Only a Small Fraction of Purified Hepatitis C RNA-Dependent RNA Polymerase Is Catalytically Competent: Implications for Viral Replication and in Vitro Assays

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ABSTRACT: The enzymatic activity of a C-terminally truncated form of the RNA-dependent RNA polymerase, termed NS5B(Δ21), of the hepatitis C virus (strain BK) has been investigated using both homopolymeric and heteropolymeric RNA templates. Incorporation of nucleotides into a heteropolymeric RNA template as catalyzed by NS5B(Δ21) is characterized by biphasic reaction time courses. At high concentrations of nucleoside triphosphate in reactions allowing a preincubation of NS5B(Δ21) and RNA template, an initial rapid phase of the reaction is followed by a slower linear phase. The amplitude of the first phase of the reaction varies directly with the concentration of the enzyme in the reaction. It is shown here that full-length copies of the template are produced during the first phase of the reaction. Our results reveal that NS5B(Δ21) is processive but only a small fraction, less than 1%, of the purified enzyme present participates productively in the reaction. Most importantly, the turnover number for the hepatitis C NS5B(Δ21) is comparable to those observed for other polymerases such as the HIV-1 reverse transcriptase. The combined results reconcile in part the apparent discrepancy of the low, observed specific activity of the purified enzyme and the rapid generation of HCV in vivo.

Hepatitis C virus (HCV)[1] is a positive single-strand RNA virus and the major cause of post-transfusion sporadic, non-A, non-B hepatitis (*1*, *2*). The viral genome encodes a polyprotein of about 3000 amino acids that is processed by cellular and virally encoded proteases into 10 proteins, designated NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (*3*−*5*). NS5B contains the sequence motifs that are characteristic of viral RNA-dependent RNA polymerases (*6*−*8*).

Recombinant NS5B has been purified from Sf9 cells infected with a recombinant baculoviral vector and has been shown to contain RNA-dependent RNA polymerase activity (*9*, *10*). NS5B has also been expressed in *Escherichia coli* (*11*), and a form of the enzyme devoid of the C-terminal 21 amino acids that are likely responsible for the association of the full length NS5B with cellular membrane (*12*) has been expressed in and purified from *E. coli* (*12*, *13*). The enzymatic activity of both the full length NS5B (*14*, *15*) and the truncated forms (*12*, *13*) has been demonstrated using both homopolymeric and heteropolymeric RNA templates. Published values for the specific activity of the enzyme vary somewhat, probably owing to differences in the various expression constructs and assay conditions. However, even the highest reported specific activity (200 pmol/(h/μg), *15*) is 6000-fold lower than the rate of nucleotide incorporation catalyzed by poliovirus RNA polymerase (*16*) and is significantly lower than specific activities for HIV-1 reverse transcriptase (*17*). It is difficult to reconcile the low turnover rates of purified HCV NS5B with the extremely rapid generation of HCV in vivo (*18*).

To improve the chances of developing an effective HCV chemotherapeutic by targeting the HCV RNA-dependent RNA polymerase, it is necessary to gain a better understanding of the catalytic activity of the enzyme. Toward that end, this work has been conducted to investigate the enzymatic activity of the soluble, C-terminally truncated form of HCV NS5B, termed NS5B(Δ21). Our results show that the enzyme is capable of carrying out processive synthesis on a single strand heteropolymeric RNA template in the presence of a trapping agent, heparin. With our preparations of the truncated enzyme and under the reaction conditions employed, only a very small fraction of the total enzyme is capable of processive synthesis in the presence of heparin.

MATERIALS AND METHODS

Reagents. [α-^{32}P] and [α-^{33}P] nucleoside triphosphates were purchased from NEN. Ultrapure NTPs, dNTPs, and ddNTPs were from Pharmacia. Heparin (Li^+ salt) was purchased from Sigma. All chromatographic materials were purchased from Amersham Pharmacia Biotech.

Template. The RNA template, t500, was generated with the use of T7 runoff transcription using the Megascript kit (Ambion). The DNA template used for the runoff transcription was generated by polymerase chain reaction (PCR) in a two step PCR method. The first step amplified a 1500 bp sequence from a cDNA copy of the NS2/3 region of the HCV

[1] Abbreviations used: HCV, hepatitis C virus; NS5B, nonstructural protein 5B; NS5B(Δ21), a form of the NS5B protein of hepatitis C virus with the C-terminal 21 amino acids genetically removed to improve solubility; EDTA, ethylenediamine tetraacetate; PEG8000, poly(ethylene glycol) with an average molecular weight of 8000 Da; DTT, dithiothreitol; t200 and t500, single strand RNA of 200 and 500 nucleotides length, respectively; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl thiogalactoside; AEBSF, [4-(2-aminoethyl)-benzenesulfonyl fluoride].

