



Universiteit
Leiden
The Netherlands

Functions and requirements of conserved RNA structures in the 3' untranslated region of Flaviviruses

Agostinho Gonçalves Costa da Silva, P.

Citation

Agostinho Gonçalves Costa da Silva, P. (2011, June 27). *Functions and requirements of conserved RNA structures in the 3' untranslated region of Flaviviruses*. Retrieved from <https://hdl.handle.net/1887/17775>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/17775>

Note: To cite this publication please use the final published version (if applicable).

ABSTRACT

Flaviviruses are small enveloped viruses with a positive, single-stranded RNA genome of approximately 11 kb in length, with a 5' cap structure and a 3' non-polyadenylated end. The Flavivirus genus has been divided into three different clusters that correlate with the vector that is used for their transmission: i) mosquito-borne, ii) tick-borne, and iii) no known vector flaviviruses. The 3' untranslated region (UTR) of flaviviruses can be roughly divided into a proximal part, which exhibits extensive heterogeneity in both length and sequence and is present immediately downstream of the stop codon of the open reading frame, and a more conserved distal part that has been defined as the core element of the 3' UTR as it contains the majority of the elements involved in viral translation, replication, and assembly. A number of small but well conserved RNA sequence elements as well as secondary and tertiary RNA structures have been identified in the flaviviruses 3' UTR. Some of these have been recognized in all flaviviruses studied thus far, whereas others are characteristic for a particular cluster of the genus. This review describes the characteristics and function of conserved RNA sequences and structures in the 3' UTR of all three Flavivirus clusters. For this purpose, the flavivirus 3' UTR was divided into five domains in a 3'-to-5' direction according to the position in the genome, sequence and structural similarity. Special emphasis has been given to the RNA structures that were reported to play a role in viral replication and pathogenicity.

INTRODUCTION

Positive-strand RNA viruses are unique in the viral world as their genome serves a dual role as both mRNA and as a template for minus-strand RNA synthesis. In general, these viral genomes are characterized by a 5' untranslated region (UTR), one or more open reading frames (ORF) and a 3' UTR. Apart from the coding information for the viral proteins present within the ORFs, the viral genome contains a substantial amount of information in the form of RNA sequences and structures that is required for RNA synthesis, translation and encapsidation. Identifying and characterizing the function of these RNA elements is important for understanding the regulation of the several, often mutually exclusive, activities in which the viral genome is involved. Research has shown that these RNA signals can in principal be anywhere in the viral genome, but that the 5' and especially the 3' UTR harbor the majority of them. This review focuses on the conserved RNA sequences and structures in the 3' UTR of viruses belonging to the genus *Flavivirus*.

The *Flavivirus* genus belongs to the *Flaviviridae* family, which also includes the *Pestivirus* and *Hepacivirus* genera¹. Flaviviruses are small enveloped viruses containing a positive, single-stranded RNA genome of approximately 11 kb in length, with a 5' cap structure and a 3' non-polyadenylated end. Nearly 80 viruses belong to the *Flavivirus* genus and many of them are considered important human pathogens, namely dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and tick-borne encephalitis virus (TBEV). Phylogenetic analysis based on the complete coding sequence divided the flaviviruses into three clusters that correlate with the vector of transmission: mosquito-borne, tick-borne and no known vector (NKV) flaviviruses^{2,3}. In general, the 3' UTR of viruses that have a similar mode of transmission show a higher similarity in terms of conserved sequences and RNA structures⁴⁻⁸. Despite these differences, certain RNA elements are, however, characteristic for the 3' UTR of every flavivirus. This review describes the characteristics and function of conserved RNA sequences and structures in the 3' UTR of all three *Flavivirus* clusters. It should be emphasized however that this review may seem rather biased towards the mosquito-borne flaviviruses; this is hard to avoid since the 3' UTR has been studied more extensively for these viruses in comparison to the NKV flaviviruses, and to a lesser extent, the tick-borne flaviviruses.

The length of the viral 3' UTR varies from approximately 350 to 800 nts depending on the virus, and it can differ even between strains of the same virus. This heterogeneity in length originates primarily from the proximal part of the 3' UTR, immediately following the stop codon of the viral ORF, where deletions, insertions, sequence repeats and even internal poly(A) tracts have been observed; in contrast, the distal part of the 3' UTR exhibits a more similar RNA topology and regions with significant sequence similarity⁹⁻¹⁵.

This distal region has been defined as the core element of the flavivirus 3' UTR in which important elements for viral translation and replication are located ⁵.

In this review, the flavivirus 3' UTR has been divided into five domains in a 3'-to-5' direction according to the position in the genome, sequence and structural similarity (fig. 1). The identified domains are usually separated from each other by U-A rich sequences predicted to be single-stranded ¹⁶.

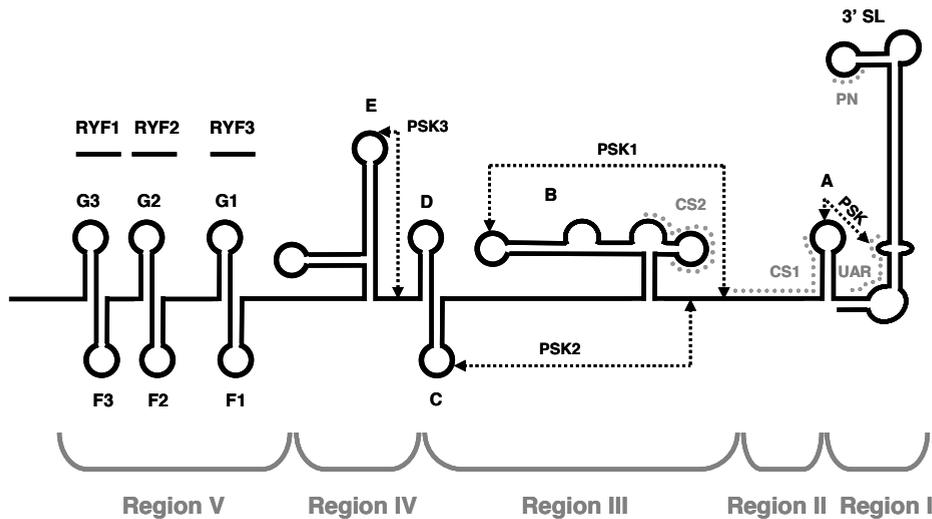


Fig. 1. Schematic model of the predicted RNA folding of the 3' UTR of the prototype flavivirus yellow fever virus (YFV). The 3' UTR was divided into five different regions. Nomenclature for the individual stem-loop (except 3' SL) and pseudoknot structures was adopted from Olsthoorn and Bol ⁶. The pseudoknot (PSK) predicted by Shi and colleagues ²² is also depicted. The pentanucleotide motif (PN), the conserved sequence 2 (CS2) and the 3' cyclization motifs CS1 and UAR (upstream AUG region) are indicated. The third 3' cyclization motif DAR (downstream AUG region) overlaps with CS1 in the stem-loop A region. The predicted pseudoknot structures (PSK) and the repeated sequences of the yellow fever virus (RYF) are also depicted.

3' UTR region I: the 3' stem-loop structures

Region I comprises the last 100 to 120 nucleotides of the viral genome. RNA structure analysis of this region revealed that it can fold into two RNA stem-loop structures. The more upstream structure is relatively small, encompassing only 14 to 20 nts, whereas the last 80 to 100 nts are predicted to form a long stem-loop structure (3' SL; fig. 1) ^{4,9,14,17-22}. Even though the general structure of 3' SL is well conserved among all flaviviruses, the sequence similarity is restricted to the pentanucleotide (PN) motif 5'-CACAG-3', that is located in the bulge at the top of 3' SL (fig. 1 and 2), and the terminal 3' dinucleotide

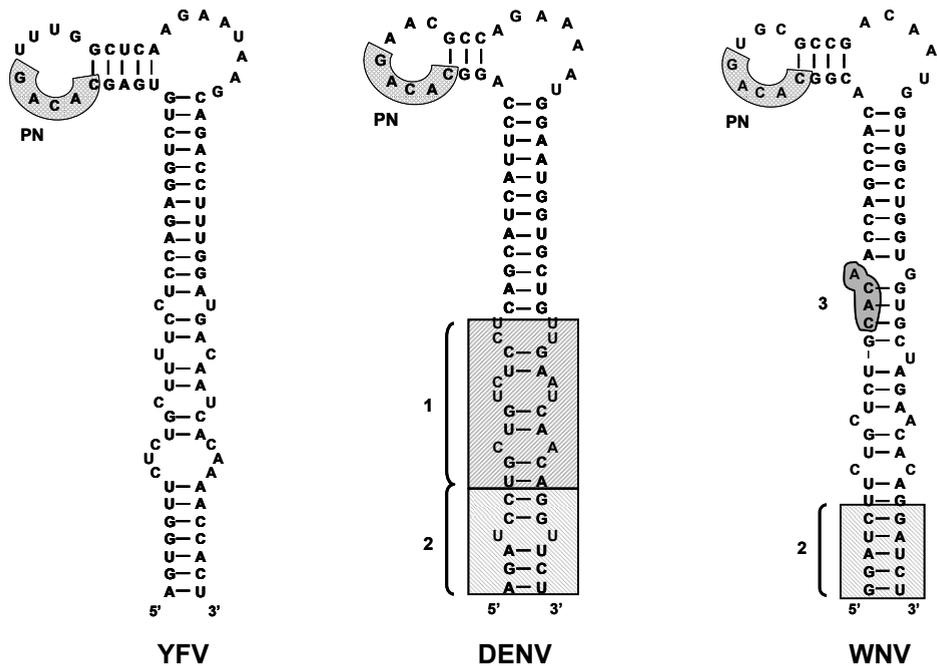


Fig. 2. Structural model of the terminal 3' SL structure of YFV, DENV and WNV. The pentanucleotide motif (PN) is indicated. The shaded box 1 represents a region important for virus viability while box 2 represents the region that is required for replication in mosquito cells. The region indicated by number 3 corresponds to the eEF-1 α -binding sequence.

