De Novo Synthesis of RNA by the Dengue Virus RNA-dependent RNA Polymerase Exhibits Temperature Dependence at the Initiation but Not Elongation Phase*

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Replication of positive strand flaviviruses is mediated by the viral RNA-dependent RNA polymerases (RdRP). To study replication of dengue virus (DEN), a flavivirus family member, an in vitro RdRP assay was established using cytoplasmic extracts of DEN-infected mosquito cells and viral subgenomic RNA templates containing 5′- and 3′-terminal regions (TRs). Evidence supported that an interaction between the TRs containing conserved stem-loop, cyclization motifs, and pseudoknot structural elements is required for RNA synthesis. Two RNA products, a template size and a hairpin, twice that of the template, were formed. To isolate the function of the viral RdRP (NS5) from that of other host or viral factors present in the cytoplasmic extracts, the NS5 protein was expressed and purified from Escherichia coli. In this study, we show that the purified NS5 alone is sufficient for the synthesis of the two products and that the template-length RNA is the product of de novo initiation. Furthermore, the incubation temperature during initiation, but not elongation phase of RNA synthesis modulates the relative amounts of the hairpin and de novo RNA products. A model is proposed that a specific conformation of the viral polymerase and/or structure at the 3′ end of the template RNA is required for de novo initiation.

The dengue virus, which is the causative agent of dengue fever, dengue hemorrhagic fever, and dengue shock syndrome, is estimated to infect 100 million people per year worldwide (1–3). The virus is spread by the mosquito, Aedes aegypti, which puts ~40% of the world at risk for dengue infection (1). Approximately 5% of infected individuals worldwide develop hemorrhagic or shock manifestations, which can commonly result in death (1). The dengue virus type 2 (DEN2) is the most prevalent of the four dengue serotypes.

The virus contains a positive strand, 5′-capped RNA, 10,723 nucleotides in length (for New Guinea-C strain; Ref. 4), which encodes a single polyprotein precursor, arranged in the order, C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (for a review, see Ref. 5). This precursor is processed in the endoplasmic reticulum by a combination of the signal peptidase and the viral serine protease to generate three structural proteins of the virion, C, prM, and E (6–8) and at least seven nonstructural (NS) proteins.

NS3, the second largest protein encoded by the virus, contains a serine catalytic triad within the N-terminal 180 amino acids, and it requires NS2B for protease activity (9–18). The crystal structures of the protease domain alone and in complex with an inhibitor have been reported (19, 20). However, the function of other nonstructural proteins in viral replication is poorly understood.

NS3 also contains conserved motifs found in several NTPase/RNA helicases (21–23). According to the current model, replication is initiated by the viral RNA-dependent RNA polymerase (RdRP) by synthesis of minus (−) strand to form a double-stranded RNA intermediate, which then serves as a template for genomic positive strand (24–27). The viral NTPase and RNA helicase activities were reported for a recombinant NS3 lacking the protease domain, expressed and purified from Escherichia coli (28). These activities may be important for replication of positive (+) strand from the double-stranded intermediate by an energy-dependent unwinding step. In addition to protease and RNA helicase activities, flavivirus NS3 protein possesses an RNA 5′-triphosphatase activity that can hydrolyze the γ-phosphate of RNA (29). This activity is the first of the three enzymatic activities required for 5′-capping, the other two being guanylyltransferase and 5′-RNA methyltransferase (30, 31).

NS5, the largest of the DEN2 viral proteins, contains the conserved motifs found in several RNA-dependent RNA polymerases encoded by positive strand RNA viruses (32, 33). Additionally, NS5 contains conserved motifs found in RNA 5′-methyltransferases (34, 35), but has not been directly demonstrated for any flavivirus NS5. In DEN2-infected cells, NS3 and NS5 exist as a stable complex, suggesting that viral replication and 5′-capping are closely linked (36). The NTPase activity of dengue virus type 1 NS3 was stimulated by NS5 (37), and this observation is consistent with the role of these proteins as a complex in viral replication.

The in vitro replication systems developed to study positive strand RNA replication have revealed that viral replicases mostly are membrane-bound complexes containing the viral RdRP as well as other cellular and viral proteins. These in vitro RdRP assays utilize exogenous RNA templates and either crude or purified components of viral replicases that catalyze...
specific synthesis of RNA (38–45). Viral replicases recognize specific elements contained within the 5′- and 3′-terminal regions of the viral genomes for initiation of viral replication (46–58). In flavivirus genomes, two conserved sequences within the 3′-untranslated region (3′-UTR) as well as stem-loop structures within the 3′- and 5′-UTRs of flavivirus genomes are thought to be important for viral RNA replication (50, 59–63). Within the 3′-conserved sequence of the dengue virus genome, 94 nucleotides (nt) from the 3′-terminus, there is a 9-nt motif that is complementary to a conserved motif within the 5′-terminal region (5′-TR), located 133 nucleotides from the 5′-terminus. These motifs are separated by 10.5 kilobases in the full-length viral RNA. It was proposed that these motifs could bring the two ends of the genome together through base pairing and play a role in viral replication. Hence, these motifs are referred to as “cyclization” (CYC) motifs (63).

Previously, we described an in vitro RdRp assay system derived from whole cell lysates isolated from DEN2-infected mosquito (C6/36) or monkey (LLC-MK2) cells. These active viral replicase complexes can utilize exogenous RNA templates that contained the 5′-TR and 3′-UTR of DEN2 RNA with the internal coding sequences deleted (hereafter referred as “subgenomic RNA”) and synthesize (−) strand RNA (64). There were two products formed in the in vitro RdRp assay; the first was a labeled template with the same size as the input RNA (770 nt), and the second was twice that of the template (−1540 nt). Using this system, we showed that there is an interaction between the two terminal regions of the viral RNA which is required for RNA synthesis (64). Subsequently, a physical interaction between the two RNAs was also demonstrated using the psoralen/UV cross-linking method, and the two CYC motifs played an essential role for both physical interaction and for RNA synthesis. These results suggested that there is cross-talk between the two terminal regions through the conserved sequence elements in the viral RNA that is required for (−) strand RNA synthesis (65).

