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Role of RNA structures present at the 3′UTR of dengue virus on translation, RNA synthesis, and viral replication

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Abstract

We have developed a dengue virus replicon system that can be used to discriminate between translation and RNA replication. Using this system, we analyzed the functional role of well-defined RNA elements present at the 3′UTR of dengue virus in mammalian and mosquito cells. Our results show that deletion of individual domains of the 3′UTR did not significantly affect translation of the input RNA but seriously compromised or abolished RNA synthesis. We demonstrated that complementarity between sequences present at the 5′ and 3′ ends of the genome is essential for dengue virus RNA synthesis, while deletion of domains A2 or A3 within the 3′UTR resulted in replicons with decreased RNA amplification. We also characterized the vaccine candidate rDEN2Δ30 in the replicon system and found that viral attenuation is caused by inefficient RNA synthesis. Furthermore, using both the replicon system and recombinant viruses, we identified an RNA region of the 3′UTR that enhances dengue virus replication in BHK cells while is dispensable in mosquito cells.

Keywords: Dengue virus; RNA replication; Translation; 3′untranslated region; Flavivirus; Viral attenuation; Subgenomic replicon

Dengue virus belongs to the *Flaviviridae* family together with other important human pathogens such as yellow fever virus, West Nile virus, and Japanese encephalitis virus. Dengue fever is the most prevalent mosquito-borne viral disease of humans (Gubler, 1998). It is estimated that more than 50 million infections occur annually and 2.5 billion people are at risk of dengue virus infection worldwide (WHO, 2004). Despite the wide morbidity and mortality associated with dengue infections, the molecular biology of this virus is not well understood and at present neither specific antiviral therapies nor a licensed vaccine exist.

Dengue is an enveloped virus with a positive single stranded RNA genome of about 11 kb. The viral RNA encodes one large open reading frame flanked by 5′ and 3′ untranslated regions (UTRs) that are required for viral replication. The 5′ UTR is relatively short (around 100 nucleotides) and has a cap structure at the 5′ end, while the 3′ UTR is longer (around 450 nucleotides), lacks a poly(A) tail, but contains a number of conserved RNA structures (Markoff, 2003). The genomic RNA is directly used as mRNA for protein synthesis. The large viral polyprotein is co- and posttranslationally processed by viral and cellular proteases into three structural proteins, capsid (C), premembrane (prM), and envelope (E); and seven nonstructural proteins (NS) that are primarily involved in replication of the viral RNA (Rice, 2001). The mechanism by which the viral replicase initiates RNA synthesis specifically at the viral 3′UTR is not clearly understood. The RNA replication complex assembles on cellular membranes and involves the viral RNA dependent RNA polymerase-methyl transferase NS5, the helicase-protease NS3, the glycoprotein NS1, the hydrophobic proteins NS2A and NS4A, and presumably host factors (Mackenzie et al., 1998; Westaway et al., 1997, 1999). The nucleotide sequence at the 3′ end of the genome and

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the presence of specific RNA structures appear to be essential for dengue and other flavivirus RNA replication (Elghonemy et al., 2005; Khromykh et al., 2003; Tilgner and Shi, 2004; Tilgner et al., 2005; Yu and Markoff, 2005; Zeng et al., 1998).

The 3′ end of the flavivirus genomes folds into a highly conserved stem–loop (3′SL). Detailed analysis of the structure–function of the 3′SL in West Nile virus, Kunjin virus, dengue virus, and yellow fever virus revealed an absolute requirement of this RNA element for viral replication (Brinton et al., 1986; Men et al., 1996; Proutski et al., 1997; Rauscher et al., 1997; Zeng et al., 1998). Upstream of the 3′SL there is another essential RNA element for viral replication, the conserved sequence CS1 (Men et al., 1996). This element contains the cyclization sequence CS that is complementary to a sequence present at the 5′ end of the genome (Hahn et al., 1987). 5′–3′ long-range RNA–RNA interactions have been proposed to be necessary for RNA replication in West Nile virus, Kunjin virus, and dengue virus (Alvarez et al., 2005; Khromykh et al., 2001; Lo et al., 2003). In addition, using recombinant dengue virus NS5 polymerase, it has been demonstrated that in vitro RNA synthesis requires sequences present at the 5′ and 3′ends of the genome (You and Padmanabhan, 1999; You et al., 2001). While the 3′SL has been extensively studied, the function of the other RNA structures and conserved motifs present within dengue virus 3′UTR remain elusive. Folding algorithms predict two almost identical structures designed A2 and A3 preceding the 3′SL. These structures contain the highly conserved sequence CS2 and the repeated CS2 (RCS2), within A3 and A2, respectively (Shurtleff et al., 2001). CS2 and RCS2 sequences are found in Japanese encephalitis, West Nile, Murray Valley encephalitis, and dengue virus types 1 to 4 (for review, Markoff, 2003). Furthermore, between the stop codon of the viral polyprotein and domain A2 resides a variable region (VR), which displays large heterogeneity in length and nucleotide sequence among different dengue virus isolates (Shurtleff et al., 2001).

Even though previous studies reported that deletions within the 3′UTR yielded seriously impaired dengue viruses (Men et al., 1996), the role of each of these RNA elements during translation and RNA synthesis has not been analyzed due to the lack of an amenable genetic system. We have developed a dengue virus replicon that allows discrimination between viral translation and RNA synthesis. Using this replicon, we performed a systematic deletion analysis of each RNA structural element of the 3′UTR on the viral processes. We found that deletion of RNA elements at the 3′UTR greatly decreased viral RNA synthesis without compromising translation initiation. In addition, using the replicon system and recombinant dengue viruses, we identified an RNA element of the 3′UTR that differentially modulates viral replication in mosquito and mammalian cells.

Results

Construction and characterization of a subgenomic dengue virus replicon

A dengue virus replicon system has been previously described (Pang et al., 2001). This replicon allows detection of viral proteins by immunofluorescence and RNA replication in transfected cells. However, this system lacks a sensitive reporter amenable to discriminate translation of input RNA and RNA replication. In an attempt to overcome this limitation, we developed a new replicon system carrying a sensitive reporter. In the context of dengue virus 2 16681 cDNA clone (Kinney et al., 1997), we introduced the firefly luciferase (Luc) coding sequence replacing the structural proteins (Fig. 1A). The *trans* membrane domain (TM) corresponding to the C-terminal 24 amino acids of E was retained in order to maintain the topology of the viral protein NS1 inside of the ER compartment. The Luc was fused in-frame to the first 102 nucleotides of the capsid protein (C), which contain the *cis*-acting element of 11 nucleotides complementary to the 3′CS sequence (Alvarez et al., 2005; Hahn et al., 1987; Khromykh et al., 2001; You et al., 2001). A similar replicon system has been recently developed for West Nile virus (Lo et al., 2003). To ensure proper release of the Luc from the viral polyprotein, we designed three alternative constructs carrying different protease cleavage sites between the C-terminus of luciferase and the beginning of the TM domain of E (Fig. 1A). Two of these constructs contain recognition sites for NS3 protease, corresponding to the C-prM and the NS4B-NS5 junctions (DVRepCprM and DVRep4B-5, respectively). The third construct contains the *cis*-acting FMDV 2A protease (DVRep) (Ryan and Drew, 1994).