5'-CU-3', which is complementary to the dinucleotide 5'-AG-3' present at the very 5' end of every flavivirus genome¹⁹⁻²¹. Limited sequence variation has been observed in the PN motif of the NKV flaviviruses, which either contain a "C" or a "U" residue at the second position. APOI virus contains an additional "C" to "U" change at the third position⁷.

Because of the absence of a poly-A tail at the 3' end of the flavivirus genome it was suggested that the 3' SL structure was functionally replacing the poly-A tail by signalling the integrity of the viral genome and therefore protecting it from degradation. Experiments using either reporters expressing WNV replicon RNAs or synthetic mRNAs containing flavivirus 5' and 3' UTRs, yielded contradicting results varying from no effect to a modest stimulation on translation by the 3' SL²³⁻²⁶, to actually inhibiting translation^{27,28}.

Deletion of the 3' SL showed that this conserved RNA structure is absolutely required for flavivirus RNA synthesis²⁹⁻³⁴. Detailed mutagenesis of the 3' SL revealed that the terminal dinucleotide 3' CU_{OH} is essential for efficient viral replication and that it probably functions as a recognition site for the replication complex to initiate RNA synthesis^{33,35}. Deletion of the conserved PN motif does not influence translation efficiency of the viral genome, but is lethal for viral RNA synthesis. Mutational analysis of the 5' CACAG 3' sequence revealed that the "G" residue at the 5th position and base pairing of the nucleo-

tide at the 1st position with a complementary nucleotide four positions downstream of the PN motif, are the most critical elements of this conserved RNA sequence. Nucleotide changes at either the 2nd or 4th positions of the WNV or YFV PN motif were shown to be well tolerated. Mutations at the 3rd position were reported to be detrimental for WNV replication, but showed only a relatively minor to no effect in YFV-17D^{25,33,36,37}. The exact role of the PN motif in flavivirus RNA replication is currently unknown. It is interesting to note that although some mutations within the PN motif seem to be well tolerated, competition experiments revealed that a virus with the wild-type PN sequence has a clear advantage over viruses with a mutated PN motif³⁷. Phosphorodiamidate morpholino oligomers (PMOs) targeted at regions including the PN motif inhibited the replication of DENV and WNV replicons^{38,39}. Interestingly, part of the loop that contains the PN motif was shown to be involved in binding eukaryotic translation elongation factor-1 α (eEF-1 α)⁴⁰. Recently, the RNA structure of the top of 3' SL was resolved by NMR for representatives of the three flavivirus groups. Surprisingly, the results implied that the structure was not well conserved and indicated clear differences in the stacking pattern of the nucleotides that form the top of the 3' SL, including the PN motif, among the different groups of flaviviruses⁴¹.

Immediately upstream the 3' SL is a smaller stem-loop structure (SL-A according to the nomenclature of Olsthoorn and Bol⁶) that comprises 14 to 18 nucleotides and that is present in every flavivirus. Biochemical and biophysical probing and *in silico* RNA modeling showed that the four nucleotides in the SL-A loop of mosquito-borne flaviviruses were involved in the formation of an RNA pseudoknot by base pairing with nucleotides in the lower part of the 3' stem of 3' SL²² (fig. 1). This pseudoknot appears well conserved and can also be predicted for TBEV and the NKV flaviviruses MMLV, MODV, RBV and APOIV. SL-A and the RNA pseudoknot were shown to be required for efficient viral RNA synthesis in *in vitro* DENV RdRp assays³⁰. Furthermore, a PMO targeting the pseudoknot interaction moderately inhibited the replication of a WNV reporter replicon³⁸. Exchanging SL-A of DENV2 by the WNV SL-A sequence was relatively well tolerated. This substitution, which is predicted to maintain the pseudoknot formation, illustrates that the formation of the structure is more important than the primary sequences²⁹. Deletion of SL-A was shown to decrease the translation efficiency of a DENV reporter construct²⁶. SL-A was suggested to be a minor binding site for the host eEF-1 α protein in WNV⁴⁰.

Previous studies demonstrated that the complete DENV and WNV 3' SL nucleotide sequences were not interchangeable^{29,34}. Specific nucleotide sequence elements within the 3' SL were found to be important for viability as illustrated by the RNA elements indicated in fig. 2 as number 1 in the DENV 3' SL²⁹, and number 3 in the WNV 3' SL (5'-CACCA-3'; the eEF-1 α -binding sequence)^{34,40}. Others were found to be important in a host cell-specific manner; for example the RNA structure represented by number 2 (fig. 2). Substitution of this sequence in DENV by the comparable region of WNV resulted in

a mutant that grew well in monkey kidney cells but was severely restricted in mosquito cells²⁹. The U-U bulge in this region of the DENV 3' SL was shown to be important for efficient replication of these WNV chimeras in C6/36 cells, suggesting that it acts as an enhancer for both DENV and WNV replication in mosquito cells, while it is dispensable for replication in mammalian cells³⁴. The results demonstrate that specific bulges and regions in the flavivirus 3' SL are critical determinants for viral RNA replication. These bulges were suggested to likely represent important binding sites for viral and cellular proteins to assemble the flavivirus replication complex^{29,34}. It should be noted that the bottom part of the right-hand side of 3' SL also contains other elements like the 3' UAR (upstream AUG region) and the 3' DAR (downstream AUG region) sequences which have been predicted to be required for cyclization of the viral genome (see a more detailed description on region II).

3' UTR region II: the cyclization sequences

Immediately upstream of the 3' SL, and partially overlapping with SL-A, is a 10 to 18 nucleotides region that is predicted to be relatively unstructured but forms the key RNA element for the initiation of viral minus-strand RNA synthesis. The importance of this region was initially suggested by Hahn et al.⁴, whom reported the presence of a conserved sequence shared by all the mosquito-borne flaviviruses. This conserved sequence was originally named CS1 (conserved sequence 1) (fig. 1). The most conserved part of CS1 is located upstream of SL-A but the functional part of this sequence is likely to involve the nucleotides of SL-A as well. The key observation has been that CS1 is complementary to a conserved sequence (5' CS) within the N-terminal coding region of the capsid protein⁴. This complementarity suggested a long-range RNA interaction that would promote circularization of the flavivirus genome resulting in the formation of a panhandle-like RNA structure required for viral RNA synthesis⁴. The critical role of CS1 in this 5'–3' RNA interaction for RNA synthesis in mosquito-borne flaviviruses has now been firmly established by using *in vitro* RdRp assays^{30,42-44} and mutational analysis of infectious flavivirus cDNAs^{32,45-50}. Physical evidence for the 5'CS-3'CS1 interaction in DENV was obtained by atomic force microscopy⁵⁰. Although formally a requirement for RNA circularization during flavivirus (+) strand RNA synthesis cannot be ruled out, current data demonstrate that it is crucial for (-) strand RNA synthesis⁴⁴. Genome cyclization is dispensable for viral translation^{26,46,49}.

Apart from the 5'CS-3'CS1 interaction, a second long-range RNA interaction was shown to be required for genome cyclization. In mosquito-borne flaviviruses this interaction is mediated by a sequence at the 5' end, located immediately upstream of the start codon of the ORF, and a sequence that is part of the bottom of the 3' SL⁵⁰⁻⁵². These complementary sequences were named 5'-3'UAR (upstream AUG region) and were shown to

be important for viral replication⁵⁰. Similar to the 5'CS-3'CS1 interaction, 5'-3'UAR base pairing was also demonstrated by atomic force microscopy⁵⁰. Current evidence suggests that the base pairing involving 5'CS and 3'CS1 initiates the circularization of the genome and promotes the 5'-3' UARs interaction to increase the stability of this long-range RNA interaction⁵³. Interestingly, additional RNA base pairing contributing to flavivirus genome circularization were recently identified between nucleotides downstream of the AUG region (5' DAR) and nucleotides downstream CS1 in the SL-A stem (3' DAR)^{54,55}. The 5' and 3' DAR motifs were shown to be important for genome circularization and RNA replication in DENV and WNV^{54,56}. In WNV, the 5'-3' DAR interaction actually consists of two stretches of complementary sequences⁵⁵. In YFV, the DAR motifs⁵⁶ were originally included in the 18 nt found to be part of the 5' CS and 3' CS1 elements⁴⁷ (see fig. 3). A general model was proposed for flaviviruses in which the 5'-3' DAR interaction extends the initial circularization between 5' and 3' CS, and together with the 5'-3' UAR interaction, unwinds the 3' SL^{54,56}. The various 5' and 3' RNA sequences that were shown to be required for RNA circularization in mosquito-borne flaviviruses are shown in figure 3.

RNA cyclization sequences have also been identified in the other two flavivirus clusters. In tick-borne flaviviruses these complementary sequences are named 5'-CS-A and 3'-CS-A. 5'-CS-A is located upstream of the translation initiation codon at approximately 100 nt from the 5' end, whereas 3'-CS-A is present in the 3' SL, approximately 80 nts from the 3' end²¹. There is no sequence similarity between the 5'- and 3'-CS-A sequences and 5'CS and CS1 of the mosquito-borne flaviviruses^{21,51} and the position of 5'- and 3'-CS-A actually resembles the location of the sequences involved in the 5'-3' UAR interaction of mosquito-borne flaviviruses. Mutagenesis of the 5'- and 3'-CSA revealed that complementarity between these RNA sequences was required for viral RNA synthesis^{21,51,57}. In addition to the 5'-3' CS-A interaction, another pair of complementary sequences (CS-B) has been identified at positions in the genome of tick-borne viruses (fig. 3). These sequences are reminiscent of the 5'-CS and CS1 in the mosquito-borne flaviviruses⁴⁵. Although the 5'- and 3'-CS-B sequences are well conserved in TBEV strains and related viruses like Powassan, Vasilchenko and louping ill virus^{10,13,21}, their interaction is not essential for viral replication⁵⁷. Sequence complementarity between 5' and 3' ends in the NKV flaviviruses MODV and MMLV has also been reported. Complementary RNA sequences that could promote circularization of the viral genome were identified at the 5' end of the genome, encompassing the AUG codon and the nucleotides encoding the N-terminus of the capsid protein; the 3' end counterpart was located immediately upstream the SL-A, resembling the location of CS1 in mosquito-borne flaviviruses^{7,58}.