Mutational analysis revealed that RNA synthesis at the 3′-UTR of the subgenomic RNA template requires the 5′-UTR and the highly conserved 5′-CYC motif, which is complementary to the 3′-CYC motif within the 3′-UTR. Also required is the 3′ stem-loop structure that includes a predicted pseudoknot structure. Furthermore, it was shown that the complementarity between the two CYC motifs rather than the actual sequences was important for RNA synthesis (64, 65). A recent study using a Kunjin viral RNA replicon cell line also revealed that the complementarity rather than actual sequence of CYC motifs are required for replication of Kunjin viral replicon RNA in vivo (66).

Previous studies on flavivirus replication intermediates using Kunjin virus, dengue virus, and West Nile virus revealed that, in flavivirus-infected cells, three RNA species were detected: a genomic RNA of 40–44 S, a double-stranded RNA-resistant replicative form of 20–22 S, and a partially RNAse-sensitive replicative intermediate of 20–28 S RNA species (24, 26, 67). The analysis of virus-specific RNAs isolated from Kunjin virus-infected cells in completely denaturing formaldehydeagarose gels (similar to the one used for analysis of in vitro RdRp products in our studies) did not reveal the presence of any species larger than genome size RNA (24). These results suggest that the copy-back mechanism of RNA synthesis resulting in dimer species, on the other hand, would result in loss of genome sequences. Because template size and hairpin RNA products were both formed in our in vitro RdRp assays, several questions remained to be addressed: How are these two species related? Are those products of specific structures of the template and/or different enzyme conformations? Is the template size product produced by de novo initiation of RNA synthesis or is it the byproduct of the hairpin RNA by nucleolytic attack at the single-stranded loop region?

To address these questions, in this study, we expressed full-length NS5 with an N-terminal histidine tag in E. coli. We purified the protein to >90% in a soluble form. The purified protein is active in the synthesis of (−) strand RNA from positive strand subgenomic RNA templates but not from an RNA containing only the 3′-UTR or from nonspecific RNA templates to any significant extent. For optimal RNA synthesis, 5′-TR and 3′-UTR are both required as well as the wild type or complementary mutant CYC motifs. These results prove that the NS5 protein alone exhibits specificity for the DEN2 viral subgenomic RNA and is able to initiate (−) strand RNA synthesis de novo without the requirement of other viral or host cofactors. At the same time, the purified RdRp also synthesizes a hairpin RNA product by 3′ end elongation. Interestingly, the ratio between de novo and hairpin products formed was directly dependent on the incubation temperature of the RdRp reaction during the initiation phase, but not during the elongation phase of viral RNA synthesis. Temperature is known to influence the structure of nucleic acid or protein. Based on the recently reported structural differences between polioviral and hepatitis C virus (HCV) RdRPs (68), we propose a model in which DEN2 RdRp enzyme assumes two conformations that are in equilibrium. According to this model, a temperature-dependent shift of the equilibrium could determine how the enzyme recognizes the conformation of the template RNA that ultimately results in RNA synthesis by de novo initiation or by 3′ end elongation.

**EXPERIMENTAL PROCEDURES**

**His Tag NS5 Expression Construct—**A BamHI restriction site was engineered into our previously described pLZ-5 plasmid (69), which contains the coding sequence for NS5. PCR was carried out using the primers 5′-GCAGGATCCCGGAACTGGCAACATAGGAGAGA-3′ and 5′-CGCACAGGGTGAGTCGCC-3′ (nt 7570–7591 and nt 7716–7735, respectively, in the DEN2 genome (Ref. 4)) and pLZ-5 as the template, producing a 175-base pair fragment. The PCR fragment contained the DEN2-encoded NruI site at the 3′ end and an engineered in-frame BamHI site at the 5′ end. The PCR product was then blunt end-ligated into pLZ-5, which had been previously digested with NruI. This intermediate plasmid was subjected to BamHI digestion, and the fragment was cloned into pQE-32 at the BamHI site to give rise to pMHA-77-3 plasmid. The plasmid contains full-length DEN2 NS5 with an N-terminal 6-histidine tag under control of the lac promoter.

**Purification of DEN2 NS5/RdRp (from E. coli)—**E. coli (XL1-Blue) cells (1-liter culture), transformed with pMHA-77-3 plasmid that were grown in LB media containing 100 μg/ml ampicillin and 0.5% glucose (w/v) at 37 °C until A_{600} nm reached 0.55. Bacteria were then centrifuged at 5,000 g for 1 h. The resin was batch-washed five times, with 12 ml of buffer A containing 50 mM NaH_{2}PO_{4}, pH 7.0, 300 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 1× Complete protease inhibitor mixture without EDTA purchased from Roche Molecular Biochemicals (Mannheim, Germany). Lysate was then incubated with 1.5 ml of Talon resin (CLONTECH, Palo Alto, CA) at 4 °C for 1 h. The resin was batch-washed five times, with 12 ml of buffer A containing 50 mM NaH_{2}PO_{4}, pH 7.0, 300 mM NaCl, 10% glycerol. After transfer of Talon resin to a disposable Bio-Rad column, nonspecific proteins were removed by washing with 40 ml of the buffer A containing 15 mM imidazole (pH 7.1), followed with 25 ml of 20 mM imidazole in the same buffer. Proteins were eluted from the Talon resin with buffer A containing 0.5 M imidazole. NS5-containing fractions were pooled, concentrated by Centricron-30 (Millipore, Bedford, MA) to ~500
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µl, and applied to a G-75 Sephadex (Sigma) column. Proteins were eluted from the column in 0.5-m l fractions at 5 ml/h. NS5 was eluted between fractions 21 and 30. Fractions were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 5 mM MgCl₂, and 40% glycerol. Purified NS5 protein was aliquoted and stored at −30 °C.

