A Locking Mechanism Regulates RNA Synthesis and Host Protein Interaction by the Hepatitis C Virus Polymerase


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Mutational analysis of the hepatitis C virus (HCV) RNA-dependent RNA polymerase (RdRp) template channel identified two residues, Trp397 and His428, which are required for de novo initiation but not for extension from a primer. These two residues interact with the Δ1 loop on the surface of the RdRp. A deletion within the Δ1 loop also resulted in comparable activity. The mutant proteins exhibit increased double-stranded RNA binding compared with the wild type, suggesting that the Δ1 loop serves as a flexible locking mechanism to regulate the conformations needed for de novo initiation and for elongation RNA synthesis. A similar locking motif can be found in other viral RdRps. Products associated with the open conformation of the HCV RdRp were inhibited by interaction with the retinoblastoma protein but not cyclophilin A. Different conformations of the HCV RdRp can thus affect RNA synthesis and interaction with cellular proteins.

All RNA viruses encode their own RNA-dependent RNA polymerases (RdRps) that direct viral RNA replication and transcription. Similar to other template-dependent polymerases, the shape of a right hand is a useful description of the structures of viral RdRps (1). The finger and the thumb subdomains guide the template, whereas the palm contains the active site for nucleotidyl transfer.

The hepatitis C virus (HCV) nonstructural protein 5B (NS5B) provides a useful model system to study viral RNA-dependent RNA synthesis. In contrast to a partially open conformation, the HCV RdRp structure has extensive interaction between the thumb and finger subdomains, resulting in the formation of a closed hand structure with a prominent template channel (2, 3). This closed conformation is also found in other viral RdRps (4–11). Modeling studies and analysis of the ternary RdRp complex of the bacteriophage Φ6 suggest that the template RNA threads into the channel. Functional studies of various template RNAs also reveal a shared requirement for single-stranded sequence in RNA-dependent RNA synthesis (12–15). Thus, viral RdRps, unlike DNA-dependent DNA polymerases and reverse transcriptases, may use a defined channel as part of the mechanism to recognize templates (16). In addition, since the channel contains the active site, it must participate in contacting the nascent RNA during elongative synthesis.

Although the template channel provides an elegant solution for the specific recognition of the initiation sequence for RNA synthesis, the diameter of this channel within the crystal structure of the HCV RdRp (ranging from 6 to 20Å; Fig. 1A) is too narrow to accommodate the duplex formed by the template and newly synthesized nascent RNA without a significant conformational change. In addition, there is growing evidence that the HCV NS5B protein undergoes a transition into an open structure to accept the template RNA (17–20). Indeed, the HCV RdRp can initiate de novo RNA synthesis from a circular template that cannot possibly be threaded into the template channel, functionally demonstrating that a transition between closed and open conformation can occur during template recognition (21).

Different conformations of the HCV RdRp may also affect its interactions with cellular proteins. The retinoblastoma tumor suppressor protein (pRb) is one interesting example, since it is targeted by DNA tumor viruses and by HCV as a means to deregulate cell cycle progression (22). Rb-binding proteins contain an LXCXE motif. In NS5B, this motif overlaps the residues that coordinate divalent metals in the catalytic pocket (23). Thus, the NS5B residues that are putatively involved in the interaction with pRb are not surface-exposed, suggesting the need for a conformational change in NS5B to accommodate the binding of pRb.

Cyclophilins are cellular peptidyl isomerases that are required for replication of several viruses, including human immunodeficiency virus and HCV (24, 25, 80). The peptide drug cyclosporin, which binds cyclophilins, was found to inhibit HCV RNA replication (26–30), and some mutations that render resistance to cyclosporin mapped to NS5B (27). Cyclophilin A (CypA) and CypB have been shown to co-precipitate with the HCV NS5B protein (27), but RNA silencing...
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experiments have yielded conflicting results as to whether CypB and/or CypA are required for HCV replication.

We have carried out a series of experiments aimed at determining how mutations in the template channel of the HCV RdRp affect RNA synthesis and its interactions with cellular proteins. Three of the mutations affected an interaction between a surface loop and the outer lining of the template channel. Furthermore, the interaction appears to regulate the transition between the conformations that are needed for de novo initiation and primer extension (PE). Furthermore, we show that RNA synthesis by the PE-competent and presumably the more open conformation is preferentially inhibited by pRb.

