F1 Motif of Dengue Virus Polymerase NS5 Is Involved in Promoter-Dependent RNA Synthesis

Running Title: dengue virus RNA synthesis

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ABSTRACT

The mechanism by which viral RNA-dependent RNA polymerases (RdRp) specifically amplify viral genomes is still unclear. In the case of flaviviruses, a model has been proposed that involves the recognition of an RNA element present at the viral 5’ untranslated region (named SLA) that serves as promoter for NS5 polymerase binding and activity. Here, we investigated requirements for specific promoter-dependent RNA synthesis of the dengue virus NS5 protein. Using mutated purified NS5 recombinant proteins and infectious viral RNAs, we analyzed the requirement of specific amino acids of the RdRp domain on polymerase activity and viral replication. A battery of 19 mutants were designed and analyzed. By measuring polymerase activity using non-specific poly(rC) templates or specific viral RNA molecules, we identified four mutants with impaired polymerase activity. Viral full-length RNAs carrying these mutations were found to be unable to replicate in cell culture. Interestingly, one recombinant NS5 protein carrying the mutation K456A and K457A located in the F1 motif, lacked RNA synthesis dependent on the SLA promoter, but displayed high activity using a poly(rC) template. Promoter RNA binding of this NS5 mutant was unaffected, while de novo RNA synthesis was abolished. Furthermore, the mutant maintained RNA elongation activity, indicating a role of the F1 region in promoter-dependent initiation. In addition, four NS5 mutants were selected to have polymerase activity in the recombinant protein but delayed or impaired virus replication when introduced into an infectious clone, suggesting a role of these amino acids in other functions of NS5. This work provides new molecular insights on the specific RNA synthesis activity of the dengue virus NS5 polymerase.
INTRODUCTION

Dengue virus (DENV) is the single most significant arthropod-borne virus pathogen in humans. It belongs to the *Flaviviridae* family together with other important pathogens such as yellow fever virus (YFV), West Nile virus (WNV), Saint Louis encephalitis virus (SLEV), and Japanese encephalitis virus (JEV). The World Health Organization continues reporting every year dengue outbreaks in the Americas and Asia. In spite of the urgent medical need to control DENV infections, vaccines and antivirals are still unavailable. Although a model for DENV RNA synthesis was previously proposed (17), molecular aspects of the mechanism by which the polymerase specifically amplifies the viral genome are still unclear for DENV and other flaviviruses. To further understand this viral process, we investigated functional properties of the viral polymerase NS5.

NS5 is the largest of the flavivirus proteins (105 kDa); it contains an N-terminal methyltransferase domain (MTase) and a C-terminal RNA-dependent RNA polymerase (RdRp) domain. The MTase is responsible for methylation of the cap structure present at the 5' end of the viral genome. This process involves methylation in two positions, guanine N-7 and ribose 2'-O (12, 13, 28, 38). The RdRp domain has primer independent (*de novo*) RNA synthesis activity (1, 26). Interaction of NS5 with a promoter element present at the 5' end of the genome is necessary for specific RNA synthesis (17, 21). This promoter element is known as stem-loop A (SLA) and corresponds to one of the two RNA structures present at the viral 5' untranslated region (UTR) (for review see (34)). The other element, stem–loop B (SLB), contains a sequence that is complementary to a region present...
at the 3'UTR, which is involved in long range RNA-RNA interactions and cyclization
of the viral RNA (3, 4). Previous studies have demonstrated that DENV genome
cyclization is necessary for relocating the promoter-NS5 complex, formed at the 5'
end, to the 3' end initiation site (17).

The crystal structure of the DENV 3 RdRp has been recently solved (35). Similar to
other template-dependent polymerases, the DENV RdRp resembles a right hand
containing fingers, palm, and thumb subdomains. In contrast to DNA polymerases,
the DENV RdRp contains an encircled active site with extensive interactions
between the fingers and thumb subdomains (named “fingertips”), resulting in a
protein that adopts a “closed” conformation (35). A similar closed conformation was
observed for different RdRps such as Hepatitis C virus (HCV), bovine viral diarrhea
virus (BVDV), bacteriophage phi6, foot-and-mouth disease virus (FMDV) and
reovirus (2, 7, 10, 11, 15, 20, 31). The general fold of the DENV RdRp is similar to
that of the reported structure of the WNV protein (22, 35). In the thumb sub-domain
a priming-loop was identified, which differentiates primer-independent RdRps
(HCV, BVDV, WNV, DENV) from primer-dependent enzymes (FMDV and rabbit
hemorrhagic disease virus (RHDV)) (14). The priming loop is thought to provide the
initiation platform stabilizing the \textit{de novo} initiation complex (7, 10). Furthermore, as
shown for other RdRps, the template tunnel in the DENV protein has dimensions
that would permit access only to single stranded RNA, suggesting a conformational
change associated to duplex RNA formation during RNA synthesis (35). Based on
biochemical and structural studies, a model was proposed in which the DENV
RdRp has a closed conformation during initiation, and after a short oligonucleotide
is synthesized, a switch to an open conformation occurs, allowing elongation to proceed (1).

DENV RdRp activity was observed using viral RNAs of 160 nucleotide-long carrying the SLA promoter (17). Longer RNAs were also active as templates, however, this activity was dependent on complementary sequences at the 5' and 3' ends of the RNA, indicating that a circular conformation of the template was necessary (17). It has been reported that poly(rC) homopolymers, but not non-viral heteropolymeric RNAs, were active as templates for DENV RdRp activity (17, 29). Nevertheless, it is unclear how specific viral RNA elements facilitate flavivirus RNA synthesis.