Preparation of RNA Templates—Contraction of the plasmid encoding DEN2 subgenomic RNA has been described (64). RNA templates were prepared by T7-RNA polymerase (Promega)-catalyzed in vitro transcription of linearized plasmids (pTM1 cut with BamHI or KpnI), or PCR products produced from the plasmid templates as described previously (64). RNA was quantified by spectrophotometry and integrity was verified by denaturing urea-polyacrylamide gel electrophoresis, followed by staining with acridine orange.

RdRP Assay—The standard reaction mixture (50 µl) contained 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, template RNA (1 µg), 500 µM each of ATP, GTP, and UTP, 10 µM unlabelled CTP, and 10 µCi of [α-³²P]CTP along with 200 ng of purified NS5 except where indicated. The reaction was carried out by incubation at 30 °C for 1 h and terminated by acid phenol/chloroform extraction, followed by ethanol precipitation after the addition of yeast tRNA (5 µg) as a carrier. The RNA pellet was collected by centrifugation and the pellet was dried. RNA was resuspended in 50 µl of nuclease-free H₂O and passed through a Bio-Rad P-30 column to remove unincorporated nucleotides. Flow-through fraction was precipitated with ethanol. RNA was analyzed by formaldehyde-agarose gel electrophoresis and visualized by autoradiography (64). The labeled bands were excised from dried gels and quantified by liquid scintillation counting and also measured by densitometry utilizing the NIH program Scion. RdRP reactions at different temperatures were carried out using a gradient thermocycler (Tgradient, Biometra, Göttingen, Germany). The reactions were terminated and analyzed as described above.

Sodium Periodate Treatment of RNA Transcripts—The 3′-hydroxyl of RNA transcripts was blocked by sodium periodate as described previously (64). Reactions were phenol-extracted and ethanol-precipitated as described above.

Heparin-trap Experiments to Distinguish Early Initiation Phase Versus Elongation Phase of RNA Synthesis—To isolate the initiation and elongation phases of RNA synthesis by NS5, we altered previously described protocols for use with our system (70–73). Subgenomic RNA labelled with [³²P]CTP was incubated at 30 °C for 10 min with 500 µM each of three nucleotides (ATP, GTP, and CTP), 10 µCi of [α-³²P]CTP, and 200 ng of purified NS5 to allow partial synthesis of RNA (based on the template sequence to be six nucleotides). Heparin (50 ng) was then added (2 µl of 25 mg/ml) and incubated for 5 min before the addition of UTP (500 µM). Reactions were then allowed to complete elongation synthesis for 60 min. To study the influence of varying the temperature of incubation on initiation phase of RNA synthesis, reactions were incubated at different temperatures with the enzyme and three nucleotides, but were moved immediately (C until after the addition of heparin; UTP was then added, and reactions were continued at 30 °C for 16 h. This resulted in

![Fig. 1. Purification of full-length N-terminal His tag NS5. A. lane 1, whole cell lysate; lane 2, 15 µl imidazole wash; lane 3, 20 µl imidazole wash; lanes 4–8, fractions 6–10 from elution with 500 mM imidazole wash. B, pooled NS5 from Talon column (lane 1) was concentrated in a Centricon-30 spin column (lane 2; 1:4 loaded on gel). Concentrated NS5 was applied to G-75 Sephadex column and eluted with wash buffer as described under "Experimental Procedures." Proteins eluted in indicated fractions 21–30. At fraction 33, the contaminating 30-kDa protein began to elute.](http://www.jbc.org/)

**RESULTS**

Purification of Full-length DEN2 NS5—Initial attempts to express full-length NS5 were unsuccessful because of toxicity of NS5 to E. coli; the bacteria were able to grow only to an A₆₀₀ of about 0.35. We made use of previous observations that growth in glucose maintains a low level of cAMP and cAMP activator protein (also known as cAMP receptor protein), allowing the repression of lac promoter, resulting in a tight regulation of T7 RNA polymerase and NS5 expression. Prior to induction of the lac promoter with isopropyl-β-d-thiogalactopyranoside, glucose was removed. The expression of full-length NS5 protein was significantly improved under these conditions; however, the protein was primarily located in the insoluble pellet fraction (data not shown). To maximize the amount of soluble NS5 and reduce cleavage of the full-length protein, expression was then performed at 18 °C for 16 h. This resulted in ~80% full-length NS5 in the soluble fraction with cleavage products of NS5 partitioning primarily in the insoluble fraction. Eluate of 6×His-tagged NS5 from the metal affinity (Talon®) matrix contained the full-length NS5 as well as additional
polypeptides in the size range of ~46–28 kDa (Fig. 1A, fractions 6–10). To separate the full-length NS5 from these contaminating proteins, gel filtration chromatography was employed. As shown in Fig. 1B, this gel filtration step yielded NS5 protein of over 90% purity (Fig. 1B, lanes 3–8) and all subsequent experiments were carried out using this protein.

**Purified NS5 Is a True RNA-dependent RNA Polymerase and Is Active in RNA Synthesis on Subgenomic Dengue Viral RNA Templates**—We then sought to determine whether the purified recombinant NS5 was enzymatically active in RNA synthesis using the subgenomic RNA770 nt template (64). This RNA contains the entire 3'-UTR and 5'-TR230 nt of the DEN2 genome, including the stem-loop structures and 3'- and 5'-CYC motifs. The RdRP assay was carried out as described under “Experimental Procedures” except that initial assays were conducted in the presence of RNasin (RNase inhibitor; 0.2 unit/μl) and actinomycin D (16 μM) to inhibit any potential contaminating RNases or bacterial DNA-dependent RNA polymerase, respectively. The purified NS5 contains no detectable RNase activity, which was determined by incubation of the RNA template with enzyme for various time periods followed by denaturing polyacrylamide gel electrophoresis analysis. There was no difference in polymerase activity in the presence or absence of actinomycin D (data not shown). Therefore, these two reagents were removed from the subsequent assays. Additionally, a DNA template containing a T7 polymerase promoter was not active with purified NS5, verifying that there is no detectable contaminating bacterial polymerase (Fig. 2, lane 3). The reaction was incubated at 30 °C for 1 h and analyzed by a completely denaturing formaldehyde-agarose gel electrophoresis followed by autoradiography.

