THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 47, Issue of November 19, pp. 33714–33722, 1999

Printed in U.S.A.

A Novel *in Vitro* Replication System for Dengue Virus

INITIATION OF RNA SYNTHESIS AT THE 3′-END OF EXOGENOUS VIRAL RNA TEMPLATES REQUIRES 5′- AND 3′-TERMINAL COMPLEMENTARY SEQUENCE MOTIFS OF THE VIRAL RNA*

(Received for publication, August 5, 1999, and in revised form, September 5, 1999)

Shihyun You and R. Padmanabhan‡

From the Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160-7421

Positive strand viral replicases are membrane-bound complexes of viral and host proteins. The mechanism of viral replication and the role of host proteins are not well understood. To understand this mechanism, a viral replicase assay that utilizes extracts from dengue virus-infected mosquito (C6/36) cells and exogenous viral RNA templates is reported in this study. The 5′- and 3′-terminal regions (TR) of the template RNAs contain the conserved elements including the complementary (cyclization) motifs and stem-loop structures. RNA synthesis *in vitro* requires both 5′- and 3′-TR present in the same template molecule or when the 5′-TR RNA was added in *trans* to the 3′-untranslated region (UTR) RNA. However, the 3′-UTR RNA alone is not active. RNA synthesis occurs by elongation of the 3′-end of the template RNA to yield predominantly a double-stranded hairpin-like RNA product, twice the size of the template RNA. These results suggest that an interaction between 5′- and 3′-TR of the viral RNA that modulates the 3′-UTR RNA structure is required for RNA synthesis by the viral replicase. The complementary cyclization motifs of the viral genome also seem to play an important role in this interaction.

The mosquito-borne dengue viruses, members of positive strand RNA viruses of Flavivirus family, are human pathogens that cause dengue fever, dengue hemorrhagic fever/dengue shock syndrome (for a review see Refs. 1 and 2). It is currently estimated that about 40% of the world population is at risk and about 5% of one million dengue hemorrhagic fever cases per year are fatal (3). Dengue virus type 2 (DEN2), the most prevalent of the four serotypes, contains a single-stranded RNA genome of positive polarity that encodes a single polyprotein precursor (3391 amino acid residues for DEN2, New Guinea C-strain) (4) arranged in the order, C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. The precursor polyprotein is processed into three structural proteins of the virion, C, prM, and E, by the host signal peptidase associated with the endoplasmic reticulum (5–7) and at least seven nonstructural proteins, NS1 to NS5, which are expressed in the infected cells (for a review see Ref. 8).

The processing of the nonstructural region of the polyprotein precursor is catalyzed by the two-component, viral serine protease, NS2B/NS3, first identified based on conserved catalytic triad of trypsine-like serine proteases (9, 10). The serine protease domain of NS3, present within the N-terminal 180 amino acid residues, requires NS2B for activity (11–18). The crystal structure of the NS3 protease domain has recently been reported (19). The C-terminal region of the NS3 protease domain contains conserved motifs found in the NTP-binding proteins and DEXH family of RNA helicases (20–22). The nucleoside triphosphatase and RNA helicase activities of DEN2 NS3 have recently been demonstrated by expression and purification of recombinant NS3 in *Escherichia coli* (23). The RNA helicase activity is thought to be involved in unwinding of a double-stranded RNA replicative intermediate formed during replication of the flavivirus genome (8, 24). NS5, the largest of the flaviviral proteins, contains conserved motifs found in many viral RNA-dependent RNA polymerases (RdRP)[1] (25, 26), implicating a role of NS5 in viral replication.

Template-dependent and template-specific *in vitro* replication systems have been developed to study mechanisms of some plant and a few eukaryotic positive strand RNA viruses. These studies have revealed that viral replicases function as membrane-bound complexes of both viral and cellular proteins and viral RNA (Refs. 27–35 and for a review see Ref. 36). However, the mechanism of initiation of viral RNA synthesis as well as RNA genome replication in general and the role of host proteins in this process remain unclear for positive strand RNA viruses.

In flavivirus-infected cells, three RNA species have been detected: a genomic RNA of 40–44 S; a double-stranded, RNase-resistant, replicative form of 20–22 S; and a partially RNase-sensitive, replicative intermediate of 20–28 S RNA (37–40). The *in vitro* RdRP assays that have been developed to study flavivirus replication utilize membrane-bound complexes isolated from the infected cell lysates (37–40). These studies have examined incorporation of radiolabeled nucleotides into the three RNA species on endogenous viral RNA templates. In these replication complexes, the endogenous replicative intermediates present in the infected cell extracts were shown to form a double-stranded replicative form. The replicative form produced endogenously from replicative intermediate or added exogenously gave rise to a 44 S genome-size RNA species, suggesting that initiation of (+)-strand RNA synthesis occurred in these *in vitro* assays (39, 40).

To study the mechanism of viral replication in molecular detail, it is crucial to develop an *in vitro* RdRP assay that can utilize exogenous RNA templates containing essential regulatory elements of the viral genome involved in viral replication. For initiation of (−)- and (+)-strand RNA synthesis, conserved RNA sequences with intrinsic stem-loop structures from the 3′

* This work was supported by National Institutes of Health Grant AI-32078. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160-7421. Tel.: 913-588-7018; Fax: 913-588-7440; E-mail: rpadmana@kumc.edu.

[1] The abbreviations used are: RdRP, RNA-dependent RNA polymerase(s); UTR, untranslated region; nt, nucleotide(s); TR, terminal region(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CS, conserved sequence; SL, stem-loop; wt, wild type.

and 5′ regions of many viral genomes are thought to play an important role in RNA replication (41–55). In flavivirus genomes, such conserved elements have been noted previously; their functions in replication remain to be established. For example, the 3′-terminal 96 nucleotides of the mosquito-borne flaviviruses within the 3′-UTR form a conserved and stable stem-loop structure, although the primary sequence is not conserved (for a review see Ref. 8). Formation of such a stable secondary structure within the 3′-terminal 373 nt of the 3′-UTR was shown to exist in solution (56). A potential secondary structure with a lesser predicted stability near the 5′ termini of several flavivirus RNAs including DEN2 was reported (57). There are also two short conserved sequences (CS1 and CS2) in 3′-UTR shared by all mosquito-borne flaviviruses; CS1, 26 nucleotides in length, is located 5′ to the stem-loop structure of the 3′-UTR (3′-CS1). A portion of the 3′-CS1 is complementary to a conserved element is located within the N-terminal coding region of the capsid protein, C, in the 5′-TR of the viral genome (5′-CS). It has been proposed that these complementary sequences might play a role in cyclization of the viral genome (Ref. 58 and for a review see Ref. 8). However, cyclization of any of the flavivirus genome has not been observed to date. Thus the role of cyclization motifs in genome replication is unknown at present.

In this study, we report the development of the first *in vitro* RdRP assay that utilizes cell-free extracts of DEN2-infected mosquito (C6/36) cells and exogenous subgenomic RNA templates containing 5′- and/or 3′-terminal regions of the viral genome. The results indicate that there is an interaction between 5′- and 3′-terminal regions of the viral RNA that is required for RNA synthesis *in vitro*. This interaction is modulated by the complementary cyclization motifs. RNA synthesis occurs by the 3′-end elongation of the template RNA to yield a predominantly double-stranded RNA hairpin with a limited single-stranded loop region. The kinetics of the formation of the RNA hairpin product indicates that the template RNA is first modified yielding a RNase A-sensitive intermediate that is then converted to the hairpin product twice the size of the template RNA. This *in vitro* RdRP assay will be useful to study the sequence and protein requirements for RNA synthesis *in vitro*.

EXPERIMENTAL PROCEDURES

Preparation of DEN2 Viral Replicase Complex from the Infected Mosquito (C6/36) Cells

Dengue virus type 2 (New Guinea Strain C) was propagated as described (59, 60). To obtain the DEN2 viral replication complex, C6/36 cells were infected with DEN2 virus (multiplicity of infection = 5) in T-150 cm^2 flasks for 36 h at 28 °C. Uninfected C6/36 cells were used as controls. Cells were harvested by centrifugation at 800 × *g* for 10 min, and the cell pellet was resuspended in 0.5 ml of TNMg buffer (10 mM Tris-HCl, pH 8.0, 10 mM sodium acetate, 1.5 mM $MgCl_2$) per T-150 cm^2 flask. After passing the cell suspension 20 times through a syringe (one ml capacity), fitted with a 27-guage needle, the cell lysates were centrifuged at 800 × *g* for 10 min at 4 °C to fractionate the cytoplasmic and nuclear fractions as described (39). The protein concentrations of the cytoplasmic extracts were determined as described (61), and the extracts were stored in aliquots at −70 °C until used.

Western Blot Analysis

An aliquot of the cytoplasmic extract was mixed with an equal volume of a 2× sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.7 M β-mercaptoethanol, 10% glycerol, 0.05% bromphenol blue). Proteins were separated by SDS-PAGE (10%) and transferred electrophoretically to a polyvinylidene difluoride membrane. The membrane was treated with nonfat dry milk (5%) for 2 h in Tris-buffered saline containing 0.1% Tween-20, pH 7.5, and then incubated overnight with polyclonal rabbit anti-NS3 or anti-NS5 antibodies (1:2000). The membranes were washed three times for 20 min each with this buffer and incubated with horseradish peroxidase-labeled goat anti-rabbit antibody (1:20000) for 2 h at room temperature. Chemiluminescence detection of the immunoreactive bands were performed as described by the manufacturer (ECL system; Amersham Pharmacia Biotech).

