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# The Helicase Activity Associated with Hepatitis C Virus Nonstructural Protein 3 (NS3)

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**To assess the RNA helicase activity of hepatitis C virus (HCV) nonstructural protein 3 (NS3), a polypeptide encompassing amino acids 1175 to 1657, which cover only the putative helicase domain, was expressed in *Escherichia coli* by a pET expression vector. The protein was purified to near homogeneity and assayed for RNA helicase activity in vitro with double-stranded RNA substrates prepared from a multiple cloning sequence and an HCV 5′ nontranslated region (5′-NTR) or 3′-NTR. The enzyme acted successfully on substrates containing both 5′ and 3′ single-stranded regions (standard) or on substrates containing only the 3′ single-stranded regions (3′/3′) but failed to act on substrates containing only the 5′ single-stranded regions (5′/5′) or on substrates lacking the single-stranded regions (blunt). These results thus suggest 3′ to 5′ directionality for HCV RNA helicase activity. However, a 5′/5′ substrate derived from the HCV 5′-NTR was also partially unwound by the enzyme, possibly because of unique properties inherent in the 5′ single-stranded regions. Gel mobility shift analyses demonstrated that the HCV NS3 helicase could bind to either 5′- or 3′-tailed substrates but not to substrates lacking a single-stranded region, indicating that the polarity of the RNA strand to which the helicase bound was a more important enzymatic activity determinant. In addition to double-stranded RNA substrates, HCV NS3 helicase activity could displace both RNA and DNA oligonucleotides on a DNA template, suggesting that HCV NS3 too was disposed to DNA helicase activity. This study also demonstrated that RNA helicase activity was dramatically inhibited by the single-stranded polynucleotides. Taken altogether, our results indicate that the HCV NS3 helicase is unique among the RNA helicases characterized so far.**

Hepatitis C virus (HCV) is a positive-stranded RNA virus belonging to the family *Flaviviridae* (8, 42). The viral genome encodes a large polyprotein consisting of 3,010 (25, 42) or 3,011 amino acids (aa) (9). The polyprotein is processed into a range of structural (C, E1, and E2/NS1) and nonstructural (NS2 to NS5) proteins by cellular as well as viral proteases (9, 17, 21, 42). The NS3 protein (72 kDa) of HCV, which contains a putative serine protease domain at its N terminus and NTPase-RNA helicase motifs at its C terminus, is predicted to possess protease and helicase activities (3, 14–16, 35). Serine protease activity—a current topic of active research—has been extensively demonstrated (1, 18, 22, 45) and shown in conjunction with NS4A to be required for optimal viral polyprotein processing (12, 19, 33). In contrast, to our knowledge biochemical data demonstrating the putative helicase or NTPase activity of HCV NS3 protein have appeared in only one report (references 26 and 41, respectively); with respect to the further characterization of RNA helicase activity, no report at all has yet been made.

Many RNA helicases in organisms as different as *Escherichia coli* and humans have been identified. Those helicases are involved in important cellular functions, such as RNA splicing, ribosome assembly, and translational initiation (for reviews, see references 13 and 39). Similarly, several known viral RNA helicases are involved in viral replication, transcription, and protein translation (4, 20, 31, 43) and are thus important for viral propagation. All the helicases characterized are intrinsically disposed to NTPase activity, which hydrolyzes nucleoside triphosphates and provides the energy source for unwinding. Sequence analysis of these helicases has revealed several conserved motifs, which form a large protein family (14, 16, 24, 34). This protein family can be further subdivided into a group containing a DEAD motif and others containing a DEAH or DEXH motif (13). This protein family contains eight domains of strong peptide sequence conservation. However, a function can be attributed to only two or three of the domains. The first (AXXGXGKX), referred to as the A motif (46), is involved in ATP binding (37). The fifth (LDEAD), referred to as the B motif (46), is possibly involved in ATP binding and/or ATP hydrolysis (16). The eighth is part of a basic domain, which suggests a possible interaction with RNA (34). Hypothetically, NS3 protein of HCV may be a member of this family since corresponding motifs are present in the carboxy-terminal two-thirds of the protein: a GXGKX motif at aa 1233 to 1237, a DECH motif at aa 1316 to 1319, and a basic domain at aa 1486 to 1493 (Fig. 1A).

While NTPase activities have been amply demonstrated in several purified viral RNA helicases (28, 40, 41, 44, 48, 49), fewer RNA helicase activities have been reported (11, 40), leaving the helicase activities of many of these proteins in supposition. Recently, RNA helicase activities in recombinant NS3 proteins of bovine viral diarrhea virus (BVDV) (47) and HCV (26) have been reported, confirming the predicted enzymatic activity. In this report, the issue is further substantiated by demonstrating that the carboxy terminus of HCV NS3 protein is indeed disposed to RNA helicase activity. In addition, the further characterization of HCV NS3 helicase indicates that the enzymatic activity here is quite different from that of the BVDV RNA helicase.

