Structural and functional analysis of dengue virus RNA

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Abstract. Sequences and structures present at the 5′ and 3′ UTRs of RNA viruses play crucial roles in the initiation and regulation of translation, RNA synthesis and viral assembly. In dengue virus, as well as in other mosquito-borne flaviviruses, the presence of complementary sequences at the ends of the genome mediate long-range RNA–RNA interactions. Dengue virus RNA displays two pairs of complementary sequences (CS and UAR) required for genome circularization and viral viability. In order to study the molecular mechanism by which these RNA–RNA interactions participate in the viral life cycle, we developed a dengue virus replicon system. RNA transfection of the replicon in mosquito and mammalian cells allows discrimination between RNA elements involved in translation and RNA synthesis. We found that mutations within CS or UAR at the 5′ or 3′ ends of the RNA that interfere with base pairing did not significantly affect translation of the input RNA but seriously compromised or abolished RNA synthesis. Furthermore, a systematic mutational analysis of UAR sequences indicated that, beside the role in RNA cyclization, specific nucleotides within UAR are also important for efficient RNA synthesis.

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The genome-length RNA of dengue virus is infectious. Delivery of this RNA molecule into a susceptible cell triggers a complete round of viral replication. Once in the cytoplasm of the host cell, the viral genome participates in at least three different processes: it serves as mRNA to direct the synthesis of viral proteins, it acts as template for genome amplification, and it is packaged along with structural proteins during viral assembly. The molecular mechanisms controlling the utilization of the viral RNA in each step of the viral life cycle are still poorly understood. Because the 5′ and 3′ ends of the viral RNA are the places where translation initiation and synthesis of positive and negative strand RNA occur, we are interested

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in investigating the function of RNA structures and sequences located at the ends of the viral RNA.

The genome of dengue and other flaviviruses is about 11 kb long and encodes one open reading frame flanked by 5′ and 3′ untranslated regions (UTRs) (Rice 2001). The 5′ UTR of dengue virus is around 100 nucleotides long and the sequence conservation is almost complete among different dengue virus serotypes (Markoff 2003). The predicted structure consists of a large stem loop (SLA) and a second short stem loop (SLB) containing at the 3′-terminal sequences the translation initiation codon (Fig. 1). It is likely that these sequences and structures influence translation initiation, which presumably takes place by a cap dependent scanning mechanism. Another major function of the 5′ UTR probably resides in the negative strand, which serves as a site for positive strand RNA synthesis. Deletions engineered into the 5′ UTR of dengue virus 4 were lethal (Cahour et al 1995), suggesting an important role of these RNA structures in viral replication.

The 3′ UTR of dengue virus is around 450 nucleotides, lacks a poly(A) tail, but contains a number of conserved RNA structures (Fig. 1) (Shurtleff et al 2001). The viral genome ends in a very conserved 3′ 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, Zeng et al 1998, Rauscher et al 1997, Proutski et al 1997, Men et al 1996, Yu & Markoff 2005, Tilgner et al 2005, Elghonemy et al 2005). 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 CS sequence, which is complementary to a sequence present within the coding region of protein C at the 5′ end of the genome (Hahn et al 1987). 5′–3′ long-range RNA–RNA interactions through these complementary sequences have been proposed to be necessary for replication of different mosquito-borne

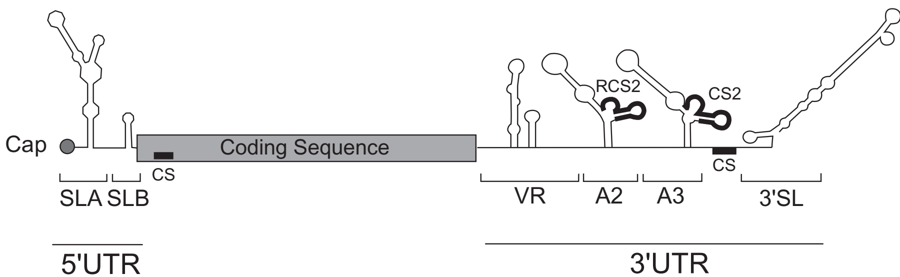

FIG. 1. Schematic representation of dengue virus genome. The predicted secondary structures of defined domains at the 5′ and 3′ UTR are indicated: stem loop A (SLA), stem loop B (SLB), variable region (VR), domain A2, domain A3, and the 3′ stem loop (3′SL). Also, the conserved sequences CS, CS2 and RCS2 are shown.

flaviviruses (Alvarez et al 2005b, 2005a, Lo et al 2003, Khromykh et al 2001, Corver et al 2003). In addition, 5′–3′ CS base pairing has also been reported to be important for *in vitro* activity of dengue and West Nile virus RNA polymerases (You et al 2001, Nomaguchi et al 2004). The mechanism by which the flavivirus replicase machinery initiates RNA synthesis specifically at the viral 3′ UTR is still not clearly understood. The RNA replication complex assembles on cellular membranes and involves the viral RNA dependent RNA polymerase-methyltransferase NS5, the helicase-protease NS3, the glycoprotein NS1, the hydrophobic proteins NS2A and NS4A, and presumably host factors (Westaway et al 1999, 1997, Mackenzie et al 1998).

