In Vitro RNA Synthesis from Exogenous Dengue Viral RNA Templates Requires Long Range Interactions between 5'- and 3'-Terminal Regions That Influence RNA Structure*

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Viral replicates of many positive-strand RNA viruses are membrane-bound complexes of cellular and viral proteins that include viral RNA-dependent RNA polymerase (RdRp). The in vitro RdRp assay system that utilizes cytoplasmic extracts from dengue viral-infected cells and exogenous RNA templates was developed to understand the mechanism of viral replication in vivo. Our results indicated that in vitro RNA synthesis at the 3'-untranslated region (UTR) required the presence of the 5'-terminal region (TR) and the two cyclization (CYC) motifs suggesting a functional interaction between the TRs. In this study, using a psoralen-UV cross-linking method and an in vitro RdRp assay, we analyzed structural determinants for physical and functional interactions. Exogenous RNA templates that were used in the assays contained deletion mutations in the 5'-TR and substitution mutations in the 3'-stem-loop structure including those that would disrupt the predicted pseudoknot structure. Our results indicate that there is physical interaction between the 5'-TR and 3'-UTR that requires only the CYC motifs. RNA synthesis at the 3'-TR, however, requires long range interactions involving the 5'-UTR, CYC motifs, and the 3'-stem-loop region that includes the tertiary pseudoknot structure.

Dengue viruses type 1–4 are members of the flavivirus family of positive-strand RNA viruses. The diseases caused by dengue viruses range from a simple form of dengue fever to a more complex form of dengue hemorrhagic fever/shock syndrome, which exhibits considerable morbidity and mortality, especially among children in the tropical and subtropical regions of the world (for reviews see Refs. 1–3). It is currently estimated that about 40% of the world’s population living in these areas is at risk for dengue viral diseases, and about 5% of about one million dengue hemorrhagic/dengue shock syndrome cases are fatal (1). Dengue virus type 2 (DEN2)1 is the most prevalent serotype identified over a 10-year period in epidemiological studies. DEN2 has a single-stranded RNA genome consisting of 10,723 nt (in New Guinea-C strain) (4) which encodes a single polyprotein arranged in the order, NH2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH. The RNA genome contains a type I cap at the 5'-end but lacks a poly(A) tail at the 3'-end (5). The polyprotein precursor is processed in the endoplasmic reticulum, where the action of the cellular signal peptidase results in three structural proteins, the components of the virion, the capsid (C), precursor membrane protein (prM), and the envelope (E) protein (6–8). The remainder of the polyprotein codes for at least seven nonstructural proteins that are generated by the combination of both the cellular signal peptidase and the virally encoded, two-component serine protease, NS2B/NS3.

The NS3 protein of flaviviruses has a serine protease catalytic triad within the N-terminal region of 180 amino acid residues (9,10) that requires NS2B for protease activity (11–20). The conserved motifs found in the NTP-binding proteins and the DEXH family of RNA helicases were also identified within a region from amino acid residue 160 to the C terminus of the NS3 protein (Refs. 10, 21, and 22 and for a review see Ref. 23). The RNA helicase activity of NS3 is thought to be required in flaviviral replication and is involved in unwinding of a putative double-stranded RNA replicative intermediate (5,24). In addition, the West Nile viral and dengue viral NS3 proteins exhibit 5'-RNA triphosphatase activity (25).

The largest of the flaviviral proteins, NS5, contains conserved motifs found in many viral RNA-dependent RNA polymerases (RdRp) (26, 27), suggesting a role for NS5 in viral RNA replication. In addition to the RdRp motifs, NS5 has conserved motifs found in RNA methyltransferases (28, 29) and also includes motifs found in RNA methyltransferases (28, 29) at the N-terminal region, although this activity has not been experimentally demonstrated for any flaviviral NS5. 5'-RNA triphosphatase, guanylyltransferase, and RNA methyltransferase are the three enzyme activities required for 5'-capping (30,31). Since NS3 and NS5 exist as a complex in dengue virus-infected cells (32), they are likely to be the components of not only the viral replicase but also the enzyme complex involved in 5'-capping.

Positive-strand viral RNA genomes contain recognition motifs for specific initiation of minus- and plus-strand RNA synthesis. These promoter elements are usually contained within the 5'- or 3'-terminal regions (TR), especially in the conserved stem-loop (SL) structures that are shown to be important for

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¶ The abbreviations used are: DEN2, dengue virus type 2; CYC, cyclization; NS, nonstructural; RdRp, RNA-dependent RNA polymerase; sl, stem-loop; TR, terminal region; WT, wild type; UTR, untranslated region; kb, kilobase pair; BMV, brome mosaic virus.

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viral RNA replication in many (+)-strand virus RNAs (33–45). In flavivirus RNAs, two conserved sequences (CS1 and CS2) within the 3′-untranslated region (UTR) and conserved SL structures and cyclization (5′-CYC and 3′-CYC) motifs within the 3′- and 5′-TRs have been identified by sequence and secondary structure prediction analyses (46–49). Recent studies suggested a possible role for the 3′-SL structure in flavivirus viral RNA replication (45). The 3′-UTR of West Nile virus is known to interact with the translation elongation factor, eF-1α (50) although the biological significance of this interaction is not clear. In addition, the 3′-UTR of the Japanese encephalitis viral RNA was found to interact with NS3 and NS5, the putative components of the viral replicase (51) that exist as a heteromeric complex in infected cells (32, 51).

To study the mechanism of viral replication in molecular detail, we reported the development of an in vitro assay system that utilized exogenous subgenomic RNA templates containing these conserved elements at the 5′- and 3′-TRs. A subgenomic RNA containing the 5′- and 3′-UTRs and the two CYC motifs was active as a template for RNA synthesis in vitro. RNA synthesis at the 3′-end of the 3′-UTR in the subgenomic RNA gave rise to two labeled products. The first was a double-stranded RNA that was resistant to RNase A under high ionic strength and had a mobility identical in size to the template RNA. The second product was twice the size of the input RNA template. This product was converted to a template size RNA species upon digestion with RNase A under high ionic strength. These results suggested that the 2× product had a hairpin-like structure with an RNase A-sensitive single-stranded region and that this product was formed by a mechanism involving a snapback of the 3′-end of the template and elongation by the viral RdRP. Moreover, when the 3′-UTR RNA alone was used as a template, it did not form a 2× hairpin product in the in vitro RdRP assay with infected C6/36 cell extracts. However, addition of 5′-TR230nt RNA in trans activated RNA synthesis from the 3′-UTR resulting in the formation of this 2× product of the 3′-UTR in the RdRP assay. Furthermore, it was shown that the CYC motifs are important for RNA synthesis in vitro because if the motif in the template 3′-UTR RNA or the activator 5′-TR230nt RNA was mutated, RNA synthesis was significantly reduced but was restored when the mutant motifs were complementary to each other (52).