The results of RdRP activity assay indicate that RNA synthesis was dependent on the addition of subgenomic RNA770 nt template and the NS5 protein in a complete system, but not when the NS5 was omitted or heat-inactivated (Fig. 2, lanes 1, 2, 4, and 5). Two products were formed in the complete system, one having the size of the input template (770 nt), and the second having a mobility consistent with twice the size of the template (~1540 nt). The formation of these two products required all four nucleotides. When labeled CTP alone was present at the same concentration as in the complete reaction, no product could be detected (lane 6), suggesting that the purified enzyme is free of any terminal transferase as a contaminant. However, when reactions contained only CTP, UTP, and ATP, the complete template length product was produced in significantly lower amounts compared with those formed when all four nucleotides were present (lane 8). Lanes 6–9 needed a 10-day exposure of the gel for the same experiment shown in panel A, which was visualized in an overnight exposure; therefore, these are shown separately in panel B. The products of similar size were also formed when either ATP or UTP alone were omitted from the reaction mixture and but these were even lower in abundance than when GTP alone was omitted (lanes 7 and 9 versus lane 8). The sensitivity of the product to RNase A under high salt conditions indicated that the labeled product is a single-stranded RNA (lanes 11 and 12). This result suggested that the template RNA was labeled in the reaction because of terminal addition of nucleotides to the 3' end by RdRP. Taken together, these results indicate that the purified NS5 is specific for RNA, acting as a true RNA-dependent RNA polymerase.

**Template Specificity and RdRP Activity on DEN2 Subgenomic RNA Template for RNA Synthesis in Vitro Requires Both 5'- and 3'-Terminal Regions**—To determine whether the purified NS5 specifically recognizes dengue viral RNA templates or is able to utilize any nonspecific RNA, we tested different RNAs as templates. Nonspecific RNA templates were produced by digestion of the pTM1 vector with either BamHI or KpnI to yield linearized plasmid templates, which were then used for T7 RNA polymerase-catalyzed *in vitro* transcription. The 3'-UTR RNA454 nt was also tested as a template for the purified RdRP, as it alone was inactive for RNA synthesis in
our previous in vitro system that utilized crude cytoplasmic extracts from DEN2-infected cells (64). The results of RdRP assays using the purified NS5 and different RNA templates shown in Fig. 3 indicated that only the subgenomic RNA\textsubscript{770 nt}, 3′-UTR alone (373 nt), nonspecific RNAs, transcribed from pTM1 vector plasmid containing the T7 promoter linearized by BamHI or KpnI digestion (expected to produce 645- and 480-nt RNAs, respectively) prior to in vitro transcription catalyzed by T7 RNA polymerase. Reactions were visualized by formaldehyde-agarose gel electrophoresis followed by autoradiography.

Fig. 3. Template specificity of the purified NS5. Each RdRP reaction contained NTPs, [α-\textsuperscript{32}P]CTP, 200 ng of purified NS5 protein, and various templates as noted. Templates tested were subgenomic RNA (770 nt), 3′-UTR alone (373 nt), nonspecific RNAs, transcribed from pTM1 vector plasmid containing the T7 promoter linearized by BamHI or KpnI digestion (expected to produce 645- and 480-nt RNAs, respectively) prior to in vitro transcription catalyzed by T7 RNA polymerase. Reactions were visualized by formaldehyde-agarose gel electrophoresis followed by autoradiography.

The purified enzyme was also analyzed for its specificity of recognition of subgenomic RNA templates in which both terminal regions of the viral genome are in the same RNA molecules as opposed to in two separate (5′-TR\textsubscript{230 nt} and 3′-UTR\textsubscript{454 nt}) RNAs as described above. Our previous study using the cell lysate system indicated that mutation of the 5′-CYC motif in the subgenomic RNA still severely affected RNA synthesis in vitro, but the mutation of the 3′-CYC motif was tolerated as the activity was only reduced by 2-fold. The results shown in Fig. 4A (lanes 2–4) also support other data that the purified polymerase exhibited similar template specificities as the viral replicase from the cell lysate system.

Characterization of the RdRP Products—Our previous assays utilizing crude cytoplasmic extracts from DEN2-infected mosquito (C6/36) cells also produced two products on a denaturing formaldehyde-agarose gel system: a template-sized RNA (1×) and a hairpin RNA, twice the size of the template (2×) (64, 65). The dimer-sized species are possibly formed by short additions of nucleotides, either by RdRP or the host terminal transferase to the 3′ end of the template RNA, which are then extended by RdRP. Alternatively, the dimer species could also have resulted from 3′-elongation of a “folded-back” structure of the RNA template. Such an intrinsic structure in the viral genome was previously suggested to be the source of cDNA species that were isolated in which sequences complementary to 3′-internal region were covalently linked to the 3′-terminal sequences by the reverse transcriptase (65).

In addition to the dimer species, we also detected template-sized (1×) RNA product using the cytoplasmic extract as the source of RdRP. The result that this template size product was resistant to RNase A digestion supported the conclusion that the labeled (−) strand RNA was annealed to the unlabeled input (+) strand RNA template. This conclusion was further supported by the results of RNase H mapping using strand-specific oligodeoxynucleotides (65). However, the question of whether the 1× product was synthesized by de novo initiation of RNA synthesis or was produced by digestion of the hairpin RNA product by a nuclease(s) present in the cytoplasmic extract was not resolved.

