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Flavivirus RNA Synthesis in vitro

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Summary

Establishment of in vitro systems to study mechanisms of RNA synthesis for positive strand RNA viruses have been very useful in the past and have shed light on the composition of protein and RNA components, optimum conditions, the nature of the products formed, *cis*-acting RNA elements and trans-acting protein factors required for efficient synthesis. In this review, we summarize our current understanding regarding the requirements for flavivirus RNA synthesis in vitro. We describe details of reaction conditions, the specificity of template used by either the multi-component membrane-bound viral replicase complex or by purified, recombinant RNA-dependent RNA polymerase. We also discuss future perspectives to extend the boundaries of our knowledge.

1. Introduction

Many RNA viruses encode an enzyme that is required for replication of their genomes without involvement of DNA intermediates. This enzyme, RNA-dependent RNA polymerase (RdRp), is an essential component of the multimeric replication complex which often includes other viral and cellular proteins performing specialized functions in the complex replication machinery. In the first part of this review we will focus on the characterization of the membrane-bound viral replicase complex containing the endogenous replicating viral RNA isolated from the dengue virus (DENV)-infected mammalian cells. In the second part of this review we describe RNA synthesis catalyzed by the DENV RdRp present in the cytoplasmic extracts from the DENV-infected cells utilizing the exogenously added specific viral RNA templates. We also describe the properties of recombinant full-length nonstructural (NS) protein 5 (NS5_{FL}) and NS5 polymerase domain alone (NS5POL) expressed and purified from *E. coli*. NS5_{FL} has the 5'-RNA methyltransferase domain at the N-terminus and NS5POL domain at the C-terminus. The polymerase activities of

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recombinant NS5 on nonspecific as well as specific viral RNA templates, the nature of the products formed and the *cis*-acting RNA elements required for RNA synthesis *in vitro* are described. In this review, NS5_{FL} and RdRp refer to the same protein and NS5POL refers to the C-terminal domain of NS5_{FL}.

1.1. DENV RNA

The DENV has a ~11-kb long positive sense RNA genome which functions as mRNA in cell-free translation systems [1–4]. The genome RNA was the only species isolated from polysomes and no subgenomic RNA was found [5, 6]. The flavivirus RNA has a single open reading frame coding for a polyprotein precursor. The precursor is processed both co-translationally and post-translationally into 10 proteins [7], three of which form the virion and the seven NS proteins are expressed only in the infected cells (for a review, see [8]). Through N- and C-terminal sequencing, metabolic labeling and immunoprecipitation using antibodies raised against viral proteins, the gene order of the structural and nonstructural proteins were established as NH₂-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A(2K)-NS4B-NS5-COOH [8]. The *in vitro* processing experiments using vaccinia virus or Semliki forest virus expression systems demonstrated that one of the seven NS proteins, NS4A protein, is cleaved at a specific site within the carboxy terminal region of the protein by the viral protease, NS2B-NS3, which is required prior to cleavage of the NS4A-NS4B precursor by the signal peptidase of the endoplasmic reticulum [7, 9, 10]. The NS4A and NS4A-2K proteins seem to play important roles in viral replication [7, 10–14]. The nonstructural proteins alone and the viral RNA containing *cis*-acting elements within the 5'- and 3'-terminal sequences are sufficient for replication of the genomic RNA. The topology and the cleavage map of the polyprotein and the sites of cellular and viral proteases involved in cleavages to yield mature proteins had previously been reviewed [15]. The cellular signal peptidase cleaves the C-prM, prM-E, E-NS1 and NS4A-NS4B sites and an unknown protease cleaves the NS1-NS2A junction site [16]. The viral protease is involved in the cleavages of membrane anchored C (anch-C), NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4B-NS5 sites. The active viral protease consists of two protein components, NS2B and the N-terminal domain of NS3 containing the trypsin-like serine protease catalytic triad [17, 18]. NS2B and NS3 form a stable non-covalent complex that can be readily purified with a His-tag inserted at the N-terminus of NS2B [19], or the C-terminus of NS3 ([20]; reviewed in [21]). The viral protease cleaves the polyprotein precursor at the junction of two basic amino acids, RR, KR, QR (at the NS2B-NS3 site) as P2-P1 residues, which are followed by A, S, or G at the P1' site. The C-terminal domain of NS3 has conserved motifs found in NTPase/RNA helicases [22]. It is active in unwinding double-stranded RNA or DNA substrate [20, 23–27]. DENV NS3 is also a 5'-RNA triphosphatase, an enzyme activity that is required at the first step in 5'-capping for hydrolysis of γ -phosphate of triphosphorylated RNA to produce diphosphorylated RNA [24, 28]. This step is followed by addition of GMP by the guanylyltransferase to form the 5' cap GpppA—RNA [29]. The N-terminal domain of NS5 protein has conserved motifs found in 5'-RNA methyltransferases [30]. 5'-cap G is methylated at the N-7 position followed by methylation of 2'-OH group of 5'-end A in S-adenosylmethionine(SAM)-dependent reactions by 5'-RNA methyltransferase activities of NS5 to form the type I cap structure, ⁷-MeGpppA_{2'}-Ome [31–33]; reviewed in [34, 35]. The C-terminal domain of NS5 (NS5POL) has conserved motifs found in RNA-dependent RNA

polymerases [36] which are required for replication of viral RNA genomes. In DENV2-infected cells, it was shown that both NS3 and NS5 are in a complex [37] suggesting that NTPase/RNA helicase and 5'-RNA triphosphatase activities of NS3, the polymerase, guanylyltransferase, and 5'-RNA methyltransferase activities of NS5 function in RNA replication and 5'-capping in a coupled fashion. In this chapter, we will review the biochemical properties of NS5 in RNA synthesis in vitro.

2. In vitro DENV RNA synthesis using endogenous viral RNA templates in membrane-bound replication complexes

2.1. Introduction

Flaviviruses have a broad host range and can replicate in a variety of mammalian and mosquito cells. It was reported that although alphavirus, Sindbis virus, can replicate and produce virus-specific antigens in enucleated chick embryo cells, Japanese encephalitis virus (JEV) was unable to produce so, and nuclear involvement for at least 2–4 hour period after infection for virus specific antigen synthesis was required [38]. The role of the nucleus for this requirement is not understood.

The endoplasmic reticulum (ER) membranes are intimately involved in translation and viral replication in the cytoplasm. In fact, studies using electron microscopy and three dimensional electron tomography methods have shown that viral replication occurs in membrane-bound replication complexes within virus-induced and extended ER membrane compartments [39, 40]. The membrane-bound replication complexes from flavivirus-infected cells have been analyzed for composition of viral RNA species by several groups. The genome-size RNA of 40–44S has type 1 cap at the 5'-end but no poly(A) at the 3'-end [1, 41–44]. In addition, double-stranded RNA of 20–22S, and heterogeneous 20–28S RNA presumably replicative form (RF) and replicative intermediate (RI) were also observed [45–48] as well as smaller RNAs of 8–12S RNA in flavivirus-infected cells [1]. It was concluded that this small RNA species were the degradation products of viral RNAs [49] as it was reported that there was no evidence for template activity of a small RNA in a previous study [1]. In the light of current knowledge, the small RNA species could likely include the subgenomic RNA that was reported to be incomplete degradation of viral genomic RNA by cellular RNases [50]. This small RNA was shown to be essential for WNV cytopathicity and pathogenicity [50, 51].

2.2. Early systems to study viral replication in vitro

The notion that cellular membranes played an important role in (+)strand RNA virus life cycle including in replication has been known at least from late 1960s to early 1970s [52, 53]. For example, picornaviral RNA synthesis is initiated in smooth membrane fraction with a sedimentation value of 130 S in isopycnic sucrose density gradient centrifugation, which then matures into late replication complex of 250 S structures. The replication complex consisted of viral RNA polymerase, ssRNA, the 20 S and 26 S replicative form (RF) and replicative intermediate (RI) form of viral RNA, respectively [53, 54]. In earlier studies, the endogenous membrane-bound replication complex has been characterized in viral RNA synthesis by Qureshi and Trent using Saint Louis encephalitis virus (SLE)-infected BHK-21

cells [55] and by Zebrovitz et al. using the JEV-infected porcine kidney cells, PS(Y-15) [47, 48]. To characterize the RNA species in the cytoplasmic membrane structures sedimenting at 250 S, the SLE-infected BHK-21 cells in the presence of actinomycin, were pulse-labeled with [³H]labeled uridine for 30 min at 16 h post-infection [55]. The 250 S membrane structures, which contained ~15% of the total RNA, were stably associated with the membranes during the isolation by sucrose density gradients containing EDTA (20 mM). Three species of RNA were identified; the largest was the RNase-sensitive, 43 S, the progeny ssRNA, then the partially RNase-sensitive, 26 S, and the RNase-resistant, 20 S RNA species [55]. The polymerase activity of gradient fractions was measured using [³H]GTP into acid precipitable labeled products. Treatment of the extracts with sodium deoxycholate (DOC) and Brij-58 inactivated the enzyme activity. The polymerase activity was dependent on Mg⁺² and 4 NTPs but inhibited by Mn⁺² and RNase. The enzyme activity was linear for an hour and declined subsequently [55].

In the study described by Zebrovitz et al. [47], cytoplasmic membranes were prepared by lysis of hypotonic suspension of JEV-infected PS(Y-15) cells and centrifuged at 1000 x g. The remaining nuclear fraction was washed extensively to remove the cytoplasmic contamination [56]. The nuclear fraction was subjected to Dounce homogenization for resuspension in 2.3 M sucrose containing 1.5 x 10⁻³ M CaCl₂ and 10 mM Tris-HCl buffer, pH 7.5. The suspension was centrifuged at 35,000 x g for 30 min. The pellet of nuclei was washed with 3 to 4 volumes of 0.23 M sucrose containing 3 x 10⁻³ M CaCl₂ and centrifuged at 600 x g for 5 min. The nuclei were swelled for 30 min in 10 volumes of 2 x 10⁻² M phosphate buffer, pH 7.2, and subjected to Dounce homogenization [56]. The nuclear suspension was centrifuged at 600 x g to remove intact nuclei and nuclear debris. The suspension of nuclear envelope fraction was centrifuged at 47,000 x g for 20 min.