The results of our previous study supported the conclusion that a functional interaction between the 5′-TR and 3′-UTR is required for RNA synthesis at the 3′-end of the positive-stranded template. In this study, we sought to determine whether there is any physical interaction between the 5′-TR and the 3′-UTR. Moreover, the 5′-TR RNA that we had used in our previous study contained the 5′-UTR as well as the 5′-CYC motif. Therefore, it remained to be established whether the 5′-UTR was required or only the 5′-CYC motif alone was sufficient for physical interaction with the 3′-UTR as well as for RNA synthesis. It also remained to be established whether the conserved 3′-SL structure of the 3′-UTR played any role in RNA synthesis in vitro. By using a psoralen-UV cross-linking method, we now show that there is physical interaction between the two ends of the dengue viral RNA that is dependent on the two cyclization motifs. However, our results also indicate that the interaction between the two ends alone is not sufficient for RNA synthesis in vitro at the 3′-UTR and that the 5′-UTR is also required for this process. For analyzing the importance of 3′-SL structure in RNA synthesis, we used the mutants that had previously been characterized for their replication efficiencies in vivo in the context of full-length infectious dengue viral RNAs (45). Some of these 3′-SL mutants were constructed by nucleotide substitutions that either maintained or disrupted base pairings within the dengue viral 3′-SL; others were constructed by substituting different regions of the dengue viral 3′-SL with corresponding regions of West Nile virus 3′-SL structures. We engineered these 3′-SL mutations into the subgenomic RNAs. Analysis of these mutant subgenomic RNA templates in the in vitro RdRP assays revealed that the secondary structure as well as tertiary pseudoknot structure (53) of the 3′-SL region were required for efficient RNA synthesis. In general, the 3′-SL mutations that interfered with the infectivity of the genomic RNAs in vivo also had similar effects on the template efficiencies in the in vitro RdRP assays. A few 3′-SL mutations that were defective in viability in vitro or exhibited "host range" phenotype (45) did not affect template efficiency in vitro suggesting that the defect in vivo could be at a step other than the initial negative-strand RNA synthesis.

**Experimental Procedures**

**Preparation of DEN2-infected Cell Lysates**

*Aedes albopictus* cells (C6/36 cells) were cultured as described (54, 55). LLC-MK2 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were infected by DEN2 (New Guinea C-strain) for 3 days using ∼5 plaque-forming units/cell. Cell lysates from infected C6/36 or LLC-MK2 cells were prepared as described (52). The viral proteins, NS3 and NS5, were detected in the cell lysates by Western blotting using rabbit polyclonal antibodies, followed by a chemiluminescence detection system (ECL system from Amersham Pharmacia Biotech) (16, 56).

**Plasmid Constructs**

**Plasmids Containing cDNAs Encoding Subgenomic RNAs with Mutation CYC Motif**—The preparation of pSY-2 plasmid containing the wild type 5′-CYC and 3′-CYC motifs, TCAATATGC and GCATATTGA, respectively, and the PCR fragment containing the 5′-TR230nt-mutCYC or the 3′-UTRmut mutCYC motif was as described (52). The PCR fragments were phosphorylated by polynucleotide kinase and cloned into pSP64 vector (Promega, Madison, WI) that was digested with SmaI and PstII and dephosphorylated at the blunt ends to prevent religation. The pSY-1 plasmid (52) was digested with two sets of double digestions; for cloning the 5′-CYC motif, the plasmid was digested with EcoRI + SalI, and for cloning the 3′-CYC motif, the plasmid was digested with NcoI + PstII. For construction of subgenomic RNA containing the 5′-CYCmut motif, the pSP64 intermediate plasmid was digested with EcoRI and SalI, and the 200-nt fragment containing the 5′-CYC was isolated; from the EcoRI + SalI digest of pSY1 plasmid, the SalI fragment (950 nt) containing the wild type 3′-CYC motif and the EcoRI-SalI vector fragment (2.9 kb) were also isolated and ligated with the 200-nt fragment to yield the pSY-1-5′-CYCmut plasmid. Similarly, the NcoI fragment (225 nt) containing the 3′-CYCmut motif was isolated from the pSP64 intermediate plasmid clone and was ligated to the two fragments derived from the NcoI + PstI digest of the pSY-1 plasmid, NcoI fragment (430 nt) and the vector fragment (3.3 kb). In both ligation reactions, the vector fragments of 2.9 and 3.3 kb were dephosphorylated prior to ligation. The pSY-2 plasmid derivatives (containing the cDNA coding for the 770-nt-long subgenomic RNA) were obtained from the pSY-1 plasmid constructs by digesting the respective plasmids with XmnI and BssUI, followed by treatment with the Escherichia coli Klenow fragment and ligation of the blunt ends. The subgenomic RNA construct containing both 5′- and 3′-CYCmut motifs was constructed by digesting the respective plasmids with XmnI and BssUI, followed by treatment with the Escherichia coli Klenow fragment and ligation of the blunt ends. The subgenomic RNA construct containing both 5′- and 3′-CYCmut motifs was constructed by digesting the respective plasmids with XmnI and BssUI, followed by treatment with the Escherichia coli Klenow fragment and ligation of the blunt ends.

**Plasmid Constructs Containing cDNAs Encoding 3′-SL Mutant Subgenomic RNAs**—In a previous study, several substitution mutations in the 3′-SL structure of DEN2 were generated by repositioning of the DEN2 sequences within the 3′-stem either without disrupting base pairing (D2-SL(a) and (b) mutants) or with disruption of base pairing within the top half of the long stem (D2-SL(c) mutant) as well as by replacing different segments of 3′-SL with corresponding regions from West Nile virus (WN) 3′-SL. The effects of these mutations in the context of DEN2-infected clones were analyzed by their replication efficiencies and infectivities in C6/36 and LLC-MK2 cells (45). To analyze the effects of these 3′-SL mutations on the exogenous RNA tem-
Plate efficiencies in the in vitro RdRP assays, plasmids encoding the 3'-SL mutant 3'-UTR RNAs and subgenomic RNAs containing the 3'-SL mutations were constructed by PCR. The 3'-SL mutants of the 3'-UTR were first constructed by using the 5'-primer consisting of the T7 promoter (underlined) on one end of the 3'-UTR primer, 5'-TAATACGACTCACTATAGGGCAAACACTACGAAAC-3', and a specific 3'-primer depending on the mutant. Four different 3'-end primers used for PCR were as follows: D2 3', 5'-GAAGACCTGTTGATTCT-CA-3', WN 3', 5'-AGATCCTGTTGCTCT-3', mutf 3', 5'-GAAGACCCTGGTTGTCT-3', and mutf 3', 5'-GAAGCCCTGGTTGTCT-CA-3'. To amplify the wild type D2, D2/WN, SL(mutA), SL(mutD), D2-SL(a), -SL(b), -SL(c), and D2-SL(mutB) plasmids, the 3'-primers were used. For the D2/WN-SL, D2/WN-SL(mutB), and -SL(mutC) primers, the WN 3'-primer was used. To amplify the D2/WN-SL(mutE) and -SL(mutF) mutants, the mutF 3'- and mutF 3' primers were used, respectively. PCR was for 30 cycles in the following order: 94°C for 1.5 min, 45°C for 1.5 min, and 72°C for 2 min. To increase the efficiency of in vitro transcription, a second PCR was carried out with a different 5'-primer containing the T7 promoter (underlined), 5'-TTACGAACTGACTCACTATAGGCAAAA-3', that corresponds to the sequences upstream of the T7 promoter, using the 3'-end primers described under “Plasmid Constructs” and the same 3'-end primer. Again four different 3'-end primers (see "Plasmid Constructs Containing cDNAs Encoding 3'-SL Mutant Subgenomic RNAs") were used for PCR. All PCR products were purified from agarose gel using the Qiagen gel extraction kit. Plasmids encoding the subgenomic RNAs were amplified from agarose gel by Qiagen gel extraction kit. Plasmids containing the subgenomic RNAs were cleaved with XbaI/ApaI to yield the plasmid encoding the subgenomic RNA 770nt (WT and CYC Mutants). The amplified PCR products were cloned into the EcoRI site, and the 3'-SL mutations was used as templates, 3'-UTR454nt RNAs containing different 3'-UTR454nt RNAs in equimolar amounts. The template efficiencies of the various 3'-SL mutants were quantitated spectrophotometrically, and their integrity was verified by electrophoresis on 3.5% polyacrylamide, 7 M urea gels followed by staining with acridine orange.

