Hepatitis C Virus RNA-dependent RNA Polymerase (NS5B) as a Mediator of the Antiviral Activity of Ribavirin*

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Ribavirin is administered in combination with interferon-α for treatment of hepatitis C virus (HCV) infection. Recently, we demonstrated that the antiviral activity of ribavirin can result from the ability of a viral RNA polymerase to utilize ribavirin triphosphate and to incorporate this nucleotide with reduced specificity, thereby mutagenizing the genome and decreasing the yield of infectious virus (Crotty, S., Maag, D., Arnold, J. J., Zhong, W., Lau, J. Y., Hong, Z., Andino, R., and Cameron, C. E. (2000) Nat. Med. 6, 1375–1379). In this study, we performed a quantitative analysis of a novel HCV RNA polymerase derivative that is capable of utilizing stably annealed primer-template substrates and exploited this derivative to evaluate whether lethal mutagenesis of the HCV genome is a possible mechanism for the anti-HCV activity of ribavirin. These studies demonstrate HCV RNA polymerase-catalyzed incorporation of ribavirin opposite cytidine and uridine. In addition, we demonstrate that templates containing ribavirin support CMP and UMP incorporation with equivalent efficiency. Surprisingly, templates containing ribavirin can also cause a significant block to RNA elongation. Together, these data suggest that ribavirin can exert a direct effect on HCV replication, which is mediated by the HCV RNA polymerase. We discuss the implications of this work on the development of nucleoside analogs for treatment of HCV infection.

Hepatitis C virus (HCV) establishes a persistent infection of the liver, which leads to the development of cirrhosis and cancer (1). Currently, at least four million Americans are carriers of HCV. The available therapies are not very effective in curing the disease. The current therapeutic regimen includes a combination of interferon-α (IFN-α) and ribavirin (2). IFN-α increases the nonadapтивantiviral response in cells (3). Ribavirin is a broad-spectrum antiviral nucleoside that is converted to the mono-, di-, and triphosphorylated forms inside of cells (4). Until this year, it was thought that the mechanism of action for ribavirin has been expanded to include lethal mutagenesis of the viral genome as a result of ribavirin triphosphate utilization by the viral RNA-dependent RNA polymerase and incorporation into viral RNA (6, 7). As shown in Fig. 1, the pseudo base of ribavirin can pair equivalently with both cytosine and uracil, thus providing a molecular basis for the mutagenetic potential of ribavirin. Given the need for more effective strategies to control HCV infection, a precise understanding of the mechanism of action of existing antivirals should provide direction for the development of new anti-HCV therapeutics.

Full-length HCV polymerase (NS5B) contains a very hydrophobic carboxyl terminus. To increase the solubility of this enzyme, the carboxyl-terminal 21 amino acids have been removed (8). This enzyme has been referred to as NS5B-CTΔ21 (8). The enzymatic properties of this derivative are similar to that of the full-length enzyme; therefore, for clarity we will refer to this enzyme as wild-type NS5B (8). The structure of this enzyme is shown in Fig. 2 (9, 16, 17). Like most nucleic acid polymerases, HCV polymerase (NS5B) has fingers, palm, and thumb subdomains (Fig. 2). Unique to this enzyme, however, is the completely encircled active site, which derives from extensive interactions between a unique amino-terminal subdomain, referred to as the fingertips, and the thumb subdomain. A second unique feature of this enzyme is the presence of a β-hairpin, referred to as the β-loop, which protrudes into the catalytic site of the enzyme (Fig. 2). This structure has some interactions with the remaining portion of the carboxyl-terminal tail, and this interaction occludes the nucleic acid-binding site (Fig. 2) (10). Deletion of 8 amino acids from the β-loop yielded an enzyme, NS5B-Δ21, with increased activity, and this derivative was now capable of utilizing stably annealed primer-templates (11), including the symmetrical primer-template substrate (sym/sub) developed to study the poliovirus polymerase (12) and used to demonstrate that ribavirin triphosphate is a substrate for this enzyme (6).

In this report, we exploit the HCV NS5B-Δ21 derivative to test the hypothesis that the antiviral activity of ribavirin against HCV can be mediated, in part, by the viral polymerase. Like poliovirus polymerase, HCV polymerase incorporates ribavirin opposite cytidine and uridine with equal efficiency. Once incorporated, however, ribavirin causes a much more significant impediment to RNA elongation by HCV polymerase than observed for poliovirus polymerase. Together, the data described herein provide evidence for a direct effect of ribavirin on HCV replication mediated by the viral polymerase and provide additional support for the use of lethal mutagenesis as a strategy for the development of antiviral agents.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-32P]ATP (>7,000 Ci/mmol) was from ICN; nucleoside 5′-triphosphates (ultrapure solutions) were from AmerSharm Pharmacia
Mechanism of Action of Ribavirin

Fig. 1. The pseudo base (1,2,4-triazole 3-carboxamide) of ribavirin pairs equivalently with cytosine and uracil. R denotes ribofuranosyl mono- or triphosphate.