In this work, we used recombinant DENV NS5 and genetically modified full-length DENV clones to investigate the requirement of positive-charged amino acids in the RdRp domain for SLA promoter-dependent RNA polymerase activity. A battery of 19 mutations in NS5 were designed and polymerase activity was evaluated using non-specific poly(rC) or specific viral RNA templates. Four mutants were found that lacked polymerase activity with both templates. Interestingly, we identified one mutant carrying a two amino acid substitution (K456A and K457A, located in the F1 motif) that lacked RNA synthesis dependent on the SLA, while displaying high activity using the poly(rC) template. This mutant was able to elongate a primer but was unable to initiate de novo RNA synthesis using the natural viral RNA. All the inactive NS5 mutants identified retained the ability to interact with the viral RNA with high affinity. In addition, these mutations impaired viral replication when introduced into a DENV2 infectious clone. Our results provide new information
about amino acids in the DENV RdRp involved in specific promoter-dependent
initiation of RNA synthesis.

MATERIALS AND METHODS

Protein expression and purification

Nucleotide fragments containing the coding region of full-length NS5 and the RdRp
domain were PCR amplified from the infectious clone of DENV2 strain 16681
(GenBank accession number U87411) and cloned into the plasmid pQE-30 at
BamHI and HindIII sites, adding a 6-histidine tag at the N-terminus of the proteins.
The proteins were expressed in *E. coli* (Rosetta [pLacI]) overnight at 20°C after
induction with 100 μM IPTG. Lysis of the cells was carried out using French-press
in binding buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 1 mM DTT,
and 10% glycerol) in the presence of DNase I and protease inhibitor cocktail
(SIGMA). After centrifugation the supernatant was loaded on a His-Trap Nickel
sepharose affinity column (GE Healthcare), washed twice with binding buffer plus
100 mM imidazole, and the proteins were eluted with binding buffer containing 300
mM imidazole. The proteins were dialyzed against 20 mM Tris-HCl buffer, pH 7.5,
300 mM NaCl, 10% glycerol, and 1 mM dithiothreitol. The dialyzed protein solution
was further purified by size exclusion chromatography using a Superdex 200
column. Proteins were stored at –20°C in dialysis buffer containing 40% glycerol.

For mutant recombinant proteins, the oligonucleotides used to introduce each
mutation are given in table below. Overlapping PCRs were performed with the
external primers AVG611 (GCGCATGGATCCCGGAACTGGCAACATAGGAGAG)
and AVG612 (CAGATTAAGCTTGTCGACCTACCACAGAACTCCTGCTT) using
the cDNA of the 16681 infectious clone. All mutants were cloned into the plasmid pQE-30 at BamHI and HindIII sites, similar to that described for the wild-type protein. And purified following the protocol described above.

Oligonucleotides used for overlapping PCRs:

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Orientation</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>R279-K282</td>
<td>For</td>
<td>GGGAAACCCATAGAAGCAATAAAGCCAGGAC</td>
<td>GCCTTGCGCTTTATGCTCCTTCCTGTTGGCTCCAAATATATC</td>
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<tr>
<td>K300</td>
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<tr>
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<td>For</td>
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<td>TGCTGACCCAGTCTGTGCTTCTTCAATACCTT</td>
</tr>
<tr>
<td>R325</td>
<td>Rev</td>
<td>CAGGTTTTGTCACGCGGAGCTTGACCATTTG</td>
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</tr>
<tr>
<td>R361-K370</td>
<td>For</td>
<td>AAAGTGCGACAGGCAACCAAAGCAGAGGAGCGACGCGGCAACTAATGAAAA</td>
<td>TTTCCTGCTCTCTCTGCTGCTGCTGTTGCTCTTTTTT</td>
</tr>
<tr>
<td>K386-K388</td>
<td>Rev</td>
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<td>CTGGTGAAATCTTGTGCGTTGTGCAATATCAAG</td>
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<tr>
<td>R391-R395</td>
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<td>CTGCTGATCCAGTCTGTGCTGTTTCATAGCTACCAT</td>
</tr>
<tr>
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<td>Rev</td>
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</tr>
<tr>
<td>R438</td>
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<td>K456-R457</td>
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<td>Rev</td>
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*For, forward; Rev, reverse.*
Construction of recombinant dengue viruses

The mutations were introduced into a DENV type 2 cDNA (19) (GenBank accession number U87411) clone by replacing the StuI-AvrII or AvrII-HpaI fragment of the wild type plasmid with the fragment obtained by overlapping PCR.