EXPERIMENTAL PROCEDURES

Reagents, Site-directed Mutagenesis, and Protein Purification—Recombinant pRb was purchased from QED Biosciences. Oligoribonucleotides were chemically synthesized by Dharmacon, Inc. Wild-type and mutant versions of the HCV RdRp were expressed with a C-terminal His6 tag in Escherichia coli and purified first by metal ion affinity chromatography, followed by a resin polyuridylate resin (Sigma) (14). The purified proteins were stored in 50 mM Tris (pH 7.9), 400 mM NaCl, 1 mM dithiothreitol, and 10% (v/v) glycerol at −20 °C. Protein concentrations were determined by staining SDS-PAGE with Coomassie Blue and comparing the bands to those of bovine serum albumin at known concentrations. Amino acid substitutions were introduced in the plasmid encoding HCV Δ21 using the QuikChange site-directed mutagenesis protocol recommended by Stratagene. The entire open reading frame was sequenced to confirm the presence of directed mutation and the absence of unintended mutations.

HCV Subgenomic Replicon Assay—All of the mutations except m26–30 were introduced into the subgenomic replicon by using the appropriate BglII DNA fragment from the pET21B plasmid that can express the mutant protein to replace the comparable fragment in the wild-type subgenomic HCV replicon (pFK/I_{389}neo/NS3-3′/5.1). To introduce the m26–30 mutation into the replicon, an EcoRI-Spel fragment from the wild-type subgenomic HCV replicon was subcloned into pBSK− plasmid (Stratagene), and mutations were introduced by site-directed mutagenesis. The subcloned fragment was reintroduced into pFK/I_{389}neo/NS3-3′/5.1, replacing the comparable DNA fragment. The presence of the mutation in the HCV subgenomic replicon was confirmed by DNA sequencing.

Subgenomic HCV replicon RNAs were transcribed using the T7 Ampliscribe kit (Epicerentc, Madison, WI) from plasmids linearized with Scal. The subgenomic HCV replicon RNAs were transfected into Huh7 cells by electroporation with a GenePulser system (Bio-Rad) as described by Krieger et al. (31). Briefly, 1 μg of the in vitro transcribed RNAs along with 9 μg of total RNA from naive Huh-7 cells were electroporated at 960 microfarads and 270 V into ~1 × 10⁶ Huh7 cells in 0.4 ml of ice-cold Cytomix. The cells were immediately transferred to 8 ml of complete Dulbecco’s modified Eagle’s medium containing 1.25% DMSO and seeded in a 10-cm dish. 24 h after transfection, the cell culture medium was replaced with complete Dulbecco’s modified Eagle’s medium supplemented with 0.5 mg/ml G418. The medium was changed every week for 3 weeks for the colony formation assay. The G418-resistant cell colonies were stained with 0.01% Coomassie Brilliant Blue.

Reverse Transcription-PCR Assay—The reverse transcription reaction used 400 ng of total RNA (harvested 72 and 96 h after electroporation with the RNeasy RNA isolation kit (Qiagen)). Reverse transcription reactions used the Superscript III reverse transcriptase (Invitrogen), incubated at 50 °C for 60 min. 15% of the reaction mixture was used directly for PCR with an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s. The final extension was at 72 °C for 5 min. PCR products were separated in a 1% agarose gel. Primers used had the sequence 5′-CTCGATGTCCCTACACATG-3′ (plus-strand primer) and 5′-ATTGACTGGAGTGTGTCTAG-3′ (minus-strand primer).

RdRp Activity Assays—Standard RdRp assays consisted of 1 pmol of linear template RNA and 0.04 μM NS5B (unless stated otherwise) in a 20-μl reaction containing Core buffer (20 mM sodium glutamate (pH 8.2), 4 mM MgCl2, 12.5 mM dithiothreitol, 0.5% (v/v) Triton X-100) amended with 200 mM GTP, 100 μM each of ATP and UTP, and 250 mM [α-32P]CTP (Amersham Biosciences). MnCl2, if used, was added to a final concentration of 1 mM. The final reaction also contained 20 mM NaCl that came from the storage buffer. RNA synthesis reactions were incubated at 25 °C for 60 min and stopped by phenol/chloroform extraction, followed by ethanol precipitation in the presence of 5 μg of glycogen and 0.5 mM ammonium acetate. Products were usually separated by electrophoresis on denaturing (7.5 M urea) polyacrylamide gels. Gels were wrapped in plastic, and radiolabel was quantified using a PhosphorImager (Amersham Biosciences). Each quantified result shown was from a minimum of three independently assayed samples.

Intrinsic Fluorescence Spectroscopy—Fluorescence measurements were done at 23–25 °C with a PerkinElmer Life Sciences LS55 spectrometer. Proteins were in a buffer of 20 mM Tris (pH 7.9), 1 mM dithiothreitol, 10% glycerol, and 400 mM NaCl. The protein concentrations were adjusted to 0.75–1.5 μM. Measurements were performed with constant stirring to minimize photobleaching. Five independent measurements were recorded per sample with 1-min intervals between scans, and the measurements were averaged. Excitation was performed at 295 nm, and the emission spectrum was collected at 1-nm intervals from 305 to 400 nm, with an integration time of 1 s and slit width of 5 nm.