In this study, we sought to address this question using the purified polymerase as the enzyme was devoid of any nuclease contamination. When the assays were performed with the purified RdRP and all four NTPs, both hairpin and template size products were formed similar to our previous observation using the cell lysate system (64), suggesting that the 1× product was more likely to be formed because of de novo initiation of RNA synthesis than from the hairpin product.

Therefore, to determine whether the 2× product was in fact a hairpin RNA formed by 3′ end elongation of the template RNA, we subjected the products to RNase A digestion under high and low salt conditions. Under high salt conditions, RNase A will digest only single-stranded RNA, whereas double-stranded RNAs will be resistant. The single-stranded loop region of a hairpin product, however, will be sensitive to RNase A digestion, and will migrate as a 1× product on a completely denaturing gel (see Fig. 5A). Under low salt conditions, both double-stranded and single-stranded RNAs are sensitive to RNase A digestion. When the products were subjected to RNase A analysis, high salt conditions produced predominantly 1× product, when related to the same reaction without addition of RNase A (Fig. 5B, lane 2 versus lane 1). Under low salt conditions, all RNA was digested (Fig. 5B, lane 3). This result establishes that the slower migrating band on the completely denaturing gel is in fact a hairpin RNA product, produced from
3' end-elongation of the input template. Further proof that (-) strand was synthesized de novo and by 3'-elongation was obtained by reverse transcriptase-mediated cDNA synthesis using strand-specific probes followed by PCR (RT-PCR) (see below).

Evidence for de Novo Initiation of Minus Strand Synthesis by the Purified RdRP—We were interested to know whether the template length product was produced through de novo initiation of the 3'-UTR. First we sought to determine whether a sodium periodate-treated subgenomic RNA_{770 nt} having a blocked 3' hydroxyl group can still serve as the template for RNA synthesis. This template should only form the 1× product, and no hairpin product would be possible, as the 3' end-elongation would be blocked.

The periodate-treated template RNA_{770 nt} produced only the 1× product, and no hairpin product could be detected (Fig. 5B, lane 4), which suggested that periodate oxidation of the 3'-terminal ribose moiety went to completion because the presence of any unmodified RNA template would have resulted in formation of hairpin RNA. This experiment also serves as an evidence against the possibility that the 1× product is formed from a hairpin intermediate by a structure-dependent "ribozyme-like" nuclease, because no hairpin product was formed in this reaction. We sought to determine whether this product is in fact the minus strand formed by de novo initiation at the 3' end of the subgenomic RNA template. We analyzed the product by RNase A digestion. As shown in Fig. 5B (lane 5), the product was RNase A-resistant under high salt, whereas it was completely sensitive under low salt conditions (lane 6). As diagrammed in Fig. 5A, resistance of the product to RNase A digestion proves that it is a double-stranded RNA. This verifies that the radioactive RNA visualized on the gel is not simply a terminally radiolabeled input template RNA, as this would be degraded by RNase A. Next, we used strand-specific oligodeoxynucleotides as primers that could anneal only to specific regions of either (+) or (-) strand polarity in the double-stranded RNA product for reverse transcriptase reaction. This step was followed by PCR amplification, and the cDNA products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The results shown in Fig. 5C indicate that cDNA products of expected sizes were formed only from the template size de novo product formed in the RdRP reaction using the sodium periodate-treated (Fig. 5C, lanes 5 and 6) and untreated samples (lanes 3 and 4); these results verify that (-) strand formed in the RdRP reaction is annealed to the (+) strand template RNA. No cDNA products were synthesized from the (+) strand template RNA alone with DNA primers of (+) polarity, serving as the control (lanes 1 and 2). These results, taken together, indicate that the purified RdRP is able to initiate primer-independent de novo synthesis of (-) strand RNA in the absence of other viral or host factors.

Temperature Dependence of de Novo RNA Synthesis by the Purified NS5/RdRP—The evidence gathered thus far using our in vitro RdRP assay systems suggests that the structure of the 3'-UTR RNA dictates its template activity. The structure is modulated by interaction between the two terminal regions of the viral genome. The CYC motifs are required for this interaction, although they are not sufficient for initiation of RNA
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Fig. 5. Characterization of RdRP products. A, schematic description of RNase A digestion of RdRP products. B, lanes 2 and 3, product from RdRP reaction using the subgenomic RNA template was incubated with RNase A in high or low salt conditions. Lane 1, no RNase A added. Subgenomic RNA was subjected to sodium periodate treatment to block the 3′-OH group and was used as a template for the RdRP reaction. Lane 4, no RNase A added; only template-length product was formed. Lanes 5 and 6, aliquots of reaction mixture was subjected to RNase A digestion under high and low salt, respectively. C, purified RNA from RdRp reactions was subjected to RT-PCR as follows. Reactions were first digested with RNase A to remove single stranded RNA. Samples were then subjected to RT-PCR utilizing primers of either (+) polarity (lanes 1, 3, and 5) or (–) polarity (lanes 2, 4, and 6). The product from reverse transcription was then utilized for PCR with the corresponding primers, as described in “Experimental Procedures.” Products utilized for RT-PCR were input subgenomic RNA, template before RdRp reaction (lanes 1 and 2), which was not digested with RNase A, products from RdRp reactions with subgenomic RNA (lanes 3 and 4), or periodate treated subgenomic RNA (lanes 5 and 6). Products were fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining. Expected products were 280 nt for negative strand RNA and 340 nt for positive strand RNA.