Mobility shift assay

RNA-protein interactions were analyzed by electrophoretic mobility shift assays (EMSA). Uniformly $^{32}$P-labeled RNA probes were obtained by in vitro transcription using T7 RNA polymerase and purified on 5% polyacrylamide gels and 6 M urea. Purified PCR products were used as templates for the in vitro transcription. The probe corresponding to the 5'DV (nucleotides 1 to 160) was PCR amplified from the 16681 DENV cDNA with the primers AVG 213 (TAATACGACTCACTATAAG) and AVG 130 (GTTTCTCTCGCGTTTCAGCATATTG). 5'DV-ΔSLA was amplified using the primers AVG 213 and AVG 130 from a plasmid containing the deletion of the first 70 nucleotides (17). Capped RNAs were transcribed using the PCR products as templates in the presence of an m7GpppA cap analog (New England Biolabs). The binding reaction mixture contained 5 mM Heps (pH 7.9), 25 mM KCl, 2 mM MgCl$_2$, 3.8% glycerol, 0.12 mg/ml heparin, 0.1 nM $^{32}$P labeled probe, and increasing concentrations of the protein. For binding competition experiments increased concentrations of purified 5'DV or 5'DVΔSLA RNAs were added to the binding reaction mixture containing 20 nM of NS5 and 0.1 nM of radiolabeled 5'DV probe. RNA-NS5 complexes were analyzed by electrophoresis through native 5% polyacrylamide gels supplemented with 5% glycerol. Gels were prerun for 30 min.
at 4°C at 120 V, and then 20 µl of sample was loaded and electrophoresis was allowed to proceed for 5 h at constant voltage. Gels were dried and visualized by autoradiography. The macroscopic dissociation constants were estimated by nonlinear regression (Sigma Plot), fitting Equation 1: Bound % = Bound$_{\text{max}}$ · [Prot] / (K$_d$ + [Prot]), where Bound % is the percentage of bound RNA, Bound$_{\text{max}}$ is the maximal percentage of RNA competent for binding, [Prot] is the concentration of purified NS5, and K$_d$ is the apparent dissociation constant.

**Polymerase activity assay**

The standard in vitro RdRp assay was carried out in a total volume of 25 µl in buffer containing 50 mM Hepes (pH 8.0), 10 mM KCl, 5 mM MgCl$_2$, 10 mM dithiothreitol, 4 U RNase inhibitor, 500 µM (each) ATP, CTP and UTP, 10 µM [$\alpha$-$^{32}$P] GTP, 0.5 µg template RNA, and 0.2 µg of recombinant purified protein. The reaction mixture was incubated at 30°C for 30 min and stopped by adding a denaturing solution to a final concentrations of TCA 7% (wt/vol) and 50 mM H$_3$PO$_4$ at 0°C. The TCA-precipitated RNAs were then collected by vacuum filtration using a V-24 apparatus, with the mixture carefully added onto the center of a Millipore filter (type HAWP, 0.45-µm pore size). The filters were washed eight times with 5 ml each of cold 7% (wt/vol) TCA-50 mM H$_3$PO$_4$, dried, and the radioactivity was measured. The polymerase assay on homopolymeric poly(rC) template was carried out in a total volume of 25 µl in buffer containing 50 mM Hepes (pH 8.0), 10 mM KCl, 5mM MnCl$_2$, 5 mM MgCl$_2$, 10 mM dithiothreitol, 4 U RNase inhibitor, 10 µM [$\alpha$-$^{32}$P] GTP, 3 µg poly(rC) RNA and 0.2 µg of recombinant purified protein. The
detection was the same as the standard RdRp assay. The elongation assay mixture contained 50 mM Hepes (pH 8.0), 10 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol, 4 U RNase inhibitor, 500 μM CTP, and UTP, 10 μM [α-³²P] ATP, 50 ng of template RNA (5'-UGUUAUAAUAUUUUAGGUUCU-3' IDT), 10 ng of primer (AGAA, Dharmacon), and 0.2 μg of recombinant purified protein, in a total volume of 30 µl. The reaction was incubated at 30°C for 60 min. The reaction was ended by phenol extraction followed by ethanol precipitation. The RNA products of the polymerase assay were resuspended in Tris-EDTA containing formamide (80%) and heated for 5 min at 65°C. The samples were then analyzed by electrophoresis on a 20% denaturing polyacrylamide gel-6 M urea and visualized by autoradiography.

**Periodate treatment and poly-(A) polymerase activity**

The periodate treatment was carried out as described previously (37). Briefly, 5'DV RNA was incubated for 1h at room temperature in 40 mM NaOAc, pH 5.0, and 20 mM NaIO₄. Then, lysine (60 mM) was added and the mixture was incubated 3h at room temperature. The treated RNA was purified using an RNeasy minikit (Qiagen, Inc.) and quantified spectrophotometrically. Its integrity was verified by electrophoresis on agarose gels. The poly-(A) polymerase reaction was carried out following the manufacturer’s protocol (New England Biolabs).

**RNA transfection and immunofluorescence**

To obtain infectious DENV RNAs, in vitro transcription by T7 RNA polymerase in the presence of an m7GpppA cap analog was used as previously described (3). BHK-21 cells were transfected with 3 μg of viral RNA using Lipofectamine 2000
(Invitrogen). The RNAs were treated with RNase-free DNase I to remove the templates and purified using an RNeasy minikit (Qiagen, Inc.) to eliminate free nucleotides. The products were quantified spectrophotometrically, and the integrity of the RNAs was verified by electrophoresis on agarose gels.

Cells transfected with WT or mutated full-length DENV RNA were used for immunofluorescence (IF) assays. BHK-21 cells were grown in 35-mm diameter tissue culture dishes containing a 1-cm² coverslip inside. The coverslips were removed and directly used for IF analysis. The transfected cells were trypsinized on day 3, and two-thirds of the total cells were reseeded to a 35-mm diameter tissue culture dish containing a coverslip. This procedure was repeated every 3 days until a cytopathic effect was observed. At each time point, a 1:200 dilution of murine hyperimmune ascitic fluid against DENV2 in phosphate buffered saline–0.2% gelatin was used to detect viral antigens. Cells were fixed in methanol. Alexa Fluor 488 rabbit anti-mouse immunoglobulin G conjugates (Molecular Probes) was used as detector antibody at a 1:500 dilution. Photomicrographs (x200 magnification) were acquired with an Olympus BX60 microscope coupled to a CoolSnap-Pro digital camera (Media Cybernetics) and analyzed with Image-Pro Plus software.