Electrophoretic Mobility Shift Assays—In vitro transcribed double-stranded RNAs treated with alkaline phosphatase were radiolabeled with [32P]ATP, using T4 polynucleotide kinase. Labeled RNA (40 fm) was incubated in a 20-μl reaction with a 40 nM final concentration of the polymerase in the Core buffer amended with 1 mM MnCl2 and 0.2 mM GTP. The reactions were incubated at 25 °C for 30 min, after which a 4-μl solution containing 10 μM Tris (pH 8.1), 1 mM EDTA, 0.1% bромphenol blue, 0.1% xylene cyanol, and 30% glycerol was added. The samples were electrophoresed on a native 10% polyacrylamide gel at 400 V for 1 h, and then the gel was autoradiographed.

Analytical Ultracentrifugation—Analytical ultracentrifugation experiments were performed at the Center for Analytical Ultracentrifugation of Macromolecular Assemblies at the University of Texas Health Science Center at San Antonio. Δ21 and W397A were analyzed by sedimentation velocity experiments.
performed in a Beckman XLA centrifuge using absorbance optics by measuring intensity scans at 280 nm of a 0.8 OD loading. The experiments were performed in two-channel epon centerpieces at 45,000 rpm in an AN50 Ti rotor using a buffer containing 20 mM MOPS, 100 mM NaCl, and 0.75% (w/w) glycerol at 10 and 25 °C. Data analysis was performed with the UltraScan software suite (32). Data were first analyzed with the two-dimensional spectrum analysis (33) with simultaneous time invariant noise subtraction according to Schuck and Demeler (34). After noise subtraction, the data were examined for heterogeneity with the enhanced van Holde-Weischet analysis (35) and fitted by the genetic algorithm Monte Carlo analysis (36) in order to obtain shape information. All computations were performed on the TIGRE cluster at the University of Texas Health Science Center at San Antonio and the Texas Advanced Computing Center at the University of Texas in Austin. Partial specific volume was estimated based on PROTEIN sequence according to Durchschlag (37) and was found to be 0.732 cm³/g for both H900421 and W397A.

**Circular Dichroism Analysis**—Δ21, W397A, and H428A were subject to thermal unfolding using an Aviv 202SF spectropolarimeter with temperature control and stirring units. The change in CD signal at 220 nm was measured from 2 to 97 °C with 3-min equilibration intervals, and the data were averaged for 5 s at each interval. The concentration of the protein samples was between 4 and 15 μM in 5 mM MOPS at pH 7.6. The data were analyzed using methods described by Grimsley et al. (38).

**Molecular Modeling**—The crystal structure of the wild-type HCV-RDRP (Protein Data Bank code 1QUV) was used to con-
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FIGURE 2. His428 and Trp397 interact with the Δ1 loop. A, RNA products made by mutant and Δ21 using templates and reaction conditions to examine de novo initiation and PE. The proteins, templates, and the GTP concentrations used in the reactions are indicated above the gel image. LE19P is a version of LE19 that has a covalently attached 3'-puromycin. The lane numbers are indicated at the bottom of the gel image. The de novo initiated (19 nt) and primer extension products (32 nt) are indicated to the left of the gel image. B, RNA synthesis by the HCV RdRp with template derived from HCV RNA H115 contains the 3′-115 nt of HCV minus-strand RNA. RNA H115 + PE46 contains the PE46 sequence (boxed) fused to the 3′ end of H115. Whereas H115 can direct de novo initiation, H115 + PE46 is capable of primer extension. The gel images show the reaction products of the enzymes using H115 or H115 + PE46 as template, which were run on a 10% urea denaturing polyacrylamide gel. The divalent metal ion manganese was left out of the reaction mix in the assays with H115 and H115 + PE46 templates. The lane numbers are shown at the bottom, and the enzyme names are shown at the top of the lanes. The lengths of the products are shown to the left of the image; the 115 nt band corresponds to a de novo initiation product of H115, and the 275 nt band corresponds to the PE product of H115 + PE46. C, interaction between His428, Trp397, and the Δ1 loop. The Δ1 loop is rendered as a blue ribbon with the network of primarily hydrophobic interaction residues below, indicated in red, except for His428 and Trp397, which are in purple. The box contains a ribbon representation of the Δ1 loop (residues underlined) interacting with the helices in the thumb domain. The apex of the Δ1 loop is colored gold.

