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

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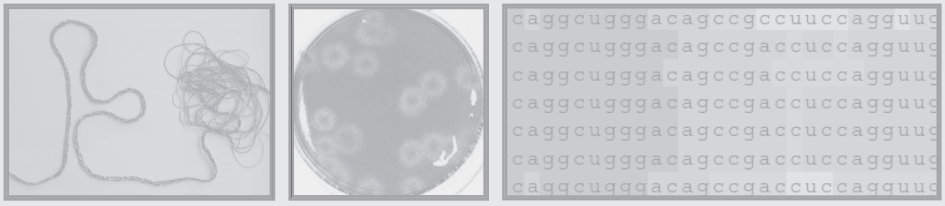
Chapter 1

General introduction



GENERAL INTRODUCTION

Infectious diseases are estimated to be the direct cause of more than 25% of all annual human deaths worldwide [1]. In fact, infection itself exerts a tremendous selective pressure that has driven the evolution of resistance mechanisms and, to a large extent, has shaped the human genome [2]. Infectious diseases are caused by many different organisms from several biological taxa. There are more than 1.400 species known to be pathogenic to humans [3]. Many of these species are associated with emerging diseases and they mainly consist of zoonotic pathogens, with 44 % of these agents being viruses and prions [3]. RNA viruses in particular, are the cause of many of the emerging and re-emerging diseases of the last decades [4]. RNA viruses have the highest mutation rate among species (estimated at 10^{-3} to 10^{-5} misincorporations per nucleotide and replication cycle) due to the lack (or low efficiency) of proof-reading activity by their RNA-dependent RNA polymerase (RdRp). As a consequence, RNA viruses replicate as complex and dynamic swarms of virus mutants known as virus quasispecies. In combination with short replication times and extremely large populations, this explains why RNA viruses can efficiently adapt to new selective pressures in the environment and are able to exploit new ecological niches and to jump between host species (reviewed in [5]). There are currently 95 virus families and unassigned genera approved by the International Committee on Taxonomy of Viruses [6]; among these, positive-stranded RNA viruses undoubtedly comprise the biggest fraction and it is within this group that we encounter the Flaviviruses.

Flavivirus genus

The family *Flaviviridae* currently consists of three genera: *Flavivirus* (from the Latin *flavus*, "yellow"), *Pestivirus* (from the Latin *pestis*, "plague"), and *Hepacivirus* (from the Greek *hepar, hepatos*, "liver"). Besides these genera, two distinct groups of viruses have tentatively been assigned to the family, GBV-A and GBV-C [7]. All members of the *Flaviviridae* family share similar characteristics in virion morphology, genome organization, and replication strategy. In contrast, members of the three genera are antigenically unrelated and exhibit different biological properties, such as host range and transmission [7]. The *Flavivirus* genus contains nearly 80 viruses and its members show a worldwide distribution. The majority of Flaviviruses is arthropod-borne and many of them are important human pathogens that can cause a variety of diseases including encephalitis and hemorrhagic fevers. Flaviviruses of major global concern include dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and tick-borne encephalitis virus (TBEV). Flaviviral infections have dramatically increased in frequency [8] and the reasons underlying this phenomenon are complex. The decrease in mosquito

control measures during the last decades together with social and environmental factors such as the unprecedented population growth, increased urbanization, travel, trade, and deforestation are believed to be the main reasons for the re-emergence of flaviviruses. DENV, for instance, has spread into new areas and is now endemic in more than 100 countries where 2.5 billion people (40% of the world's population) are at risk of infection and an estimated 50 million people are infected every year [9]. YFV appeared to be under control in the middle of the previous century due to mass vaccination campaigns and eradication of the principal urban vector, *Aedes aegypti*. However, YFV is re-emerging as numerous outbreaks have been registered during the last decades in both Africa and South America, due to the declining vaccination coverage and mosquito reinfestation [10-12]. Furthermore, the vectors used by flaviviruses have the ability to infest alternative favourable habitats where the viruses can eventually cause an epidemic. WNV had never been isolated in the Americas until 1999, when it emerged in New York City [13]. By the end of 2003, it was present in almost every state of the United States, Mexico, and the Caribbean. It has now been detected as far south as Argentina (reviewed in [14]). Climate changes can also affect vector distribution implying that global warming, for instance, could significantly increase the potential for flavivirus dispersal (reviewed in [14,15]). As an example, *Ixodes ricinus*, the main vector of TBEV, used to be found in Europe up to 700-750 m above sea level in the early 1980s, but in 2001 it was found up to 1.000 m [16], and in 2009 ticks infected with TBEV were detected up to 1.140 m [17]. Global warming was suggested to be responsible for this shift in the habitat of ticks.

