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# Biochemical properties of full-length hepatitis C virus RNA-dependent RNA polymerase expressed in insect cells

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Abbreviations: HCV, hepatitis C virus; IRES, internal ribosomal entry site; NS5B, nonstructural protein 5B; RdRp, RNA-dependent RNA polymerase; TNTase, terminal transferase; UTR, untranslated region

## Abstract

**The hepatitis C virus (HCV) RNA-dependent RNA polymerase, NS5B protein, is the key viral enzyme responsible for replication of the HCV viral RNA genome. Although several full-length and truncated forms of the HCV NS5B proteins have been expressed previously in insect cells, contamination of host terminal transferase (TNTase) has hampered analysis of the RNA synthesis initiation mechanism using natural HCV RNA templates. We have expressed the HCV NS5B protein in insect cells using a recombinant baculovirus and purified it to near homogeneity without contaminated TNTase. The highly purified recombinant HCV NS5B was capable of copying 9.6-kb full-length HCV RNA template, and mini-HCV RNA carrying both 5'- and 3'-untranslated regions (UTRs) of the HCV genome. In the absence of a primer, and other cellular and viral factors, the NS5B could elongate over HCV RNA templates, but the synthesized products were primarily in the double stranded form, indicating that no cyclic replication occurred with NS5B alone. RNA synthesis using RNA templates representing the 3'-end region of HCV minus-strand RNA and the X-RNA at the 3'-end of HCV RNA genome was also initiated *de novo*. No formation of dimer-size self-primed RNA products resulting from extension of the 3'-end hydroxyl group was observed. Despite the internal *de novo* initiation from the X-RNA, the NS5B could not initiate RNA synthesis from the internal region of oligouridylic acid $(U)_{20}$, suggesting that HCV RNA polymerase initiates RNA synthesis from the selected region in the 3'-UTR of HCV genome.**

**Keywords:** Baculovirus; HCV; *de novo* initiation; NS5B; RdRp; terminal transferase

## Introduction

Hepatitis C virus (HCV) is the major etiologic agent of non-A, non-B hepatitis. Persistent infection by this virus is often associated with chronic hepatitis, liver cirrhosis, and in some cases hepatocellular carcinoma (Choo *et al*., 1989; Saito *et al*., 1990; Major and Feinstone, 1997; Moriwaki *et al*., 2002). It is estimated that chronic HCV infection affects more than 170 million people worldwide (WHO, 1999).

HCV is an enveloped RNA virus in the *Flaviviridae* family with a positive sense single-strand RNA genome of about 9.6 kb. The viral genome consists of one long open reading frame (ORF) that is flanked by untranslated regions (UTRs) at both the 5' and 3' ends of the genome (Brown *et al*., 1992; Chen *et al*., 1992; Yamada *et al*., 1996). The ORF encodes a single polyprotein of 3,010 amino acids that is proteolytically processed into 10 polypeptides by cellular and viral proteases (Major and Feinstone, 1997). The 5'-UTR of 341 nucleotides (nt) contains an internal ribosomal entry site (IRES) that consists of four stem-loop structures followed by a translational initiation codon for the polypeptides (Tsukiyama- Kohara *et al*., 1992; Wang *et al*., 1994; Major and Feinstone, 1997). The 3'-UTR consists of variable regions, pyrimidine-rich tract carrying variable length of U residues, and sequence-conserved 98-nt X-RNA at the far 3'-end (Kolykhalov *et al*., 1996; Tanaka *et al*., 1996; Yamada *et al*., 1996). The (-) 3'-UTR, complementary sequence of IRES, and the X-RNA serve as promoters for HCV RNA polymerase to initiate RNA synthesis. Their secondary structures have been determined (Blight and Rice, 1997; Schuster *et al*., 2002). The promoter in the minus-strand HCV RNA has been shown to be stronger than the promoter in the 3'-UTR of plus-strand HCV genome (Reigadas *et al*., 2001).

The 65-kDa HCV NS5B protein carrying RNA-dependent RNA polymerase (RdRp) activity is the key player in HCV RNA replication. It is a 67×63×68

Å compact globular protein consisting of three subdomains called the palm, finger, and thumb, and resembles a right hand, similar to other related enzymes (Ollis *et al*., 1985; Poch *et al*., 1989; Ago *et al*., 1999; Bressanelli *et al*., 1999; Lesburg *et al*., 1999; Butcher *et al*., 2001; Bressanelli *et al*., 2002). Several conserved amino acid sequence motifs (A, B, C, D, E, and F) have been identified as crucial for RdRp activity (Ago *et al*., 1999; Bressanelli *et al*., 1999). Motif C contains the Gly-Asp-Asp (GDD) motif, the hallmark of the RNA virus RNA polymerases, is involved in binding $Mg^{2+}$ and is essential for RNA polymerase function (Lesburg *et al*., 1999; Cheney *et al*., 2002; Labonte *et al*., 2002).