Transfection into BHK cells of the replicon RNAs carrying the NS3 recognition sites yielded low levels of Luc activity and no amplification of the RNA was detected, likely due to the slow processing by the NS3 protein (data not shown). In contrast, the processing by FMDV 2A was fast and Luc activity was readily detected few hours after transfection. To examine whether the DVRep was capable to autonomously replicate in cells, we transfected the RNA into BHK cells and assayed for Luc activity as a function of time. The levels of Luc activity peaked between 8 and 10 h after transfection. Around 20 h, the Luc signal dropped but rebounded exponentially after 30 h (Fig. 1B). To confirm that the observed time increase in Luc signal was the result of replicon RNA amplification by the viral replicase activity, a replication defective RNA was designed. We replaced the essential GDD motif of the RNA dependent RNA polymerase NS5 by AAA (DVRepNS5Mut). Similar mutations of the GDD motif have been shown to have a lethal effect on flavivirus replication (Khromykh et al., 1998). We transfected BHK cells with equal amounts of RNA of DVRep WT and NS5Mut and monitored Luc activity. During the first 20 h, the Luc signal obtained from cells transfected

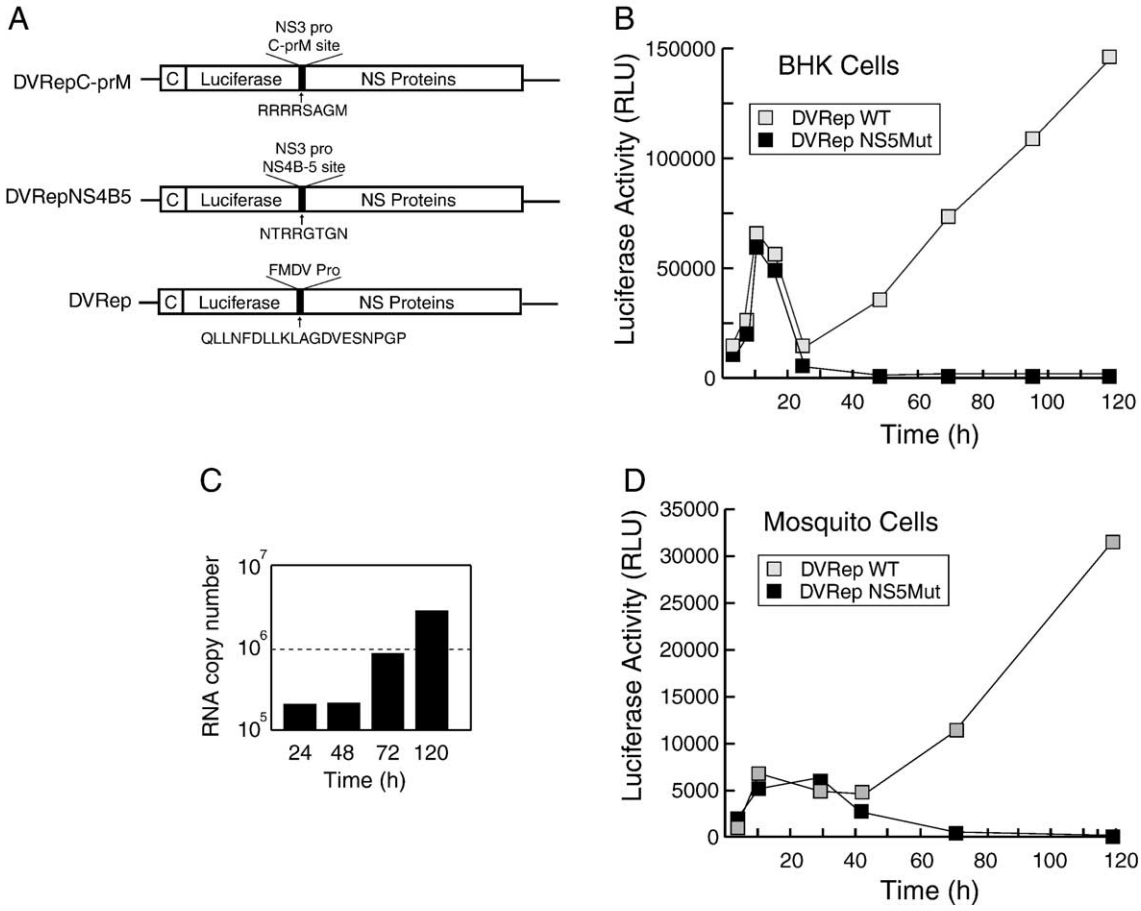


Fig. 1. Luciferase containing dengue virus replicon allows monitoring RNA translation and RNA replication in BHK and C6/36 mosquito cells. (A) Schematic representation of dengue virus replicons. Boxes denoting coding sequences of capsid (C), Luciferase, and non-structural (NS) proteins are shown. Amino acids corresponding to NS3 protease recognition sites C-prM and NS4B-5 are indicated in the DVRepC-prM and DVRepNS4B-5, respectively. The amino acid sequence of FMDV2A protease is also shown. (B) Replication of dengue virus replicon in BHK cells. Time course luciferase activity was detected in cytoplasmic extracts prepared from BHK cells transfected with DVRep or replication-incompetent DVRepNS5Mut RNAs. (C) Quantification of DVRep RNA as a function of time by real time RT-PCR. RNA copy numbers are shown at different times post-transfection of BHK cells with DVRep RNA. (D) Replication of dengue virus replicon in mosquito C6/36 cells. Time course luciferase activity was detected in cytoplasmic extracts prepared from C6/36 cells transfected with DVRep or replication-incompetent DVRepNS5Mut RNAs.

with the two RNAs was indistinguishable (Fig. 1B). However, after 24 h, the levels of Luc obtained from cells transfected with the NS5Mut RNA maintained background levels while the replication competent RNA increased more than 60-fold. The increase of DVRep RNA was also detected by real time RT-PCR using TaqMan technology (Fig. 1C).

To further characterize the dengue virus replicon system, we analyze the ability of the DVRep RNA to replicate in mosquito cells. We transfected C6/36 cells with both the WT and the NS5Mut DVRep RNAs and monitored the Luc signal as a function of time. After transfection, the Luc signal increased reflecting translation of the input RNA (Fig. 1D). After about 45 h, the Luc signal increased exponentially in cells transfected with the WT replicon but not in cells transfected with the NS5Mut RNA. The kinetics of Luc activity in the mosquito cells differed from those of BHK cells (compare Figs. 1B and D). Translation of the input RNA in C6/36 cells increased in the first 10 h and then was maintained almost constant up to 40 h. This was presumably due to the higher stability of the input RNA in mosquito cells at 28 °C, the temperature used to grow insect cells, as compared with 37 °C used for BHK cells.

Taken together, the results indicate that DVRep RNA translation and amplification can be monitored through the expression of Luc as a function of time in replicon-transfected mosquito and BHK cells. We conclude that the levels of Luc obtained around 10 h after transfection reflects translation of the input RNA, while the Luc signal after 60 h of transfection can be used to assess RNA replication.

RNA elements of the 3′UTR of dengue virus greatly enhance RNA synthesis

It has been previously reported that partial deletions within the 3′UTR of infectious dengue virus 4 yield viruses with impaired replication in cell culture and rhesus monkeys (Men et al., 1996). However, it is not clear how the different

RNA elements at the 3′UTR participate during viral replication.

To investigate the role of conserved structures and sequences at the 3′UTR of dengue virus during viral translation and RNA synthesis, we performed deletions and mutations of defined RNA domains using the replicon system. Based on conserved secondary structures previously predicted for dengue and other flaviviruses (Shurtleff et al., 2001), four domains can be defined in dengue virus 3′UTR: VR, A2, A3, and the 3′SL (Fig. 2A). We constructed 7 different replicons carrying the following modifications: (i) complete deletion of the 3′UTR (DVRepΔ3′UTR), (ii) deletion of A2 domain including the conserved sequence RCS2 (DVRepΔA2), (iii) deletion of A3 domain including the conserved sequence CS2 (DVRepΔA3), (iv) deletion of both A2 and A3 structures (DVRepΔA2A3), (v) deletion of 30 nucleotides from the top of domain A3 (DVRepΔ30), (vi) mutation of the complementary sequence 3′CS (DVRepCSMut), which generates mismatches between the 5′ and 3′CS complementarity region, and (vii) deletion of the 155 nucleotides of VR (DVRepΔVR) (Fig. 2A).