Preparation of in Vitro RNA Transcripts
In vitro transcription was performed at 37°C for 3 h in a 100-μl reaction volume as described by the manufacturer (Promega, Madison, WI; also see Ref 52). 30 μg of each of the RNAs were incubated 30 °C for 1.5 h using the infected C6/36 extracts, and the RNA products were precipitated by the same 3'-end primer and EcoRI (underlined) as described above. The PCR product was treated with T4 polynucleotide kinase and subcloned into the Smal-digested and dephosphorylated pSP64 vector. Clones that contained the 3'-UTR inserts upstream of the Sp6 promoter were selected (series pUP7T-X clones where “X” is a specific 3'-SL mutation). Each intermediate clone was cleaved with ApoI and XbaI, and the ~200-nt fragment was cloned into the AapI/xbai-digested pSY-2 plasmid (52). The regions of the 3'-SL structure replacements in the subgenomic RNA constructs were confirmed by DNA sequencing.

Plasmid Constructs Containing the Pseudoknot Mutation in the 3'-SL—In order to disrupt the potential pseudoknot structure in the WN-3'-SL structure (WN-3'-SLC73G), a site-directed mutagenesis was performed on the wild type 770nt subgenomic RNA containing the pseudoknot. Two PCR products were generated with overlapping sequences using two sets of primers. PCR1 was obtained using the 5'-primer (T7-3'-UTR), 5'-TTAATACGACTCACTATAGGGCAAAAACACTACGAAAC-3', and the 3'-primer (underlined) T7 promoter (T7-3'-UTR), 5'-TTAATACGACTCACTATAGGGCAAAAACACTACGAAAC-3', that corresponds to the sequences upstream of the T7 promoter (underlined) followed by the 21 nt of 3'-UTR, the 3'-primer (C73G(A)), 5'-TTGCTCTGCACAA-3', that corresponds to the sequences downstream of the T7 promoter (underlined) followed by the 21 nt of 3'-UTR, the 3'-primer (C73G(A)), 5'-TTGCTCTGCACAA-3', and the region beginning with the downstream capsid coding sequence. The 5'-primer contains the complementary sequence of the 3'-end of the 3'-UTR. PCR was performed using the 5'-primer described under “Plasmid Constructs” and the same 3'-primer as mentioned above. PCR Product Encoding the Subgenomic RNA 770nt (without 5'-UTR)—PCR was carried out with the same template as the first two primers: the 5'-primer, same as under “Preparation of DNA Fragments for Use as Templates in the in Vitro RNA Transcription by T7 RNA Polymerase,” and the 3'-primer, 5'-CTGTTTGAAGGTCCATGGCAGCCG-3'. PCR Fragment for the 5'-TR 454nt RNA Transcript—PCR was performed with the same 5'- and 3'-primer, 5'-CTGTTTGAAGGTCCATGGCAGCCG-3'.

Measurement of polynucleotide loading in vitro transcription assays were carried out directly through the culture supernatant from infected C6/36 cells. A. albopictus (C636) or the LLC-MK2 cell extracts and the various exogenous RNA templates were carried out as described (52). In the RdRP assays, where the various 5'-TR RNAs were added in trans to the reaction mixtures containing the 3'-UTR 454nt RNA template (3 μg), the RNAs were added in equimolar amounts. The template efficiencies of the various 3'-UTR 454nt RNAs containing different 3'-SL mutations were assayed by incubating 3'-UTR 454nt RNAs (3 μg each) with 1.2 μg of the 5'-TR 454nt RNA. The reactions were terminated by phenol extraction (phenol: chloroform, 24:1, pH 4.5), and the unincorporated radiolabeled CTP was removed by passage through a G-30 Spin Column (Bio-Rad). After ethanol precipitation, the RdRP assay products were analyzed on a 1.5% agarose-formaldehyde gel followed by autoradiography (52). In the RdRP assays where the subgenomic RNA 770nt containing the various 3'-SL mutations were used as templates, 3 μg of each of the RNAs were used per assay.

Analysis of the Polarity of the RdRP Product by RNase A Digestion followed by RNase H Mapping
The RdRP reaction was first carried out under standard conditions at 30°C for 1.5 h using the infected C6/36 extracts, and the RNA products were purified by phenol:CHCl3 extraction and ethanol precipitation. RNA pellet was then resuspended in 20 μl of H2O and purified by using the Qiagen gel extraction kit. The RNase A digestion was carried out directly through the culture supernatant from infected C6/36 cell extracts. A. albopictus (C636) or the LLC-MK2 cell extracts and the various exogenous RNA templates were carried out as described (52). In the RdRP assays, where the various 5'-TR RNAs were added in trans to the reaction mixtures containing the 3'-UTR 454nt RNA template (3 μg), the RNAs were added in equimolar amounts. The template efficiencies of the various 3'-UTR 454nt RNAs containing different 3'-SL mutations were assayed by incubating 3'-UTR 454nt RNAs (3 μg each) with 1.2 μg of the 5'-TR 454nt RNA. The reactions were terminated by phenol extraction (phenol: chloroform, 24:1), and the unincorporated radiolabeled CTP was removed by passage through a G-30 Spin Column (Bio-Rad). After ethanol precipitation, the RdRP assay products were analyzed on a 1.5% agarose-formaldehyde gel followed by autoradiography (52). In the RdRP assays where the subgenomic RNA 770nt containing the various 3'-SL mutations were used as templates, 3 μg of each of the RNAs were used per assay.