Vectors of members of the Flavivirus genus

Phylogenetically, the Flavivirus genus is grouped into three clusters based on the vector involved in transmission: (i) mosquito-borne, (ii) tick-borne, and (iii) no known vector (NKV) viruses [18,19]. The evolutionary relationship between these three clusters is not clear. Initial phylogenetic analysis using the amino acid sequences of the envelope gene established that mosquito- and tick-borne viruses represent two different evolutionary lineages [20]. In another study, the flaviviruses NS5 amino acid sequence was used for phylogenetic analysis [18]. This study included the NKV flaviviruses and postulated that the NKV and then the vector-borne flaviviruses have emerged from an ancestral insect-borne flavivirus. These vector-borne viruses later diverged into the tick-borne and then the mosquito-borne virus clusters, suggesting that arthropod-mediated transmission is a derived trait within the genus. Other studies supported this topology based on the NS5 gene [21]. However, an alternative phylogenetic tree based on the amino acid sequence encoded by either the NS3 gene or the entire open reading frame (ORF) demonstrated that the tick-borne and NKV viruses have diverged together and independently from the mosquito-borne flaviviruses, suggesting a common ancestor for the tick-borne and NKV viruses [19,22,23]. The mosquito-borne cluster can be further subdivided into two

epidemiologically distinct groups: the *Culex* and the *Aedes* mosquito clades. A strong correlation was observed between the mosquito clade, the mammalian host, and the type of disease caused by the virus. In general, flaviviruses transmitted by the *Culex* mosquito are neurotropic viruses associated with neurological diseases in both humans and livestock. These viruses usually cycle between mosquitoes and birds. In contrast, flaviviruses transmitted by the *Aedes* mosquito are associated with hemorrhagic fevers and are non-neurotropic in humans. These viruses usually cycle between mosquitoes and primate hosts [21].

Besides the viruses assigned to one of the three clusters within the Flavivirus genus, there are a few viruses that are currently considered tentative species of the flavivirus genus. This group includes viruses like cell fusing agent virus (CFAV), Kamiti River virus (KRV) and *Culex* flavivirus (CxFV), which have all been exclusively isolated from mosquitoes or insect cell lines [24-26]. There is no evidence that these viruses are able to infect a vertebrate host and therefore they are also referred to as insect flaviviruses. Interestingly, they have been suggested to represent the primordial forms of the Flavivirus genus [18-20,27], and CFAV- and KRV-related genomic fragments have been found integrated in a DNA form in the genome of Aedes mosquitoes [28,29].

Flaviviruses transmission by arthropods, such as mosquitoes or ticks, requires infection of the vector's midgut tissue after the ingestion of an infected blood meal and subsequent release of progeny virus. Subsequently, the virus escapes from the midgut and spreads to the hemocele, from where the virus is able to infect several other tissues, including the salivary glands. After infection of the salivary glands, the arthropod vector is able to transmit the virus via the infected saliva while feeding on a susceptible host (reviewed in [30]). The arthropod vector usually becomes persistently infected [31]. Vertebrate hosts that survive a flavivirus infection usually develop lifelong immunity, implying that flaviviruses need a constant supply of immunologically naïve hosts. As a result, the majority of the flaviviruses is enzootic and infects vertebrate hosts with a high reproductive rate like birds or small mammals (reviewed in [32]). In the case of DENV, which has four distinct serotypes, recovery from infection by one of the serotypes provides lifelong immunity against that particular serotype, but only partial and transient protection against the other three serotypes [9]. Except for dengue, humans are only infected accidentally when they intrude in the natural cycle of flaviviruses. For most flaviviruses, humans are in fact considered "dead end" hosts as these infections do not result in a significant viremia; humans are therefore unable to transmit the virus when bitten by arthropod vectors. Legendary exceptions are the dengue virus, for which humans actually seem to be the natural vertebrate host [9], and yellow fever virus (reviewed in [32]).

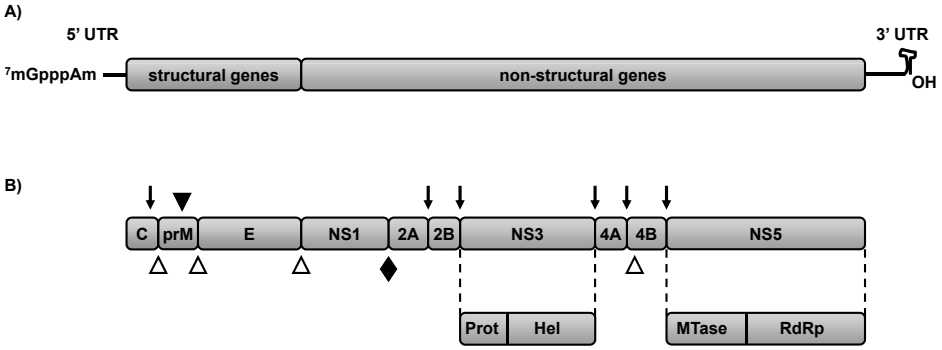


Fig. 1. Flavivirus genome organization.
A) Schematic representation of the flavivirus genome structure. The viral RNA encodes one large open reading frame (ORF). The 5'-terminal region of the ORF encodes three viral structural proteins whereas the remaining region encodes seven non-structural (NS) proteins. The ORF is flanked by 5' and 3' untranslated regions (UTR). **B)** Polyprotein processing and cleavage products. The cleavage sites for the host signal peptidase (Δ), the viral serine protease (↓), the furin-like protease (▾), as well as a yet unknown protease responsible for the NS1-NS2A cleavage (♦) are indicated. Prot and Hel in NS3 refer to the serine-like protease and helicase domains respectively. MTase and RdRp in NS5 reflect the position of the methyltransferase/ RNA capping enzyme activity and RNA-dependent RNA polymerase domains respectively.