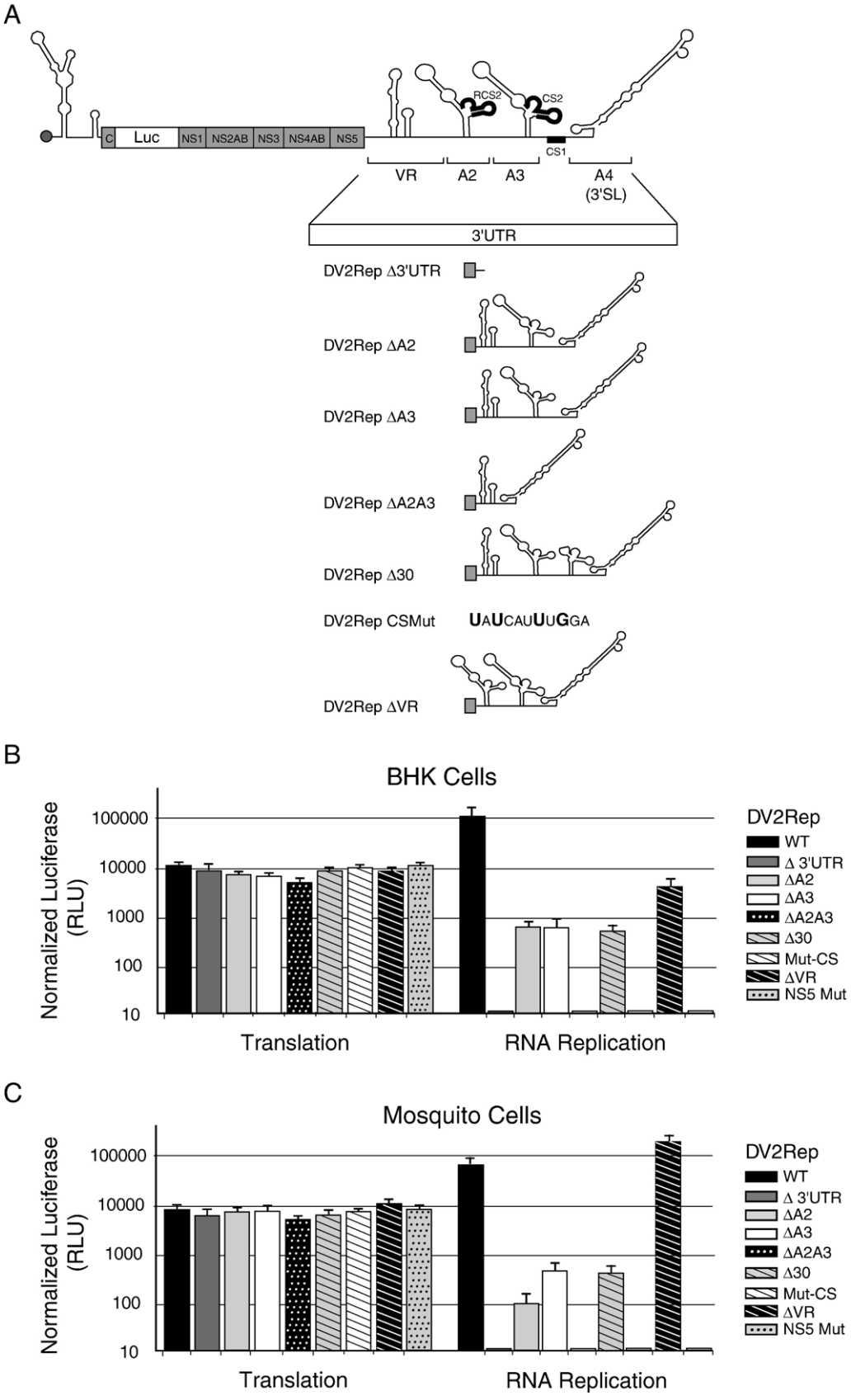
Replicon RNAs corresponding to the WT, NS5Mut, and the 7 mutants within the 3′UTR described above were in vitro transcribed and equal amounts of RNA were transfected into both BHK and C6/36 cells. *Renilla* Luc mRNA was cotransfected with all the replicons and both Luc activities were monitored. After RNA transfection, *Renilla* activity increased, reached a maximum around 24 h, and then decreased as a function of time depending on the stability of its mRNA and the half-life of the protein, which was equivalent for all co-transfections. *Renilla* activity was used to standardize the transfection efficiency in each time point. We monitored Luc activities at 4, 10, 24, 48, 72, 96, and 120 h after transfection. The normalized Luc signal at 10 h was representative of the translation activity. To assess RNA amplification, we analyzed Luc activity at 3 days post-transfection in BHK cells and at 4 days post-transfection in C6/36 cells.

With the exception of the DVRepΔA2A3 RNA, which showed a slight but significant 30–40% lower translation activity when compared to the WT, the levels of Luc at 10 h were no significantly different between cells transfected with DVRepWT or mutant RNAs in both cell types (Figs. 2B and C). The complete deletion of the 3′UTR resulted in a replicon that was translated efficiently in BHK and C6/36, suggesting that translation initiation is not dependent on the 3′UTR. In contrast, profound effects were observed on RNA amplification with replicons carrying modifications at the 3′UTR. Deletion of the complete 3′UTR, mutation of 3′CS, or deletion of domains A2A3 (DVRepΔ3′UTR, DVRepCS-Mut, and DVRepΔA2A3) yielded RNAs with undetectable RNA amplification in BHK or mosquito cells (Figs. 2B and C). Deletion of either domain A2 or A3 decreased RNA amplification more than 100-fold. A similar decrease of RNA amplification was observed with the replicon RNA carrying a deletion of 30 nucleotides at the top of domain A3 (DVRepΔ30). It has been well documented that this deletion in the infectious clones of dengue virus 1, 2, and 4 caused attenuation of viral replication and viruses with this deletion are currently been evaluated as vaccine candidates (Blaney et al., 2004; Durbin et al., 2001; Hanley et al., 2004; Troyer et al., 2001; Whitehead et al., 2003). Furthermore, deletion of the region just downstream of the stop codon (VR) resulted in a replicon that was amplified about 10-fold less efficiently than the WT RNA in BHK cells but was efficiently amplified in mosquito cells, reaching levels slightly higher that the WT replicon (Figs. 2B and C), suggesting that viral sequences can be responsible for differential viral RNA synthesis in the two host cells.

Sequence complementarity between 5′ and 3′CS of dengue virus are essential for RNA synthesis

Cyclization of flavivirus genomes through long range RNA–RNA interactions have been previously proposed. Using Kunjin and West Nile virus replicons, it has been shown that sequence complementarities between 5′ and 3′ CS regions of the viral RNA rather than the sequence per se are important for RNA replication (Khromykh et al., 2001; Lo et al., 2003). For dengue virus, we have previously found that two pairs of complementary sequences present at the 5′ and 3′ ends of the genome (5′–3′CS and 5′–3′UAR) are necessary for RNA–RNA interaction and RNA cyclization (Alvarez et al., 2005). Furthermore, using recombinant viruses, we demonstrated that 5′–3′UAR complementarity is essential for viral viability (Alvarez et al., 2005). However, the importance of 5′–3′CS complementarity in dengue virus replication has not been previously examined. Here, we found that point mutations within the CS region present upstream of the 3′SL of dengue virus RNA abolished replicon RNA synthesis without altering translation efficiency. To analyze whether the lack of RNA synthesis was due to a mismatch between the 5′ and 3′ CS or to the nucleotide changes in 3′CS, we generated a replicon carrying point mutations at the 5′CS that restored sequence complementarity with the mutated 3′CS, and analyzed the ability of this 5′–3′CS double mutant RNA to replicate (Fig. 3A).