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genome (BK strain, supplied by Paul Darke, Merck Research Laboratories) subcloned in pET11a using the following DNA oligonucleotide primers: 5′-CTA GCT AGC CAT CAC CAT CAC CAC CAT GCG CCC ATC ACG GCC TAC TCC CAA C-3′; 5′-CGG GAT CCA AGC TGA AAT CGA CTG TCT GGG TGA CAC-3′. A second round of PCR was performed on the product from the first round to introduce the promoter sequence recognized by T7 RNA polymerase using the following two primers: 5′-TAA TAC GAC TCA CTA TAG GGA GGT TCA GGT GGT TTC CAC CGC AAC ACA ATC C-3′; 5′-TTG AAA TGA CT GCG GTA CGG CCG G-3′ (named 3′500NoT7). After overnight transcription reactions, RNA was treated with proteinase K, phenol/chloroform extracted, and precipitated by the addition of $^1/_{10}$ volume of 3 M ammonium acetate, pH 5, and 1 volume of 2-propanol. Alternately, the RNA was precipitated by the addition of LiCl as per the instructions of the manufacturer of the Megascript kit. After centrifugation, the pellet was redissolved in distilled H_2O and the RNA concentration was estimated by absorbance at 260 nm (1 OD at 260 nm = 40 μg/mL). The sequence of the RNA template corresponded to base numbers 3504−4004 of the BK genome (*19*). The molecular weight of t500 was assumed to be 165 000 Da. The DNA template used in runoff transcription reactions to generate template t200 was created in a similar manner using PCR, the same 1500 bp DNA template as for t500, and the PCR primers, 5′-TAA TAC GAC TCA CTA TAG GGT CTC CTA CTT GAA GGG CTC TTC GGG TGG TCC ACT G- 3′ and 3′500NoT7.

Enzyme. A form of the HCV (BK strain, *21*) NS5B protein devoid of the C-terminal 21 amino acids was expressed in *E. coli* BL21(DE3) cells via transformation with plasmid pT7(NS5BΔ21) which contained the gene encoding the truncated NS5B gene and allowed for IPTG-inducible expression. Cells were cultured at 37 °C to an optical density of 0.8−0.9 at 600 nm in LB broth containing 50 μg/mL ampicillin. The temperature was lowered to 30 °C to allow for induction with 1 mM isopropyl-D-(−)-thio galactopyranoside (IPTG, Boehringer Mannheim). The cells were further grown for 2 h, at 30 °C with shaking at 225 rpm, prior to pelleting via centrifugation for storage at −70 °C.

Packed cells from a 30-L culture were suspended at 4 °C in 180 mL of lysis buffer (20 mM Tris HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 0.1 M KCl, 10 mM β-mercaptoethanol, 100 μM amino ethyl benzyl sulfonyl fluoride AEBSF, 10 μg/mL of leupeptin). The cells were homogenized in a Dounce homogenizer and immediately disrupted with three passes through a microfluidizer (Microfluidics, model 110-S) at 6 bar pressure. The cells were cooled on ice between each pass. The lysate was centrifuged at 15 000 rpm in a Beckman JA17M rotor for 30 min at 4 °C. The pellet was resuspended in lysis buffer and homogenized in a Dounce homogenizer to solubilize additional enzyme from the pellet. The supernatant was retained after the solubilized pellet was centrifuged at 15 000 rpm, for 30 min. The two supernatants were pooled and loaded onto 150-mL DEAE Fast Flow column (Amersham Pharmacia Biotech) at 20 mL/min. The DEAE Fast Flow column was washed with two column volumes of 25 mM K−Hepes pH 7.5, 5% glycerol, 1 mM EDTA, and 10 mM β-ME (buffer A) containing 0.1 M KCl. The flow-through and washes from the DEAE Fast Flow column were combined, and the KCl concentration was adjusted to 0.3 M before loading onto an 80 mL HiTrap heparin column (Amersham Pharmacia Biotech) at 7 mL/min which was preequilibrated with buffer A containing 0.3 M KCl. The enzyme was eluted with a 0.3−1.0 M KCl gradient. Eluent fractions were analyzed with SDS−PAGE and Coomassie Blue staining. Fractions containing ∼80% pure NS5B(Δ21) were pooled and diluted with buffer A to a final concentration of 0.3 M KCl before rechromatographing on a 25 mL HiTrap heparin column preequilibrated with buffer A containing 0.3 M KCl. The protein was eluted at 3 mL/min with a gradient containing 0.3−0.7 M KCl. Fractions that contained >90% pure NS5B(Δ21) by SDS−PAGE were pooled and diluted to 0.3 M KCl with buffer A and loaded at 1 mL/min onto a 40 mL polyuridylic acid column (Sigma) preequilibrated with buffer A containing 0.3 M KCl. The protein was eluted with a gradient in buffer A containing 0.3−1.0 M KCl. Fractions containing >95% pure NS5B-(Δ21) were pooled and concentrated to 25−40 mg/mL with use of Centriprep-10 concentrators (Amicon). The enzyme was aliquoted, quick frozen on dry ice, and stored at −70 °C until use. N-terminal sequence analysis was carried out by Edman degradation on an Applied Biosystems model 494A protein sequencer. Protein concentrations were determined with use of quantitative amino acid analyses with a postcolumn ninhydrin derivatization method on a Beckman 6300 analyzer.

Full length NS5B protein (BK strain) with an additional C-terminal His_6 tag was overexpressed with the use of a baculoviral vector and purified from Sf9 insect cells using metal chelate chromatography as previously described (*14*).