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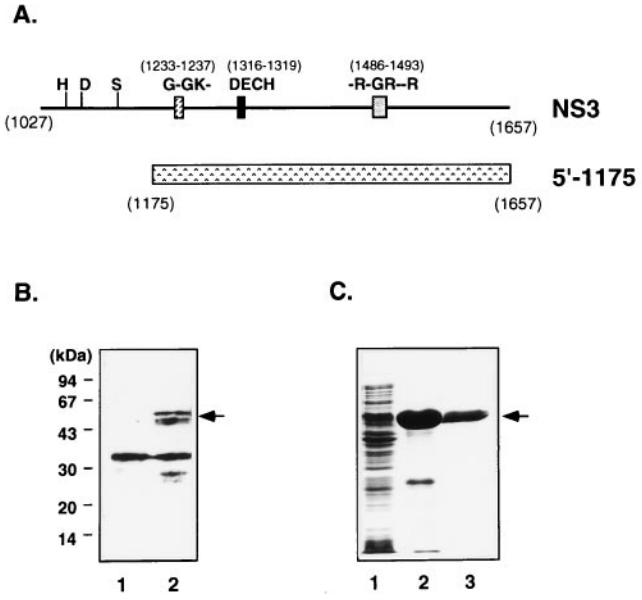


FIG. 1. Expression and purification of clone 5′-1175. (A) Schematic diagrams of clone 5′-1175, which was expressed in *E. coli*. The full-length NS3 protein ranges from aa 1027 to 1657 (32), whereas clone 5′-1175 ranges from aa 1175 to 1657. H, D, and S represent the important triad amino acid residues His, Asp, and Ser, respectively, for serine protease activity; G-GK-, DECH, and -R-GR--R are the three consensus motifs for RNA helicase. (B) Western blot analysis of the proteins from induced cultures containing the pET 21a vector (lane 1) or plasmid 5′-1175 (lane 2). An arrow to the right indicates the expected size of the protein. (C) Purification of recombinant NS3 protein from *E. coli*. Total lysates from induced culture containing plasmid 5′-1175 (lane 1) were subjected to an Ni column (lane 2) and then to a poly(U)-Sepharose 4B column (lane 3). Proteins purified from each step were analyzed by SDS-PAGE and stained with Coomassie blue.

## MATERIALS AND METHODS

**Construction and expression of NS3 protein.** A truncated NS3 clone, 5′-1175, encompassing aa 1175 to 1657 was obtained by PCR from a cDNA clone of a Taiwanese HCV strain (7). Two restriction sites, *Eco*RI and *Xho*I were created at the 5′ and the 3′ ends of the PCR product, respectively. The resulting PCR fragment was cloned into a pET21a expression vector (Novagen, Madison, Wis.). The clone was subsequently introduced into *E. coli* BL21(DE3) by transformation. Transformants were grown in M9ZB medium (each liter contains 10 g of N-Z-Amine A and 5 g of NaCl, autoclaved and cooled, to which is added 100 ml of 10× Mg salts, 1 ml of 1 M $MgSO_4$, and 10 ml of 40% glucose) at 37°C until an optical density at 600 nm of 0.6 to 1.0 was reached. One millimolar isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to induce expression. After a 3-h induction, the cells were harvested and disrupted with a Microfluidizer (Microfluidic Corp., Christison Scientific Equipment).

**Western blot (immunoblot) analysis.** Proteins from a total bacterial lysate were resolved by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE) (27) and transferred to a nitrocellulose filter (6). The filter was reacted first with rabbit anti-NS3 polyclonal antiserum directed against the middle portion (aa 1188 to 1493) of the NS3 protein expressed in *E. coli* (unpublished results) and subsequently with a second antibody, goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratory, West Grove, Pa.) conjugated with horseradish peroxidase. The blot was developed with an enhanced chemiluminescence detection reagent (Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

**Purification of the recombinant protein.** Upon induction, the recombinant protein of clone 5′-1175 was present in a soluble fraction. Cells were disrupted in the binding buffer (20 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 5 mM imidazole) and the lysate was directly applied to an Ni column preequilibrated with the same binding buffer. The column was washed sequentially with buffers containing 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and imidazole stepwise in increasing concentrations (10, 20, 30, 40, and 50 mM). After being washed, the bound proteins were eluted with elution buffer (20 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 60 mM imidazole). The eluted fractions were pooled and dialyzed in a TNE buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA). The dialyzed proteins were further applied to a poly(U)-Sepharose 4B column (Pharmacia, Uppsala, Sweden) which was preequilibrated with TNE buffer. After extensive washing of the column with TNE buffer, proteins were eluted with elution buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA). The eluted fractions were pooled, dialyzed in TNE, and concentrated with Amicon Centriprep-10.

**Preparation of helicase substrates.** Representative structures of all the helicase substrates are depicted in Fig. 2, and the nucleotide lengths in each region for individual substrates are indicated. These substrates are composed of two

| | Substrate | Structure | Nucleotide length in the region of | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | MCS | | | | HCV 5'-NTR | | | | HCV 3'-NTR | | | |
| | | | *x* | *y* | *z* | *z′* | *x* | *y* | *z* | *z′* | *x* | *y* | *z* | *z′* |
| RNA/RNA* | | | | | | | | | | | | | | |
| (1) | 5'-x-y-z-3' / 3'-z′-5' | standard | 31 | 34 | 39 | 7 | 31 | 34 | 39 | 7 | 31 | 34 | 39 | 7 |
| (2) | 5'-x-y-3' / 3'-z-5' | 5′/5′ | 35 | 10 | 31 | | 31 | 34 | 39 | | 31 | 34 | 39 | |
| (3) | 5'-y-z-3' / 3'-x-5' | 3′/3′ | 27 | 34 | 37 | | 31 | 34 | 39 | | 25 | 65 | 25 | |
| (4) | 5'-y-3' / 3'-5' | blunt | 0 | 34 | 0 | | 0 | 34 | 0 | | 0 | 34 | 0 | |
| RNA/DNA* | | | | | | | | | | | | | | |
| (5) | 5'-x-y-z-3' / 3'-z′-5' | standard | 35 | 30 | 39 | 24 | | ND | | | | ND | | |
| DNA/RNA* | | | | | | | | | | | | | | |
| (6) | 5'-x-y-z-3' / 3'-z′-5' | standard | 49 | 29 | 20 | 10 | | ND | | | | ND | | |
| DNA/DNA* | | | | | | | | | | | | | | |
| (7) | 5'-x-y-z-3' / 3'-z′-5' | standard | 49 | 27 | 20 | 27 | | ND | | | | ND | | |