A particular stem-loop structure (SLA) in the flavivirus 5' UTR was found to be important for RNA replication^{30,59-61}. In fact, it was shown that this SLA structure was the responsible for promoting viral RNA synthesis and not the cyclization sequences per se⁵⁹. This was supported by the fact that the SLA was found to be specifically recognized by the viral

YFV	5' CS: 5' – CCCU <u>GG</u> CGUCAUAUUGGU – 3'	3' CS1: 5' – ACCAUAUUGAG CCAGGG – 3'
	5' UAR: 5' – AGCAGAGAACUG – 3'	3' UAR: 5' – UGGUUCUCUGCU – 3'
	5' DAR: 5' – CCUGG – 3'	3' DAR: 5' – CCAGG – 3'
DENV	5' CS: 5' – UCAAUAUGCUG – 3'	3' CS1: 5' – CAGCAUAUUGA – 3'
	5' UAR: 5' – AGAGAGCAGAU <u>CUCUG</u> – 3'	3' UAR: 5' – CAGAGAU <u>CCUGCUGUCU</u> – 3'
	5' DAR: 5' – CCA <u>ACG</u> – 3'	3' DAR: 5' – CG <u>CUGG</u> – 3'
WNV	5' CS: 5' – UGUCAUAUUGCU – 3'	3' CS1: 5' – AGCAUAUUGACA – 3'
	5' UAR: 5' – AGCA <u>CGA</u> GAAGAU <u>CUC</u> – 3'	3' UAR: 5' – GAGAU <u>CUUCUGCU</u> – 3'
	5' DAR: 5' – GUCUA.....CCAGG – 3'	3' DAR: 5' – CCUGG..UAGAC – 3'
JEV	5' CS: 5' – UCAAUAUGUG – 3'	3' CS1: 5' – CACAUAUUGA – 3'
	5' UAR: 5' – UAGA <u>ACGGA</u> AGAU <u>AACCAUG</u> – 3'	3' UAR: 5' – UGGG <u>AGAU</u> CUUCUG <u>CCUCUA</u> – 3'
	5' DAR: 5' – CCAGG – 3'	3' DAR: 5' – CCUGG – 3'
TBEV	5' CS-A: 5' – GGAGACAAGAGCUG – 3'	3' CS-A: 5' – CGGUUCUUGUUCUCC – 3'
	5' CS-B: 5' – GGGG <u>CGGU</u> CCC – 3'	3' CS-B: 5' – GGGAG <u>CCCC</u> – 3'
MODV	5' CS: 5' – AAU <u>GUCG</u> GAAAAUAACAGGA	3' CS: 5' – UCCUGUUAUUU <u>UCCAAAUU</u> – 3'

Fig. 3. Potential 5' and 3' cyclization motifs in the mosquito-borne flaviviruses YFV, DENV, WNV and JEV, the tick-borne flavivirus TBEV and the no known vector MODV. The YFV nucleotides presented in bold in the 5' CS and 3' CS1 sequences⁴⁷ correspond to nucleotides that were afterwards described as the DAR elements⁵⁶. The underlined nucleotides indicate unpaired nucleotides. The dots in the DAR elements of WNV represent the nucleotides between the two stretches of nucleotides characteristic of the DAR interaction of WNV.

RdRp NS5^{59,60,62}. These data have led to a model for the initiation of viral (-) strand RNA synthesis in which the viral RdRp would bind to a conserved stem-loop structure at the 5' end of the genome. This binding would initiate genome circularization by the 5'CS-3'CS1 interaction, which is subsequently extended and therefore stabilized by base pairing of the additional RNA UAR and DAR motifs. Because the 3'UAR is located at the bottom of the 3'SL, the 5'UAR-3'UAR interaction might be responsible for destabilizing the 3'SL structure, which enforces the 3' end of the genome in a single-strand conformation, thereby making it accessible for the RdRp to use it as a template for initiation of viral (-) strand RNA synthesis^{59,60,63}. This model implies that the interaction between the 5'- and 3' ends of the viral genome functions as a riboswitch that can be induced by the binding of NS5 to the 5' end of the genome. This ensures refolding of the RNA from a "linear" conformation that is used for translation and packaging, into a circular conformation that is required for the initiation of viral RNA synthesis^{26,50,64,65}. Genome circularization might be beneficial for viral replication for several reasons: (i) as a control mechanism to amplify only full-length templates, (ii) to regulate transcription versus translation in space and time, (iii) to bring the replication complex in close vicinity of the transcription initiation site at the 3' end of the viral genome, and (iv) to control the level of (-) strand RNA synthesis^{4,59,65}.

3' UTR region III: RNA structures required for enhancement of RNA synthesis

This region essentially involves the nucleotides downstream of the XRN1 stalling signal (see region IV) and upstream of the CS1 or the circularization sequences in the mosquito-borne and NKV flaviviruses or 3' CS-B in the TBE-like viruses. Even flaviviruses that belong to the same cluster show a significant variation in the length of this region due to duplication of RNA sequences. Region III varies in the mosquito-borne flaviviruses from approximately 100 nts for YFV to 170 nts in DENV and in the NKV viruses from \pm 150 nts for MODV to 225 nts for RBV. In terms of RNA structure, this region is characterized by the predicted formation of one or two so-called dumbbell-like or Y-shaped RNA stem-loop structures^{6,7,13}. The dumbbell structure of the mosquito-borne flaviviruses contains a conserved sequence that has originally been named CS2 and has \sim 24 nucleotides in length (fig. 1). Mosquito-borne flaviviruses like JEV, WNV and DENV, which show a duplication of this dumbbell-like structure, contain two CS2 elements (CS2 and RCS2⁴). In NKV flaviviruses an RNA sequence with significant sequence homology to CS2 can be found in a similar position in the predicted Y-shaped RNA structures as to CS2 of the mosquito-borne flaviviruses^{7,58}. So far a CS2-like sequence has not been identified within region III of TBEV or related viruses. Deletion of CS2 has a relatively minor effect on viral RNA synthesis and virus production, but seems to decrease viral pathogenicity^{32,46,48,49,66}.

In addition to CS2, the region III of flaviviruses is characterized by two predicted RNA pseudoknots (fig. 1)⁶ (Jiang, Silva, Dalebout and Bredenbeek unpublished results). These RNA pseudoknots, named PSK1 and PSK2, involve four to five nts of the loop on the left-hand side of the dumbbell structures. These nucleotides are predicted to base pair with a complementary sequence downstream of the dumbbell structures. Experimental evidence for the formation of both these pseudoknots in DENV, YFV and WNV has been obtained by RNA structure probing and mutational analysis^{67,68} (Molenkamp, Dalebout and Bredenbeek, unpublished results). Disruption of either PSK1 or PSK2 significantly impairs viral RNA synthesis and virus production. This effect is enhanced when neither of the two pseudoknot structures can be formed. Formation of PSK1 and PSK2 is not required for efficient viral translation (Molenkamp, Dalebout and Bredenbeek, unpublished results).

Many other deletion mutants involving sequences of region III have been described for several flaviviruses^{46,48,49,66}. Unfortunately, most of these deletions were not guided by the predicted RNA structures and/or did not take into account the potential redundancy of the effect of the introduced deletions due to duplication of conserved RNA elements. Therefore, and also due to the differences in experimental design, it is rather difficult to truly compare the outcome of the various studies. In general, the results support a

role for CS2 and the RNA pseudoknots in viral RNA synthesis. Deletion of the (nearly) complete dumbbell structure that contains CS2 has a more dramatic effect on viral RNA synthesis than deleting only CS2. This can be explained by the fact that such deletions exhibit the cumulative effect of disrupting one of the RNA pseudoknots and the loss of the CS2 sequence. Removing both dumbbell structures of region II of DENV results in very crippled to none-viable viruses when analyzed in mammalian cells^{49,66}. Surprisingly, one of these DENV deletion mutants was able to replicate in C6/36 mosquito cells. Another mutant, in which the left part of the dumbbell structures was deleted but that maintained CS2 and RCS2 sequences, exhibited the reverse phenotype as it was able to replicate efficiently in Vero cells but not in C6/36 cells⁶⁶. The molecular basis of these apparently host-specific effects of deletions in region III of DENV is unknown. YFV mutants that are either unable to form PSK1 or PSK2 or lack CS2 yield a similar phenotype in the mammalian BHK and SW13 cells and in the C6/36 mosquito cells (Molenkamp, Dalebout and Bredenbeek; unpublished results).

Of particular interest is a 30-nucleotide deletion (nucleotides 10,478-10,507 in the dumbbell structure 1; involving PSK1) in the 3' UTR of DENV4 genome. This rDENV4 Δ 30 mutant was shown to be attenuated in rhesus monkeys⁴⁸ and well tolerated and highly immunogenic in human volunteers^{69,70}. In addition, rDENV4 Δ 30 also exhibited a limited ability to infect the midgut of mosquitoes⁷¹. Introduction of the Δ 30 mutation into the homologous region of DENV1 yielded similar results as for rDENV4 Δ 30^{72,73}. Unfortunately, introduction of the Δ 30 mutation in DENV2 and DENV3 did not result in significant attenuation when tested in rhesus monkeys^{74,75}. The molecular basis of the different phenotypes caused by this deletion in either DENV1 and 4 versus DENV2 and 3 is currently unknown, but it should be the subject of further studies in order to produce a safe and effective tetravalent DENV vaccine based on this Δ 30 deletion.