The cytoplasmic and nuclear-envelope membrane fractions prepared from uninfected and JEV-infected PS(Y-15) cells at 40 h post-infection were analyzed in a RdRp assay as follows. The reaction mixture consisted of 10 mM Tris-HCl (pH 8.2), 50 μM sodium phosphoenolpyruvate, 4 μM magnesium acetate, 5 μM each of unlabeled ATP, CTP, and UTP, 1 μCi of [³H]GTP and 0.5 ml of cell-membrane suspension from infected and uninfected cells. The reaction mixtures were incubated for 30 min at 37°C. The RNA products were analyzed by sucrose density gradients. The major RNA species formed in the in vitro reaction was an RNase-resistant 20S RNA species produced by membrane fractions from JEV-infected cells. The nuclear envelope membrane fraction from infected cells contained 1.7- to 4.3-fold higher levels of viral RNA polymerase activities than the cytoplasmic membrane fraction [48]. The sucrose density gradient centrifugation method chosen to fractionate the products of RNA species synthesized in vitro by the viral polymerase lacked the resolution and was cumbersome to perform detailed analyses.

2.3. The Westaway and Brinton systems

The Westaway and Brinton groups developed in vitro RNA replication systems to study flavivirus RNA synthesis ([57–61]; for a review see [62] and the references therein). The protocols used by these two groups with details of modifications and improvements of their systems are given below.

2.3.1. Protocol for isolation of membrane-bound Kunjin virus (KUN) replication complex (the Westaway system)

2.3.1.1. Preparation of Cytoplasmic and nuclear extracts from KUN-infected Vero cells:

Vero cells were infected with KUN virus (strain MRM61C) for isolation of the cytoplasmic membrane-bound replication complex [57]. Extracts were prepared at various times post-infection as described [63]. Briefly, pelleted cells were resuspended in 10 mM Tris-HCl, pH 8.0, 10 mM sodium acetate (2.5×10^7 cells/ml). Cells were disrupted by passing 20 x through a 20-gauge syringe needle, followed by 20 x through 26-gauge syringe needle. The cell suspension was centrifuged at $800 \times g$ for 7 min. The nuclear pellet was washed with the same Tris-HCl/sodium acetate buffer at 1×10^8 cells/ml and centrifuged at $800 \times g$. The combined supernatant fractions from two centrifugations were aliquoted and stored at -70°C as the cytoplasmic fraction. The nuclear pellet fraction was resuspended in the same buffer as mentioned above at 2×10^7 cells/ml, centrifuged at $500 \times g$ to clarify the supernatant and stored in aliquots as nuclear material at -70°C . The fractions retained enzyme activities for more than 12 months [58].

2.3.1.2. RdRp assay and analysis of RNA products formed in vitro: The reaction mixture (50 μl) consisted of 50 mM Tris-HCl, pH 8.0, 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, 6 $\mu\text{g/ml}$ actinomycin D to inhibit DNA-dependent RNA polymerase, the ATP-regenerating system consisting of 5 mM phosphoenolpyruvate and 3 U/ml pyruvate kinase, 1 mM each of CTP, ATP, and UTP, 50 μM GTP and 5 μCi of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ or 5 μCi of $[\text{5,6-}^3\text{H}]\text{UTP}$ and 25–50 μl of the cytoplasmic extracts (or nuclear materials) containing 7.5–9 mg/ml protein prepared from infected Vero cells collected at various time points post-infection (e.g. 8, 16, 24, and 32 h). After optimization of RdRp enzyme assays, addition of 7.5 mM K-acetate, 0.5U/ μl RNase inhibitor (RNasin; Promega Biotech) and only 0.5 mM NTPs and 25 μM cold GTP were included in the reaction mixture [58]. After one hour incubation at 37°C , 100 μl of TNE buffer (50 mM Tris-acetate, pH 7.6, 0.1 M sodium acetate, and 1 mM EDTA) containing 4 % SDS was added. After mixing, the RNA was isolated by phenol and CHCl_3 extraction. The labeled RNA products were analyzed by partially denaturing polyacrylamide-urea gel electrophoresis and visualized by autoradiography. Three species of RNA were detected and separated from each other in the following order from the slowest (top) to the fastest migrating species (bottom): partially RNase-sensitive replication intermediate (RI), RNase-resistant replicative form (RF) and completely RNase-sensitive 44S single-stranded virion RNA (44 S vRNA). The RI was predominant band seen as early as 8 h whereas RF was faint. RF reached the maximum level at 24 h and 32 h post-infection time points and 44S RNA appeared only at 32 h post-infection membrane fraction as a faint band. The sequence of accumulation of RNA species in a pulse-chase experiment was seen as $\text{RI} \rightarrow \text{RF} \rightarrow 44\text{S RNA}$ [57, 58]. In a later study [58], addition of 0.5U/ μl RNasin was reported to increase the accumulation of 44 S RNA under standard incubation period (60 min). The temperature of incubation was also reduced from 37°C to 28°C [58]. When the products of RdRp assay were fractionated by LiCl precipitation (described in detail under 2.3.3. below) and analyzed by aqueous agarose gel electrophoresis, RI and RF were not well separated but the 44S single-stranded vRNA was the fastest migrating species [57, 58]. Based on the kinetics of formation of the RdRp products, Chu and Westaway [57] proposed a model consisting of four phases for replication

of flavivirus RNA. In Phase I, semi-conservative synthesis initiated on RF as the template to form RI which becomes the first radiolabeled species in the process of serving as the template for rapid synthesis of mainly (+)strand on the (–)strand template. In Phase 2, release of RdRp and fully displaced unlabeled 44S RNA occur after a short delay. At this stage, RF is labeled in the 3'-end regions. In Phases 3 and 4, subsequent rounds of (+)strand synthesis on labeled RF and displacement of labeled 44 S RNA result in accumulation of labeled 44S (+)strand RNA. In addition, a small increase in labeled RF may be due to small amount of (–)strand synthesis (~10% of the total; [64]). This model does not account for the mechanisms involved in initial synthesis of (–)strand RNA by the viral replicase complex but does provide a molecular basis for the recycling role for RF and relatively high RNase-resistance and slow decay of RI [57].

2.3.2. Protocol for isolation of membrane-bound WNV replication complex in Brinton system

2.3.2.1. Preparation of cytoplasmic and nuclear outer membrane fractions (S1 and S1) from WNV-infected BHK-21 cells: Grun and Brinton [59] reported a detailed study regarding the properties of the cytoplasmic membrane-bound replication complex isolated from WNV-infected BHK-21/WI2 cells [65]. Cells were harvested by scraping into phosphate-buffered saline (PBS) and collected by centrifugation at 800 x g. Cells were allowed to swell for 10 min in the hypotonic buffer with slightly different composition containing 10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM PMSF (Sigma), and 100 kIU of aprotinin/ml, a potent inhibitor of trypsin as well as trypsin-like serine proteases including flavivirus proteases. Cells were lysed by 20 strokes with a Dounce homogenizer. The disrupted cells were pelleted by centrifugation at 1,000 x g for 5 min. The supernatant (S1) fraction containing cytoplasmic and plasma membrane materials was saved on ice. The nuclear pellet was resuspended in 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 100 Kallikrein Inhibitor Unit (KIU) aprotinin/ml and 0.5 mM PMSF (TNAP). The outer nuclear membranes were sheared by passing the suspension of nuclei through a 20-gauge followed by 26-gauge syringe needle 6 x times each. Nuclei and cell debris were pelleted by centrifugation at 1000 x g for 10 min. The supernatant, S2, containing fragments of outer nuclear membranes and S1 fraction (see above) were concentrated in some cases by centrifugation at 200,000 x g for 30 min. The pellets were resuspended in TNAP (100 µl/10⁸ cell equivalents) supplemented with 300 U of RNasin.

2.3.2.2. RdRp assay: One difference between the Westaway and Brinton systems is the storage of the aliquoted cytoplasmic membrane fractions containing RdRp and endogenous RNA. Grun and Brinton reported that storage of the cellular membrane fractions at –70°C for 7 days in the presence or absence of glycerol reduced the polymerase activity more than 50%. Therefore, the membrane preparations were made fresh each time for enzyme activity assays [59]. The reaction mixtures contained Tris-HCl or HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4–8.2, an aliquot of S1 or S2 fraction as the source of viral polymerase and endogenous template, NTPs, one of which [α -³²P]UTP or [α -³²P]GTP, was radiolabeled, monovalent cation, reducing agent, and an ATP regenerating system. The incubations were carried out at 30°C for 15, 30, 60, 90, and 120 min. The radiolabeled RNA products were phenol extracted, precipitated with ethanol,

and analyzed by electrophoresis on agarose gels under non-denaturing conditions. Faint heterogeneous species of viral RNAs appeared at 15 min which became more intense at 30 and 60 min incubation periods [59]. These species represent RI and RF which were not well separated in the gel system employed. A faster migrating genome-length RNA was apparent only after 30–60 min incubation period. By varying the incubation temperature as 4, 20, 30, 40, or 50°C, the optimum temperature was found to be 30°C [59]. The optimum pH was 8.0 using 50mM HEPES or Tris-HCl buffer. Mg^{+2} at 3mM was required but Mn^{+2} at 1.0, 0.5, 0.05 mM in the presence of 3 mM Mg^{+2} inhibited the polymerase activity by about 75, 50, and 25%, respectively. The optimum NTP concentration was next determined. When labeled UTP (17.5 μ Ci of [α - ^{32} P]UTP, 6 pmol, 3000 Ci/mmol) and varying concentrations of unlabeled UTP (0 to 0.5 mM) and 1 mM each of ATP, GTP, and CTP were used, maximum incorporation of [^{32} P]UMP occurred at unlabeled UTP concentration of 10 μ M. The low concentration of unlabeled UTP was not favorable for formation of genome-size RNA. Therefore, unlabeled UTP was used at 25 to 50 μ M [59]. In this study, the optimum parameters of the RdRp assay were established as shown in Table 1. A notable difference between this study and the Westaway system is that the ATP-regenerating system had no effect in the RdRp activity and was therefore omitted [59]. Moreover, when the RI and RF RNA species were eluted from the non-denaturing agarose gel and were re-run on completely denaturing 0.8% agarose gel, a prominent genome-size RNA species along with heterogeneous RNA species migrating slower as well as faster than the genome-size RNA were seen. The smaller heterogeneous RNAs were thought to be the growing nascent strands of RNA from RI species [59]. The origin of the slower migrating RNA species is not clear.