Flavivirus RNA genome and life cycle

Flaviviruses are small (~50 nm), enveloped animal viruses containing a single positive-strand RNA genome of approximately 11 kb with a 5'-cap structure and a 3' non-polyadenylated terminus. The genomic RNA serves as the messenger RNA for translation of a single open reading frame (ORF) into a large polyprotein that is subsequently co- and post-translationally processed into the functional viral proteins by cellular and viral proteases (fig. 1). The flavivirus ORF is flanked by 5' and 3' untranslated regions (UTRs) of approximately 100 nts and 400 to 700 nts, respectively. The N-terminal region of the polyprotein encodes the viral structural proteins core (C), membrane (prM/M), and envelope (E), which are involved in the formation of the virus particle (reviewed in [33]). The core or capsid protein is a small (≈11 kDa) basic protein that forms the icosahedral nucleocapsid in which the virus genome is packaged. Nascent C (or anchored C) protein contains a COOH-terminal hydrophobic anchor that serves as a signal sequence for ER translocation of the prM protein. This hydrophobic domain is cleaved from anchored C protein by the viral protease to produce C protein for capsid assembly (reviewed in [33]). The prM protein is a glycoprotein precursor of the viral M protein. It serves as chaperone for the E protein and forms prM-E heterodimers at the envelope of the newly formed, immature virions. This prM-E interaction prevents acid-induced conformational changes in the E protein during transit through the secretory pathway [34,35]. The conversion of immature to mature virions requires the cleavage of the prM protein into pr and M

fragments by the Golgi-resident protease furin [36]. The E protein (≈ 53 kDa) is the most prominent protein on the flavivirus surface. It mediates receptor binding and membrane fusion and is an important target for the humoral immune response. The E protein structure as present in the mature virion as well as that of the post-fusion form have been determined and the combination of these structural data with cryo-EM studies have resulted in fairly detailed models for flavivirus maturation and entry (reviewed in [37]). The furin-mediated maturation of the virion (see above) catalyzes a major rearrangement of the interactions and structure of the E protein. During entry, upon exposure to low pH, the E protein homodimers dissociate into monomers which then form trimers. This reconfiguration of the E proteins exposes the previously buried fusion peptide that is subsequently inserted into the host endosomal membrane to mediate fusion between the viral envelope and the endosomal membrane; after fusion the virion RNA is released into the cytoplasm (reviewed in [37]).

The C-terminal two-thirds of the polyprotein include seven nonstructural (NS) proteins (fig. 1) that are primarily involved in viral RNA replication. NS1 is a glycoprotein of approximately 46 kDa that can be excreted from infected cells. The role of this protein in the viral life cycle is poorly understood although there is compelling evidence that it is required for RNA replication [38-41]. NS2A is a small (22 kDa) hydrophobic transmembrane protein that is important for assembly and/or release of the newly formed virus particles [42-44]. NS2B serves as an essential cofactor for the viral serine protease activity that is associated with the N-terminal region of NS3. This protease activity mediates the cleavage of the viral polyprotein at the C-terminal side of two highly conserved basic residues located at the C-terminal of the capsid protein and at the junctions NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 (reviewed in [45,33]) (fig. 1). In addition, the C-terminal half of this protein functions as RNA helicase during viral RNA transcription. NS4A is a small (16 kDa) integral membrane protein which has been shown to induce membrane rearrangements [46,47]. An interaction between NS4A and NS1 was reported to be important for RNA replication [41]. NS4B is a small (27 kDa) hydrophobic protein that colocalizes with NS3 and viral double-stranded RNA in membrane-associated replication complexes [48]. NS5 is the largest viral protein (approximately 103 kDa). The N-terminal region of this protein has methyltransferase (MTase) activity and is required for the capping of the newly synthesized genomic RNA. The C-terminal part of NS5 contains the viral RdRp (reviewed in [33]) (fig. 1). The protein structure has been determined for the full-length NS3 [49,50] and for both MTase and RdRp NS5 domains [51-56] of several flaviviruses. A model for the full-length WNV NS5 structure has been proposed based on an *in silico* docking approach [52]. These structures are currently used for the rational design of inhibitors to block the essential function of these proteins in the virus life cycle. Moreover, NS2A, NS4A, NS4B and NS5 were found to be able to inhibit the host-antiviral interferon response [57-68].