3' UTR region IV: the XRN1-stalling region

Region IV of the flavivirus 3' UTR varies in length due to sequence duplication especially in members of the JEV subgroup that have a third conserved sequence (CS3), which is also repeated (RCS3)^{4,76}. These sequences were shown to be important for efficient RNA replication in KUNV and WNV^{46,77}. DENV mutants with deletions in region III also exhibited reduced growth properties, suggesting that this region is essential for an efficient viral replication⁷⁸.

Recently it has become evident that the major characteristic of this region is an RNA pseudoknot structure that can be formed in all flaviviruses (fig. 4). Viruses that contain a sequence duplication within region IV are predicted to form two pseudoknots. This RNA pseudoknot structure is often followed by a small stem-loop structure (see fig. 1) with

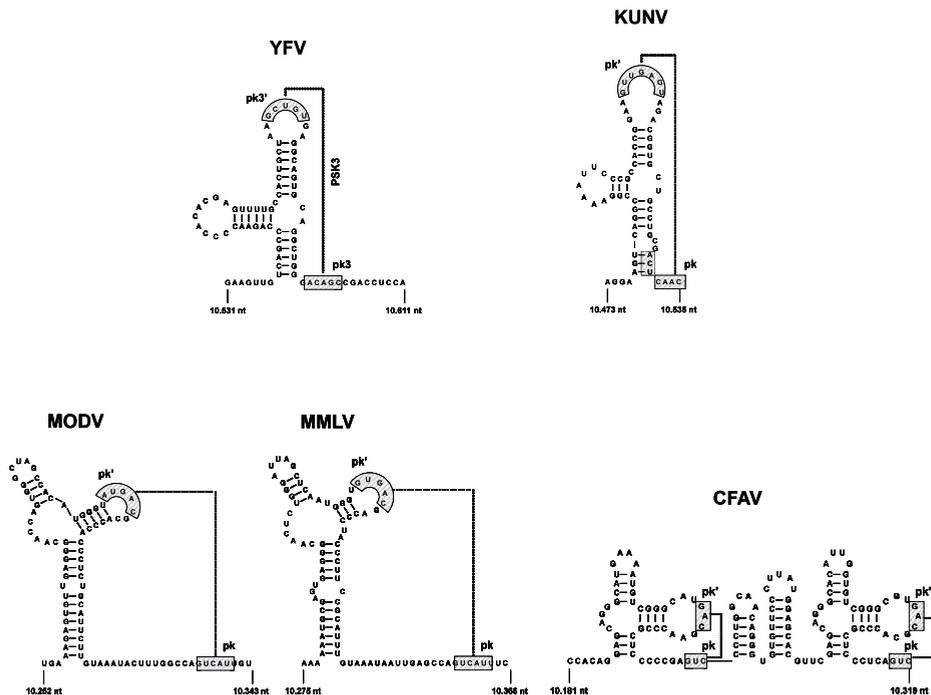


Fig. 4. RNA sequence and predicted RNA structures for the part of region III that is required for stalling XRN1. RNA structures were predicted using a combinatorial approach involving Mfold, phylogeny and manual sequence-structure analysis. The predicted RNA pseudoknot formation by interaction of the pk and pk' sequences are indicated by grey boxes and dotted lines. The NCBI accession numbers are X03700 for YFV-17D, D00246.1 for KUNV, AJ242984 for MODV, AJ299445 for MMLV, and NC_008604 for CFAV.

unknown function. The RNA pseudoknot has been identified as an important determinant for the synthesis of a small flavivirus subgenomic RNA (sfrNA) that is colinear with the 3' end of the viral genome^{68,79,80}. This sfrNA is not produced by viral transcription, but by 5' to 3' degradation of the viral genome by the host exoribonuclease XRN1^{79,81}. XRN1 is the main host RNase associated with cellular 5' to 3' mRNA decay (reviewed in^{82,83}). The RNA pseudoknot has been shown to serve as a stalling site for XRN1-mediated RNA decay, resulting in the production of the sfrNA^{68,79,81}. Interestingly, this sfrNA was shown to be an important determinant for virus pathogenicity as recombinant flaviviruses that are unable to produce the sfrNA show an attenuated phenotype. Disruption of the RNA pseudoknot structure has only a moderate effect on flavivirus genome RNA synthesis^{68,79,81}.

These results provide a prime example of how an RNA structure can (in)directly be involved in viral pathogenesis. The mechanism by which the sfrNA influences the viral pathogenicity is not known. It has been suggested that it may act as decoy for host miRNAs or proteins that are directed against the viral genome to decrease replication

^{81,84}. Another intriguing hypothesis is that the sRNA itself may serve as a precursor for the production of a virus-encoded miRNA ⁷⁹. It is interesting to note that XRN1-mediated RNA decay occurs within the subcellular P bodies that among others also contain Dicer and Argonaute proteins, which are required for the production of functional miRNAs (reviewed in ^{82,83}). It is important to realize that the sRNA contains all the RNA sequences and most likely the RNA structures that are characteristic for the distal part of the flavivirus 3' UTR. Therefore, the effect of mutations within this region is not necessarily linked to the viral genome, but can be the result of their effect on the function of the sRNA.

3' UTR region V: the variable region

The RNA sequence that starts immediately downstream of the stop codon of the viral ORF up to the XRN1-stalling site is known as the variable region (VR). Natural isolates of arthropod-borne flaviviruses often show a significant sequence and size variability due to relatively large nucleotide insertions and deletions in this region of the 3' UTR ^{9-12,15,85}. The heterogeneity of the VR in natural isolates is well documented for YFV. The originally reported YFV-17D VR corresponds to a unique set of three closely spaced repeated sequences (RYF1, RYF2 and RYF3), each of approximately 40 nucleotides in length ¹⁷ (fig. 1) that were shown to be characteristic for West-African strains of YFV ⁸⁶. It was subsequently demonstrated that Central and East-African strains had only two repeats (RYF1 and RYF3), whereas South America genotypes had one single copy (RYF3) ^{12,87}. Interestingly, passaging of an YFV strain harboring all three repeats in mice and cell culture resulted in the deletion of both RYF1 and RYF2 ⁸⁷. Similar results were reported for TBEV isolates, as they were also shown to accumulate deletions in the VR region during propagation in either cell lines or mice ^{13,88}. These results suggest that the majority of the 3' VR is not essential for efficient replication of natural virus isolates. Deletions in the background of infectious cDNA clones of YFV-17D, KUNV, and TBEV demonstrated that actually the complete 3' VR could be deleted without significant impact on replication or virulence for these mutant viruses in either cell culture or mice ^{32,77,88-90}. Mutagenesis studies on the 3' VR of DENV resulted in a slightly more complicated picture. Relatively small deletions up to 19 nts did not in general have any significant effect on virus replication in either insect or mammalian cell lines ⁹¹. However, larger deletions in the VR resulted in a significant decrease in RNA synthesis and virus production especially in mammalian cells ^{49,78}. The significance of these often subtle differences is currently unknown and may in part result from the use of different cell lines and virus isolates as well as the rationale of the introduced deletions.

No characteristic conserved RNA structure has been predicted in the VR. It has been suggested that the VR acts as a spacer element to allow proper folding of the RNA struc-

tures in the distal part of the 3' UTR ¹⁶. However, in the case of NKV flaviviruses, the VR sequences are in general rather short for serving as a spacer element (e.g. 20 and 41 nts for MODV and MMLV, respectively), although they are relatively A-U rich and therefore likely to be unstructured ⁷.

In order to truly understand the importance of the VR in the 3' UTR of flaviviruses, recombinant deletion mutants should be assessed *in vivo* in mosquitoes/ticks and animal models. The fact that this region is present in almost every flavivirus known indicates that it serves a purpose in the viral life cycle in nature. Furthermore, the fact that natural isolates tend to have a longer VR suggests a selective advantage that is either associated with replication or pathogenicity in the natural situation.

Flavivirus 3' UTR-binding proteins

Many host proteins have been found to play an important role in flavivirus infection (for a review see ⁹²). Several of these proteins were shown to interact with the viral 5' and 3' UTRs. Unfortunately, for only a few of these host factors the function of the interaction has been identified. This section briefly describes the host and viral proteins that were reported to interact specifically with the flavivirus 3' UTR and summarizes our limited knowledge about their role in the flavivirus life cycle.

Host proteins interacting with the 3' UTR of the viral genome

Apart from the usual suspects like polypyrimidine tract-binding protein (PTB), La and to some extent Poly(A)-binding protein (PABP), only a few host proteins that specifically interact with the 3' UTR of the flavivirus genome have been identified. These include the eukaryotic translation elongation factor-1 α (EF-1 α), Y box binding protein-1 (YB-1) and the protein Mov34.

The eukaryotic translation elongation factor-1 α (EF-1 α) was shown to bind specifically to the right-hand side of the 3' SL region of WNV ⁴⁰ and DENV4 ⁹³. The binding of EF-1 α to the 3' SL is primarily determined by an only four nucleotides long 5'-CACAA-3' motif, although additional sequences that are located in the top 3' SL loop and in the smaller adjacent stem-loop SL-A are also involved ⁴⁰. Mutational analysis of a WNV infectious cDNA clone revealed that the interaction between eEF-1 α and the WNV 3' SL was required for viral (-) strand RNA synthesis ⁹⁴. In addition, in WNV and DENV4 infected cells, eEF-1 α colocalized with dsRNA and the viral proteins NS3 and NS5, suggesting that this host protein was required for specific recognition of the 3' SL by the RdRp to promote (-) strand RNA synthesis ⁹⁴. It is currently unknown whether, apart from DENV4, eEF-1 α also binds to the 3' UTR of other flaviviruses.