2.3.3. Protocol for isolation and characterization of membrane-bound DENV2 replication complex in RdRp assay

2.3.3.1. Protocol for isolation of membrane-bound DENV2 replication complex: The Westaway's protocol [57] was adopted for isolation of DENV2 replication complex in our laboratory with some modifications and is given below.

1. Grow monkey kidney (LLC-MK2) cells in T x150 cm² flasks in M199 medium supplemented with 10–20% fetal bovine serum (FBS) (higher serum when the stock vial comes out of liquid nitrogen) and 1% L-glutamine at 37°C in a humidified CO₂ (5%) incubator until ~70% confluence is reached. For subculturing, use 0.25% trypsin (w/v)-0.53mM EDTA following a standard protocol (described in ATCC^R manual for LLC-MK2 CCL-7^{TM}).
2. Grow mosquito (C6/36) cells in Dulbecco's Modified Eagle medium supplemented with 10% heat-inactivated (56°C/30 min) FBS, 1% L-glutamine, and 1% non-essential amino acids. For preparation of virus stocks, C6/36 cells are infected with DENV2 virus at a MOI of 10 and incubated in the medium containing 2–5% heat-inactivated serum for ~ 7 days. The supernatant is removed at 7th day post-infection and cells are incubated with fresh growth medium containing 2–5% serum for additional 7 days. The combined supernatants containing DENV2 are aliquoted and stored at –80°C and a small aliquot is used to titer the virus by a plaque assay using LLC-MK2 monolayer of cells. Vero or BHK-21 cells could also be used for plaque assays as described in literature.

3. For preparation of replicase complex, grow LLC-MK2 cells to ~80% confluence in two T-150 flasks. Infect cells with DENV2 (New Guinea C strain) at a multiplicity of infection (MOI) of 5–10. Incubate infected cells for 48 h at 37 °C in a humidified chamber containing 5% CO₂.
4. Remove the growth medium and add 10 ml phosphate-buffered saline (PBS). Using a cell scraper, collect the cells in a 50 ml conical polypropylene centrifuge tube, and spin down at 800 x g for 10 min.
5. Wash once with ice-cold PBS and then with 5 ml cold buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM sodium acetate and 1.5 mM MgCl₂ (TNMg buffer) and spin down at 800x g for 10 min.
6. Resuspend the cell pellet with 1.0 ml cold TNMg buffer (containing 0.5 mM PMSF and 0.5 mM DTT) per T-150 cm² flask in a 1.5 ml Eppendorf tube. Incubate for 10 min on ice. Since this is a hypotonic buffer, cell swelling can be observed under a microscope.
7. Pass the cell suspension through a 1 ml syringe fitted first with a 22-gauge needle followed by a 26-gauge needle, 20 x each, while keeping the Eppendorf tube on ice. This step disrupts the integrity of cells releasing the cytoplasm along with plasma membrane. Monitor cell disruption by a microscope. Centrifuge at 800 x g to pellet nuclei.
8. Aliquot the cytoplasmic extracts, 20µl each, in 0.5 ml Eppendorf tubes. Store the cytoplasmic extract at –70° to 80 °C freezer. A smaller aliquot is used to measure the protein concentration. We have used Bio-Rad protein assay kits (based on modified Bradford or Lowry methods) following the manufacturers' recommended protocols.

2.3.3.2. In vitro DENV2 RdRp assay

1. The components of the in vitro RdRp assay that utilizes membrane-bound replicase complex are given in Table 2. The final reaction mixture (50 µl) contains 20 µg cytoplasmic extract from DENV2-infected cells prepared and aliquoted as described in Section 1.2.1. ATP-regenerating system (by addition of phosphoenol pyruvate and pyruvate kinase; Sigma), Mg⁺² and K⁺, actinomycin D (AMD) to inhibit DNA-dependent RNA polymerase present in the cytoplasmic extracts (viral RdRp is resistant to AMD), 4 NTPs in which [α -³²P]-labeled GTP (or CTP in some experiments) and freshly prepared β -mercaptoethanol (β -ME). The reaction mixture is incubated for one hour at 37°C.
2. Terminate the reaction by the addition of 5 µl of 100 mM EDTA.
3. RNAs associated with membranes are extracted using the RNeasy kit (Qiagen) as follows. Add 55 µl of TNE-SDS buffer (50 mM Tris-acetate, pH 7.6, 0.1 M sodium acetate, 1 mM EDTA and 2% SDS) to disrupt membranes in the reaction. Mix well with a plastic tip by pipetting up-and-down three times and let stand for 10 min.

4. Add 350 μ l RLT (a guanidine-thiocyanate-containing lysis buffer) supplemented with β -ME; mix by pipetting a few times.
5. Add 250 μ l of 95–100% ethanol; mix by pipetting three times.
6. Apply 700 μ l to the column, spin for 30s in small centrifuge; discard flow-through.
7. Transfer the spin column to a new tube and wash with 500 μ l buffer RPE; spin for 30s.
8. Add 500 μ l buffer RPE; spin for 4 min and discard flow-through.
9. Transfer to a new 1.5 ml-tube and add 50 μ l nuclease-free water and spin for 1 min in a refrigerated centrifuge at 4°C. Add water 50 μ l and spin for 1 min. Add 10 μ l of 3M sodium acetate and 275 μ l of 95–100% ethanol. Cool on dry ice for ~30 min.
10. Spin at 4°C for 15 min and discard the supernatant; wipe excess ethanol with a Kimwipe paper and air-dry the RNA pellet.
11. Resuspend the RNA in 10 μ l loading buffer (1 x TBE buffer containing 7M urea and 0.5% Bromophenol blue).
12. Run on 7M Urea/3% PAGE gel at 150V for 2.0 h; no pre-run is required. The gel apparatus is from Bio-Rad (Mini-Protean II Electrophoresis Cell) with specifications of 16 cm (L) x 12 cm (W) x 18 cm (H). The gel size is 7 cm (L) x 8 cm (W) and the glass plate size for casting the gel, (inner) 7.3 cm x 10.2 cm and (outer) 8.3 cm x 10.2 cm.
13. Dry the gel at 80 °C for 1.30 h and visualize radiolabeled RNAs by autoradiography.

2.4. Results and Discussion

2.4.1. Utility of endogenous viral replication complex in screening antiviral compounds—

We have utilized this system in our laboratory to study the effect of antiviral compounds, mycophenolic acid (MPA) and ribavirin, on DENV replication in monkey kidney (LLC-MK2) cells. These antiviral compounds had a profound effect on RNA replication and assembly, and hence the level of membrane-bound replication complex in the DENV2-infected cells treated with the compounds [66]. We showed by performing plaque assays that mycophenolic acid is a very potent inhibitor of DENV replication with an EC50 value of 0.4 ± 0.3 μ M whereas ribavirin had a modest antiviral activity with an EC50 value of 50.9 ± 18 μ M, results consistent with two previous studies on the effect of MPA in inhibition of DENV2 replication in a hepatoma (Hep3B) cell line (EC50 value of 0.3 μ M [67]) as well as inhibition of yellow fever virus 17-D RNA synthesis in Vero cells (EC50 value of 0.625 ± 2504 μ M [68]). The MPA inhibition of viral replication was reversed by addition of guanosine [66, 67] suggesting that the MPA acts by depletion of intracellular pool of GTP by targeting the IMP dehydrogenase [66, 67]. Moreover, we extended the mechanistic study and examined whether MPA affected the activity of the viral replicase to produce the three intracellular RNA species in vitro [66]. Our results showed that RNA synthesis in DENV2-infected and MPA-treated LLC-MK2 cells was reduced at MPA concentration as low as 0.1 μ M and a significant reduction was seen at concentrations of 1

and 2 μM suggesting that MPA reduced the levels of membrane-bound viral replication complex [66]. However, the full potential of the Westaway system in unraveling the steps involved in assembly of the viral replication complex and the sequence of steps involved in the synthesis of flavivirus RNA and nature of the products formed are yet to be explored.

2.4.2. In vitro endogenous membrane-bound DENV2 RdRp assay—The RdRp assays were carried out as described in Section 2.3.3.2. The labeled viral RNA species were extracted from the reaction mixture and analyzed as described above in Section 2.3.3.2. (step 12). At the start of the RdRp assay, the RI was the predominantly labeled species compared to vRNA and RF (Fig. 1, lane 1). In 10–30 min incubation time, vRNA and RF were formed from RI to an almost equal level. As the incubation time was increased to 60–120 min, increased levels of RF were seen at the expense of RI with a modest increase in the level of vRNA (lane 4 versus lane 6). These results suggest that at the point of isolation of membrane-bound viral replicase complex from DENV2-infected cells, the RI species is actively synthesizing and releasing vRNA and concomitantly forming RF species. The re-initiation of RNA synthesis from RF $\rightarrow$ RI appears to be the rate-limiting step under the experimental conditions used resulting in accumulation of RF and a steady decrease in RI species. This observation could be explained due to depletion of ATP during long incubation period in spite of having ATP regenerating system and/or the depletion of other unlabeled NTPs added in the reaction mixture. Continuous supply of ATP may be required for displacement and release of (+) strand RNA (vRNA). Further work is necessary to test this possibility. Our results are similar to the patterns of in vitro RNA synthesis by the replicase complex isolated from KUN virus-infected Vero cells and JEV-infected porcine kidney cell line [58, 69].

Next, we performed pulse-chase experiments in which reaction mixture containing [α - ^{32}P]GTP and the three unlabeled NTPs was incubated for 5 min (Fig. 2A) and 2 min (Fig. 2B), followed by the addition of excess unlabeled GTP. Incubation was continued up to various time points as indicated, and RNAs were extracted and analyzed as described in Fig. 1. As shown in Fig. 2A and 2B, RI that was formed in 5 min and 2 min (lane 1), respectively, was chased to the RF in progressively increasing amounts (lanes 2–6 in Fig. 2A; lanes 2–7 in Fig. 2B), with a modest increase in the formation of vRNA. In the experimental conditions used in Fig. 1, the viral replicase complex from the cytoplasmic fraction was incubated with [α - ^{32}P]-labeled GTP (10 μCi) in the presence of 3 cold NTPs along with the endogenous pool of unlabeled NTPs, the [α - ^{32}P]-labeled GMP incorporation into the products was chased from RI to RF and vRNA species (Fig. 1). However, in the experiments shown in Fig. 2A and 2B, the viral replicase complex from the cytoplasmic fraction was incubated with labeled GTP (10 μCi) in the presence of 3 cold NTPs for 5 and 2 min, respectively, followed by the addition of 164 μM unlabeled GTP (300-fold excess), the [α - ^{32}P]-labeled GMP incorporation into the products was chased from RI to RF and vRNA species. In either scenario, the vRNA formed from RI is rapidly converted to RF resulting in a steady decrease in the level of labeled RI and accumulation of RF.