FIG. 2. Substrates employed for the helicase reaction. The second column depicts schematic diagrams of the substrates employed for the helicase reaction. Thick lines represent RNA strands, thin lines represent DNA strands, and vertical lines represent the duplex region. The italicized letters *x*, *y*, *z*, and *z′* indicate the lengths in nucleotides (or base pairs) of the left overhang regions, the duplex regions, the right overhang regions, and the overhang regions on the release strand of standard substrates, respectively. Asterisks indicate the labeled strands which are referred to as release strands. ND, experiment not done.

annealed complementary RNA or DNA strands. The unlabeled strand is referred to as the template strand, and the $^{32}$P-labeled strand is referred to as the release strand.

**(i) RNA-RNA substrates.** For the double-stranded RNA (dsRNA) substrates, both strands were prepared by the in vitro transcription of multiple cloning sequences (MCS) derived from a pGEM vector (Promega, Madison, Wis.) or of PCR products consisting of desired lengths of HCV 5′ nontranslated region (5′-NTR) or 3′-NTR sequences with either SP6 or T7 RNA polymerase (Promega). The transcripts were treated with DNase (Boehringer GmbH, Mannheim, Germany), extracted with phenol-chloroform, and then purified with a Chromaspin 10 column. The transcripts were combined at a molar ratio of release strand to template strand of approximately 1:10 in a solution containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.6), 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS. The mixture was boiled for 10 min, transferred to 65°C for 30 min, and then incubated at 25°C overnight. The hybridized products were mixed with 5× RNA loading dye (0.1 M Tris-HCl [pH 7.4], 20 mM EDTA, 0.5% SDS, 0.1% bromophenol blue, 0.1% xylene cyanol, 50% glycerol) and electrophoresed on an 8% native polyacrylamide (acryl-bis, 30:1)–1× Tris-borate-EDTA gel. The duplex substrate band was localized by autoradiography. The gel slice was excised, pulverized, and extracted with 0.5 M ammonium acetate (pH 7.0)–0.1% SDS–10 mM EDTA for 2 h at 25°C. The eluted substrate was then extracted with phenol-chloroform, ethanol precipitated, and resuspended in storage buffer (20 mM HEPES [pH 7.6], 0.1 mM EDTA).

**(ii) DNA-DNA substrate.** For the dsDNA substrates, the MCS region derived from an M13mp18 vector was initially amplified by PCR with flanking sequences as primers. The resulting PCR product, 98 bp in length, was further amplified by a PCR with a single primer to amplify the single-stranded DNA. Primers were chosen at specific sites along the substrates in order to generate template or release strands of desired lengths. Duplex DNA substrates were then prepared and gel purified, as described for the dsRNA substrates.

**(iii) DNA-RNA and RNA-DNA substrates.** Single-stranded RNAs or DNAs derived from the MCS were synthesized in a manner similar to those described above for RNA-RNA and DNA-DNA substrates, the heteroduplex substrates being prepared and gel purified also as described above.

**Helicase assay.** After serial titrations (see Results), unless otherwise specified, the reaction condition for HCV NS3 helicase activity was optimized with 20 μl of reaction mixture containing 20 mM HEPES–KOH (pH 7.0), 2 mM dithiothreitol, 1.5 mM $MnCl_2$, 2.5 mM ATP, 0.1 mg of bovine serum albumin (BSA) per ml, 2 U of RNasin, 1 μg of purified protein, and 0.66 pmol of substrate. The reaction was carried out at 37°C for 1 h and then terminated by adding 5 μl of 5× RNA loading dye. An aliquot (12.5 μl) of each reaction mixture was loaded onto an 8% native polyacrylamide gel and electrophoresed. The gel was dried and autoradiographed. The ratio of single-stranded products to double-stranded substrates was quantified by PhosphorImage analysis with FUJIX BAS1000.

**RNA binding assay.** A microgram of NS3 protein and 1.3 pmol of each dsRNA substrate were incubated in 20 μl of the helicase reaction buffer lacking ATP at 37°C for 15 min. For competitive binding, cold single-stranded release strands or polynucleotides, whose concentrations are indicated in respective figures, were preincubated with the enzyme for 5 min; labeled dsRNA substrates were then added to the reaction mixture. The binding reaction was terminated by adding 5× RNA loading dye containing 0.5% Nonidet P-40 instead of SDS and electrophoresed on a 4% polyacrylamide (acryl-bis, 80:1)–1/3× Tris-borate-EDTA gel containing 5% glycerol. The bound complexes were visualized by autoradiography.

**ATPase activity assay.** Standard analytical ATPase assays were conducted in a total volume of 10 μl containing 20 mM HEPES-KOH (pH 7.0), 2 mM dithiothreitol, 1.5 mM $MgCl_2$, 5 μCi of [α-$^{32}$P]ATP (3,000 Ci/mmol; Amersham), and 0.1 μg of enzyme. Reaction mixtures were incubated at room temperature for 60 min, and then the reactions were terminated by adding EDTA to a final concentration of 20 mM. Effects of polynucleotides were studied by directly adding 500 μg (each) of poly(A), poly(U), and poly(G) per ml to the reaction mixture. Reaction products were analyzed by thin-layer chromatography. Half a microliter of the reaction mixture was spotted onto plastic-backed polyethyleneimine cellulose F sheets (Merck) and developed by ascending chromatography in 0.375 M potassium phosphate (pH 3.5). The sheets were dried and exposed to an FUJIX imaging plate, and the conversion rates were quantitated by PhosphorImage analysis (FUJIX BAS1000).