Y box binding protein-1 (YB-1) is a member of the highly conserved Y box proteins and functions as pleiotropic transcription factor that is mainly involved in the regulation of the expression of stress induced genes. YB-1 was shown to bind specifically to the DENV 3' SL and to repress replication. This antiviral effect can in part be explained by YB-1 mediated inhibition of DENV RNA translation⁹⁵. In addition, it is speculated that the YB-1 serves as a transcription factor to promote activation of innate immune response genes such as ISG54 and ISG56, which can down-regulate translation. It is currently unknown whether YB-1 binds to the 3' UTR of other flaviviruses, or whether its role as virus repressor is limited to DENV4 virus.

Mouse Mov34 protein is another cellular protein that was reported to bind to the 3' SL RNA of JEV⁹⁶. It belongs to a family of proteins that share a so-called MPN-like domain. Mov34 serves as regulatory subunit of the 26 proteasome and it is therefore not clear why this protein would interact with the flavivirus 3' SL.

In addition to the above proteins, various nuclear ribonucleoproteins like hnRNP A1, hnRNPA2/B1 and hnRNP Q were also shown to bind to the DENV 3' SL by RNA affinity chromatography⁹⁵. However, no *in vivo* data that demonstrate the relevance of these hnRNP- 3' SL interactions is available.

The promiscuous RNA-binding proteins polypyrimidine tract-binding (PTB) and La protein have also been shown to interact with the 3' UTR of several flaviviruses like DENV and JEV^{93,97}. These proteins are normally restricted to the nucleus, but were shown to translocate to the cytoplasm upon infection^{98,99}. Binding for both La and PTB is mapped in the CS1, SL-A and 3' SL RNA structures in which putative binding sites have been suggested⁹³. However, the precise binding sites for these proteins in the viral 3' UTR remain to be determined. Several studies suggest that PTB is part of the viral replication complex^{93,99-101}. However, the experimental data on the role of PTB in the viral life cycle is often conflicting as illustrated by experiments in which inhibition of the expression of PTB by siRNA did not have a significant effect on YFV replication, whereas it severely inhibited DENV production¹⁰⁰. There appears to be consensus that La is required for efficient virus replication, although the suggested functions are rather diverse^{93,97,98,102,103}. Several studies suggest that La serves as an RNA chaperone or an indirect factor that by interacting with NS3 and NS5 helps in the transition from the "linear" translation competent RNA structure to the circular RNA structure that is required for transcription/replication^{93,98,102}. Other studies suggest that the La-associated helicase activity is required in the flavivirus replication complex^{97,103}. This would be rather surprising since the viral NS3 protein, which is present in the viral replication complex, also contains a helicase activity.

Poly(A)-binding protein (PABP) is a unique translation initiation factor that stimulates translation by promoting mRNA circularization through simultaneous interactions with eIF4G and the 3' poly(A) tail (reviewed in¹⁰⁴). Despite the fact that the genome RNA of

flaviviruses is not polyadenylated, PABP was shown to bind to the DENV 3' UTR¹⁰⁵. This binding most likely involves the relatively A-rich sequences flanking the RNA dumbbell structures in region III of the viral 3' UTR. PABP interaction with the 3' UTR appears to be required for efficient translation initiation of the viral genome¹⁰⁵.

Viral proteins interacting with the 3' UTR of the viral genome

Purified recombinant NS5 protein that contains the viral RdRp activity was shown to interact *in vitro* with the 3' UTR, especially the 3' SL, of JEV and DENV^{106,107}. In addition to NS5, purified recombinant NS3 that contained the viral RNA helicase activity, was also reported to interact with the 3' UTR of DENV¹⁰⁸. The NS3 and NS5 interactions with the 3' SL were also demonstrated by UV cross-linking of proteins from JEV-infected cell lysates with RNA probes mimicking the viral 3' UTR¹⁰⁹. The studies cited above suggested that the interaction of NS3 and NS5 with RNA sequences or structures of region I or II reflect the (partial) formation of the viral replication complex on the 3' UTR to initiate viral (-) strand RNA synthesis. However, these data are contradicted by recent data on the formation of DENV replication complex, which shows that DENV NS5 has a strong affinity for stem-loop A at the 5' UTR of the viral genome⁵⁹. The binding of NS5 to and/or the formation of a replication complex at the 5' UTR is postulated to serve as a trigger to initiate the circularization of the viral genome that is required for flavivirus RNA synthesis. This resulted in a very attractive model that explains many of the experimental data on the initiation of flavivirus (-) strand RNA replication (reviewed in^{65,110}). Taking into account their function as viral RNA helicase and RdRp it is not surprising that both NS3 and NS5 demonstrated affinity for RNA, but the biological relevance of these interactions with RNA elements in the 3' UTR is in our opinion questionable. Specific binding of KUNV recombinant NS2A with the 3' UTR of this virus has also been reported¹¹¹.

A FINAL NOTE

Understanding the biology of flaviviruses at the molecular level is crucial for the development of new vaccines and the rational design of novel antiviral strategies. Although the E protein is recognized as a major determinant for the tropism and virulence of flaviviruses (reviewed in¹¹²), it is evident that RNA sequences and structures located in the 3' UTRs can also influence their virulence^{48,88,113-118}. Several of these RNA elements within the viral 3' UTR represent interesting targets for an antiviral strategy based on antisense oligonucleotides that either inhibit translation or replication, or serve as siRNA^{38,39,119-123}.

The overall structural integrity of the flavivirus 3' UTR is clearly important for efficient replication of the virus¹⁶. Deletion or mutation of RNA elements that do not appear to result in a significant decrease in viral replication when analyzed in cell culture, may

actually show a crippled replication or pathogenicity of the virus when analyzed in a more relevant model system or in competition with the wt-virus^{37,68,79,81}.

The biological significance and molecular function of many of the RNA structures within the viral 3' UTR, as well as the interactions between host and/or viral proteins with the viral 3' UTR, are still unknown. Future research should verify the predicted RNA structures and determine their role in the virus life cycle, as well as the role of certain protein interactions with the flavivirus RNA under *in vivo* conditions. Furthermore, the composition and assembly of the viral replication complex and the mechanism by which this complex initiates the transcription of viral RNA should also be studied in more detail. The results from such studies will increase our understanding over the role of RNA structures in flavivirus replication, and help us in identifying and validating new targets for antiviral strategies and/or for the development of attenuated viruses that can be used as vaccines.

REFERENCE LIST

1. **ICTV.** 2005. Virus Taxonomy - Eight Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego.
2. **Kuno, G., G. J. Chang, K. R. Tsuchiya, N. Karabatsos, and C. B. Cropp.** 1998. Phylogeny of the genus Flavivirus. *J. Virol.* **72**:73-83.
3. **Cook, S. and E. C. Holmes.** 2006. A multigene analysis of the phylogenetic relationships among the flaviviruses (Family: Flaviviridae) and the evolution of vector transmission. *Arch. Virol.* **151**:309-325.
4. **Hahn, C. S., Y. S. Hahn, C. M. Rice, E. Lee, L. Dalgarno, E. G. Strauss, and J. H. Strauss.** 1987. Conserved elements in the 3' untranslated region of flavivirus RNAs and potential cyclization sequences. *J. Mol. Biol.* **198**:33-41.
5. **Proutski, V., E. A. Gould, and E. C. Holmes.** 1997. Secondary structure of the 3' untranslated region of flaviviruses: similarities and differences. *Nucleic Acids Res.* **25**:1194-1202.
6. **Olsthoorn, R. C. and J. F. Bol.** 2001. Sequence comparison and secondary structure analysis of the 3' noncoding region of flavivirus genomes reveals multiple pseudoknots. *RNA.* **7**:1370-1377.
7. **Charlier, N., P. Leyssen, C. W. Pleij, P. Lemey, F. Billoir, L. K. Van, A. M. Vandamme, C. E. De, L. de, X, and J. Neyts.** 2002. Complete genome sequence of Montana Myotis leukoencephalitis virus, phylogenetic analysis and comparative study of the 3' untranslated region of flaviviruses with no known vector. *J. Gen. Virol.* **83**:1875-1885.
8. **Gritsun, T. S. and E. A. Gould.** 2007. Origin and evolution of 3'UTR of flaviviruses: long direct repeats as a basis for the formation of secondary structures and their significance for virus transmission. *Adv. Virus Res.* **69**:203-248.
9. **Mandl, C. W., C. Kunz, and F. X. Heinz.** 1991. Presence of poly(A) in a flavivirus: significant differences between the 3' noncoding regions of the genomic RNAs of tick-borne encephalitis virus strains. *J. Virol.* **65**:4070-4077.
10. **Wallner, G., C. W. Mandl, C. Kunz, and F. X. Heinz.** 1995. The flavivirus 3'-noncoding region: extensive size heterogeneity independent of evolutionary relationships among strains of tick-borne encephalitis virus. *Virology* **213**:169-178.
11. **Poidinger, M., R. A. Hall, and J. S. Mackenzie.** 1996. Molecular characterization of the Japanese encephalitis serocomplex of the flavivirus genus. *Virology* **218**:417-421.
12. **Wang, E., S. C. Weaver, R. E. Shope, R. B. Tesh, D. M. Watts, and A. D. Barrett.** 1996. Genetic variation in yellow fever virus: duplication in the 3' noncoding region of strains from Africa. *Virology* **225**:274-281.
13. **Gritsun, T. S., K. Venugopal, P. M. Zanotto, M. V. Mikhailov, A. A. Sall, E. C. Holmes, I. Polkinghorne, T. V. Frolova, V. V. Pogodina, V. A. Lashkevich, and E. A. Gould.** 1997. Complete sequence of two tick-borne flaviviruses isolated from Siberia and the UK: analysis and significance of the 5' and 3'-UTRs. *Virus Res.* **49**:27-39.
14. **Rauscher, S., C. Flamm, C. W. Mandl, F. X. Heinz, and P. F. Stadler.** 1997. Secondary structure of the 3'-noncoding region of flavivirus genomes: comparative analysis of base pairing probabilities. *RNA.* **3**:779-791.
15. **Shurtleff, A. C., D. W. Beasley, J. J. Chen, H. Ni, M. T. Suderman, H. Wang, R. Xu, E. Wang, S. C. Weaver, D. M. Watts, K. L. Russell, and A. D. Barrett.** 2001. Genetic variation in the 3' non-coding region of dengue viruses. *Virology* **281**:75-87.