In addition to the 3′ SL, other RNA structures and conserved motifs are present within dengue virus 3′ UTR. Folding algorithms predicts two almost identical structures designed A2 and A3 preceding the 3′ SL. Recent experiments using recombinant dengue virus 2 carrying deletions of domain A2 and/or A3 showed viral attenuation with defects in RNA synthesis, suggesting an important role of these RNA structures in viral replication (Alvarez et al 2005a). Within domains A3 and A2 there are highly conserved regions known as CS2 and repeated CS2 (RCS2), respectively (Shurtleff et al 2001). A recombinant virus with a deletion of 30 nucleotides between CS2 and RCS2 is currently under study as a dengue virus vaccine candidate (Durbin et al 2001). CS2 and RCS2 sequences can be found in Japanese encephalitis virus, West Nile virus, Murray Valley encephalitis virus, and dengue virus types 1 to 4 (for review see Markoff 2003), suggesting a conserved function of these elements in flavivirus replication. However, it is not clear the mechanisms by which these RNA structures participate in viral replication.

Here, we discuss the nature and requirements of long-range RNA–RNA interactions in the viral genome during dengue virus replication. Using genomic and subgenomic dengue virus RNAs together with biochemical tools, we analysed the role of secondary and tertiary structures of the viral RNA during translation and RNA synthesis.

Results and discussion

Flavivirus genomes possess inverted complementary sequences at the ends of the RNA, similar to that observed in the negative strand RNA viruses (bunya-, arena- and orthomyxoviruses) (Kohl et al 2004, Barr & Wertz 2004, Hsu et al 1987, Raju & Kolakofsky 1989, Mir & Panganiban 2005, Ghiringhelli et al 1991). These complementary sequences have been suggested to allow the ends of the genome to associate through base pairing, leading to circular conformations of the RNA (panhandle-like structures). Do flavivirus genomes acquire a circular conformation? Is the long-range RNA–RNA interaction required for dengue virus replication?

To investigate a possible association between the 5′ and 3′ ends of dengue virus genome, we analyzed the formation of RNA–RNA complexes using electrophoresis mobility shift assays (EMSA) with *in vitro* transcribed radiolabelled RNA molecules. We found that an RNA molecule corresponding to the first 160 nucleotides of dengue virus RNA (5′UTRC62 RNA) specifically binds to a second RNA molecule carrying the last 106 nucleotides of the viral genome (3′SL Probe, Fig. 2A). This RNA–RNA interaction shows high affinity (K_d about 8 nM) and is absolutely dependent on the presence of Mg^{2+}. In dengue and other mosquito-borne flaviviruses it was proposed that the complementary sequences 5′–3′ CS are a potential cyclization element. Folding predictions of the sequences representing the RNA–RNA complex formed by the ends of the dengue virus genome show two pairs of complementary regions (Fig. 2B). One of these regions is the 5′–3′ CS, the second is located at the 5′ end just upstream of the initiator AUG and at the 3′ end within the stem of the 3′ SL (named UAR, <u>u</u>pstream <u>A</u>UG <u>r</u>egion, Fig. 2B). To investigate the RNA determinants for complex formation, we generated 5′ UTRC62 RNA molecules with substitutions generating mismatches in either 5′–3′ CS or 5′–3′ UAR. We tested the binding ability of these mutated 5′RNAs in EMSA using a 3′ SL wild-type probe. Mutations in 5′ CS or 5′ UAR greatly decreased the binding of the mutated 5′UTRC62 RNAs to the radiolabelled 3′ SL, confirming that both complementary sequences were necessary for RNA–RNA complex formation (Fig. 2C).

To investigate whether the RNA–RNA contacts observed between two RNA molecules representing the ends of dengue virus genome also occur in a single RNA molecule as long-range interactions, we analysed the conformation of individual molecules by atomic force microscopy (AFM). The *in vitro* transcribed full-length dengue virus RNA was deposited on mica, dried and visualized by tapping mode AFM. The single-stranded RNA molecules acquire compact structures, precluding visualization of intramolecular contacts (Fig. 3A). To overcome this problem, we hybridized the viral RNA with an antisense molecule of 3.3 kb (complementary to a region encompassing NS4B–NS5), which yielded an extended double stranded region flanked by the single stranded ends of the viral RNA. These molecules were observed in both circular and linear conformations in the absence of proteins (Fig. 3B and C). Statistical analysis of this and other model RNA molecules carrying specific mutations confirmed that viral RNA circularizes through direct RNA–RNA contacts involving CS and UAR sequences (Alvarez et al 2005b).

