Elongation of Synthetic RNA Templates by Hepatitis C Virus NS5B Polymerase*

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Here we examine the ability of seven, 3’-related, short synthetic RNAs to serve as templates for the hepatitis C virus (HCV) polymerase, non-structural protein 5B (NS5B). These RNAs, termed HL, range from 8 to 16 nucleotides in length, each with ACC at the 3’ terminus. Interestingly, HL12 and longer templates have a predicted secondary structure. Those with one or two unpaired adenylates at the 5’-end of a stem were increased in size by one or two nucleotides, respectively, following incubation with NS5B and UTP. Using labeled template RNA and cold UTP, extension in size could be inhibited by addition of non-labeled template of the same size. This template elongation was not inhibited by cold linear HL10 template unless pGpG was added. Fluorescence anisotropy demonstrated HL14, a template with secondary structure, bound with an apparent $K_d$ of 22 nM. A linear template, HL10, plus pGpG primer was bound by NS5B with a $K_d$ of 45 nM, whereas HL10 alone bound with a $K_d$ of 182 nM. The amplification of the template extension product was increased by a brief preincubation at 4 °C followed by incubation at 23 or 30 °C. The nucleotide-mediated increase in size occurred for both templates that required a mismatch or bulge at the 3’-end as well as for those without the mismatch. These results suggest an NS5B active site pocket can readily accommodate short templates with four or five base stems and initiate copy-back replication in the presence of a one nucleotide mismatch.

About 3% of the world’s population is chronically infected with hepatitis C virus (HCV). Many of these chronically infected patients develop life threatening cirrhosis and hepatocellular carcinoma (1, 2). Current therapy, interferon-based products plus ribavirin, has a broad side-effects profile and is marginally effective especially in HCV genotype 1. An effective vaccine is also not available.

HCV, a flavivirus, is a positive strand RNA virus that encodes at least 10 proteins, including a viral polymerase, NS5B, an RNA-dependent RNA polymerase (RdRp) is one of the key enzymes involved in replication and a primary target for the development of antiviral drugs.

The crystal structures for NS5B protein in the absence of RNA, determined by three different laboratories independently (3–5), show this enzyme to contain like other polymerase proteins the classic structural domains, denoted as palm, fingers, and thumb. The thumb subdomain plays a role in positioning the RNA for initiation and elongation. Unique to NS5B is the space between the fingers and the thumb domains is small with a short loop connecting these two domains. This loop is likely responsible for the closed conformation of HCV NS5B, as observed in the crystal structure of φ6 RNA-dependent RNA polymerase (6). The β-hairpin, seen in the crystal structure overlapping the active site of NS5B, blocks RNA entry to the active site. It has been proposed that the β-hairpin functions to position the 3’ terminus of RNA templates into the correct position for replication (7). Because the β-hairpin obstructs the active site of the enzyme and limits the entry of double-stranded RNA, it is not clear that NS5B can bind double-stranded RNA and efficiently initiate template transcription (7, 8).

Although HCV NS5B polymerase activity has been extensively studied (8–14), the catalytic mechanism of NS5B at a molecular level, including possible subunit interactions in virally infected cells, has not been established. Reports suggest that only a low percentage of protein purified from bacterial expression systems is active (15). The RNA synthesis catalyzed by NS5B is a nucleotide transfer elongation reaction, where nucleotidyl residues are transferred to the 3’-hydroxyl group of the last nucleotide of the primer-region, hybridized to the template. The replication process can occur by extension of an exogenous primer or copy-back extension where the input RNA forms a hairpin or dimer and supplies both primer and template (16–19). In the presence of high levels of NTP, NS5B is capable of de novo initiation (9, 12, 20, 21). There have been reports of terminal nucleotidyl transferase activity associated with NS5B (8, 10, 16, 22–24). This activity catalyzes the non-specific addition of nucleotides to the 3’-end of template in the absence of primer.