Plasmid Constructs

3′-UTR$_{373nt}$/wtCYC Plasmid—The p190-24 plasmid contains the 3′-terminal 373 nt from the DEN2 cDNA and 26 nt from the pGEM7Zf$^+$ vector (TCAAGCTATGCATCCAACGCGTTGGG) under the control of SP6 promoter (56). When this plasmid is linearized with *Xba*I, the cDNA of (−)-strand polarity was extended by 4 additional nucleotides of the *Xba*I-5′ overhang. The RNA synthesized from this template strand would include the 4 additional nucleotides complementary to this overhang at its 3′-end. To generate 3′-UTR$_{373nt}$ RNA, which contains the wild type cyclization sequence but without these 4 additional nucleotides, two approaches were followed. Firstly, a PCR approach was followed using the two oligonucleotide primers: the upstream 5′-primer (A), 5′-TGACCATGATTACGCCAAGCTATTTAGGTG-3′ anneals a region upstream of the SP6 promoter and a part of the promoter (underlined); the downstream 3′ primer (B), 5′-AGAACCTGTTGATTCAACAGCACC-3′ is complementary to the 3′-end of the viral genome. The PCR fragment was purified and used for *in vitro* transcription (see below). In the second approach, the *Xba*I-linearized p190-24 plasmid (10 μg in each reaction) was treated with increasing amounts of S1 nuclease (0, 3, 10, 30, 75, and 150 units) in 100 μl of reaction mixtures containing 50 mM sodium acetate, pH 4.5, 28 mM NaCl, and 4.5 mM $ZnSO_4$ at room temperature for 30′, extracted with phenol, and precipitated with ethanol. The S1 nuclease-treated plasmids were used for the *in vitro* transcription catalyzed by SP6 RNA polymerase. To ensure that the S1 nuclease precisely removed the *Xba*I overhang, the blunt ends were ligated by T4 DNA ligase and cloned. DNA sequencing was carried out using an automated DNA sequencer (Biotech Facility, University of Kansas Medical Center).

cDNA Constructs Encoding Subgenomic RNA Containing the 5′- and 3′-Terminal Regions of DEN2 Genome—A 230-nt DNA fragment from the 5′-terminal region that includes the 5′-UTR (96 nt), and the 5′-CS1 was generated by PCR using pMK4 as a template and the upstream primer (C), 5′-CGGAATTCGGATCGATCCCCCCTAATAC-3′ (containing the T7 promoter), and the downstream primer (D) 5′-CAGTTCCTGAGGTCCTCGTCCCTGCAG-3′ (complementary to 217–233 nt of the viral genome, which includes the 5′-CS1 element). The primers, C and D, contain the *Eco*RI and *Bsu*36I sites (underlined), respectively. The PCR product was digested with *Eco*RI and *Bsu*36I and purified using a Qiagen cartridge. The 3′-terminal fragment (800 nt) that includes the 3′-UTR was derived from the plasmid clone pGEM-PCR1.3 (4) by digestion with *Bsu*36I (nt position 9885) and *Xba*I (nt position 10723). The pSP64 vector plasmid was digested at the multiple cloning site with *Eco*RI and *Xba*I. A three-fragment ligation and cloning yielded pSY-1 plasmid containing DEN2 cDNA sequences under the control of T7 promoter. To construct a plasmid encoding a shorter subgenomic RNA, the pSY-1 plasmid was partially digested with *Xmn*I, followed by digestion with *Bsu*36I. The overhang from the *Bsu*36I-cut plasmid was blunt-ended by treatment with *E. coli* Klenow DNA polymerase fragment and was cloned to yield the pSY-2 plasmid (720 nt) in which 348 nt from the pSY-1 was deleted but retaining all of 3′-UTR region. The pSY-1 and pSY-2 plasmids were used to produce the subgenomic RNAs of 1.0 kilobase and 720 nt, respectively, by *in vitro* transcription (Fig. 1).

5′-TR/mutCYC and 3′-UTR$_{373nt}$/mutCYC Plasmids—To construct a PCR fragment of 5′-TR/mutCYC (which contains substitution mutations within the cyclization sequence), a PCR-based mutagenesis protocol was followed. Two PCR products were generated with overlapping sequences using two sets of primers. PCR1 was obtained using the 5′-primer (E), containing the *Eco*RI site, 5′-AGCTATGACCATGATTACGAATTC-3′ that corresponds to the upstream region of the T7 promoter in the pSP64 vector and the 3′-primer (F), 5′-TTTCACAGAGAGAGAAGGCGTATTTCTCGCCTTT-3′. PCR2 was produced using the 5′ primer (G), 5′-GCCTTCTCTCTCTGTGAAACGCGAGAGAAACCG-3′, and the 3′ primer (H), 5′-TGAGGTCCTCGTCCTG-3′. The underlined complementary sequences in F and G primers represent the mutated cyclization element (mutCYC). The primer H shares identical sequences with pSY-1 plasmid in the vicinity of *Bsu*36I site (underlined). The two products, PCR1 and PCR2, produced using the primer sets E/F and G/H were purified, mixed, and used for a third PCR in the presence of the primer set E/H. This final PCR product was purified and used for *in vitro* transcription to generate 5′-TR/mutCYC RNA.

To construct a PCR fragment containing the 3′-UTR$_{373nt}$/mutCYC that contains mutations within the cyclization sequence complementary to that in 5′-TR/mutCYC), a PCR-based mutagenesis protocol was

followed. PCR1 was obtained using the 5′-primer (A), the 3′-primer (I), 5′-CAGCGCTCTCTCTGTGTTTTTTTGTTTTGGGGGGG-3′. PCR2 was produced using 5′ primer (J), 5′-AAACACAGAGAGAGCGCTGGGAAAGACCAGAGAT-3′ and the 3′-primer (B). The underlined complementary sequences in I and J primers represent mutated cyclization element (mutCYC). p190-24 plasmid was used as the template for PCR. The two products, PCR1 and PCR2, produced using the primer sets A/I and J/B were purified, mixed, and used for a third PCR in the presence of the primer set A/B. This final PCR product was purified and used for *in vitro* transcription to generate the 3′-UTR/mutCYC RNA.

Preparation of RNA Templates

The RNA templates used in the *in vitro* RdRP assays are shown in Fig. 1. To synthesize RNA templates containing 3′-terminal 373 nt (3′-UTR_{373nt}) or the subgenomic RNA templates, the plasmid constructs, p190.24 (56) and pSY-2, respectively, were linearized with *Xba*I. The supercoiled plasmids were used as templates for PCR. The PCR products or the *Xba*I-linearized plasmids were used in the *in vitro* transcription reaction catalyzed by either SP6 (for the 3′-UTR_{373nt} RNA) or T7 RNA polymerase (for subgenomic RNAs) using conditions supplied by the manufacturer (Promega). Briefly, the *in vitro* transcription reactions were carried out at 37 °C for 3 h in reaction mixtures (100 μl) containing the 1× buffer (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 5 μg of the DNA templates, a mixture of four rNTPs (0.5 mM each), 100 units of RNasin, 10 mM dithiothreitol, and 5 units of T7 or SP6 RNA polymerase. Then the reaction mixtures were digested with 5 units of RNase-free DNase I (Promega) at 37 °C for 20 min to remove the DNA templates. The RNA transcripts were extracted with phenol at pH 4.5 and precipitated with ethanol. The quality of RNA products was analyzed electrophoretically and quantified spectrophotometically. Because of the 26 nt from the vector sequence at the 5′-end, the 3′-UTR_{373nt} RNA is actually 399 nt in length but is referred to in this study as 3′-UTR_{373nt}. Nonviral exogenous RNAs were prepared by linearizing three different vector plasmids (pSP64, pSP70, and pTM1) with six different restriction enzymes. RNAs were prepared by *in vitro* transcription and purified as described above.

In Vitro RdRP Assay

The *in vitro* RdRP assay was performed in 50-μl reaction mixtures containing 50 mM Hepes, pH 7.3, 3 mM magnesium acetate, 6 μM zinc acetate, 25 mM potassium acetate, 60 units of RNase inhibitor, 10 mM β-mercaptoethanol, 0.5 mM each ATP, GTP, and UTP, and 10 μM of CTP mixed with 10 μCi of [α-^{32}P]CTP (800 Ci/mmol), 0.1 mg/ml actinomycin D, 10 μg of cytoplasmic extract from infected cells, and 5 μg of an exogenous RNA template. As a control, a parallel reaction mixture containing all the components except the cytoplasmic extract from the uninfected C6/36 cell lysate (10 μg) was included. Controls in which cytoplasmic extracts from infected or uninfected cells but without any exogenous RNA were also included. Reaction mixtures were incubated at 30 °C for 1.5 h. To carry out the kinetics of RdRP reaction, seven separate RdRP reactions that included DEN2-infected cytoplasmic extracts and the subgenomic RNA template were initiated. At time intervals of 0, 5, 10, 15, 20, 30, and 40 min, reactions were terminated by the addition of EDTA (10 mM). The samples were extracted with phenol, pH 4.5, and precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol in the presence of 5 μg of yeast tRNA (Ambion). The RNA products were analyzed using either polyacrylamide, 7 M urea or by formaldehyde-agarose gel electrophoresis, followed by autoradiography.