Preparation of DNA Fragments for Use as Templates in the in Vitro RNA Transcription by T7 RNA Polymerase
PCR Products Encoding the Subgenomic RNA 770nt (WT and CYC Mutants)—To generate the subgenomic RNA 770nt WT as well as the CYC motif mutants without any additional 3'-terminal nucleotides, PCR was performed using a plasmid construct encoding a specific subgenomic RNA as a template and two primers as follows: the 5'-primer containing the EcoRI site, 5'-AGCTATGACCATGGATTACGAATTC-3', and the 3'-primer, 5'-AGCTATGACCATGGATTACGAATTC-3', that corresponds to the sequences upstream of the T7 promoter, and the pSP64 vector sequence in each plasmid and the 3'-primer, 5'-AGACCTCGATCTTGGTTATTGCAAGACC-3', that anneals with the 3'-end of the 3'-UTR. PCR was performed for 35 cycles of each step as follows: 94°C for 1.5 min, 68°C for 1.5 min, and 72°C for 2 min. PCR Product Encoding the Subgenomic RNA 770nt (without 5'-UTR)—The DNA template for preparation of the subgenomic RNA 770nt that lacks the 5'-UTR (96 nt) is otherwise identical to the subgenomic RNA 770nt was prepared by PCR using pSP2-3 as the template and the two primers as follows: the 5'-primer, 5'-TAATACGACTCACTATAGGA-
for 30 min. The reaction was terminated by phenol:CHCl₃ extraction followed by ethanol precipitation. RNA products were analyzed by denaturing formaldehyde/agarose gel electrophoresis (52). The sequences of oligodeoxynucleotides were selected from a 19-nt region (between 231 and 250 nt from the 3′-end of 3′-UTR) for annealing. RNase H digestion would generate two fragments, 530 and 230 nt in length.

RNA:RNA Interaction Analysis
Preparation of a radiolabeled RNA Template—The various 5′-TR RNAs were radiolabeled during in vitro transcription. DNA template (1 μg) was mixed with 20 μl of 1× reaction buffer for in vitro transcription as mentioned above, except containing 12.5 μC TTP, 30 μCi of [α-32P]ATP (3000 Ci/mmol), and 10 units of T7 RNA polymerase. The reaction mixture was incubated at 37 °C for 1 h. Then, RNase I (1 unit) was added, and the reaction was incubated at 37 °C for 15 min to digest the DNA template. Labeled RNA was purified by phenol:chloroform extraction and by using a Micro-spin P-30 column (Bio-Rad) followed by ethanol precipitation. The radiolabeled RNA transcript was run on a polyacrylamide (4%), 7 M urea gel and eluted from the gel as described (52). The radioactivity of the eluted RNA was measured by a scintillation counter.

Psoralen/UV Cross-linking—To study the physical interaction between the 5′-TR RNA and 3′-UTR RNA, the psoralen/UV cross-linking method was used (57). Briefly, the radiolabeled 5′-TR RNA (0.3×10⁶ cpm) and 5 μg of the unlabeled 3′-UTR RNA were denatured at 90 °C for 2 min and chilled quickly on ice. Then, 25 μl of annealing buffer (50 mM sodium cacodylate, 300 mM KCl) was added, and the mixture was incubated at 70 °C for 20 min. The samples were supplemented with 5 mM MgCl₂, and were incubated at 37 °C for an additional 15 min. The samples were kept on ice until 2 μl of AMT-psoralen (4′-aminomethyl-4′,5′,8′-trimethylpsoralen, 1 mg/ml in RNase-free water; Sigma) was added, followed by further incubation on ice for 15 min in the dark. The samples were transferred to a 96-well flat bottom plate on ice. The plate was exposed to UV irradiation (365 nm) at a distance of 2–3 cm for 30 min. The samples were transferred back to microcentrifuge tubes, followed by phenol extraction and ethanol precipitation. The precipitated and cross-linked RNA samples were analyzed on a formaldehyde (2.2 M)-agarose (1.5%) gel. The gel was dried under vacuum and was subjected to autoradiography.

Secondary Structure Modeling of RNA
The predicted secondary structures of the mutant subgenomic RNAs were analyzed by using the RNA structure algorithm developed by Zuker et al. (58). Among the predicted structures for each RNA, the most stable forms based on ΔG values were chosen for comparisons.

RESULTS
Analysis of the Polarity of the RdRP Product by RNase H Digestion—In a previous study (52), we reported the first in vitro RdRP assay that utilizes exogenous subgenomic DEN2 RNA templates and membrane-bound cytoplasmic extracts from the DEN2-infected mosquito (C6/36) cells. By using a subgenomic RNA template that contained wild type 5′- and 3′-UTRs and CYC motifs in this assay system, we found that two RNA products were formed as follows: one had the same polarity, which was resistant to RNase H digestion on a completely denaturing RNA strand. RNase A treatment would produce a double-stranded hairpin RNA hairpin with a size twice that of the template RNA or it was formed by a cleavage of the 2× hairpin product by a nuclease present in the cytoplasmic extract at the single-stranded loop region. The predominant RNA product that was formed in the in vitro RdRP assay was the 2× hairpin product, the structural requirements of the template for the formation of the 2× product were analyzed in this study.

First, the polarity of the radiolabeled RNA formed in the RdRP reaction carried out using the subgenomic RNA template of (+) polarity was determined by RNase H mapping. The oligodeoxynucleotides were designed to anneal with the region 231–250 nt from the 3′-end of the genomic RNA so that RNase H digestion would be expected to yield fragments of 520 and 230 nt in length. However, denaturation of the covalently linked double-stranded RNA hairpin followed by annealing with an oligodeoxynucleotide of either polarity followed by RNase H digestion did not produce any discrete RNA products suggesting that complete denaturation of the highly structured RNA was not attained under these conditions. Therefore, the 2× RNA product formed in the standard RdRP reaction was first digested with RNase A under high salt conditions in order to remove the single-stranded loop region that connects the unlabeled input RNA with the newly synthesized complementary RNA strand. RNase A treatment would produce a double-stranded RNA not covalently linked which would facilitate denaturation and annealing with the oligodeoxynucleotides (Fig. 1, panel A). Inclusion of 30% formamide in the annealing reaction was also found to be necessary for optimum annealing of RNA-DNA hybrids and that under these conditions RNase H activity was not affected.

The RdRP product (Fig. 1, panel B, lane 1) was digested by RNase A under high salt conditions. Equal aliquots were digested with RNase H after annealing with oligodeoxynucleotide of either (+) or (−) polarity or in the absence of any oligodeoxynucleotide as a negative control. The RNA product without the addition of any oligodeoxynucleotide was not susceptible to RNase H (Fig. 1, panel B, lane 2). However, the RNA
product upon digestion with RNase H gave rise to essentially two distinct fragments of 520 and 230 nt after annealing with oligodeoxynucleotide of (+)-polarity (panel B, lane 3) but not with (−)-oligodeoxynucleotide (panel B, lane 4). These results indicated that the labeled strand from the RdRP reaction was of (−)-polarity, which is complementary to the input (+)-strand RNA template. The radiolabeled (+)-RNA annealed with the (−)-oligodeoxynucleotide served as a positive control for RNase H digestion (data not shown).