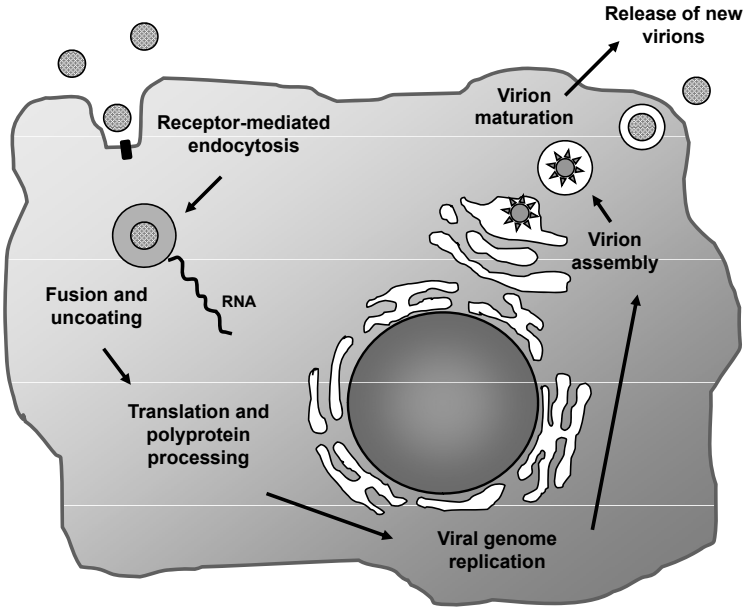


Fig. 2. Schematic representation of the Flavivirus life cycle.
See the text for more details.

Flaviviruses enter the cells by receptor-mediated endocytosis via clathrin-coated vesicles (fig. 2). They are then transported to a prelysosomal endocytic compartment. As explained in more detail above, acidification of this compartment induces a conformational change in the envelope protein that activates the fusion domain resulting in the fusion between the virus and the endosomal membrane, and ultimately resulting in the release of the viral genomic RNA into the cytoplasm. Once in the cytoplasm, the positive-strand viral RNA serves as mRNA and is translated by the host ribosomes (reviewed in [33]). Subsequent replication of the viral genome takes place in close association with virus-induced intracellular membrane structures. These membranes appear to be wrapped around the RNA amplification machinery. This replication complex (RC) is associated with unique perinuclear structures termed "vesicle packets" (VP) [69]. VPs are enriched in viral NS proteins (NS1, NS2A, NS3, NS4A, and NS5), dsRNA, and presumably some host factors [69-74]. While RNA replication takes place in these vesicle packets, translation and processing of the flavivirus polyprotein is thought to occur in association with different membrane structures designated convoluted membranes/paracrystalline arrays [72,70]. The suggested shielding of the RC by membranes is thought to prevent or reduce the exposure to cytoplasmic sensors like RIG-I or MDA5, and to dsRNA-induced host defence mechanisms, like protein kinase R, RNase L or RNA interference [75]. Additionally, it could also provide a stable and confined surface area for the RC to assemble and function [76].

A role for NS4A was demonstrated in the induction of these membrane alterations [46,47]. More recently, NS2A was also proposed to be involved in the induction of virus-specific membrane structures [44]. After the formation of the replication complex, negative-sense genome-length RNA is synthesized, which serves as a template for new positive-strand genomic molecules. Flavivirus RNA replication is an asymmetric process in which the positive-strand RNA is synthesized in 10- to 100-fold excess over the negative-strand RNA [77,78]. The newly synthesized positive-strand is subsequently used for (i) translation into new viral proteins, (ii) synthesis of negative-strand RNA, and/or (iii) encapsidation into new viral particles. Virus assembly is thought to occur by budding into the endoplasmatic reticulum (ER). The immature viral particles transit through the trans-Golgi network. Upon prM cleavage by the Golgi-resident protease furin [36], the immature viral particles turn into mature virions, which are released from the cell by the host secretory pathway (reviewed in [37], [33]) (fig. 2).

Three major viral RNA species have been detected in cells infected with flaviviruses: the genomic positive-strand RNA, a double-stranded replicative form (RF), and a heterogeneous population of replicative intermediate (RI) RNAs [77,78]. Surprisingly, an additional positive-sense small viral RNA species was reported to accumulate in both mammalian and insect cells and also in mouse brains infected with flaviviruses [79-82]. This small RNA was found to correspond to the 3′ terminal region of the viral genome [80] and to be generated by a mechanism independent of the endoribonuclease RNase L [81]. Recently, it was shown that this small flavivirus (sf) RNA is actually a product of incomplete degradation of the viral genomic RNA by the host 5′-3′ exoribonuclease XRN1 [83], the main mediator of the 5′ to 3′ mRNA decay that takes place in cytoplasmic processing bodies [84,] (reviewed in [85-87]). Interestingly, production of this sfRNA was shown to be an important parameter for viral pathogenicity [83]. The molecular basis for the role of the sfRNA in pathogenicity has not yet been elucidated.