synthesis. The conserved secondary structures at the 5′-UTR and the 3′-stem-loop region that includes the tertiary pseudoknot structure are also important for RNA synthesis in vitro (64, 65). Several studies on RNA folding reveal that varying the temperature could influence RNA secondary structure as well as tertiary interactions such as pseudoknot structures (see, for example, Refs. 74–76). To study the effect of temperature on the template efficiency and the products formed in the in vitro RdRP assay, we incubated the enzyme with subgenomic RNA template at different temperatures in the range between 20 and 40 °C for 90 min, as described under “Experimental Procedures.” Products of RdRP reactions were analyzed by completely denaturing formaldehyde gel electrophoresis and autoradiography. The results shown in Fig. 6A indicate that the 1× de novo initiation product was predominantly formed at lower temperatures, whereas at higher temperatures a predominance of hairpin product was produced. We quantitated the amount of each product by excising the corresponding radioactive band from the gel followed by scintillation counting. The autoradiograms of labeled RNA were also quantitated by densitometry using the NIH software Scion (Scion Corp). The experiments were repeated three times, and the quantitative data were plotted. The results shown in Fig. 6B indicate that there is a good correlation between the formation of the hairpin product and an increase in the temperature of incubation of the RdRP reaction.

Temperature Dependence of de Novo RNA Synthesis Is at the Stage of Initiation and Not Elongation—With the knowledge
that de novo minus strand RNA synthesis by RdRP is dependent on temperature, we set forth to determine at which step, initiation or elongation, temperature plays a role in RNA synthesis. Heparin, which binds to free RNA polymerase and inhibits its activity, has been used previously to study promoter occupancy by the enzyme (71, 77–79). It is also known that heparin inhibits initiation of transcription but not elongation by the RNA polymerase already bound to the newly initiated RNA (77, 80). We used a strategy of partial incorporation of nucleotides to allow limited synthesis of nascent chain after initiation (70–73, 79). In this way, we could examine the temperature dependence of initiation events by the RdRP, independently from the elongation step, by addition of the missing nucleotide and heparin to inhibit new initiation events. By incubating the template RNA with the viral polymerase and three nucleotides (ATP, CTP, and GTP), based on the sequence at the 3' end, the enzyme was expected to add 6 nucleotides and pause; then, heparin and, 5 min later, UTP were added. This stable complex was able to carry out elongation of RNA synthesis, which was resistant to the presence of heparin in the reaction mixture. We were able to achieve a single round of RNA synthesis from this protocol, because the viral polymerase is inactivated by heparin once it releases the template, such as in the case of abortive transcription or runoff from the template. By segregating the binding and initiation phases from the elongation phase, we were able to study the effect of temperature during these two processes.

We varied the temperature between 20 and 40 °C during initiation by incubating the template and the RdRP together with ATP, CTP, and GTP. After the addition of heparin, reactions were moved to a constant 30.8 °C temperature, and then UTP was added. This effectively varied the temperature during the initiation phase of the RdRP activity, but kept the elongation phase constant at 30.8 °C. The products were analyzed by formaldehyde-agarose gel electrophoresis followed by autoradiography, B, labeled bands were excised, quantitated by liquid scintillation counting, and verified by densitometry. The average ratio of de novo (1X) to 3' end elongation (2X) products from four separate experiments was plotted as shown.

FIG. 6. De novo synthesis of RNA by the RdRP is temperature-dependent. A, Purified RdRP was incubated with subgenomic RNA for 90 min at varying temperatures as described under “Experimental Procedures.” Products were resolved by formaldehyde-agarose gel electrophoresis and subjected to autoradiography. B, bands were excised from dried gel and subjected to liquid scintillation counting and verified by densitometry using an NIH software (Scion) as described under “Experimental Procedures.” The average ratio of de novo (1X) to 3' end elongation (2X) products from three separate experiments were plotted as shown.

FIG. 7. Temperature dependence of de novo synthesis of RNA is at the initiation but not elongation phase. A, purified NS5 was incubated with subgenomic RNA and three nucleotides (A, G, C and [α-32P]CTP for 10 min at varying temperatures. 50 ng of heparin was added and incubated for an additional 5 min before addition of UTP. At this time, temperatures for all reactions were held constant at 30.8 °C. Products were analyzed by formaldehyde-agarose gel electrophoresis followed by autoradiography. B, labeled bands were excised, quantitated by liquid scintillation counting, and verified by densitometry. The average ratio of 1X:2X products from four experiments was plotted as shown.
is dependent on temperature, the initiation step was carried out at a constant temperature (30.8 °C) in the presence of three nucleotides (A, G, C), and [α-32P]CTP for 10 min at 30.8 °C. 50 ng of heparin was added to the reaction mixtures and incubated for an additional 5 min before addition of UTP. At the time of UTP addition, reaction mixtures were moved to varying temperatures. Products were visualized by formaldehyde-agarose gel electrophoresis followed by autoradiography. B, labeled bands were excised and quantitated by liquid scintillation counting and verified by densitometry. The average ratio of 1×2× products from four experiments was plotted as shown.

The results shown in Fig. 8 (A and B) indicate that there is no difference in the relative amounts of de novo versus 3′ end “fold-back” synthesis of RNA. A, purified RdRp was incubated with subgenomic (770 nt) RNA, three nucleotides (A, G, C), and [α-32P]CTP for 10 min at 30.8 °C. 50 ng of heparin was added to the reaction mixtures and incubated for an additional 5 min before addition of UTP. At the time of UTP addition, reaction mixtures were moved to varying temperatures. Products were visualized by formaldehyde-agarose gel electrophoresis followed by autoradiography. B, labeled bands were excised and quantitated by liquid scintillation counting and verified by densitometry. The average ratio of 1×2× products from four experiments was plotted as shown.