RESULTS

Mutational Analysis of the Template Channel in the HCV RdRp—An unusual aspect of the crystallographic structures of viral RdRps is the presence of a RNA template channel that can lead the template RNA to the active site (2, 39, 40). The template channel has areas that are more constricted, with the narrowest part having a diameter of ~5.5 Å when measured from the surfaces of the channel (Fig. 1A). To probe how the template channel might regulate RNA synthesis, we mutated many of the polar or aromatic residues that line the channel, typically to alanine. The locations of these mutations and their relationship to the channel are shown in Fig. 1A.

Template LE19 was used to assess the effects of the mutant proteins on RNA synthesis. The 19-nt LE19 can exist either as a hairpin with a single-stranded 3′ sequence that directs de novo initiation from the 3′-terminal cytidylate or as a partially duplexed dimer that can be primer-extended to result in a 32-nt product (Fig. 1B). Higher molecular mass bands that are multiples of 19 nt are also produced and have been characterized as template switch products (41, 42). Given that these are extended from the nascent RNA, they should be considered primer extension products.

Compared with the wild-type Δ21 NS5B protein, several mutants demonstrated significantly decreased RNA synthesis, including D225A, T389A, and Q436A. Asp225 and Thr389 are residues that are 6 and 16 Å, respectively, from the active site (the measurement was to the center of the respective side chains from the side chain of Asp225), but Gln436 resides at a distance of 32.4 Å from the active site, at the tip of the thumb domain. Unexpectedly, 14 of 33 mutants demonstrated increased RNA synthesis. When mapped onto the template channel, these mutations tend to be located at the entry and exit portions as well as the constricted region of the channel (Fig. 1A, blue residues). Thus, the observed increase in RNA synthe-
protein failed to initiate RNA synthesis de novo without GTP (Fig. 2A, lane 3). To demonstrate that the 32-nt products resulted in PE, we used LE19P, which is identical to LE19 but has a puromycin blocking the 3’ terminus. H428A and W397A did not produce the 32-mer from LE19P (Fig. 2A, lanes 6 and 10), confirming that products were made by extension from the 3’ terminus of LE19.

Next, we used the 115 nt from the 3’ end of minus-strand HCV RNA as a template to confirm the defect in de novo initiation with W397A and H428A. This RNA, designated H115, forms extensive secondary structures (Fig. 2C) but can direct de novo RNA synthesis (44). H428A and W397A failed to generate the expected 115-nt products from this template (Fig. 2B, compare lane 1 with lanes 2 and 3). However, a related template RNA, H115 + PE46, which provides a “looped back” primer allowing for PE, restored RNA synthesis by W397A and H428A to 34 and 9%, respectively, of the level seen with Δ21. Truncated products from H115 + PE46 were not observed, suggesting that the low level of synthesis was not due to premature termination. The reduction in PE observed with the H115 + PE46 template suggests that mutants W397A and H428A may have additional defects in RNA synthesis that are not as evident with shorter templates.

Δ1 Loop Participates in the Open and Closed Transition States by the HCV RdRp—Trp397 and His428 have their side chains pointing away from the active site and are part of a hydrophobic surface overlaid with a structure named the Δ1 loop. In the closed RdRp conformation, the Δ1 loop protrudes at the tip of the fingers domain and contacts the thumb domain by packing its short α-helix (helix A) against the α-helices O (residues 388–401) and Q (residues 418–437) of the thumb domain. The Trp397 residue is shown in purple. The active site is indicated by green stars. B, superposition of the Trp397 mutant model (red and sky blue) of NS5B and the WT structure (yellow and cyan). Only Δ1 loop and its contact regions are shown. The side chain of Trp397 stacks into the Δ1 loop. In the W397A mutant model, the key interactions are lost, and the Δ1 loop moves significantly away from its contact region. The red arrow indicates the movement. C, superposing a mutant in helix A of the Δ1 loop (L26A/S27A/N28A/S29A/L30A) and the wild-type protein; the Δ1 loop moves away from the thumb domain, and the helix A widens into a loop. The arrow indicates the direction of the movement.

sis suggests that these residues lining the channel may function to restrict access of inappropriate templates.

Two substitutions, W397A and H428A, resulted in severely decreased de novo initiation but had little impact on PE (Fig. 1C). Both Trp397 and His428 are more than 20 Å from the GDD motif that coordinates divalent metals and therefore should not affect binding of the initiation GTP (43). Our reactions contained 1 mM Mn²⁺, the presence of which generally decreases the effects of mutations of the NTPi-binding residues. Therefore, these residues are not directly involved in NTPi recognition (43).

