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

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Chapter 6

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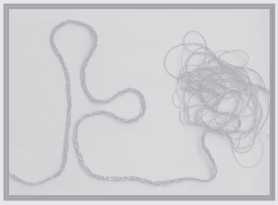
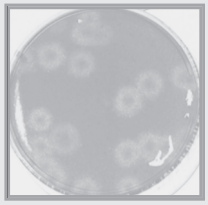
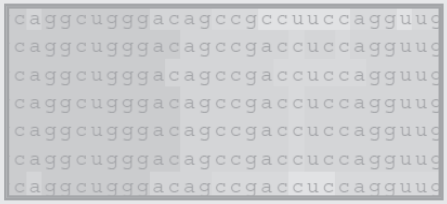

ABSTRACT

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 genera [1]. 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 structures [4-8]. 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 similarity [9-15].

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].

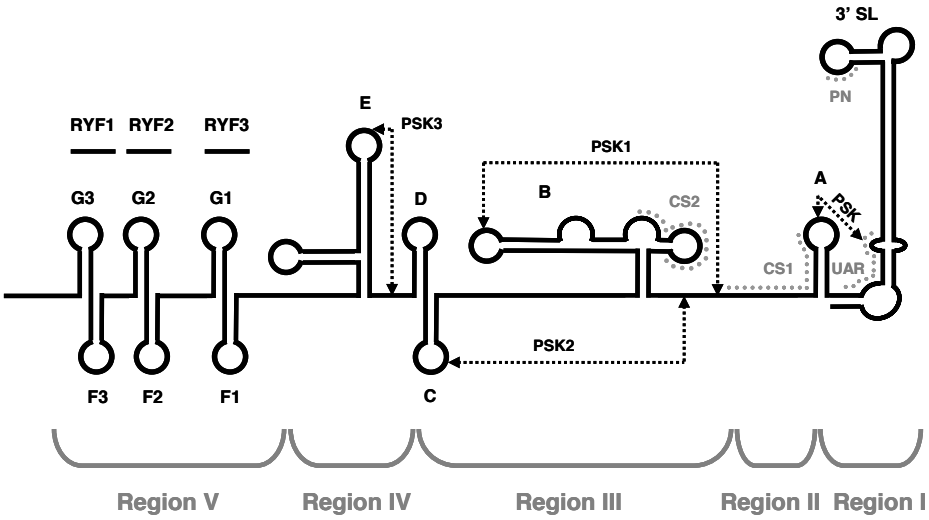

Fig. 1. Schematic model of the predicted RNA folding of the 3′ UTR of the prototype flavivirus yellow fever virus (YFV). The 3′ UTR was divided into five different regions. Nomenclature for the individual stem-loop (except 3′ SL) and pseudoknot structures was adopted from Olsthoorn and Bol [6]. The pseudoknot (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