Previous reports have suggested that base pairing between 5′ and 3′ CS of flavivirus genomes is necessary for viral replication. Using Kunjin and West Nile virus replicons it has been shown that substitution mutations in either 5′ or 3′ CS that disrupted base pairing were lethal for RNA replication (Khromykh et al 2001, Lo et al 2003). However, when both CS sequences were mutagenized with respect

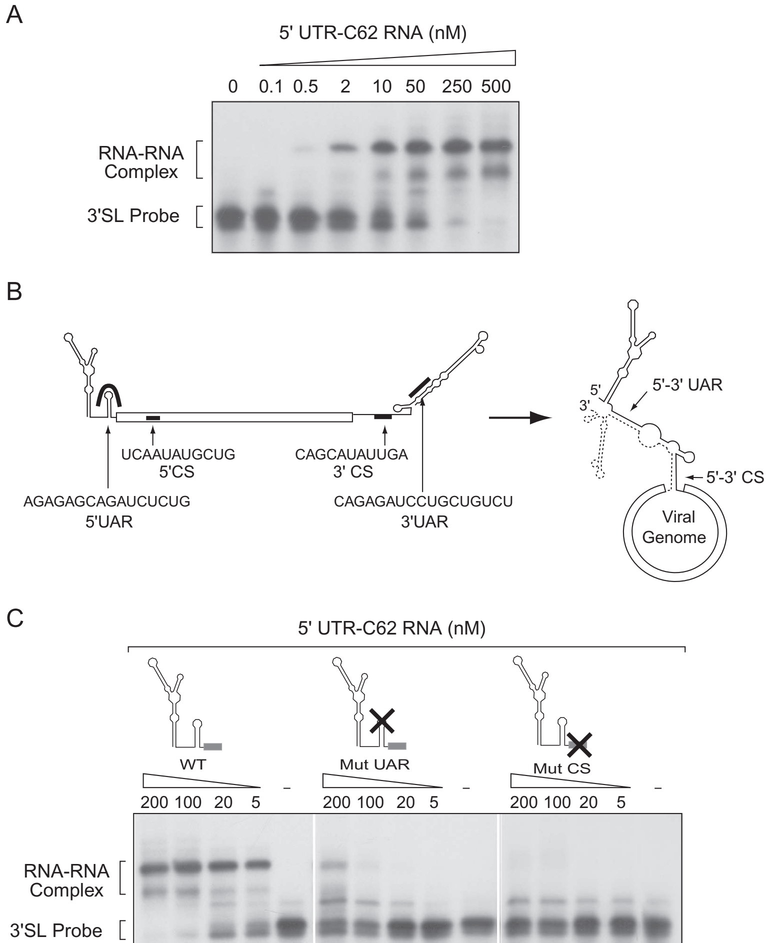

FIG. 2. RNA–RNA complex formation between the end sequences of dengue virus RNA. (A) Mobility shift assays shows RNA–RNA complex formation. Uniformly labelled 3′ SL RNA (1 nM, 30 000 cmp), corresponding to the last 106 nucleotides of dengue virus 2, was incubated with increasing concentrations of the 5′UTR–C62 RNA corresponding to the first 160 nucleotides of the viral genome, as indicated at the top of the gel. (B) Schematic representation of dengue virus genome showing the location and nucleotide sequence of 5′ CS, 5′ UAR, 3′ CS, and 3′ UAR. Folding prediction of the proposed circular conformation of the RNA is also shown. (C) RNA mobility shift analysis showing the effect of mutations in CS or UAR within the 5′ UTR–C62 RNA on binding to the 3′ SL RNA. Uniformly labelled 3′ SL RNA was incubated with increasing concentrations of wild-type and mutated 5′ UTR–C62 RNAs as indicated at the top of the gel.