The experimental evidence that RF that is formed in the vitro replication system is active species to form RI is provided by two studies. First, Bartholomeusz and Wright [71] showed that RF isolated from the DENV2 or KUN replication complex could be converted to RI by

extracts from DENV2 or KUN virus-infected Vero cells and antibodies against NS3 or NS5 inhibited the conversion [71] suggesting that NS3 and NS5 play an important role in replication and that this in vitro system lacks specificity regarding the viral RNA template for RNA synthesis in vitro. This conclusion needs to be further explored in future studies. In the second study, Raviprakash et al. [72] purified RF from RI and vRNA by using LiCl precipitation protocol [58, 71] and showed that labeled RF species could be converted to RI and vRNA by incubation with unlabeled NTPs and cytoplasmic extracts from DENV2-infected Vero cells [72]. We have used LiCl fractionation protocol in our laboratory and works well to isolate RF (see below).

2.4.3. Protocol for isolation of RF

1. Perform RdRp assay in the presence of 10 μ Ci GTP as described in Section 2.2. up to step 9, the first elution with water 50 μ l.
2. Elute RNA species with 50 μ l sterile RNase-free water.
3. Add one volume of 4M LiCl and mix well. Incubate at 4°C 16–24 h.
4. Spin down in a bench top centrifuge (4°C) at 14,000 x g. Both RI and vRNA will be in the pellet fraction and the RF will be in the supernatant.
5. Precipitate the RF fraction in the supernatant by adding one volume of isopropanol.
6. Centrifuge at 14,000 x g. Wash the pellet with 70% ethanol and resuspend in a desired volume of RNase-free water for further characterization.

In our experience, although the supernatant after LiCl precipitation contains relatively pure RF with only traces of RI and vRNA, the pellet fraction could contain ~15% RF co-precipitating with RI and vRNA. If necessary, the mixture in the LiCl pellet fraction could be further purified by repeating the steps 3–6.

2.4.4. Current state of our knowledge and Future Perspectives—Using KUN virus as a model system, Westaway and co-workers reported a number of studies directed toward a detailed characterization of the components of the virus-induced membrane-bound replicase complex (reviewed in [62, 73]). The antibodies against NS1, NS2B, NS3 and double-stranded (ds) RNA were used for immunofluorescence (IF) and cryo-immuno electron microscopy (CIEM) [39]. These proteins and dsRNA were associated with the virus-induced intracellular membrane structures in the perinuclear region. Two types of distinct induced intracellular membrane structures were identified; one of them consisted of dsRNA and NS1 or NS3 and the second consisted of adjacent paracrystalline arrays or convoluted smooth membranes which were detected only with anti-NS2B and anti-NS3 antibodies [74, 75]. The results of incorporation of bromouridine (BrU) into replicating KUN RNA in infected Vero cells treated with actinomycin D and analysis by IF and anti-BrU antibody revealed that the nascent Br-U RNA was localized to the same sites as the dsRNA, and was not accessible to RNase digestion under high salt conditions [75]. Adjacent to these distinct membrane structures, there were other membranes to which none of the antibodies reacted that contained virions [76].

Elegant studies by Bartenschlager's group by using high resolution three dimensional electron tomography (ET) of DENV2-infected cells provided novel insight into the sites of viral replication and assembly into virions within the intracellular virus-induced membrane structures [77]. ET showed the virus-induced membranous structures as a part of an expanded ER-derived network, which also shows vesicle pores through which the newly synthesized RNAs are possibly released. Furthermore, ET revealed that vesicle pores are juxtaposed to virion assembly sites [77].

The body of evidence derived from these studies suggests that translation, polyprotein processing, assembly of membrane-bound viral replication complex, are sequential and coupled processes (reviewed in [40, 62, 73]). The in vitro RdRp assay using membrane bound replication complex containing replicating viral RNA remains an attractive system for studying the biochemical mechanisms involved in assembly of viral replication complex, active synthesis of viral RNA, virion assembly and release of immature virus particles from the ER membrane. However, at present the utility of this system to study steps involved in assembly of replicase complex, virion assembly, release of immature particles, the effects of lethal or temperature-sensitive mutations of *cis*-acting elements and/or of proteins that play critical roles in viral replication has not been explored. Future studies could be focused on development of in vitro methods for assembly of membrane-bound viral replicase complex in the absence of genomic RNA, followed by recruitment of WT and mutant RNAs into those complexes. On the other hand, perhaps the use of the current system is feasible to study the mechanism(s) of viral replication of conditionally defective (e.g. temperature-sensitive) DENV mutants [78–82] by preparing cytoplasmic extracts under permissive conditions and carrying out RNA synthesis at permissive as well as non-permissive conditions to understand the step(s) that is blocked in replication using in vitro assays as well as by high resolution 3D electron tomography [77]. Moreover, the components of the viral replicase complex consisting of viral and host proteins can be dissected and reconstituted to an active form in future studies. In summary, future studies could focus on exploring the full potential of in vitro replication systems that employs membrane-bound replication complexes.

3. In vitro RNA synthesis using exogenous RNA templates

We sought to develop an in vitro RdRp assay that could utilize exogenous positive-sense viral RNA templates that are specific for binding and de novo synthesis of minus-strand RNA. We describe below the construction of cDNA encoding the mini DENV2 RNA containing the *cis*-acting elements that are sufficient for minus-strand synthesis by the cytoplasmic extract from the DENV2-infected cells [83] as well as the purified recombinant full-length DENV2 NS5 [84]. This in vitro system has been very useful in the identification and characterization of the role of *cis*-acting RNA elements that are involved in long distance interaction between conserved sequences at the terminal regions involved in circularization of the viral RNA. Subsequently, this RNA-RNA interaction was shown to be essential for minus-strand RNA synthesis in vitro as well as in replication of the reporter replicons and flavivirus genome RNAs in virus-infected mammalian and mosquito cells. In this review, we will focus on the details of the in vitro systems that utilize a variety of exogenous viral RNA templates developed in several laboratories with emphasis on

experimental conditions, the nature of the products formed and information gained from those systems.

3.1. Conserved sequences (CS) at the 5'- and 3'-terminal ends

Sequence analysis of flavivirus genomes revealed the presence of conserved sequences (CS) near the 5'- and 3'-terminal ends. These sequences, about 11 nucleotides long, are complementary to each other with a potential to form a “pan-handle”-like structure proposed by Hahn et al. [85].

3.2. Construction of DENV2 cDNA encoding subgenomic RNA template for RdRp assay

To explore the role of 5'-CS and 3'-CS1 in RNA synthesis, an *in vitro* RdRp assay was developed. To this end, a DENV2 (New Guinea C strain) cDNA of 720 nt encoding the 5'-terminal 230 nt containing the 5'-CS fused to the 3'-terminal 490 nt was cloned under the T7 promoter control [83, 86]. The RNA of (+) polarity was generated as a run-off transcript of the linearized plasmid clone containing the DEN2 cDNA or by transcription of a PCR product obtained with specific primers. In the former method, depending on the restriction enzyme used for linearization, extra nonviral nucleotides may be present at the 3'-end of the DENV transcript. However, the *in vitro* transcription of cDNA obtained by PCR with specific primers, or by insertion of hepatitis delta virus ribozyme sequence at the 3'-end, one could generate RNA with an authentic 3' end. In our laboratory, we have used both methods to generate *in vitro* transcripts for RdRp assays [84, 87].

3.3. In vitro transcription for preparation of RNA templates

The *in vitro* transcription reactions were carried out at 37 °C for 3 h in reaction mixtures (100 µl) containing the 1X reaction buffer (40 mM Tris-Cl, pH 7.5, 6 mM MgCl₂, 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 DTT, 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. RNA transcripts were extracted with an equal volume of the acid phenol (pH 4.5), followed by addition of an equal volume of chloroform: isoamylalcohol (24:1). The aqueous phase was collected into a new tube and precipitated with 1/10 volume of 3M sodium acetate (pH 5.2) and 3 volumes of 100% ethanol. The precipitated RNA was dissolved in 30–40 µl of RNase-free water. The quality of RNA transcripts was analyzed electrophoretically and quantified spectrophotometrically.

3.4. Preparation of cytoplasmic extract from DENV2-infected C6/36 cells

To prepare the cytoplasmic extract containing DENV2 replication complex for the RdRp assay, C6/36 cells were infected with DENV2 (MOI of 5) in T-150 cm² flasks for 2–3 days at 28°C. Uninfected C6/36 cells were used as a control. Cells were harvested by centrifugation at 800 x g for 10 min and the cell pellet was resuspended very well in 0.5 ml of TNMg buffer (10 mM Tris-HCl, pH 8.0, 10 mM sodium acetate, 1.5 mM MgCl₂) per T-150 cm² flask. After passing the cell suspension 20 x through a syringe (1 ml) fitted with a 27-gauge needle, the cell lysate was centrifuged at 800 x g for 10 min at 4°C to isolate the

cytoplasmic and nuclear fractions. The protein concentration of the cytoplasmic fraction was determined by Bradford method. The extracts were stored at -80°C until use.

3.5. Detection of NS3 and NS5 proteins in the cytoplasmic extract from DENV2-infected cells

An aliquot of the cytoplasmic extract was mixed with an equal volume of a 2X sample-loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.7 M β -mercaptoethanol, 10% glycerol, 0.05% bromophenol blue). Proteins were separated by SDS-PAGE (10%) and transferred electrophoretically to a polyvinylidene difluoride (PDVF) membrane. The membrane was treated with nonfat dry milk (5%) for 2 hours in Tris-buffered saline (TBS) containing 0.1% Tween-20, pH 7.5, and then incubated overnight with polyclonal rabbit anti-NS3 or anti-NS5 antibodies (1:2000). The production and characterization of rabbit polyclonal anti-NS3 and anti-NS5 antisera raised against full-length NS3 and NS5, expressed using recombinant vaccinia virus expression system, has been described elsewhere [88] and the details will be provided on request. The membranes were washed three times for 20 min each with Tris-buffered saline buffer and incubated with horseradish peroxidase-labeled goat anti-rabbit antibody (1:20000) for 2 h at room temperature. NS3 and NS5 bands were detected by chemiluminescence as described by the manufacturer (ECL system; Amersham Pharmacia Biotech).