In this report we examine the ability of NS5B to interact with several short synthetic RNA templates, comparing binding of enzyme to template and the generation of products. NS5B activity was compared for enzyme with a truncation of 21 or 55 of the C-terminal amino acids. We report that an RNA template with secondary structure is preferentially bound and more efficient at generating template-directed product via a template elongation reaction compared with binding to a linear RNA with the same 3’ region. It is of interest that templates with secondary structure without and with a mismatch at the 3’-end will yield products formed by template elongation. These results should aid in developing highly spe-
cific templates for NS5B as well as potent inhibitors of viral RdRp.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The NS5B 1b-BKΔ21-His gene was constructed from hybridized DNA oligomers and PCR products, using standard molecular biology techniques. The PCR template was plasmid pRC-CMV-HCV-BK, which contains the genotype 1b BK genomic HCV cDNA, similar to HCV-1b GenBank™ accession number M58335. Oligomers and PCR primers were based upon the M58335 sequence. The NS5B gene was altered by deleting the 21 C terminus amino acids at 37 °C with 3′-prime nucleotide-specific degenerate 5′-amino modifier linker followed by a 6-histidine tag, and adding an 5-prime Ndel site to provide the initiating bacterial ATG. Other restriction sites were added or removed silently to facilitate cloning. Several codons at the beginning and end of the gene were changed, to high frequency bacterial codons. This resulted in a 5′ sequence of atg tct atg tca gtc ggt gct atc acc acg for both Δ21 and Δ55. The 3′ sequence for Δ21 was cgg gta ggc cac cat cat cac taa tag. The assembled gene was inserted into Escherichia coli expression vector pSETb (Invitrogen, Carlsbad, CA) between the Ndel and EcoR1 sites, and sequenced. The predicted amino acid sequence in the non-oligomer, non-PCR primer derived regions differed from M58335 only at position 544, where the R commonly found in 1b isolates was converted to a Q in this BK isolate. Sequencing of the pRC-CMV-HCV-BK PCR template around this position of the difference confirmed that the template encodes Q at this position. The NS5B 1b-BKΔ55 gene was constructed from restriction and PCR fragments and inserted into pSETb, using the constructed NS5B 1b-BKΔ21-His gene as template, deleting the His tag region and 34 additional C terminus amino acid codons. The resulting plasmids were sequenced and found to have the predicted amino acid sequence.

BL21 DE3 cells transformed with the expression clone were grown to saturation at 37 °C in 1 liter of LB medium. A 10-liter LB fermentation vortexed overnight. Precipitated chloride gradients of the 1-liter preculture were preincubated for 5 min, and the reaction mixture was initiated by the addition of NTP solution at the indicated temperature for 30 min or as stated. The product were resolved on denaturing 20% polyacrylamide/urea gels and visualized as stated. The gel bands containing the dissociation constant value using, 2 pmol of RNA, 100 μCi of [γ-32P]ATP, and 30 units of T4 polynucleotide kinase (in buffer 70 mm Tris-HCl, 10 mm MgCl2, 5 mm DTT, pH 7.6). After labeling, the reaction mixture was extracted twice with equivalent volume of phenol-chloroform. The 5′-radioactively labeled RNA was precipitated with ethanol by incubation at –20 °C overnight in the presence of glycerol as carrier, and 10% of 3 M NaOAc (pH 5.2). This was centrifuged at 4 °C, for 15 min at 13,000 × g, and the labeled RNA pellet washed with 70% ethanol to remove residual salt and aid rehydration. The pellet was dissolved in diethylpyrocarbonate-treated water and the concentration of 5′-labeled RNA determined by the 260-nm absorbance.

**[32P]UTP Incorporation Assay Using Unlabeled Template**—Reactions were carried out with 20 μl of buffer containing 25 mm HEPEs (pH 7.8), 1 mm MgCl2, 0.25 unit of RNasin/ml (Promega), 100 μg of bovine serum albumin/μl, 5 mM MgCl2, and 2% glycerol. 1 μM RNA template, 1.25 μM NS5B, 10 μM pGpG (oligonucleotides etc.), 100 μM UTP, 2.5 μCi of [α-32P]UTP (PerkinElmer Life Sciences) label. A mixture of NS5B and RNA template was preincubated for 5 min prior to the initiation by the addition of NTP solution. The reaction was carried out at room temperature (23 °C) for 30 min and then quenched by the addition of 20 μl quenching buffer (90% formamide, 0.025% bromophenol blue, and 0.025% xylene cyanol) or 2× TBE-urea sample buffer (1× TBE, Tris base, boric acid, EDTA, Ficoll 1.2 g/ml, 0.01% bromophenol blue, 0.05% xylene cyanol, 7 mM urea). The quenched reaction mixture was heated to 80 °C for 3 min prior to loading 3 μl onto a denaturing 20% polyacrylamide/7 M urea/Tris borate/EDTA (TBE) gel. Electrophoresis was performed in 1× TBE at 70–90 watts. Following vacuum drying of gels, bands containing isoform were visualized and analyzed using a phosphorimaging device (Bio-Rad).

**Time Course Reaction and Data Analysis**—Reactions were run similarly as described above. 2 μM RNA template and 2.5 μM NS5B in the absence of standard buffer were preincubated for 5 min at 23 °C and initiated by the addition of a mixture of 10 μM pGpG and 200 μM UTP, 2.5 μCi of [α-32P]UTP (values are final concentrations). The reaction time data points were recorded from initiation and to the addition of quenching buffer. The amount of product formed was analyzed by denaturing 20% polyacrylamide/7 M urea/Tris borate/EDTA (TBE) gel electrophoresis. Rates were fit to a burst model (Product(t) = A*(1 – exp(–k_obs*t)) + k_steady-state*A), where A is the maximal amplitude and k_steady-state is the steady-state rate constant of the reaction. k_steady-state is the steady-state rate constant. 