Analysis of RdRP Products by RNase A Digestion

The RdRP products separated on PAGE, 7 M urea gels were cut out and eluted in 400 μl of elution buffer (2 M ammonium acetate, 1% SDS, 1 mM EDTA, and 25 μg/ml tRNA) either at 37 °C for overnight or at 65 °C for 4 h. After briefly spinning down gel slices, the supernatants were precipitated overnight with 1 ml of 100% ethanol at −20 °C. The eluted products were treated with or without RNase A (Sigma; 5 ng/μl) in 20 μl of either 2× SSC (300 mM NaCl and 30 mM sodium citrate, pH 7.2) or 0.1× SSC (15 mM NaCl and 1.5 mM sodium citrate, pH 7.2) at 37 °C for 30 min. The reactions were stopped by adding 30 μl of TES stop buffer (10 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 0.2% SDS), followed by phenol extraction and ethanol precipitation in the presence of 5 μg of yeast tRNA. The RNase A-treated samples were analyzed on formaldehyde agarose gel, followed by autoradiography.

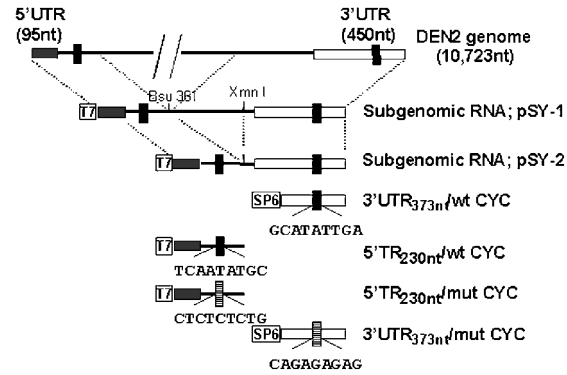

FIG. 1. **Plasmid constructs used for the synthesis of RNA templates.** Details regarding the construction of these plasmid constructs are given under "Experimental Procedures." The 5′- and 3′-UTR are depicted as *filled*, and *open horizontal rectangular boxes*, respectively. T7 or SP6 promoters are shown as indicated. Wild type and mutant complementary cyclization sequences are shown as *closed* and *shaded vertical boxes*, respectively.

Sodium Periodate Oxidation of RNA

In vitro transcribed RNAs were dissolved in 200 μl of 50 mM NaOAc, pH 5.0. 50 μl of 0.1 M $NaIO_4$ (20 mM) was added and then incubated for 1 h at room temperature. Lysine (60 mM) was added to saturate the excess periodate and further incubated for 3 h at room temperature. The reactions were phenol extracted and precipitated with ethanol. The pellets were washed with 70% EtOH and dissolved in water and subsequently desalted with Bio-Gel P gel column (Bio-Rad). The periodate-treated RNAs were quantified spectrophotometrically and visualized on PAGE, 7 M urea gel by acridine orange.

RESULTS

Cytoplasmic Extracts from DEN2-infected Mosquito (C6/36) Cells Are Active in the Synthesis of RNA from the Exogenous Templates—Previous studies showed that flavivirus RdRP activity was tightly associated with intracellular membranes in the cytoplasmic fractions of flavivirus-infected mammalian (monkey kidney cell line (Vero), or baby hamster kidney cell line (BHK-21)) cells (37–40, 62, 63). The earlier studies examined the incorporation of labeled nucleotides into the three RNA species that were synthesized from endogenous viral RNA templates. We sought to develop an *in vitro* RdRP assay that could utilize exogenous RNA templates that contain either 3′- or 5′-untranslated regions or both to determine the sequence requirements for RNA synthesis and to characterize the proteins that interact with these elements. We chose to use an established *Aedes albopictus* (C6/36) cell line for preparation of cytoplasmic extracts from DEN2-infected cells (see "Experimental Procedures") because the viral titers are significantly higher in C6/36 cells than in vertebrate cells (64).

We constructed cDNA clones containing the putative regulatory regions of the viral genome at the 5′-TR and 3′-UTR under the control of either the T7 or SP6 promoter as described under "Experimental Procedures." pSY-2 contains the subgenomic cDNA (720 nt) corresponding to both 5′-TR_{230nt} and 3′-UTR (full-length) of the genome linked together in the same molecule. The 5′-TR_{230nt}/wtCYC plasmid contains the cDNA corresponding to the 5′-terminal region (230 nt) of the genome; both cDNAs were downstream of the T7 promoter. The 3′-UTR_{373nt}/wt CYC plasmid contains the SP6 promoter along with 26 nt from the vector followed by the 3′-UTR_{373nt}. Plasmids containing mutations in the 5′-CYC or 3′-CYC motif within either the 5′-TR_{230nt} or the 3′-UTR_{373nt} were constructed as described under "Experimental Procedures" (Fig. 1).

First, we analyzed whether our cytoplasmic extracts contained the putative viral replicase components, NS3 and NS5, by Western blot analysis using rabbit polyclonal anti-NS3 and -NS5. Both NS3 and NS5 proteins were readily detected in the

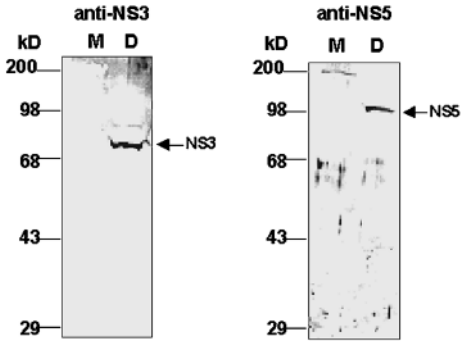

FIG. 2. **Detection of NS3 and NS5 in cytoplasmic extracts from infected C6/36 cells.** C6/36 cells were infected with dengue virus type 2 (DEN2) and at 36 h post-infection, cytoplasmic extracts were prepared as described under "Experimental Procedures." The extracts were fractionated by SDS-PAGE and analyzed by Western blot using polyclonal antibodies against NS3 and NS5 and a chemiluminescence detection system. *M*, control uninfected cell lysate; *D*, DEN2-infected cell lysate.

cytoplasmic extracts prepared from DEN2-infected cells but not from the uninfected cells (Fig. 2).

For the *in vitro* RdRP assay, exogenous RNAs were synthesized as run-off transcripts in the *in vitro* transcription reactions on either *Xba*I-linearized plasmids or the PCR DNAs as described under "Experimental Procedures." The 3′-UTR_{373nt} RNA synthesized from the *Xba*I-linearized plasmid contains 4 additional nucleotides complementary to the 5′ overhang (5′-CTAG-3′) extending the authentic 3′-end of viral RNA (referred as 3′-$UTR_{373+4nt}$ RNA-*Xba*I). However, the RNA synthesized by the SP6 RNA polymerase on the PCR template would lack this 3′ extension of 4 nt (3′-UTR_{373nt} RNA-PCR) and maintain the authentic 3′-end of viral genome. Both 3′-UTR RNAs include the highly conserved cyclization motif located from nucleotide positions 90 to 100 from the 3′-end (3′-wtCYC). The subgenomic RNA containing both 5′-TR and full-length 3′-UTR was synthesized as a run off transcript by T7 RNA polymerase either from the *Xba*I-linearized pSY-2plasmid (720 + 4 nt) or the PCR product (720 nt). The subgenomic RNA includes the wild type cyclization motifs, which are complementary to each other (Fig. 1).

The RdRP reactions were carried out using the 3′-$UTR_{373+4nt}$ RNA or the 3′-UTR_{373nt} RNA as the exogenous RNA template and cytoplasmic lysates from either the DEN2-infected or uninfected cells (Fig. 3*A*, *lanes 2* and *3*). Control RdRP assays lacking the exogenous RNA template were also included (Fig. 3*A*, *lanes 4* and *5*). The labeled RdRP products were analyzed on partially denaturing 4% polyacrylamide gels containing 7 M urea (PAGE, 7 M urea). The results of the *in vitro* RdRP assay shown in Fig. 3 indicate that both uninfected and DEN2-infected cell lysates gave rise to a labeled product of the same size as the input 3′-$UTR_{373+4nt}$ RNA (Fig. 3*A*, *lanes 2* and *3*, respectively, and also Fig. 3*B*, *lane 1*). However, DEN2-infected cell lysates produced a distinctly labeled product that migrated faster than the size expected for the input 3′-$UTR_{373+4nt}$ RNA (Fig. 3, *A*, *lane 2*, and *B*, *lane 1*). In contrast, the RdRP assays carried out in the absence of an exogenous RNA template but in the presence of cytoplasmic extracts from infected or uninfected cells did not produce either of the products, indicating that these two products were formed only when the exogenous RNA template was added (Fig. 3*A*, compare *lanes 4* and *5* with *lanes 2* and *3*). Analysis of six nonviral RNA templates generated from vector plasmids as controls indicated that only two produced faster migrating RNA species like the 3′-$UTR_{373+4nt}$ RNA (data not shown). The labeled products with mobilities slower than the input exogenous RNA were also formed with cytoplasmic extracts from DEN2-infected cells