5′-TR RNA and 3′-UTR RNA Physically Interact via the Cyclization Motif—In a previous study, we showed that in vitro RNA synthesis by the cytoplasmic extract from dengue virus-infected cells that required specifically the 5′- and 3′-TRs of the dengue virus RNA be present in the same template RNA molecule (in cis) or that the 5′-TR230nt RNA be added in trans to the 3′-UTR454nt RNA. However, the 3′-UTR130nt RNA alone was not active to form the 2× RNA product. The results of that study suggested that there is a functional interaction between the 5′- and 3′-TRs that conferred to the inactive 3′- and 5′-UTR RNA the ability to serve as an efficient template for RNA synthesis resulting in the formation of the 2× product in vitro. Similar results were also obtained when the full-length 3′-UTR454nt and the 5′-TR230nt RNA were both present in the RdRP assay (data not shown).

In this study, we investigated whether there is any physical interaction between the 5′-TR and 3′-UTR RNA by using the psoralen-UV cross-linking method. Psoralen intercalates between base pairs, and upon UV treatment it cross-links opposite strands especially at pyrimidine bases. The psoralen-UV method has been used extensively to study RNA-RNA and RNA-protein interactions (57, 59–64). To determine whether there is a physical interaction between the two ends of DEN2 RNA, the 5′-TR230nt RNA was radiolabeled with 32P in the in vitro transcription reaction and purified from the polyacrylamide 7 M urea gel. It was then mixed with unlabeled 3′-TR454nt RNA and treated with psoralen, followed by UV irradiation. The cross-linked products were analyzed by formaldehyde-agarose gel electrophoresis and autoradiography. The results shown in Fig. 2, panel A indicate that the formation of cross-linked products was observed only when the 5′-TR230nt and the 3′-UTR454nt RNAs containing the wild type or mutant CYC motifs, which were complementary to each other, were present in the psoralen/UV cross-linking reaction (lanes 3 and 10). The cross-linked products were significantly reduced under conditions in which one of the CYC motifs was mutated which would disrupt complementarity between the two CYC motifs (lanes 5 and 8). No cross-linked products were formed in control reactions omitting the psoralen (lanes 2, 4, and 9) or reactions containing only the 5′-TR230nt RNA but treated with psoralen/UV (lanes 1 and 6). In the reaction mixtures containing only the labeled 5′-TR RNAs, treatment with psoralen/UV resulted in loss of radioactivity probably due to degradation, and the loss was greater for 5′-TR230nt RNA than for 5′-TR130nt RNA (lanes 1 and 6, panel A versus B). These results taken together indicated that there was a physical interaction between the 5′-TR and 3′-UTRs in the viral subgenomic RNA and that the CYC motifs played an important role in facilitating this interaction. Moreover, this interaction still occurred even if CYC motifs were mutated as long as the complementarity between them was maintained.

Next, we investigated whether a shorter RNA from the 5′-TR containing the CYC motifs but without the 5′-UTR could still physically interact with the 3′-UTR454nt RNA. The results of the psoralen/UV cross-linking experiment shown in Fig. 2, panel B (lane 3) indicated that the 5′-TR130nt RNA containing the 5′-CYC motif alone was sufficient for interaction with the 3′-UTR454nt RNA. Again, substitution mutations in the 5′-CYC motif did not abolish interaction as long as the complementary mutations were introduced into the 3′-CYC motif (lane 10). In control experiments, it was found that omission of either 3′-UTR454nt RNA (lanes 1 and 6) or psoralen/UV treatment (lanes 2, 4, 7, and 9) did not give any cross-linked products. Substitution mutations of either of the CYC motifs in the two RNAs significantly reduced the ability to form cross-linked products although they did not abolish cross-linking completely (see lanes 5 and 8). In both experiments shown in Fig. 2, panels A and B, there were two major species of cross-linked products formed suggesting that psoralen cross-linking occurred at more than one site. The results presented thus far indicated that the...
interaction between the 5'- and 3'-TR was facilitated by the CYC motifs, and that the 130-nt region containing the CYC motif alone in the absence of the 5'-UTR was sufficient for physical interaction with the 3'-UTR. The 5'-UTR RNA alone, lacking the 5'-CYC motif, did not interact with the 3'-UTR RNA to form the psoralen cross-linked product (data not shown).

5'-UTR Is Crucial for RdRP to Initiate RNA Synthesis—Next, we sought to determine whether this 5'-UTR RNA can transactivate the 3'-UTR RNA in the in vitro RdRP assay. To address this question, in addition to the 5'-UTR RNA, we used three different RNA templates in the in vitro assays as follows: the 5'-UTR RNA containing 5'-UTR RNA and the CYC motif but shorter at the 3'-end, the 5'-UTR RNA lacking the 5'-UTR region, and the 5'-UTR lacking the CYC motif. The 3'-UTR RNA alone was inactive in the formation of the two products in the RdRP assay as reported earlier (Fig. 3, lane 1). However, a labeled RNA species of the same size as the input species was formed (lanes 2, 4, 6, and 8). The labeled RNA species was analyzed on a formaldehyde-agarose gel (1.8%), followed by autoradiography. Lane 1, 3'-UTR RNA alone. Lanes 2, 4, 6, and 8, 5'-UTR RNA alone. Lanes 3, 5, 7, and 9, 3'-UTR RNA in the presence of the 5'-UTR RNA. Lanes 3, 5, 7, and 9. The labeled 3'-UTR RNA band was not well resolved from the 2× product (lanes 3 and 5). In control RdRP assays containing only 5'-UTR RNA species as templates, the labeled and 2× products of the 5'-UTR RNA (lanes 2, 4, 6, and 8) were also formed, and the former was more predominant than the latter species. This observation was consistent with our previous study (52) which also reported that blocking the 3'-end of the 5'-UTR RNA abolished its template activity but not its ability to transactivate the 3'-UTR RNA template for 3'-end elongation. However, the presence of 3'-UTR and 5'-UTR RNAs together in the RdRP assay resulted in significant suppression of RNA synthesis at the 5'-UTR RNA templates for reasons unknown (lanes 2 and 4 versus 3 and 5). Our results shown in Fig. 3, taken together, indicated that although the 5'-UTR RNA containing the CYC motif alone was sufficient for physical interaction with the 3'-UTR RNA (Fig. 2, panel B), it was not sufficient for activation of RNA synthesis at the 3'-end of 3'-UTR RNA template, and both the 5'-UTR and the 5'-CYC motifs are required.

In the previous study, we demonstrated a functional interaction between the 5'-TR and the 3'-UTR for RNA synthesis at the 3'-end when both regions are in the same RNA template such as the subgenomic RNA770nt that includes the CYC motifs. To confirm further the role of 5'-UTR and CYC motifs, four mutant subgenomic RNA770nt templates were used in the RdRP assays as follows: subgenomic RNA670nt with a deletion of the 5'-UTR, two subgenomic RNA670nt containing the same substitution mutations as described before within either the 5'-CYC or the 3'-CYC motif which would disrupt base pairings, and the subgenomic RNA700nt in which both CYC motifs were mutated such that the complementarity was restored (Fig. 4).