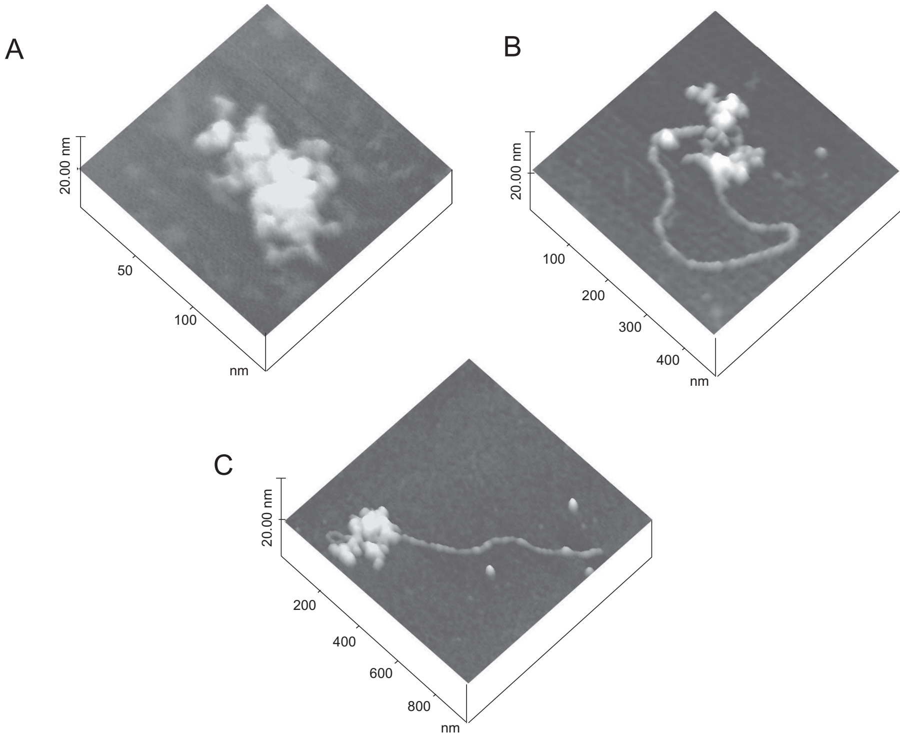

FIG. 3. Visualization of genome–length dengue virus RNA of 10.7 kb by tapping mode AFM. (A) Visualization of a representative single stranded dengue virus RNA molecule. (B, C) Images of circular and linear conformations of individual dengue virus RNA molecules, respectively. The 10.7 kb RNA molecule was hybridized with an antisense RNA of 3.3 kb resulting in a linear double stranded region with single stranded regions of 6970 and 451 nucleotides at the 5′ and 3′ ends, respectively.

to the wild-type sequence such that their capacity to base pair was maintained, RNA replication was restored. We performed similar experiments using recombinant dengue viruses to address the importance of 5′–3′ UAR complementarity during viral replication. Our data showed that specific substitutions within 5′ or 3′ UAR, in the context of the infectious dengue virus 2, yielded no viable viruses. Importantly, mutations at the 5′ and 3′ UAR that restored complementarity were sufficient to rescue viral replication. The replication of this recombinant virus displayed slow growth and small plaque phenotype when compared with the wild-type virus, suggesting that the nucleotide sequence/structure of 5′ and/or 3′ UAR is also important for efficient viral replication (Alvarez et al 2005b).

Our results indicate that long range RNA–RNA interactions result in circular conformation of the viral genome. In addition, the functional studies on dengue

virus together with previous reports obtained with other flaviviruses strongly suggest that sequence complementarity is required for viral replication. However, many questions remain open: what is the role of the long-range 5′–3′ end interactions during dengue virus replication? Is the cyclization of the viral genome necessary for efficient translation, similar to that observed for cellular mRNAs? Is the complex between the 5′ and 3′ ends of the genome required for NS5 polymerase binding during RNA synthesis? Does the structure of the RNA involving both ends of the genome play a role in coordinating translation and RNA synthesis? Does the long range RNA–RNA interaction constitute a signal for RNA encapsidation?

To dissect the role of the cyclization sequences during the viral processes, we constructed a dengue virus replicon that allows discrimination between translation of the input RNA and RNA synthesis (Alvarez et al 2005a). Similar replicons have been recently developed for West Nile and yellow fever viruses (Lo et al 2003, Jones et al 2005). In the context of dengue virus 2, we replaced the viral structural proteins by the firefly luciferase coding sequence (Luc). The trans membrane domain (TM) corresponding to the C-terminal 24 amino acids of E protein was retained in order to maintain the topology of the viral protein NS1 inside of the ER compartment (Fig. 4A). The Luc was fused in-frame to the first 102 nucleotides of the capsid protein (C), which contain the 5′ CS sequence. To ensure proper release of the Luc from the viral polyprotein, we introduced the *cis*-acting FMDV 2A protease (Fig. 4A).

Dengue virus replicon was efficiently translated and amplified in transfected BHK and mosquito cells. After RNA transfection with lipofectamine, Luc activity increases as a function of time reaching the highest levels between 8 and 10 h, reflecting the translation of the input RNA. After 24 h of transfection, Luc activity increases exponentially as a result of replicon RNA amplification (Fig. 4A). A replicon with a mutation in the GDD active site of the RNA dependent RNA polymerase NS5 (MutNS5) showed the translation peak, but after 24 h the levels of Luc were indistinguishable from the background, showing the lack of RNA amplification. These results indicate that replicon RNA amplification by the viral replicase machinery can be monitored through the expression of Luc as a function of time in transfected cells.