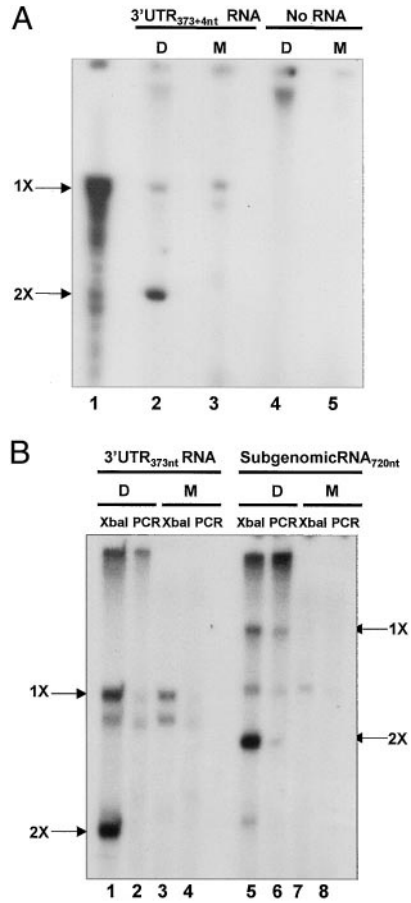

FIG. 3. **Analysis of RNA products formed *in vitro*.** Exogenous RNA templates (5 μg) were synthesized from either *Xba*I-linearized plasmids or PCR-generated DNA. The RdRP assays were carried out with either DEN-2 infected (*D*) or uninfected (*M*) cell lysates for 1.5 h at 30 °C as described under "Experimental Procedures." The products were fractionated by PAGE (4%) containing 7 M urea and analyzed by autoradiography. *1*× denotes the labeled RNA product which is of the same size as the input template RNA. *2*× denotes the RNA product with a faster mobility in this gel system. Later experiments showed that this RNA has a double-stranded hairpin-like structure with a size twice the size of input RNA. *A*, exogenous 3′-UTR_{373nt} RNA produced from the *Xba*I-linearized plasmid which would contain 4 additional nucleotides complementary to the *Xba*I overhang was used. *Lane 1*, labeled $3UTR_{373+4nt}$ RNA as a marker; *lanes 2* and *3*, exogenous 3′-$UTR_{373+4nt}$ RNA added; *lanes 4* and *5*, no exogenous RNA added. *B*, the exogenous 3′-UTR_{373nt} RNA (*lanes 1–4*) or the subgenomic RNA (*lanes 5–8*) was synthesized from either *Xba*I-linearized plasmids (marked as *XbaI* in *lanes 1*, *3*, *5*, and *7*) or from the PCR-generated DNAs (marked as *PCR* in *lanes 2*, *4*, *6*, and *8*).

(Fig. 3*A*, *lanes 2* and *4*). These are likely to be the products formed by the viral replicase utilizing the endogenous viral RNA templates as reported previously (37). However, these products did not fractionate into distinct species observed previously (37), probably because the gel system we used is selected to fractionate products formed from the small exogenous RNA templates.

However, the 3′-UTR_{373nt} RNA template generated from the PCR product did not produce the faster migrating species in the RdRP assay with DEN2-infected extracts (Fig. 3*B*, *lane 2*). The faster migrating species were also not formed when uninfected cell lysates were used with either of the 3′-UTR RNA templates (Fig. 3*A*, *lane 3*, and *B*, *lanes 3* and *4*). In contrast, the subgenomic RNA template, which contained the 4 additional nucleotides complementary to the *Xba*I-5′ overhang or generated from the PCR DNA lacking these 4 nt at its 3′ end, formed the faster migrating species (marked as *2*×) as well as the unit-sized RNA product (marked as *1*×). The faster migrating RNA

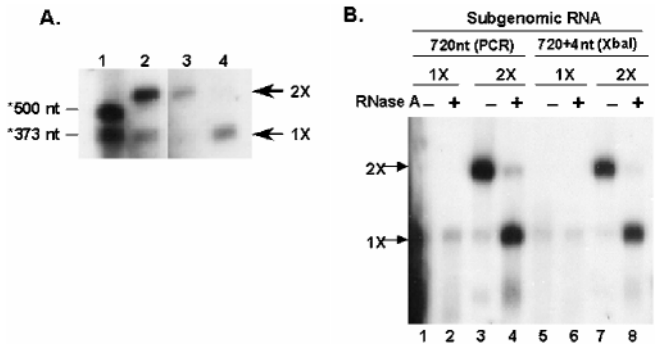

FIG. 4. **Analysis of RNA products formed *in vitro* by RNase A digestion.** The faster migrating (2×) and the input template-sized RNA (1×) products shown in Fig. 3 were eluted from the PAGE/urea gel and digested with RNase A under high salt conditions. The digested products and the controls were analyzed by formaldehyde-agarose gel as described under "Experimental Procedures." *A*, *lane 1*, RNA markers; *lane 2*, the products formed in the *in vitro* assay; *lanes 3* and *4*, eluted 2× product before and after RNase A digestion, respectively. To detect the low amounts of radioactivity in *lanes 3* and *4*, the same autoradiogram was developed by longer exposure. A composite picture is shown. *B*, an *in vitro* assay was performed with subgenomic RNA (720 nt) synthesized from either *Xba*I-linearized or PCR-generated DNA, and the RNA products were analyzed by RNase A digestion under high salt conditions. *1*× and *2*× are as defined in Fig. 3.

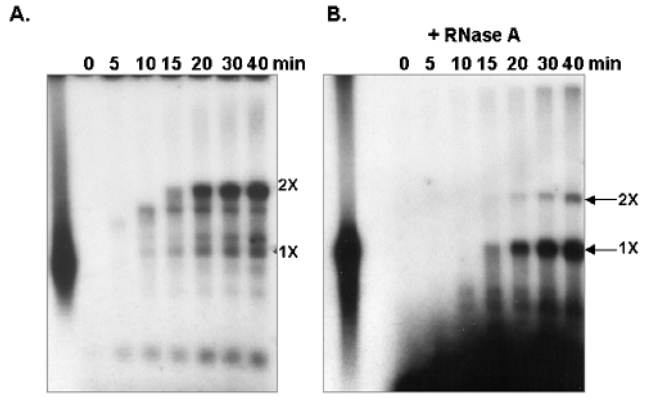

FIG. 5. **Kinetics of RdRP activity with the exogenous subgenomic RNA template.** *A*, seven RdRP reactions were initiated using DEN2-infected cell lysates and exogenous subgenomic RNA template as described under "Experimental Procedures." Each of the reactions was terminated by the addition of EDTA (10 mM). RNA products were purified by phenol extraction and ethanol precipitation. One-half of each sample was digested with RNase A. The untreated (*A*) and RNase A-treated (*B*) samples were analyzed by electrophoresis on two separate formaldehyde-agarose gels, followed by autoradiography. The *first lane* indicates the labeled marker subgenomic RNA (720 nt).

product was observed in the RdRP reaction that contained only the DEN2-infected cell lysates (Fig. 3*B*, *lanes 5* and *6*). These experiments have been repeated six times with consistent results.

Because both DEN2-infected and uninfected cell lysates produced RNA products of unit size (1×) with the exogenous RNA templates, it is possible that this RNA was the product of 3′-terminal labeling by the host-encoded terminal nucleotidyl transferase in both cases. A second possibility is that *de novo* initiation by the DEN2-infected lysate at the 3′-end of template strand produced the (−)-strand RNA, which could migrate as 1× in the partially denaturing gel electrophoresis system. On the other hand, the faster migrating RNA could be either a prematurely terminated (−)-strand product or an RNA that is highly compact in structure, such as a double-stranded RNA hairpin.

To characterize the labeled RNA products of the RdRP assays further, the faster migrating product formed with 3′-$UTR_{373+4nt}$ RNA template was eluted from the PAGE, 7 M urea gel and digested by RNase A under conditions of high ionic strength (see "Experimental Procedures"). The products of RNase A digestion were analyzed by electrophoresis on a formaldehyde-agarose gel (Fig. 4*A*). Under conditions of high ionic strength, the double-stranded regions in RNA are resistant to RNase A, whereas single-stranded regions are susceptible. The results showed that the faster migrating RNA product of 3′-$UTR_{373+4nt}$ RNA template had twice the size of the input RNA template (2×) prior to RNase A digestion (Fig. 4*A*, *lane 3*) but migrated as a unit-sized RNA (1×) after RNase A digestion under conditions of high ionic strength (Fig. 4*A*, *lane 4*). These results indicated that the faster migrating product is a double-stranded hairpin that had a single-stranded region susceptible to RNase A. The RNA size markers and an aliquot of the RdRP reaction mixture were loaded in *lanes 1* and *2* of Fig. 4*A*, respectively. The faster migrating RNA products formed from the subgenomic RNA templates (generated from the PCR DNA and *Xba*I-linearized plasmid) also had twice the size (2×) of input RNAs before RNase A digestion (Fig. 4*B*, *lanes 3* and *7*) but migrated as a unit-sized (1×) product after RNase A digestion (*lanes 4* and *8*).