**Viral RNA extraction and sequencing**

Viral RNA was extracted with TRIzol (Invitrogen) from a 300 μl aliquot of the medium from transfected cells. The RNA was reverse transcribed by Superscript II reverse transcriptase (Invitrogen) for 1 h at 42°C using random primers (Promega).

In each case, reverse transcription-PCR (RT-PCR) products were sequenced

RESULTS

NS5 binding to capped and uncapped RNA molecules

The RdRp domain of NS5 binds specifically to the DENV 5'UTR (17, 21). In addition, the MTase domain recognizes the cap structure present at the 5' end of the genome (12, 13). Thus, we hypothesized that NS5 could bind capped 5'UTR molecules with higher affinity than uncapped RNAs. To investigate this possibility, we expressed the recombinant DENV NS5 and the RdRp domain and performed gel shift assays with radiolabeled RNAs corresponding to the first 160 nucleotides of the viral genome (5'DV, Fig. 1A).

The 5'DV RNA was titrated with increasing concentrations of NS5 or RdRp protein. As we previously reported, the RdRp domain bound the RNA with an apparent Kd of 12 ± 4 nM, which was similar to the affinity observed for the full NS5 protein (14 ± 3 nM) (Fig. 1A). Then, we analyzed the binding of NS5 to capped and uncapped 5'DV RNA. Protein binding was similar for both RNAs (Fig. 1B), suggesting that MTase interaction with the cap structure did not contribute significantly to RNA binding affinity of NS5. It is possible that the contribution of the MTase domain to RNA binding is not detectable due to the high affinity of the RdRp domain to the SLA. To examine this possibility, we deleted the 70-nucleotide SLA and tested the binding of NS5 to the capped and uncapped 5'DVΔSLA RNA. In our experimental conditions, no binding of NS5 to the RNA was observed (Fig. 1C). We conclude...
that binding of NS5 to capped or uncapped RNAs is mainly driven by interaction of
the protein with the SLA structure.

**Different requirements for NS5 RNA synthesis using specific and non-
specific templates**

It has been previously shown that flavivirus RdRps use specific viral RNA
templates carrying the SLA promoter but they also use non-specific poly(rC)
templates (17, 29). In order to investigate the requirements for specific and non-
specific activity of the DENV RdRp, we compared RNA synthesis activity in
different conditions. The presence of Mg\(^{2+}\) is crucial for catalysis, however, it was
previously reported that poly(rC) RNA only functions as template in the presence of
Mg\(^{2+}\) plus Mn\(^{2+}\) (29). Mn\(^{2+}\) usually alters biochemical properties of polymerases,
decreasing the stringency of the substrate selection and incorporation fidelity (5). In
the presence of Mg\(^{2+}\) and absence of Mn\(^{2+}\), the DENV NS5 showed polymerase
activity using the 5'DV RNA as template while it was almost undetectable when the
poly(rC) RNA was used (Fig. 2A). We also analyzed RNA synthesis in the
presence of Mg\(^{2+}\) but absence of Mn\(^{2+}\) at different concentrations of GTP using the
viral RNA and as control a ΔSLA RNA (Fig. 2B). In these conditions, the 5'DV-
dependent polymerase activity reached a maximum at 12 µM GTP (Fig. 2B). We
also compared the RdRp activity using poly(rC) as template, in the presence or
absence of Mn\(^{2+}\), using increasing concentrations of GTP (Fig. 2C). In the
presence of Mn\(^{2+}\), we observed high polymerase activity even at low GTP
concentrations, while in the absence of Mn\(^{2+}\), polymerase activity was only
observed at concentrations of GTP above 200µM. The results indicate that NS5
requires either Mn$^{2+}$ or high GTP concentration for RNA synthesis using the non-
specific poly(rC) template. Although Mn$^{2+}$ increases polymerase activity (29), this
cation was not a requirement for RNA synthesis using the viral 5’DV RNA carrying
the promoter SLA.

It has been previously reported that in vitro RNA synthesis of flavivirus RdRps
display two activities; one corresponding to de novo RNA synthesis and the second
one consistent with elongation of the template (1, 26, 29, 30). To analyze whether
NS5 initiated de novo RNA synthesis using the 5’DV RNA, we treated the 5’DV
RNA with sodium periodate to block the 3-OH group of the template (37). This
treatment avoids elongation of the template mediated by a terminal nucleotidyl
transferase activity (32). As a control, the templates treated or not with sodium
periodate were incubated with a commercial poly(A) polymerase. This enzyme was
able to elongate efficiently the untreated template but was inactive with the
periodate-treated RNA (Fig. 2D, left panel) indicating that the treatment was
efficient. Then, the two RNAs were used as templates for NS5. Both RNAs yielded
a similar product (Fig. 2D, right panel). We conclude that, in our experimental
conditions, the DENV NS5 carried out de novo initiation of RNA synthesis using the
viral 5’DV RNA as template.