To further characterize the defects in de novo initiation manifested by H428A and W397A mutants, we manipulated the concentration of the initiation GTP. LE19P has a cytidylate only at the 3’ initiation site; hence, GTP is required for initiation but not PE (Fig. 1B). The absence of GTP in the reaction mix did not affect the abundance of the 32-nt PE product produced by the mutants (Fig. 2A, lanes 7 and 11). In contrast, the wild-type Δ21 and Closed Transition States by the HCV RdRp
To determine whether the Δ1 loop is required for de novo initiation, we deleted the five residues of helix A within the Δ1 loop. The resultant protein, designated m26–30, was capable of PE when tested with the LE19 template (Fig. 2A, lanes 13–16). This result confirms that de novo initiation requires the interaction between the Δ1 loop and the underlying residues that help to form the template channel. Furthermore, the presence of higher levels of the template in the reaction increased in preferential accumulation of the primer extension products (supplemental Fig. 1). Two additional mutants, I432W/H428A and A396W/W397A, were made in an attempt to restore this interaction. However, although both of these mutants were found to be competent for PE, they were defective for de novo initiation (Table 1), perhaps due to a requirement for additional interactions between the outer surface of the template channel and the Δ1 loop that will not be addressed in this work.

W397A, H428A, and the m26–30 and HCV Replication in Cells—We next introduced several of these mutations into cDNA encoding a subgenomic HCV replicon in order to assess their effects on replication of HCV RNA in cultured human hepatoma cells. In vitro transcribed RNAs produced from these mutant plasmids were transfected into HuH7 cells, and colonies supporting replication of the viral RNA were selected by growth in the presence of G418. The H428A mutant failed to form colonies, whereas the wild-type replicon and an unrelated mutant replicon formed a significant number of colonies (supplemental Fig. 2A). To further examine the functional impact of the NS5B mutations, subgenomic replicons containing the H428A and W397A substitutions and the m26–30 deletion were transfected into HuH7 cells, and the level of replicon RNAs was examined semiquantitatively using reverse transcription-PCR. The WT subgenomic replicon RNA could be detected at 72 and 96 h after transfection, but an active site GAA mutant and all three mutants of interest failed to produce a detectable signal with two independent sets of primers specific to HCV cDNA (supplemental Fig. 2B). These results demonstrated that the in vitro competence of NS5B for de novo initiation is correlated to HCV RNA replication in cells.

Molecular Modeling of the Mutant Polymerases—We used molecular modeling to examine the effects of the W397A substitution. Residues within a 15-Å radius of W397A were subjected to a medium level of simulated annealing optimization using the variable target function of Modeler. W397A resulted in the Δ1 loop moving away from the thumb domain, with a maximum deviation of 12 Å. In fact, helix A unwinds into a loop during the energy optimization and loses contact to the neighboring helix Q (Fig. 3, A and B). Similar results were obtained when we analyzed the structure of an HCV RdRp that had the helix A residues 26–30 in the Δ1 loop (sequence LSNSL) replaced with alanines (Fig. 3C). These changes in the Δ1 loop were predicted to alter the structure of the template channel, making it more accessible to solvents. These predicted changes in the structure of the HCV RdRp could account for the functional differences we observed between the mutant RdRps and the wild-type Δ21 polymerase.

Biophysical Characterizations—We sought biophysical evidence in support of these hypothesized differences in the conformations of Δ21 and the mutant RdRps generated by computational modeling. The HCV RdRp has been reported to form oligomers (46, 47). Therefore, we examined the masses of Δ21 and W397A in solution at 10 and 25 °C using velocity sedimentation analytical centrifugation. Enhanced van Holde–Weischet analyses indicated that both protein samples were homogeneous and had S values between 4.1 and 4.2 (Fig. 4A). Results from a global genetic algorithm Monte Carlo analysis of each sample suggested that the proteins are globular in shape with frictional coefficients between 1.1 and 1.25, which correspond to an average molecular mass of 67.7 kDa, an excellent fit with monomers. Slight differences in the sedimentation of the two proteins exist at 25 °C, but it is difficult to assess whether this is due to significant conformational changes within the monomers. Nonetheless, these results establish that the mutation W397A, which renders it unable to initiate de novo, is not through an effect on the oligomerization state of the protein.

Next, we characterized the intrinsic fluorescence of the wild-type and mutant RdRps. The environment of aromatic residues, especially tryptophans, can affect their fluorescence (48). The intrinsic fluorescence spectra of several independent preparations of the H428A and m26–30 mutants exhibited a pronounced red shift in comparison with the wild-type Δ21 protein (Fig. 4B, both

### Table 1

A summary of the effects of mutations in or near the template channel

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<th>Functional subclasses</th>
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<th>PE</th>
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<tr>
<td>H428A</td>
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conformations (Fig. 4). The profiles for the two proteins largely overlap, suggesting that both are homogeneous and monomeric in solution. The intrinsic fluorescence of the mutant RdRps demonstrated multiple transitions, with 

However, this red shift was not seen with the mutant W397A, despite its sharing a common defect in de novo initiation. These results suggest that the three mutant RdRps are not in identical conformations (Fig. 4B, graph on the left).