For replication of the HCV genome *in vivo*, a specific sequence and/or structures of RNA at the 3' ends of both plus- and minus-strands of HCV RNA must be recognized by the HCV RdRp or RNA replicase consisting of other cellular and/or viral factors for *de novo* RNA synthesis. Mapping of *cis*-acting HCV RNA required for RNA synthesis both *in vitro* and *in vivo* indicated that the X-RNA is a minimal *cis*-acting RNA essential for HCV minus-strand RNA synthesis (Oh *et al*., 2000; Friebe and Bartenschlager, 2002). Furthermore, synthesis of minus-strand HCV RNA was shown to be initiated from the internal region of X-RNA with N-terminal $(His)_6$ tagged full-length NS5B and *E. coli* lysyl-tRNA synthetase-fused NS5B (Oh *et al*., 2000; Kim *et al*., 2002). Similarly, 21 C-terminal amino acid truncated NS5B purified from *E. coli* without terminal transferase activity (TNTase) also copied X-RNA by internal initiation at 20-25 nt inside from the 3'-end even though the RNA synthesis efficiency was extremely low (Kashiwagi *et al*., 2002). However, different mechanisms for the RNA synthesis initiation, which can be represented by RNA products synthesized from the plus-strand HCV RNA, have also been proposed. For example, C-terminal 21 amino acid truncated NS5B fused with glutathione S transferase (GST) generated two RNA products, one longer and one shorter than the template X-RNA (Yamashita *et al*., 1998). In addition, insect cell-expressed full-length NS5B generated approximately template-size labeled X-RNA by action of TNTase associated with NS5B (Ishii *et al*., 1999). Other previous studies have also shown that HCV NS5B proteins expressed in insect cells are associated with host TNTase (Behrens *et al*., 1996; Lohmann *et al*., 1997). The NS5B with TNTase activity generated dimer-size RNA product using the full-length 9.6-kb HCV RNA template, which is probably the RNA product synthesized by TNTase-mediated priming and/or copy-back RNA synthesis mechanism from the X-RNA. Furthermore, two recent studies have demonstrated that *E. coli*- and insect cell-expressed full-length HCV NS5B proteins utilize a pyrimidine base in poly (U) and the 3'-UTR of the HCV genome for RNA synthesis initiation (Sun *et al*., 2000; Pellerin *et al*., 2002). One reason for the discrepancy with respect to RNA synthesis initiation using natural HCV *cis*-acting element at the 3'-UTR might be due to intrinsic TNTase activity of HCV NS5B or host origin TNTase co-purified with HCV NS5B. It is still unclear whether HCV NS5B has intrinsic TNTase activity as indicated by recent data shown with the C-terminus truncated NS5B expressed in *E. coli* and the full-length NS5B expressed in insect cells (Ranjith-Kumar *et al*., 2001).

HCV RNA synthesis initiation might also be affected by hydrophobic amino acids at the C-terminus of NS5B. Even though the C-terminal 21 hydrophobic amino acids are involved in membrane anchorage of the HCV NS5B *in vivo* (Ivashkina *et al*., 2002), HCV RNA synthesis initiation seems to be influenced *in vitro* by this hydrophobic C-terminal domain of HCV NS5B. *E. coli*-expressed C-terminal 21 amino acid truncated NS5B generated a longer-than-template sized product with X-RNA (Reigadas *et al*., 2001). Another TNTase-free HCV NS5B with deletion of the 21 C-terminal hydrophobic amino acids copied HCV X-RNA inefficiently by internal initiation (Kashiwagi *et al*., 2002). Tomei *et al.* reported that the full-length NS5B has a slightly enhanced processivity compared to the truncated form of NS5B (Tomei *et al*., 2000). Since all the HCV NS5B proteins used for structure determination were missing these hydrophobic residues (Bressanelli *et al*., 1999; Lesburg *et al*., 1999; Butcher *et al*., 2001), knowledge of the topological characteristics of the C-terminal hydrophobic domain of NS5B is still needed to understand the role of this region in enzymatic function of NS5B. However, experimental data with X-RNA and different forms of NS5B described above suggest the importance of C-terminal region amino acids in polymerase activity and in determining the properties of the polymerase.

In this study, we have expressed HCV NS5B in insect cells as a full-length form and purified it near homogeneity. Using this highly TNTase-free purified NS5B, we investigated the RNA synthesis initiation mechanism with both artificial and HCV genome-derived RNA templates and further characterized the biochemical properties of HCV RNA polymerase.

## Materials and Methods

### Recombinant baculovirus expressing HCV NS5B protein

The NS5B cDNA in pThNS5B and pThNS5B$_{D318H}$ (Oh *et al*., 1999) was subcloned between the *Nhe*I and *Eco*RI sites of the pBlueBacHis2B transfer vector (Invitrogen) to obtain pBacHis-NS5B or pBacHis-NS5B$_{D318H}$. Selection of a recombinant baculovirus expressing

HCV NS5B proteins was carried out using a Bac-N-Blue transfection kit (Invitrogen). Briefly, total $10^6$ *Spodoptera frugiperda* (Sf9) cells per 35 $cm^2$ dish seeded 1 h before transfection were washed twice with 1.5 ml of a fetal bovine serum (FBS; Gibco-BRL)-free medium, then transfected with 4 μg of either pBacHis-NS5B or pBacHis-NS5B$_{D318H}$ mixed with 0.5 μg of linearized Bac-N-Blue DNA (Invitrogen) using the supplied liposome. The transfected cells were incubated for 72 h at 27°C and plaque purification was performed as described previously (Lee *et al*., 1998) to obtain pure recombinant baculoviruses expressing NS5B proteins.