## RESULTS

**Producing recombinant HCV NS3 protein in bacteria.** To assess the RNA helicase activity of HCV NS3 protein, a clone, 5′-1175 (aa 1175 to 1657), encompassing all the putative helicase consensus motifs (13, 39) but lacking the protease domain, was expressed in *E. coli* (Fig. 1A). The clone was expressed by a pET-21a expression vector which provided an additional 12 aa at the N terminus and 6 histidine residues at the C terminus of the expressed protein. Upon induction, although the corresponding band was not detectable by Coomassie blue staining (data not shown), possibly because of low-level expression, it was clearly detected by Western blot analysis (Fig. 1B, lane 2). The expected band and several other smaller fragments were observed in the induced cultures containing the 1175 plasmid but not in the culture containing the pET21a control plasmid (Fig. 1B, lane 1). The band at around 32 kDa may possibly be an *E. coli* protein cross-reacting with the antiserum, since it was also present in lane 1.

For purification, an affinity Ni column specific to His-tailed proteins was employed. Most of the recombinant proteins from clone 5′-1175 were present in soluble fractions. The proteins from bacterial lysate were therefore directly subjected to an Ni column and then to a poly(U)-Sepharose 4B column. The purity of the final product was around 97% (Fig. 1C, lane 3). The identities of all the purified proteins were confirmed by rabbit anti-NS3 antiserum (unpublished data).

**Analysis of NS3 RNA helicase activity.** To evaluate the RNA helicase activity of the purified protein, initially a standard dsRNA substrate containing both the 5′ single-stranded and the 3′ single-stranded regions was tested (Fig. 2, substrate 1). The substrate was incubated with purified protein under conditions that had been previously employed to assay cellular RNA helicase A (30). However, under such conditions the recombinant HCV NS3 protein displayed very weak helicase activity. The conditions for the purified protein of clone 5′-1175 were, therefore, calibrated. As Fig. 3A illustrates, increasing concentrations of potassium ion dramatically decreased the level of helicase activity; in fact, the maximum activity was obtained in the absence of potassium ion. The reaction conditions were adjusted to totally exclude potassium ion, and the optimal concentration of divalent ion was then determined. It was found that low concentrations (up to 1.5 mM) of divalent ions, either $Mn^{++}$ or $Mg^{++}$, could stimulate activity but that increased concentrations produced an adverse effect (Fig. 3B). At lower concentrations of divalent ion, HCV RNA helicase exhibited a higher level of enzymatic activity with $Mn^{++}$ than with $Mg^{++}$. This finding is similar to that reported for the BVDV NS3 RNA helicase (47). Reaction conditions were further modified to contain 1.5 mM $MnCl_2$. Under these modified conditions, the pH optimal for the reaction was 7.0 (Fig. 3C). Finally, when the optimal concentration of ATP was determined, it was found that for HCV NS3 helicase activity, ATP was an absolute prerequisite and that activity was maximal at concentrations higher than 1 mM (Fig. 3D). To ensure a complete reaction, 2.5 mM ATP was used. Under each condition tested, the optimal condition was finally determined as described in Materials and Methods and used in all subsequent experiments. At optimized conditions, the greatly improved unwinding activity was around 96% for clone 5′-1175 (Fig. 3E, lane 3). However, proteins of the equivalent fractions purified from the control plasmid culture did not exhibit any enzymatic activity (Fig. 3E, lane 4), which indicated that the observed RNA helicase activity was not derived from *E. coli* contaminants.

**Directionality of HCV NS3 RNA helicase.** As previously described (30), the directionality of a helicase is defined by the strand to which the enzyme binds or translocates. To determine what kind of directionality the HCV NS3 helicase possesses, three more dsRNA substrates were prepared (Fig. 2, substrates 2 to 4). They contained single-stranded RNA regions exclusively at either their 5′ (5′/5′) or their 3′ (3′/3′) ends or no single-stranded RNA region at either end (blunt). These duplex RNA substrates were tested for unwinding under the optimized conditions. As Fig. 4A illustrates, only the standard