**Nucleotide Incorporation Assay Using 5′ -End-labeled Template**—Using cold nucleotide the assay was performed as above with the following modifications. The reaction volume was 20 μl standard replication buffer with 1 μM of 5′-labeled RNA template, 1.25 μM NS5B, and 100 μM cold UTP with/without 10 μM pGpG primer. The template and enzyme were preincubated for 5 min, and the reaction mixture was initiated by the addition of NTP solution at the indicated temperature for 30 min or as stated. The product were resolved on denaturing 20% polyacrylamide/7 M urea/Tris borate/EDTA (TBE) gel and RNA bands were quantified by phosphorimaging.

**Fluorescence Anisotropy**—Tetramethylrhodamine-labeled oligonucleotides (Dharmacon Research Co.) were generated by attaching the fluorophore to the 5′-end of RNA via a 5′-amino-modifier linker, post synthesis. Labeled RNAs were purified by denaturing gel electrophoresis. Fluorescence experiments were performed using a QM-1 Photon Technology International 7-formate fluorometer with excitation monochromator set to 560 nm with a 3- to 5-nm slit width and emission monochromator set to 585 nm with slit width of 6–8 nm. All measurements were carried out at 23 °C. Titration of the 1b-BKΔ55 were carried out in a standard replication buffer described above. We obtained the dissociation constant value using,

\[
y = \frac{(x + K_d + R) - \sqrt{(x + K_d + R)^2 - 4Rx}}{2R}
\]

where \(y\) is the fraction of the RNA bound complex, \(R\) is the total concentration of fluorescently labeled RNA, \(x\) is the total concentration of added enzyme, and \(K_d\) is the dissociation constant. 

**RESULTS**

**Use of Synthetic RNA Templates**—To better understand the molecular mechanism for NS5B initiation we derived seven short RNA templates, six containing identical 8-nucleotide sequence from the 3′-end and all having two 3′-cytidylates. Starting with
HL8, two nucleotides were added to the 5′-end forming HL10, HL12, HL14-nb or HL14 and HL16 (Table I). HL13-nb has a different stem region. HL16, HL14, and HL12, can form an off-set homo-dimer consisting of a 4-bp stem/3-bp loop/4-bp stem or a hairpin, consisting of an intramolecular fold of 4-bp stem/3-bp loop; each with a single unpaired C at the 3′-end (see Fig. 2) (25). HL14-nb has a five base stem but identical to HL14 except for the two 5′ bases. HL13-nb has a different sequence but also possesses a 4-bp stem/hairpin. HL10 and HL8 form a linear structure only. HL10 and HL8 form a linear structure only. HL13-nb and HL14-nb lack the unpaired C at the 3′-end, whereas HL14 and HL16 will need to form a bulge or bubble out of one C to form a template from the stem.

We examined the products catalyzed by the NS5B polymerase 1b-BKΔ55 when incubated with HL templates, pGpG primer, and α-32P-labeled UTP for 30 min at room temperature. A 3-mer RNA product was likely initiated from the two cytidylates at the 3′ terminus serving as the priming site for pGpG; with the UTP base paired to the adjacent adenosine added to the 3′-end yielding the product, pGpGpU. The amount of 3-mer product measured was highest for HL10 and HL8 templates, reduced in amount for HL12, HL14, and HL16 templates (Fig. 1A). The 3-mer band was only seen for HL14-nb and a 4-mer formed for the HL13-nb template upon prolonged exposure of the gel (Fig. 1A and data not shown). Because the HL12 and longer templates can form secondary structure (Fig. 2 and Table I), this could interfere with the ability of pGpG to prime with the 3′-end of these templates. Following this reasoning, one would expect HL14-nb, having the lowest free energy for secondary structure, to give lowest amount of a 3-mer product (Fig. 2). However, the free energy for all other templates with secondary structure is similar and should, therefore, all give the same level of 3-mer formation, which was not seen. There were no 3-mer bands observed when templates were incubated with labeled UTP in the absence of pGpG (Fig. 1A), and there were no products observed following incubation of NS5B and labeled UTP in the absence of template (data not shown).