Assays. The incorporation of radiolabeled nucleotides catalyzed by NS5B or NS5B(Δ21) on t500 was determined in reactions at 23 °C including 20 mM Tris, pH 7.5, 50 μM EDTA, 2 mM $MgCl_2$, 80 mM KCl, 5 mM DTT, 0.4 unit/μL of RNAsin (Promega), unless otherwise noted. The concentration of template t500 was 90 nM, unless otherwise noted. Reactions utilizing oligodT_{16}/poly(rA) as the primer template were carried out in the same reaction buffer as for reactions including t500 except the concentration of KCl was 10 mM. The ratio of oligo(dT_{16}) to poly(rA) was approximately 6:1 in strands. The concentrations of enzyme and NTPs employed in activity assays varied as noted in figure legends and table footnotes. Unless otherwise noted, reactions allowed a preincubation of enzyme and template in reaction buffer and were initiated by the addition of a mixture of NTPs. Reactions were quenched by addition of a reaction aliquot to 20 μL of 0.5 M EDTA. Aliquots were spotted onto DE-81 filter disks (Whatman) and dried. Filters were washed in 0.3 M ammonium formate for six 150-mL washes or until 1 mL of wash contained <100 cpm. Filters were counted in 5 mL Beckman Ready Safe scintillation fluid in a Beckman LS6000 scintillation counter. Radiolabel specific activity was determined by spotting an aliquot of quenched time point on a DE-81 filter and counting without washing the filter (*20*). Michaelis−Menten parameters were determined from rates calculated from single-time point reactions (2 h).

Enzyme-Trapping Experiments. Reactions were initiated either by addition of enzyme to a mixture of template and NTPs in reaction buffer or by addition of a mixture of heparin (1 mg/mL final concentration in the reaction) and NTPs to reaction buffer containing NS5B(Δ21) and template t500.

Data Analysis. Rate data were fit to either the Michaelis−Menten equation or to an exponential increase in product

Table 1: Purification of HCV NS5B(Δ21)

purification steps	concn (mg/mL)	tot. protein (mg)	activity[a] (nmol/(mg/h))	relative activity[b]
supernatant	15.7	6990	0.065	1
DEAE flow through	6.0	3860	0.125	1.9
heparin 1	0.3	69	305	4690
heparin 2	1.6	44	664	10220
polyU	45.6	29	1507	23180

[a] [UTP] = 10 μM. [b] Activity of cell lysate supernatant was used as the standard.

formation for single-turnover kinetic reactions using Kaleidagraph (Synergy Software) with fitting based on the algorithm of Marquardt (*21*).

RESULTS

Purification of NS5B(Δ21). Table 1 summarizes the NS5B(Δ21) purification procedure and yields. To achieve homogeneous NS5B(Δ21) enzyme, the initial purification steps were designed to remove quickly from the crude lysate *E. coli* proteases and nucleic acids. The chromatography on the first heparin column was carried out at a higher flow rate to achieve a ~1500-fold increase in specific activity. The second heparin column was utilized to achieve higher protein peak resolution to remove impurities that were not separable in the subsequent polyU chromatography. The polyU chromatography removed several low abundance impurities and increased the specific activity by another 2-fold. During the entire purification no detergent was required to keep the protein soluble. We were able to achieve concentrations of ~40 mg/mL in 0.1 M KCl containing buffer in the absence of detergents. The N-terminal sequence analysis showed a single sequence starting with Ser-Met-Ser-Tyr-Thr-Trp-Thr ..., and the amino acid composition from quantitative amino acid analysis correlated well with the theoretical composition (data not shown). An identical purification was carried out with use of a construct in which the active site residue D318 was mutated to an Asn. The resulting preparation displayed no RNA-dependent RNA polymerase activity (data not shown), revealing that in the wild-type preparation there was no contaminating host cell RNA polymerase activity.

Homopolymeric Templates. Reaction kinetics were studied using as primer/template oligo(dT_{16})/poly(rA), and product formation was determined with the use of DE-81 filter binding. Initial velocity reaction rates correlated linearly with the concentration of enzyme over the range of 30−150 nM. The steady-state kinetic parameters for the incorporation of UTP are summarized in Table 2. The k_{cat} value as determined for NS5B(Δ21) was higher than that determined with the full length enzyme under the same buffer conditions and was higher than values previously reported for a different version of the truncated enzyme (*13*). The reaction buffer was optimized for NS5B(Δ21), which could explain the increased efficiency of catalysis (k_{cat}/K_M) relative to that of the full-length enzyme. Whether the full length NS5B is equally efficient as its truncated version in steady-state reactions under a different buffer condition is being evaluated.

Heteropolymeric Template. A heteropolymeric single strand RNA template, t500, was generated using T7 runoff transcription of a DNA fragment containing the cDNA sequence of HCV strain BK from the NS2/3 region of the genome. An analysis of the products formed during extension of t500 catalyzed by the NS5B(Δ21) (BK strain) was carried out using radiolabeled GTP and gel electrophoresis on acrylamide−8 M urea gels. The results are shown in Figure 1. At low concentrations of GTP (<1 μM), the predominant product migrated on the gel with approximately the same mobility as the input RNA, and the products were very heterogeneous in size. At higher concentrations of GTP (10 μM), there was predominantly one product which migrated significantly faster on the gel than the substrate t500. By analogy with previous results (*9*, *14*), the faster migrating species corresponded to a hairpin RNA generated by a "copy-back" mechanism, with the enzyme making use of the 3′-end of the template as the primer for RNA synthesis. Addition of a DNA primer, pD500, with the sequence 5′-TTG AAA TGA CTG CGG TAC GGC-3′, designed to anneal to the 3′-end of t500, did not significantly change

Table 2: Kinetic Constants for Synthesis on Primer/Template dT16/Poly(rA) Catalyzed by Full Length and C-Terminally Truncated Hepatitis C NS5B Protein, NS5B(Δ21)[a]

	k_{cat}[b] (pmol/(h/μg) or h^{-1})	K_M^{UTP} (μM)	k_{cat}/K_M (M^{-1} s^{-1})
HCV NS5B(Δ21)	1550 or 98	5.7	4540
full length NS5B	90 or 6	13	128

[a] Reactions included 0.4 μM primer dT_{16}, 10 μg/mL poly(rA) (average chain length of 307 nucleotides), 30 nM NS5B, or NS5B(Δ21) and from 1 to 30 μM UTP, in reaction buffer. Kinetic parameters were determined by a direct fit of the data to the Michaelis−Menten equation. [b] If the fraction (0.14%) of competent NS5B in reactions were the same for both homopolymeric and heteropolymeric substrates and for both enzyme forms, the adjusted values would be 70 000 and 4300 h^{-1} for the truncated and full-length enzymes, respectively (see text for details).