Identification of elements in NS5 necessary for SLA-dependent RNA
synthesis

DENV NS5 binds to the SLA RNA and this interaction promotes RNA synthesis.
Specific structures of the SLA that include a top loop and a side stem loop were
found to be necessary for genome replication in infected cells and polymerase
activity in vitro (16, 17, 21). Although there is substantial information about RNA elements necessary for SLA promoter function, it is still unknown which domain(s) of NS5 are responsible for promoter-dependent RNA synthesis. In a recent study, it has been proposed that binding of NS5 to the viral RNA is not sufficient for polymerase activity (16). We hypothesized that specific SLA-NS5 contacts could induce conformational changes in the protein that would facilitate initiation of RNA synthesis. To investigate whether there are amino acids in NS5 that would be involved in SLA-dependent activity, a mutational analysis was performed substituting conserved positively charged residues present on the surface of NS5 to alanine. RNA synthesis activity was analyzed in parallel using the 5'DV RNA or the poly(rC) template. Nineteen substitutions were designed mainly based on the crystal structure of the DENV3 RdRp (35) (Fig. 3). The mutations were introduced into a bacterial expression vector to produce the recombinant NS5 proteins, and into the DENV2 infectious clone to analyze viral phenotypes.

The recombinant proteins were purified using metal affinity and size exclusion chromatography. Five mutants were insoluble (M6, M7, M9, M14, and M16), while the remaining 14 NS5 mutants were soluble and stable (Fig. 4A). Eight NS5 mutants were active with both 5'DV and the poly(rC) templates (M2, M3, M4, M13, M15, M17, M18, and M19, Fig. 4B and C). M5 showed about 60% of the WT activity with both templates. In addition, M1, M8, M11, and M12 lacked polymerase activity. Interestingly, M10 including the substitution K456A-R457A displayed selective polymerase activity. This mutant showed almost WT levels of polymerase activity using the poly(rC) template, whereas it was unable to synthesize RNA
using the 5'DV RNA (compare M10 in Fig. 4B and 4C). Amino acids K456 and 
R457 are located in the F1 motif of the F region, which has not been solved in the 
DENV3 RdRp structure (see Fig 8) (35). It has been previously proposed that the 
F1 motif of viral RdRps could be involved in interacting with the RNA template 
and/or incoming nucleotides (8, 11, 15). This motif has been also defined in 
different flavivirus RdRps (22, 29). However, its functional role during RNA 
synthesis remains unknown. The high polymerase activity observed with the 
poly(rC) RNA indicated that the M10 protein was properly folded, suggesting that 
K456 and R457 are involved in promoter-dependent RNA synthesis.

Viral replication of the 19 NS5 mutants was also analyzed in BHK cells by 
transfecting full-length mutated DENV RNAs. Viral replication was assessed by 
immunofluorescence as a function of time using specific anti-DENV2 antibodies. 
Cells transfected with the WT RNA were nearly 100% antigen-positive at day 3 and 
showed cytopathic effect and cell death after day 4. The five NS5 mutants that 
lacked in vitro polymerase activity with the 5'DV template (M1, M8, M10, M11, and 
M12) and four of the five insoluble mutants (M7, M9, M14, and M16), in the context 
of the infectious clone, abolished DENV replication (Fig. 5). NS5 M6 mutant that 
was insoluble when expressed in E. coli, in the context of the DENV genome, 
resulted in a virus with a delayed replication phenotype, indicating that the protein 
was folded and active in mammalian cells. The lower in vitro polymerase activity 
observed for the mutant M5 correlated well with its slow replicating phenotype (Fig. 
5). In addition, four NS5 mutants (M4, M13, M18, and M19) that were fully active in 
the in vitro assay were delayed or impaired in vivo, indicating that the mutations
could affect other important roles of NS5 in infected cells. To investigate whether spontaneous mutations were associated to the replication of mutants with delayed propagation, viruses M4, M5, M6 and M18 were recovered at 6 and 9 days after transfection, and the purified viral RNA was used for sequencing analysis. The replicating viruses retained the original mutations within NS5 and showed wild type sequences at the 5' and 3'UTRs. Because M10 was particularly interesting, we also searched for spontaneous mutations that would revert the phenotype by passing the transfected cells up to 21 days. To this end, we transfected the RNAs corresponding to WT, mutant M10 (K456A-R457A), and the individual mutants M10.1 (K456A) and M10.2 (R457A). Viral replication was not detected with the mutated RNAs and no revertant viruses were rescued (data not shown).

Based on our mutational studies, we conclude that amino acids in the F1 motif of NS5 are necessary for SLA-dependent RNA synthesis in vitro and for viral replication in transfected cells.

**RNA binding and elongation activity of NS5 mutants with impaired RNA synthesis activity**

We further analyzed the defects of the mutants in NS5 that lacked RNA synthesis. The NS5 mutants have substitutions in basic amino acids. Because these residues could be involved in template recognition, we tested the ability of mutants M1, M8, M10, M11, and M12 to bind the viral 5'DV RNA using gel shift assays. Increasing concentrations of each of the recombinant purified proteins was incubated with the radiolabeled 5' DV RNA. The estimated dissociation constants were $16 \pm 2$ nM, $13 \pm 4$ nM, $14 \pm 3$ nM, $15 \pm 2$ nM, and $18 \pm 2$ nM for the M1, M8, M10, M11, and M12,
respectively. Representative gels of EMSAs and the data fitting are shown in Figure 6A. The estimated Kds were similar to that observed with the WT NS5 protein, indicating that the mutations did not affect binding to the promoter RNA. Because M10 showed a promoter dependent defect, we further analyzed its binding specificity to the viral RNA in a competitive binding mode. To this end, we incubated a radiolabeled 5′DV RNA with the amount of WT or M10 NS5 to bind about 50% of the probe, and mixed it with increasing concentrations of competitor RNA. Two RNA molecules were used as competitors, the 5′DV RNA containing the first 160 nucleotides of the viral genome and the same molecule with a deletion of the promoter SLA (5′DVΔSLA). Mobility shift assays showed that the 5′DV RNA competed in a concentration-dependent manner for binding the WT or the M10 protein (Fig. 6B). The 5′DVΔSLA RNA did not compete even at 500 fold excess, indicating that the RNP complex formed between the 5′DV and the WT or the M10 protein was specific (Fig. 6B).