Fig. 2. Structural model for hepatitis C virus RNA-dependent RNA polymerase (NS5B). The β-loop (residues 445–454) is shown in gray. This element interacts with the portion of the carboxyl-terminal tail (residues 546–569) shown in black. This interaction occludes the nucleic acid-binding pocket of the enzyme (9, 16, 17).

Biotech; ribavirin 5'-triphosphate was from Moravek Biochemicals, Inc. (Brea, CA); all RNA oligonucleotides were from Dharmacon Research, Inc. (Boulder, CO); T4 polynucleotide kinase was from New England Biolabs, Inc. All other reagents were of the highest grade available from Sigma or Fisher.

Methods—Expression and purification of NS5B and NS5B-Δ8 was performed as described previously (8, 10, 11). Purification, 5'-32P labeling and annealing of sym/subs were performed as described previously (12). Reaction conditions were exactly as described previously for poliovirus polymerase (6, 12). Details for experimental design have been presented under Results and in the appropriate figures or figure legends. Rapid mixing/quenching experiments were performed using a Model RQF-3 chemical quench-flow apparatus (KinTek Corp., Austin, TX). Product analysis (gel electrophoresis, imaging, and quantitation) was as described previously (6, 12). Data were fit by nonlinear regression using the program KaleidaGraph (Synergy Software, Reading, PA).

RESULTS

Characterization of HCV NS5B-Δ8—We developed a primer extension assay to study the activity of the wild-type enzyme (10) and employed the present assay to compare the two enzymes directly. This assay employs a 10-nt RNA template and a dinucleotide primer (Fig. 3A). NS5B (2.5 μM) was mixed with template (100 μM) and primer (10 μM) and incubated at 22 °C for 90 s in order to assemble productive complexes. The reaction was initiated by addition of ATP (500 μM), and aliquots were removed and quenched at various times. The product (pGpGpA) formed was resolved from primer by electrophoresis through a denaturing 20% polyacrylamide gel, visualized using a PhosphorImager, and quantified by using ImageQuant software. A linear accumulation of product was observed; the velocity of the reaction was 0.00095 ± 0.00019 μm/s (Fig. 3B). A similar experiment was performed with NS5B-Δ8. Again, a linear accumulation of product formation was observed, and the velocity of the reaction was 0.0160 ± 0.0013 μm/s. These data support the conclusion that deletion of the β-loop does not impair the catalytic activity of this derivative relative to wild-type enzyme. Surprisingly, this deletion caused a 17-fold increase in the observed steady-state rate of product formation relative to the wild-type enzyme. Although we do not know the step(s) that limits the steady-state turnover of the enzyme, the steady-state rate measured is too slow to include phosphoryl transfer as a key contributor to the observed rate. As a result, it is unlikely that the observed 17-fold increase in the observed steady-state rate of the Δ8 enzyme relative to wild-type enzyme reflects a substantial difference in the chemical or kinetic mechanism of this derivative relative to wild-type NS5B. However, additional experiments will be required to test this hypothesis directly. It is plausible the Δ8 enzyme merely establishes more productive complexes than the wild-type enzyme. Consistent with this possibility is the finding that there was a demonstrable increase in the steady-state rate measured is too slow to include phosphoryl transfer as a key contributor to the observed rate. As a result, it is unlikely that the observed 17-fold increase in the observed steady-state rate of the BL derivative relative to wild-type enzyme. Surprisingly, this deletion caused a 17-fold increase in the observed steady-state rate of the BL derivative relative to wild-type enzyme. Although we do not know the step(s) that limits the steady-state turnover of the enzyme, the steady-state rate measured is too slow to include phosphoryl transfer as a key contributor to the observed rate. As a result, it is unlikely that the observed 17-fold increase in the observed steady-state rate of the Δ8 derivative performs primer extension more efficiently than the wild-type enzyme. A, the pGpG dinucleotide primer and 10-nt template employed in this experiment. B, kinetics of pGpGpA formation by NS5B (○) and NS5B-Δ8 (□). The kinetics of product formation fit best to a line. For NS5B the slope was 0.00095 ± 0.00019 μm/s, and the intercept was 0.053 ± 0.017 μm; for NS5B-Δ8 the slope was 0.016 ± 0.0013 μm/s, and the intercept was 0.27 ± 0.12 μm.