Flavivirus genomic 3′ UTR

The 3′ UTR of flavivirus genomes is predicted to fold into a complex structure in which, despite the generally large sequence variability, a number of small, but well conserved RNA sequence elements as well as secondary and tertiary RNA structures have been identified (reviewed in [88]) (fig. 3.A). Some of these have been identified in all flaviviruses studied thus far, whereas others are characteristic for a particular cluster of the genus. The flavivirus 3′ UTR can be divided into a proximal part, immediately following the stop codon of the NS5 protein, which exhibits extensive heterogeneity in both length and sequence, 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 [89-96]. The 3′ end of the flavivirus genome is not polyadenylated; instead, all flavivirus genomes analyzed to date terminate with a large, stable stem-loop

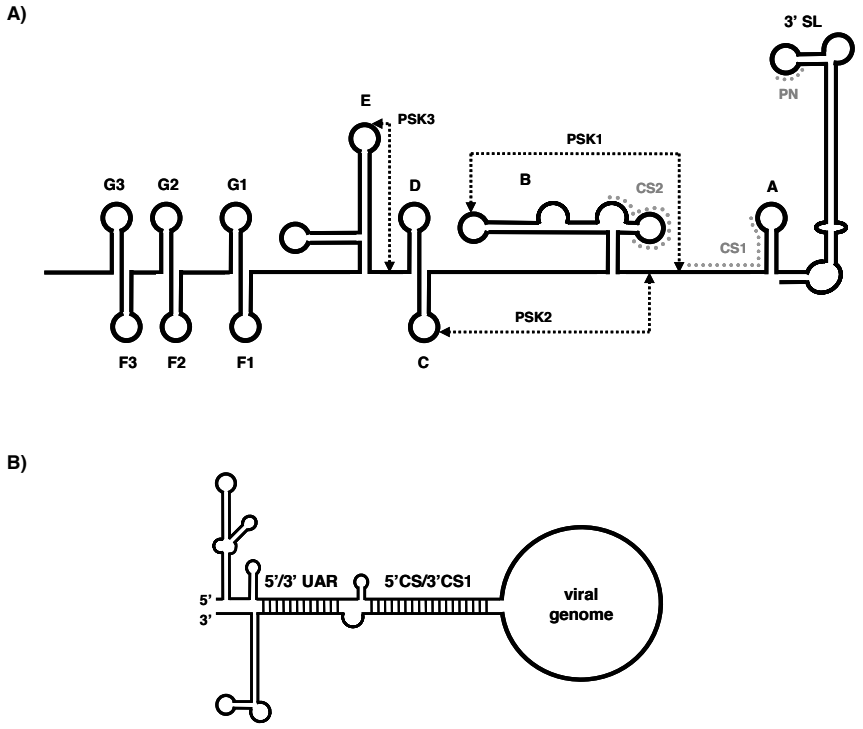


Fig. 3. Schematic model of the predicted RNA structure of the complete 3′ untranslated region (UTR) of the prototype flavivirus YFV and of the genome circularization.
A) The large 3′ terminal stem-loop structure is termed 3′ SL, other secondary RNA structures are indicated A through G as in Olsthoorn and Bol [122]. Sequences that are predicted to base pair and form an RNA pseudoknot (PSK) are connected by a dashed line. The location of conserved RNA sequences within the 3′ UTR is indicated by dots. PN, pentanucleotide motif; CS1, conserved sequence 1; CS2, conserved sequence 2. **B)** The circular conformation promoted by the long-range RNA interactions between the YFV 5′ and 3′ ends is schematically shown. The 5′/3′ UAR and 5′CS/3′CS1 interactions are indicated.

structure (3′ SL) involving 90 to 120 nts. Within the 3′ SL, two small conserved sequence motifs were identified and found to be required for viral RNA synthesis. One of these motifs is the dinucleotide 5′-CU-3′ at the 3′ end of the genome [97-99]. The other conserved sequence is the pentanucleotide motif 5′-CACAG-3′ in the top loop of the 3′ SL [97,100,101]. Upstream of the 3′ SL, there are two conserved sequence elements designated CS1 and CS2 that are well conserved among mosquito-borne flaviviruses (fig. 3.A). CS1 is found immediately adjacent to the 3′ SL and is involved in a long range RNA-RNA interaction with a complementary conserved sequence (5′ CS) near the 5′ end of the genome, downstream of the translation initiation codon in the capsid gene [102]. Base pairing of these two sequence elements allows the formation of a panhandle-like structure that mediates circularization of the viral genome (fig. 3.B). The base pairing between 3′ CS1

and 5′ CS has been shown to be critical for viral RNA synthesis [103-109]. Recent studies have demonstrated that, apart from the 5′CS – 3′CS1 interaction, another long-range RNA interaction also plays a role in promoting genome cyclization. This interaction involves complementary sequences at the 5′ end, located immediately upstream the AUG start codon region (UAR) of the ORF, and at the 3′ end within the bottom part of the 3′ SL (3′ UAR) [109] (fig. 3.B). This pair of complementary sequences has been shown to be important for viral replication [108,110-114]. In DENV and WNV yet a third interaction important for genome circularization and RNA replication was recently identified and involves nucleotides downstream of the AUG region (5′ DAR) and nucleotides downstream CS1 (3′ DAR) [115-117]. Similar long-distance RNA interactions involving elements in the terminal regions of the genome, but at a different location from 5′CS and CS1, have also been shown for the tick-borne [118,104,110,119] and NKV flaviviruses [120,121].