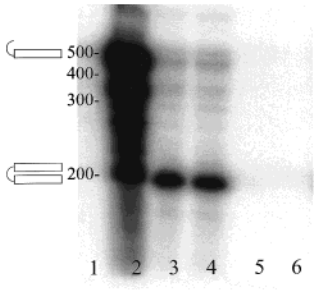

FIGURE 1: PhosphorImage of gel electrophoretic analysis of the products from reactions on template t500. Reactions included 0.5 μM NS5B(Δ21) and 0.75 μg of t500 (90 nM) in a total volume of 50 μL of reaction buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 3 mM MgCl2, 80 mM KCl, 0.4 unit/μL RNasin (Promega), 1 mM DTT, 500 μM ATP, UTP, and CTP, and 0.5 μCi [α-^{32}P]-GTP) with either no nonradiolabeled GTP (lane 2) or 10 μM GTP (lanes 3−6). Reactions proceeded for 2 h, after which 50 μL of proteinase K buffer (300 mM NaCl, 100 mM Tris, pH 7.5, 0.1% w/v SDS) and 50 μg proteinase K (Gibco/BRL) were added. The reaction was incubated at 37 °C for 30 min and then was extracted once with phenol/$CHCl_3$. The RNA was precipitated by the addition of 2 μg of tRNA, ammonium acetate, and one volume of 2-propanol, followed by incubation on dry ice for 1 h. RNA was pelleted by centrifugation at 14K rpm, and the pellet was rinsed with 70% ethanol. The RNA was resuspended in 90% formamide, 1 × Tris borate/EDTA buffer and electrophoresed on 6% acrylamide−8 M urea gels. Bands were visualized after staining with ethidium bromide. Radioactivity was determined with PhosphorImaging. Lane 1 gives the location of RNA century markers (Ambion). Lane 4 represents the products of a reaction containing 5 pmol of DNA primer, pD500, that anneals to the 3′ end of t500. Lane 5 is a control reaction without t500, and lane 6 was from a reaction without any NS5B(Δ21) added.