We used the replicon system to generate specific substitutions disrupting 5′–3′ CS or 5′–3′ UAR complementarity. To this end, we incorporated substitutions within the 3′ CS sequence, generating 4 mismatches (the wild-type 3′ CS CAGCAUAUUGA was replaced by **U**A**U**CAU**U**U**G**GA, Mut.3′CS replicon). In addition, a second replicon was designed carrying point mutations at the 5′ CS that restored sequence complementarity with the mutated 3′CS (Rec.5′–3′CS replicon). RNAs corresponding to the wild-type, Mut.NS5, Mut.3′CS, and the double mutant Rec.5′–3′CS were *in vitro* transcribed and equal amounts of the RNA were trans-

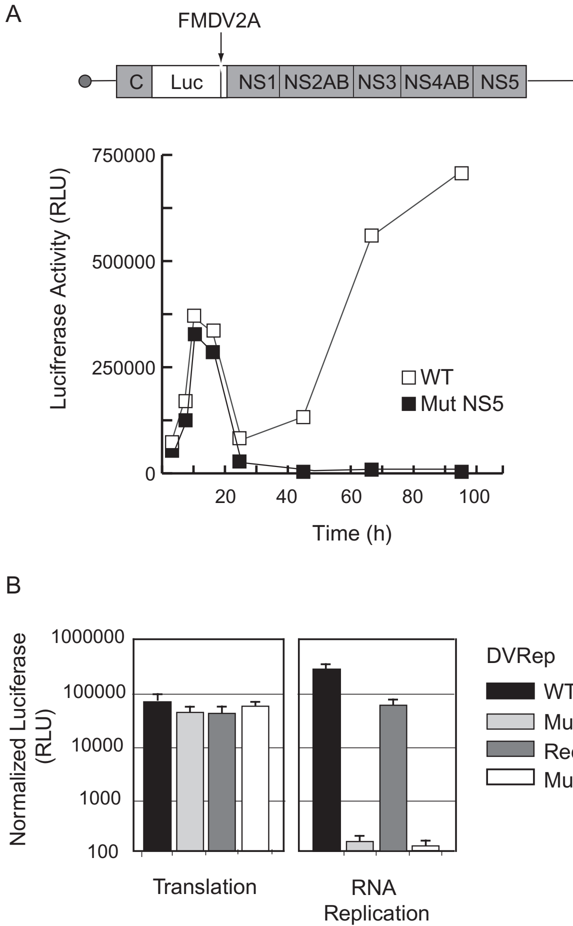

FIG. 4. Dengue virus replicon allows discrimination between translation and RNA replication in transfected cells. (A) At the top, schematic representation of dengue virus replicon. Boxes denoting coding sequences of capsid (C), Luciferase (Luc), and non–structural proteins (NS) are shown. Also, the position of FMDV2A protease is indicated by an arrow. At the bottom, replication of dengue virus replicon in BHK cells is shown. Time-course of luciferase activity was detected in cytoplasmic extracts prepared from BHK cells transfected with wild-type dengue virus replicon (WT) or replication-incompetent MutNS5 RNAs. (B) Translation and RNA replication of WT, 3′ CS mutant (3′CSMut), double mutant at the 3′ and 5′ CS (Rec.5′–3′CS), and replication-incompetent MutNS5 were analysed 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.

fected into BHK cells (Fig. 4B). Renilla Luc mRNA was cotransfected with all the replicons and used to standardize the transfection efficiency in each time point. After 10 h of transfection no significant differences in Luc activity were observed between cells transfected with the wild-type or mutated replicons, suggesting that translation of the input RNA was not dependent on 5′–3′ CS interactions. In contrast, RNA synthesis of the Mut 3′CS replicon was undetectable. Reconstitution of 5′–3′ base pairing in the Rec.5′–3′CS replicon also restored RNA synthesis (Fig. 4B). The level of RNA replication detected with this replicon was lower than the one observed with the wild-type RNA, suggesting that the highly conserved WT sequences within CS provide an advantage during RNA amplification.