### Expression and purification of recombinant HCV NS5B protein from insect cells

To express recombinant NS5B proteins, Sf9 insect cells were adherently cultured at 27°C. Complete TNM-FH media (Sigma) supplemented with 10% FBS and penicillin-streptomycin were used for cultivation of Sf9 cells. The Sf9 cells infected with recombinant baculovirus were harvested 72 h post-infection and washed once with phosphate-buffered saline (pH 7.4). The washed cell pellet was resuspended in 10 ml of binding buffer (50 mM Na-phosphate [pH 8.0], 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 10% glycerol, 1% Nonidet P-40) supplemented with EDTA-free protease inhibitor cocktail (Roche), then frozen at -80°C. After thawing on ice, cells were sonicated on ice and crude cell extracts were centrifuged at 10,000 rpm for 30 min to obtain cleared lysates, which were incubated for 2 h on ice with Ni-nitrilotriacetic acid (NTA)-Sepharose resin pre-equilibrated with the binding buffer. After washing with 10 column volumes of the binding buffer, bound NS5B proteins were step-eluted with the binding buffer containing 50 to 450 mM imidazole. NS5B-containing fractions were collected and dialyzed against buffer A (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 10% glycerol), and then applied to a heparin-Sepharose CL-4B column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The column was washed with buffer A and the bound proteins were eluted using a NaCl gradient of 0.1 to 1 M. The peak fractions containing pure NS5B were pooled and dialyzed against buffer A. The proteins were further purified by passing them through an SP-Sepharose column (Amersham Pharmacia Biotech). Adsorbed proteins were then eluted with a 10-ml linear gradient of NaCl from 0.1 to 1 M in buffer A. Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin as a standard.

### Preparation of RdRp template RNA

RNA templates derived from HCV genome were prepared as descried previously using MEGAscript T7 kits (Ambion) (Oh *et al*., 1999). Mini-HCV RNA was prepared using the PCR product amplified from pT7Mini-HCV that was constructed by joining the cDNAs for full-length 5'- and 3'-UTRs of the HCV genome. After *in vitro* transcription, DNA templates were digested by treating the transcription mixture with RNase-free DNase I for 15 min at 37°C. Runoff transcripts were then phenol-chloroform extracted to remove T7 RNA polymerase and free ribonucleotides were removed using a Sephadex G-25 column (Amersham Pharmacia Biotech). The flow-through fractions were precipitated with 2.5 volumes of 5 M ammonium acetate and isopropanol (1:5). RNA concentration was determined by measuring the $OD_{260nm}$.

### RdRp activity assay

*In vitro* RdRp activity assay was carried out with 200 ng of purified NS5B in a total volume of 25 μl containing 50 mM Tris-HCl (pH 8.0), 50 μM NaCl, 100 μM potassium glutamate, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 20 units of RNase inhibitor (Promega), 0.5 mM each ATP, CTP, and GTP, 5 μM UTP, 10 μCi of [α-$^{32}$P] UTP (3,000 Ci/mmol; Amersham Pharmacia Biotech). For small RNA template, 200 ng of RNA was routinely used. RdRp reactions with the poly (A) template (1 μg) were conducted in the absence or presence of 10 pmol of oligonucleotide $(U)_{20}$. The reaction mixture was incubated for 2 h at 25°C unless otherwise specified. After RdRp reactions, 35 μl of deionized $H_2O$ containing 20 μg of glycogen (Roche) and 60 μl of an acidic phenol emulsion (phenol, chloroform, 10% SDS, 0.5 M EDTA [1:1:0.2:0.04]) were added to the reaction mixtures to stop the reactions. RNAs were then precipitated with 2.5 volumes of 5 M ammonium acetate-isopropanol (1:5) and washed with 70% cold ethanol. The products were resuspended in a denaturing loading buffer containing 95% formamide with 10 mM EDTA and 0.025% each of xylene cyanol and bromophenol blue. After heat denaturation and quick chilling on ice, the products were resolved on 8 M urea, 5% polyacrylamide gel for poly (A), (-) 3'-UTR, and X-RNA, or on 1% agarose gel for HCV full-length genomic RNA and mini-HCV RNA. The denaturing sequencing gel was pre-run at 300 V for 1 h before samples were loaded. The gels were stained with ethidium bromide, photographed to locate the template positions, and then dried after fixing. Dried gels were exposed to X-ray film (BioMax MS, Kodak) for autoradiography.

### 5'-end labeling of RNA and assay of terminal transferase activity

Labeling of HCV X-RNA with T4 polynucleotide kinase (New England Biolabs) and [γ-$^{32}$P] ATP (6,000 Ci/

mmol; NEN Life Science Products) was performed as described previously (Oh *et al.*, 2000). TNTase activity assay was carried out with the labeled X-RNA ($10^5$ cpm) and 200 ng of unlabeled X-RNA as templates using 0.5 mM each ATP, CTP, GTP, and UTP as substrates. Oligouridylic acid $(U)_{20}$ (10 pmol) was also used as a template to detect TNTase activity of purified NS5B by incubation with 200 ng of purified NS5B and 10 μCi of [$\alpha$-$^{32}$P] ATP (3,000 Ci/mmol; Amersham Pharmacia Biotech) as a single substrate for up to 100 min.

### SDS-PAGE and Western blot analysis

Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked with 5% skim milk in TBST (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20), reacted with either anti-His antibody (Qiagen) at a 1:1,000 dilution or anti-NS5B monoclonal antibody kindly provided by Dr. S. B. Hwang at Hallym University, Korea. Proteins were detected using goat anti-mouse IgG conjugated with peroxidase (Sigma) and enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

## Results

### Expression and purification of recombinant HCV NS5B protein in insect cells

In order to investigate HCV RNA synthesis initiation mechanism and to analyze the biochemical properties of HCV NS5B expressed in eukaryotic cells as a full-length form, both wild-type and mutant ($NS5B_{D318H}$) forms of recombinant HCV NS5B were expressed in insect cell Sf9 using recombinant baculoviruses (Figure 1A, lanes 3 and 4). For comparison of biochemical properties of eukaryotic cell-expressed NS5B with *E. coli*-expressed one, the cDNA of the HCV NS5B was used whose protein was characterized previously (Oh *et al.*, 1999) for the generation of recombinant baculoviruses. Western blot analysis for detection of the six histidine residues tagged at the N-terminus of NS5B confirmed the expression of approximately 65-kDa HCV NS5B in insect cells infected with recombinant baculoviruses 3 days post-infection (Figure 1B, lanes 3 and 4). Authenticity of NS5B proteins were also confirmed by Western blot analysis with anti-NS5B monoclonal antibody (data not shown).