The enzyme also exhibited a primer-template-dependent RNA synthesis. Previous studies on other viral polymerases demonstrated initiation of RNA synthesis was primer-dependent and the enzymes acted on RNA templates without any specificity. For example, poliovirus, encephalomyocarditis virus, rhinovirus, brome mosaic virus, or dengue virus RdRPs all required a primer, which can be either the 3′ end of the template that is then extended by a fold-back mechanism or an exogenous RNA primer (81–86). In poliovirus replication, the genome-linked VPg is first uridylylated by the 3D polymerase, which requires polioviral RNA; the uridylylated VPg then serves as a primer for initiation of negative strand RNA synthesis on the polyadenylated polioviral RNA (87). An RNA hairpin within the coding region of poliovirus 2C protein is the site specifically used for uridylylation of VPg, and this hairpin structure, which is well conserved in Enterovirus family of Picornaviridae, is required for replication of the viral genome (88, 89). An internal RNA structural element has also been identified that is required for rhinovirus14 replication (90). It is possible that 5′-TR RNA of dengue viral genome might also contain a cis-acting replication element that includes the 5′-CYC motif. Mutation of this motif has been shown to affect RNA synthesis in vitro (Ref. 64 and this study) and for another flavivirus, Kunjin virus replicon, in vivo (66).

The HCV RdRp (NS5B protein), expressed and purified from E. coli or insect cells, has been characterized in great detail. The enzyme also exhibited a primer-template-dependent RNA synthesis on homopolymeric or heteropolymeric HCV-derived “D” RNA templates without any specificity (91–94). In the absence of a primer, the polymerase catalyzed 3′ end elongation to produce a hairpin RNA (91, 95–97). However, template size product was formed only in the presence of a primer complementary to its 3′-terminus of an RNA template, which multiple rounds of synthesis were permitted within the time period of incubation. Therefore, our results, taken together, indicate that temperature of the initiation step of the RdRP reaction directly influences the ratio of the products formed by de novo synthesis versus 3′ end elongation from the dengue viral subgenomic RNA template. To our knowledge, this is the first report describing the effect of temperature on RNA synthesis by de novo initiation versus 3′ end elongation by a viral RdRP.

**DISCUSSION**

**Expression and Purification of DEN2 Viral RdRp from E. coli**—Previously, we described an in vitro RNA-dependent RNA polymerase assay system using cytoplasmic extracts from DEN2-infected mosquito (C6/36) and monkey kidney (LLC-MK2) cells (64). To isolate the function of DEN2 NS5 from that of other viral and cellular proteins present in our previous assay system and study the biochemical properties of the viral RdRp, we set out to purify NS5 from E. coli. Purified viral polymerase enabled us to accurately assess the enzymatic functions of NS5 in the absence of any intervening host or viral proteins as well as endogenous viral replicative intermediate RNA species that were present in the previously characterized system (64).

**Template Specificity of the Purified RdRp**—The purified NS5 is a true RdRp, and it requires all four NTPs. It exhibited selective template specificity, as it is active optimally only on dengue viral-derived subgenomic RNA templates containing wild type CYC motifs but not on RNA containing only the 3′-UTR and a significantly reduced activity on the subgenomic RNA containing the mutant 5′-CYC motif, or on other nonspecific RNA templates. These results suggest that the enzyme recognizes some specific secondary and/or tertiary structure at the 3′-terminal region of RNA template for initiation of RNA synthesis.

Previous studies on other viral polymerases demonstrated initiation of RNA synthesis was primer-dependent and the enzymes acted on RNA templates without any specificity. For example, poliovirus, encephalomyocarditis virus, rhinovirus, brome mosaic virus, or dengue virus RdRPs all required a primer, which can be either the 3′ end of the template that is then extended by a fold-back mechanism or an exogenous RNA primer (81–86). In poliovirus replication, the genome-linked VPg is first uridylylated by the 3D polymerase, which requires polioviral RNA; the uridylylated VPg then serves as a primer for initiation of negative strand RNA synthesis on the polyadenylated polioviral RNA (87). An RNA hairpin within the coding region of poliovirus 2C protein is the site specifically used for uridylylation of VPg, and this hairpin structure, which is well conserved in Enterovirus family of Picornaviridae, is required for replication of the viral genome (88, 89). An internal RNA structural element has also been identified that is required for rhinovirus14 replication (90). It is possible that 5′-TR RNA of dengue viral genome might also contain a cis-acting replication element that includes the 5′-CYC motif. Mutation of this motif has been shown to affect RNA synthesis in vitro (Ref. 64 and this study) and for another flavivirus, Kunjin virus replicon, in vivo (66).
was blocked at the 3' end to prevent "copy-back" synthesis (96).

**De Novo Initiation of RNA Synthesis by the Purified RdRP**—In contrast to the properties of these picornaviral RdRPs, previous studies on Q-β RdRP (98) and some plant viral enzymes (43, 45, 70, 99–101), and more recent work on purified HCV NS5B (102–104), bovine viral diarrhea virus RdRP (105), and Kunjin RdRP (106) have shown that these enzymes can initiate RNA synthesis de novo. The HCV NS5B RdRP was able to utilize the HCV-derived "D" RNA and yielded both template size and hairpin products (103). After blocking the 3'-OH of the template by incorporation of the chain terminator, cordycepin, the formation of the hairpin product was blocked without affecting the formation of the template size product. From these results the authors concluded that both de novo and the copy-back mechanism of RNA synthesis were responsible for the formation of these two products.