To further characterize the polymerase activity of the M10 mutant, RNA synthesis was measured using the 5′DV RNA as template under optimal conditions observed for the poly(C) dependent non-specific activity (Fig. 2C). We determined polymerase activity using increasing GTP or Mn²⁺ concentrations. The M10 mutant was inactive even at 500 µM GTP (Fig. 7A). In contrast, RNA synthesis was detected in the presence of Mn²⁺. The highest activity was observed at 4 mM Mn²⁺, reaching about 40% of the activity observed for the WT protein, suggesting that the phenotype of this enzyme was partially suppressed by Mn²⁺ (Fig. 7A).
The M10 mutant carries two amino acid substitutions (K456A and R457A). To investigate which residue was responsible for the defect in polymerase activity, recombinant NS5 proteins carrying the individual substitutions, mutant M10.1 (K456A) or M10.2 (R457A), were designed, expressed, and purified (Fig. 7B, left panel). The individual mutants conserved full activity using poly(rC) RNA as template (Fig. 7B, middle panel). In contrast, using the specific 5'DV RNA both mutated proteins showed reduced activity (Fig 7B, right panel), indicating that amino acid substitutions in both positions greatly decrease promoter-dependent polymerase activity.

Previous studies support a model for DENV RdRp RNA synthesis that involves an initiation step, which includes the synthesis of a short oligonucleotide primer, followed by a conformational change necessary for the elongation step (1, 23). Thus, we investigated whether the NS5 M10 mutant was able to elongate a primer. In addition, because mutations M1, M8, M11, and M12 impaired NS5 polymerase activity, they were included in the analysis. To this end, we optimized an elongation activity assay using an RNA template of 24 nucleotides and primers of different lengths (Fig. 7C). Primers of 2, 3, 4, and 5 nucleotides were used. Only the 4-nucleotide long primer was efficiently used by the DENV NS5 protein for elongation (Fig. 7C). This result is in agreement with a previous observation (26) and indicates that the elongation activity is independent on the promoter element (SLA). Using this assay, the elongation activity of M1, M8, M10, M11, and M12 mutants together with the WT protein was evaluated. Interestingly, only M10 was able to elongate the primer, while the other mutants were inactive (Fig. 7D).
These studies indicate that the NS5 mutants are able to bind the 5’DV RNA. In addition, the M10 NS5 was the only mutant with elongation activity, suggesting that amino acids in the F1 motif of NS5 are involved in a promoter dependent pre-elongation step during viral RNA synthesis.

DISCUSSION

The mechanism by which flavivirus RdRps initiate RNA synthesis is still unclear. Although in vivo and in vitro studies have demonstrated the requirement of the SLA promoter element at the 5’ end of the genome for RNA replication, it is unknown how binding of this RNA structure to the RdRp facilitates the initiation process. It was originally proposed that the promoter would provide specificity by bringing the polymerase to the viral RNA; however, we have recently reported that binding of the SLA to the RdRp is not sufficient for polymerase activity (16). Specific contacts between the SLA and the RdRp were necessary, suggesting a post-binding activation of the polymerase. Here, we found differential cation and GTP concentration requirements when the DENV RdRp used a homopolymeric or the viral RNA as template (Fig. 2). Previous studies with the HCV RdRp revealed a specific GTP-binding site in a shallow pocket at the molecular surface of the enzyme at the interface between fingers and thumb. The position of this site suggested a possible role of GTP either in triggering a conformational change or in stabilizing an active conformation for efficient initiation (7). We found that high GTP concentration or Mn^{2+} was necessary for DENV NS5 RNA synthesis using poly(rC) but not with the viral RNA carrying the SLA element (Fig. 2). Previous studies...
showed that DENV RdRp required 500 µM GTP concentration for *de novo* initiation but not for the elongation step (26). In our studies, we were able to detect initiation of RNA synthesis at 12 µM GTP concentration with the 5′DVRNA (Fig. 2B and 2D).

A mutational analysis of the RdRp domain of DENV2 NS5, in which basic regions on the surface of the protein were substituted to alanine, identified NS5 mutants with high RNA synthesis activity in vitro but delayed or impaired replication in vivo (M4, M13, M18, and M19, Fig. 4 and 5). M4 included the substitution of R325, which comprises part of the α2 helix, a region that connects the fingers with the thumb sub-domain. This region is part of a nuclear localization signal (NLS) of NS5, which could be affected by the mutation (9, 18, 27). M13 contains two amino acids changes (R519 and 523) which are located in the α12 helix present in the palm domain, while M18 and M19 include substitutions in the α23 and α26 respectively, for which roles in NS5 function have not been previously investigated.