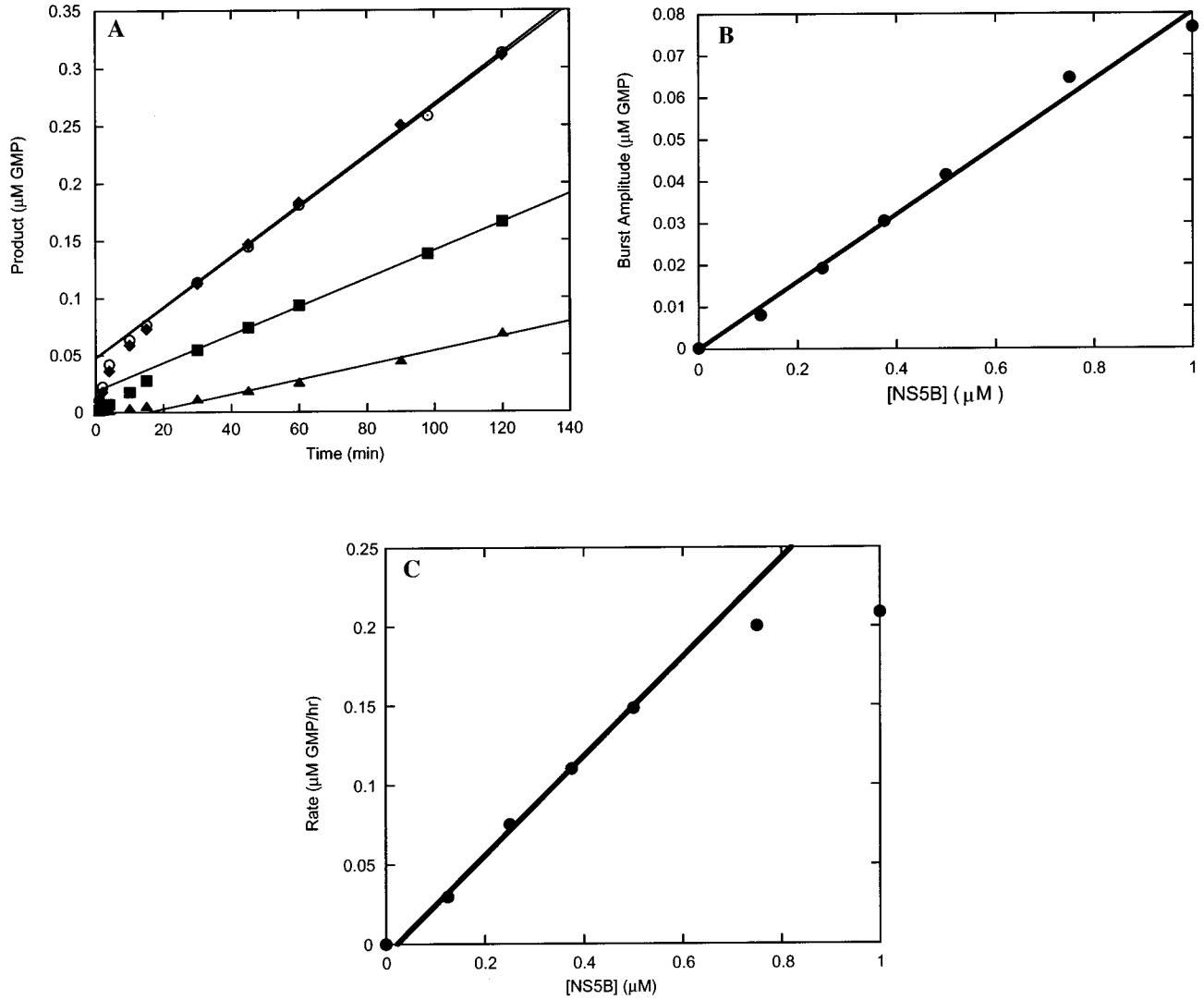

FIGURE 2: (A) Reaction time course with template t500. Reaction time courses included different concentrations of nucleoside triphosphates. NS5B(Δ21)-catalyzed reactions were carried out on template t500, as described in Materials and Methods, in reactions including 20 mM Tris, pH 7.5, 50 μM EDTA, 2 mM $MgCl_2$, 80 mM KCl, 0.4 U/μL RNasin, 5 mM DTT, 0.02 μg/μL t500 (120 nM) and either 20 μM GTP, 500 μM ATP, UTP, and CTP (◆); 10 μM GTP, 500 μM ATP, UTP, CTP (○); 1 μM GTP, 10 μM ATP, UTP, and CTP (■); or 1 μM GTP, 1 μM ATP, UTP, and CTP (▲). NS5B(Δ21) and t500 were preincubated in reaction buffer for 30 min prior to initiation of the reaction by addition of a mixture of NTPs. Reaction aliquots were quenched at the indicated times and product formation was analyzed by DE-81 filter binding. The data from reaction times of 30−120 min were fit to a straight line. The slopes were 2.2×10^{-3} (◆), 2.2×10^{-3} (○), 1.2×10^{-3} (■), and 6.4×10^{-4} (▲) μM GMP incorporated/min. (B) Burst amplitude as a function of the concentration of NS5B(Δ21) in the reaction. Template t500 (0.02 μg/mL, 120 nM) was preincubated with varying concentrations of NS5B(Δ21) in reaction buffer for 30 min followed by initiation of a reaction by addition of a mixture containing 10 μM [α-^{32}P]-GTP and 500 μM each of ATP, CTP, and UTP. Reaction time courses were carried out, and the burst amplitude was determined by extrapolation to zero reaction time of a linear fit of the product vs time plot. Results were plotted as concentration of GMP incorporated during the burst amplitude. The line represents a linear fit to the data. (C) Reaction rates as a function of the concentration of NS5B(Δ21) in the reaction. The slopes of the linear fits to the reaction time courses as described for (B) were plotted. The line represents a linear fit to the data for NS5B(Δ21) concentrations from 0.125 to 0.5 μM.

either the mobility or amount of the hairpin product. Control reactions without either t500 or NS5B(Δ21) showed no significant incorporation of radiolabel into the product.

Reaction Time Courses. The time course of reactions catalyzed by NS5B(Δ21) with template t500 was examined by following the incorporation of [α-^{32}P]-GTP. The formation of product was quantified with use of DE-81 filter binding. As shown in Figure 2A, at high concentrations of NTPs, reaction time courses were biphasic with an initial rapid phase followed by a slower linear phase that began at approximately 20 min of reaction time. In reactions containing a lower concentration of NTP (1 μM), a lag in product formation was evident followed by a linear phase that began at approximately 30 min of reaction time. A lag in product formation has previously been detected in reactions catalyzed by the full length NS5B using template poly(rC)-oligo(dG) (*15*). The amplitude of the initial phase of the reaction, determined by extrapolation of the linear phase to zero time, was equivalent to the incorporation of 0.04 μM GMP with a concentration of 0.5 μM NS5B(Δ21) in the reaction and with a 30 min preincubation of enzyme and template. The amplitude of the initial phase varied linearly with enzyme concentration in the range of 0.125 to 0.5 μM NS5B(Δ21), as shown in Figure 2B. The rate of the linear phase of the reaction also varied linearly with the concentration of NS5B-(Δ21) in the range of 0.125−0.5 μM with 10 μM GTP and