Next, we sought to analyze the 1× product, which was formed in reactions containing lysates from either uninfected or DEN2-infected C6/36 cells. The *in vitro* assays using uninfected cell lysates with the 3′-UTR_{373nt} and the subgenomic RNA templates did not produce appreciable 1× labeled product. The 1× labeled RNA products formed from the 3′-$UTR_{373+4nt}$ and the subgenomic RNAs as templates with DEN2-infected cell lysates were isolated from the PAGE/urea gel and digested with RNase A. The results showed that the 1× products formed with the subgenomic RNA templates synthesized from *Xba*I-linearized plasmid and the PCR DNA were resistant to RNase A under conditions of high ionic strength (Fig. 4*B*, *lanes 2* and *6*). This result suggested that the 1× product contained the input template RNA and the complementary (−)-strand RNA synthesized in the RdRP reaction, which would anneal when isolated from the gel and become resistant to RNase A. These results did not distinguish between the possibilities that the 1× product was formed by *de novo* initiation of RNA synthesis at the 3′-end of the template or arising from the digestion of the 2× hairpin product by a nuclease (see below). However, the 1× product formed with the 3′-$UTR_{373+4nt}$ RNA template and uninfected cell lysate (Fig. 3*A*, *lane 3*) was sensitive to RNase A under these conditions, suggesting that it is a single-stranded RNA (data not shown). This RNA product is likely to be formed by the action of the host terminal nucleotidyl transferase, which has been observed in earlier studies as a membrane-associated enzyme (38, 65, 66).

Kinetics of the Formation of RNA Products in the RdRP Assays—To determine the pathway for the formation of the double-stranded (2×) hairpin product and whether the unit-sized RNA was formed independent of the hairpin product, a time course of RdRP reaction was performed. The standard RdRP reactions were initiated and at the end of each indicated time point, EDTA was added (10 mM) to terminate the reaction. The RNA products formed at various time points were analyzed by formaldehyde-agarose gel electrophoresis prior to or after digestion with RNase A (Fig. 5). The results shown in Fig. 5*A* indicate that in 10 min of incubation time, the 1× product was formed first at detectable levels that increased gradually with time. In contrast, the 2× product was formed with a lag of 15 min, and the levels increased significantly over the time course of incubation. In addition, there were other RNA products formed starting at 10 min whose mobilities were between those of 1× and 2× products. In comparison, the results obtained after RNase A digestion of these time course samples (Fig. 5*B*)

indicated that the 1× product formed at 10 min of incubation was predominantly sensitive to RNase A, suggesting that this RNA species is likely to be the product of a terminal nucleotidyl transferase. However, the 2× product formed between 15 and 40 min, upon digestion with RNase A, was converted predominantly to RNase A-resistant 1× product. The RNA species of intermediate mobilities were essentially sensitive to RNase A, which yielded two small RNA fragments as seen first with the 10-min sample. These results revealed a pathway for the formation of the double-stranded hairpin product as follows. The RNA template is initially modified by the 3′-end elongation to an RNase A-sensitive product by either terminal nucleotidyl transferase or the viral replicase or both. This first intermediate is further elongated to a second intermediate (with a mobility between 1× and 2×), which is predominantly a RNase A-sensitive product but contains some double-stranded region to yield small RNase A-resistant products (Fig. 5*B*, 10 min lane). Further incubation of the reaction between 15 and 40 min results in a RNA product having a hairpin (2×) structure. This RNA product has a predominantly RNase A-resistant, double-stranded region and a limited single-stranded loop region sensitive to RNase A. These results suggest that a small amount of the RNase A-resistant 1× product, formed in a 90-min standard RdRP reaction with the subgenomic RNA template (Fig. 4*B*), originated from the double-stranded hairpin (2×) product during the course of incubation.

5′-terminal Region of DEN2-RNA Is Required for Self-primed RNA Synthesis at the 3′-End—Our results presented in Figs. 3 and 4 suggested that self-priming at the 3′-end of the RNA template by viral RdRP activity to produce a hairpin-like structure was dependent on the nature of the RNA template used. The 3′-$UTR_{373+4nt}$ RNA was active, whereas the 3′-UTR_{373nt} generated from the PCR fragment was essentially inactive. This result was independently confirmed by removal of the *Xba*I 5′ overhang from the linearized plasmid by digestion with S1 nuclease at two different concentrations (3 and 10 units/5 μg). Sequence analysis at the 3′-end of the S1 nuclease-treated plasmids indicated that at 3 units of enzyme/5 μg of plasmid DNA, the 3′-UTR_{373nt} cDNA end corresponded to the authentic 3′ terminus, whereas at 10 units of the enzyme/5 μg of DNA, it had lost three additional nucleotides from the 3′ terminus. The 3′-UTR_{373nt} RNA templates produced from the S1 nuclease-treated plasmids by the SP6 RNA polymerase-catalyzed *in vitro* transcription were also inactive like the RNA produced from the PCR product (data not shown). On the other hand, the subgenomic RNAs generated from either the S1 nuclease-treated plasmids under identical conditions used for the 3′-$UTR_{373+4nt}$ plasmid or from the PCR fragment (which would correspond to the authentic 3′ terminus) were active in the RdRP assays in producing the hairpin-like products (data not shown).

These results indicated that the subgenomic RNA is intrinsically active in the RdRP reaction to produce the self-primed product, whereas the 3′-UTR_{373nt} RNA is not. The data suggested that the 5′-terminal sequences of DEN2 RNA are likely to play a role in modulating the structure at the 3′-end to confer a self-priming activity to the subgenomic RNA in the RdRP assay. To address this hypothesis, we tested whether the addition of 5′-terminal RNA containing 230 nt (5′-TR_{230nt}) would confer the self-priming activity to the 3′-UTR_{373nt} RNA in our RdRP assay. The RdRP assay was carried out using the 3′-UTR_{373nt} RNA alone or in the presence of 5′-TR_{230nt} RNA as templates. The results shown in Fig. 6 indicated that the 3′-UTR_{373nt} RNA alone was inactive as a template in the RdRP assay (*lane 2*), confirming the previous observations; however, the 5′-TR_{230nt} added in *trans* could activate the 3′-UTR_{373nt} RNA to have the self-primed RNA synthesis activity in the RdRP reaction to produce the faster migrating 2× product (*lane 3*). Moreover, the 5′-TR_{230nt} RNA by itself had self-priming activity (*lane 4*). The faster migrating products were confirmed to be 2× by running the samples in formaldehyde-agarose gels (data not shown and Fig. 7).

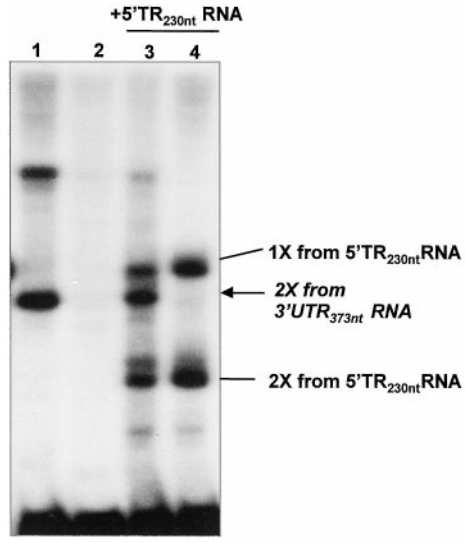

FIG. 6. **5′-TR_{230nt} RNA mediates RNA synthesis at the 3′-end of the inactive 3′-UTR_{373nt} RNA template.** The *in vitro* replication assay was carried out using exogenously added RNA templates synthesized from the *Xba*I-linearized plasmid (3′-$UTR_{373+4nt}$ RNA; *lane 1*), the PCR-generated DNA (3′-UTR_{373nt} RNA; *lane 2*), 5′-terminal 230-nt RNA (5′-TR_{230nt} RNA; *lane 4*), or a mixture of 3′-UTR_{373nt} and 5′-TR_{230nt} RNAs (*lane 3*). RNA products formed were fractionated by PAGE (5.5%), 7 M urea gel system and analyzed by autoradiography. The input template-sized and dimer-sized RNA products formed from the 3′-UTR_{373nt} or the 5′-TR_{230nt} RNAs are indicated.

The conserved sequence elements within the 5′- and 3′-terminal regions of flavivirus RNA genomes (5′-CS1, 3′-CS1, 3′-CS2, and RCS2) include the complementary motifs, "cyclization sequences" (5′-CYC and 3′-CYC; Fig. 1) (58). The role of 5′- and 3′-CYC motifs or the other conserved sequence elements in the viral life cycle is unknown at present. The results shown thus far indicated that the 3′-UTR_{373nt} RNA, which includes the 3′-CYC element, is inactive in the RdRP assay; however, the presence of the 5′-terminal region containing the entire 5′-UTR (96 nt) with conserved stem-loop structure (57) and the 5′-CS1 element along with the 3′-UTR in the same molecule (for example, in the subgenomic RNA) or its addition in *trans* to the 3′-UTR RNA activated RNA synthesis at the 3′-end of the 3′-UTR RNA.