The template efficiencies of these mutant subgenomic RNAs were analyzed using the in vitro RdRP assay. The results indicated that the subgenomic RNA74nt (without the 5'-UTR) had significantly reduced template activity for RNA synthesis (Fig. 4, panel A). These results support the conclusion reached from the transactivation assays that the 5'-UTR is necessary for the viral RdRP to initiate RNA synthesis at the 3'-end of the RNA template (Fig. 3).

Next, the contribution of the two CYC motifs for RNA synthesis at the 3'-end of subgenomic RNA templates was examined. The subgenomic RNA720nt, containing either of the CYC motifs mutated or both mutated, were used as templates in RdRP assays. The results indicate that the double mutant subgenomic RNA containing complementary mutant CYC motifs was an equally efficient template as the wild type RNA for RNA synthesis (Fig. 4, panel B). The subgenomic RNA with the mutated 5'-CYC motif showed a dramatic reduction in template efficiency compared with the wild type (lane 2). These results are consistent with the conclusion reached in the previous study based on the transactivation assays (52). However, the subgenomic RNA with the 3'-CYC mutation showed a reduced template activity (about 60% of the wild type) (lane 3 versus lane 1). But this result was contrary to the results obtained in the transactivation assays in that the 5'-TR with wild type CYC motif was essentially inactive in promoting RNA synthesis from the 3'-UTR RNA containing the mutant 3'-CYC motif (52). This experiment was repeated four times, and similar results were obtained. One
MK2) cells were electroporated with the genome length RNA shown in Fig. 5, into the full-length DEN2 infectious clone (diagrammatically secondary structure. These substitution mutations were cloned -SL, without disrupting the overall regions of the West Nile 3 9 3 9 3 9 part of the 3 9 -SL of the DEN2 genome with the corresponding 3 9 3 9 disruption or maintain secondary structure or by replacing all or 3 9 3 9 constructed several substitution mutations within the DEN2 -SL structure in DEN2 viral replication and infectivity. They 3 9 3 9 template efficiency of the subgenomic RNA 674nt (without the 5 9 -UTR). Lane 1, subgenomic RNA 770nt WT. Lane 2, subgenomic RNA770nt containing the deletion of 5 9 -UTR. Panel B, analysis of the template efficiency of the subgenomic RNA 770nt WT, 5 cyc MUT, 3 cycMUT, and 5 3 double cycMUT (lanes 1–4, respectively).

fig. 4. In vitro RdRP assay with various subgenomic mutant RNA templates. RNA templates were produced by in vitro transcription as described under “Experimental Procedures.” The integrity of RNAs were verified by formaldehyde, 1.8% agarose gel electrophoresis and visualized by staining with acridine orange. Panel A, analysis of the template efficiency of the subgenomic RNA 770nt (without the 5 9 -UTR). Lane 1, subgenomic RNA 770nt WT. Lane 2, subgenomic RNA 770nt containing the deletion of 5 9 -UTR. Panel B, analysis of the template efficiency of the subgenomic RNA 770nt WT, 5 cyc MUT, 3 cycMUT, and 5 3 double cycMUT (lanes 1–4, respectively).

possible explanation for this difference is that the interaction between the 5 9 -TR containing the wild type 5 9 -CYC motif with the 3 9 -UTR RNA containing the mutant 3 9 -CYC motif is intramolecular and may be more favorable as the two ends are in the same molecule compared with the scenario in which the interaction between the two terminal regions of RNAs is intermolecular as is the case with 5 9 -TP 230nt/wtCYC and the 3 9 -UTR 230nt/mutCYC used in the transactivation assays.

importance of 3 9 Stem-loop Structure in the 3 9 -UTR for RNA Synthesis in Vitro by RdRP—The 3 9-most 90–100 nucleotides of the flavivirus RNA genomes have been predicted to form a stable stem-loop (SL) structure (Fig. 5, panels A and B) (40, 45, 46, 48, 53, 65–68). Although the primary sequences of the region are less homologous, the 3 9- SL structures are highly conserved throughout the flavivirus family. This implies that the conserved 3 9- SL structure might have functional importance for viral replication.

Recently, Zeng et al. (45) reported the functional role of the 3 9- SL structure in DEN2 viral replication and infectivity. They constructed several substitution mutations within the DEN2 3 9- SL either by rearrangement of base pairings that would disrupt or maintain secondary structure or by replacing all or part of the 3 9- SL of the DEN2 genome with the corresponding regions of the West Nile 3 9- SL, without disrupting the overall secondary structure. These substitution mutations were cloned into the full-length DEN2 infectious clone (diagrammatically shown in Fig. 5, panel C). In that study, monkey kidney (LLC-MK2) cells were electroporated with the genome length RNA transcript from each mutant clone, and virus production and the spread of infection was followed over time by immunofluorescence assay (45). The ability of the viable mutant viruses to replicate in the mosquito (C6/36) cells and monkey kidney cells (LLC-MK2) was also examined. From the analysis of infectivity of these RNA transcripts, it was concluded that the stem structure of the top half and the nucleotide sequence of the 11 base-paired region in the uppermost portion of the bottom half of the 3 9- SL are important for efficient viral replication (45). Furthermore, a point mutation that would disrupt the predicted pseudoknot structure within the 3 9- SL (53) in the context of infectious clone was lethal for viral replication and infectivity in vivo C71G; see Fig. 5, panel A). Four of the first 6 nt of the WN small loop region also had the potential to form a pseudoknot structure with nucleotides 71–74 in the long stem (53), suggesting an interesting possibility that disruption of this tertiary structure interaction could account for the loss of replication potential of the chimeric mutants in this part of the viral genome.

3 L. Zeng, B. Falgout, and L. Markoff, personal communication.
To study the effects of these substitutions within the DEN2 3'-SL structures on their template efficiencies in the in vitro RdRP assay system, we subcloned these 3'-SL mutations into the plasmid coding for the subgenomic RNA. RNAs from these constructs were produced by in vitro transcription and were used as templates in the in vitro RdRP assays using DEN2-infected mosquito (C6/36) as well as monkey kidney (LLC-MK2) cell lysates. The rationale for using these two cell lysates was to examine whether there are any differences in host restriction for RNA synthesis in vitro as was found previously with one mutant (mutF) in the in vivo infectivity assays (45).