While 3-mer was the only product observed for HL8, HL10 and HL12, the other templates, HL13-nb, HL14, HL14-nb and HL16, each generated higher molecular weight bands (Fig. 1A). HL13-nb and HL14-nb gave a band of the same size migrating above 5′ (kinase plus γ-[32P]ATP)-labeled HL14 and below 5′-labeled HL16 (Fig. 1A, lanes 11–14). The product from HL14 template migrated at the same position as the 5′-labeled HL16 (Fig. 1A, lanes 16 and 17), and HL16 gave a band larger than the starting template (Fig. 1A, lanes 19 and 20). In some replicate experiments we observed two high molecular weight bands for the HL14 and HL16 with the upper band migrating at the same position as shown in Fig. 1A and a lower band just above the 5′ (kinase)-labeled template. The discrete bands for these templates, i.e. without significant laddering or higher molecular weight bands, strongly suggested these products were template derived.

When the NS5B 1b-BKΔ21 form of the polymerase was used under the same conditions as in Fig. 1A, the level of 3-mer formation, in the presence of pGpG, was very low for all templates (Fig. 1B). However, high molecular weight bands were readily seen for templates HL13-nb and HL14-nb, with products migrating as observed using 1b-BKΔ55 (Fig. 1B, lanes 4 and 5). For templates HL14 and HL16 each yielded two faint bands, the upper band migrating at the size seen in Fig. 1A, and the lower band migrating about one base lower (Fig. 1B, lanes 6 and 7).

To determine if these higher molecular weight bands were due to template extension of homo-dimer or hairpin, we generated conditions that would disrupt secondary structure of RNA. HL16 was heated above its melting temperature followed by cooling in the absence or presence of an 8-nt primer (5′-GGUCUAU-3′) complementary to the 3′-end of the HL templates. The annealed and non-annealed HL16 templates were incubated with 1b-BKΔ55 and labeled UTP for 30 min and products analyzed. The HL16 template, pre-annealed with the 8-nt primer, did not give the high molecular weight bands (Fig. 1C, lane 2), whereas HL16, heated in the absence of 8-nt primer, gave the same size of high molecular weight bands seen in A and B of Fig. 1 (Fig. 1C, lane 1). Thus, formation of high molecular weight bands appears dependent upon the secondary structure of the template.

T+n Generation—Because all templates with secondary structure and at least one free 5′ A gave the higher molecular weight bands, it is likely this occurs by the enzyme utilizing the stem region of dimer or hairpin as template (Fig. 2). Template extension would require enzyme binding to the double-stranded region and addition of labeled UTP to the 3′ C. For HL14-nb one incoming UTP base pairs with the 5′-adenylate and forms a phosphodiester bond with the 3′-cytidylate (Fig. 2). A time course for HL14-nb revealed rapid accumulation of the 15-mer with an exponential rate of 0.015/s and ~45 s of incubation to generate the half-maximal amount of product (Fig. 3A). For HL14-nb two nucleotides were incorporated yielding a 15-mer with the same exponential rate, 0.015/s, seen for adding one nucleotide to HL14-nb; ~45 s was also the time required to generate half of the final level of product (Fig. 3B). Differences in rate were seen when templates with secondary structure and containing the unpaired C at the 3′-end were used. Utilizing HL14 as the template resulted in the rapid appearance of the T+1 band, followed by the generation of a T+2 band, which co-migrated with the kinase labeled HL16 marker (Fig. 3C). The time required to generate half of the final level of T+2 was ~4 min, and the exponential rate for T+2 generation from HL14 was 0.003/s. The timed reaction demonstrates that there is a rapid addition of one nucleotide, and formation of a 15-mer with an exponential rate of 0.01/s. However, the addition of the second UMP was much slower. The addition of two UTPs to HL13-nb appears to occur as one step; the addition to HL14 (or HL16 data not shown) appears to occur in a two-step process. Nucleotide addition to the existing 3′-ending generating T+1 product was followed by generation of the T+2 product or 16 mer (Fig. 3C).

In the absence of the stem region from hairpin or dimer a high molecular weight product was not observed for HL8 (Fig. 3D). The initial rate of 3-mer production, for HL8 template and pGpG primer, was 0.015/s, similar to that seen for nucleotide addition (T+1) to the templates with secondary structure (Fig. 3). About 45 s was required to generate half the maximal level of 3-mer product observed (Fig. 3D).
Figs. 1. Products from HL series of templates. Incorporation of \([\alpha^{32}P]UTP\) into RNA products catalyzed by NS5B 1b-BKΔ55 using templates HL8, HL10, HL12, HL13-nb, HL14-nb, HL14, and HL16. A 30-min reaction carried out at 25 °C contained: 1 μM template, 200 μM UTP, 1.25 μM 1b-BKΔ55 in the absence of (first lane of each set) or presence of (second lane of each set) 10 μM pGpG (detailed under “Experimental Procedures”). Samples were resolved on denaturing 20% TBE-urea (polyacrylamide gels by electrophoresis). Autoradiograms of gels were analyzed using a PhosphorImager.