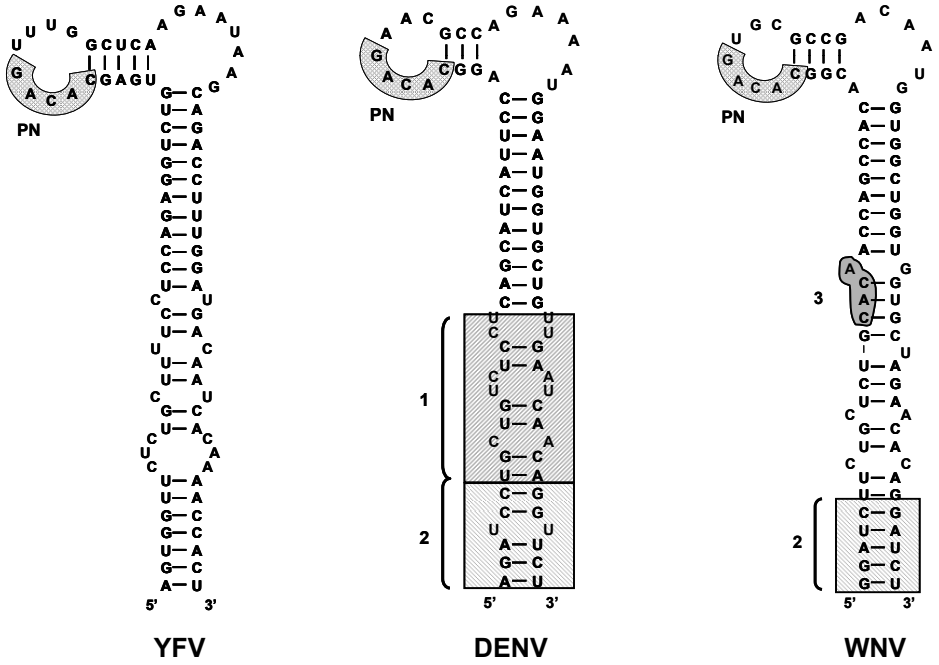

Fig. 2. Structural model of the terminal 3′ SL structure of YFV, DENV and WNV. The pentanucleotide motif (PN) is indicated. The shaded box 1 represents a region important for virus viability while box 2 represents the region that is required for replication in mosquito cells. The region indicated by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

5′-CU-3′, which is complementary to the dinucleotide 5′-AG-3′ present at the very 5′ end of every flavivirus genome [19-21]. 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 position [7].

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′ SL [23-26], to actually inhibiting translation [27,28].

Deletion of the 3′ SL showed that this conserved RNA structure is absolutely required for flavivirus RNA synthesis [29-34]. 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 paring 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 motif [37]. 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 show to be involved in binding eukaryotic translation elongation factor-1α (eEF-1α) [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 flaviviruses [41].

Immediately upstream the 3′ SL is a smaller stem-loop structure (SL-A according to the nomenclature of Olsthoorn and Bol [6]) that comprises 14 to 18 nucleotides and that is present in every flavivirus. Biochemical and biophysical probing and *in silico* RNA modelling 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′ SL [22] (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 assays [30]. Furthermore, a PMO targeting the pseudoknot interaction moderately inhibited the replication of a WNV reporter replicon [38]. 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 sequences [29]. Deletion of SL-A was shown to decrease the translation efficiency of a DENV reporter construct [26]. SL-A was suggested to be a minor binding site for the host eEF-1α protein in WNV [40].

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′ SL [29], and number 3 in the WNV 3′ SL (5′-CACA-3′, the eEF-1α-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 cells [29]. 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 cells [34]. 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 protein [4]. 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 synthesis [4]. 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 microscopy [50]. 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 synthesis [44]. 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′ SL [50-52]. These complementary sequences were named 5′-3′UAR (upstream AUG region) and were shown to

be important for viral replication [50]. Similar to the 5'CS-3'CS1 interaction, 5'-3'UAR base pairing was also demonstrated by atomic force microscopy [50]. 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 interaction [53]. 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 sequences [55]. In YFV, the DAR motifs [56] were originally included in the 18 nt found to be part of the 5' CS and 3' CS1 elements [47] (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' end [21]. 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 flaviviruses [45]. 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 replication [57]. 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 se [59]. This was supported by the fact that the SLA was found to be specifically recognized by the viral

YFV	**5' CS:** 5' – C**CCUGG**GCGUCAAUAUGGU – 3'	**3' CS1:** 5' – ACCAUAUUGACG**CCAGG**G – 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' – AGAGAGCAGAUCUCUG – 3'	**3' UAR:** 5' – CAGAGAUCCUGCUGUCU – 3'
	5' DAR: 5' – CCAACG – 3'	**3' DAR:** 5' – CGCUGG – 3'
WNV	**5' CS:** 5' – UGUCAAUAUGCU – 3'	**3' CS1:** 5' – AGCAUAUUGACA – 3'
	5' UAR: 5' – AGCACGAAGAUCUC – 3'	**3' UAR:** 5' – GAGAUCUUCUGCU – 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' – UAGAACGGAAGAUAACCAUG – 3'	**3' UAR:** 5' – UGGGAGAUCUUCUGCUCUA – 3'
	5' DAR: 5' – CCAGG – 3'	**3' DAR:** 5' – CCUGG – 3'
TBEV	**5' CS-A:** 5' – GGAGAACAAGAGCUG – 3'	**3' CS-A:** 5' – CGGUUCUUGUUCUCC – 3'
	5' CS-B: 5' – GGGGCGGUCCC – 3'	**3' CS-B:** 5' – GGGAGGCCCC – 3'
MODV	**5' CS:** 5' – AAUGUGCGAAAAUAACAGGA	**3' CS:** 5' – UCCUGUUAUUUUCCAAAUU – 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 sequences [47] correspond to nucleotides that were afterwards described as the DAR elements [56]. 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 ± 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 ~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 RCS2 [4]). 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 cells [66]. 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 rDENV4Δ30 mutant was shown to be attenuated in rhesus monkeys [48] and well tolerated and highly immunogenic in human volunteers [69,70]. In addition, rDENV4Δ30 also exhibited a limited ability to infect the midgut of mosquitoes [71]. Introduction of the Δ30 mutation into the homologous region of DENV1 yielded similar results as for rDENV4Δ30 [72,73]. Unfortunately, introduction of the Δ30 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 Δ30 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 replication [78].