It is likely that these amino acid changes in NS5 that result in active enzymes in the in vitro assay alter other protein functions in the infected cell. It has been reported that during DENV infection NS5 interacts with host and viral proteins. It has been demonstrated that NS5 interferes with the innate antiviral cell response by binding and inducing STAT2 degradation (6, 24). In addition, NS5 interacts with the viral NS3 protein. NS3 has RNA helicase activity, which could modulate RNA synthesis in the replication complex. Also, in vitro studies reported that NS5 stimulates the NTPase and RNA triphosphatase activities of NS3 (36). More information about the specific regions of NS5 involved in protein-protein interactions during viral replication is necessary to better understand the structural requirements of NS5.
The mutant M5 showed both delayed viral replication and reduced polymerase activity in vitro. Therefore, the slow replication phenotype observed (Fig. 5) can be explained by a defect in the enzymatic function of the protein. However, because the mutated amino acids 361 and 370 are located in the loop connecting $\alpha$5 and $\alpha$6 included in the NLS region, they may also cause a defect in NS5 localization (35). Five NS5 mutants were found to lack polymerase activity in vitro and impair viral replication in transfected cells (M1, M8, M10, M11, and M12, Fig. 4 and 5). These mutants bound the viral RNA with high affinity (Fig. 6A). The lack of polymerase activity observed with M1 is in agreement with a previous report with WNV NS5, which demonstrated an important role of the region encompassing amino acids 273-316 for RdRp activity. Structural analysis indicated that these amino acids were involved in maintaining the integrity of a 3 stranded $\beta$ sheet of the fingers domain (35). M8 contains the substitution R400A and K401A, which are amino acids located in the $\alpha$7 connecting helix between the fingers and thumb domains that could have a structural function. Importantly, mutant M10, which included the substitution K456A-R457A in the F1 motif, displayed altered promoter-dependent RNA synthesis. Amino acids 454 to 466 were not observed in the electron density map and were not included in the reported model of the DENV3 RdRp structure (35). Interestingly, the ordered part of the F region observed in the structure in DENV and WNV run perpendicular to that observed for the F region of other related RdRps such as HCV and BVDV (22, 35) (Fig. 8). If we assume that this region plays a similar role in these polymerases, a conformational change
would be necessary to create the proposed NTP or template-binding site in the DENV RdRp (for review see (23)).

It has been proposed that viral RdRps adopt a catalytically inactive form, which can be transformed to a catalytically competent state by binding primers, templates, divalent metal ions and/or nucleotide triphosphates (25). Thus, two conformational changes in the protein can be associated with RNA synthesis. The first change would yield a catalytically active protein, and the second one would occur during the transition between the initiation and the elongation modes. The mutant M10 was active for elongation, indicating that mutation K456A-R457A is not affecting catalysis but is altering a promoter dependent pre-elongation step. The defect observed with this mutant was partially suppressed by Mn$^{2+}$ but not by 500 μM GTP, suggesting that Mn$^{2+}$ is not functioning by increasing the affinity of the enzyme for GTP. It has been previously reported that Mn$^{2+}$ promotes nucleotide misincorporation, which could explain the activity of the mutant (5). However, we speculate that binding of Mn$^{2+}$ could facilitate a conformational change in the mutant protein that occurs naturally in the WT enzyme.

In our working model, we propose that interaction of specific nucleotides of the promoter SLA with NS5 induces a conformational change in the protein, which involves the F1 motif, yielding a catalytically active enzyme. Crystallization of the SLA-RdRp complex will be necessary to provide the molecular information necessary to understand in depth this viral process. Structural and functional studies of the flavivirus NS5 protein in the last few years provided a great deal of information about viral RNA synthesis (for review see (23)). However, molecular
details of the specific interactions between NS5 and the viral genome during the initiation process are still missing. We have recently provided a new model for the dynamic nature of the DENV genome during viral replication (33). It is possible that structures or transient conformations in the RNA genome could facilitate or repress the initiation of RNA synthesis (33). We believe that understanding at the molecular level the process of flavivirus genome replication will provide new avenues for antiviral intervention.

ACKNOWLEDGMENTS

The authors thank Richard Kinney for DENV cDNA infectious clone and members of Gamarnik’s laboratory for helpful discussions. This work was supported by grants from HHMI and Agencia Nacional de Promoción Científica y Tecnológica (Argentina). AVG and CF are members of the Argentinean Council of Investigation (CONICET). NGI was funded by CONICET.
REFERENCES


FIGURE LEGENDS

**Figure 1.** Specific binding of NS5 to the 5’DV RNA. (A) EMSA showing the interaction between the 5’DV RNA probe (schematically represented on the left) and the full length NS5 or the RdRp domain. Uniformly $^{32}$P-labeled 5’DV RNA (0.1 nM) was titrated with increasing concentration of NS5 (0, 2.5, 5, 10, 20, 50, and 100 nM) (top panel) or the RdRp domain (0, 2.5, 5, 10, 20, 50, and 100 nM) (bottom panel). (B) EMSA showing the interaction of NS5 with the pppA-5’DV RNA (left) or the m7G-pppA-5’DV RNA (right). (C) EMSA showing the lack of interaction of NS5 with a pppA-5’DV ΔSLA RNA (left) or an m7G-pppA-5’DV ΔSLA RNA (right) carrying deletions of the promoter element SLA.