To study the importance of 5′–3′ UAR complementarity during viral translation and RNA synthesis we introduced specific mutations in these regions in the replicon system. UAR sequences are located within very conserved stem loops of the viral 5′ and 3′ UTRs, therefore, it is difficult to introduce mutations in 5′ or 3′ UAR sequences that disrupt complementarity without altering secondary structures. We designed point substitutions in both sides of the stem or loop of SLB (5′ UAR) and/or in their complementary sequences within the stem of the 3′SL (3′ UAR) (Fig. 5A). We generated three groups of mutations (Mut 1, Mut 2 and Mut 3), each group was composed of three different replicons: (a) with mutations in 5′ UAR, (b) with mutations in 3′ UAR, and (c) with mutations a + b in the same replicon, restoring 5′–3′ UAR complementarity with sequences that differed from the wild-type sequences (Fig. 5A). The RNAs corresponding to the wild-type and the nine mutated replicons described above were transfected into BHK cells and Luc activity was measured as a function of time. The translation efficiency was determined measuring Luc activity after 10 h of transfection. The results showed that all mutant replicons were translated with similar efficiencies to that observed with the wild-type replicon (data not shown). To evaluate RNA synthesis, we compared the RNA amplification of each mutated replicon with the wild-type levels and expressed the results as a percentage of the wild-type (Fig. 5B). From this analysis we observed that: (i) single mutations disrupting 5′–3′ UAR base pairing decreased or abolished RNA synthesis; (ii) in the three groups of mutants the reconstitution of the 5′–3′ complementarity (5′3′Mut 1, 5′3′Mut 2 and 5′3′Mut 3) also increased the levels of RNA synthesis; (iii) not all base pairings within 5′–3′ UAR are equally important for RNA synthesis (compare the replication levels of 3′Mut-2 with 3′Mut-3, both with only one mismatch); and (iv) in some cases reconstitution of 5′–3′ complementarity is not sufficient to rescue RNA synthesis to wild-type levels, suggesting that specific nucleotides within the 5′ or 3′ UAR sequences can be critical. In this regard, it is important to mention that the sequence of 5′ and 3′ UAR are absolutely conserved in all dengue virus serotypes, suggesting that even though co-evolution of the two complementary sequences could occur there must be a growth advantage to preserve the wild-type nucleotide sequences.

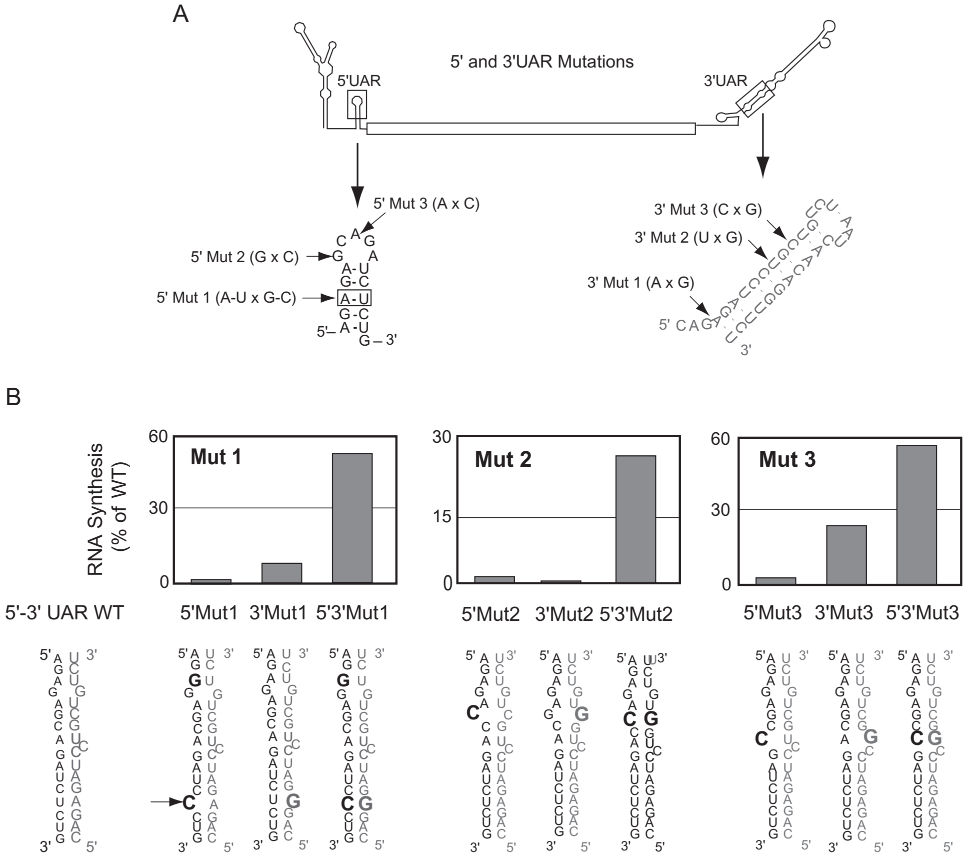

FIG. 5. Mutations within 5′ or 3′ UAR alter replicon RNA amplification (A) Schematic representation of dengue virus genome showing the predicted secondary structure of the RNA elements containing 5′ and 3′ UAR (shown in boxes). Nucleotide sequences of wild-type and the substitutions introduced at the 5′ UAR (5′Mut1, 5′Mut2, and 5′Mut3); and at the 3′ UAR (3′Mut1, 3′Mut2, and 3′Mut3) are shown. (B) RNA replication of mutant replicons disrupting and restoring 5′–3′ UAR complementarity in transfected BHK cells. Normalized luciferase levels determined 3 days after transfection are shown as a percentage of the wild-type replicon for the three groups of mutants within UAR sequences (Mut1, Mut2 and Mut3). Underneath of each plot, base pairing between sequences corresponding to 5′–3′UAR for the mutants are compared with the wild-type sequences.