3.6. The *in vitro* RdRp assay

The *in vitro* RdRp assay [83] was performed with 5 μ g of unlabeled *in vitro* RNA transcript, 10 μ g of the cytoplasmic extract from DENV2-infected C6/36 cells in 50 μ l of reaction volume containing 50 mM HEPES, pH 7.3, 3 mM magnesium acetate, 6 μ M zinc acetate, 25 mM potassium acetate, 60 units of RNase inhibitor, 10 mM β -mercaptoethanol (or 4 mM dithiothreitol), 10 μ M of CTP mixed with 10 μ Ci of α -32P CTP (800 Ci/mmol), 0.5 mM each of ATP, GTP, and UTP, and 0.1 mg/ml actinomycin D to inhibit DNA-dependent RNA polymerase. The control reaction contained all components except the cytoplasmic extract or uninfected cell extract without any exogenous RNA. The reaction mixtures were incubated at 30°C for 1.5 h. The reaction mixtures were phenol-extracted and passed through the Micro Bio-Spin P-30 column in 10mM Tris buffer, pH 7.4 (Bio-Rad, USA) to remove the unincorporated labeled nucleotides. After ethanol precipitation, the RdRp assay products were analyzed by electrophoresis on either a partially denaturing PAGE/7M urea gel or a completely denaturing 1.5% agarose-formaldehyde gel, followed by autoradiography.

3.7. PAGE/7M urea gel electrophoresis

A partially denaturing, 4% PAGE/7M urea gel was used to separate 400–1000 nt RNAs [4% acrylamide:bisacrylamide (19:1), 1X TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA), 0.1% ammonium persulfate, 7M urea, 0.1% TEMED]. After polymerization, the gel was pre-run at room temperature at 200 V for 30 min in 1X Tris-borate-EDTA/7 M urea buffer. After the RdRp assay, RNA products were dissolved in 30 μ l of 10 M urea, followed by mixing with 3 μ l of RNA loading dye (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol). After boiling for 3 minutes, RNA samples were loaded onto a pre-run gel and the gel was run at 230V for ~2.5 hours until the xylene cyanol dye reached

one fourth of the way from the bottom of the gel. The gel was wrapped with saran wrap and exposed overnight to an X-ray film.

3.8. Completely denaturing formaldehyde/agarose gel electrophoresis

To prepare 1.8% agarose gel in 80 ml, 1.44 g of RNase-free agarose was heated in a microwave oven in 1X MOPS/EDTA buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, pH 8.0) before adding 14.4 ml of 37% formaldehyde (final concentration = 2.2 M). The formaldehyde was added after the gel was cooled down to about 50 °C. After polymerization, the gel was pre-run in 1X MOPS/EDTA buffer at 70 V for an hour at room temperature. The RdRp products were dissolved in 2.5 µl of buffer A (3X MOPS/EDTA). Then, 5 µl of formaldehyde/formamide buffer (89 µl formaldehyde, 250 µl formamide) was mixed with the sample, and heated at 70°C for 10 min. The samples were chilled on ice and 2 µl of gel loading buffer was added (322 µl buffer A, 5 mg xylene cyanol, 5 mg bromophenol blue, 400 mg sucrose, then add 178 µl formaldehyde and 500 µl formamide). Then samples were loaded onto the gel and run at 100 V for about three hours at room temperature. The gel was dried and exposed to an X-ray film overnight. To visualize unlabeled RNAs, the gel was rinsed in a large volume of 10 mM phosphate buffer, pH 7.0 for 20 min. The gel was stained with acrydine orange (final concentration of 30 µg/ml) at room temperature for 3 min. After destaining the gel in water for 30 to 60 min with several changes of water, the RNAs were visualized under UV light.

3.9. Analysis of RdRp assay product 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 mg/ml tRNA) either at 37 °C for overnight or at 65 °C for 4 h. After briefly spinning down the 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/ml) in 20 ml of either 2X SSC (300 mM NaCl and 30 mM sodium citrate, pH 7.2) or 0.1X 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 gels, followed by autoradiography.

3.10. Requirement of RNA-RNA interaction between 5'- and 3'-terminal regions of DENV2 subgenomic RNA containing the 5'-CS and 3'-CS1 motifs for RNA synthesis in vitro

An in vitro RdRp assay that could use the exogenous viral RNA templates will be very useful to characterize the sequence and RNA structural requirements for RNA synthesis as well as the proteins that recognize these RNA elements for initiation of RNA synthesis. Initially, the cytoplasmic extracts prepared from DENV2- infected C6/36 cells were used as the source of the viral replicase containing the NS5 RdRp and associated factors. The presence of the two major components of the viral replicase, NS3 and NS5, in the cytoplasmic extract was verified by Western blotting using polyclonal rabbit anti-NS3 and anti-NS5 antibodies [83, 87]. The cytoplasmic extracts from DENV2-infected C6/36 cells were active on exogenously added viral subgenomic RNA template containing 5'-CS and 3-

CS1 RNA elements. The products of the RdRp assay were fractionated on a partially denaturing 4% PAGE/7 M urea gel. The template-size and the hairpin products, the latter twice the size of the template-size RNA, as well as other minor species of labeled RNA, were detected by autoradiography (Fig. 5A in [83]). The time-course analysis revealed the rapid formation of the template-size product, presumably the de novo initiation product, followed by the formation of twice the size of the template (2X) through a number of intermediates within 20–30 min incubation time (Fig. 5A in [83]). These experiments confirmed that the template-size product was formed independent of the 2X product. The template-size (1X) and the hairpin products were further characterized by electrophoresis on a completely denaturing formaldehyde-agarose gel before and after RNase A digestion which converted the hairpin product to the template size product (Fig. 5B in [83]). The polarity of the newly synthesized RNA as the complementary minus-strand was established by hybridization of strand-specific deoxyoligonucleotide primers followed by RNase H digestion [83].

Furthermore, RNA-RNA interactions between the 5'- and 3'-terminal sequences of DENV2 RNA containing the two complementary cyclization motifs, 5'-CS and 3'-CS1, respectively, their respective substitution mutants within the 5'-CS and 3'-CS1 motifs, and a 5'-UTR deletion mutant, were studied by in vitro RdRp assay in the presence of cytoplasmic extracts from DENV2-infected cells [87].

The results showed that deletion of 5'-UTR (96 nt) from the 5'-end of subgenomic RNA template significantly reduced RNA synthesis (Fig. 4A in [87]). The 5'-UTR of DENV2 contains the conserved 5'-stem loop A (5'-SLA), an RNA element that was shown to be important for binding RdRp using a gel shift assay and atomic force microscopy (AFM) [89]. Furthermore, substitution of nonviral nucleotides within the 5'-CS to disrupt basepairing interaction with 3'-CS1 also significantly affected RNA synthesis supporting the conclusion that the conserved cyclization motifs play an important role in RNA-RNA interaction and is required for RNA synthesis from the subgenomic RNA template (Fig. 4B in [87]). The physical interaction between the 5'-CS and 3'-CS1 were studied by using the psoralen-UV cross-linking experiments [87].

3.11. In vitro RNA synthesis using purified RdRp either full-length NS5 or NS5POL domain

3.11.1. Purification of full-length NS5 from *E. coli*—A recombinant DENV1 NS5 protein was expressed as a GST fusion protein cloned into pGEX-KG vector in *E. coli* and was purified using glutathione-Sepharose 4B beads. The NS5 protein was released from the fusion protein after digestion with thrombin [90]. The recombinant protein was active in the in vitro RdRp assay using viral and nonviral RNA templates only after thrombin cleavage [90]. DENV2 NS5 RdRp with an N-terminal His-tag was expressed in *E. coli* and purified by cobalt affinity (Talon^R resin; Clontech, Palo Alto, CA) and gel filtration chromatography [84].

3.11.2. RdRp assay—The polymerase assay using the DENV1 RdRp [90] was performed in 50 µl containing 0.6 to 1 µg of purified NS5, 60 units of human placental RNase inhibitor, 50 mM HEPES pH 7.3, 0.4 mM each GTP, ATP, and CTP, 100 µM UTP and 10 µCi of

[α - ^{32} P]UTP (800 Ci/mmol), 4 mM DTT, 3 mM magnesium acetate, 6 μ M ZnCl₂ and 1 μ g of an in vitro transcribed RNA template. The RNA template sequences with or without poly(A) tails were of varied length and were generated by T7 RNA polymerase-catalyzed in vitro transcripts from cDNA clones from NS5 gene [90].

For the DENV2-full-length NS5 RdRp assay [84], the enzyme reaction contained 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, template RNA (1 μ g), 500 μ M each of ATP, GTP, and UTP, 10 μ M unlabeled CTP, and 10 μ Ci of [α - ^{32} P]CTP along with 200 ng of purified NS5 in 50 μ l. The incubation was at 30 °C for 1 h and terminated by acid phenol/chloroform extraction, followed by ethanol precipitation in the presence of yeast tRNA (5 μ g) as a carrier. The RNA was purified by centrifugation, the pellet was dried, and was resuspended in 50 μ l of nuclease-free H₂O. The unincorporated nucleotides were removed from RNA by passing through a Bio-Rad P-30 column. RNA was precipitated with ethanol and was analyzed by formaldehyde-agarose gel electrophoresis and visualized by autoradiography. The labeled bands were quantified by liquid scintillation counting and/or analyzed by densitometry. RdRp assays at different temperatures were carried out using a gradient thermocycler (Tgradient, Biometra, Gottingen, Germany).