The replicon RNA carrying simultaneous mutations at the 5′ and 3′ CS (DVRepCS DoubleMut) as well as the DVRepWT, DVRepCSMut, and DVRepNS5Mut were transfected into BHK and C6/36 cells. At 10 h post-transfection, the Luc levels observed with the 4 RNAs were similar (Figs. 3B and C). Restoration of the complementary sequences 5′–3′CS in replicon DVRepCSDoubleMut also restored RNA synthesis in both BHK and C6/36 cells (Figs. 3B and C). The RNA replication of the double mutant was efficient, however, it did not reach the same replication levels of the WT RNA. These results indicate that 5′–3′CS base pairing is not required for translation of the input RNA but is an essential element for dengue virus RNA synthesis. In addition, the lower levels of RNA amplification of the


A
C
Luc
NS1
NS2AB
NS3
NS4AB
NS5
RCS2
CS2
CS1
VR
A2
A3
A4
(3'SL)
3'UTR
DV2Rep Δ3'UTR
DV2Rep ΔA2
DV2Rep ΔA3
DV2Rep ΔA2A3
DV2Rep Δ30
DV2Rep CSMut
UAUCAUUUGGA
DV2Rep ΔVR
B
BHK Cells
Normalized Luciferase (RLU)
100000
10000
1000
100
10
Translation
RNA Replication
DV2Rep
WT
Δ 3'UTR
ΔA2
ΔA3
ΔA2A3
Δ30
Mut-CS
ΔVR
NS5 Mut
C
Mosquito Cells
Normalized Luciferase (RLU)
100000
10000
1000
100
10
Translation
RNA Replication
DV2Rep
WT
Δ 3'UTR
ΔA2
ΔA3
ΔA2A3
Δ30
Mut-CS
ΔVR
NS5 Mut

DVRepCSDoubleMut when compared with WT replicon suggest that the nucleotide-sequences of 5′ and/or 3′CS are required for efficient dengue virus RNA synthesis.

RNA sequences present downstream of the stop codon differentially modulate viral replication in mammalian and mosquito cells

Using the replicon system, we found that deletion of the VR in the 3′UTR decreases RNA synthesis in BHK cells more than 10-fold but has no effect on RNA replication in mosquito cells. In addition, we observed that deletions within domains A2 and A3 greatly decreased RNA synthesis in both cell types. To further investigate a possible differential role of the VR in dengue virus replication in mosquito and BHK cells, and to confirm the importance of domain A2 and A3 during viral replication, we generated the following recombinant full-length dengue virus cDNAs: (i) deletion of 155-nucleotides corresponding to the VR (pDVΔVR), (ii) complete deletion of domain A2 (pDVΔA2), (iii) deletion of the top 30 nucleotides of domain A3 (pDVΔ30), and (iv) deletion of both A2 and A3 (pDVΔA2A3). All constructs were derived from the 16881 cDNA clone of dengue virus 2 (Kinney et al., 1997).

In vitro transcribed RNAs from pDVWT, pDVΔVR, pDVΔA2, pDVΔ30, and pDVΔA2A3 were transfected into BHK and C6/36 cells. Initially, the infectivity of the RNAs was assessed by immunofluorescence assays (IFA) for dengue virus antigens, using murine-anti-dengue 2 antibodies. All the RNA transfections tested positive by IFA within 5 days and produced infectious particles in both cell types. With the exception of virus DVΔA2A3, which replicated very poorly, viral stocks of recombinant viruses were generated and titered by plaque assay in BHK cells. Delayed replication of recombinant viruses DVΔVR, DVΔA2, and DVΔ30 was evident by plaque morphology. The plaque size of these three recombinant viruses was small as compared with plaques obtained with the parental virus (data not shown). Virus DVΔA2A3 was viable, however, less than 5% of the cells were positive by IFA on day 10 post-transfection and the higher titers achieved in the media of both cell types was about 100 PFU/ml. Therefore, due to the slow replication, DVΔA2A3 was not included in further studies.

To characterize the recombinant viruses recovered from transfected cells, we performed IFA as a function of time (Fig. 4). To this end, BHK and C6/36 cells were infected at MOI of 0.1. At day 2 after infection, around 50% of BHK cells were IFA positive for DVWT, 10% for DVΔVR, and less than 5% were positive for DVΔA2, and DVΔ30 viruses. At day 4, the WT virus killed the complete monolayer. In contrast, the cells infected with DVΔVR were nearly 100% IFA positive showing profound cytopathic effect (CPE), while cells infected with DVΔA2 and DVΔ30 viruses had an intact monolayer with moderate CPE, accompanied by almost 100% IFA positive cells (Fig. 4), confirming a delayed replication of the three recombinant viruses in BHK cells. The IFA performed in C6/36 cells at day 3 and day 5 showed similar amounts of positive cells with the WT and DVΔVR viruses (Fig. 4). With these two viruses, nearly 50% of cells were positive within 5 days. Viruses DVΔA2 and DVΔ30 replicated less efficiently and at day 5 became around 5% IFA positive (Fig. 4). These results further confirm the requirement of intact domains A2 and A3 for efficient viral replication. In addition, the data suggest that replication of a virus with deletion of the VR is less efficient in BHK cells while it replicates similarly to the WT virus in C6/36 cells.

To further characterize the differential replication of DVWT and DVΔVR in BHK and mosquito cells, we analyzed the kinetic of replication by one-step growth curves. Cells were infected at MOI of 0.01 using plaque-titered stocks. The amount of virus secreted into the medium was determined as a function of time. The comparative growth of the two viruses in BHK cells indicated delayed replication kinetics of the recombinant virus. The amount of DVΔVR viruses produced after 24 h of infection was almost two orders of magnitude lower than that obtained with the parental virus. This differential growth was observed for up to 72 h (Fig. 5A). Interestingly, the same analysis performed in C6/36 cells indicates that both viruses replicated with similar efficiencies (Fig. 5B), suggesting that the VR is dispensable for dengue virus replication in this cell type and confirming a differential role of VR in mosquito and mammalian cells.

Discussion

We developed a dengue virus replicon system that can be used to dissect RNA elements of the viral genome involved in translation and/or RNA replication. This replicon has a Luc gene fused in frame to the viral polyprotein in place of the viral structural proteins. Using this system, we analyzed the functional role of defined RNA structures present at the 3′UTR of dengue virus. Our results show that deletion of individual domains of the 3′UTR did not significantly affect translation of the input RNA but seriously compromised or

Fig. 2. Deletion analysis of RNA structures at the 3′UTR of DVRep. (A) Schematic representation of DVRep RNA. The predicted secondary structures of defined domains at the 3′UTR are indicated: variable region (VR), A2, A3, and A4 (3′SL). Also, the conserved sequences CS1, CS2, and RCS2 are shown. Underneath, schematic representation of mutations within the 3′UTR introduced in the DVRep are shown with the respective names. Nucleotide substitutions at 3′CS are indicated in bold case. (B) Translation and RNA replication of WT and mutant dengue virus replicons in BHK cells. Normalized luciferase levels are shown in logarithmic scale at 10 h after transfection to estimate translation of input RNA and at 3 days after transfection to evaluate RNA replication. (C) Translation and RNA replication of WT and mutant dengue virus replicons in mosquito C6/36 cells. Normalized luciferase levels are shown in logarithmic scale at 10 h after transfection to estimate translation of input RNA and at 4 days after transfection to evaluate RNA replication.