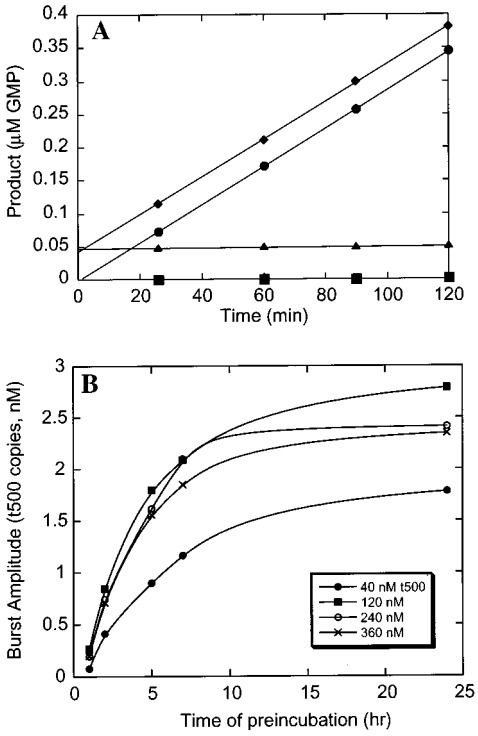

FIGURE 3: (A) Effect of addition of an enzyme-trapping reagent on reaction time courses. Reactions included 0.5 μM NS5B(Δ21) and 4 μg t500 in a final volume of 200 μL (120 nM t500). Reactions either included a 30 min preincubation of NS5B(Δ21) and t500 (◆, ▲) or did not include a preincubation (●, ■). Reactions were initiated either by the addition of a mixture of NTPs (500 μM ATP, UTP, CTP, 20 μM [α-^{32}P]-GTP final concentration; ◆, ●) or a mixture of NTPs at the same final concentration and heparin (1 mg/mL final; ▲, ■). (B) Burst amplitude as a function of time of preincubation of NS5B(Δ21) and various concentrations of t500. NS5B(Δ21) (0.5 μM) and t500 (40, ●; 120, ■; 240, ○; 360 nM, ×) were preincubated in reaction buffer for varying lengths of time. A reaction was initiated by the addition of a mixture of 500 μM ATP, CTP, and UTP and 20 μM [α-^{32}P]-GTP. Reaction time courses proceeded for up to 2 h. The amount of product formed was determined by DE-81 filter binding. The amount of product formed during the burst phase of the reaction was determined by linear extrapolation of the reaction time course to zero time. The lines represent smooth curves drawn through the data points.

500 μM ATP, CTP, and UTP (Figure 2C). The rates of enzyme-catalyzed reactions were determined from the linear region of the reaction time courses, unless otherwise noted.

Effect of a Trapping Agent on Reaction Time Courses. To investigate whether enzyme dissociation occurred during steady-state RNA synthesis catalyzed by HCV NS5B(Δ21), reactions were carried out in the presence and absence of heparin. A concentration of 1 mg/mL heparin was determined to be sufficient to inhibit completely RNA synthesis in reactions that were initiated by addition of enzyme to a mixture of heparin, t500, and NTPs, as shown in Figure 3A. Reactions in which NS5B(Δ21) was preincubated with template t500, prior to the addition of a mixture of heparin and NTPs to initiate the reaction, showed that the initial rapid phase of product formation was still evident. However, the linear phase of product formation was eliminated in the presence of heparin. In addition, it was evident (Figure 3) that the presence of the initial rapid phase of product formation required the preincubation of NS5B(Δ21) and t500. Increasing the concentration of t500 from 120 to 360 nM at a fixed concentration (0.5 μM) of NS5B(Δ21) did not increase the amplitude of the burst phase of the reaction

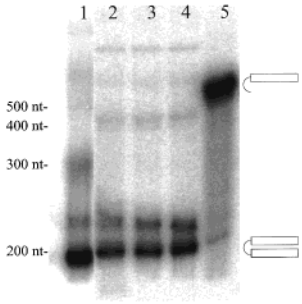

FIGURE 4: Gel analysis of products formed in the presence of heparin: lane 1, products at 30 min of reaction in the absence of heparin; lanes 2−4, products formed in the presence of heparin at 30, 60, and 90 min reaction, respectively; lane 5, product formed at 30 min reaction time in the absence of heparin treated with 5 ng/μL RNase A at 500 mM NaCl for 2 h at room temperature. Reaction products were precipitated by the addition of 2.5 M ammonium acetate and one volume of 2-propanol and analyzed on 6% polyacrylamide/8 M urea gels. Gels were briefly stained in ethidium bromide to visualize the size markers and photographed, and then radiolabeled products were visualized with the use of a PhosphorImager. The lower figure on the right is a schematic representation of a hairpin RNA product, and the upper figure represents the radiolabeled product after treatment with RNase A.

time course, suggesting that the active enzyme approached saturation with template at 120 nM t500 and demonstrating that the concentration of the template did not limit the burst amplitude.

The length of time required for the preincubation of NS5B-(Δ21) and t500 to achieve the maximal burst amplitude was investigated by starting a preincubation including enzyme and template and then, after varying times, initiating the reaction with the addition of a mixture and NTPs. As shown in Figure 3B, the amplitude of the initial phase of the reaction continues to increase even after several hours of preincubation of NS5B(Δ21) and t500.

Reaction time courses were also carried out on a 200-nt template, t200, in the presence and absence of 1 mg/mL heparin. The results of synthesis on t200 were qualitatively similar to the results of synthesis on t500 in that there was a burst of synthesis followed by a linear reaction phase that was eliminated in the presence of heparin.

Product Analysis. The products synthesized in the presence of heparin in reactions including t500 were examined with the use of gel electrophoresis on gels containing 6% polyacrylamide and 8 M urea, as shown in Figure 4. The major product synthesized in the presence of heparin (lanes 2−4) was similar in mobility to the major product synthesized in the absence of heparin (lane 1). Treatment of the product synthesized in the presence of heparin with RNase A at high salt concentration (500 mM NaCl) converted the major product band to a mobility on the gel similar to that of the template, t500 (data not shown).