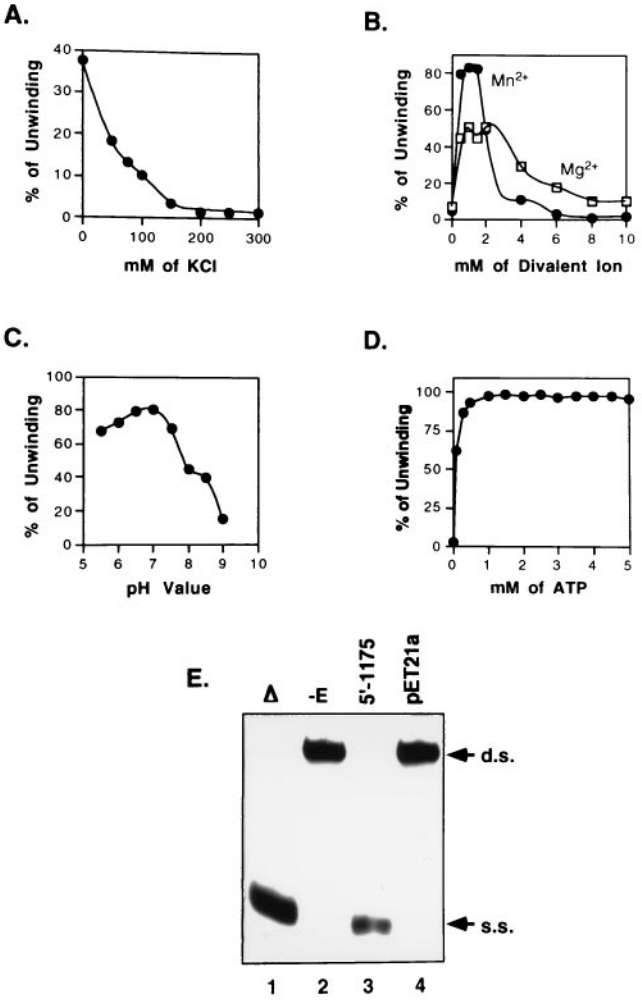


FIG. 3. Optimizing reaction conditions for HCV NS3 RNA helicase. The helicase reactions were optimized for clone 5′-1175 with the MCS standard dsRNA substrate. The activity was quantified by determining the percentages of unwinding. (A) Determinations of $K^+$ concentrations were conducted under previously described conditions (30). (B) Determinations of the concentrations of two different divalent ions, $Mn^{++}$ and $Mg^{++}$, were conducted at 0 mM $K^+$ and 1 mM ATP at pH 7.6. (C) Determinations of optimal pH were conducted at 0 mM $K^+$, 1.5 mM $MnCl_2$, and 1 mM ATP. Buffers for preparing different pHs were as follows: PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]-HCl for pHs 5.5 and 6.0, HEPES-HCl for pHs 6.5 and 7.0, and Tris-HCl for pHs 7.5 to 9.0. (D) Determinations of ATP concentrations were conducted at 0 mM $K^+$ and 1.5 mM $MnCl_2$ at pH 7.0. (E) The helicase activity assay was performed under the optimized conditions described in Materials and Methods. Products were analyzed by electrophoresis. Lane 1 (Δ), the reaction did not contain the enzyme and the substrates were heat denatured; lane 2 (−E), the reaction did not contain the enzyme and the substrate was left native; lane 3, the reaction mixture contained the protein 5′-1175; lane 4, the reaction mixture contained the proteins purified from equivalent fractions of culture harboring the pET21a control plasmid. d.s., double-stranded; s.s., single-stranded.

and the 3′/3′ dsRNA substrates were efficiently unwound; the 5′/5′ and the blunt dsRNA substrates were not.

The helicase activities on dsRNA substrates derived from HCV sequences, i.e., the 5′-NTR (nucleotide [nt] 1 to 104) or the 3′-NTR (nt 9322 to 9425), were also tested. Like the MCS dsRNA substrates, the standard and the 3′/3′ substrates of both sequences were efficiently unwound; the 5′-tailed or blunt-ended substrates were not (data not shown), except for the 5′/5′ substrate of the HCV 5′-NTR, which was partially unwound (Fig. 4B, first panel). The efficiency was around 26%. The duplex nature of the 5′/5′ substrate of the HCV 5′-NTR was therefore examined by labeling on the template strand instead of the release strand. The annealed hybrids from such synthesized transcripts migrated at the same position as the previous 5′/5′ substrate (data not shown), thus excluding the possibility that the previously isolated 5′/5′ substrate was a self-folded release strand, which would have led to its partial unwinding.

To investigate further whether the partial unwinding activity was of a nature unique to the single-stranded region of the 5′/5′ substrate of the HCV 5′-NTR, the single-stranded regions were shortened to 21 or 10 nt at either end and the new substrates were reexamined for unwinding activities. Figure 4B indicates that partial unwinding activity was still observed in the substrate containing the 21-nt single-stranded region (33%, second panel) but was no longer detected in the substrate containing the 10-nt single-stranded region (third panel). The results suggest that the partial unwinding activity occurring in the 5′/5′ substrates of the HCV 5′-NTR might be a propensity unique to the single-stranded regions of this substrate.