These results raised the interesting question of whether the cyclization motifs play any role in conferring the template activity of 3′-UTR_{373nt} RNA in the RdRP assay. To address this question, a mutant cDNA containing point mutations in the 5′-CYC motif that was designed to disrupt base pairing with the 3′-CYC motif was constructed (5′-TR/mutCYC; Fig. 1). A second construct containing complementary mutations in the 3′-CYC motif within the 3′-UTR_{373nt} cDNA (3′-UTR_{373nt}/mutCYC) was also made. These mutant cDNAs were used to generate the 5′-TR_{230nt}/mutCYC and 3′-UTR_{373nt}/mutCYC RNAs, which were then used as templates in the RdRP assays. The results shown in Fig. 7 indicate that the wild type or mutant 3′-UTR_{373nt} RNA was inactive in the self-priming activity (*lanes 2* and *3*; *lane 1* contained the control, 3′-$UTR_{373+4nt}$ RNA, that has this activity). However, the addition of 5′-TR_{230nt} RNA containing the wild type 5′-CYC element activated the 3′-UTR_{373nt} RNA containing the wild type 3′-CYC element (*lane 4*) but not the mutant 3′-CYC element (*lane 5*).

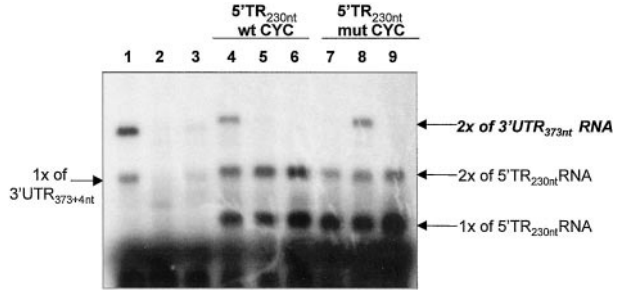

FIG. 7. **Role of cyclization motifs in RNA synthesis mediated by the 5′-TR$_{230nt}$ RNA at the 3′-end of 3′-UTR$_{373nt}$ RNA template.** The 9-nt cyclization motifs (CYC) were mutated either individually to disrupt or together to restore complementarity. The mutant plasmids were used for *in vitro* transcription to synthesize 5′-TR$_{230nt}$ and 3′-UTR$_{373nt}$ RNAs containing either wild type or mutant CYC motifs (*wtCYC* or *mutCYC*). RNAs were used in the *in vitro* assays, and the products were analyzed by formaldehyde-agarose gel system. *Lane 1*, 3′-UTR$_{373nt+4nt}$ RNA; *lane 2*, 3′-UTR$_{373nt}$ RNA with wtCYC; *lane 3*, 3′-UTR$_{373nt}$ RNA with mutCYC; *lane 4*, 3′-UTR$_{373nt}$ RNA + 5′-TR$_{230nt}$ RNA containing wtCYC motifs; *lane 5*, 3′-UTR$_{373nt}$ RNA with mutCYC + 5′-TR$_{230nt}$ RNA with wtCYC motifs; *lane 6*, 5′-TR$_{230nt}$ RNA with wtCYC motif alone; *lane 7*, 3′-UTR$_{373nt}$ RNA with wtCYC motif + 5′-TR$_{230nt}$ RNA with mutCYC motif; *lane 8*, both 3′-UTR$_{373nt}$ RNA and 5′-TR$_{230nt}$ RNA with complementary mutCYC motifs; *lane 9*, 5′-TR$_{230nt}$ RNA with mutCYC motif alone. In this gel system, the labeled input template-sized (1×) 3′-UTR$_{373nt}$ RNA, which has an additional 26 nt from the vector sequences, appears to have migrated close to the 2× of 5′-TR$_{230nt}$ RNA product.

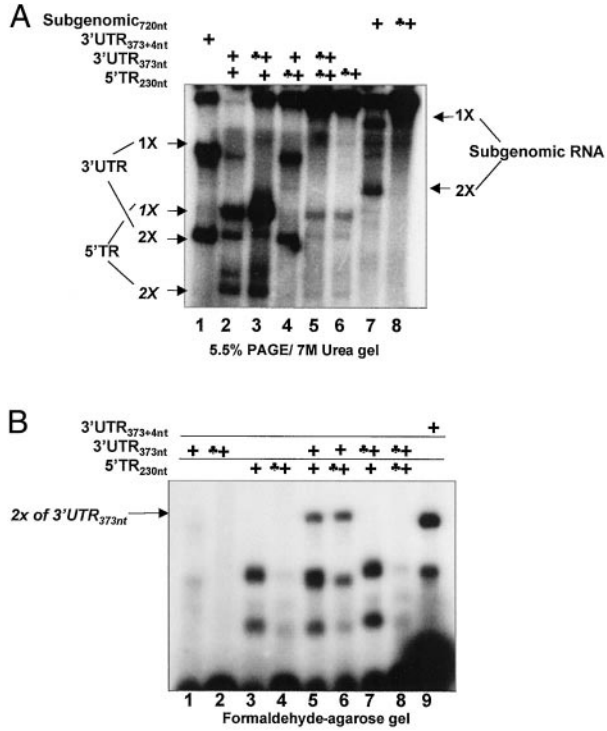

FIG. 8. **3′-hydroxyl group of the subgenomic RNA or the 3′-UTR$_{373nt}$ RNA is required for RNA synthesis mediated by the 5′-TR$_{230nt}$ sequences.** RNAs were synthesized by *in vitro* transcription and treated with sodium periodate (indicated as ♣) followed by lysine to block the 3′-OH group. Purified RNAs were used in the *in vitro* RdRP assays as described under "Experimental Procedures." *Lane 1* in *A* and *lane 9* in *B* refer to the periodate-untreated 3′-UTR$_{373nt+4nt}$ RNA as control. RNAs 3′ and 5′ refer to 3′-UTR$_{373nt}$ RNA and 5′-TR$_{230nt}$ RNA, respectively. *A*, analysis of products was carried out by PAGE/urea system. *B*, analysis of products by formaldehyde-agarose gel system. The products were detected by autoradiography.

Similarly, the mutant 5′-TR$_{230nt}$/mutCYC RNA could not transactivate the wild type 3′-UTR$_{373nt}$ RNA (*lane 7*), whereas it could transactivate the mutant 3′-UTR$_{373nt}$/mutCYC RNA (*lane 8*). We also observed that both mutant and wild type 5′-TR$_{230nt}$ RNAs were active by themselves in the RdRP assay (*lanes 6* and *9*). This template activity was probably due to a specific structure of these RNAs recognized by the RdRP.

These results taken together indicated that the 5′- and 3′-terminal regions of the viral genome do functionally interact with each other. This interaction is required for the template activity of 3′-UTR$_{373nt}$ RNA in self-primed RNA synthesis by a copy-back mechanism at the 3′-end of viral RNA catalyzed by the viral-specific RdRP. In addition, the CYC motifs, which are complementary to each other, are important in modulating this interaction.

The Role of 3′-OH Moieties of 3′-UTR/wtCYC and 5′-TR/wtCYC RNAs in Self-primed RNA Synthesis in Vitro—We sought to determine whether the 3′-OH group of the 5′-TR/wtCYC RNA was required for activation of the template activity of the 3′-UTR/wtCYC RNA in the self-primed RNA synthesis. The 3′-OH group of each of the RNA template was oxidized by sodium periodate followed by treatment with lysine to remove the excess periodate. The RNAs were purified and used in the RdRP assays. The results shown in Fig. 8 indicate that the 5′-TR/wtCYC and 3′-UTR/wtCYC RNAs, when present together, are active in self-primed RNA synthesis from the 3′-OH moiety of each template molecule (Fig. 8, *A*, *lane 2*, and *B*, *lane 5*). When the 3′-UTR/wtCYC RNA was treated with periodate, its self-priming activity was nearly abolished in the presence of the untreated 5′-TR/wtCYC RNA, whereas the untreated 5′-TR/wtCYC RNA by itself was still active to produce the 1× and 2× products (Fig. 8, *A*, *lane 3*, and *B*, *lane 7*). Similarly, when the 5′-TR/wtCYC RNA was treated with periodate, its self-priming activity was nearly abolished, but its ability to activate the 3′-UTR/wtCYC RNA in *trans* for self-primed RNA synthesis was still intact (Fig. 8, *A* and *B*, *lanes 4* and *6*, respectively). When both RNA templates were treated with periodate, the self-priming activity was severely reduced (Fig. 8, *A* and *B*, *lanes 5* and *8*, respectively). Similar results were observed by treatment of the subgenomic RNA with sodium periodate (Fig. 8*A*, *lanes 7* and *8*). These results suggested that the self-priming RNA synthesis at the 3′-OH end of the RNA templates is very specific and is not modified by the cell extracts during the *in vitro* RdRP reactions. In addition, the *trans* activation of 3′-UTR/wtCYC RNA by 5′-TR/wtCYC RNA for RNA synthesis does not require the 3′-OH group of the latter. These results are also consistent with the conclusion derived from the results of Fig. 5 because the formation of both 1× and 2× products were abolished by blocking the 3′-OH group of the subgenomic RNA (Fig. 8*A*, *lanes 7* and *8*).