These results indicated that synthesis in the presence of heparin lead to the formation of a copy-back hairpin RNA product, indistinguishable from the product synthesized in the absence of heparin. The fact that the amplitude of the initial phase of the reaction, as determined from extrapolation of the linear phase of the reaction to zero time in the absence of heparin, was equal to the amount of product made in the presence of heparin indicated that very little of the active enzyme dissociated from t500 prior to completion of the full length copy of the template. The amplitude of the initial phase of the reaction with a 24 hr preincubation of 120 nM

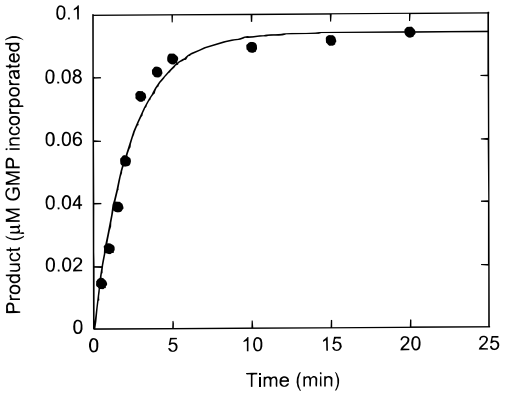

FIGURE 5: Determination of the rate of the initial phase of the reaction in the presence of heparin. NS5B(Δ21) (0.5 μM) was preincubated with template t500 (4 μg in a 200 μL reaction, 120 nM) in reaction buffer for 2 h. A reaction was then initiated by the addition of a mixture of 500 μM ATP, CTP, and UTP, 40 μM [α-^{32}P]-GTP, and heparin (1 mg/mL final concentration). Aliquots were quenched after various reaction times and the amount of product formed was determined with DE-81 filter binding. The reaction progress curve was fit to the equation product $(t) = A[1 - \exp(-k_{obs}t)]$, where A is the maximal amplitude and k_{obs} is the observed exponential rate of the reaction. A was determined to be 0.09 μM GMP incorporated, and k_{obs} was determined to be 0.42 min^{-1}. Since the product formed in the presence of heparin was determined to be a full-length, hairpin copy of the input template, t500, the observed rate of the reaction is 0.4 × 500 = 200 nucleotides/min.

t500 and 0.5 μM NS5B(Δ21) was 0.35 μM GMP incorporated. Because the product formed during the initial phase of the reaction was a full-length copy of t500 containing 125 guanines, 2.8 nM of full-length copies of t500 was synthesized during the initial phase of the reaction. This quantity corresponded to approximately 0.56% of the total NS5B(Δ21) present in the reaction.

The rate of the burst phase of the reaction was determined in a reaction that included 0.5 μM NS5B(Δ21), preincubated with 4 μg of t500 in a volume of 200 μL (120 nM). The reaction was initiated by the addition of a mixture of 40 μM GTP and 500 μM each of ATP, CTP, and UTP and heparin at a final concentration of 1 mg/mL. The results are shown in Figure 5. Under these single turnover conditions, the reaction progress curve was fit to an exponential rate equation to give an observed turnover rate of 0.4 min^{-1}, which corresponded to a rate of incorporation of 200 nucleotides/min for copying the 500-nucleotide template. This value is in line with previous estimates of nucleotide incorporation onto heteropolymeric RNA templates catalyzed by the full-length NS5B (*15*).

The rate of the steady-state reaction displayed saturation kinetics with respect to variable NTP concentrations. Kinetic parameters were determined for each nucleoside triphosphate in reactions in which the other three NTPs were held fixed at a concentration greater than their respective K_M values and the concentration of the template, t500, was at a concentration near saturation; the results are shown in Table 3. The values of k_{cat}/K_M for GTP, CTP, and UTP were somewhat higher than the value for ATP. If the steady-state rate were limited by dissociation of the enzyme from the completed copy of the template, the K_M values for the NTPs would necessarily be lower as measured in these reactions than in reactions in which dissociation is not limiting, such as reactions using much longer templates.

Table 3: Kinetic Constants for Synthesis on the Heteropolymeric Template T500 Catalyzed by NS5B(Δ21)

	experimental[b]			
NTP	k_{cat}[a] (h^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} hr^{-1})	adjusted[c] k_{cat}/K_M (M^{-1} s^{-1})
UTP	0.62	0.6	1.0	2×10^5
CTP	0.48	0.6	0.8	1.6×10^5
ATP	0.38	2.9	0.13	2.6×10^4
GTP	1.4	1.4	1.0	2×10^5

[a] The concentration of NS5B(Δ21) was 0.5 μM. The concentrations of the other three NTPs were held at 500 μM. Kinetic parameters were determined from a direct fit of the rate data to the Michaelis−Menten equation. [b] Uncorrected experimental values from initial velocity, steady-state kinetic data. [c] Adjusted values taking into account that only 0.14% of the purified NS5B(Δ21) is participating in the reaction (see text for details).