CS2 is approximately 24 nts in length and is located upstream of CS1. YFV contains only one copy of CS2 but the sequence is duplicated (RCS2) in members of the JEV and DENV subgroups [102]. A sequence motif with high sequence identity and a position that resembles the one of CS2 in mosquito-borne flaviviruses has also been identified in the 3′ UTR of NKV flaviviruses [120,121]; while it is apparently absent in tick-borne flaviviruses. Deletion of CS2 has little effect on viral RNA synthesis but seems to affect pathogenicity of at least YFV and DENV, as mutants lacking CS2 form turbid plaques [107,103]. Dengue viruses lacking this sequence are attenuated in rhesus monkeys [103]. The viral 3′ UTR region encompassing CS2 in mosquito-borne flaviviruses is predicted to fold into dumbbell-like structures of which a loop is thought to be involved in the formation of an RNA pseudoknot with downstream sequences [122] (fig. 3.A).

Yellow fever

Yellow fever was the first human disease shown to be caused by a virus and the third viral infection (after smallpox and rabies) to be controlled by vaccination [123]. Yellow fever is a mosquito-borne, viral hemorrhagic fever that is endemic in tropical regions of Africa and South America where it affects 45 countries with a combined population of over 900 million people [124]. WHO estimated 200,000 cases of yellow fever worldwide every year, resulting in 30.000 deaths. However, as with many diseases in rural Africa, underreporting of the actual number of infections is likely. More than 90% of the YFV cases occur in Africa and, according to the WHO, one single confirmed case of YFV in an unvaccinated population should be considered an outbreak [124].

YFV probably evolved from ancestral mosquito-borne viruses over 3.000 years ago [125]. It is postulated that the virus originated in Africa and was subsequently introduced from the Old World into the Americas during the slave trade period in the 16th century

(reviewed in [10]). In 1900, an American commission headed by Walter Reed proved that yellow fever was caused by a filterable agent and transmitted to humans by mosquitoes [126,] (reviewed in [127]). In 1927 the Rockefeller Foundation's West Africa Yellow Fever Commission isolated the virus by inoculation of a rhesus monkey with blood of an YFV-infected Ghanian male named Asibi [128]. Theiler and Smith [129] attenuated the YFV Asibi strain by serial passage in cultures of mouse brain and modified chick embryo tissues, and demonstrated the use of the resulting attenuated YFV-17D strain as a vaccine to protect humans from yellow fever infection. In 1951, Theiler was awarded with the Nobel Prize in medicine for this groundbreaking work. In 1985, the complete genome sequence of YFV-17D was reported [130]. Shortly thereafter, the nucleotide sequence of YFV-Asibi was determined and it was shown that the Asibi and 17D strains differ at 68 nucleotide positions resulting in 32 amino acid changes [131]. Despite the fact that infectious cDNA clones for both YFV-17D [132,107] and YFV-Asibi [133] (Bredenbeek, Dorner, Ploss and Rice, unpublished results) are available, the precise genetic determinants for attenuation of YFV-17D are still unknown. Several studies have shown that the flavivirus envelope protein contains important determinants for cell tropism, virulence, as well as immunity (reviewed in [134]). Therefore, it has been suggested that either one or several of the eight amino acid differences between the Asibi and 17D E proteins are critical for the attenuation of the YFV-Asibi.

Unfortunately, despite the availability of a very successful vaccine, yellow fever is still a major public health concern. Because of the variable clinical presentation of the infected individuals, yellow fever can be difficult to differentiate from other hemorrhagic fevers (e.g. Lassa fever, Ebola) and diseases like malaria, influenza, and typhoid fever, which often also occur in areas where YFV is endemic [12]. The liver is the target organ in humans and liver dysfunction results in patient's skin turning yellow, a characteristic from which the name of the disease was derived (reviewed in [135]). YFV infection presents a broad clinical spectrum varying from mild symptoms to a fatal hemorrhagic fever, with a biphasic pattern. The onset of the disease is typically within 3 to 6 days after a bite from an infected mosquito. The symptoms during this first phase of the infection include fever, headache, backache, myalgia (muscle pain), chills, malaise, nausea, dizziness and vomiting. During this phase, patients are viremic and infectious to mosquitoes. This period will last for several days and may be followed by a "period of remission", with mitigation of symptoms lasting up to 24 h. In this phase, the virus is cleared by antibodies and the cellular immune response of the individual. Most patients recover at this point without further signs or symptoms. However, in approximately 15 to 25% of those infected, symptoms reappear in a more severe form with high fever, vomiting, epigastric pain, jaundice, renal failure, hemorrhagic diathesis ("black vomit") and coma. Bleeding can occur from the mouth, nose, eyes, or stomach. This is the "period of intoxication". Viremia is generally absent, and anti-YFV antibodies appear during this phase. Depending on

the virulence of the YFV strain, up to 50% of the patients do not survive this second phase and die within 7 to 10 days after the onset of symptoms. Patients that survive the infection usually recover without significant organ damage or other lasting effects (reviewed in [10,12,124]).