In the experiment shown in Fig. 4, the reaction was initiated by addition of NS5B-Δ8 (5 μM) to a mixture containing the symmetrical primer-template substrate, sym/sub, (0.5 μM duplex) shown in Fig. 4A and ATP (500 μM). Product analysis was as described above. The result of this experiment is shown in Fig. 4A, B and C. The kinetics of AMP incorporation into sym/sub were biphasic (Fig. 4C), consisting of an exponential phase followed by a linear phase. The observed rate of the first phase was 0.0048 ± 0.0001 s⁻¹, and the amplitude was 0.45 ± 0.1 μm, consistent with utilization of 90% of the input substrate. The observed rate of the second phase was 0.000071 ± 0.000005 s⁻¹ (calculated as the slope of the line divided by the amplitude of the exponential). The observed rate of the exponential phase likely reflects assembly of NS5B-Δ8-sym/sub complexes rather than the rate of nucleotide incorporation (12). The observed rate of the linear phase likely reflects dissociation of NS5B-Δ8 from sym/sub to bind to the unextended side of the substrate for a second round of incorporation (12).
The NS5B-BL8 derivative utilizes primer-template substrates with stable duplexes. A, the symmetrical primer-template substrate employed in this experiment. This substrate is referred to as sym/sub-U (the letter after sym/sub indicates the base of the templating nucleotide). B, AMP incorporation into sym/sub-U by NS5B-BL8. C, kinetics of assembly of H5b8-sym/sub complexes. The solid line represents a fit of the data to a single exponential followed by a linear phase. The observed rate of the exponential phase is 0.0048 ± 0.0001 s⁻¹, and the amplitude is 0.45 ± 0.01 μM. The observed rate of the linear phase is 0.000071 ± 0.000005 s⁻¹.

To determine the rate constant for dissociation (Fig. 5A), NS5B-BL8 (1 μM) was mixed with labeled sym/sub (0.1 μM) for 5 min followed by the addition of a 1000-fold molar excess of unlabeled sym/sub as a trap for unbound and dissociating enzyme. At various times after addition of trap, ATP was added, and incorporation was permitted for 30 s, a time sufficient for quantitative incorporation of nucleotide. Product formation was monitored as described above. The amount of product formed is a reflection of the number of intact complexes remaining. The percent of complexes remaining was plotted as a function of time after the addition of trap (Fig. 5B). The data fit best to an equation describing the sum of two exponentials. Two complexes exist: the first represents 37% of the total and dissociates at a rate of 0.013 s⁻¹; the second represents 63% of the total and dissociates at a rate of 0.00008 s⁻¹ (Fig. 5B).

To measure the rate constant for nucleotide incorporation (Fig. 5C), NS5B-BL8 (1 μM) was mixed with sym/sub (0.5 μM duplex) for 10 min, mixed rapidly with ATP (100 μM), and then quenched by the addition of EDTA (0.3 M). Product formation was determined as described above. Product formed was plotted as a function of incubation time (Fig. 5D). The data were fit best by an equation describing the sum of two exponentials. Again, two complexes exist: the first represents 24% of the total and incorporates nucleotide at a rate of 136 s⁻¹; the second represents 76% of the total and incorporates nucleotide at a rate of 8.1 ± 0.5 s⁻¹.

![Figure 4](https://example.com/figure4.png)

**Fig. 4.** The NS5B-BL8 derivative utilizes primer-template substrates with stable duplexes. A, the symmetrical primer-template substrate employed in this experiment. This substrate is referred to as sym/sub-U (the letter after sym/sub indicates the base of the templating nucleotide). B, AMP incorporation into sym/sub-U by NS5B-BL8. C, kinetics of assembly of H5b8-sym/sub complexes. The solid line represents a fit of the data to a single exponential followed by a linear phase. The observed rate of the exponential phase is 0.0048 ± 0.0001 s⁻¹, and the amplitude is 0.45 ± 0.01 μM. The observed rate of the linear phase is 0.000071 ± 0.000005 s⁻¹.

