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

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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

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Chapter 7

Epilogue

Positive-stranded RNA viruses represent the biggest fraction of all known viruses [1] and are responsible for many human and animal diseases. RNA viruses have the highest mutation rate among living species, which explains why they can adapt relatively easy to new environments (reviewed in [2]). As Stuart Nichol [3] wrote "There is a sense that RNA viruses enjoy life in the evolutionary fast lane; however we, slow-moving DNA-based life forms, may have some opportunities to outwit them yet". In order to achieve this, a thorough understanding of the virus life cycle in general and the molecular interactions between the virus and the host is required. Certain steps in the viral life cycle were found to correlate closely with conformational changes in the viral RNA [4]. RNA elements present in the 3′ untranslated region (3′ UTR) of the genome in particular, are known to undergo such dynamic conformational changes and to play a role in critical steps such as translation and replication. In this thesis, RNA structures and motifs present in the 3′ UTR of flaviviruses were studied in more detail, including their role in the virus life cycle.

The 3′ UTR of the flavivirus genome is predicted to fold into a complex structure that includes well conserved primary sequences, multiple RNA stem-loop structures as well as a number of RNA pseudoknots [5,6] (chapter 6 of this thesis). Some of these RNA structures have been studied extensively, while others have only been predicted based on phylogenetic studies and computer-aided RNA folding and still require functional characterization.

As shown in chapter 2 and discussed in chapter 6, the well conserved pentanucleotide (PN) sequence CACAG at the top bulge of the 3′ terminal stem-loop (3′ SL) is an essential element for viral RNA synthesis [7-9]. Unfortunately, the function of this PN motif is not known. Several studies have indicated that it is part of the binding site for certain host proteins such as the eukaryotic translation elongation factor-1α (eEF-1α) in West Nile virus (WNV) [10,11], Y box binding protein-1 (YB-1) in dengue virus (DENV) [12] and La protein in Japanese encephalitis virus (JEV) [13]. Although none of these studies claim that the PN motif is the major interface for this protein-RNA interaction, it is rather surprising that such a conserved sequence appears to be part of the binding site for these different and functionally unrelated proteins. An alternative explanation is that the PN motif is actually part of an RNA kissing or pseudoknot interaction; however, the fact that the yellow fever virus (YFV) PN motif tolerates point mutations at almost every position would also argue against this hypothesis. Recently, the structure of the top of the 3′ SL including the PN motif was solved for mosquito-borne, tick-borne and no known vector (NKV) flaviviruses by nuclear magnetic resonance (NMR) spectroscopy [14]. Different from what was expected, the NMR analysis did not yield a conserved RNA structure for the studied viruses. This lack of conservation can be explained by the current technical limitations associated to NMR. Only the very top part of the 3′ SL was analyzed, therefore i) the folding might have been incorrect in comparison with the natural situation, ii) relevant long-range RNA-RNA interactions involving the top of the 3′ SL structure could not be taken into account. Alternatively, the absence of particular host or viral proteins may

have prevented the RNA from adopting the natural conformation. Our study and those of others have clearly demonstrated that the PN motif is crucial for flaviviruses RNA synthesis and showed that the wild-type CACAG sequence confers a selective advantage. The mutational analysis of this region mainly involved point mutations. Therefore, it will be interesting to perform an *in vivo* RNA SELEX experiment to reveal the actual sequence requirements of the PN motif as well as of the nucleotides immediately downstream. Viable virus mutants could be selected by their ability to form plaques. Continued passaging of poorly replicating mutants isolated from such a screen can potentially identify efficiently replicating "second-site revertant" viruses that may yield valuable information about the function and interaction partners of the PN motif.