DISCUSSION

In this study, we describe the first flaviviral replicase assay system that utilizes cytoplasmic extracts from dengue virus-infected mosquito (C6/36) cells and exogenously added subgenomic RNA templates to study the mechanism of viral replication *in vitro*. The RNA templates used in this *in vitro* system contain the conserved sequence (CS) elements found near or within the 5′- and 3′-UTR of many flavivirus RNA genomes examined to date (Ref. 58 and for a review see Ref. 8). These sequences also include those that are predicted to form potential stem-loop (5′- and 3′-SL) structures (56–58, 67, 68). Biochemical evidence for the formation of SL structures was provided by enzymatic RNA structure probing studies (56, 68) and from the sequence data of some rare cDNA clones derived from three flaviviral RNAs, which could only be explained by self-primed cDNA synthesis by reverse transcriptase at the 3′-end of these viral RNAs (58).

Our results shown in this study indicate that the self-primed

RNA synthesis producing a hairpin-like structure (2×) occurs in the viral replicase-catalyzed reaction, which appears to be the major species formed *in vitro*. A minor product formed in a RdRP assay carried out using either infected or uninfected cell extracts has the same mobility as the input RNA in a partially denaturing gel electrophoresis system. However, analysis of the product formed with uninfected cell lysates indicated that it was sensitive to RNase A digestion, whereas the product formed with infected cell lysates was RNase-resistant, suggesting that the latter product was double-stranded RNA. These results suggested that the RNase A-sensitive product formed in the reaction containing the uninfected cell lysates is likely to be formed by the host terminal nucleotidyl transferase by addition of nucleotides to the 3′-end of the RNA template.

The kinetics of the RdRP reaction catalyzed by the viral replicase reveals that the initial RNA product formed is also sensitive to RNase A, suggesting that it was the product of limited 3′-end elongation by either the host terminal nucleotidyl transferase or by the viral replicase or both. This RNA intermediate is then converted to the hairpin-like product by a copy-back mechanism. The results of sodium periodate treatment that abrogated the formation of both 1× and 2× products are also consistent with this conclusion. The RNase A-resistant 1× product observed in a 90-min RdRP reaction could arise from digestion of the single-stranded region of the 2× product by nuclease(s) during the RdRP reaction. However, formation of this product at least in part by *de novo* synthesis by the viral replicase at the 3′-end of RNA template could not be ruled out.

The role of the conserved sequence elements 3′-CS1, 3′-CS2, 3′-RCS2, and 5′-CS1 in flaviviral life cycle is unknown. The 9-nt complementary sequence elements, known as cyclization sequences (3′-CYC and 5′-CYC), are thought to cyclize viral RNA to form a panhandle structure with 11–12 contiguous base pairs that include the adjacent sequences (58). The 3′-UTR_{373nt} RNA, as well as the subgenomic RNA used in this study, include these conserved sequence elements. Whether these CYC elements in flaviviral RNAs are involved in formation of cyclic intermediates during replication or in some other event in the viral life cycle is yet to be determined. The free energy of cyclization was calculated to be −9 to −11 kcal at 25 °C, supporting the possibility that cyclization could indeed occur (58). We analyzed the potential secondary structure within the 3′-UTR and predicted base pairing between 5′- and 3′-CYC motifs in the 0.7-kilobase subgenomic RNA using the MFOLD program (69) (data not shown). The predicted structure for the 3′-SL alone up to CS1 showed that the 3′-CYC motif is in a loop region, whereas the predicted structure for the subgenomic RNA showed the 5′- and 3′-CYC motifs form base pairings in a stable stem containing 11 base pairs (from 134 to 144 nt from the 3′-end). Studies using RNA structure probing methods are essential to determine the secondary structure of the subgenomic template RNA active in our *in vitro* assay. Interestingly, Alphaviral and Bunyaviral RNAs have been reported to cyclize under physiological conditions (70, 71). It was proposed that the cyclization event would fulfill a putative requirement for binding of viral RNA replicase to both 5′- and 3′-terminal regions prior to initiation of viral replication (58).

The results of our study suggest that interactions between the 5′-and 3′-terminal regions, mediated by conserved CYC motifs, result in a conformational change that is a prerequisite for self-primed RNA synthesis at the 3′-end of the 3′-UTR by the viral replicase. Cellular proteins that are shown to bind the 3′-stem-loop structure of a flavivirus RNA (48, 49, 72) could play an important role in modulating the stability of this interaction. Although the complementarity between the CYC motifs might be the driving force in bringing the ends of the genome together, it is possible that there are additional interactions, mediated by other conserved motifs such as 3′-CS2 and RCS2, which could play a role in conferring a specific conformation for initiation of RNA synthesis.

In a recent report, a functional interaction *in vivo* between two RNA components, RNA-1 and RNA-2 of the plant virus, red clover necrotic mosaic virus genome, was demonstrated (73). In that study, a 34-nt segment of RNA-2 component was shown to be required for subgenomic RNA synthesis from genomic RNA-1 component *in vivo*. Interestingly, within the RNA-1 and RNA-2 components, two 8-nt motifs complementary to each other were shown to be important for subgenomic RNA synthesis. Again, mutations that maintained complementarity were active, suggesting that base pairing interactions between the two motifs are likely to be involved in subgenomic RNA synthesis (73).

Complete replication of RNA genomes of the positive strand viruses, cucumber mosaic virus, tobacco mosaic virus, and nodavirus by either a partially purified or by a crude membrane-bound replicase complex have been reported (28, 34, 74). In other *in vitro* template-dependent replication systems, only synthesis of the negative strand to form the double-stranded RNA intermediate has been demonstrated (35). In some *in vitro* RdRP reactions, the template RNA gave rise to not only the complementary (−)-strand RNA but also RNA species larger than the input RNA templates. For example, poliovirus RdRP gave rise to dimer-sized RNA products *in vivo* and *in vitro* (75–78). The evidence suggested that the 3′-ends were generated by endonucleolytic attack of the 3′-SL structure(s) of polioviral RNA, which then served as templates for the RdRP giving rise to a variety of self-primed products. Moreover, blocking the 3′-OH by sodium periodate did not have any effect on the generation of dimer-sized products, because new 3′-OH was presumably generated by the endonucleolytic cleavage (77). However, the RNA synthesis at the 3′-end of the RNA templates used in our system appears to be specific to RNA structure. In contrast, purified preparations of viral RdRP of poliovirus (79), hepatitis C virus (80) or dengue virus (81) can utilize exogenous viral or synthetic RNA templates for RNA synthesis *in vitro* but without any template specificity or structure dependence.

A few other viral RdRP such as cucumber mosaic virus (28), brome mosaic virus (31, 82), and turnip crinkle virus (32) showed template and structure-specific RNA synthesis. The turnip crinkle virus RdRP utilized the (−)-strand of satellite C RNA as a template and produced two non-template-sized products, a panhandle-like structure and a hairpin-like structure (L- and S-RNAs, respectively) by terminal elongation of the 3′-end. The formation of the L-RNA had specific sequence and structural requirements, whereas the S-RNA was formed from all (−)-strand RNAs tested (50). These RdRP preparations had some cellular proteins that perhaps contributed to the template specificity (for a review see Ref. 36). Cellular proteins from the DEN2-infected extracts are also likely to contribute to the exogenous template specificity observed in this study.

Other studies have shown that *cis*-acting signals required for viral RNA replication are noncontiguous in the viral genome of the brome mosaic virus (44, 83, 84), tobacco mosaic virus (85), and the double-stranded RNA virus of yeast (L-A virus) (Ref. 41 and for a review see Ref. 36). Moreover, in the single-stranded negative sense RNA genome of the Rhabdoviridae family (the vesicular stomatitis virus RNA (86)) and in the segmented influenza virus RNA genome (87), interactions between 5′- and 3′-terminal sequences involve self-complementary sequences. The mechanisms of viral replication and the specificity of interactions of the viral replicases and viral RNA genomes are

likely to be distinct in these different viruses. Development of an *in vitro* RdRP assay for a flavivirus that can utilize exogenous RNA templates and identification of a functional interaction between conserved sequence elements within the 5′- and 3′-terminal regions that play a role in viral RNA synthesis *in vitro* are important steps toward unraveling the mechanism of flavivirus replication.