HCV NS5B proteins were purified by Ni-affinity chromatography using cell extracts prepared 3 days post-infection by lysis and sonication of infected cells in a buffer containing a high salt content, a non-ionic detergent, and glycerol. NS5B bound to the Ni-NTA column was then eluted with 250-350 mM imidazole

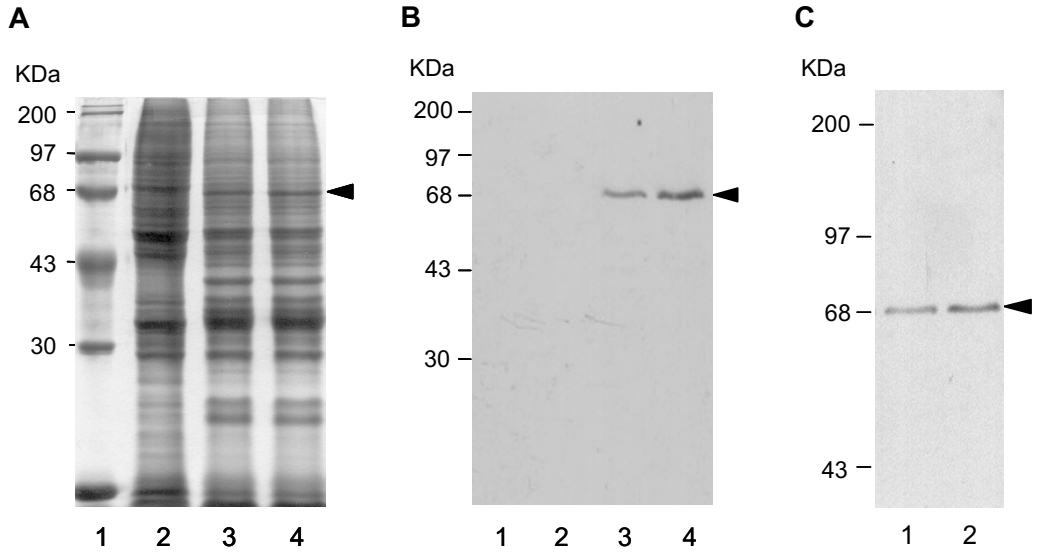


**Figure 1.** SDS-PAGE and Western blot analysis of HCV the full-length NS5B protein derived from purification steps. Sf9 insect cells were infected with recombinant baculoviruses expressing the wild-type NS5B or the mutant NS5B ($NS5B_{D318H}$) and harvested 3 days post-infection. Crude insect cell extracts were separated by SDS-10% PAGE followed by Coomassie brilliant blue staining (A) or Western blot analysis with monoclonal anti-his antibody (B). (A and B) Lane 1, molecular weight markers; lane 2, crude extract of non-infected insect cells; lane 3, crude extract of insect cells expressing the wild-type NS5B; lane 4, crude extract of insect cells expressing mutant $NS5B_{D318H}$. (C) Silver staining of the purified wild-type NS5B (lane 1) and mutant $NS5B_{D318H}$ (lane 2) resolved by SDS-10% PAGE.

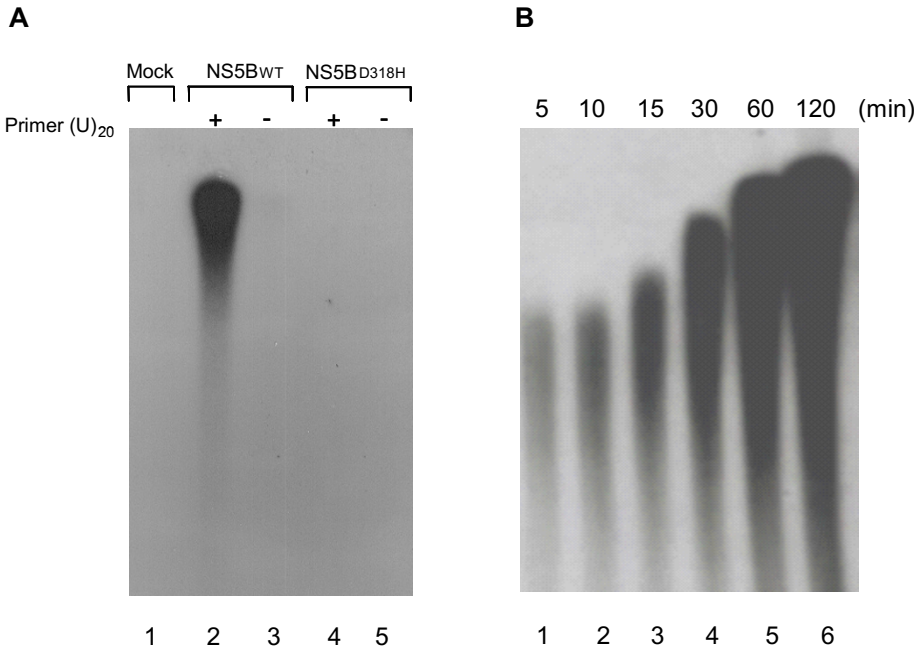


**Figure 2.** RdRp activity assay with a poly(A) template and an oligo$(U)_{20}$ primer. (A) *In vitro* RdRp assays were performed with the purified wild-type NS5B ($NS5B_{WT}$) and mutant $NS5B_{D318H}$ using a poly(A) RNA template in the presence (+) or absence (-) of the primer $(U)_{20}$. (B) *In vitro* RdRp reactions with poly(A) and primer $(U)_{20}$ were stopped at the time points indicated above the gel (in minutes). Products were separated on 5% acrylamide/8 M urea gel after heat denaturation.