We attempted to examine the relative stabilities of Δ21 and the mutant proteins using circular dichroism spectroscopy with increasing temperature. H428A and W397A demonstrated smooth two-state transitions, with 

respectively. In contrast, Δ21 demonstrated multiple transitions, probably due to some of the protein precipitating during the experiment (supplemental Fig. 3). Altogether, these biophysical and modeling results show that the mutant proteins have physical properties that differ from the wild-type Δ21. Furthermore, the mutant proteins are not in identical conformations.

Additional Functional Characterizations—We hypothesized that the different conformations assumed by the mutant and wild-type HCV RdRps are related to the conformational states required for distinct stages of RNA synthesis by the HCV RdRp. The closed conformation with a defined template channel could be responsible for de novo initiation, whereas the open conformation would permit elongation. The template channel will expand to accommodate the duplex of the nascent and template RNA by releasing the interaction with the Δ1 loop.

To examine this hypothesis, we characterized how Δ21 and the mutant RdRps interacted with RNA. Dinucleotides can replace the NTPI and can improve the stability of the ternary complex and rescue some defects in RNA synthesis (50). We investigated whether RNA synthesis by the mutants could be restored with the dinucleotide (GpU in place of GTP). GpU is complementary to the first two nucleotides at the 3′ terminus of LE19. The presence of GpU increased de novo initiated RNA synthesis by W397A and H428A by 27- and 12-fold, respectively, in comparison with GTP (Fig. 4C). However, GpU failed to rescue de novo initiation by m26–30.

To examine whether template binding by the mutant RdRps was affected, we performed RNA synthesis in the presence of heparin, a template mimic (51). RNA PE46 was selected for this analysis, because it is capable of directing only PE, thus eliminating possible effects on de novo initiation. The enzymes were preincubated with PE46 for 5 min to form a binary complex before the addition of heparin and rNTPs. Heparin reduced PE by the wild-type protein, Δ21, to ~10%, whereas W397A, H428A, and m26–30 were capable of PE at less than 0.5% of the amount seen in the absence of heparin (Fig. 4D, lanes 2, 5, 7, and 9).

However, when heparin and PE46 were added simultaneously to Δ21 before rNTP addition, no RNA synthesis was observed (Fig. 4D, lane 3). These results show that Δ21 can form a more stable binary complex with RNA than the mutant RdRps and suggest that the mutant polymerases might have reduced affinity for single-stranded RNA as compared with Δ21 and/or a reduced capacity to form a productive complex as compared with Δ21. The mutant RdRps were also found to be more sensitive to inhibition by NaCl in the reaction buffer, consistent with a decreased ability to interact with the single-stranded template (supplemental Fig. 3). Together, these results suggest
that the interaction between the Δ1 loop and the thumb subdomain can affect binary complex formation with single-stranded RNA, probably accounting for the defect in RNA synthesis.

Last, we compared the abilities of Δ21 and the mutant RdRps to bind double-stranded RNAs in an electrophoretic mobility shift assay. For these experiments, we used the RIII46 RNA, which can form a stable 21-nt hairpin (52) (Fig. 4E). Δ21 failed to form a shifted band. However, the three mutant enzymes bound detectably to RIII46, in increasing quantities from W397A to H428A and m26–30 (Fig. 4E). These results are consistent with the model that the mutant RdRps are in more open conformations when compared with Δ21.

Interaction with Cyclophilins and Retinoblastoma—Several cellular proteins have been shown to interact with the HCV NS5B in pull-down and co-immunoprecipitation assays, including CypB and CypA and the cell cycle regulator, pRb (23, 27, 30, 53). CypB interacts with the C-terminal domain of NS5B and has been reported to increase template binding in pull-down assays (27, 53). However, Robida et al. (27) did not observe a significant effect on HCV replication with the silencing of CypB, suggesting that another cyclophilin, perhaps CypA, has a role. An et al. (80) found that polymorphisms in CypA affected HCV replication.