A, visualization of RNA products using templates: HL8 (lanes 2 and 3), HL10 (lanes 5 and 6), HL12 (lanes 7 and 8), HL13-nb (lanes 11 and 12), HL14-nb (lanes 13 and 14), HL14 (lanes 16 and 17), HL16 (lanes 19 and 20). 5'-Kinase-labeled molecular weight markers (in bold) are as follows: HL8, lane 1; GACC, lane 4; HL13-nb, lane 9; HL14-nb, lane 10, HL14, lane 15; HL16, lane 18. B, visualization of RNA products catalyzed by BK-Δ21 (lanes 1–7) using HL series of templates under identical replication condition as A, except all were in the presence of 10 μM pGpG. HL8 (lane 1), HL10 (lane 2), HL12 (lane 3), HL13-nb (lane 4), HL14-nb (lane 5), HL14 (lane 6), and HL16 (lane 7). 5'-Kinase-labeled molecular weight markers (in bold) are as follows: HL14, lane 8; HL16, lane 9; GACC, lane 10. C, template HL16 was pre-annealed by heating to 80 °C and cooling to room temperature in the absence (lane 1) and presence (lane 2) of an equimolar amount of 8-nt primer (5'-GGUCUCAU-3'). Once cooled these were used as templates. Thick horizontal line indicates migration of high molecular weight band; the dotted line indicates migration of approximately one nucleotide shorter “high molecular weight band.” These two high molecular weight bands co-migrate with HL16 high molecular weight bands seen in A.

Fig. 2. Schematic representation of the template extended (T+1 and T+2) by NS5B using HL series of templates (HL12, HL14, HL16, HL13-nb, and HL14-nb) in possible homo-dimer secondary structure only. The free energy was reported at 25 °C and 50 mM salt (25). HL12, hairpin, ΔG = −1.2 kcal/mol, dimer, ΔG = −3.6 kcal/mol; HL13-nb, hairpin, ΔG = −1.0 kcal/mol, dimer, ΔG = −3.4 kcal/mol; HL14-nb, hairpin, ΔG = −4.5 kcal/mol, dimer, ΔG = −6.9 kcal/mol; HL14: hairpin, ΔG = −1.2 kcal/mol, dimer, ΔG = −3.6 kcal/mol; HL16: hairpin, ΔG = −1.2 kcal/mol, dimer, ΔG = −3.6 kcal/mol. For the pGpG primer interacting with HL8 (and others) the ΔG = 0.1 kcal/mol.

Products from Labeled Template—Experiments described to this point were performed in the presence of unlabeled RNA template and radiolabeled substrate ([α-32P]UTP). To directly observe the amount of template modified and to examine the T+1 and T+2 sequence for higher molecular weight bands for templates with the 3' mismatch, the HL templates were 5'-end-labeled (γ-32P) plus kinase and used as the template for either 1b-BKΔ21 or 1b-BKΔ55 in the presence/absence of pGpG primer and cold UTP (detailed under “Experimental Procedures”). The size of the starting template HL14 and HL16 is shown in lanes 1 and 2 of Fig. 4. Consistent with data in Figs. 1 and 3, the initial template shifted up one or two nucleotides in size generating T+1 and T+2 for the HL14 and an apparent T+1 and T+2 for the HL16 template (Fig. 4). The T+2 size of
the product was consistent with NS5B binding to the template stem and adding two UTPs to the 3'-end by base pairing with the two adenosines. Template elongation by both forms of NS5B for stem-containing templates did not depend upon the presence of pGpG primer (Fig. 4). The 1b-BKΔ55 polymerase converted about 85% of the HL14 template to a T+2 product with the remainder divided between the starting template and T+1 product (Fig. 4, lanes 5 and 9). The 1b-BKΔ55 catalyzed about 50% of the HL16-labeled template to the T+2 product with very little T+1 product and about 50% unmodified template (Fig. 4, lanes 6 and 10). The NS5B 1b-BKΔ21 form of the enzyme was much less active, converting the HL14 template to