and the pooled NS5B fractions were loaded onto a heparine-Sepharose CL-4B column after dialysis in buffer A. Bound NS5B proteins were eluted by a NaCl gradient in buffer A. Main peak fractions (~400-600 mM NaCl-eluted fractions) were pooled and loaded onto an SP-Sepharose column after adjusting the NaCl concentration to 100 mM. Most NS5B proteins were eluted from the SP-Sepharose column with buffer A containing 500-700 mM NaCl. Both wild-type NS5B and mutant $NS5B_{D318H}$ proteins purified in parallel were obtained with near homogeneity, as shown by silver staining (Figure 1C). Highly purified full-length recombinant HCV NS5B carrying a hexahistidine tag at the amino terminus was used in subsequent experiments.

### Primer-dependent RNA synthesis using poly(A) RNA template

RdRp activity assays were carried out in the absence or presence of primer $(U)_{20}$ to confirm the enzymatic activity of purified HCV NS5B and to test the primer dependency of RNA synthesis using a poly(A) RNA template. In the absence of NS5B, no products were produced (Figure 2A, lane 1). Purified NS5B was able to synthesize RNA products using a poly(A) template only with the oligo$(U)_{20}$ primer (compare lane 2 with 3). Mutant $NS5B_{D318H}$, which has a histidine at the position of the first aspartate of the GDD motif, completely lost the RNA polymerase activity (lanes 4 and 5), confirming the enzyme activity of recombinant NS5B purified by three steps of column chromatography. The kinetics of RNA synthesis using a poly (A) template/oligo$(U)_{20}$ substrate were investigated. *In vitro* RdRp reactions were stopped at 5, 10, 15, 30, 60, and 120 min and heat-denatured products were separated on 5% acrylamide/8 M urea gel. The product size increased steadily up to a range of 60 and 120 min, and both high and low molecular weight RNA products gradually accumulated (Figure 2B), indicating that RNA synthesis from the primer bound to the template is not strictly position-dependent.

### *De novo* initiation of RNA synthesis using HCV genomic RNA and mini-HCV genome containing 5'- and 3'-UTRs of HCV genome

RNA synthesis initiation on authentic HCV RNA templates was characterized. RdRp activity assays were performed with full-length HCV RNA and mini-HCV RNA carrying both 5'- and 3'-UTRs in the absence of a primer. For the full-length HCV RNA template, RdRp reactions were stopped at 5, 10, 15, 30, 60, 120, 180, 240, 300, and 360 min. Products were separated on 1% agarose gel after heat denaturation. RNA product size increased steadily up to 120 min

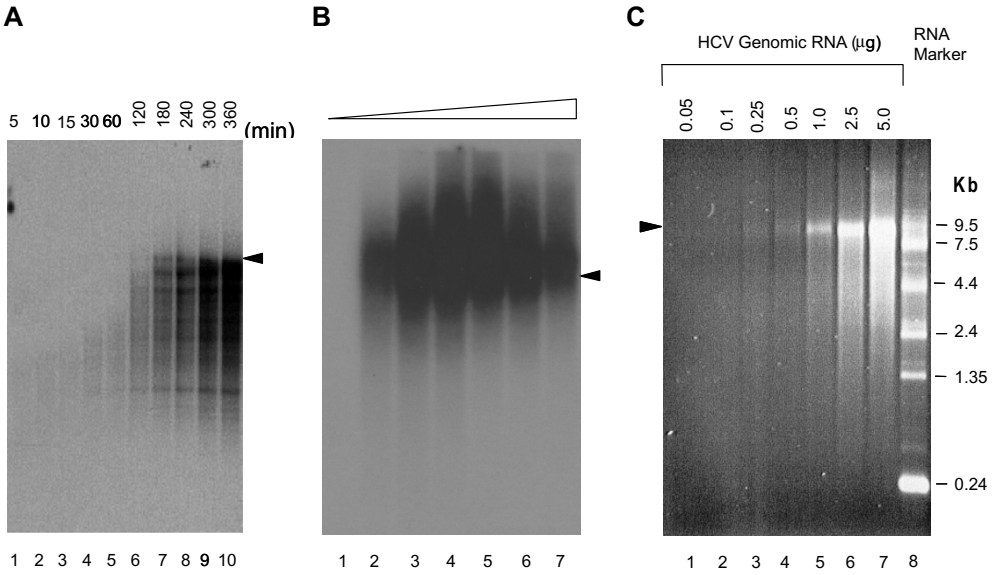


**Figure 3.** RdRp activity assay with a full-length HCV genomic RNA template. *In vitro* RdRp assays were performed with a full-length HCV RNA template. (A) *De novo* initiation of RNA synthesis with a full-length HCV RNA template. *In vitro* RdRp reactions were stopped at the time points indicated above the gel (in minutes). Products were separated on 1% agarose gel after heat denaturation. (B) Increasing amounts of full-length HCV RNA templates as in (C) were added in the RdRp reactions. The products were resolved in non-denaturing agarose gel without heat-denaturation. (C) The same agarose gel as in (B) was stained with ethidium bromide to localize the template position. RNA size markers are indicated in kilobases on the right. The amounts of the RNA templates used in the reactions are indicated above the gel. The arrowheads indicate the positions of the RNA templates.

when the product reached the template size (Figure 3A, lane 6). The kinetics of RNA synthesis using a full-length HCV RNA template over 360 min time-course indicated that RNA synthesis is not initiated from the 3'-snap-back structure, in contrast to previous reports using TNTase-associated HCV NS5B proteins purified from insect cells (Behrens *et al*., 1996; Lohmann *et al*., 1997; Ishii *et al*., 1999).