As shown in Fig. 6, the 3'-SL mutant subgenomic RNAs showed template efficiency at different levels. Subgenomic RNAs with the D2/WN-SL(mutC) and D2-SL(mutB), D2/WN-SL, D2/WN-SL(mutB), and D2/WN-SL(mutF) mutations exhibited template efficiency either close to or only slightly reduced compared with the wild type template in the in vitro RdRP assays that utilized either C6/36 or LLC-MK2 cell extracts for the source of viral replicase (Fig. 6, panels B and C, lanes 1, 3, 4, 7, 8, and 11 versus 12). Of these mutants, mutagenesis of the top half of the DEN2-3'-SL either completely or partially, as in D2/WN-SL(mutA), D2-SL(mutA), and D2-SL(mutB) (see Fig. 5, panel C), had only modest effects on virus growth compared with the wild type DEN2 genome (45). The in vitro template efficiencies also support this finding (Fig. 6, panels B and C, lanes 1, 3 and 4). In contrast, D2/WN-SL and D2/WN-SL(mutB) behaved like the wild type in the in vitro assays, whereas they were severely affected for growth in vivo (45). Moreover, the mutants D2/WN-SL(mutD) and D2/WN-SL(mutF) exhibited similar template activities with the C6/36 extract which were closer to that of the wild type template (Fig. 6, panel B, lanes 2 and 11 versus lane 12). On the other hand, with the LLC-MK2 cell extract, the (mutD) was less active than (mutF), but both mutants were less active than the wild type template in the in vitro assay (Fig. 6, panel C, lane 2 versus 11; compare lanes 2 and 11 with lane 12). The (mutF) exhibited a “host-range” phenotype in vivo in that it was severely restricted for replication in C6/36 cells but grew like the wild type in LLC-MK2 cells (45). In contrast, the results of the in vitro RdRP assays showed little difference in template efficiencies with the extracts from LLC-MK2- or C6/36-infected cells.

Template efficiency of the subgenomic RNAs containing D2/WN-SL(mutC) and -(mutE) mutations exhibited a reduced template efficiency for RNA synthesis using either the LLC-MK2- or the C6/36-infected cell extract (Fig. 6, panels B and C, lanes 9 and 10). Consistent with these results, these mutants exhibited either no DEN-positive antigens (not viable) or only 10% of the cells were positive for viral antigen (barely viable) as visualized by immunofluorescence of DEN2-transfected LLC-MK2 cells (45). In (mutC) and (mutE) mutants, the mutations were introduced at the bottom half of the 3'-stem (Fig. 5, panel C). In addition, the mutant subgenomic RNAs containing the D2-SL(c) and D2-SL(C71G) mutations lost their template activities dramatically when either of the two infected cell extracts was used in the in vitro assays (Fig. 6, panels B and C, lanes 5 and 6) which are consistent with their lethal phenotypes in vivo. In the D2-SL(c) mutant, the lethal phenotype was attributed to the disruption of the top half of the 3'-SL (45). The loss of template activity of the D2-SL(C71G) mutant is particularly interesting because this subgenomic RNA contains a mutation that would disrupt the predicted pseudoknot structure based on the comparison to the homologous structure in the West Nile viral RNA 3'-SL (53) (see Fig. 5, panels A and B). When the mutant genomic RNA was electroporated into LLC-MK2 cells, the mutation reverted to wild type, and only the latter grew out of the transfected cells suggesting that this pseudoknot mutant was lethal in vivo.4 In order to prove that the tertiary interaction involving a pseudoknot structure in the 3'-SL is important for template efficiency for RNA synthesis in vitro, we engineered the C73G mutation to disrupt the pseudoknot base pairing in the D2/WN-SL subgenomic RNA mutant in which the entire D2-SL was replaced with WN-SL. These subgenomic RNA templates containing the wild type or mutant pseudoknot base pairs within 3'-D2-SL or 3'-WN-SL were analyzed for RNA synthesis in vitro. The results from Fig. 7 indicate that the templates harboring a single base pair change that would disrupt the pseudoknot structure within either of the two 3'-SLs significantly affected the template efficiency for RNA synthesis in vitro. Based on these results, the strong template activity of D2/WN-SL(mutB) with either of the two extracts could be explained by the possibility that the pseudoknot structure of the West Nile 3'-SL is not affected in this mutant. These results presented in this study, taken together, support the conclusion that RNA synthesis in vitro at the 3'-end of the 3'-UTR is not only dependent on the conserved CYC motifs and the 5'-UTR but also, more importantly, on the determinants of the conserved 3'-SL structures of the subgenomic RNA template that are influenced by the conserved elements at the 5'-TR of the viral genome.

**FIG. 6. Comparison of template efficiencies of the subgenomic RNAs with specific 3'-SL mutations using the in vitro RdRP assays.** Each 3'-SL mutation was subcloned into the plasmid encoding the subgenomic RNA in vitro. In vitro transcribed 3'-SL mutant subgenomic RNA templates were analyzed on formaldehyde, 1.8% agarose gels, followed by staining with acridine orange prior to their use in the in vitro RdRP assays. Panel A, in the previous study, the viabilities of the 3'-SL mutations in the monkey kidney (LLC-MK2) cells in the context of full-length DEN2 RNA genome were determined by immunofluorescence assays and quantitated as the percent of DEN antigen-positive cells (45). These semiquantitative values are presented here together with the template efficiencies of the 3'-SL mutant subgenomic RNAs in the in vitro RdRP assay carried out using infected LLC-MK2 extracts (see panel C). Panel B, DEN2-infected C6/36 cell lysates were used for the in vitro RdRP assay. Panel C, DEN2-infected LLC-MK2 cell lysates were used for the in vitro RdRP assay. Panel D, RNA templates used stained by acridine orange.

*Discussion*

**Functional Interaction between the 5'-TR and 3'-UTR Required for RNA Synthesis in Vitro**—In this study, we have further established that the newly synthesized RNA from the subgenomic RNA template is of (−)-polarity. Furthermore, the

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4 L. Zeng, B. Falgout, and L. Markoff, unpublished results.
results of analyses of the structural requirements for RNA synthesis indicate that both 5'-UTR and 3'-SL structures contribute to template efficiency in addition to the conserved CYC motifs. The results of psoralen-UV cross-linking experiments further establish that there is physical interaction between the 5'- and 3'-TRs that require complementarity between the CYC motifs.

A number of studies have employed computer algorithms that are capable of analyzing predictive secondary structures as a guide or as a prelude for chemical and/or enzymatic probing to understand RNA-RNA interactions. Therefore, we sought to compare the predictive secondary structures of the subgenomic RNAs having wild type and mutant CYC motifs using the software described by Zuker et al. (58) (version 3.0). Among several structures that were produced from each RNA molecule, the most stable structures were selected for comparison and with its in vitro template efficiency (Fig. 8). As shown in Fig. 8, the subgenomic RNAs containing the CYCwt motifs and the complementary, double-mutant CYC (5'-CYCmut) motifs seem to maintain a similar predicted secondary structure (panels A and D). Interestingly, the subgenomic RNA/3'CYCmut also seems to maintain the same overall predicted secondary structures of the above two subgenomic RNAs (Fig. 8, panel C), although there is no predicted base pairing between the wild type 5'-CYC and the mutated 3'-CYC motifs. On the other hand, the predicted structures of the subgenomic RNA/5'CYCmut and the subgenomic RNA at (without 5'-UTR) are quite different from that of the wild type (panels E and B versus panel A). Thus, the predictive analyses of RNA secondary structure suggest that mutations in the conserved 3'-CYC motifs are tolerated, and the template efficiency in RNA synthesis is not affected because the overall RNA structure is maintained. Moreover, the overall conformation of RNA influenced by the interaction of the 5'- and 3'-terminal SL structures seems more important for RNA synthesis than the CYC motifs which seem to facilitate this interaction. Further work using chemical and/or enzymatic RNA structure probing methods and by site-directed mutagenesis is required to understand the RNA structure-template activities of these subgenomic RNA molecules using our in vitro RdRP assay.