REFERENCES

1. Monath, T. P. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2395–2400
2. Kautner, I., Robinson, M. J., and Kuhnle, U. (1997) *J. Pediatr.* **131,** 516–524
3. Gubler, D. J., and Clark, G. G. (1995) *Emerg. Infect. Dis.* **1,** 55–57
4. Irie, K., Mohan, P. M., Sasaguri, Y., Putnak, R., and Padmanabhan, R. (1989) *Gene* (*Amst.*) **75,** 197–211
5. Svitkin, Y. V., Lyapustin, V. N., Lashkevich, V. A., and Agol, V. I. (1984) *Virology* **135,** 536–541
6. Markoff, L. (1989) *J. Virol.* **63,** 3345–3352
7. Nowak, T., Farber, P. M., Wengler, G., and Wengler, G. (1989) *Virology* **169,** 365–376
8. Chambers, T. J., Hahn, C. S., Galler, R., and Rice, C. M. (1990) *Annu. Rev. Microbiol.* **44,** 649–688
9. Bazan, J. F., and Fletterick, R. J. (1989) *Virology* **171,** 637–639
10. Gorbalenya, A. E., Donchenko, A. P., Koonin, E. V., and Blinov, V. M. (1989) *Nucleic Acids Res.* **17,** 3889–3897
11. Chambers, T. J., Weir, R. C., Grakoui, A., McCourt, D. W., Bazan, J. F., Fletterick, R. J., and Rice, C. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8898–8902
12. Preugschat, F., Yao, C. W., and Strauss, J. H. (1990) *J. Virol.* **64,** 4364–4374
13. Falgout, B., Pethel, M., Zhang, Y. M., and Lai, C. J. (1991) *J. Virol.* **65,** 2467–2475
14. Chambers, T. J., Grakoui, A., and Rice, C. M. (1991) *J. Virol.* **65,** 6042–6050
15. Wengler, G., Czaya, G., Farber, P. M., and Hegemann, J. H. (1991) *J. Gen. Virol.* **72,** 851–858
16. Zhang, L., Mohan, P. M., and Padmanabhan, R. (1992) *J. Virol.* **66,** 7549–7554
17. Cahour, A., Falgout, B., and Lai, C.-J. (1992) *J. Virol.* **66,** 1535–1542
18. Clum, S., Ebner, K. E., and Padmanabhan, R. (1997) *J. Biol. Chem.* **272,** 30715–30723
19. Murthy, H. M., Clum, S., and Padmanabhan, R. (1999) *J. Biol. Chem.* **274,** 5573–5580
20. Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1989) *Nucleic Acids Res.* **17,** 4713–4730
21. Koonin, E. V. (1992) *Trends Biochem. Sci.* **17,** 495–497
22. Kadare, G., and Haenni, A. L. (1997) *J. Virol.* **71,** 2583–2590
23. Li, H., Clum, S., You, S., Ebner, K. E., and Padmanabhan, R. (1999) *J. Virol.* **73,** 3108–3116
24. Westaway, E. G. (1987) *Adv. Virus Res.* **33,** 45–90
25. Poch, O., Sauvaget, I., Delarue, M., and Tordo, N. (1989) *EMBO J.* **8,** 3867–3874
26. O'Reilly, E. K., and Kao, C. C. (1998) *Virology* **252,** 287–303
27. Blumenthal, T., and Carmichael, G. G. (1979) *Annu. Rev. Biochem.* **48,** 525–548
28. Hayes, R. J., and Buck, K. W. (1990) *Cell* **63,** 363–368
29. Wu, S. X., and Kaesberg, P. (1991) *Virology* **183,** 392–396
30. Molla, A., Paul, A. V., and Wimmer, E. (1991) *Science* **254,** 1647–1651
31. Quadt, R., Kao, C. C., Browning, K. S., Hershberger, R. P., and Ahlquist, P. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1498–1502
32. Song, C., and Simon, A. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8792–8796
33. Barton, D. J., Black, E. P., and Flanegan, J. B. (1995) *J. Virol.* **69,** 5516–5527
34. Osman, T. A., and Buck, K. W. (1996) *J. Virol.* **70,** 6227–6234
35. Lemm, J. A., Bergqvist, A., Read, C. M., and Rice, C. M. (1998) *J. Virol.* **72,** 6546–6553
36. Lai, M. M. (1998) *Virology* **244,** 1–12
37. Chu, P. W., and Westaway, E. G. (1985) *Virology* **140,** 68–79
38. Grun, J. B., and Brinton, M. A. (1986) *J. Virol.* **60,** 1113–1124
39. Chu, P. W., and Westaway, E. G. (1987) *Virology* **157,** 330–337
40. Bartholomeusz, A. I., and Wright, P. J. (1993) *Arch. Virol.* **128,** 111–121
41. Esteban, R., Fujimura, T., and Wickner, R. B. (1989) *EMBO J.* **8,** 947–954
42. Nakhasi, H. L., Cao, X. Q., Rouault, T. A., and Liu, T. Y. (1991) *J. Virol.* **65,** 5961–5967
43. Pardigon, N., and Strauss, J. H. (1992) *J. Virol.* **66,** 1007–1015
44. Pogue, G. P., and Hall, T. C. (1992) *J. Virol.* **66,** 674–684
45. Rohll, J. B., Percy, N., Ley, R., Evans, D. J., Almond, J. W., and Barclay, W. S. (1994) *J. Virol.* **68,** 4384–4391
46. Rohll, J. B., Moon, D. H., Evans, D. J., and Almond, J. W. (1995) *J. Virol.* **69,** 7835–7844
47. Carpenter, C. D., Oh, J. W., Zhang, C., and Simon, A. E. (1995) *J. Mol. Biol.* **245,** 608–622
48. Blackwell, J. L., and Brinton, M. A. (1995) *J. Virol.* **69,** 5650–5658
49. Shi, P. Y., Li, W., and Brinton, M. A. (1996) *J. Virol.* **70,** 6278–6287
50. Song, C., and Simon, A. E. (1995) *J. Mol. Biol.* **254,** 6–14
51. Hsue, B., and Masters, P. S. (1997) *J. Virol.* **71,** 7567–7578
52. Hsue, B., and Masters, P. S. (1998) *Adv. Exp. Med. Biol.* **440,** 297–302
53. Schuppli, D., Miranda, G., Qiu, S., and Weber, H. (1998) *J. Mol. Biol.* **283,** 585–593
54. Stewart, S. R., and Semler, B. L. (1998) *Nucleic Acids Res.* **26,** 5318–5326
55. Zeng, L., Falgout, B., and Markoff, L. (1998) *J. Virol.* **72,** 7510–7522
56. Mohan, P. M., and Padmanabhan, R. (1991) *Gene* (*Amst.*) **108,** 185–191
57. Brinton, M. A., and Dispoto, J. H. (1988) *Virology* **162,** 290–299
58. Hahn, C. S., Hahn, Y. S., Rice, C. M., Lee, E., Dalgarno, L., Strauss, E. G., and Strauss, J. H. (1987) *J. Mol. Biol.* **198,** 33–41
59. Yaegashi, T., Vakharia, V. N., Page, K., Sasaguri, Y., Feighny, R., and Padmanabhan, R. (1986) *Gene* (*Amst.*) **46,** 257–267
60. Smith, T. J., Brandt, W. E., Swanson, J. L., McCown, J. M., and Buescher, E. L. (1970) *J. Virol.* **5,** 524–532
61. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
62. Grun, J. B., and Brinton, M. A. (1987) *J. Virol.* **61,** 3641–364
63. Grun, J. B., and Brinton, M. A. (1988) *J. Gen. Virol.* **69,** 3121–3127
64. Mussgay, M., Enzmann, P. J., Horzinek, M. C., and Weiland, E. (1975) *Prog. Med. Virol.* **19,** 257–323
65. Dasgupta, A., Zabel, P., and Baltimore, D. (1980) *Cell* **19,** 423–429
66. Andrews, N. C., Levin, D., and Baltimore, D. (1985) *J. Biol. Chem.* **260,** 7628–7635
67. Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L., and Strauss, J. H. (1985) *Science* **229,** 726–733
68. Brinton, M. A., Fernandez, A. V., and Dispoto, J. H. (1986) *Virology* **153,** 113–121
69. Zuker, M., Jaeger, J. A., and Turner, D. H. (1991) *Nucleic Acids Res.* **19,** 2707–2714
70. Hsu, M. T., Kung, H. J., and Davidson, N. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **38,** 943–50
71. Hewlett, M. J., Pettersson, R. F., and Baltimore, D. (1977) *J. Virol.* **21,** 1085–1093
72. Blackwell, J. L., and Brinton, M. A. (1997) *J. Virol.* **71,** 6433–6444
73. Sit, T. L., Vaewhongs, A. A., and Lommel, S. A. (1998) *Science* **281,** 829–832
74. Wu, S. X., Ahlquist, P., and Kaesberg, P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11136–11140
75. Young, D. C., Tuschall, D. M., and Flanegan, J. B. (1985) *J. Virol.* **54,** 256–264
76. Lubinski, J. M., Kaplan, G., Racaniello, V. R., and Dasgupta, A. (1986) *J. Virol.* **58,** 459–467
77. Hey, T. D., Richards, O. C., and Ehrenfeld, E. (1987) *J. Virol.* **61,** 802–811
78. Richards, O. C., Hey, T. D., and Ehrenfeld, E. (1987) *J. Virol.* **61,** 2307–2310
79. Neufeld, K. L., Richards, O. C., and Ehrenfeld, E. (1991) *J. Biol. Chem.* **266,** 24212–24219
80. Behrens, S. E., Tomei, L., and De Francesco, R. (1996) *EMBO J.* **15,** 12–22
81. Tan, B. H., Fu, J., Sugrue, R. J., Yap, E. H., Chan, Y. C., and Tan, Y. H. (1996) *Virology* **216,** 317–325
82. Siegel, R. W., Adkins, S., and Kao, C. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11238–11243
83. Lahser, F. C., Marsh, L. E., and Hall, T. C. (1993) *J. Virol.* **67,** 3295–3303
84. Quadt, R., Ishikawa, M., Janda, M., and Ahlquist, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4892–4896
85. Leathers, V., Tanguay, R., Kobayashi, M., and Gallie, D. R. (1993) *Mol. Cell. Biol.* **13,** 5331–5347
86. Wertz, G. W., Whelan, S., LeGrone, A., and Ball, L. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8587–8591
87. Luo, G. X., Luytjes, W., Enami, M., and Palese, P. (1991) *J. Virol.* **65,** 2861–2867