Taken altogether, the results are consistent with those of the

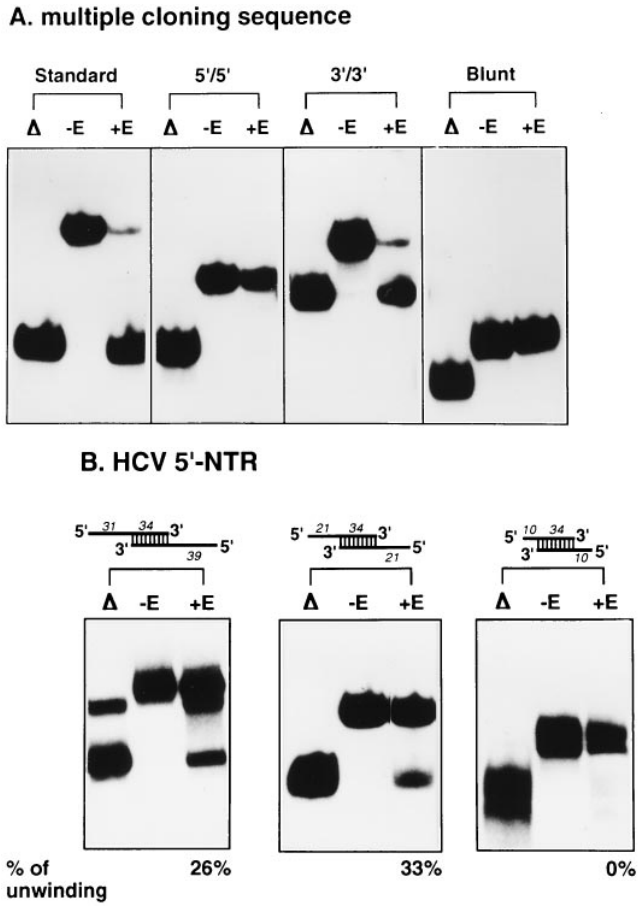


FIG. 4. Direction of RNA unwinding by HCV NS3 RNA helicase. The dsRNA substrates depicted in Fig. 2 (substrates 1 to 4) were prepared as described in Materials and Methods. Unwinding reactions were conducted with each substrate under the optimized conditions. (A) dsRNA substrates derived from MCS. (B) 5′/5′ dsRNA substrates derived from the HCV 5′-NTR (nt 1 to 104). Unwinding activities on the three substrates containing the same duplex region but with 5′ overhangs of different lengths were compared. Control assays involved incubating the substrates in a reaction mixture lacking the enzyme in which the substrates were either heat denatured (Δ), or left native (−E). +E, the reaction mixture contained the enzyme. The numbers in italics indicate the lengths in nucleotides (or base pairs) of the individual regions.

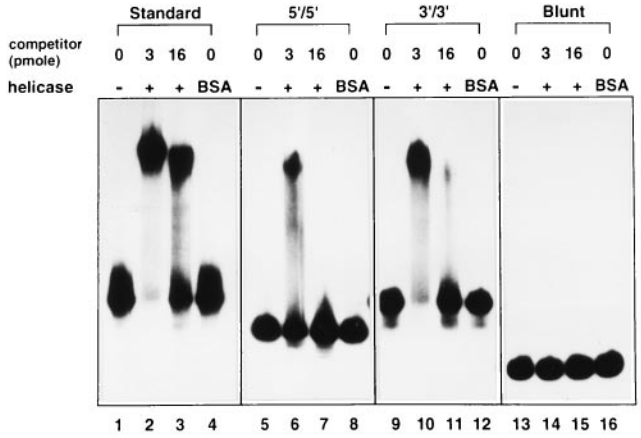


FIG. 5. Binding activities of 5′-1175 protein to dsRNA substrates. A binding reaction was performed in a reaction mixture lacking ATP with 1.3 pmol of each of the indicated substrates. Reaction mixtures contained either no NS3 RNA helicase (lanes 1, 5, 9, and 13), 1 μg of 5′-1175 (lanes 2, 3, 6, 7, 10, 11, 14, and 15), or 1 μg of BSA (lanes 4, 8, 12, and 16) in the presence of 3 pmol (lanes 2, 6, 10, and 14) or 16 pmol (lanes 3, 7, 11, and 15) of the unlabeled single-stranded release strand. The binding complexes were analyzed by electrophoresis on a 4% polyacrylamide gel.

BVDV NS3 RNA helicase, indicating a 3′ to 5′ directionality with respect to the template strand for HCV NS3 helicase. The enzyme seemed to unwind substrates without sequence specificity.

**Binding properties of the HCV NS3 helicase.** The inability of HCV NS3 helicase to unwind the 5′/5′ and the blunt dsRNA substrates was further investigated by detecting whether the enzyme could interact with them. The $^{32}$P-labeled dsRNA substrates depicted in Fig. 2 (substrates 1 to 4) were incubated with the enzyme in the helicase reaction mixture lacking ATP. The formation of complexes was analyzed by RNA gel mobility shift assay (Fig. 5). Our results revealed that HCV NS3 helicase could interact with all but the blunt dsRNA, whereas the same amount of BSA failed to bind to any of the dsRNA substrates. However, as little as 3 pmol of the cold single-stranded release strand were needed in the binding reaction in order to avoid the complex being retained in the well. Furthermore, the interaction could be diminished by an excess amount (16 pmol) of the unlabeled single-stranded release strand (Fig. 5, lanes 3, 7, and 11) but not of the blunt substrates (data not shown). The results thus indicated that the binding activity of HCV NS3 helicase is single strand specific. However, despite the finding that HCV NS3 helicase could bind to the 5′/5′ substrate, the enzyme was unable to unwind it, implying that the directionality of template strand was more important in determining the enzymatic function.

**Substrate specificity of the HCV NS3 RNA helicase.** The substrates described above were all dsRNA. To examine whether the HCV NS3 helicase could act on substrates containing DNA strands, three additional double-stranded substrates were prepared (Fig. 2, substrates 5 to 7). These included an RNA template strand annealed with a DNA release strand (RNA/DNA*), a DNA template strand annealed with an RNA release strand (DNA/RNA*), and a DNA release strand (DNA/DNA*). They were all of standard structure and of sequences derived from MCS. Unwinding reactions were performed under optimal conditions. Interestingly, all the substrates tested were efficiently unwound by the enzyme (Fig. 6). The results thus suggest that the HCV NS3 helicase was able to function on a DNA template strand, a property that was quite different from that of BVDV NS3 helicase (47).

**Inhibition effect of polynucleotides on HCV NS3 helicase activity.** The enzymatic activity that is always associated with helicase activity is NTPase activity. In the clone of this study, 5′-1175, strong ATPase activity was indeed observed when [α-$^{32}$P]ATP served as a substrate. The activity was 2.5-fold stimulated by poly(A) or poly(U) but was 4-fold inhibited by poly(G) (Fig. 7A). The effect of polynucleotides was further tested on RNA helicase activity. It was found that, at the level of concentrations of polynucleotides that affected ATPase activity, all polynucleotides inhibited HCV NS3 RNA helicase activity (Fig. 7A). The binding activity of 5′-1175 protein to the dsRNA substrates in the presence of these polynucleotides was, therefore, examined. It was noted that poly(A) and poly(U) completely inhibited the binding of the enzyme to the substrates whereas poly(G) abolished the original protein-RNA complex formation while inducing a new intermediate-sized complex (Fig. 7B). The nature of this new complex was not further characterized. These results indicated that these polynucleotides might inhibit RNA helicase activity through competitive binding to the 5′-1175 protein.