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

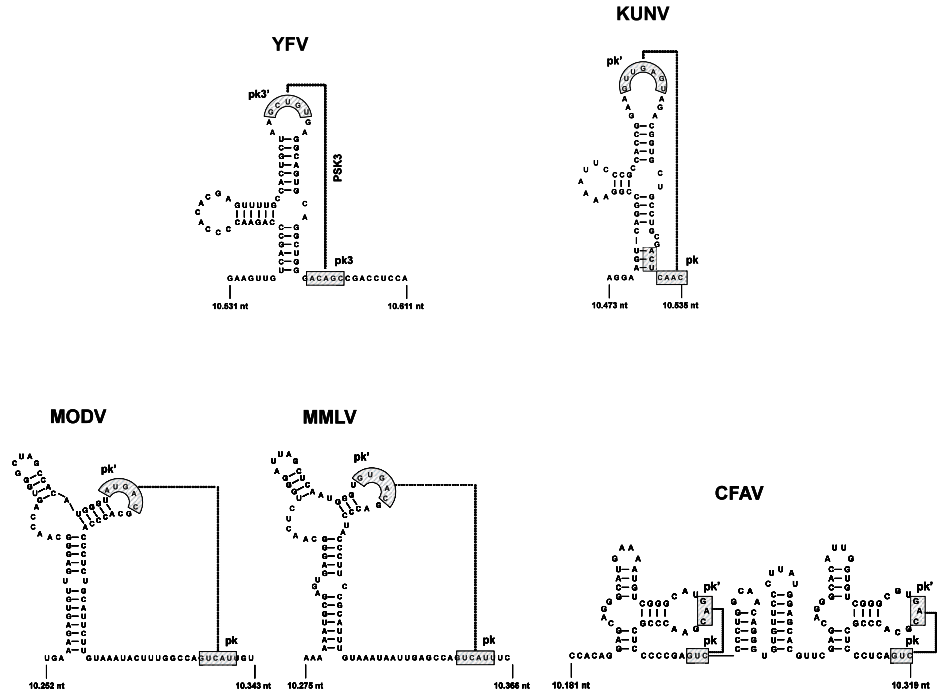

Fig. 4. RNA sequence and predicted RNA structures for the part of region III that is required for stalling XRN1. RNA structures were predicted using a combinatorial approach involving Mfold, phylogeny and manual sequence-structure analysis. The predicted RNA pseudoknot formation by interaction of the 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 sfRNA 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 sfRNA 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 sfRNA.

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 extend 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-1α (EF-1α), Y box binding protein-1 (YB-1) and the protein Mov34.

The eukaryotic translation elongation factor-1α (EF-1α) 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-1α to the 3′ SL is primarily determined by an only four nucleotides long 5′-CACA-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-1α and the WNV 3′ SL was required for viral (-) strand RNA synthesis [94]. In addition, in WNV and DENV4 infected cells, eEF-1α 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-1α 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 translation [95]. 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 JEV [96]. 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 chromatography [95]. 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 suggested [93]. 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 production [100]. 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 in [104]). Despite the fact that the genome RNA of

flaviviruses is not polyadenylated, PABP was shown to bind to the DENV 3′ UTR [105]. 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 genome [105].

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 DENV [108]. 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′ UTR [109]. 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 genome [59]. 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 reported [111].

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 in [112]), 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 virus [16]. 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.

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