16. **Proutski, V., T. S. Gritsun, E. A. Gould, and E. C. Holmes.** 1999. Biological consequences of deletions within the 3'-untranslated region of flaviviruses may be due to rearrangements of RNA secondary structure. *Virus Res.* **64**:107-123.
17. **Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss.** 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* **229**:726-733.
18. **Grange, T., M. Bouloy, and M. Girard.** 1985. Stable secondary structures at the 3'-end of the genome of yellow fever virus (17 D vaccine strain). *FEBS Lett.* **188**:159-163.
19. **Wengler, G. and E. Castle.** 1986. Analysis of structural properties which possibly are characteristic for the 3'-terminal sequence of the genome RNA of flaviviruses. *J. Gen. Virol.* **67 (Pt 6)**:1183-1188.
20. **Brinton, M. A., A. V. Fernandez, and J. H. Disposito.** 1986. The 3'-nucleotides of flavivirus genomic RNA form a conserved secondary structure. *Virology* **153**:113-121.
21. **Mandl, C. W., H. Holzmann, C. Kunz, and F. X. Heinz.** 1993. Complete genomic sequence of Powassan virus: evaluation of genetic elements in tick-borne versus mosquito-borne flaviviruses. *Virology* **194**:173-184.
22. **Shi, P. Y., M. A. Brinton, J. M. Veal, Y. Y. Zhong, and W. D. Wilson.** 1996. Evidence for the existence of a pseudoknot structure at the 3' terminus of the flavivirus genomic RNA. *Biochemistry* **35**:4222-4230.
23. **Tilgner, M. and P. Y. Shi.** 2004. Structure and function of the 3' terminal six nucleotides of the west nile virus genome in viral replication. *J. Virol.* **78**:8159-8171.
24. **Holden, K. L. and E. Harris.** 2004. Enhancement of dengue virus translation: role of the 3' untranslated region and the terminal 3' stem-loop domain. *Virology* **329**:119-133.
25. **Tilgner, M., T. S. Deas, and P. Y. Shi.** 2005. The flavivirus-conserved penta-nucleotide in the 3' stem-loop of the West Nile virus genome requires a specific sequence and structure for RNA synthesis, but not for viral translation. *Virology* **331**:375-386.
26. **Chiu, W. W., R. M. Kinney, and T. W. Dreher.** 2005. Control of translation by the 5'- and 3'-terminal regions of the dengue virus genome. *J. Virol.* **79**:8303-8315.
27. **Li, W. and M. A. Brinton.** 2001. The 3' stem loop of the West Nile virus genomic RNA can suppress translation of chimeric mRNAs. *Virology* **287**:49-61.
28. **Wei, Y., C. Qin, T. Jiang, X. Li, H. Zhao, Z. Liu, Y. Deng, R. Liu, S. Chen, M. Yu, and E. Qin.** 2009. Translational regulation by the 3' untranslated region of the dengue type 2 virus genome. *Am. J. Trop. Med. Hyg.* **81**:817-824.
29. **Zeng, L., B. Falgout, and L. Markoff.** 1998. Identification of specific nucleotide sequences within the conserved 3'-SL in the dengue type 2 virus genome required for replication. *J. Virol.* **72**:7510-7522.
30. **You, S., B. Falgout, L. Markoff, and R. Padmanabhan.** 2001. In vitro RNA synthesis from exogenous dengue viral RNA templates requires long range interactions between 5'- and 3'-terminal regions that influence RNA structure. *J. Biol. Chem.* **276**:15581-15591.
31. **Shi, P. Y., M. Tilgner, and M. K. Lo.** 2002. Construction and characterization of subgenomic replicons of New York strain of West Nile virus. *Virology* **296**:219-233.
32. **Bredenbeek, P. J., E. A. Kooi, B. Lindenbach, N. Huijckman, C. M. Rice, and W. J. Spaan.** 2003. A stable full-length yellow fever virus cDNA clone and the role of conserved RNA elements in flavivirus replication. *J. Gen. Virol.* **84**:1261-1268.
33. **Khromykh, A. A., N. Kondratieva, J. Y. Sgro, A. Palmenberg, and E. G. Westaway.** 2003. Significance in replication of the terminal nucleotides of the flavivirus genome. *J. Virol.* **77**:10623-10629.

34. **Yu, L. and L. Markoff.** 2005. The topology of bulges in the long stem of the flavivirus 3' stem-loop is a major determinant of RNA replication competence. *J. Virol.* **79**:2309-2324.
35. **Nomaguchi, M., M. Ackermann, C. Yon, S. You, and R. Padmanabhan.** 2003. De novo synthesis of negative-strand RNA by Dengue virus RNA-dependent RNA polymerase in vitro: nucleotide, primer, and template parameters. *J. Virol.* **77**:8831-8842.
36. **Elghonemy, S., W. G. Davis, and M. A. Brinton.** 2005. The majority of the nucleotides in the top loop of the genomic 3' terminal stem loop structure are cis-acting in a West Nile virus infectious clone. *Virology* **331**:238-246.
37. **Silva, P. A., R. Molenkamp, T. J. Dalebout, N. Charlier, J. H. Neyts, W. J. Spaan, and P. J. Bredenbeek.** 2007. Conservation of the pentanucleotide motif at the top of the yellow fever virus 17D 3' stem-loop structure is not required for replication. *J. Gen. Virol.* **88**:1738-1747.
38. **Deas, T. S., I. Binduga-Gajewska, M. Tilgner, P. Ren, D. A. Stein, H. M. Moulton, P. L. Iversen, E. B. Kauffman, L. D. Kramer, and P. Y. Shi.** 2005. Inhibition of flavivirus infections by antisense oligomers specifically suppressing viral translation and RNA replication. *J. Virol.* **79**:4599-4609.
39. **Holden, K. L., D. A. Stein, T. C. Pierson, A. A. Ahmed, K. Clyde, P. L. Iversen, and E. Harris.** 2006. Inhibition of dengue virus translation and RNA synthesis by a morpholino oligomer targeted to the top of the terminal 3' stem-loop structure. *Virology* **344**:439-452.
40. **Blackwell, J. L. and M. A. Brinton.** 1997. Translation elongation factor-1 alpha interacts with the 3' stem-loop region of West Nile virus genomic RNA. *J. Virol.* **71**:6433-6444.
41. **Lescrinier, E., N. Dyubankova, K. Nauwelaerts, R. Jones, and P. Herdewijn.** 2010. Structure determination of the top-loop of the conserved 3'-terminal secondary structure in the genome of flaviviruses. *Chembiochem.* **11**:1404-1412.
42. **You, S. and R. Padmanabhan.** 1999. A novel in vitro replication system for Dengue virus. Initiation of RNA synthesis at the 3'-end of exogenous viral RNA templates requires 5'- and 3'-terminal complementary sequence motifs of the viral RNA. *J. Biol. Chem.* **274**:33714-33722.
43. **Ackermann, M. and R. Padmanabhan.** 2001. De novo synthesis of RNA by the dengue virus RNA-dependent RNA polymerase exhibits temperature dependence at the initiation but not elongation phase. *J. Biol. Chem.* **276**:39926-39937.
44. **Nomaguchi, M., T. Teramoto, L. Yu, L. Markoff, and R. Padmanabhan.** 2004. Requirements for West Nile virus (-) and (+)-strand subgenomic RNA synthesis in vitro by the viral RNA-dependent RNA polymerase expressed in *Escherichia coli*. *J. Biol. Chem.* **279**:12141-12151.
45. **Khromykh, A. A., H. Meka, K. J. Guyatt, and E. G. Westaway.** 2001. Essential role of cyclization sequences in flavivirus RNA replication. *J. Virol.* **75**:6719-6728.
46. **Lo, M. K., M. Tilgner, K. A. Bernard, and P. Y. Shi.** 2003. Functional analysis of mosquito-borne flavivirus conserved sequence elements within 3' untranslated region of West Nile virus by use of a reporting replicon that differentiates between viral translation and RNA replication. *J. Virol.* **77**:10004-10014.
47. **Corver, J., E. Lenches, K. Smith, R. A. Robison, T. Sando, E. G. Strauss, and J. H. Strauss.** 2003. Fine mapping of a cis-acting sequence element in yellow fever virus RNA that is required for RNA replication and cyclization. *J. Virol.* **77**:2265-2270.
48. **Men, R., M. Bray, D. Clark, R. M. Chanock, and C. J. Lai.** 1996. Dengue type 4 virus mutants containing deletions in the 3' noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. *J. Virol.* **70**:3930-3937.