3.11.3. RNA synthesis by purified NS5 RdRp on the exogenous viral and nonviral RNA templates—The results of DENV2 RdRp activity assay indicated that RNA synthesis was dependent on the exogenous addition of subgenomic RNA template. Similar to the results of the RdRp assay that is described in Section 3.6 in which cytoplasmic extracts from DENV2-infected C6/36 cells were used, two products were also formed with the purified RdRp. One had the size of the input RNA template, and the second had a mobility consistent with twice the size of the template [84] as analyzed by formaldehyde-agarose gel electrophoresis. The NS5 RdRp had essentially no template activity in the presence of non-specific RNA in vitro transcripts from pTM1 vector [84] or the DENV2 3'-UTR RNA template [84, 89]. On the other hand, the in vitro transcript from the DENV2 3'-UTR with a 4-nt extension at the 3'-end complementary to the 5'-XbaI overhang produced RNA product suggesting the template RNA conformation at the 3'-terminus could potentially influence binding and RNA synthesis by RdRp [83]. The 5'-terminal 230nt RNA (5'TR_{230nt}) alone was active in RNA synthesis by the RdRp [83, 84, 91]. When the 5'-terminal 230nt RNA was mixed with 3'-UTR RNA, the latter gained the template activity for RNA synthesis by RdRp (Fig. 4B, lane 3 in [84]). These results suggested that functional RNA-RNA interaction between the 5'TR_{230nt} containing 5'-CS) and 3'-UTR_{451nt} RNA trans-activated the inactive 3'-UTR to an active conformation suitable for initiation of RNA synthesis by RdRp. Mutational analysis of 5'-CS and 3'-CS1 motifs individually to disrupt basepairing between the 5'-CS and 3'-CS1 RNA elements or together to restore basepairing showed that complementarity between these two sequences is important for RNA synthesis from 3'-UTR [84]. Filomatori et al. showed that the 5'-TR folds into a conserved 5'-stem-loop A (5'-SLA), an RNA element that recruits the RdRp and promotes RNA synthesis, and that 3'-UTR containing the conserved 3'-step-loop structure (3'-SL) represses RNA synthesis at the 3'-end. The interaction between the two elements relieves the repression for RNA synthesis by changing the conformation of the 3'-UTR RNA [89, 91].

The 2X product obtained using DENV2 subgenomic RNA [83, 84] was converted to the template-size product by RNase A digestion supporting that the RdRp product was a 3'-fold-back synthesis from the template RNA. The polarity of the de novo (1X) and 2X products was determined by RNase H digestion after annealing with strand-specific deoxyoligonucleotides (Fig. 4) [83, 84].

Other studies showed that both full-length NS5 as well as NS5 polymerase domain alone were able to utilize homopolymeric RNA template poly (rC) with [³H]GTP in the presence or absence of rGG primer to produce [³H]GMP labeled poly(rG) [92]. Moreover, a subgenomic DENV2 (NGC strain) RNA template containing 224 nt from the 5'-end fused to 492 nt from the 3'-end of viral RNA was active in the in vitro RdRp assay producing a template-size and 2X template-size products [92]. Baculovirus-expressed KUN NS5 was also active in utilizing 3'-terminal ~3kb RNA and full-length 9 kb KUN replicon RNA as well as a 8300 nt 'non-specific' Semliki Forest virus (SFV) replicon RNA template [93].

The RdRp assay with the purified NS5 was used to confirm the functional RNA-RNA interaction between the 5'-CS and the 3'-CS1 by substitution mutagenesis of the two motifs either individually (to disrupt the interaction) or together to restore base-pairing with non-viral nucleotides. The mutation of the 5'-CS significantly affected the RNA synthesis and the template activity [84]. However, the substitution mutagenesis of 3'-CS1 was somewhat tolerated as the activity was reduced by ~50%. When the basepairing between both 5'-CS and 3'-CS1 motifs were restored with nonviral sequences, the RNA synthesis was also restored supporting the conclusion that a functional interaction between 5'-CS and 3'-CS1 is required for initiation of RNA synthesis in vitro at the 3'-end of DENV2 RNA.

Similar results were obtained with West Nile virus subgenomic (+) strand RNA templates containing 5'- and 3'-terminal sequences that included 5'-CS and 3'-CS1 [94]. The polarity of the RNA product synthesized using the (+) strand RNA template was determined by a slightly modified protocol from the one previously described for DENV2 [83, 84] (see Fig. 4A and 4B). On the other hand, there were no differences in level of RNA synthesis from the subgenomic (–) strand RNA templates containing WT or mutant sequences complementary to 5'-CS and 3'-CS1 motifs [94].

3.11.4. Protocol to determine the polarity of the RdRp products

1. Perform WNV RdRp assay as described in Section 3.6 for DENV2 RdRp [84] with slight modifications. The assay mixture (50 µM) contained 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl₂, template RNA (0.3 µg), 500 µM each of ATP, GTP, and UTP, 10 µM unlabeled CTP, and 10 µCi of [α-³²P]CTP along with 270 ng of purified WNV NS5 (2.6 pmol). The incubation temperature was 25 °C, to favor de novo synthesis of RNA over 3'-fold back synthesis by elongation [95].
2. Extract with phenol/chloroform and pass through P-30 gel filtration column to remove unincorporated NTPs.
3. Divide the RdRp reaction mixture after step 2 equally into two fractions. To one fraction, add 200 ng of RNase A (Sigma) in 20 µl of 2 X SSC and incubate at 30 °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). This step removes excess unlabeled single-stranded (+) strand RNA template used for RdRp assay. It also digests the single-stranded loop from the hairpin RNA product as well as any 3'-labeled single-stranded RNA product of putative terminal transferase activity of NS5 RdRp (Fig. 4A).

4. Digest with proteinase K (Sigma; 40 µg) for 30 min at 37°C and extract with phenol/chloroform and precipitate with ethanol in the presence of 10 µg of yeast tRNA (Ambion).
5. Mix with 1.5 µg of either unlabeled WNV (+)RNA_{1051nt} or WNV (–)RNA_{1051nt} complementary to the template used in the RdRP reaction, denatured at 95 °C for 5 min, and reanneal in a hybridization buffer (80% formamide, 40 mM PIPES, pH 6.8, 1 mM EDTA, 400 mM NaCl) at 42°C overnight. RNA samples are then precipitated with ethanol and resuspended with 20 µl of RNase H buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 10 units of RNasin (Promega), 4 units of RNase H (Thermostable; Epicenter Technologies), 200 µM oligodeoxynucleotide of either (+)- or (–)-strand polarity).
6. Incubate the reaction mixture at 45 °C for 1 h. Terminate the reaction by addition of one volume of 2X stop buffer (100 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.5% SDS). RNA was extracted with phenol/chloroform; precipitate with ethanol, and analyze by formaldehyde agarose gel electrophoresis as described in Section 3.8.

3.11.5. RNA synthesis by de novo initiation and elongation is temperature-dependent—The influence of temperature in RNA synthesis by RdRp in vitro was studied using DENV2 subgenomic RNA as a template [84, 95]. The rationale for this approach is that flavivirus RNAs replicate very efficiently in mosquitoes or in cells from the established cell line, C6/36 at 28 °C, whereas virus replication in mammalian hosts occurs at 37°C. The conformation of RdRp in the viral replicase complex is likely to be different in these two hosts.

Flavivirus RdRps perform de novo synthesis of RNA without using a primer. The enzyme exists in a “closed” conformation in which a “priming loop” in the thumb domain closes the active site precluding template RNA binding ([96, 97]; reviewed in [98]). De novo initiation by RdRp involves the formation of a stable complex by binding of initiating NTP(s) and the template RNA and synthesis of short primers de novo, complementary to the 3'-end of template RNA [99]. Then, the enzyme switches to “open” conformation and continues RNA synthesis in the elongation mode. As shown in Fig. 5, NS5 RdRp produced 2-fold more de novo (1X) product from the subgenomic RNA at 28°C than at 37–40°C and the formation of hairpin RNA by self-primed synthesis predominates at higher temperatures [84, 95]. The conformation of NS5 could change at the priming loop allowing binding of template RNA in the “self-priming” mode.

Recently, it was shown that DENV2 NS5 RdRp synthesizes AG dinucleotide primer de novo in the presence of DENV2 10-mer of (+) or (–) strand polarity as the template in the presence of ATP and GTP and Mg⁺² ion. However, in the presence of other 10-mer

templates of (+) sense polarity with different dinucleotide sequences at the 3'-end, pppAG was not formed in the presence of Mg^{+2} ion. However, in the presence of Mn^{+2} ion, pppAG was formed even in a template-independent manner. In addition, the RdRp could still form pppAG in the presence of Mn^{+2} and the 10-mer templates of (+) polarity with incorrect 3'-terminal nucleotides, thus able to maintain the correct dinucleotide sequence [100]. From the crystal structure of DENV RdRp [101], the priming loop consists of residues 792–804 providing the priming site for de novo initiation [100]. The priming site mutants were created by deletion of residues between T793 and K800 and substitution of two glycines (T₇₉₃-G-G-K₈₀₀) as well as by substitution of W795A or H798A [100]. The active site occlusion was relieved in all the NS5 mutants. The mutants adopted a more “open” conformation and produced 4- to 5-fold more 2X hairpin product than the WT NS5 on DENV2 mini genome RNA template in the presence of either Mg^{+2} or Mn^{+2} ion [100]. The TGGK and H798A mutants were also unable to synthesize pppAG in the absence or presence of a DENV2 10-mer of 3' (–) strand template in the presence of Mg^{+2} ion in contrast to WT RdRp. In the presence of DENV2 10-mer 3' (+) strand template and Mn^{+2} ion, TGGK mutant NS5 was unable to initiate de novo and form pppAG but was able to form pppGA, an internal initiation product from the 2nd nucleotide. Thus, the capability of specific de novo initiation with ATP as the priming nucleotide from the template CU-3' was lost [100]. These results are consistent with the observations reported in a previous study that short deletions from the 3'-end of DENV2 genome or substitution mutant (pppGAG→pppAG) at the 5'-end were repaired to WT sequence in infected LLC-MK2 cells [102].

It was reported that NS5POL domain alone encoding 272–900 amino acid residues expressed in *E. coli* had low de novo initiation activity in vitro and was also thermolabile. However, the NS5POL domain containing amino acids 265–900 which included the linker region between the methyltransferase and RdRp domains was more stable and active in de novo synthesis of RNA [103]. In that study, a fluorescence-based alkaline phosphatase-coupled polymerase assay (FAPA) was employed using a subgenomic RNA template containing 5'-UTR, 66 nt from capsid gene, 19 nt nonviral linker sequence followed by 3'-UTR [104, 105]. The RdRp generated predominantly template-size de novo product [105] suggesting that nature of the DENV2 mini genome template influences the product(s) formed in the in vitro RdRp assay.