Next, we investigated whether HCV NS5B can perform cyclic replication without help of cellular and/or viral factors; synthesis of complementary RNA using the input RNA template and subsequent use of the RNA products for the synthesis of original input RNA template. To this end, polymerase reactions were programmed with increasing amounts of full-length HCV RNA template to visualize labeled plus-strand RNA synthesized from the double-stranded replication intermediate. The RNA products that were not heat-denatured before loading were mainly in the double-stranded form and thus migrated slowly than the RNA template in non-denaturing agarose gel (Figure 3B). This result is consistent with a previous report showing that the RNA products synthesized using full-length HCV genomic RNA are resistant to the nuclease S1 degrading single-stranded RNA (Oh *et al*., 1999). Since RNA synthesis was inhibited at concentrations higher than 1.0 μg of the RNA substrate in the reaction (lanes 6 and 7), and non-denatured RNA products migrated close to the template position, it was difficult to detect a discrete band co-migrating with the template, even on the briefly-exposed X-ray film. Thus, a similar analysis was performed with the mini-HCV genome consisting of both 5'- and 3'-UTRs of HCV RNA. This RNA template contains all the known promoters for HCV RdRp (Oh *et al*., 1999). By analysis of denatured RNA products in 1% agarose gel, we found that a majority of RNA products migrated to the template position. Longer-than-template products, possibly generated by extension of nascent RNA products without template or by polymerase slippage and/or stuttering at the pyrimidine-rich tract at the 3'-UTR, were also detected (Figure 4A). In contrast, non-denatured RNA products run in parallel on agarose gel migrated slower than the template, indicating the products were in the double-stranded form (Figure 4B). Since this RNA template was well separated from the double-stranded product in non-denaturing gel, any labeled template-size product synthesized by cyclic replication could be detected. But no newly synthesized plus-strand mini-HCV RNA co-migrating with the template was detected (Figure 4B). The RNA product smaller than the template also appeared, but the nature of this RNA band is not yet known. Since the minus-strand form of mini-HCV RNA co-migrates with the plus-strand form in non-denaturing agarose gel (data not shown),

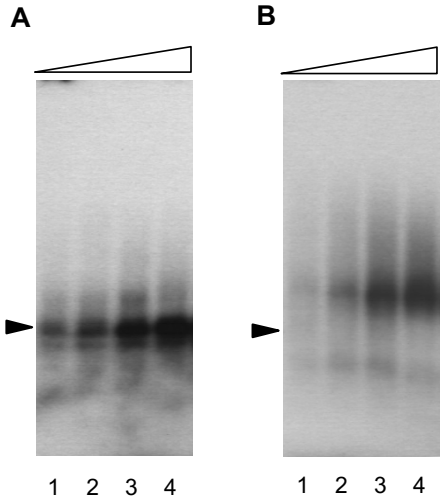


**Figure 4.** RdRp activity assay with mini-HCV RNA. *In vitro* RdRp assays with mini-HCV RNA were performed. Products were resolved on 1% agarose gel with heat-denaturation (A) or without heat-denaturation (B). Increasing amounts (0.1, 0.25, 0.5, and 1.0 µg) of RNA template were included in the RdRp activity assay reactions. Arrowheads indicate the positions of the RNA templates.

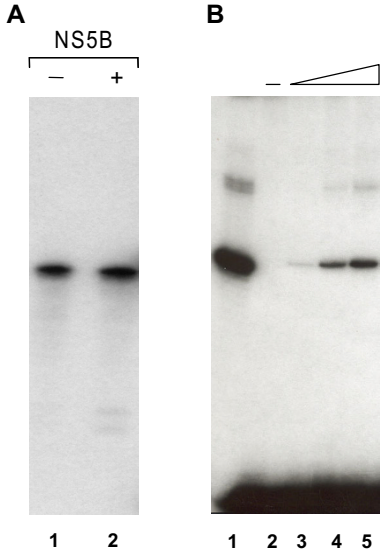


**Figure 5.** Assay of TNTase activity. TNTase activity assays were performed using labeled HCV X-RNA or unlabeled oligouridylic acid $(U)_{20}$. (A) 5'-labeld HCV X-RNA was reacted with purified HCV NS5B (+). Products were separated on 5% acrylamide/8 M urea gel after heat denaturation. (-) indicates the reaction performed without the enzyme. (B) Reactions were performed with 10 µCi of [$\alpha$-$^{32}$P] ATP as a single substrate and oligouridylic acid $(U)_{20}$ template for 25 min (lane 3), 50 min (lane 4), and 100 min (lane 5). Products were separated on 20% denaturing polyacrylamide gel after heat denaturation. An end-labeled template is shown in lane 1 as a size marker. (-) indicates the reaction without the enzyme.

the smaller-than-template product is unlikely to be the full-length minus-strand mini-HCV genome. It might represent the pre-terminated RNA products not in a stable double-stranded form corresponding to the smaller-than-template product detected when the products were denatured (Figure 4A). All together, our results shown in Figures 3 and 4 indicate that HCV NS5B alone is not capable of carrying out cyclic replication *in vitro*.