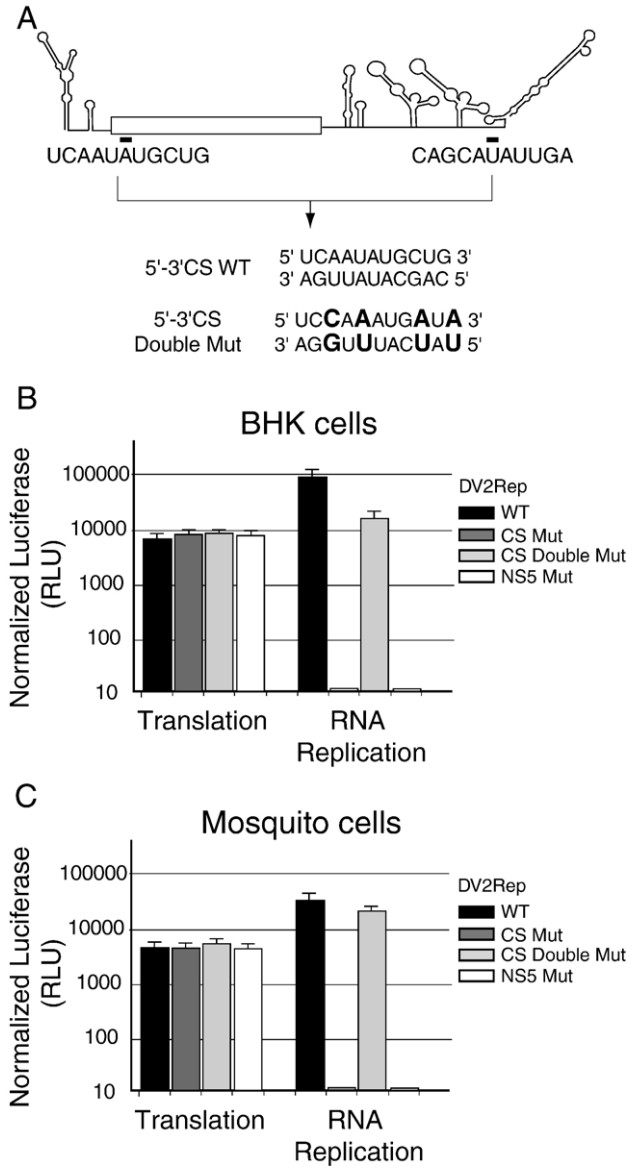


Fig. 3. 5′–3′CS complementarity is dispensable for translation but essential for dengue virus RNA replication. (A) Schematic representation of dengue virus RNA showing the location and sequence of 5′ and 3′ CS. Nucleotide changes within 5′ and 3′ CS in DVRep that restores complementarity are shown. (B) Translation and RNA replication of WT (DVRep), 3′CS mutant (DVRepCSMut), double mutant at the 3′ and 5′CS (DVRepDoubleMut), and replication-incompetent DVRepNS5Mut were analyzed in transfected BHK cells. Normalized Luc levels are shown in logarithmic scale at 10 h after transfection to estimate translation of input RNA and at 3 days after transfection to evaluate RNA replication. (C) Translation and RNA replication of WT and 5′ and 3′CS mutant replicons described in (B) were analyzed in transfected C6/36 mosquito cells. Normalized Luc levels are shown in logarithmic scale at 10 h after transfection to estimate translation of input RNA and at 4 days after transfection to evaluate RNA replication.

abolished RNA synthesis. We demonstrated that sequence complementarity between the 5′ and 3′ CS is essential for dengue virus RNA synthesis, while deletion of domains A2 or A3 resulted in replicons with decreased RNA replication. Furthermore, using both the replicon system and recombinant dengue viruses, we identified an RNA region present just downstream of the stop codon of the viral polyprotein that enhances viral replication in BHK cells while is dispensable in mosquito cells.

It is generally accepted that dengue viruses initiate translation of the polyprotein by a cap-dependent mechanism, similar to that observed in most cellular mRNAs. Dengue virus contains a cap structure at the 5′ end and lacks a poly(A) tail at the 3′ end. Because translation initiation of cellular mRNAs is enhanced by the poly(A) tail, it has been speculated that RNA structures at the 3′ end of dengue virus could functionally replace the poly(A) tail. It was previously reported that translation initiation of RNAs carrying the 5′ and 3′UTRs of dengue virus flanking a Luc gene, was stimulated by the presence of 3′UTR sequences (Holden and Harris, 2004). Using a comparable experimental design, we have observed similar stimulation effects by dengue virus 3′UTR sequences (A. Gamarnik et. al unpublished data). Here, in the context of RNAs that were competent for both translation and RNA replication, a significant effect of 3′UTR elements on translation initiation was not evident. Similar results were previously reported for other members of the *Flaviviridae* family (Kong and Sarnow, 2002; Tilgner et al., 2005). For dengue virus, removal of the complete 3′UTR or individual elements A2, A3, or VR, did not significantly alter translation efficiency (Fig. 2). Surprisingly, we observed that deletion of both A2 and A3 reduced the levels of translation 30 to 40% when compared with the WT levels. Using our experimental system, we cannot rule out a role of A2A3 on RNA stability. Therefore, the lower Luc activity observed for the mutant ΔA2A3 could be the result of lower translation efficiency or destabilization of the input RNA. The lack of a noticeable effect on translation when the complete 3′UTR was deleted makes the interpretation of the effect of A2A3 deletion difficult. However, it is possible that deletion of the last 452 nucleotides could include sequences that compensate the effect caused by deleting the 175 nucleotides of A2A3.

The viral RNA has to be translated in different conditions throughout the viral life cycle. Upon infection, translation of the input RNA depends on *cis*-acting elements of the RNA and the host translation machinery. After several rounds of translation and RNA synthesis, new RNA molecules have to be translated in the presence of viral proteins in a host cell modified by viral products. In this last scenario, a complex interaction between viral and host factors will influence viral RNA translation. Taking this into account, our data strongly suggest that elements at the 3′UTR of dengue virus do not participate in regulating translation initiation of the input RNA but we cannot rule out a possible regulatory role during late stages of infection.

Several studies have previously demonstrated that the sequence and structure of the terminal 3′SL are critical for flavivirus replication (Elghonemy et al., 2005; Khromykh et al., 2003; Men et al., 1996; Tilgner and Shi, 2004; Tilgner et al., 2005; Yu and Markoff, 2005; Zeng et al., 1998). The molecular details of how the 3′SL participates in the viral