The RNA pseudoknot that serves as a stalling site for XRN1, resulting in the generation of a small flavivirus (sf) RNA, is another RNA element in the flavivirus 3′ UTR that was described in detail in this thesis [15]. An RNA pseudoknot is essentially an RNA structure that is minimally composed of two helical segments, which are connected by single stranded regions [16]. These tertiary RNA structures were initially recognized in the 3′ UTR of turnip yellow mosaic virus [17] and subsequently found to be one of the most widespread structural RNA domains. Currently, pseudoknots have been shown to be essential in the catalytic center of the hepatitis delta virus and related ribozymes, telomerases, the regulation of gene expression by ribosomal frameshifting, IRES-driven translation initiation, and to be involved in the transcription and replication of many RNA viruses (reviewed in [16,18]). The stalling of the host ribonuclease XRN1 by an RNA pseudoknot in the flavivirus 3′ UTR (chapters 3 and 4 of this thesis and by others [19,20]), points yet to another function of these RNA structures. Although the role of XRN1 in 5′ to 3′ RNA decay is well documented (reviewed in [21-24]), these particular RNA pseudoknots are the first tertiary RNA structures that are capable of stalling XRN1. In addition, these results provide an interesting link between RNA structure and pathogenicity, as the sfRNA that results from the incomplete degradation of the viral genome has been demonstrated to play an important role in the pathogenicity of mosquito-borne flaviviruses in cell culture and in mice [15,19,20]. The precise role of the sfRNA in the viral life cycle is currently unknown. It has been speculated that the sfRNA is involved in evading the host innate immune response, for instance by antagonizing cellular RNA sensors like RIG-I and MDA5 [25]. Alternatively, it has been suggested that the sfRNA could serve as a decoy to protect the genomic RNA from cellular proteins or miRNAs that would otherwise bind to the 3′ UTR of the viral genome and inhibit viral RNA synthesis or translation [25]. There is currently no evidence that supports any of the above hypotheses. It is, however, interesting to note in this context that the cellular YB-1 protein, which binds to the DENV 3′ SL structure, has been suggested to mediate antiviral activity [12].

An intriguing alternative hypothesis is that the sfRNA itself serves as a precursor for a virus-encoded miRNA. The ribonuclease XRN1, that is required for the production of the

sfRNA, is enriched in cytoplasmic P bodies that also harbor proteins like Dicer, GW182 and Argonaute, which are involved in RNA interference [21-23,26]. Fluorescence *in situ* hybridization (FISH) analysis of Kunjin-infected cells suggested accumulation of the sfRNA in these P bodies [19]. Recent experiments have shown that recombinant flaviviruses containing the sequence of a cellular miRNA can produce functional miRNAs upon infection [27,28]. These results demonstrate that flavivirus RNA is somehow able to enter the miRNA biogenesis pathway. However, in apparent contradiction with the above hypothesis, is a progressive decrease in the number of P bodies that has been observed in cells during the time course of DENV-2 and WNV infections [29]. To increase our understanding regarding sfRNA production, a more detailed analysis of the kinetics and the subcellular sites of sfRNA synthesis is required. Important questions that need to be address are: i) How do flavivirus genomes end up in P bodies and are these important for sfRNA generation ii) Are the sfRNAs the final product or are they intermediates that undergo further processing iii) Do sfRNAs or their derivatives leave the P bodies and is this required for their function? Various experiments can be envisioned to deal with these questions. "Pulse-chase"-like experiments using temperature-sensitive viruses impaired in viral RNA synthesis or small molecule inhibitors of flavivirus replication are required to determine the kinetics of sfRNA production and turn-over. Reagents that either induce or disrupt P bodies and the associated stress granules should be tested as they can be useful in determining the role of these subcellular structures in sfRNA synthesis. In addition, expanding the RNA silencing experiments that so far have been limited to XRN1, by including other targets like stress granules markers [e.g. Ras-GTPase-activating protein SH3-domain-binding protein (G3BP), T-cell intracellular antigen 1 (TIA-1) or TIA-1-related protein (TIAR)], P body markers (e.g. RNA decapping enzymes DCP1 and 2) and various proteins involved miRNA production (Dicer as an obvious candidate).