3.11.6. Heparin trap experiment to distinguish *de novo* initiation versus elongation phase of RNA synthesis using subgenomic DENV2 RNA templates

—To isolate the requirements for formation of stable de novo initiation complex consisting of NTPs, NS5 RdRp, and template RNA from those required for elongation reaction, we modified the heparin trap protocol [106] for use in our system [84, 86]. The principle of this method is to mix NS5 RdRp, RNA template, and a partial set of NTPs required for formation of a stable de novo initiation complex by omission of one or two NTPs from a complete system. After incorporation of a few nucleotides, the RdRp stalls due to the absence of missing nucleotide(s). Addition of heparin and the missing nucleotides, any free polymerase that is not bound to the template RNA is “trapped” and becomes inactive in RNA synthesis whereas the enzyme in a stable complex switches to elongation mode and

continue RNA synthesis. To identify the optimum temperature that promotes initiation versus elongation phase of RNA synthesis by NS5, the RdRp assay was modified as follows.

In the first protocol, subgenomic RNA template was incubated for 10 min with 500 μ M each of ATP, GTP, and CTP, 10 μ Ci of [α - ^{32} P]CTP, and 200 ng of purified NS5 at various temperatures to allow addition of 6 nucleotides. Heparin (50 ng) was then added (2 μ l of 25 ng/ μ l) and incubated for 5 min before the addition of UTP (500 μ M). Reactions were then allowed to complete elongation synthesis by incubation at 30.8°C for 60 min. Addition of heparin will inactivate the RdRp not bound to the template RNA in a stable complex and only one round of RNA synthesis occurs by the processive RdRp.

- 2 In the second protocol, RNA template was incubated at a constant temperature of 30.8 °C for 10 min with 500 μ M each of ATP, GTP, and CTP, 10 μ Ci of [α - ^{32} P]CTP, and 200 ng of purified until the addition of heparin.
- 3 Incubate for 5 min and then add 500 μ M UTP. Continue incubations at differing temperatures for 60 min [84].

3.11.7. Heparin trap protocols to establish the right combination of NTPs and their concentrations required for *de novo* initiation versus elongation phase of RNA synthesis—The approach described in Section 3.11.6 showed the feasibility of using partial incorporation of NMPs during initiation phase before the addition of heparin and follow up on the processive RNA synthesis to completion [84]. We used this approach to identify that addition of NS5, RNA, ATP, GTP (500 μ M each) alone followed by incubation at lower temperatures that were needed to form a stable *de novo* initiation complex before the sequential addition of 10 μ M [α - ^{32} P]CTP, heparin, and UTP producing 2-fold higher level of 1X product (Fig. 1 in ref. [95]).

3.11.8. Results and Discussion—The results of the first protocol described in Sections 3.11.6. step 1 showed that at lower temperatures of incubation with template RNA, NS5, and limiting NTPs that allowed initial phase of RNA synthesis prior to addition of heparin favored *de novo* RNA synthesis. Increasing temperatures of incubation favored self-primed RNA synthesis from the template RNA. On the other hand, in the second protocol, incubation with template RNA, NS5, and limiting NTPs at a constant temperature of 30.8°C, followed by addition of heparin and UTP and incubation at various temperatures resulted in equal amounts of *de novo* and 3'-foldback RdRp products (Fig. 8 in [84]). These results support the conclusions that temperature of incubation in the RdRp assay influenced the nature of the products formed by the polymerase from an RNA template, which has a propensity to assume different conformational states at different temperatures (see for example, [107, 108]).

Additionally, RdRp could assume two distinct conformational states and use *de novo* or 3'-fold back template. The flavivirus RdRp have roughly the shape of a right hand with fingers, palm, and thumb subdomains, and the inner surfaces of the three subdomains form a central template-binding channel. Flavivirus RdRps that initiate RNA synthesis via a *de novo* mechanism have large thumb subdomains and a C-terminal loop (residues 790–804) within the same domain that encircles the active site. This arrangement restricts access to the

template-binding channel, allowing only ssRNA to access the active site during *de novo* initiation. This structure likely represents a ‘closed’ polymerase form, that needs to ‘open’ during elongation, where the C-terminal loop has moved away to allow template and nascent RNA to exit the active site [98]. Consistent with the idea, the biochemical studies described above could be explained by two distinct conformational states of the RdRp. The RdRp can adopt a ‘closed’ conformation around the 3′-end of the template in the *de novo* initiation complex, generating a 1X product. When the RdRp switches to an ‘open’ conformation, it can accommodate the 3′-fold back template and generate the 2X product.

A series of RdRp assays were performed to determine the NTP concentrations that are required for *de novo* initiation versus 3′-end elongation synthesis of RNA from the subgenomic RNA template [95]. The RdRp assays were performed in which three NTPs are kept at fixed concentrations while the fourth NTP concentration was varied. The results showed that while high GTP concentrations (500 μM) were required, 10 μM ATP, UTP, and CTP, are sufficient, to produce equal amounts of *de novo* (1X) and 3′-end elongation (2X) product. Under lower concentrations of GTP, only 3′-end elongation (2X) product was preferentially formed. At GTP concentrations of 50 and 100 μM, the ratio of 1X to 2X was only 0.04 and 0.15, respectively. Previous studies with RdRps of Brome Mosaic Virus (BMV) [109], Qβ [110] and HCV NS5B [111–113] revealed that high concentrations of GTP were required for *de novo* initiation of RNA synthesis whereas for influenza virus RdRp high concentration of ATP was required [114].

Selisko et al. [100] reported in an elegant study that DENV NS5 RdRp has a specific priming site that binds ATP and synthesizes in a template-independent manner, a dinucleotide primer, AG, in the presence of Mn^{+2} ion. The priming loop residues, T794–A799, especially W795 and H798, in the thumb domain that are conserved in all flavivirus RdRps, are involved in the primer synthesis. H798 is required for *de novo* initiation and stabilization of ATP binding. Flavivirus RNA genomes have highly conserved dinucleotide sequences AG at the 5′-end and CU at the 3′-end. Selisko et al. showed that a 10-mer sequence from the 3′-end of (+) and (–) strand RNA, which lack any secondary sequence, can serve as templates for *de novo* synthesis of RNA.

3.11.9. Deletion of nucleotides sequentially from the 3′-end of subgenomic RNA template reduces the foldback synthesis of RNA—Next, we investigated the effect of deletion of nucleotides sequentially from the 3′-end of the subgenomic RNA on *de novo* and 3′-foldback synthesis of RNA. As shown in Fig. 6, deletion of 10 nucleotides (nt) from the 3′-end of the template RNA reduced significantly the template activity in 3′-foldback synthesis of RNA without affecting the *de novo* RNA synthesis, whereas deletion of 14–22 nt significantly reduced RNA synthesis from the template. Sequence analysis of the minus strand synthesized by NS5 could reveal the site of *de novo* initiation from the 3′-end of deletion mutant templates. In the context of DENV2 infectious clone, we showed that deletion of up to 10-nt was not lethal for replication and the virus was able to repair the deletion from the 3′-end during viral replication using the complementary RNA strand in the 3′-SL structure whereas deletion of 14–22 nt were lethal for virus growth [102].

3.11.10. RNA-RNA Interaction and cyclization of viral RNA mediated by 5'- and 3'-terminal cis-acting elements studied by other biochemical and cell-based assays

1. Gel mobility shift assays: Alvarez et al. [115] studied the RNA-RNA interaction between the 5'- and 3'-terminal ends using Electrophoretic Mobility Shift Assay (EMSA). Four fragments from the 3'-UTR of DENV2 (strain 16681), variable region, VR, A2, A3, and 3'-SL, were uniformly radiolabeled with [³²P] using in vitro transcription and purified by 5% PAGE containing 7M urea. The binding reaction with the increasing amounts of unlabeled 5'-UTR-C62 (nt 1–160 containing the 5'-CS) and constant amount of uniformly labeled 3'-SL RNA fragment from the 3'-UTR (last 106 nt of the DENV2 RNA containing 3'-CS1) in 5 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl₂, .8% glycerol, 2.5 µg tRNA as described in detail [115]. The results of EMSA indicated that the high affinity between the two fragments with a *K_d* of 8 nM. The 5'-UTR-C62 carrying a deletion of 5'-CS or point mutations containing more than 4 mismatches that disrupted basepairing were unable to form stable interaction with 3'-SL RNA.

3. Cyclization of the RNA fragment containing 5'-CS and 3'-CS1 by atomic force microscopy (AFM): Alvarez et al. [115] showed using atomic force microscopy (AFM) for the first time that a model artificial RNA containing a double-stranded region of 1 kb flanked by 5'-single-stranded region of 863 nt that includes 5'-terminal 160 nt of DENV2 RNA and 3'-UTR of 451 nt was able to circularize in the absence of any proteins. To test if full-length DENV2 RNA can be visualized in a circular form, DENV2 RNA was transcribed in vitro and made partially double-stranded in the region encoding nucleotides 6971 to 10272. AFM analysis showed that both the model RNA and the full length RNA could exist in both linear and circular forms [115].

4. Role of other cis-acting elements, 5'-3' UARs and 5'-3'-DARs in RNA-RNA interaction and viral replication: The cyclization of flavivirus RNA requires RNA-RNA interaction between 5'- and 3'-terminal sequences containing 5'-CS and 3'-CS1 and also another pair of cis-acting elements, known as 5'-Upstream of AUG Region (5'-UAR) and its complementary 3'-UAR. The 5'-UTR encodes an RNA structural element, 5'-stem loop A (5'-SLA), which was shown to be important for RNA synthesis and recruits the RdRp by binding at SLA and positions the 3'-end in close proximity for initiation of RNA synthesis [89].

These RNA elements play a crucial role for RNA synthesis in vitro and viral replication in mammalian and/or mosquito cells [91, 115–117].

Using flavivirus reporter replicon and infectivity (plaque) assays, it was subsequently shown that RNA-RNA interaction between the 5'-CS and 3'-CS1 is essential for RNA synthesis and viral replication in mammalian or mosquito cells [115, 118–122]. Although substitutions with nonviral sequences within the 5'-CS and 3'-CS1 were somewhat tolerated, the growth of mutant viruses were much slower than WT supporting that specific viral sequences besides the complementarity between 5'-CS and 3'-CS1 are required for viral replication

[118–120, 123]. In addition, a motif named as 5'-downstream of AUG region (5-DAR) and its complementary sequence, 3'DAR ([124], are also required for viral replication.