## DISCUSSION

On the basis of sequence motif analysis, the NS3 protein of HCV is predictive of a multifunctional protein containing serine protease, NTPase, and RNA helicase activities (3, 14–16, 35). This report has experimentally demonstrated that the carboxyl terminus of NS3 protein is indeed disposed to RNA helicase activity, thus supporting the prediction from the amino acid sequence motifs. Similar results were also reported recently by Kim et al. (26), who noted that a clone ranging from aa 1193 to 1658 was expressed. However, comparing the results of our clone and their clone revealed different enzyme/substrate ratios (27:1 versus 2:1, respectively) in their reactions for achieving equivalent unwinding activities, implying that the level of specific enzymatic activity in the clone of Kim et al. appeared to be higher. To understand whether this is an inhibition effect derived from the additional 18 aa residues present in our clone, we expressed and purified the same-sized clone as they did, and compared it in parallel with clone 5′-1175. However, in our experiments both clones functioned at about the same level of efficiency (data not shown). Thus, we excluded the possibility that the difference was due to different clones

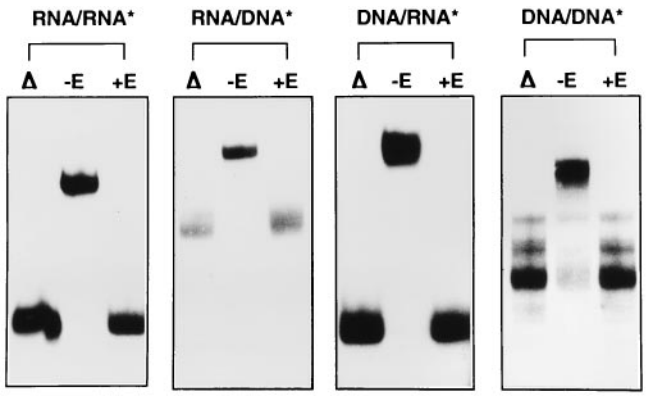


FIG. 6. Unique substrate specificity for HCV NS3 helicase. The double-stranded duplexes consisting of dsRNA, dsDNA, or RNA-DNA hybrids, as depicted in Fig. 2 (substrates 1 and 5 to 7), were prepared as described in Materials and Methods. Unwinding reactions of these four substrates were performed under the optimal condition. Δ, substrate was heat denatured; −E, substrate was left native; +E, the reaction mixture contained the enzyme.

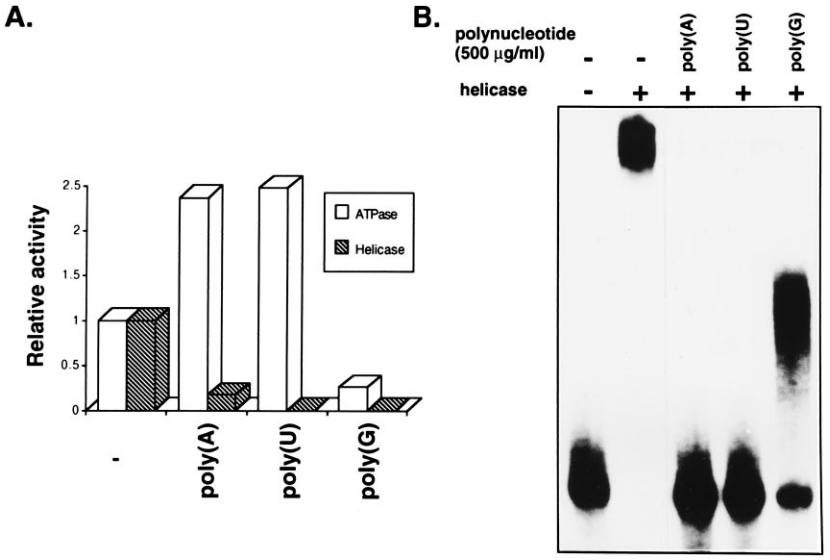


FIG. 7. Effects of polynucleotides on HCV NS3 ATPase and RNA helicase activities. (A) Effects of polynucleotides on both enzymatic activities. The relative activity of ATPase or RNA helicase in the absence of polynucleotides was defined as 1. Effects of polynucleotides were evaluated after adding 500 μg of poly(A), poly(U), or poly(G) per ml to the reaction mixture. (B) Effects on the binding activities. The binding assays described in Fig. 5 were performed in the presence of 500 μg (each) of poly(A), poly(U), and poly(G) per ml. The products were analyzed on a 4% polyacrylamide gel.

and suggest that it may be due to the experimental variations in different laboratories.

Even accepting that the enzymatic activity was detected from this N-terminus-truncated protein and suggesting that the N terminus of HCV NS3 protein is nonessential for helicase activity, it remains possible that a full-length NS3 protein is disposed to a higher level of enzymatic activity or that another cofactor(s) further stimulates NS3 RNA helicase activity in vivo. It is established that NS4A, by interacting with NS3 protein, can significantly stimulate the protease activity of NS3 protein in vitro and in vivo (12, 19, 33). Likewise, it may also stimulate the RNA helicase activity of the same protein complex. To address these issues, a full-length protein should first be produced. However, our initial attempts to obtain a functional full-length NS3 protein from *E. coli* failed (unpublished results). So, we now seek an alternative method of producing the full-length NS3 or NS3 plus NS4A proteins. Therefore, the questions raised are so far unanswered.