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** The NS5B-BL8 derivative forms stable complexes with sym/sub that are competent for fast rates of nucleotide incorporation. Dissociation of NS5B-BL8-sym/sub-U complexes: A, experimental design; B, kinetics of dissociation. The percent of complex remaining was plotted as a function of time and the data were fit best by the sum of two exponentials. The inset expands the first 2% of the time course. The first complex (37%) dissociated at a rate of 0.013 ± 0.011 s⁻¹; the second complex (63%) dissociated at a rate of 0.00008 ± 0.00001 s⁻¹. Incorporation of AMP into sym/sub-U: C, experimental design; D, kinetics of incorporation. The product was plotted as a function of time, and the data were fit best by the sum of two exponentials. The first complex (24%) incorporates nucleotide at a rate of 136 ± 23 s⁻¹; the second complex (76%) incorporates nucleotide at a rate of 8.1 ± 0.5 s⁻¹.

**Table I**

Ribavirin is a guanine and adenine mimic

Experiments were performed as described in the text and the legend to Fig. 3C. The boldface letters in the sym/sub sequences indicate the templating residues.

| Primer/template | Nucleotide  | $K_{d, app}$ | $k_{pol}$ |
|-----------------|-------------|--------------|-----------|
| sym/sub-U       | ATP         | 25 ± 11      | 12.3 ± 2.2 |
| sym/sub-C       | GTP         | 6.2 ± 1.1    | 10.4 ± 0.4 |
| sym/sub-R       | UTP         | 313 ± 60     | 0.047 ± 0.004 |

$a$ = “R” denotes ribavirin.
of both nucleotides was equivalent based upon the uridine and cytidine into RNA. The efficiency of incorporation presence of ribavirin in the template supported incorporation of either sym/sub-C or sym/sub-U was employed (Table I). The 500-fold increase in the 

FIG. 6. The presence of ribavirin in the RNA template impedes elongation of nascent RNA. A, sym/sub-UR substrate employed in this experiment. The R denotes ribavirin. B, RNA synthesis by HCV polymerase, NS5B-BLΔ8 (upper panel), or poliovirus polymerase 3Dpol (lower panel) on templates lacking (S/S-U) or containing (S/S-UR) ribavirin. Enzyme (5 μM) was incubated with primer-template (0.5 μM duplex) prior to initiating the reaction by the addition of ATP (lanes 1, 4, 7, and 10), ATP and UTP (lanes 2, 5, 8, and 11), or ATP, UTP, and GTP (lanes 3, 6, 9, and 12). Each nucleotide was present at a final concentration of 1 mM. Nucleotide incorporation was permitted for 30 s, and then reactions were quenched by the addition of EDTA (50 mM). In each case for NS5B-BLΔ8, there was a 2–4-fold increase in the 11-mer product band when ribavirin was present in the template.

residue (Table I). The $K_{d, app}$ value for RTP was in the 500–700 μM range and the $k_{pol}$ value in the 0.03–0.04 s$^{-1}$ range when either sym/sub-C or sym/sub-U was employed (Table I). The presence of ribavirin in the template supported incorporation of uridine and cytidine into RNA. The efficiency of incorporation of both nucleotides was equivalent based upon the $k_{pol}/K_{d, app}$ values (Table I). The $k_{pol}$ values for uridine and cytidine incorporation template by ribavirin were en par with those measured for ribavirin incorporation template by uridine or cytidine (Table I). This result was surprising because experiments performed previously with poliovirus polymerase showed a 500-fold increase in the $k_{pol}$ value measured when ribavirin was in the template relative to that measured when ribavirin was the incoming nucleotide (6).

We evaluated a substrate in which ribavirin was placed as the second templating nucleotide in the substrate (sym/sub-UR, Fig. 6A). Complexes containing NS5B-BLΔ8 and sym/sub-UR were assembled and mixed with the following for 30 s: ATP (Fig. 6B, lane 4); ATP and UTP (Fig. 6B, lane 5); or ATP, UTP, and GTP (Fig. 6B, lane 6). Control experiments were performed in which sym/sub-U was employed instead of sym/sub-UR (Fig. 6B, lanes 1–3). The elongation efficiency of a significant fraction of enzyme was reduced by the presence of ribavirin in the template relative to control (compare lanes 2 and 5 in Fig. 6B). However, nucleotide incorporated opposite ribavirin was efficiently extended into full-length RNA (com-

DISCUSSION

Poliovirus and its polymerase have been employed as a model system to explore alternative hypotheses for the antiviral activity of the broad-spectrum antiviral nucleoside, ribavirin (6). These studies have suggested that the viral polymerase mediates the antiviral activity of ribavirin by utilizing ribavirin triphosphate as a substrate and incorporating this nucleotide into RNA (6). The ambiguous nature of the pseudo base of ribavirin (Fig. 1) forces polymerase to incorporate ribavirin with decreased specificity leading to increased mutagenesis of the viral genome and production of defective virus (6, 7). Whether this mechanism of action of ribavirin is unique to poliovirus is important to determine. Should the next generation of antiviral nucleosides target inosine monophosphate dehydrogenase or the viral polymerase?