Taken together, the results discussed here indicate that the complementary sequences 5′–3′ CS and 5′–3′ UAR are not necessary for efficient translation of the input RNA but they are essential during RNA synthesis. To better define the role of sequence complementarity during the process of RNA synthesis, we are currently investigating NS5 polymerase association to circular and linear

conformations of the RNA and the impact of RNA conformation on enzyme activity. Disruption of 5′–3′ interactions did not alter significantly translation initiation of the input RNA, however, we cannot rule out a possible regulatory role of RNA-RNA interactions on translation after several rounds of protein synthesis had taken place in the infected cell. It is possible that dynamic RNA–RNA and RNA–protein interactions involving cellular and viral factors, induce conformational changes of the viral RNA rendering the viral genome more competent for translation, RNA synthesis, or encapsidation at different stages of viral infection. Understanding the role of secondary and tertiary structures of the viral RNA during the viral life cycle will help to clarify molecular details of dengue virus replication. At present neither specific antiviral therapy nor licensed vaccine exists to control dengue virus infections. Therefore, it is of crucial interest to investigate the biology of this virus at the molecular level as an essential step on designing novel antiviral strategies.

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DISCUSSION

Harris: When you add NS5, does it increase the percentage of circular RNA molecules?

Gamarnik: That's one of the key questions. We need to do AFM in solution. The images of NS5 bound to the viral RNA shown here were obtained in air. However, we would like to see the RNA–protein interaction in solution. Using different buffer conditions, we can monitor the percentage of RNA cyclization in the presence of the viral protein. We would like to see the polymerase activity in real time. Some people have been able to visualize transcription in real time using AFM (*http://alice.berkeley.edu/~cjblab/transcription.html*).

Harris: Presumably you never see NS5 binding to the linear molecule?

Gamarnik: That is not the case. We indeed see linear molecules binding to NS5. However, it is difficult to discriminate whether the protein binds to the 5′ or the 3′ end of the RNA molecules. What we most commonly see is that the circles are decorated with polymerase.

Padmanabhan: When we did gel shift assays with NS3, we also used NS5 and found exactly the same thing: it binds to subgenomic RNA which has both ends, but not to the 5′ or 3′ separately. This is consistent with the requirement for RNA synthesis.

Jans: I'm curious as to how you did some of these AFM experiments. What sort of buffers did you use? Did you look at ionic strength? And what control did you do for NS5?

Gamarnik: We haven't used other proteins as controls, but we need to do this. It is necessary to include Mg^{2+} in the buffers. If we add other divalent cations, such as Ni^{2+}, we don't see RNA–RNA interactions. In addition, a divalent cation is necessary to immobilize the RNA on the mica. For this purpose people use Ni^{2+}, however, in our case the Ni^{2+} interferes with RNA–RNA complex formation and we do not observe circular forms of the RNA in this condition. Also if we include both Ni^{2+} and Mg^{2+} in the mix, we observe less RNA–RNA interaction.

Jans: Do you dry rapidly?

Gamarnik: We heat the samples to 80 °C, mix the sense and antisense molecules, dry them with nitrogen on a freshly cleaved mica, and then look at them right away.

Jans: To what extent do you think these 5′ and 3′ structures are dynamic?

Gamarnik: The RNA is very dynamic. The presence of viral and cellular proteins could also modulate RNA conformations.

Jans: Do you estimate the free energies of mutant RNAs?

Gamarnik: When we generate mutant RNAs, we try to maintain the free energy.

Jans: To get RNA–RNA complexes using gel shift assays, do you need heating?

Gamarnik: We can see the RNA–RNA complexes with or without heating. However, the standard assay includes heating and refolding. We use the same conditions to compare binding affinities of mutants and wild-type RNA.

Canard: Did you use capped and uncapped RNAs?

Gamarnik: That's a very good question. We are currently working on that.

Canard: The two anchor points for the polymerase. One is the 3′ end of the genome and the other is the 5′ cap.