3.12. Concluding remarks

The RdRp is a remarkable enzyme encoded by a number of viruses that have both (+) or (–) sense single-stranded as well as double-stranded RNA genomes. It is absolutely required for replication of their respective genomes in their natural hosts. In this review, we focused on its enzymatic role in RNA synthesis as a component of multimeric complex, which includes other viral and cellular proteins in association with membranes (reviewed in [40, 62]). In some cases, it has also been reported that the flavivirus RdRp is localized in cellular compartment(s) other than the site of viral replication and/or in phosphorylated form. For example, in DENV2- and Yellow fever virus-infected cells, NS5 is phosphorylated and localized in the nucleus of infected mammalian cells [37, 125–128] whereas WNV NS5 is phosphorylated but is localized in the cytoplasm [129]. The role of nuclear NS5 or the function of phosphorylated form in viral replication is unknown at present. Mosquito-borne flavivirus NS5 N-terminal domain is also endowed with enzyme activities, 5'-guanylyltransferase [29] and 5'-RNA methyltransferase [31–33] that are required for 5'-capping. Our knowledge regarding how these enzyme activities are coordinated in the genome replication complex in membrane compartments is far from complete. Further work concomitant with development of elegant assays is necessary to unravel the mechanisms of NS5 in these multiple functions in flavivirus-infected cells.

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Highlights

- Flavivirus RNA replicates in the virus-induced membrane compartments in the cytoplasm.
- Methods are given to measure activity of flavivirus RNA polymerase in RNA synthesis in vitro.
- We discuss future research perspectives to expand our knowledge in this field.

RdRp assay: Incubation time of complete system

Fig. 1. DENV2 RdRp assay on endogenous membrane-bound viral replicase complex containing viral RNA

The reaction conditions are described in the text and the components of the RdRp assay are given in Table 1. Total protein concentration in the cell lysate was 20 $\mu\text{g}/\text{assay}$. The incubation temperature was 37°C. After addition of 10 μCi [α - ^{32}P]-GTP, samples were removed at various time points and the reaction was terminated by addition of 5 μl of 100 mM EDTA. RNA products were extracted, fractionated by a partially denaturing polyacrylamide gel electrophoresis and visualized by autoradiography as described in Section 1.2.2. Lanes 1–6 represent samples removed at 0, 10, 30, 60, 90, and 120 min and termination of RNA synthesis. The faint bands of RI and vRNA were probably due to rapid formation of products before termination of the reaction. Three distinct species of RNA were detected by autoradiography as reported for KUN [57] and JEV [69]. RI, partially RNase-sensitive, the slowest migrating replicative intermediate, vRNA, completely RNase-sensitive single-stranded vRNA, and RF, fastest migrating, RNase-resistant, double-stranded replicative form.

Fig. 2A RdRp assay: Pulse for 5 min and chase with cold GTP

Fig. 2B

RdRp assay: Pulse for 2 min and chase with cold GTP

Fig. 2. Pulse-chase experiment to study the interconversion of RNA species

A. The complete RdRp reaction mixture was incubated with radiolabeled [α - ^{32} P]GTP for 5 min and in B, for 2 min, followed by the addition of 300-fold excess of unlabeled GTP (164 μ M) to each reaction. The reaction continued to indicated time point, followed by the addition of 5 μ l of 100 mM EDTA to terminate the reaction. The three species of RNA were analyzed as described in Fig. 1 legend. The lanes 1–6 represent 0, 5, 15, 30, 45, and 60 min samples removed after the addition of unlabeled GTP. In B, lane 7 represents 120 min time point.

Fig. 3. Template specificity of the purified DENV2 NS5

“This figure was originally published in Journal of Biological Chemistry. Matt H. Ackermann and R. Padmanabhan. J. Biological Chemistry. 2001; 276: pp. 39926–39937. © the American Society for Biochemistry and Molecular Biology.” Each RdRp reaction contained NTPs, [α - ^{32} P]CTP, 200 ng of purified NS5 protein, and indicated templates. Lanes 1 and 2: Sg (subgenomic) RNA, and 3'-UTR RNA. Lanes 3 and 4: 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. The products of RdRp assay were fractionated by agarose-formaldehyde gel electrophoresis and detected by autoradiography as described [84].

Figure 4A

Figure 4B

Fig. 4. RNase H mapping of newly synthesized RNA

"This figure and the legend were originally published in Journal of Biological Chemistry. Masako Nomaguchi, Tadahisa Teramoto, Li Yu, Lewis Markoff, and R. Padmanabhan. J. Biological Chemistry. 2004; 279: pp. 12141–12151. © the American Society for Biochemistry and Molecular Biology." A: Schematics for RNase H mapping. RdRP products (step 1) were synthesized from (+)-strand (or (-)-strand; not shown) RNA template by WNV NS5. RdRP products were incubated either with or without RNase A under the high salt conditions (step 2). After RNase A treatment, the mixtures were denatured and annealed with the WNV (-)-strand RNA (step 3). The (+)-strand RNA template used for the RdRP reaction will be displaced from the labeled (-)-strand RNA and anneal with the excess unlabeled (+)-strand RNA. The free single-stranded labeled (-) RNA product was annealed to the single-stranded oligodeoxynucleotide primer of either (+)- or (-)-strand polarity and digested with RNase H as described under "Experimental Procedures" (step 4). The products of RNase H digestion were analyzed by formaldehyde-agarose gel electrophoresis followed by autoradiography. The template RNA is shown by a thin line and the labeled newly synthesized RNA as a thick line. TTase, terminal transferase. B. RdRP reactions contained 270 ng (2.6 pmol) of NS5, 0.3 µg each of WNV(+)/RNA_{1051nt} as a template (lanes 1–5) and all other components as described under "Experimental Procedures" in [94]. The reactions were incubated at 25 °C for 1 h. Then, the four steps outlined in A were carried out. Lanes 1–5, RNase H was added to all RdRP reaction

products. Lane 1, no further treatment; lane 2, RNase A treatment; lane 3, annealing with unlabeled (–)-strand RNA_{1051nt} to remove (+)-strand RNA template annealed to the labeled product RNA; lane 4, all of the components as in lane 3 and oligodeoxynucleotide primer of (+)-strand polarity was added; lane 5, same as in lane 4 except the DNA primer of (–)-strand polarity was added. Products of RNase H were analyzed by formaldehyde-agarose gel and visualized by autoradiography. The mobility of RNA size markers are indicated on the left-hand side of the gel.

Effect of temperature on de novo initiation versus elongation by "copy-back" mechanism

Fig. 5. De novo synthesis of RNA by RdRp is temperature-dependent

“This figure was originally published in Journal of Biological Chemistry. Matt H. Ackermann and R. Padmanabhan. J. Biological Chemistry. 2001; 276: pp. 39926–39937. © the American Society for Biochemistry and Molecular Biology.” A, Purified RdRp was incubated with subgenomic RNA for 90 min at varying temperatures as described under “Experimental Procedures” in [84]. Lanes 1–8: Incubation temperatures were, 20°C, 23.6°C, 27.3°C, 29.1°C, 30.9°C, 32.7°C, 36.4°C, 40.0°C, respectively. Products were separated 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”

in [84]. The average ratio of de novo (1X) to 3'end foldback synthesis product (2X) from three separate experiments were plotted as shown.

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Fig. 6. In vitro template activity of sgRNAs containing WT or 3'-end deletions

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Authors: Tadahisa Teramoto, Yukari Kohno, Praveena Mattoo, Lewis Markoff, Barry Falgout, and R. Padmanabhan. Title: Genome 3' end repair in dengue virus type 2. in *RNA* 14(12):2645- 56, 2008. Epub Oct 30, 2008. A. sgRNAs containing WT or 3'-end deletions were analyzed for template activities in vitro using purified NS5. (A) sgRNAs containing 4, 8, 10, 14, 17, and 22 nt deletions (lanes 1–6, respectively) from the 3'-end of subgenomic RNA. Lane 7 is a no-RNA control. The products of the reaction were separated by electrophoresis and visualized by autoradiography. 1x: De novo product. 2X: ds RNA (hairpin) product. EtBr stained gel shows approximately equal amount of RNA loading. B–D: Quantification of total RNA (B), 2X product (C), and de novo product (D).

Table 1 Reaction conditions for in vitro WNV RdRp activity[§]

Components	conditions
HEPES or Tris	50 mM; pH 8.0
MgCl ₂	3–10 mM (3)
Ammonium acetate	50–100 mM (50)
KCl	5–10 mM (5)
Dithiothreitol	5–10 mM (10 mM)
NTPs	
[α - ³² P]NTP	(17.5 μ Ci; 6 pmol)
competing unlabeled NTP	25–50 μ M (50 μ M)
Non-competing NTPs	2 mM each
Aprotinin	100 kIU/ml
PMSF	50 μ M
RNase inhibitor (RNasin)	4 U

[§]These parameters are from ref. [59].

The range for optimum RdRp activity is given with the amount used in a standard reaction.

Table 2

In vitro RdRp assay using membrane-bound DENV2 replicase complex

Stock	Reagent (final)	Volume: μ l
500mM [¶]	50mM Tris-HCl, pH8.0	
100mM [¶]	10mM magnesium acetate	5
75mM [¶]	7.5mM potassium acetate	
1mg/ml	60 μ g/ml AMD (Sigma)	3
500mM	10mM β -mercaptoethanol ¹	1
200mM	5mM phosphoenolpyruvate (Sigma)	1.25
1351.35units/ml	3 units/ml pyruvate kinase (Sigma)	0.2
40U/ μ l	40 units of RNasin (Promega)	1
2.5mM each	0.4mM rATP, CTP, UTP	8
10 μ Ci/ μ l	10 μ Ci ³² P-GTP	1
	Nuclease-free water	4.55

¹ β -mercaptoethanol was prepared fresh.

[¶] A master mix of the three buffers was prepared and 5 μ l was used/tube.

All the components were mixed in the quantities given above in a total of 25 μ l/tube. Cell extracts (20 μ g/tube) and nuclease-free water were added to a final volume of 50 μ l. The reaction was incubated at 37°C for 60 min.