The addition of purified CypA resulted in a concentration-dependent inhibition of RNA synthesis by the HCV RdRp from Δ21, m26–30, and I432V, a cyclosporin A-resistant mutation in NS5B (Fig. 5B and supplemental Fig. 5) (27). However, both de novo initiation and PE were inhibited, indicating that CypA does not preferentially target a particular mode of RNA synthesis. The presence of purified CypB resulted in a more pronounced shut-off of RNA synthesis than CypA. A molar ratio of three CypA to one RdRp reduced RNA product accumulation to approximately half, whereas the same amount of CypB reduced product formation 10-fold (Fig. 5C). In addition, the inhibitory effect of CypB was slightly stronger on the PE products than the de novo initiation products, although both were inhibited at higher CypB concentrations. These results demonstrate that both CypA and CypB can affect RNA synthesis by the HCV RdRp in vitro, although the effects are distinct. Several other proteins, such as bovine serum albumin and the RNA-binding proteins, PusA and App1, that affected brome mosaic virus RNA replication (54) did not affect HCV RNA synthesis.3

3 C. C. Kao, unpublished data.
The putative binding site for pRb lies deep within the catalytic pocket of the HCV RdRp (Fig. 5A, green residue) (22, 23). This location suggests that the closed conformation of the HCV RdRp should be more resistant to inhibition by pRb. Purified pRb inhibited RNA synthesis by $^21/_{9004}$ but demonstrated a substantially stronger inhibitory effect on the PE products than on the de novo initiated products (Fig. 5D, graph). PE by m26–30 and W397A were also inhibited by pRb (data not shown). A substitution of Cys$^{316}$ to an alanine within the putative pRb binding site (Fig. 5A) decreased the inhibitory effect of pRb on de novo initiation and also on PE (Fig. 5E, lanes 7 and 8). The differential effect of pRb on these two modes of RNA synthesis suggests that it preferentially interacts with and inhibits the open conformation of the HCV RdRp. Furthermore, mutant C316A was less affected by pRb for primer extension compared with $^21/_{9004}$, consistent with the idea that $^21/_{9004}$ exists in dynamic equilibrium between open and closed conformations.

To determine whether RNA synthesis by other RdRPs is also sensitive to pRb, NS5B derived from the genotype 2A JFH-1 strain of HCV, named 2a-$^21/_{9004}$, and from the pestivirus bovine viral diarrhea virus (BVDV) were tested (55, 56). Both 2a-$^21/_{9004}$ and the BVDV RdRp were sensitive to the presence of pRb at levels comparable with that exhibited by $^21/_{9004}$ (Fig. 5F). Furthermore, the PE products of both RdRPs were preferentially inhibited. These results suggest that all three of these related RdRPs exist in equilibrium between a de novo initiation-competent closed form and a PE-competent open form. Consistent with this, the RdRPs from BVDV and several other members of the Flaviviridae possess loops equivalent to the HCV $^21/_{9004}$ both in location and in primarily hydrophobic interactions with the surface of the template channel (Fig. 6).

**FIGURE 6. Δ1 looplike structures in other viral RdRPs.** The space-filling models show the locations of the loops that could have a role comparable with that of the HCV Δ1 loop in the RdRp structures of HCV, Dengue virus, West Nile Virus (WNV), and BVDV. The schematic to the left shows the potential interactions that allow the comparable loops to interact with the outer surface of the template channels in the RdRPs. Residues in the apex of the loop are in **boldface** type, and residues in the D2 loop are **underlined**. The key in the box shows the **lines** used for the different interactions.

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infection but may also contribute to perturbations of cellular processes.

Several lines of evidence suggest that mutant proteins W397A, H428A, and m26–30 exist in a more open, PE-compatible conformation. First, molecular dynamic studies predict that mutations in either the residues on the outside of the template channel or the $\Delta l/1$ loop decrease contact with helices Q and O and increase access to the catalytic pocket of the RdRp (Fig. 3). Second, the mutated RdRp proteins have discernable spectroscopic properties when compared with $\Delta l21$ and with each other, indicating that there are changes in the conformations of these proteins. Third, in comparison with $\Delta l21$, all three mutants are more sensitive to heparin and higher salt concentrations in the reaction buffer in the presence of a primed single-stranded template, suggesting that they are defective in the formation of the binary complex (Fig. 4D and supplemental Fig. 4). Fourth, and perhaps the most direct evidence in support of the open conformation, the mutants have an increased ability to bind to double-stranded RNA relative to $\Delta l21$ (Fig. 4E).

The interaction between the $\Delta l1$ loop and the outer surface of the template channel provides an elegant mechanism to regulate the initiation and elongation phases of HCV RNA synthesis (Fig. 7). The closed conformation is required for de novo initiation (Fig. 7, form i). In this conformation, the template channel can orient the single-stranded template to the catalytic site (form ii). In fact, since substitutions at the most constricted portion of the template channel increased RNA synthesis, the template channel may function to restrict access to more structured or otherwise inappropriate RNAs. The addition of the NTPi and a nucleotide complementary to the second template nucleotide will stabilize the ternary complex (Fig. 7, form iii). The nascent RNA will then grow with polymerization until the duplex reaches a length where steric constraints force the release of the $\Delta l$ loop from the thumb domain (Fig. 7, form iv). The open form of the polymerase allows elongation to proceed in a processive manner until the entire template is copied. At this point, the $\Delta l1$ loop may regain the ability to interact with the thumb subdomain.