There is no cure for yellow fever; vaccination is the single most important preventive measure against yellow fever. The YFV-17D vaccine is effective against all African and South American YFV genotypes and has an unmatched safety record [12]. This excellent safety record might be in part explained by the fact that the virus is quite stable as it accumulates mutations at a very low frequency in healthy vaccinees [136]. On the other hand, it has been well documented that YFV-17D vaccine preparations consist of a heterogeneous population containing a mixture of variants with distinct biological properties, such as plaque size in Vero cells, virulence for mice [137,138], and antigenicity [139-141].

The YFV-17D vaccine is an affordable, highly effective vaccine that is thought to provide protection for 30-35 years or more. Severe adverse reactions to vaccination have been reported but are extremely rare (reviewed in [135,124,142]). Despite its success, the mechanisms by which YF-17D induces protective immunity are not completely understood. Vaccination is followed by a rapid activation of both the cellular and humoral arms of the adaptive immune response. Long term protection against YFV infection appears to be exclusively mediated by neutralizing antibodies (protective levels of neutralizing antibody are found in 90% of vaccines within 10 days and in 99% within 30 days) [12]. In trying to understand the molecular basis of the efficacy of the YFV-17D vaccine, recent research has focused on the innate immune response upon infection of dendritic cells [143] and in vaccines [144] using gene expression profiling. From these studies it can be concluded that YFV-17D activates multiple Toll-like receptors (TLRs) which are likely to activate several arms of the innate immune response. A robust activation and upregulation of a complex network of genes involving innate sensing receptors (e.g. TLR7, RIG-I, MDA5), IFN-β stimulated transcription factors, and pro- and anti-inflammatory cytokines, ultimately culminates in a protective immune response [143,144] (reviewed in [142]).

YFV-17D as a platform for developing recombinant vaccines

Despite our expanding knowledge of the molecular biology, immunology and pathology of flaviviruses, relatively little progress has been made with respect to treatment of infected individuals. Currently, only a limited number of licensed vaccines to protect humans against flavivirus infections is available. These include the already discussed YFV-17D vaccine, a live attenuated as well as an inactivated JEV vaccine, and a TBEV vaccine based on inactivated virus. An inactivated WNV vaccine is also available but only licensed for use in livestock [145,146]. As for DENV, despite numerous efforts, there is no vaccine available; the situation is especially aggravated by the fact that a DENV vaccine should be tetravalent, inducing protection against the four dengue serotypes (reviewed

in [145]). Recent data indicate an even bigger challenge. DENV-infected cells apparently secrete high levels of particles containing prM instead of M due to inefficient cleavage. Antibodies against prM are generated and were found to be highly cross-reactive and able to promote the antibody-dependent enhancement (ADE) that is often associated with severe secondary infections by a different serotype [147,148]. These observations imply that an effective DENV vaccine should not only be able to offer protection from the four different serotypes but also minimize the anti-prM response.

The ability for genetic manipulation of the flavivirus genome by using available infectious cDNAs allowed the construction of live attenuated chimeric viruses. These novel recombinant vaccines make use of the fact that prM and E proteins of a particular flavivirus can be exchanged for the corresponding genes of another flavivirus, without significantly affecting the replication of the new recombinant virus. The expressed prM and E of the donor virus will drive the efficient assembly and budding of an enveloped virion in which the recombinant RNA is packaged, and will trigger an immune response against the donor virus upon vaccination. YFV-17D has been used as a vector backbone for the construction of such chimeric viruses due to its unique safety record and efficacy as a vaccine for humans. YFV-based chimeric candidate vaccine marketed as Chimerivax™ have been constructed for DENV, JEV, and WNV and have now been extensively tested in clinical trials with results that demonstrate their immunogenicity and excellent safety profile in humans [149] (reviewed in [150]).

Apart from being used as a vector for the construction of chimeric flavivirus vaccine candidates, YFV-17D has also been exploited as a vector for the expression of heterologous genes to develop recombinant vaccines against pathogens like malaria [151-153], tumours [154], Lassa virus [155,156], and HIV [157,158]. Although most of these recombinants show promising results in small scale animal experiments, genetic stability is often an issue especially with longer inserts [156,159].

SCOPE AND OUTLINE OF THIS THESIS

It is generally accepted that the 3' UTR of positive-strand RNA viruses has an important role in several steps of the virus life cycle. RNA sequences and/or structures have been implicated in the regulation of translation and replication, as well as encapsidation (reviewed in [160,161]). Flaviviruses are no exception regarding the importance of the 3' UTR for the virus cycle. Several motifs and RNA structures have been identified in the flavivirus 3' UTR (see fig. 3); most have been predicted based on phylogenetic analysis and RNA folding algorithms. Unfortunately, RNA probing data to support the predicted structures is scarce. In addition, our knowledge of the biological function of most of these predicted RNA elements is still rather vague and often limited to the biological

effect of deleting the predicted RNA structures (e.g., [107]). The only exception are the RNA sequences involved in genome circularization that have been studied in detail using a variety of techniques (e.g. *in vitro* RdRp assays, mutagenesis, atomic force microscopy, RNA structure probing) [103-110,112,113,118,119,162-167].