### No intrinsic TNTase activity of HCV NS5B and *de novo* RNA synthesis using oligouridylic acid $(U)_{20}$

In order to demonstrate that the HCV NS5B used in this study for the characterization of HCV RNA synthesis initiation is free of host or intrinsic TNTase activity, TNTase activity assay was carried out with 5'-labeld X-RNA and unlabeled ribonucleotide triphosphates as substrates. No ladder-like products above the labeled X-RNA template were generated (Figure 5A), indicating that highly purified HCV NS5B from insect cells is not associated with a detectable level of TNTase. No intrinsic TNTase activity of the NS5B was observed using oligouridylic acid $(U)_{20}$ template and [$\alpha$-$^{32}$P] ATP as a single substrate. Even with the single labeled substrate, no longer-than-template products migrating above the template were generated, indicating that the HCV NS5B proteins expressed and purified from insect cells have no detectable level of intrinsic TNTase activity (Figure 5B). Furthermore, the RNA products synthesized were mainly template-size and no abortive products were observed in 20% denaturing polyacrylamide gel, indicating that HCV RNA polymerase preferentially initiates RNA synthesis from the 3'-end rather than starting RNA synthesis internally using this small artificial RNA template.

### RNA synthesis initiation from X-RNA and minus-strand 3'-UTR of HCV genome

Previously, insect cell-expressed full-length NS5B proteins have been shown to initiate RNA synthesis by snap-back intramolecular priming (Behrens *et al.*, 1996; Lohmann *et al.*, 1997) or by internal initiation in the poly(U) and 3'-UTR of the HCV genome (Sun *et al.*, 2000; Pellerin *et al.*, 2002), whereas the full-length HCV RNA polymerase expressed in *E. coli* was shown to start RNA synthesis *de novo* from internal single strand region in X-RNA loop I and from the 3'-end of (-) 3'-UTR of HCV genome (Blight and Rice, 1997; Oh *et al.*, 1999). To clarify this discrepancy, RdRp reactions was performed using X-RNA and the (-) 3'-UTR of HCV. In consistent with results obtained with the *E. coli*-expressed HCV RNA polymerase (Oh *et al.*, 1999), NS5B purified from insect cells without TNTase contamination preferentially initiated RNA synthesis from the internal region in

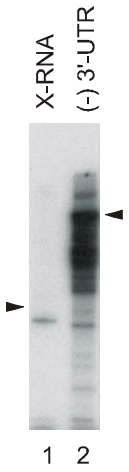


**Figure 6.** RdRp activity assay with HCV minimal *cis*-acting RNA templates. RdRp activity assays were performed with HCV X-RNA (lane 1) or (-) 3'-UTR (lane 2). Products were separated on 5% acrylamide/8 M urea gel after heat denaturation. Arrowheads indicate the positions of the RNA templates.

X-RNA loop I, specifically from U78, resulting in products smaller than the X-RNA template of 98-nt in size (Figure 6, lane 1). Moreover, major RNA products synthesized using the (-) 3'-UTR co-migrated with template. These results underscore that both *E. coli*- and eukaryotic cell-expressed HCV NS5B proteins initiate RNA synthesis *de novo* with HCV *cis*-acting RNA elements in both strands of HCV genome. We also found that HCV NS5B proteins apparently utilize a *cis*-acting RNA element in the (-) 3'-UTR more efficiently than X-RNA, a minimal *cis*-acting RNA required for the synthesis of minus-strand HCV RNA.

## Discussion

In this study, recombinant HCV NS5B proteins with a hexahistidine tag at the N-terminus were expressed in insect cells and purified by sequential chromatography using Ni-NTA-, heparin-, and SP-Sepharose columns in order to investigate RNA synthesis initiation mechanism and biochemical properties of eukaryotic cell-expressed full-length HCV RNA polymerase. Previously, Kashiwagi *et al.* reported that the NS5B proteins with a $(His)_6$ tag at the N-terminus lose the enzymatic activity (Kashiwagi *et al*., 2002). In poliovirus, similarly, N-terminal residues of poliovirus RNA polymerase has been reported to be critical for elongation activity and oligomerization of polymerase (Plotch *et al*., 1989; Hobson *et al*., 2001). However, both HCV NS5 proteins expressed in this study as a fusion protein with an extra amino sequence of N-Met-Gly-Ser-(His)6-Gly-Met-Ala-C at the N-terminus and the HCV NS5B expressed previously in *E. coli* as a fusion protein with a similar length peptide of N-Met-Pro-Arg-Gly-Ser-(His)6-Gly-Met-Ala-C at the N-terminus (Oh *et al*., 1999) were functionally active. Furthermore, NS5B with GST or *E. coli* tRNA synthetase fused to the N-terminus of NS5B still had polymerase activity (Yamashita *et al*., 1998; Kim *et al*., 2002). Therefore, short peptide or fusion protein tagged at the N-terminus of HCV NS5B apparently does not interfere with RNA polymerization.