49. **Alvarez, D. E., A. L. De Lella Ezcurra, S. Fucito, and A. V. Gamarnik.** 2005. Role of RNA structures present at the 3'UTR of dengue virus on translation, RNA synthesis, and viral replication. *Virology* **339**:200-212.
50. **Alvarez, D. E., M. F. Lodeiro, S. J. Luduena, L. I. Pietrasanta, and A. V. Gamarnik.** 2005. Long-range RNA-RNA interactions circularize the dengue virus genome. *J. Virol.* **79**:6631-6643.
51. **Thurner, C., C. Witwer, I. L. Hofacker, and P. F. Stadler.** 2004. Conserved RNA secondary structures in Flaviviridae genomes. *J. Gen. Virol.* **85**:1113-1124.
52. **Song, B. H., S. I. Yun, Y. J. Choi, J. M. Kim, C. H. Lee, and Y. M. Lee.** 2008. A complex RNA motif defined by three discontinuous 5-nucleotide-long strands is essential for Flavivirus RNA replication. *RNA.* **14**:1791-1813.
53. **Polacek, C., J. E. Foley, and E. Harris.** 2009. Conformational changes in the solution structure of the dengue virus 5' end in the presence and absence of the 3' untranslated region. *J. Virol.* **83**:1161-1166.
54. **Friebe, P. and E. Harris.** 2010. Interplay of RNA elements in the dengue virus 5' and 3' ends required for viral RNA replication. *J. Virol.* **84**:6103-6118.
55. **Zhang, B., H. Dong, H. Ye, M. Tilgner, and P. Y. Shi.** 2010. Genetic analysis of West Nile virus containing a complete 3'CSI RNA deletion. *Virology* **408**:138-145.
56. **Friebe, P., P. Y. Shi, and E. Harris.** 2010. The 5' and 3' Downstream of AUG region (DAR) elements are required for mosquito-borne flavivirus RNA replication. *J. Virol.*
57. **Kofler, R. M., V. M. Hoenninger, C. Thurner, and C. W. Mandl.** 2006. Functional analysis of the tick-borne encephalitis virus cyclization elements indicates major differences between mosquito-borne and tick-borne flaviviruses. *J. Virol.* **80**:4099-4113.
58. **Leysen, P., N. Charlier, P. Lemey, F. Billoir, A. M. Vandamme, C. E. De, L. de, X, and J. Neyts.** 2002. Complete genome sequence, taxonomic assignment, and comparative analysis of the untranslated regions of the Modoc virus, a flavivirus with no known vector. *Virology* **293**:125-140.
59. **Filomatori, C. V., M. F. Lodeiro, D. E. Alvarez, M. M. Samsa, L. Pietrasanta, and A. V. Gamarnik.** 2006. A 5' RNA element promotes dengue virus RNA synthesis on a circular genome. *Genes Dev.* **20**:2238-2249.
60. **Dong, H., B. Zhang, and P. Y. Shi.** 2008. Terminal structures of West Nile virus genomic RNA and their interactions with viral NS5 protein. *Virology* **381**:123-135.
61. **Li, X. F., T. Jiang, X. D. Yu, Y. Q. Deng, H. Zhao, Q. Y. Zhu, E. D. Qin, and C. F. Qin.** 2010. RNA elements within the 5' untranslated region of the West Nile virus genome are critical for RNA synthesis and virus replication. *J. Gen. Virol.* **91**:1218-1223.
62. **Lodeiro, M. F., C. V. Filomatori, and A. V. Gamarnik.** 2009. Structural and functional studies of the promoter element for dengue virus RNA replication. *J. Virol.* **83**:993-1008.
63. **Zhang, B., H. Dong, D. A. Stein, P. L. Iversen, and P. Y. Shi.** 2008. West Nile virus genome cyclization and RNA replication require two pairs of long-distance RNA interactions. *Virology* **373**:1-13.
64. **Edgil, D. and E. Harris.** 2006. End-to-end communication in the modulation of translation by mammalian RNA viruses. *Virus Res.* **119**:43-51.
65. **Villordo, S. M. and A. V. Gamarnik.** 2009. Genome cyclization as strategy for flavivirus RNA replication. *Virus Res.* **139**:230-239.
66. **Blaney, J. E., Jr., N. S. Sathe, L. Goddard, C. T. Hanson, T. A. Romero, K. A. Hanley, B. R. Murphy, and S. S. Whitehead.** 2008. Dengue virus type 3 vaccine candidates generated by introduction of deletions in the 3' untranslated region (3'-UTR) or by exchange of the DENV-3 3'-UTR with that of DENV-4. *Vaccine* **26**:817-828.

67. **Romero, T. A., E. Tumban, J. Jun, W. B. Lott, and K. A. Hanley.** 2006. Secondary structure of dengue virus type 4 3' untranslated region: impact of deletion and substitution mutations. *J. Gen. Virol.* **87**:3291-3296.
68. **Funk, A., K. Truong, T. Nagasaki, S. Torres, N. Floden, M. E. Balmori, J. Edmonds, H. Dong, P. Y. Shi, and A. A. Khromykh.** 2010. RNA structures required for production of subgenomic flavivirus RNA. *J. Virol.* **84**:11407-11417.
69. **Durbin, A. P., R. A. Karron, W. Sun, D. W. Vaughn, M. J. Reynolds, J. R. Perreault, B. Thumar, R. Men, C. J. Lai, W. R. Elkins, R. M. Chanock, B. R. Murphy, and S. S. Whitehead.** 2001. Attenuation and immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30 nucleotide deletion in its 3'-untranslated region. *Am. J. Trop. Med. Hyg.* **65**:405-413.
70. **Durbin, A. P., S. S. Whitehead, J. McArthur, J. R. Perreault, J. E. Blaney, Jr., B. Thumar, B. R. Murphy, and R. A. Karron.** 2005. rDEN4delta30, a live attenuated dengue virus type 4 vaccine candidate, is safe, immunogenic, and highly infectious in healthy adult volunteers. *J. Infect. Dis.* **191**:710-718.
71. **Troyer, J. M., K. A. Hanley, S. S. Whitehead, D. Strickman, R. A. Karron, A. P. Durbin, and B. R. Murphy.** 2001. A live attenuated recombinant dengue-4 virus vaccine candidate with restricted capacity for dissemination in mosquitoes and lack of transmission from vaccinees to mosquitoes. *Am. J. Trop. Med. Hyg.* **65**:414-419.
72. **Whitehead, S. S., B. Falgout, K. A. Hanley, J. E. J. Blaney Jr, L. Markoff, and B. R. Murphy.** 2003. A live, attenuated dengue virus type 1 vaccine candidate with a 30-nucleotide deletion in the 3' untranslated region is highly attenuated and immunogenic in monkeys. *J. Virol.* **77**:1653-1657.
73. **Durbin, A. P., J. McArthur, J. A. Marron, J. E. Blaney, Jr., B. Thumar, K. Wanionek, B. R. Murphy, and S. S. Whitehead.** 2006. The live attenuated dengue serotype 1 vaccine rDEN1Delta30 is safe and highly immunogenic in healthy adult volunteers. *Hum. Vaccin.* **2**:167-173.
74. **Blaney, J. E., Jr., C. T. Hanson, K. A. Hanley, B. R. Murphy, and S. S. Whitehead.** 2004. Vaccine candidates derived from a novel infectious cDNA clone of an American genotype dengue virus type 2. *BMC. Infect. Dis.* **4**:39.
75. **Blaney, J. E., Jr., C. T. Hanson, C. Y. Firestone, K. A. Hanley, B. R. Murphy, and S. S. Whitehead.** 2004. Genetically modified, live attenuated dengue virus type 3 vaccine candidates. *Am. J. Trop. Med. Hyg.* **71**:811-821.
76. **Khromykh, A. A. and E. G. Westaway.** 1994. Completion of Kunjin virus RNA sequence and recovery of an infectious RNA transcribed from stably cloned full-length cDNA. *J. Virol.* **68**:4580-4588.
77. **Khromykh, A. A. and E. G. Westaway.** 1997. Subgenomic replicons of the flavivirus Kunjin: construction and applications. *J. Virol.* **71**:1497-1505.
78. **Tajima, S., Y. Nukui, T. Takasaki, and I. Kurane.** 2007. Characterization of the variable region in the 3' non-translated region of dengue type 1 virus. *J. Gen. Virol.* **88**:2214-2222.
79. **Silva, P. A., C. F. Pereira, T. J. Dalebout, W. J. Spaan, and P. J. Bredenbeek.** 2010. An RNA Pseudoknot Is Required for Production of Yellow Fever Virus Subgenomic RNA by the Host Nuclease XRN1. *J. Virol.* **84**:11395-11406.
80. **Silva, P. A. G. C., T. J. Dalebout, and P. J. Bredenbeek.** 2010. Characterization of the sRNAs that are produced in cells infected with flaviviruses with no known vector and cell fusing agent. -
81. **Pijlman, G. P., A. Funk, N. Kondratieva, J. Leung, S. Torres, L. van der Aa, W. J. Liu, A. C. Palmenberg, P. Y. Shi, R. A. Hall, and A. A. Khromykh.** 2008. A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity. *Cell Host. Microbe* **4**:579-591.