One of the important findings of the research described in this thesis is that sfRNA production is not limited to arthropod-borne flaviviruses, but instead is a rather unique feature of all flaviviruses irrespective of the nature of their transmission cycle. This does not only imply that sfRNA production can be used as an unique additional criterion to define currently unassigned and newly discovered RNA viruses to the Flavivirus genus, but also indicates that the sfRNA production is required in the natural life cycle of all these viruses. It remains to be established whether the sfRNA of the different flaviviruses serves a similar function in the different hosts or whether it has evolved to meet specific virus requirements related to (a) particular host(s). The fact that CFAV produces an sfRNA hints at the possibility that the sfRNA of the arthropod-borne flaviviruses may not only be required in the vertebrate host but also in mosquitoes or ticks. One way to address these questions is by expression of sfRNAs in *trans* in cells that are infected with mutant viruses that no longer produce the sfRNA [19]. Such a system can be used not only to determine whether a heterologous sfRNA is able to complement the defect in pathogenicity

of an sfRNA-minus flavivirus mutant, but will also allow to determine which part of the sfRNA is required for its biological function.

It will be interesting to discover whether any of the conserved sequences or RNA structures in the flavivirus 3' UTR is actually required for sfRNA function. One possible candidate is the conserved sequence CS2 which currently has no clearly defined function. Deletion of CS2 in mosquito-borne flaviviruses was shown to yield viable mutants, albeit with a slightly delayed replication [30-33]. It is interesting to note that in YFV, deletion of CS2 results in turbid plaques on SW13 cells when compared to the clear plaques observed with the wild-type virus [30]. This turbidity in the plaques can be interpreted as a sign of decreased pathogenicity, similar to what is observed with mutants deficient in sfRNA production. However, it should also be noted that CS2 is absent from TBEV and CFAV.

Flaviviruses are not the only RNA viruses that apparently use a host ribonuclease for the production of viral subgenomic RNA. Plants and protoplasts infected with the positive-strand RNA Red clover necrotic mosaic virus (RCNMV) produce a small viral noncoding RNA, designated SR1f, by incomplete degradation of the genomic RNA 1 possibly by a host enzyme with 5'-3' exoribonuclease activity [34]. SR1f has no effect on the pathogenicity of the virus but it inhibits translation of the viral proteins resulting in a decrease in negative-strain RNA synthesis [34]. A 58-nucleotide sequence at the 5' end of this RCNMV is sufficient to stall the host ribonuclease and yield SR1f. There is currently no evidence that XRN1 is involved in SR1f production, but it is interesting to note that the 5' end of SR1f can fold into a pseudoknot structure that could act as a stalling site for the involved ribonuclease (P.A.G.C. Silva and P.J. Bredenbeek; data not shown).

The involvement of XRN1 in the life cycle of viruses is not without precedent. XRN1 has also been shown to act as a suppressor of viral RNA recombination by rapidly degrading 5' truncated RNAs that can serve as substrates for viral recombination in tomato bushy stunt virus, a positive-stranded plant RNA virus belonging to Tombusviridae [35].

The last experimental chapter of this thesis describes the construction and characterization of a full-length MODV cDNA that can be used to transcribe infectious MODV RNA. The development of these tools is important in order to truly understand the molecular biology of the flaviviruses and to identify the key factors that determine host range and tropism.

Many unanswered questions still remain. In the end, a full understanding of the 3' UTR structures and specific conformation adopted during the virus life cycle and the function they perform will require the determination of their three-dimensional structure. In this respect, X-ray crystallography and NMR spectroscopy are major challenges to be conquered in future studies. Such knowledge will provide us with further insight into the regulatory mechanisms behind the viral life cycle and can ultimately reveal targets and offer us, "DNA-based life forms", tools to outwit infection by the rapidly evolving RNA viruses.

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