG located downstream of the one typically used for polyprotein translation.

A 5' terminal fragment of WNV containing SLA, SLB and cHP was shown to bind to a recombinant p42 isoform of human 2'–5' oligoadenylate synthetase (Oas1) and activate 2'–5' oligo A synthesis *in vitro* (Deo et al., 2014). 2'–5' oligo A binds to inactive RNase L in the cytoplasm activating RNase L to dimerize and cleave cell and viral single-stranded RNA. Activation of this pathway has an antiviral effect on flavivirus infections. However, in mouse embryo fibroblasts, low levels of RNase L activation were observed only at late times after infection with WNV (Scherbik et al., 2006). Stronger RNase L activation by a DENV infection was observed in cells transiently overexpressing p42 (Lin et al., 2009). Mice lacking RNase L had higher virus titers in the CNS and an increased mortality compared to wild type mice after footpad inoculation with WNV but they were less susceptible than PKR^{–/–}, RNase L^{–/–} mice (Samuel et al., 2006).

Intracellular RNA from WNV-infected cells was shown to contain two types of pathogen-associated molecular patterns (PAMPs), 5' triphosphate (cleaved RNA fragments and uncapped full length genome RNA) and dsRNAs, that were able to activate the cytoplasmic sensors RIG-I and MDA5 (Errett et al., 2013). In this study, no activation was detected after transfection of 3' or 5' genome RNA fragments. A different study found that several different short, *in vitro* transcribed WNV genome fragments were able to induce low level activation of RIG-I after transfection into cells, with the 5'

genome fragment showing the highest level of activation (Shipley et al., 2012). However, when these regions were part of a longer segment of viral RNA they induced much less RIG-I activation.

In addition to its essential function in translation elongation, eEF1A has multiple other cell functions (Mateyak and Kinzy, 2010). One of these functions is enhancement of sphingosine kinase 1 (SphK1) enzymatic activity through protein-protein interaction (Leclercq et al., 2008, 2011). Initial studies in DENV2-infected cells showed that TNF- α was not able to activate pro-survival nuclear factor kappa B (NF κ B)-driven signals and that SphK1 activity was reduced at late times after infection (Wati et al., 2007, 2011). Based on these findings it was proposed that the reduced cellular SphK1 activity was a consequence of eEF1A binding to the viral 3' SLs in both genomic and sfRNA which reduced the amount available for SphK1 binding/activation (Carr et al., 2013). However, it is not known whether eEF1A binds to the sfRNA, which accumulates with time after infection but is not translated. Binding of eEF1A to genome RNA would be expected to decrease with time after infection as minus strand synthesis decreases (Blackwell and Brinton, 1997; Davis et al., 2007).

13. Discussion

During the early stages of the infection cycle, the replication vesicles have not yet formed and inefficient viral RNA replication occurs in association with ER membranes. Genome translation is predominant over replication at this stage to produce sufficient proteins to remodel the ER membranes and counteract the cell innate responses and to keep viral RNA levels low to avoid detection by the cell RNA sensors (Courtney et al., 2012; Scherbik et al., 2013b). eEF1A brings charged tRNAs to ribosomes involved in translating the viral genome ORF and occasionally, an eEF1A leaving a ribosome located near the end of the genome ORF may bind to the 3' SL. Even though the terminal region nts involved in 3'–5' base pairing have been identified, little is known about the mechanisms regulating the formation of the long distance interaction. However, the finding that eEF1A binds to three sites on the 3' RNA structures and that the interaction between eEF1A and the WNV 3' SL facilitates viral minus strand synthesis strongly suggest that eEF1A is involved in mediating the transition from terminal region base pairing to 3'–5' RNA–RNA base pairing. The conservation of a cis-acting metastable structural feature in the terminal 3' SLs of divergent flaviviruses located at the top of the region that unpairs to form the long distance 3'–5' RNA–RNA interaction suggests that this structural feature is also involved in regulating the switch from a 3' SL RNA–RNA interaction to a 3'–5' RNA–RNA interaction. Interaction of eEF1A with the 3' SL may initiate the structural changes in the 3' SL. Alternatively, the pairing of the 3'–5' CYC sequences by an unknown mechanism may occur prior to the opening of the bottom of the 3' SL. eEF1A bound to the 3' SL may also help to recruit NS3 and/or NS5 to the 3' end of the cyclized genome. The formation of the 3'–5' RNA–RNA interaction would inhibit further translation initiation leading to the clearing of ribosomes from the cyclized genome. Because ribosomes and eEF1A are not expected to be present in the viral replication vesicles, nascent genomes being produced there would not become cyclized and consistent with this, minus strand synthesis decreases as genome synthesis increases later in the replication cycle. The complexity of the mechanism required to create a template for the initiation of a single minus strand is likely to be the main factor limiting the amount of minus strand RNA made. Functionally important terminal flavivirus RNA structures, viral sequences involved in long distance 3'–5' RNA interactions and a few cell proteins involved in regulating viral RNA synthesis have been identified but there is still much that is not known about the detailed mechanisms regulating the switch

from genome terminal region base pairing to long distance 3'–5' sequence pairing and the possible involvement of terminal region conserved sequences and RNA structures in regulating early versus late genome RNA synthesis, viral genome translation and possibly also, genome localization and encapsidation. Viral and cell protein interaction partners of the genome RNA may also be involved in regulating these additional mechanisms. However, the likelihood of multiple overlapping functions for terminal region RNA structures and sequences is expected to make study of these mechanisms challenging.

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