**FIG. 3.** Time course for product formation using HL templates. Autoradiograms following [α-32P]UMP incorporation are shown for denaturing gels as in Fig. 1. Sample aliquots were quenched at the indicated time point, and product formation was determined. Products from the time course were fit to the equation

\[
\text{Product}(t) = A \left[ 1 - \exp(-k_{\text{obs}}t) \right] + k_{ss}t,
\]

where \(A\) is the maximal amplitude of UMP incorporation, \(k_{\text{obs}}\) is the observed burst rate constant of the reaction, and \(k_{ss}\) is the steady-state reaction rate constant. NS5B 1b-BKΔ55 was used to generate: products with HL14-nb template (A); products with HL15-nb (B); products with HL14 (C); and products with HL8 (D). Visualizations of RNA are shown on the left panel. Quantitative analysis of A–D reaction products were shown on the right panel of each corresponding gel. The y-axis was scaled as relative UMP incorporation to the first time point. Conditions were identical as indicated in Fig. 1 except 2 μM RNA templates and 2.5 μM enzyme were used.
a low level of T+1 and very low level to the T+2 form (Fig. 4, lanes 3 and 7) and with the HL16 template only a very small amount of the T+1 product was observed (Fig. 4, lanes 4 and 8). The lower production of T+2 for 1b-BKΔ21 is consistent with the lower specific activity seen for BK Δ21 versus Δ55 with homopolymeric templates. Under identical conditions, 5’-labeled templates HL8, HL10, or HL12 were not elongated by the addition of UTP (data not shown). Higher molecular weight products were not seen when the 5’-labeled HL14 template was incubated with 1b-BKΔ55 under similar conditions in the absence of UTP and presence of 100 μM of single nucleotides ATP, CTP, and GTP. The lack of linear template elongation and the elongation of the HL14 and HL16 templates only in the presence of UTP support the specificity of template extension for these NS5B polymerases.

Effect of Temperature on Template Extension—To examine the optimal temperature required for template elongation, 5’-labeled templates and 1b-BKΔ55 were preincubated at different temperatures prior to addition of UTP. Generation of the T+2 product from both HL14 and HL16 was clearly favored by the 23 °C incubation, compared with 4 °C, 30 °C, and 37 °C (Fig. 5A). We reasoned that, if lower temperatures were needed for either template, secondary structure and/or enzyme binding, then preincubation of enzyme and template at 4 °C, followed by addition of UTP, and an increase in incubation temperature, should increase the percentage of product generated.

1b-BKΔ55 and the isotope-labeled template were preincubated at 4 °C, followed by UTP addition at which point the samples were moved to a higher temperature. Following the 4 °C preincubation, the formation of T+2 RNA product for HL14 was increased to 95% at 23 °C, 93% at 30 °C, and 58% at 37 °C compared with 20% when kept at 4 °C (Fig. 5B).

Reversibility of Template Binding—To determine if the preinitiation complex formed at 4 °C was reversible we preincubated 1b-BKΔ55 with labeled template followed by addition of cold template along with UTP. Without the addition of cold HL14, there was 12% conversion to T+1, 82% conversion to T+2, and 6% left as unmodified HL14. Addition of 1 μM (1×) unlabeled HL14 template resulted in 20% converted to T+2 and 26% unmodified HL14 (an inhibition of 62 and 21%, respectively); addition of 6 μM (6×) of unlabeled template resulted in 3% converted to T+2 and 83% unmodified HL14 (an inhibition of 94 and 82%, respectively, Fig. 6A).

Next we asked if the binding of template by polymerase was still reversible in the presence of nucleotide substrate, UTP.

![Diagram](Image)

**Fig. 4.** HL RNAs were [γ-32P] 5’-labeled (see “Experimental Procedures”) and used as templates for NS5B. A 25-min reaction carried out at 23 °C contained: 1 μM labeled template, (±) 10 μM pGpG, 200 μM cold UTP, 1.25 μM 1b-BKΔ55, or 1.25 μM 1b-BKΔ21 (see “Experimental Procedures”). Lane 1, 5’-labeled HL16 without enzyme; lane 2, 5’-labeled HL14 without enzyme; lanes 3, 5, 7, and 9, 5’-labeled HL14 used as template; lanes 4, 6, 8, and 10, 5’-labeled HL16 used as template; lanes 3 and 4, 1b-BKΔ21 without pGpG; lanes 7 and 8, 1b-BKΔ21 with pGpG; lanes 5 and 6, 1b-BKΔ55 without pGpG; lanes 9 and 10, 1b-BKΔ55 with pGpG.

![Diagram](Image)

**Fig. 5.** Effect of temperature upon product formation by NS5B 1b-BKΔ55. Conditions similar to Fig. 4 except the reaction between NS5B and 5’-labeled template was carried out at the indicated temperature in the presence of UTP without pGpG. A, template and enzyme were preincubated at each indicated temperature for 5 min followed by addition of cold UTP and incubation continued for 30 min at the indicated temperature. B, 5’-labeled HL14, was preincubated with 1b-BKΔ55 for 15 min at 4 °C. Cold UTP was added at this temperature and then raised to the indicated temperature and incubation continued for another 25 min. Lane 4 represents the 5’-labeled HL14 (marker) was incubated in the presence of cold UTP without enzyme.