HCV NS5B proteins purified from insect cells herein initiate RNA synthesis *de novo* from the single stranded region in X-RNA loop I (Figure 6). RNA synthesis from (-) 3'-UTR likewise started *de novo*, but mainly from the 3'-end of template RNA, resulting in prominent formation of a template-size product. In addition, the (-) 3'-UTR was found to serve as a better template for NS5B, supporting the observation that more plus-strand HCV genome accumulates in the infected cells than the minus-strand HCV RNA (Lohmann *et al*., 1999). Similar result has also been obtained with C-terminal 21 amino acid truncated NS5B (Reigadas *et al*., 2001), but the NS5B produced a longer than template sized product using X-RNA as a template. This result suggests that C-terminal region of NS5B might play a role in the initiation of RNA synthesis from 3'-end of HCV genome. To date, HCV NS5B polymerase has been shown to initiate RNA synthesis by snap-back or *de novo* mechanisms using a natural HCV RNA template (Behrens *et al*., 1996; Lohmann *et al*., 1997; Yamashita *et al*., 1998; Ishii *et al*., 1999; Oh *et al*., 2000; Sun *et al*., 2000; Ranjith-Kumar *et al*., 2001; Reigadas *et al*., 2001; Kashiwagi *et al*., 2002; Kim *et al*., 2002; Pellerin *et al*., 2002). Our results shown with several HCV RNA templates using TNTase-free NS5B are in agreement with the previous result obtained with *E. coli*-expressed NS5B (Oh *et al*., 1999), indicating that HCV NS5B proteins initiate RNA synthesis in a primer-independent manner.

We have demonstrated that NS5B proteins purified from insect cells do not have intrinsic TNTase activity, in contrast to previous reports (Behrens *et al*., 1996; Ranjith-Kumar *et al*., 2001). In addition, any 3'-terminally extended products was not detected from oligouridylic acid $(U)_{20}$. Thus, this results strongly suggest that HCV NS5B-associated TNTase activity previously reported by others (Behrens *et al*., 1996; Lohmann *et al*., 1997) is likely originated from insect cells. Interestingly, we did not find that NS5B could copy a small homopolymeric oligonucleotide $(U)_{20}$ by initiating RNA synthesis from the 3'-end of the template, suggesting that RNA synthesis from the 3'-UTR of HCV genome is not likely to initiate from the U-rich tract upstream of X-RNA in 3'-UTR. This result argues against a

recent report showing that HCV RNA polymerase can start RNA synthesis internally from a pyrimidine base in poly(U) and the 3'-UTR of the HCV genome (Sun *et al*., 2000; Pellerin *et al*., 2002).

Even though the full-length HCV NS5B purified from insect cells in this study copied the full-length HCV genome *de novo* in the absence of other cellular and viral factors, HCV RNA polymerase alone could not accomplish cyclic replication of the RNA substrate (Figure 3B). Analysis of RNA products synthesized from the mini-HCV genome also revealed that the HCV polymerase alone could not perform cyclic replication (Figure 4B). The presence of both 5'- and 3'-UTRs in the mini-HCV genome increases the polymerase processivity or prevents the early termination of RNA synthesis. The amount of abortive or shorter-than-template ladder-like RNA products, which were routinely generated when the 3'-UTR RNA template was used as a template (Oh *et al*., 1999), was decreased as shown in Figure 4B. This result suggests that the interaction of both ends of the HCV viral genome, probably *via* NS5B, has an enhancing effect for the synthesis of minus-strand HCV RNA. Long range *cis*-effect of the 5'-UTR on RNA synthesis initiation and elongation remains to be determined.

Lack of cyclic replication of HCV RNA by HCV RNA polymerase alone indicates that other cellular and/or viral factors, or a certain cellular compartment, are required for reconstitution of true HCV RNA replicase *in vitro*. Several partial reconstitutions of HCV RNA replicase were attempted using HCV non-structural proteins to investigate their functions in HCV RNA synthesis. HCV NS3 helicase with an RNA-duplex unwinding activity and a binding affinity to NS5B polymerase (Du *et al*., 2002) has also been shown to interact with HCV X-RNA and 3'-UTR (Kanai *et al*., 1995; Banerjee and Dasgupta, 2001). Addition of NS3 helicase to the polymerase reaction with the 3'-UTR of HCV genome yielded high molecular weight products without supporting cyclic replication of RNA template (Piccininni *et al*., 2002). It is still remained to be answered whether the NS3 helicase can unwind the heavily structured X-RNA at the 3'-end of the HCV genome and the double-stranded replication intermediate generated during HCV RNA replication to allow the NS5B to efficiently copy the RNA template in a cyclic manner. HCV NS5A, which can also form a complex with NS5B, was also tested in RdRp reaction, but it only slightly enhanced RNA synthesis efficiency (Shirota *et al*., 2002). These results all together suggest that individual HCV non-structural protein might not be sufficient for enabling NS5B to support cyclic replication. Interestingly, Lai *et al*. recently showed that the HCV RNA replicase complex isolated from the subgenomic replicon cells can synthesize both double-stranded RNA products and single-stranded RNA product served as a template for further round of RNA synthesis. This indicates the possibility that HCV replicase can also undergo cyclic replication *in vitro* when the other nonstructural viral proteins are present in the HCV RNA replicase complex (Lai *et al*., 2003). Since the cellular factors present in the replicase complex have not been identified, *in vitro* reconstitution for HCV RNA replicase still requires analyses of functional cellular and/or viral proteins directly involved in the replication process. Further stoichiometrical analyses using all the HCV nonstructural proteins will be necessary for understanding the interrelationship and functions of these proteins in a putative HCV RNA replicase complex.

Because HCV NS5B is the key viral enzyme for HCV RNA replication, functional analysis of HCV RNA polymerase as a component of RNA replicase can reveal information regarding the factors that regulate this key event in virus life cycle. Furthermore, a more complete knowledge of the role of cellular and/or viral factors involved in the regulation of viral RNA synthesis is of great importance for understanding of the enzymology of HCV RNA replication.

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