In this study, we show that dengue viral RdRP, purified from E. coli, catalyzes both de novo initiation of RNA synthesis and 3’ end elongation from the viral template RNA. The enzyme exhibits high template specificity and is able to utilize, in the absence of any other viral or host factors, the dengue viral-derived subgenomic RNA template containing either wild type or complementary mutant CYC motifs for optimal RNA synthesis. Substitution mutations in either of the CYC motifs reduced the template activity, but more severely with the mutation of the 5'-CYC motif. The mutation of the 3'-CYC motif was tolerated and the activity was reduced only by about 2-fold. Because the complementary mutations in both CYC motifs restored the near-wild type level of activity, this result argues against the role of 5'-CYC motif in a structure-specific initiation event similar to the requirement of an internal RNA hairpin for uridylylation of VPg primer as in the case of poliovirus RNA (88); on the other hand, these motifs could play a role in bringing the two ends of the dengue viral RNA genome together and the resulting RNA conformation could influence the template recognition by the viral polymerase for de novo initiation of RNA synthesis at the 3’ end. We have shown previously that physical interaction facilitated by wild type CYC motifs per se is not sufficient and other motifs such as 5'-and 3'-stem loop structures within the UTR regions are also required for RNA synthesis (65). Analysis of the secondary structures of the four subgenomic RNA templates by using the software described by Zuker et al. (version 3.0) (107) revealed that, although the overall predictive structures of the wild type, complementary double CYC mutant, and the 3’ CYC mutant are similar, the 5’-CYC mutant has a strikingly different structure (65). Experimental verification of the differences in the structures of the subgenomic RNA by enzymatic and/or chemical probing methods could reveal an insight regarding differences in their template efficiencies in both cell lysate and purified RdRP assay systems.

Interestingly, when RNA synthesis was carried out at various temperatures of incubation in the presence of all four NTPs, total incorporation of nucleotides into both template size and dimeric RNA species was much greater at higher temperatures than at lower temperatures (Fig. 6A, lanes 3 and 4). This increased synthesis of RNA by the enzyme at higher temperatures is perhaps attributable to the removal of pauses on the template RNA for the enzyme, as the incubation temperature is known to influence the secondary and tertiary structures of RNA (74–76). The optimum temperature for RNA synthesis by the enzyme was also found to be between 29.1 and 30.9 °C, above which it falls off (Figs. 6A and 7A).

When RNA synthesis by partial incorporation was carried out in the presence of only three NTPs (ATP, GTP, and CTP) at various temperatures (20–40 °C), the enzyme pauses after limited synthesis of RNA, which is either in the form of a short primer or as the 3' extension of the template RNA by a few nucleotides. The ratio between these two forms of short RNA products is shown to depend on the initial temperature of

**FIG. 9. Model for temperature-dependent switch between two conformational states of dengue viral RdRP.** A model is proposed in which the viral polymerase exists in two conformational states: open and closed forms, which are in equilibrium. The equilibrium is shifted toward an open form at higher temperatures in which the enzyme can bind to a fold-back structure at the 3’ end of RNA template and carry out 3’-elongation giving rise to a dimeric RNA. At lower temperatures, the binding to the fold-back structure is less efficient because the enzyme exists predominantly in closed conformation. This form of enzyme binds more efficiently to single-stranded region at the 3’-terminus of the template RNA and initiates de novo synthesis of RNA to yield a template size double-stranded RNA product. Further details of this model are described in the text.
incubation. For example, when the initial temperature was 27.2 °C and then shifted to 30.8 °C after the heparin and UTP were added, the template-sized (1×) product was 2-fold over the dimer species compared with the ratio of products formed when the temperature of both initiation and subsequent elongation were kept at 30.8 °C (Fig. 7A, lane 3 versus lane 4).

These results seem to suggest that conformation of the enzyme may play a key role in the template recognition by the enzyme and RNA products formed. This notion is supported by a recent report, which has shed much light on the structure-function aspect of RNA polymerases (68). Although the active site of 3Dpol exists in an open conformation, HCV RdRP contains a single β-hairpin consisting of 12 amino acid residues occluding the active site (68, 108, 109). A comparison of the crystal structures from the poliovirus 3Dpol and HCV RdRP revealed an important difference at the active site of the two enzymes. HCV RdRP has an extra β-hairpin consisting of 12 amino acid residues in the thumb subdomain, which is absent in the poliovirus 3Dpol. The template requirements for 3Dpol and NSSB are quite different. Although the 3Dpol is active on double-stranded templates, HCV NS5B is unable to bind double-stranded RNA templates productively (68, 110). When this β-hairpin was shortened by 8 nucleotides (4 nucleotides on either side of the turn), NS5B was able to initiate synthesis on a double-stranded RNA as efficiently as the poliovirus 3Dpol. It has also been demonstrated that HCV NSSB produces dinucleotide primers through abortive initiation that are able to effectively prime RNA synthesis (111). The β-turn prevents the RNA from sliding through the RNA active site “hole” and may aid in the formation of the dinucleotide primers through steric hindrance effects on abortive initiation.

If the dengue virus RdRP structurally resembles the HCV enzyme in having an active site occlusion by a β-hairpin, then temperature may strongly influence its conformation. The dengue viral RdRP may exist in two conformational states, which are in equilibrium (Fig. 9). The active site of the enzyme itself is likely to exist in an equilibrium between a more rigid “closed” conformation at lower temperatures and a more mobile “open” conformation at higher temperatures. The template RNA may also assume either a “fold-back” hairpin or single-stranded RNA region at the 3′-terminal sequences which leads to 3′-end elongation or de novo initiation of RNA synthesis, respectively. Moreover, regardless of temperature, the viral RNA templates may be in an equilibrium between these two forms. At higher temperatures, the equilibrium is shifted to predominantly an open conformation of the enzyme and a very few abortive primers are produced; the enzyme, on the other hand, binds to a 3′-fold-back region of RNA and synthesizes a predominance of dimeric RNA product by 3′-elongation. However, at lower temperatures, the closed conformation of the enzyme may predominate and in this state it may be unable to bind to the fold-back structure of viral template RNA; the enzyme may therefore initiate de novo synthesis on a single-stranded region of RNA. It is possible that the conformational states of the RdRP and/or the viral template RNA may be stabilized by other viral and/or host proteins and by association with the endoplasmic reticulum membranes in the infected cells where viral replication has been localized (5, 25, 112). Our in vitro RdRP assay using the purified NS5 described in this study is likely to be useful for identification of other factors involved in viral replication.

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