Our model (Fig. 7) is consistent with results from studies of benzimidazole/indole-based nonnucleoside inhibitors that have been shown to specifically bind to the pocket in the thumb subdomain and inhibit the polymerase at a step before the elongation phase of RNA synthesis (57–63). Interestingly, pyranoindole small molecule inhibitors that bind to a site different from the $\Delta l$ loop-thumb domain interface inhibit elongation but not de novo initiation and

![Diagram](attachment:image.png)
cause an accumulation of 5-mer products, indicating that elongation might involve additional changes in the overall structure of the polymerase (20).

There is evidence that supports the concept that the HCV RdRp normally exists in equilibrium between closed and open conformations. Biswal et al. (19) crystallized two distinct conformations of the 2a form of the HCV RdRp, which retains the \( \Delta 1 \) loop, consistent with the wild-type RdRp existing in at least two distinct conformations. Furthermore, \( \Delta 21 \) can initiate RNA synthesis from circular templates, an observation that is best explained by the open conformation of the protein wrapping itself around the template (21).

The closed and open conformations of the HCV RdRp may regulate other activities of the NS5B protein that are only indirectly related to RNA synthesis. Viral replicates are usually capable of a high level of RNA synthesis despite low amounts of RdRp being present, thus making biochemical characterization of the replicase challenging (64). This raises the possibility that most of the RdRp molecules and other replicase subunits are not involved in RNA synthesis but serve to modulate the activities of other viral and also cellular proteins (22, 23, 25, 65–67).

In an interaction that is likely to have significant consequences for the association of liver cancer with HCV infection, NS5B can bind pRb, inducing its ubiquitination and subsequent proteasome-mediated degradation and thereby perturbing regulation of the cell cycle (22, 23). In vitro, such a protein-protein interaction resulted in the inhibition of RNA synthesis, perhaps by affecting template binding and/or catalysis. Interestingly, pRb preferentially inhibited PE by the wild-type HCV RdRp, \( \Delta 21 \), as well as the PE-competent mutants (Fig. 5). pRb is not known to have nucleic acid binding domains (68), and our finding that a C316A substitution resulted in escape from inhibition by pRb and an increase in the PE product (Fig. 5E) confirms earlier observations of Munakata et al. (22, 23), who demonstrated that this substitution near the active site of the RdRp inhibits its interaction with pRb. The inhibitory activity of pRb contrasts with the effects of CypA, which did not differentially affect the production of the de novo initiated versus primer-extended products of the HCV RdRp (Fig. 5B). Given that the recombinant pRb protein made in E. coli was capable of inhibiting the RdRp, post-translational modification of pRb is not required for this function (69, 70).

The differential inhibitory effects of pRb on different steps in RNA synthesis raise the interesting possibility that other cellular and/or viral proteins may interact with NS5B in a conformation-dependent manner. A screen for such interacting proteins or drugs that affect these interactions is now possible with the availability of mutant HCV RdRps that exist in different conformations.

The HCV RdRp has served as a model for the polymerases of other RNA viruses (71, 72). Indeed, some common themes are emerging now that the structures of a number of RdRps are available. Notably, the RdRp of BVDV can also exist in an open conformation, with a displaced loop that regulates access to the active site (7). A comparison of the activities of the RdRps from other members of the family Flaviviridae revealed that they have generally similar properties with respect to RNA synthesis in vitro (14, 41, 73–75). Consistent with this, we also demonstrated that pRb could preferentially inhibit the synthesis of elongation products made by the BVDV RdRp (Fig. 5F), suggesting that similar interactions may exist between pRb and RdRps from other positive strand RNA viruses.

DNA-dependent RNA polymerases, DNA-dependent DNA polymerases, and reverse transcriptases are all known to undergo transitions from open to closed states (76–79). Our results indicate that RdRps from positive strand RNA viruses can undergo similar transitions. Whether these changes go in the same order in vivo depends on how the RdRp-replicase complex locates the appropriate viral template RNA. In the case of HCV, it is possible that the RdRp first binds an internal sequence within the HCV genomic RNA and then scans for the 3’ terminus. In this scenario, the RdRp will initially bind RNA in the open form. On the other hand, it is also possible that the replicase is threaded with the 3’ terminus of the HCV RNA. In this case, the RdRp will first function in RNA synthesis in its closed conformation, followed by a transition to the open conformation during elongation. It is also possible that cellular protein(s), such as a cyclophilin, could mediate template loading by manipulating the RdRp conformation.

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REFERENCES

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