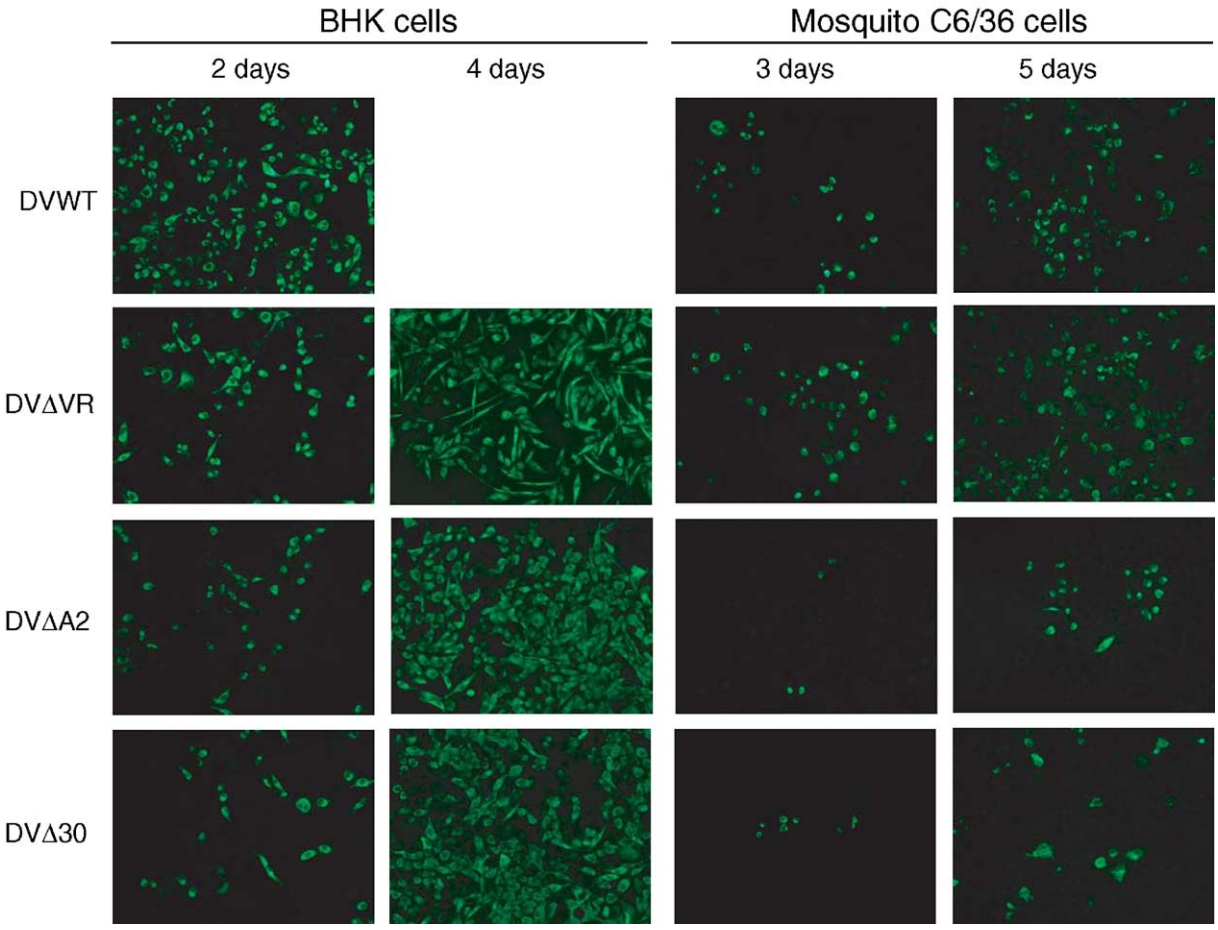


Fig. 4. Delayed replication of recombinant dengue viruses with deletions at the 3′UTR. Immunofluorescence assays (IFA) of infected BHK and C6/36 cells with dengue virus WT (DVWT), and recombinant viruses with deletion of VR (DVΔVR), domain A2 (DVΔA2), or deletion of the top of domain A3 (DVΔ30). IFA were performed at 2 and 4 days after infection of BHK cells, and 3 and 5 days after infection of C6/36 cells as indicated. Photomicrograph were taken at 200× for BHK and 400× for C6/36 cells.

processes are still not well understood. Using RNA binding assays and atomic force microscopy, we have reported that sequences within and upstream of the 3′SL of dengue virus (3′UAR and 3′CS, respectively) are necessary for long-range RNA–RNA interactions and RNA cyclization (Alvarez et al., 2005). In addition, using in vitro assays with dengue and West Nile virus RNA dependent RNA polymerases, it has been proposed that efficient RNA synthesis requires both the 5′ and 3′CS (Nomaguchi et al., 2004; You and Padmanabhan, 1999; You et al., 2001). Here, using a self replicating RNA, we found that mutations in 3′CS did not alter the efficiency of translation initiation,

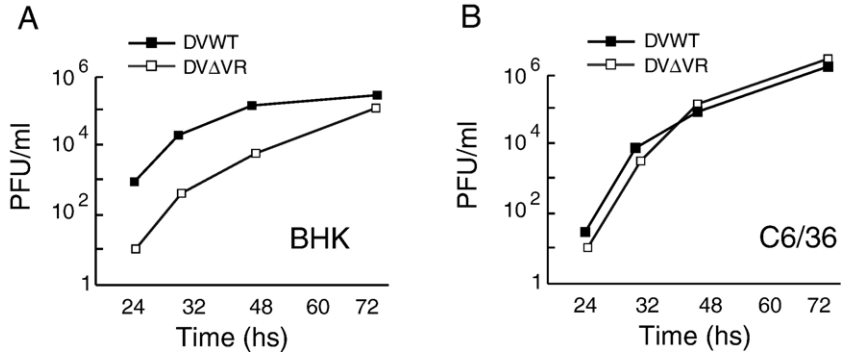


Fig. 5. Comparative growth analysis of dengue virus WT and VR deletion mutant in BHK and C6/36 cells. (A) One step growth curves of DVWT and DVΔVR in BHK cells. Cells were infected at M.O.I. of 0.01 and plaque-forming units (PFU) were determined at each time point by plaque assay in BHK cells. (B) One step growth curves of DVWT and DVΔVR as described in (A) in C6/36 cells.

suggesting that long-range 5′–3′ interactions might not be necessary during early stages of viral translation (Fig. 2). In contrast, these mutations abolished RNA synthesis. Reconstitution of sequence complementarity by mutating 5′CS with foreign sequences restored the ability of this RNA to replicate (Fig. 3). Similar results were previously reported with Kunjin and West Nile replicons (Khromykh et al., 2001; Lo et al., 2003). Taken together, the data indicate that 5′–3′CS complementarity is necessary for RNA synthesis and is a common feature shared by different flaviviruses.

Upstream of the 3′SL there are two highly conserved sequences CS2 and RCS2, which are located within side stem loops of domains A3 and A2, respectively (Fig. 2A). In order to study the role of these sequences on RNA replication without causing rearrangements of the predicted structures at the 3′UTR, we deleted the complete domains A2 and/or A3. Using full-length viral RNAs lacking both A2 and A3, we were able to recover a virus with a sub-lethal phenotype, suggesting that the presence of these RNA elements are not essential but are required for efficient replication. Using the replicon system, we observed that deletion of individual domains, A2 or A3, results in replicons with inefficient RNA synthesis, about 100-fold lower than the WT levels. Furthermore, deletion of both A2 and A3 domains resulted in RNAs that replicated below the detection limit of the system, which was estimated to be three orders of magnitude below the WT levels (Fig. 2). Because A2 and A3 have similar structures and expose similar sequence motifs in the loops, it is likely that they perform similar functions and that the loss of one can be compensated by presence of the other but deletion of both greatly impairs viral replication.

A model for replication of dengue virus proposes that RNA structures at the 3′ UTR contain elements that, concomitantly with elements at the 5′ end of the genome serve as signals for initiation of negative strand synthesis. Taking together our results with previous reports (Alvarez et al., 2005; Men et al., 1996; Proutski et al., 1999), two types of RNA elements at the 3′ end of dengue virus can be defined: (i) RNA elements that are essential for RNA replication, and (ii) RNA elements that function as enhancers of the replication process and for which their removal cause viral attenuation.

Mutations within dengue virus UTRs have been explored to obtain live attenuated vaccine candidates. However, little is know about the molecular details of viral attenuation. A potential vaccine that is currently being tested in clinical trials carries a deletion of 30 nucleotides in domain A3 (Blaney et al., 2004; Durbin et al., 2001; Hanley et al., 2004; Troyer et al., 2001; Whitehead et al., 2003). Recombinant viruses carrying this deletion resulted in reduced replication in rhesus monkeys and a restricted capacity for dissemination from the mid-gut to the head of infected mosquitoes. In order to analyze the cause of attenuation of this virus, we introduced the same deletion in domain A3 in the replicon system and in the full-length virus. Delayed replication of recombinant dengue virus carrying this mutation was observed in both mosquito and mammalian cells by IFA analysis (Fig. 4). The Luc signal at 10 h after replicon RNA transfection in BHK and C6/36 cells were similar for the WT and Δ30 RNAs, suggesting that the vaccine candidate is not attenuated in the translation process. However, the efficiency of RNA replication was 50- to 100-fold lower in BHK and C6/36 cells transfected with the DVRep Δ30 than that with the WT replicon, indicating that viral attenuation can be a consequence of defects in RNA synthesis (Fig. 2).