To begin addressing this question, we developed an HCV polymerase derivative that permits direct evaluation of single nucleotide incorporation (11). This derivative, NS5B-BLΔ8, contains a truncated β-loop that opens the nucleic acid-binding pocket providing access to double-stranded nucleic acid (Fig. 2). The NS5B-BLΔ8 enzyme likely uses the same mechanism as wild-type NS5B (Fig. 3). It is worth noting that this derivative does support some degree of replication relative to inactive polymerase derivatives when evaluated in the context of sub-genomic replicons.2 The NS5B-BLΔ8 derivative utilizes the symmetrical sym/sub system developed for poliovirus polymerase (12) (Fig. 4), establishing very stable complexes that are competent for fast rates of nucleotide incorporation (Fig. 5).

The NS5B-BLΔ8-sym/sub complexes that assemble are heterogeneous, composed of at least two or three somewhat different species having different biochemical properties. When evaluating complex dissociation, nucleotide incorporation, or thermal stability, evidence for two complexes was obtained in each case (Fig. 5 and data not shown). The relationship between these complexes is not clear; however, we speculate that the observed heterogeneity is a consequence of the remaining portion of the carboxyl-terminal tail (Fig. 2) binding in various conformations in or around the nucleic acid-binding pocket. We are currently testing this hypothesis by constructing NS5B polymerase derivatives containing additional deletions in the carboxyl terminus. Nevertheless, the largest fraction of enzyme (∼76%) behaves as a single species with a half-life of ∼2.5 h, apparent binding constants for nucleotide in a physiologically relevant range (Table I), and maximal rates of nucleotide incorporation in the 10–12 s$^{-1}$ range (Table I). The rates of nucleotide incorporation reported here are consistent with those measured by others when employing the full-length enzyme and homopolymeric substrates (13). Together, these data suggest that HCV polymerase should be capable of replicating its genome in a single binding event requiring no longer than 15 min. Moreover, given the low $K_{d, app}$ value for GTP, it is unlikely that the enzyme would be sensitive to the 2-fold reduction in intracellular GTP pools observed when cells are treated with ribavirin (14).

By using the NS5B-BLΔ8 derivative and the sym/sub sys-

$^2$D. Maag, C. Castro, Z. Hong, and C. E. Cameron, unpublished results.
tem, we demonstrated that ribavirin triphosphate is a substrate for the HCV polymerase and should be mutagenic to the viral genome (Table I). However, ribavirin incorporation should occur at best only once/7000 nucleotides incorporated; that is, approximately once per genome. As a result, several cycles of replication may be necessary to cause a significant drop in production of infectious virus, consistent with the reduced efficacy of ribavirin monotherapy for treatment of HCV infection (15). We also observed a substantial block to elongation of nascent RNA when ribavirin was present in the template (Fig. 6). In this case, a single incorporation per genome may be more than sufficient to cause a substantial antiviral effect. If this is the case, then the reduced efficacy of ribavirin monotherapy may be a reflection of the use of reverse transcriptase-polymerase chain reaction as an indicator of viral load instead of a direct analysis of virus infectivity (15). HCV RNA may be stable for substantial periods of time in cells and defective RNA may be packaged if the appropriate signals remain intact. Whether the effect of ribavirin is on genome fitness or integrity, the effect can be direct and mediated by the viral RNA polymerase. Moreover, in either case, IFN-α treatment should increase the efficiency of RNA clearance by activating the RNase L pathway among others (3).

Together, the data presented herein support the hypothesis that antiviral nucleosides can be developed that can be converted intracellularly to active substrates for the viral RNA polymerase, causing either lethal mutagenesis of the viral genome or premature termination of nascent viral RNA. By using a combination of the NS5B-BLΔ8 derivative, or related enzymes, and the sym/sub system, it is now possible to evaluate systematically and quantitatively the specificity and fidelity of the HCV RNA polymerase. This information should be useful in providing structure-activity relationships to assist in the rational design of antiviral nucleosides. In addition, it is now possible to interrogate diverse chemical libraries, including nucleotide libraries, for the desired effects on active elongation complexes, thus diminishing the noise associated with high-throughput screens that identify myriad compounds that bind to free enzyme or double-stranded nucleic acid that would have very weak, if any, biological activity.

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