Nevertheless, the helicase activities already characterized for HCV NS3 protein are interesting. First, as far as is known, most RNA helicases act unidirectionally in a 3′ to 5′ direction (23, 29, 30, 38, 40, 47). The exceptions are nuclear RNA helicase I (10) and eIF-4A plus eIF-4B (36), which have been demonstrated to displace dsRNA in a bidirectional mode. In this study, HCV NS3 helicase could unwind the standard and the 3′/3′ substrates but not the 5′/5′ and the blunt substrates, which confirms a 3′ to 5′ directionality. But HCV NS3 helicase also partially unwound the 5′/5′ dsRNA substrate of the HCV 5′-NTR (Fig. 4B, first two panels), which appeared to act in a bidirectional mode. This possibility, however, was refuted by demonstrating that the same heteroduplex with the 10-nt single-stranded region was not unwound (Fig. 4B, third panel). Therefore, it is most likely that unique propensities inherent in the 5′/5′ substrate of the HCV 5′-NTR caused the partial unwinding. Whether the presence of secondary structures or the sequences per se in the single-stranded regions led to this partial unwinding remains to be investigated. In light of the complicated secondary structures of the HCV 5′-NTR (5), the observed helicase activity might imply an important function involved in HCV viral RNA replication. Second, HCV NS3 helicase did unwind DNA-RNA and DNA-DNA substrates (Fig. 6), indicating that the enzyme is also disposed to DNA helicase activity. To date, only three helicases disposed to both RNA and DNA helicase activities have been described, simian virus 40 large T antigen (38), nuclear DNA helicase II (NDH II) (50), and vaccinia virus protein 18 R, i.e., a nucleotide triphosphate phosphohydrolase II (NPH II) (2). HCV NS3 helicase represents a fourth enzyme disposed to both RNA and DNA helicase activities. By comparison, simian virus 40 large T antigen does not have the helicase motifs typical of the helicase superfamily proteins (16). It unwinds dsDNA at the expense of ATP but unwinds dsRNA with UTP, CTP, or GTP as the cofactor; ATP is not an efficient energy source for the RNA unwinding reaction. Thus, it appears that the bound nucleotide determines whether T antigen acts as an RNA helicase or as a DNA helicase (38). In contrast, HCV NS3 helicase unwound both dsRNA and dsDNA substrates with similar levels of efficiency at the optimized conditions with ATP as the energy source (Fig. 6). In addition, our unpublished data also indicated that HCV NS3 protein employed all hydrolyzable deoxynucleoside triphosphates for its RNA helicase and DNA helicase activities alike with similar levels of efficiency (data not shown).

NDH II is a helicase from calf thymus tissues (50) which might be the bovine homolog of RNA helicase A, a protein purified from the nuclei of HeLa cells that was demonstrated to display RNA helicase activity but not DNA helicase activity (30). The NPH II helicase of vaccinia virus was initially identified as an RNA helicase (40). However, recent data from Bayliss and Smith further demonstrate the DNA helicase activity of purified NPH II (2). Both NDH II and NPH II have strong sequence homology within the domains characteristic of the helicase superfamily (2, 16). Therefore, these proteins are speculated to represent a new subgroup of helicases disposed to both DNA and RNA helicase activities (2). The HCV NS3 helicase and these helicases share directionality and nucleotide usage (references 40 and 50 and our unpublished data); however, the former still differs from the latter in some aspects. (i)

NDH II helicase binds single-stranded RNA as well as dsRNA (50), whereas HCV NS3 helicase binds only single-stranded RNA. (ii) NDH II RNA helicase activity is slightly stimulated by NaCl or KCl in concentrations up to 75 mM and is thus less salt sensitive (50). In contrast, HCV NS3 RNA helicase activity is highly salt sensitive even at 50 mM KCl (Fig. 3A). (iii) Comparing the HCV NS3 sequence with that of NDH II or NPH II indicates a lower degree of similarity within the helicase domains than that between NDH II and NPH II (data not shown).

Taken altogether, the data indicate that the HCV NS3 protein may be a unique example among all the RNA helicases characterized so far. The significance of the DNA helicase activity present in this protein is currently not understood. Though this activity is not essential to HCV itself, it may affect cellular nucleic acid structures or activities and is thus relevant to pathogenesis. On the other hand, it is not established whether the full-length NS3 protein also exhibits the same characteristics, and so this question remains for further investigation.

Finally, while poly(U) and poly(A) significantly stimulated HCV NS3 ATPase activity (reference 41 and our results), they both inhibited helicase activity (Fig. 7A). However, poly(G) inhibited ATPase activity in the first place (reference 41 and our results) and further inhibited RNA helicase activity (Fig. 7B). Therefore, while different polynucleotides, for unknown causes, may exert different effects on ATPase activity, they all inhibited RNA helicase activity. In corroboration of these findings, an RNA gel mobility assay also revealed reduced binding of 5′-1175 protein to dsRNA substrates in the presence of these polynucleotides. The results thus imply that the single-stranded polynucleotides diminished the binding of enzyme to the substrates, which reduced the level of helicase activity.

The common roles of RNA helicases in many RNA viruses are to cause RNA strands to separate during viral transcription and replication and to resolve the secondary structure preceding the ribosome to facilitate protein translation. The experimental results in this study also demonstrate that the potential secondary structures present in HCV 5′-NTR may, however, influence the directionality of RNA unwinding (Fig. 4B), thereby bringing to light the issue of how an HCV helicase actually works on its natural substrate to facilitate RNA replication as well as protein translation. Furthermore, the unusual characteristics of unwinding DNA substrates make the HCV NS3 helicase a subject most worthy of further investigation.

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