The major aim of the research described in this thesis was to characterize and further understand the sequence and structural requirements as well as the biological function of some of these predicted RNA elements in the flavivirus 3′ UTR. The work described in chapters 2 and 3 has been performed with the mosquito-borne YFV. The experiments in chapter 4 and 5 were carried out with several of the NKV flaviviruses that lack an arthropod vector and with the tentative flavivirus CFAV, which is thought to be an insect virus unable to infect vertebrate hosts. CFAV and the NKV flaviviruses are particularly interesting from the perspective that, as far as it is currently known, they do not cycle between different hosts. As a consequence of this apparently simpler life cycle, their 3′ UTR and the conserved RNA elements within it, are predicted to have evolved towards an optimal function in only one type of host, e.g. mosquitoes, bats, or rodents. This could potentially have resulted in a less complex 3′ UTR. These relatively unknown and poorly studied NKV- and insect flaviviruses may therefore be excellent tools to provide a better understanding of the function of conserved RNA structures and could yield valuable insight into virus-host interactions, host range restrictions or specific requirements for replication in different hosts.

Chapter 2 describes the importance and the sequence requirements of the pentanucleotide motif in the 3′ SL of YFV (see fig. 3). Of the five nucleotides (5′-CACAG-3′), only the G nucleotide at the 5th position was indispensable for viral replication. Mutations at the other positions were tolerated, although the nucleotide at the 1st position had to be able to base pair with the nucleotide four positions downstream of the PN sequence (9th position). This result provided experimental support for the predicted structure at the top of 3′ SL. Strikingly, YFV replication was found to be less dependent on sequence conservation of the pentanucleotide motif than reported for West Nile virus. Nonetheless, despite the fact that the majority of the mutations in the YFV PN motif did not seem to affect viral RNA synthesis, a clear preference for the wild-type sequence was observed when the fitness of these mutant viruses was analyzed in a competition experiment against the parental YFV-17D.

Chapter 3 presents a detailed description of the characteristics of the YFV sfRNA and the RNA structure within the viral 3′ UTR that is required for its production. Complementary *in vitro* and cell culture experiments confirmed the 5′ – 3′ RNase XRN1 as the host protein responsible for sfRNA generation. A predicted RNA pseudoknot with hitherto unknown function was shown to be essential for the production of the YFV sfRNA. Evidence to support the formation of this particular pseudoknot was obtained by RNA structure probing and mutagenesis studies.

From published data [79-81,83] and our work it became evident that all arthropod-borne flaviviruses produce at least one sfRNA in infected mammalian as well as insect cell lines. In addition, sfRNA production was shown to be an important determinant of virulence, as viruses that are unable to produce the sfRNA are less pathogenic in mice [83]. These data provide evidence that the sfRNA has at least a function in the vertebrate host, but do not necessarily exclude a function in the arthropod host. If the sfRNA has no function in the arthropod host it could be hypothesized that the (tentative) insect flavivirus CFAV would not produce an sfRNA whereas the bat- and rodent-infecting NKV flaviviruses would produce an sfRNA. Alternatively, sfRNA synthesis could be a unique hallmark of arthropod-borne flaviviruses or a characteristic feature of all flaviviruses irrespective of their host range. **Chapter 4** describes the experiments that were done to verify the various possibilities concerning sfRNA production in flaviviruses without a vector. From the data presented, it was concluded that all flaviviruses, including the tentative species CFAV, produce an sfRNA, suggesting that sfRNA generation is indeed a feature of the Flavivirus genus. The mechanism by which these sfRNAs are produced was shown to be similar to that of the arthropod-borne flaviviruses.

In contrast to most of the arthropod-borne flaviviruses, studies with NKV flaviviruses are hampered by the lack of infectious cDNA clones. **Chapter 5** describes the construction and characterization of a MODV full-length infectious cDNA clone. The clone was constructed in the low copy number vector pACNR that had been used before as a stable acceptor for the often "toxic" sequences of the *Flaviviridae* in *Escherichia coli*. MODV genome-length transcripts were shown to be highly "infectious" when transfected into BHK cells. The virus obtained from the transfected cells showed similar characteristics as the parental virus in terms of growth kinetics and plaque morphology. This clone can be used to study the function of predicted 3' UTR elements putatively important for NKV flaviviruses. Furthermore, the infectious MODV clone offers the possibility to construct chimeras with arthropod-borne flaviviruses in order to understand the molecular determinants required for a virus to be able to replicate in insect cells.

Chapter 6 presents an extensive literature review describing the characteristics and function of the RNA structures that were predicted within the Flavivirus 3' UTR. Data available for the well-studied arthropod-borne flaviviruses, as well as for the poorly studied NKV flaviviruses, is summarized and discussed. Emphasis was given to structures that were shown to be involved in viral replication and pathogenicity.

Chapter 7 is an epilogue in which the results of the experimental work are briefly summarized and discussed in a broader context. Potential functions and future research directions for the RNA structures that were studied in the work presented in this thesis are suggested.

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