Gamarnik: In order to study binding of the polymerase and the methyltransferase domains to the RNA, we are planning to use the full-length NS5 protein. In the results shown here, we used just the polymerase domain of NS5 without the methyltransferase. Perhaps if we use a capped RNA and full length NS5 we will be able to study the binding of the protein to both the cap structure and the RNA.

Rice: Have you looked at the influence of the capsid protein on any of your 5′/3′ interactions?

Gamarnik: We cloned and expressed a recombinant C protein, but haven't yet done anything with it.

Rice: Even in terms of your replicon assays?

Gamarnik: We have made one replicon that contains the first 34 amino acids of C fused to the reporter. Also, we have constructed monocistronic and bicistronic full-length genomes with reporters. So, hopefully we will be able to study the role of C on RNA replication.

Vasudevan: Have you looked at natural variations in this region?

Gamarnik: Yes. Dengue 1, 2, 3 and 4 are identical. All the isolates that we have analyzed showed 100% conservation of the complementary regions Sequence complementarity was also observed in other mosquito-borne flaviviruses.

Vasudevan: Earlier I was talking to Paul Young about the ease of making replicons or infectious clones with different virus strains. Not all strains seem to be the same in terms of maintaining them stably as a plasmid.

Gamarnik: They are not. It seems that we will have to test whether the reporter works in different strains. We have used DENV-2 (16681). Since the viral polyprotein is processed both in the cytoplasm and in the ER, the topology of the polyprotein fused to the reporter is critical to get replicating RNAs.

Harris: We have generated several DENV reporter replicons, and it seems to make a difference whether we place an HDVr ribozyme at the end versus cleaving with a restriction enzyme. In one case, the replicons generate a higher translation

(first) peak and less replication (second peak); and in the other, the replicons produce a lower translation peak and greater replication.

Gamarnik: We tried putting a ribozyme at the end, but we did not see improvement in the replication efficiency of our replicon.

Harris: Interestingly, the West Nile virus replicons (constructed by P.-Y. Shi), by chance recapitulated the same two systems, and they found the same differences in translation versus replication depending on the cleavage at the end (HDVr versus restriction enzyme).

Vasudevan: When Ricco-Hesse and colleagues did the strain severity work, they also looked at the 5′/3′ region and the free energy of stem loop structures. There was some sort of correlation with severity. This is why it would be interesting to look at full-genomes (at least 500) to see if there is much statistical correlation between virus sequence and disease severity.

Gamarnik: Yes, there are some differences in the stem loop A structure. There are two stem loops in the 5′ UTR of all dengue viruses, stem loop A and stem loop B. In DENV-4 from the end of the stem loop B to the AUG there are five nucleotides, while in DENV-1, -2 and -3 the AUG is right after stem loop B. We speculate that 5′–3′UAR hybridization would decrease translation efficiency since this structure is right upstream of the initiator AUG. However, we did not see an effect on translation when these sequences were mutated. In these experiments, we monitored translation efficiency only of the input RNA. It will be interesting to analyse what happens with translation when the polymerase, the viral protein NS3, and the other components of the replication machinery are present. In these conditions, it is possible that RNA cyclization could have an effect on translation. To study this, we are setting up an *in vitro* system to see translation and RNA synthesis in the presence of the viral non-structural proteins. We are testing a *Xenopus* oocyte system, where we can inject both the viral RNA and viral proteins. We have previously developed a similar system to study poliovirus replication.

Rice: Do you get initiation of minus strand synthesis in the oocytes?

Gamarnik: So far we haven't. But we haven't worked much in this system yet.

Rice: Are you just looking for a double-stranded product?

Gamarnik: We are looking for viruses now. We are injecting the RNA and making whole cytoplasmic extract and searching for viral particles.

Rice: The polio experience.

Gamarnik: We had a very good experience with polio! Actually, with that system we could discriminate between synthesis of negative and positive strand RNA.

Gu: Because both the 5′ and 3′ UTR interact with cellular proteins do you think you can try to see RNA–RNA interactions in the presence of protein?

Gamarnik: Absolutely. We haven't tried this with cellular proteins. We know that the viral genome interacts with viral and cellular proteins. We examined first RNA–RNA interactions in the absence of proteins but now we need to move to

a more complex system and add other factors. We recently performed preliminary experiments adding infected and uninfected cell extracts to the RNA–RNA complex, and we observed a mobility shift in native gels. In addition, we observed some differences between the infected and uninfected extracts, which doesn't tell us much at this point. We have done some purification and separation of fractions, but we did not identify any protein yet.

Canard: It is always tempting to get more complicated with proteins from the cell, but we can't be sure that this is telling us more: you will never know whether you have all the proteins from the cells.

Gamarnik: I agree. I think one has to choose a strategy; we started with a simple system using viral RNA and purified viral proteins. Next, the idea is to include more viral and cellular proteins to get closer to the natural environment.