DISCUSSION

NS5B(Δ21) Processivity. The incorporation of nucleoside monophosphates into RNA templates as catalyzed by the HCV NS5B(Δ21) protein has been investigated. Product analysis with gel electrophoresis reveal that NS5B(Δ21) is capable of generating hairpin RNA products from linear heteropolymeric RNA templates, as previously described (*7*, *19*). Observation of reaction time courses has revealed that, at high concentrations of NTPs, a burst of product formation occurs after preincubation of NS5B(Δ21) and RNA templates. This product corresponds to the synthesis of one copy of the template by the active enzyme in the reaction. The amount of product formed in the burst phase of the reaction is the same as the amount of product formed in the presence of a trapping agent, heparin, indicating that NS5B(Δ21) does not dissociate significantly from the template during the burst phase of the reaction under our assay conditions. On the other hand, in the presence of heparin the linear phase of the reaction time course is eliminated indicating that the enzyme dissociates from the template during the linear, steady-state phase of the reaction. The rate-limiting step of the kinetic scheme during the linear phase of the reaction is not defined by these experiments, but it is clear that during the linear phase of the reaction time course free enzyme must exist that can be trapped by heparin. Thus, the data presented in Figures 2 and 3 are consistent with the interpretation that once synthesis has begun, NS5B(Δ21) does *not* dissociate from the template until a complete double strand copy of the RNA is made.

Catalytically Productive NS5B(Δ21). The burst amplitude of product formation as a function of NS5B(Δ21) concentration (Figure 2) reveals that only a very small percentage of the purified enzyme is competent in catalyzing RNA polymerization on a single strand RNA template during the burst phase. Assuming that only one enzyme molecule is involved in polymerization per single-strand RNA molecule, then only 0.14% of the purified NS5B(Δ21) is participating in the burst phase of synthesis under our reaction conditions with a 2 h preincubation of enzyme and t500. This finding explains in part the apparent low efficiency of synthesis by NS5B. For heteropolymeric substrates, the apparent turnover rate is 0.4−1.4 h^{-1} (Table 3). If the fraction of productive enzyme that actually participates in the reaction (Figure 3) is factored in, the turnover rate of the hepatitis NS5B(Δ21) is 285−1000 h^{-1}. For homopolymeric substrates such as dT_{16}/poly(rA), the apparent turnover rate is 98 h^{-1} (Table

2). Assuming the same fraction of NS5B(Δ21) is productive in polymerization of homopolymeric substrates, the turnover rate of the hepatitis C NS5B(Δ21) is 70 000 h^{-1}. In comparison, the turnover rate of the HIV-1 reverse transcriptase is ~7200 h^{-1} in steady-state reactions where dT_{12-18}/poly(rA) is the primer/template (*17*). Thus, the fraction of NS5B(Δ21) that participates in polymerization is, in fact, comparable in catalytic efficiency to other polymerases. The turnover and pseudo-second-order rate constants of the catalytically competent NS5B(Δ21) are also given in Tables 2 and 3.

There are several possible reasons for a small percentage of the total enzyme present in the reaction to be capable of processive synthesis in the presence of heparin. There may be only a small fraction of productive protein due to incorrect folding of the majority of the NS5B(Δ21), or there may be a tightly bound inhibitor that arises during either the expression or purification of the protein. There may be a significant amount of nonproductive binding to the single-strand RNA template. It is also possible that the presence of a missing viral or cellular cofactor for the NS5B may correct the problem(s). Secondary structure(s) existing in the t500 may only allow for a fraction of the enzyme in the reaction to be active. Experiments to determine whether any of these possibilities exist are underway.

Recent studies have indicated that only a small fraction of poliovirus 3D polymerase, a viral RNA-dependent RNA polymerase, when expressed in *E. coli* and purified in the absence of cofactors, is capable of processive synthesis via a template switching mechanism in the presence of a trapping reagent, heparin (*22*). The similar conclusion regarding HCV NS5B(Δ21) from this work indicates these two viral RNA polymerases have this feature in common, so a large proportion of purified enzyme being apparently inactive is not unique for NS5B(Δ21).

Polymerization Rate of NS5B(Δ21) *and HCV Replication*. Analysis of single-turnover product formation catalyzed by NS5B(Δ21) has revealed that a full length copy of the t500 template is made at a rate of 200 nucleotides/min (Figure 3).[2] Assuming that transcription of the entire genome is processive and the rate of transcription measured in this work holds constant during replication, about 1 h would be required for one enzyme to generate a copy of the 9.4 kb HCV genome. Cellular or other viral factors and differences in the conditions employed for in vitro reactions from those that exist in vivo would, of course, affect the length of time required. The steady-state, pseudo-second-order rate constant[3] for NS5B(Δ21) is also comparable to other polymerases such as the HIV-1 reverse transcriptase which supports HIV replication at an in vivo rate comparable to that of the hepatitis C (*18*).

Current efforts are aimed at extending these results to substrates mimicking the 3′-end of the HCV genome to study the initiation of synthesis of the (−)RNA strand and substrates mimicking the 3′-end of the (−)RNA strand to investigate the initiation of (+) strand synthesis. One of the hallmarks of picornavirus and, by inference, HCV replication is the preponderance of positive RNA strands made (*23*). A better understanding of the interactions of NS5B and RNA templates may lead to a quantitative explanation of this effect.

[2] This value is independent of the fraction of enzyme productively participating in the reaction and is in agreement with previous estimates of the rate of incorporation of nucleotides onto heteropolymeric RNA templates catalyzed by the full length NS5B (*15*). In comparison, the single turnover incorporation rate for the poliovirus RNA polymerase is 1250 nucleotides/min (*16*).

[3] The steady-state rate constant, defined by k_{cat}/K_M (concentration^{-1}·time^{-1}) represents the pseudo-second-order rate constant for the turnover of substrate to product and should not be confused with the single turnover rate (nucleotides·unit time^{-1}).

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