Like most arthropod borne viruses, dengue viruses can cause significant damage when they infect vertebrate cells, yet in most cases mosquito cells sustain persistent dengue virus infection. It is plausible that both host and viral factors are responsible for differential growth of dengue viruses in mosquito and mammalian cells. Here, we found that RNA sequences within the VR of the 3′UTR enhances replicon RNA synthesis in BHK cells but not in mosquito cells. Furthermore, recombinant viruses carrying a deletion of VR showed delayed replication in BHK cells, as determined by IFA, plaque morphology, and growth curves, while replication in C6/36 cells was as efficient as for the parental virus (Figs. 4 and 5). In agreement with these observations, a large deletion in the 3′UTR of dengue virus 4 including the VR was previously reported to cause differential growth in mosquito and LLC-MK2 cells (Men et al., 1996). Moreover, chimeric viruses carrying the 3′UTR of an American dengue virus 2 genotype in the context of an Asian isolate yielded viruses with impaired replication in Vero cells but efficient replication in mosquito cells (Cologna and Rico-Hesse, 2003). Taken together, these results suggest that RNA elements present at the 3′UTR of dengue viruses differentially modulate viral replication in mosquito and mammalian cells, presumably by interacting with specific host factors.

Because the VR sequences of different dengue virus genotypes show large heterogeneity and they appear not to be essential for replication, investigators have paid little attention to this region, and a possible significance of these sequences on dengue virus pathogenesis has not been examined. Interestingly, the American genotypes, which contain a 10 nucleotide deletion following the stop codon of NS5, have not been previously associated to dengue hemorrhagic fever (DHF) (Leitmeyer et al., 1999), while the 1990 dengue virus 2 isolate from Venezuela associated with DHF retains those 10 nucleotides in the VR. Retention of those 10 nucleotides in VR was also observed in the Asian isolates, which are associated with more severe disease. Association of specific viral sequences to pathogenesis has been previously proposed (Cologna and Rico-Hesse, 2003; Gubler et al., 1978; Leitmeyer et al., 1999; Rico-Hesse et al., 1997). Differences in the sequence of NS5, envelope E protein, and the 5′ and 3′UTRs have been previously observed between low and high virulent genotypes (Cologna and Rico-Hesse, 2003; Leitmeyer et al., 1999; Rico-Hesse et al., 1997). Therefore, it will be interesting to pursue a systematic analysis of sequences

within VR in clinical isolates with different disease outcome in the context of the replicon system.

There is little evidence that transmission of dengue viruses will slow or cease during the beginning of this century. The World Health Organization continues reporting outbreaks of severe forms of the disease in the Americas and Asia (WHO, 2004). Therefore, there is an urgent need to control dengue virus infections in at least two continents. We believe that understanding the biology of dengue virus at the molecular level is an essential step on designing rational antiviral strategies. In this regard, the replicon system described here should aid investigation of various aspects of dengue virus life cycle.

Materials and methods

Dengue virus replicon construction

The cDNA DVRep was constructed from pD2/IC-30P-A, a dengue virus 2 full-length cDNA clone kindly provided by R. Kinney (Kinney et al., 1997). To facilitate insertion of the luciferase gene (Luc) into pD2/IC-30P-A, we generated an intermediate plasmid derived from pGL-3Basic (Promega). Using unique *Sac*I and *Nco*I restriction sites present upstream of Luc in pGL-3Basic, we introduced the complete 5′UTR followed by the first 102 nucleotides of the coding sequence of dengue virus. The resulting plasmid was used to introduce downstream of Luc the FMDV2A protease coding sequence (QLLNFDLLKLAGDVESNPGP) fused to the last 72 nucleotides of the envelope (E) protein followed by dengue virus sequences up to a unique *Spe*I restriction site (nucleotide 3587 in pD2/IC-30P-A). The fragment carrying FMDV2A fused to dengue virus sequences was generated by overlapping PCR using the following primers: *PCR1* sense AVG-107 (5′-CATAAAGAAAGGCCCGGCGC-3′) and antisense AVG-197 (5′-GACGTCTCCCGCAAGCTTGAGAAGGTCAAAATTCAACAGCTGCACGGCGATCTTTCCGCC-3′) and *PCR2* sense AVG-198 5′CTTCTCAAGCTTGCGGGAGACGTCGAGTCCAACCCTGGGCCATCCACCTCACTGTCTGTG-3′ and antisense AVG-104 (5′-TACGCGGATCCGCGGCAACTAGTAGTATTGCA-3′). The resulting construct was named pGL5′DVLucFMDV.

The DNA fragment containing the Luc gene flanked by dengue virus sequences was removed from pGL5′ DVLucFMDV by digestion with *Sac*I–*Spe*I restriction enzymes and introduced into homologous restriction sites within pD2IC/30P-A to obtain pDVRep.

To facilitate mutations within the 3′UTR of dengue virus in the pDVRep, a unique *Afl*II restriction site was introduced downstream of the stop codon of the viral polyprotein. To this end, the PCR product generated with primers sense AVG-62 (5′-CACCAATGTTGAGACATAGCATTGA-3′) and antisense AVG-91 (5′-GTTTCATCTTAAGTTTTGCTTTCTA-3′), and the product of a second PCR obtained with primers sense AVG-90 (5′-TAGAAAGCAAAACTTAAGATGAAAC-3′) and antisense AVG-63 (5′-ACTGGTGAGTACTCAACCAAGTCAT-3′), were fused by overlapping PCR. This PCR product was cloned into pGEM-T Easy (Promega) generating pGEM-3′DV*Afl*II. The *Avr*II–*Cla*I fragment of pDVRep was replaced with the *Avr*II–*Cla*I fragment of pGEM-3′UTR*Afl*II to generate pDVRep *Afl*II. A 3′UTR cassette between unique *Afl*II and *Xba*I restriction sites allowed us to exchange the wild type sequence by mutant 3′UTRs as follow:

pDVRepΔVR deletion mutant was generated using primer sense AVG-95 (5′-GTAATTCTTAAGCTCCACCTGAGAAGGTGT-3′) and antisense AVG-263 (5′-GCTTATCATCGATAAGCTTG-3′).

pDVRepΔA2-3 was generated by overlapping PCR. PCR1: primer sense AVG-90 and antisense AVG-66 (5′-AGCTCCACCTGAGAAGGTGCGGCCGCAGCATATTGACGCTGGGAA-3′), and PCR2 primer sense AVG-67 (5′-TTCCCAGCGTCAATATGCTGCGGCCGCACCTTCTCAGGTGGAGCT-3′) and AVG-263.

pDVRepΔA2 was generated by overlapping PCR. PCR1 primer sense AVG-90 and antisense AVG-96 (5′-GAGAAGGTGTAAAAAATCCTTACAAATCGCAGCAACAA-3′), and PCR2 primer sense AVG-97 (5′-TTGTTGCTGCGATTTGTAAGGATTTTTTACACCTTCTC-3′) and antisense AVG-263.

pDVRepΔA3 was generated by overlapping PCR. PCR1 primer sense AVG-90 and antisense AVG-99 (5′-TACAAATCGCAGCAACAATGAAACAAAAAACAGCATAT-3′), and PCR2 primer sense AVG-100 (5′-ATATGCTGTTTTTTGTTTCATTGTTGCTGCGATTTGTA-3′) and antisense AVG-263.

pDVRepΔ30 was generated by overlapping PCR. PCR1 primer sense AVG-90 and antisense AVG-374 (5′-CAACAATGGGGGCCCAAGTTAACTAGAGGTTAGAGGAGA-3′), and PCR2 primer sense AVG-375 (5′-TCTCCTCTAACCTCTAGTTAACTTGGGCCCCCATTGTTG-3′) and antisense AVG-263.

pDVRepCSMut recombinant DNA containing the mutation 10618-TATCATTTGGA was obtained by cassette substitution in the 3′UTR.