![Diagram](Image)

**Fig. 6.** A, the ability of cold HL14 to block 5’-labeled HL14 was examined. 1 μM of 5’-labeled template HL14 was preincubated with 1.5 μM NS5B 1b-BKΔ55 at 4 °C for 15 min. The solution was brought to 23 °C for 5 min, unlabeled HL14 was added, and the mixture was incubated for another 5 min. The addition of 100 μM UTP initiated the polymerase reaction, and the reaction was quenched after 25 min at 23 °C. Lane 1, 5’-labeled HL14 (marker); lane 2, generation of T+1 and T+2 in the absence of unlabeled template; generation of T+1 and T+2 in the presence of 1 μM unlabeled HL14 (lane 3) or 6 μM of unlabeled HL14 (lane 4). B, effect of UTP on inhibition by cold template. 1 μM of 5’-labeled template, HL14, was preincubated with 1.25 μM NS5B 1b-BKΔ55 for 15 min at 4 °C followed by the addition of 100 μM UTP, and the reactants were held for another 5 min at 4 °C. To this mixture was added an aliquot containing the indicated final concentration of unlabeled HL14 and the incubation continued for 25 min at 23 °C followed by quenching. In the first lane is 5’-labeled HL14 in the absence of enzyme.

C. Lima, X. Jiang, S. Swanberg, C. Liu, and S. Herrmann, unpublished data.
The HCV polymerase, NS5B, provides mRNA for the generation of viral protein and genomic RNA for the formation of new virions. The overall molecular structure of NS5B is similar to other well characterized polymerase enzymes (6, 26), with the exception of the C-terminal region, which may have some regulatory role and shares homology with a limited number of other viral polymerases (27, 28). Here we examine the interaction of truncated forms of NS5B with a series of short related RNA templates, some able to form secondary structure. These studies were undertaken to shed light upon the template requirements for NS5B and to develop RNAs that bind tightly to the active site of the enzyme.

Two routes can be used by NS5B to catalyze template-directed nucleotide incorporation: primer extension, where exogenous primer base paired to template is extended, and template extension, where a template-formed stem acts as primer and template (Fig. 2). We initiated this study examining a related series of short RNA template molecules that in the presence of the pGpG primer should all give a primer extension product. The linear templates, HL8 and HL10, in the presence of added primer and UTP gave a single product migrating at the position of a 3-mer (Fig. 1A). In the presence of primer and all four required NTPs, the linear templates yield a product equal in size to the starting template (data not shown). Although HL12 gave a 3-mer, the level of product was reduced in
Template Elongation by NS5B

amount compared with 3-mer from the linear templates (Fig. 1A). The HL13-nb and HL14-nb templates supported the generation of very little 3-mer product. With the exception of the HL14-nb, the level of 3-mer produced should be the same for all templates with secondary structure if stability of secondary structure alone determined the yield of 3-mer produced. Instead the level of 3-mer generated ranges from greatest to least as follows: HL12 > HL16 > HL14 > HL13-nb > HL14-nb (Fig. 1). The level of 3-mer generated for these templates is likely to depend upon another variable such as the binding preference of the enzyme.

Fluorescence anisotropy studies indicated 1b-BKΔ55 bound weakly to HL10, whereas the inclusion of pGpG primer increases the binding to HL10, with a 4-fold decrease in apparent $K_d$ (Fig. 7). However, much greater binding of NS5B was observed for HL14, with an apparent $K_d$ of 22 nM, half of that seen for HL10 plus pGpG (Fig. 7). The level of 3-mer production when pGpG primer was added to templates with secondary structure, therefore, appears linked to the preferential form of NS5B to bind the stem present in the secondary structure. Thus given the choice of binding to pGpG-template or binding to a 4- to 5-base stem, the enzyme shows a preference for the secondary structure of the short RNA.

There was much less 3-mer produced by 1b-BKΔ32 for all templates (Fig. 1B). Others have noted a lower activity for 5Δ21 forms of NS5B compared with 5Δ5 forms and attempted to link this difference in activity to the folding back of the C-terminal domain (amino acids 545–570) of 5Δ21 onto the template binding site (27, 28). The 1b-BKΔ55 form of NS5B lacks this region. The level of product generated for the 1b-BKΔ21 form of the enzyme does appear to directly correlate with the level of affinity for the template. This could indicate a certain level of template binding is required to displace or compete with the C-terminal region of NS5B.

HL12 and longer templates all form secondary structure, and all templates provide the same 3'-cytidylate. For all templates longer than HL12, the addition of UTP in the presence or absence of primer resulted in products that were longer than the initial template. We believe these products are arising from template-directed extension, as indicated in Fig. 2. We do not know if the enzyme is binding to the homo-dimer or hairpin form, or both forms of template to add nucleotide(s) to the 3' terminus of the template.