The important role of the 3'-SL structure of the viral RNA genome in dengue viral replication in vivo was analyzed in an earlier study by Zeng et al. (45). The chimeric virus D2/YN-SL, which had the 96-nt sequence of the WN 3'-SL as a substitute for the 93-nt DEN2 3'-SL, showed a phenotype severely defective but not lethal in monkey kidney LLC-MK2 cells (defined as “sublethal” in Ref. 45). It was suggested that the possible mechanisms involving RNA/RNA or protein/RNA interaction might have been affected which could have slowed down the replication of the transfected genome compared with that of the wild type (45).

There was a good correlation between the template efficiency of these 3'-SL mutants in the in vitro assays and the in vivo viabilities in the context of full-length infectious clones (45) for lethal mutations, D2-SL(c), D2/YN-SL/mut(C), and D2-SL(C71G), as well as for the sublethal mutant, D2/YN-SL/mut(E). Moreover, this correlation could also be extended for the mutants D2-SL(a) and D2-SL(b) and the D2/YN-SL/mut(A) in which substitutions were made within the upper half of the 3'-SL (Fig. 5, panel C). The good accord between the in vivo and the in vitro results with regard to the latter three mutants support the previous conclusion that the secondary structure rather than DEN2-specific nucleotide sequence of the top half of the 3'-SL is a major determinant for viral replication.

Notable exceptions to this good correlation are the two mutants, D2/YN-SL/mut(B) and the D2/YN-SL (mut(E) and WN in Fig. 6, panel A), which exhibited significant template activity in vitro, but were lethal or sublethal, respectively, in vivo (45). It was previously reported that the 3'-SL structures of the DEN and WN viral genome contain a pseudoknot structure between the “G” in the loop of the small SL and the “C” in the bulge of the bottom half of the 3'-SL although the length of the base-paired regions involved in the 3'-SL structures of the DEN2 and WN RNA genomes are different (53) (illustrated in Fig. 5, panels A and B). Our study clearly indicates that the template efficiency of the pseudoknot mutant D2-SL(C71G) with a single nucleotide substitution was significantly reduced, suggesting that the pseudoknot structure within the 3'-SL could play an important role in RNA synthesis in vitro. This result also suggests that perhaps the near-wild type template activities of the mutants, D2/YN-SL (mut(B) and the D2/YN-SL, might be due to the possibility that the potential pseudoknot structure of the West Nile 3'-SL could be preserved in these mutants. This hypothesis was confirmed by analysis of the C73G mutation in the D2/YN-SL RNA, which would be expected to disrupt this pseudoknot structure, and the resultant mutant subgenomic RNA had significantly reduced template efficiency for RNA synthesis in vitro. The same explanation could be considered for the mutant subgenomic RNA with D2/YN-SL (mut(F); the regions containing the DEN2 sequences are presumably involved in the pseudoknot structure although the bottommost half of the 3'-SL is from the West Nile RNA. Thus, this (mut(F)) subgenomic RNA template had significant reduction of template efficiency in vitro. There is good correlation between the template efficiency in vitro and the in vivo viability of the mutant D2/YN-SL (mut(D)) which replicated well in both LLC-MK2 and C6/36 cells (45). However, the template efficiency of this (mut(D)) mutant RNA was greater in the extracts from the infected C6/36 cells compared with the LLC-MK2 cells. Although our in vitro results are consistent with the notion that tertiary pseudoknot interaction might play an important role for RNA synthesis, they do not explain why mutants, D2/YN-SL (mut(B), D2/YN-SL, and D2/YN-SL (mut(F)), that are active as templates in vitro are defective in vivo. One possible explanation for the disparity is that other steps in the viral life cycle that contribute to the overall viability such as defects in re-initiation of positive-strand RNA synthesis from the double-stranded RNA intermediate or in viral assembly might be affected in vivo.
All of the 3'-SL mutant RNAs were also able to interact physically with the 5'-TR RNA after psoralen/UV treatment (data not shown), suggesting that substitutions within the 3'-SL do not affect the ability of the 5'-TR RNA to interact with the 3'-UTR. These results indicated that although both 5'-TR and 3'-UTR RNAs could interact with each other, possibly mediated by the wild type CYC motifs that are upstream of the 3'-SL, the mutations at the 3'-SL domains of the 3'-UTR RNAs per se must have contributed to their differences in template efficiency for RNA synthesis in vitro. These results also emphasize that the overall conformation of the 3'-SL is more important for RNA synthesis than the CYC motifs alone.

Possible Role of Cross-talk between the 5' and 3' Terminal Regions of the Viral RNA Genome in RNA Synthesis—There is increasing evidence from studies of other positive single-strand RNA viruses that the 3'-end of the viral genome has to maintain a certain secondary and tertiary structure for RNA synthesis (69–74). The 3'-UTR of brome mosaic virus (BMV) RNA-3 is predicted to have five pseudoknots at the upstream end of the 3'-end tRNA-like structure (70). The deletion mutants of RNA-3 within these regions replicated poorly, yielding no detectable RNA-3 or RNA-4 progeny. This result suggested that the pseudoknot regions of the RNA-3 at the 3'-end contribute significantly to the overall replication of the BMV genome. Deiman et al. (72) also reported that the disruption of the stable pseudoknot structure at the 3'-end of the Turnip yellow mosaic virus genome gave rise to a drop in transcription efficiency to about 50%, indicating that the stable pseudoknot structure is crucial for initiation of negative-strand RNA synthesis.

Our results suggest that the cross-talk between the 5'-TR and 3'-UTR of the viral genome might define a precise structure at the 3'-end involving tertiary pseudoknot interaction such that the initiation of negative-strand RNA synthesis could be carried out by the viral replicase complex. In fact, possible cross-talk between specific regions of the viral genome of RNA viruses has been suggested to participate in the viral replication of Qβ virus (75), BMV (76), rhinovirus type 14 (77), poliovirus (78), hepatitis C virus (79, 80), and tobacco etch virus (81).

Proteins interacting with the flavivirus terminal regions of the RNA genome remain to be studied. The results of this study indicate that using our in vitro RdRP assay and the wild type and mutant subgenomic RNA templates, it is possible to study the higher order RNA structural interactions and identify the viral and cellular factors that contribute to the stability and functional long range interactions between the ends of the viral genome.

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In Vitro RNA Synthesis from Exogenous Dengue Viral RNA Templates Requires Long Range Interactions between 5'- and 3'-Terminal Regions That Influence RNA Structure

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