**Figure 2.** *In vitro* polymerase activity of NS5. (A) *In vitro* polymerase activity of NS5 using specific (5’DV RNA), non-specific (poly(rC), and a negative control 5’DV ΔSLA) templates in a reaction containing 10 µM GTP in the absence of Mn$^{2+}$. (B) *In vitro* polymerase activity of NS5 using viral RNA templates in the absence of manganese and with increasing concentrations of GTP (0, 0.5, 1, 2, 4, 12, 30, 60, 125 and 250 µM) (left). GMP incorporation up to 12 µM GTP is shown in detail (right). (C) Polymerase activity of NS5 using poly(rC) as template in the presence or absence of manganese and with increasing concentrations of GTP (0, 15, 30, 60, 125, 250 and 500 µM). (D) *De novo* polymerase activity. Poly-A polymerase (PAP) activity using 5’DV RNA and periodate treated 5’DV RNA as templates (left). Polymerase activity of NS5 using 5’DV RNA or periodate treated 5’DV RNA as templates (right).

**Figure 3.** Amino acid position of mutations introduced in the RdRp domain of NS5. “Back” view of the structure of DV3 RdRp domain (PDB 2J7U) in surface representation (left) and “front” view of the structure of DV3 RdRp domain rotated 180° (right). Surfaces are
represented with the electrostatic potential in blue for positive charges and red for negative charges. Figure was drawn using the PyMOL program.

**Figure 4.** Polymerase activity of the recombinant NS5 mutant proteins. (A) SDS-PAGE of purified WT and mutants NS5 proteins (B) Polymerase activity of mutated NS5 proteins using poly(rC) as template. (C) Polymerase activity of mutated NS5 proteins using 5’DV RNA as template. Error bars indicate the standard deviations of results from three experiments.

**Figure 5.** Dengue viruses carrying the mutations in NS5. Immunofluorescence assays (IF) of transfected BHK cells with WT or NS5 mutant full-length RNAs. IF were performed at 3, 6 and 9 days after transfection.

**Figure 6.** (A) EMSA showing the interaction of 5’DV RNA with the mutated NS5 recombinant proteins. Uniformly 32P-labeled 5’DV RNA (0.1 nM) was titrated with increasing concentration of proteins (0, 6, 12, 25, 50, 100 and 200 nM). Quantification of the percentage of RNA probe bound was plotted as a function of NS5 concentration, and equation 1 was fitted (see materials and methods). (B) Competition binding experiment with NS5 WT and NS5 M10 proteins. Uniformly 32P-labeled 5’DV RNA–WT NS5 or 5’DV RNA-M10 mutant complexes were titrated with increasing concentration of 5’DV RNA or 5’DVΔSLA RNA (0, 0.1, 0.5, 1, 2, 4, 8 and 50 nM), as indicated.

**Figure 7.** (A) Polymerase activity of WT and NS5 M10 using 5’DV RNA as template under conditions for non-specific activity. (B) SDS-PAGE of purified M10.1 and M10.2 NS5 proteins (left). Polymerase activity of NS5 M10 individual mutants using 5’DV RNA or Poly(rC) as template (right). Errors bars indicate the standard deviations of results from three independent experiments. (C) Elongation activity of NS5 with RNA primers of different length. NS5 was incubated with a 24 nt RNA template and the indicated primers. RNA products of the reaction were run in a 20% polyacrylamide gel. (B) Elongation activity
of mutated NS5 recombinant proteins, the reaction was carried out using a 4 nt RNA primer (5’-UUCU-3’) and the 24 nt RNA template indicated on the top panel.

Figure 8. Position of the motif F on HCV and DENV RdRps. Structure of HCV (PDB 1NB6) and DENV (PDB 2J7U) RdRps (“back” view) in ribbon representation. The position of the motif F is indicated in black, for DENV RdRp the region of the motif F1 and F2 is missing in the structural model (from amino acid 454 to 466). The position of the amino acid 453 is indicated by narrow. Figure was drawn using the PyMOL program.
Figure 2

A

GTP (µM) GTP (µM)
100 5 10 200
5'DV 5'DV
∆SLA

B

GTP (µM)

C

Poly(rC) ± Mn²⁺

D

5'DV

NaIO₄

- + - +
PAP NS5
Figure 4

A

MW (kDa)

WT M1 M2 M3 M4 M5 M8 M10 M11 M12 M13 M15 M17 M18 M19

B

Relative activity (% of WT)

poly(rC) template

WT M1 M2 M3 M4 M5 M8 M10 M11 M12 M13 M15 M17 M18 M19

C

5'DV RNA template

Relative activity (% of WT)

WT M1 M2 M3 M4 M5 M8 M10 M11 M12 M13 M15 M17 M18 M19
Figure 5

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K456-R457 (M10)
K459-K460 (M11)
R481 (M12)
R519-K523 (M13)
R540 (M14)
K561-K562 (M15)
K575-R578 R581 (M16)
R648-R651 (M17)
R769 (M18)
K840-R841 (M19)
Figure 6

A

NS5 WT
NS5 M1
NS5 M8
NS5 M10
NS5 M11
NS5 M12

B

NS5 WT
NS5 M10

% Bound
NS5 (nM)
5' DV-NS5
5' DV
5' DV
5' DV
5' DV
5' DV
5' DV
5' DV
5' DV
5' DV
5' DV
5' DV
Figure 7

A

NS5 WT  NS5 M10

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Mn²⁺ (mM)

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B

Poly(rC)

Template

5'DV RNA

Template

MW (kDa)

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C

5'-UGUUAUAAUUAUUAGGUUCU-3'

Template

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D

5'-UGUUAUAAUUAUUAGGUUCU-3'

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MW (kDa)

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Figure 8

HCV RdRp

Motif F

Thumb

Fingers

Palm

DENV RdRp

Motif F

Thumb

Fingers

Palm