Multiple lines of evidence indicate the T+1 and T+2 products are not due to a NS5B-associated terminal transferase activity. These higher molecular weight bands for templates longer than HL12 were seen with UTP and not with other single nucleotides. In the absence of UTP but with high concentrations of other nucleotides (GTP, CTP, and ATP) high or low molecular weight bands for HL10, HL14, or HL16 were not seen. T+1 and/or T+2 products of the same size were found using cold template plus radiolabeled UTP or radiolabeled template and cold UTP. The annealing of an 8-mer primer to the HL16 template blocked the appearance of the higher molecular weight bands seen in the presence of UTP (Fig. 1C). Addition of cold template blocks template elongation and generation of T+1 and/or T+2 from labeled template (Figs. 5, 6, and 8). All experimental evidence indicates the secondary stem is an excellent substrate for both the 1b-BKΔ21 and 1b-BKΔ55 forms of the NS5B polymerase.

The addition of one UTP to HL14-nb occurs rapidly with near complete utilization of template during the first 3 min of incubation (Fig. 3A). For HL13-nb the incorporation of two UTPs appears to occur in rapid succession, and formation of the T+2 product is occurring at a similar rate of formation as T+1 for HL14-nb (Fig. 3B).

For HL14 to undergo template extension the mismatch in base pairing at the 3'-position must be resolved. One path for forming the T+1 product has the template mismatching the 3' C with the penultimate adenylate and adding the first UMP following base-pairing with the 5'-terminal adenylate. Formation of the T+2 would require a bulging out of the mismatched C and the (T+1) 3'-UMP now base pairing with the penultimate adenylate. Addition of the second UTP would occur across from the adenylate at the 5'-terminal end of the template for HL14 yielding the T+2 product. A second path would require the bulge or resonance bulge to occur first giving a terminal cytidylate base paired with the guanidylate (3 nt from the 5'-end), and the first incoming UTP base pairs with the penultimate adenylate on the 5'-single strand portion of the template yielding the T+1 product. Following this the addition of a second UTP to the 5'-adenosine would occur yielding the T+2 product. In contrast to HL13-nb, the addition of T+1 and T+2 to HL14 appear to occur as separate events (Figs. 1 and 3). For HL14 the exponential rate of reaction for T+2 formation is 0.003/s, one-fifth the initial rate found for HL13-nb (or generation of 3-mer from linear template). A similar scenario would apply to elongation of the HL16 template. For HL14 and HL16 we have not determined which path the enzyme favors, a mismatch first followed by the formation of the bulge or if the bulge formed before the incorporation of the first UTP. The question of how large a bubble would be tolerated on the 3'-end of the double strand of RNA and if polymerase activity would tolerate a bubble on the 5'-side of the double strand, are outside the scope of this report.

For both HL14 and HL16 the lower conversion to T+2 seen at 37 °C is possibly due to stem melting or conformational changes in the enzyme. For the HL14 template there is the formation of a clear T+1 product followed by a clear T+2 product (Fig. 3). However, for HL16, although the generation of T+2 may be somewhat slower, compared with HL14, there is very little accumulation of T+1. This needs to be examined further and could point to an increased stability or possessivity for templates with longer 5’ regions.

For template elongation we cannot differentiate between a hairpin type structure or a dimeric structure as the template acted upon by NS5B. The generation of nearly 100% T+2 product would indicate both sides of the dimer would need to serve as template. Others have reported a low percentage of the purified protein is active in their studies (15, 18). Although primer-dependent elongation can occur with one template yielding multiple products, provided primer is not limiting, the generation of elongated templates occurs once per template. Using a 1:1 ratio of NS5B to 5'-labeled HL14 template we observed a rapid T+1 production and the formation of 80–95% template modification to T+2. If the enzyme remains bound after template elongation this would strongly argue that a high percentage of enzyme must be active for the 1b-BKΔ55 construct. It was noteworthy that, under optimal conditions, primer extension did not yield greater levels of product, on a molar basis, compared with template extension. It is likely that given the choice of template extension or de novo initiation NS5B would find template extension thermodynamically more favorable.

We report here that short synthetic RNAs with secondary structure are tightly bound by NS5B and serve as templates that undergo elongation. The correlation of binding with increased activity strongly suggests the measured NS5B template interaction is occurring at the active site of the enzyme. Short RNA templates are needed to obtain co-crystals of the RNA-enzyme complex as well as to serve as templates in assays designed to identify potent inhibitors of HCV-NS5B polymerase.
Future experiments are needed to determine the tolerance level of NS5B for template bulges during initiation and the effect of longer 5' regions on initiation and elongation. This and future studies should help clarify the role of template secondary structure for viral replication.

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