PDVRepCSDoubleMut recombinant DNA containing the mutation 134-TCCAAATGATA at the 5′CS was generated replacing the fragment *Sac*I–*Sph*I in the pDVRepCSMut.

All constructs were confirmed by DNA sequencing analysis using a ABI 377 automated DNA sequencer and a Big Dye terminator chemistry (Applied Biosystems).

RNA transcription, transfection, and quantification

pDVRep, pDVRepΔVR, pDVRepΔA2, pDVRepΔA3, pDVRepΔA2A3, pDVRepΔ30, pDVRepCSMut, and pDVRepCSDoubleMut DNAs were linearized by digestion

with *Xba*I enzyme and used as templates for transcription by T7 polymerase in the presence of m^7GpppA cap structure analog. DVRepΔ3′UTR RNA was generated by transcription of pDVRep linearized by digestion with *Afl*II restriction enzyme. *Renilla* luciferase mRNA was obtained from in vitro transcription using as a template pRLCMV (Promega) linearized by digestion with *Bam*HI restriction enzyme. Linearized plasmids were phenol-chloroform extracted, ethanol precipitated, and resuspended in RNase-free water at a concentration of 100 ng/μl. In vitro transcriptions were performed in a 40-μl reaction volume using 0.5 μg of DNA template, 2 mM m^7GpppA cap structure analog, 0.8 mM ATP, and 2 mM of UTP, CTP and GTP. The transcription reaction was incubated at 37 °C for 2 h. DNA templates were removed from the reaction mix by DNase I digestion, RNAs were purified using RNeasy Mini Kit (Qiagen Inc.), and quantified spectrophotometrically.

For RNA transfections, mammalian BHK-21 and mosquito C6/36 cells were grown to 60–70% confluence in 35 mm culture dishes. Lipofectamine 2000 (Invitrogen) was used to cotransfect replicon RNA transcripts (300 ng) along with 100 ng of mRNA encoding *Renilla* luciferase. We prepared complexes of RNA (in μg): Lipofectamine 2000 (in μl) in a 1:6 ratio for both cell lines and followed the transfection procedure according to manufacturer's instructions. Each transfection was performed in triplicates and the experiments were repeated independently at least 3 times.

BHK-21 cell line was maintained at 37 °C in minimum essential medium alpha (Life Technologies, Inc.) and *Aedes albopticus* C6/36 cell line was maintained at 28 °C in Leibovitz's L-15 medium (Life Technologies, Inc.). Both media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.). To analyze Luc levels, at each time point, the medium was removed, cells were washed with PBS, harvested by scrapping with PBS, centrifuged, and lysed by adding 80 μl of cell culture lysis buffer (Promega). To quantify both firefly and *Renilla* luciferases, a dual-luciferase assay kit was used (Promega) following manufacturer's instructions. Luciferase activity was measured with a Tecan GENios luminometer.

For quantification by real time RT-PCR, replicon RNAs were Trizol-extracted (Invitrogen) at various time points post-transfection. We used an iCycler IQ (Bio-Rad) employing TaqMan technology. The primers and probe were targeted to amplify nucleotides 10419 to 10493 within the viral 3′UTR. Each 50 μl reaction mix contained 3 μl of RNA sample and final concentrations of 1× RT-PCR buffer (10 mM Tris–HCl pH 8.4, 50 mM KCl, 0.01% w/v gelatin, and 10 mM DTT), 2.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates, 100 nM primer 5′ (5′-CCTGTAGCTCCACCTGAGAAG-3′), 100 nM primer 3′ (5′-CACTACGCCATGCGTACAGC-3′), 100 nM probe (5′-/56-FAM/CCGGGAGGCCACAAACCATGG/36-TAM/-3′), and 100 units M-MLV RT (Promega). Reverse transcription was allowed to proceed for 1 h at 37 °C, and then 2 units of Taq DNA polymerase (Invitrogen) were added to each reaction tube. PCR amplification and detection were performed using the following conditions: 95 °C for 3 min (1 cycle), and then 40 cycles of 95°C for 15 s and 61 °C for 1 min. DVRep WT RNA copy number was expressed after subtracting the amount of DVRep NS5Mut RNA for each time point. A standard curve was generated using in vitro transcribed DVRep RNA.

Construction of recombinant dengue virus cDNAs

The pD2/IC-30P-A was modified to introduce a unique *Afl*II restriction site to generate pDVWT with a 3′UTR cassette between *Afl*II and *Xba*I sites using the same procedure described above for the pDVRep. pDVWT, pDVΔVR, pDVΔA2, pDVΔ30, and pDVΔA2A3 recombinant DNAs were obtained by cassette substitution of the 3′UTR following the same strategy described above for the respective replicon.

Virus recovery

Plasmids pDVWT, pDVΔVR, pDVΔA2, pDVΔ30, and pDVΔA2A3 were linearized with *Xba*I restriction enzyme and used as templates for in vitro transcription by T7 polymerase as described above. Recombinant dengue virus RNAs (2 μg) were transfected with Lipofectamine 2000 (Invitrogen) into BHK-21 and C6/36 cells. Viruses were harvested from BHK and C6/36 cells 5 to 7 days post-transfections. These viruses were used to infect fresh cells to obtain high titer viral stocks.

Viral stocks were quantified by plaque assays. To this end, 3.0×10^4 to 4.0×10^4 BHK-21 cells were seeded per well in 24-well plates and allowed to attach overnight. Viral stocks were serially diluted and 0.1 ml was added to the cells and incubated for 1 h. Afterwards, 1 ml of overlay (1× MEM alpha medium, 2% NCS, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 0.8% methyl cellulose) was added to each well. Cells were fixed 7 days post-infection with 10% formaldehyde and stained with crystal violet.

Immunofluorescence assay

BHK-21 and C6/36 cells that had been seeded to a 24-well plate on a 1-cm^2 coverslip were infected with 10^4 PFU of DVWT or mutant viral stocks that were recovered from BHK cells and immunofluorescence assay (IFA) was performed at 2, 3, 4, 5, and 6 days post-infection. At each time point a 1:200 dilution of murine hyperimmune ascitic fluid against dengue-2 in phosphate-buffered saline–0.2% gelatin was used to detect viral antigens. Cells were fixed in paraformaldehyde. Alexa Fluor 488 rabbit anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG conjugates (Molecular Probes) were used as detector antibodies at 1:500 dilution. Photomicrographs (200× for BHK and 400× for C6/36 cells) were

acquired in an Olympus BX60 microscope coupled to a CoolSnap-Pro digital camera (Media Cybernetics) and analyzed with the Image-Pro Plus software.

One step growth curves

Subconfluent BHK-21 and C6/36 cells in a six-well plate were infected with equal amounts of DVWT or DVΔVR recovered from BHK cells. A multiplicity of infection (MOI) of 0.01 in 500 μl of PBS was used. After 1 h adsorption period, the cells were washed 3 times with PBS and 2 ml of growth media were added. At each time point after infection, cell supernatants were collected and frozen at −70 °C. For virus quantification at each time point, supernatants were serially diluted and plaque assay performed on BHK-21 cells as described above.

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