82. **Anderson, P. and N. Kedersha.** 2006. RNA granules. *J. Cell Biol.* **172**:803-808.
83. **Garneau, N. L., J. Wilusz, and C. J. Wilusz.** 2007. The highways and byways of mRNA decay. *Nat. Rev. Mol. Cell Biol.* **8**:113-126.
84. **Fernandez-Garcia, M. D., M. Mazzon, M. Jacobs, and A. Amara.** 2009. Pathogenesis of flavivirus infections: using and abusing the host cell. *Cell Host. Microbe* **5**:318-328.
85. **Jan, L. R., K. L. Chen, C. F. Lu, Y. C. Wu, and C. B. Horng.** 1996. Complete nucleotide sequence of the genome of Japanese encephalitis virus ling strain: the presence of a 25-nucleotide deletion in the 3'-nontranslated region. *Am. J. Trop. Med. Hyg.* **55**:603-609.
86. **Hahn, C. S., J. M. Dalrymple, J. H. Strauss, and C. M. Rice.** 1987. Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. *Proc. Natl. Acad. Sci. U. S. A* **84**:2019-2023.
87. **Mutebi, J. P., R. C. Rijnbrand, H. Wang, K. D. Ryman, E. Wang, L. D. Fulop, R. Titball, and A. D. Barrett.** 2004. Genetic relationships and evolution of genotypes of yellow fever virus and other members of the yellow fever virus group within the Flavivirus genus based on the 3' noncoding region. *J. Virol.* **78**:9652-9665.
88. **Mandl, C. W., H. Holzmann, T. Meixner, S. Rauscher, P. F. Stadler, S. L. Allison, and F. X. Heinz.** 1998. Spontaneous and engineered deletions in the 3' noncoding region of tick-borne encephalitis virus: construction of highly attenuated mutants of a flavivirus. *J. Virol.* **72**:2132-2140.
89. **Bryant, J. E., P. F. Vasconcelos, R. C. Rijnbrand, J. P. Mutebi, S. Higgs, and A. D. Barrett.** 2005. Size heterogeneity in the 3' noncoding region of South American isolates of yellow fever virus. *J. Virol.* **79**:3807-3821.
90. **Hoenninger, V. M., H. Rouha, K. K. Orlinger, L. Miorin, A. Marcello, R. M. Kofler, and C. W. Mandl.** 2008. Analysis of the effects of alterations in the tick-borne encephalitis virus 3'-noncoding region on translation and RNA replication using reporter replicons. *Virology* **377**:419-430.
91. **Tajima, S., Y. Nukui, M. Ito, T. Takasaki, and I. Kurane.** 2006. Nineteen nucleotides in the variable region of 3' non-translated region are dispensable for the replication of dengue type 1 virus in vitro. *Virus Res.* **116**:38-44.
92. **Pastorino, B., A. Nougairède, N. Wurtz, E. Gould, and L. de, X.** 2010. Role of host cell factors in flavivirus infection: Implications for pathogenesis and development of antiviral drugs. *Antiviral Res.* **87**:281-294.
93. **De Nova-Ocampo, M., N. Villegas-Sepulveda, and R. M. del Angel.** 2002. Translation elongation factor-1alpha, La, and PTB interact with the 3' untranslated region of dengue 4 virus RNA. *Virology* **295**:337-347.
94. **Davis, W. G., J. L. Blackwell, P. Y. Shi, and M. A. Brinton.** 2007. Interaction between the cellular protein eEF1A and the 3'-terminal stem-loop of West Nile virus genomic RNA facilitates viral minus-strand RNA synthesis. *J. Virol.* **81**:10172-10187.
95. **Paranjape, S. M. and E. Harris.** 2007. Y box-binding protein-1 binds to the dengue virus 3'-untranslated region and mediates antiviral effects. *J. Biol. Chem.* **282**:30497-30508.
96. **Ta, M. and S. Vratsi.** 2000. Mov34 protein from mouse brain interacts with the 3' noncoding region of Japanese encephalitis virus. *J. Virol.* **74**:5108-5115.
97. **Vashist, S., M. Anantpadma, H. Sharma, and S. Vratsi.** 2009. La protein binds the predicted loop structures in the 3' non-coding region of Japanese encephalitis virus genome: role in virus replication. *J. Gen. Virol.* **90**:1343-1352.
98. **Yocupicio-Monroy, M., R. Padmanabhan, F. Medina, and R. M. del Angel.** 2007. Mosquito La protein binds to the 3' untranslated region of the positive and negative polarity dengue virus RNAs and relocates to the cytoplasm of infected cells. *Virology* **357**:29-40.

99. **Agis-Juarez, R. A., I. Galvan, F. Medina, T. Daikoku, R. Padmanabhan, J. E. Ludert, and R. M. del Angel.** 2009. Polypyrimidine tract-binding protein is relocated to the cytoplasm and is required during dengue virus infection in Vero cells. *J. Gen. Virol.* **90**:2893-2901.
100. **Anwar, A., K. M. Leong, M. L. Ng, J. J. Chu, and M. A. Garcia-Blanco.** 2009. The polypyrimidine tract-binding protein is required for efficient dengue virus propagation and associates with the viral replication machinery. *J. Biol. Chem.* **284**:17021-17029.
101. **Jiang, L., H. Yao, X. Duan, X. Lu, and Y. Liu.** 2009. Polypyrimidine tract-binding protein influences negative strand RNA synthesis of dengue virus. *Biochem. Biophys. Res. Commun.* **385**:187-192.
102. **Garcia-Montalvo, B. M., F. Medina, and R. M. del Angel.** 2004. La protein binds to NS5 and NS3 and to the 5' and 3' ends of Dengue 4 virus RNA. *Virus Res.* **102**:141-150.
103. **Yocupicio-Monroy, R. M., F. Medina, V. J. Reyes-del, and R. M. del Angel.** 2003. Cellular proteins from human monocytes bind to dengue 4 virus minus-strand 3' untranslated region RNA. *J. Virol.* **77**:3067-3076.
104. **Derry, M. C., A. Yanagiya, Y. Martineau, and N. Sonenberg.** 2006. Regulation of poly(A)-binding protein through PABP-interacting proteins. *Cold Spring Harb. Symp. Quant. Biol.* **71**:537-543.
105. **Polacek, C., P. Friebe, and E. Harris.** 2009. Poly(A)-binding protein binds to the non-polyadenylated 3' untranslated region of dengue virus and modulates translation efficiency. *J. Gen. Virol.* **90**:687-692.
106. **Tan, B. H., J. Fu, R. J. Sugrue, E. H. Yap, Y. C. Chan, and Y. H. Tan.** 1996. Recombinant dengue type 1 virus NS5 protein expressed in *Escherichia coli* exhibits RNA-dependent RNA polymerase activity. *Virology* **216**:317-325.
107. **Kim, Y. G., J. S. Yoo, J. H. Kim, C. M. Kim, and J. W. Oh.** 2007. Biochemical characterization of a recombinant Japanese encephalitis virus RNA-dependent RNA polymerase. *BMC. Mol. Biol.* **8**:59.
108. **Cui, T., R. J. Sugrue, Q. Xu, A. K. Lee, Y. C. Chan, and J. Fu.** 1998. Recombinant dengue virus type 1 NS3 protein exhibits specific viral RNA binding and NTPase activity regulated by the NS5 protein. *Virology* **246**:409-417.
109. **Chen, C. J., M. D. Kuo, L. J. Chien, S. L. Hsu, Y. M. Wang, and J. H. Lin.** 1997. RNA-protein interactions: involvement of NS3, NS5, and 3' noncoding regions of Japanese encephalitis virus genomic RNA. *J. Virol.* **71**:3466-3473.
110. **Simon, A. E. and L. Gehrke.** 2009. RNA conformational changes in the life cycles of RNA viruses, viroids, and virus-associated RNAs. *Biochim. Biophys. Acta* **1789**:571-583.
111. **Mackenzie, J. M., A. A. Khromykh, M. K. Jones, and E. G. Westaway.** 1998. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. *Virology* **245**:203-215.
112. **McMinn, P. C.** 1997. The molecular basis of virulence of the encephalitogenic flaviviruses. *J. Gen. Virol.* **78 (Pt 11)**:2711-2722.
113. **Leitmeyer, K. C., D. W. Vaughn, D. M. Watts, R. Salas, I. Villalobos, C. de, C. Ramos, and R. Rico-Hesse.** 1999. Dengue virus structural differences that correlate with pathogenesis. *J. Virol.* **73**:4738-4747.
114. **Pletnev, A. G.** 2001. Infectious cDNA clone of attenuated Langkat tick-borne flavivirus (strain E5) and a 3' deletion mutant constructed from it exhibit decreased neuroinvasiveness in immunodeficient mice. *Virology* **282**:288-300.
115. **Gritsun, T. S., A. Desai, and E. A. Gould.** 2001. The degree of attenuation of tick-borne encephalitis virus depends on the cumulative effects of point mutations. *J. Gen. Virol.* **82**:1667-1675.

116. **Chiou, S. S. and W. J. Chen.** 2001. Mutations in the NS3 gene and 3'-NCR of Japanese encephalitis virus isolated from an unconventional ecosystem and implications for natural attenuation of the virus. *Virology* **289**:129-136.
117. **Blaney, J. E., Jr., D. H. Johnson, G. G. Manipon, C. Y. Firestone, C. T. Hanson, B. R. Murphy, and S. S. Whitehead.** 2002. Genetic basis of attenuation of dengue virus type 4 small plaque mutants with restricted replication in suckling mice and in SCID mice transplanted with human liver cells. *Virology* **300**:125-139.
118. **Edgil, D., M. S. Diamond, K. L. Holden, S. M. Paranjape, and E. Harris.** 2003. Translation efficiency determines differences in cellular infection among dengue virus type 2 strains. *Virology* **317**:275-290.
119. **Kinney, R. M., C. Y. Huang, B. C. Rose, A. D. Kroeker, T. W. Dreher, P. L. Iversen, and D. A. Stein.** 2005. Inhibition of dengue virus serotypes 1 to 4 in vero cell cultures with morpholino oligomers. *J. Virol.* **79**:5116-5128.
120. **Deas, T. S., C. J. Bennett, S. A. Jones, M. Tilgner, P. Ren, M. J. Behr, D. A. Stein, P. L. Iversen, L. D. Kramer, K. A. Bernard, and P. Y. Shi.** 2007. In vitro resistance selection and in vivo efficacy of morpholino oligomers against West Nile virus. *Antimicrob. Agents Chemother.* **51**:2470-2482.
121. **Zhang, B., H. Dong, D. A. Stein, and P. Y. Shi.** 2008. Co-selection of West Nile virus nucleotides that confer resistance to an antisense oligomer while maintaining long-distance RNA/RNA base pairings. *Virology* **382**:98-106.
122. **Stein, D. A., C. Y. Huang, S. Silengo, A. Amantana, S. Crumley, R. E. Blouch, P. L. Iversen, and R. M. Kinney.** 2008. Treatment of AG129 mice with antisense morpholino oligomers increases survival time following challenge with dengue 2 virus. *J. Antimicrob. Chemother.* **62**:555-565.
123. **Yoo, J. S., C. M. Kim, J. H. Kim, J. Y. Kim, and J. W. Oh.** 2009. Inhibition of Japanese encephalitis virus replication by peptide nucleic acids targeting cis-acting elements on the plus- and minus-strands of viral RNA. *Antiviral Res.* **82**:122-133.

