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C-TERMINAL DOMAIN OF THE HEPATITIS C VIRUS NS3 PROTEIN CONTAINS AN RNA HELICASE ACTIVITY

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The Hepatitis C Virus (HCV) NS3 protein contains amino acid motifs of a serine proteinase, a nucleotide triphosphatase (NTPase), and an RNA helicase based on amino acid sequence analysis. Proteinase and NTPase activities of the HCV NS3 protein were reported by several investigators. Here, we show that the recombinant HCV NS3 protein purified from a T7 promoter and His-tag expression system possesses an RNA helicase activity. The recombinant HCV NS3 protein consists of 466 amino acids from the carboxy terminal of a HCV NS3 open reading frame and 25 additional residues from the vector. The recombinant HCV NS3 protein was purified by metal-binding chromatography. The helicase activity requires ATP and divalent cations such as Mg^{2+} and Mn^{2+}. The helicase activity was abolished by monoclonal antibody specific to the HCV NS3 protein.

The Hepatitis C Virus (HCV) was first reported in 1989, and is the major etiologic agent of transfusion non-A, non-B hepatitis (1). It is now known that HCV is an enveloped virus that contains a positive strand RNA genome of about 9400 nucleotides. With comparative sequence analysis studies, HCV is classified as a new genus of the *Flaviviridae* family of which the other two genera are pestivirus and flavivirus (2). The RNA genome of HCV contains a single open reading frame which encodes a viral polyprotein of about 3010 residues. This polyprotein is processed by the host and viral proteases during or after translation. The genetic map of HCV constructed by a number of researchers is N'-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-C' (3, 4, and 5).

NS3 proteins of the flavivirus, pestivirus and HCV have conserved sequence motifs of serine type proteinase and of nucleoside triphosphatase (NTPase)/RNA helicase as shown in Fig. 1A. One third of the N'-terminal of the HCV NS3 protein shows that it is a trypsin like serine proteinase which cleaves the NS3-NS4A, NS4A-

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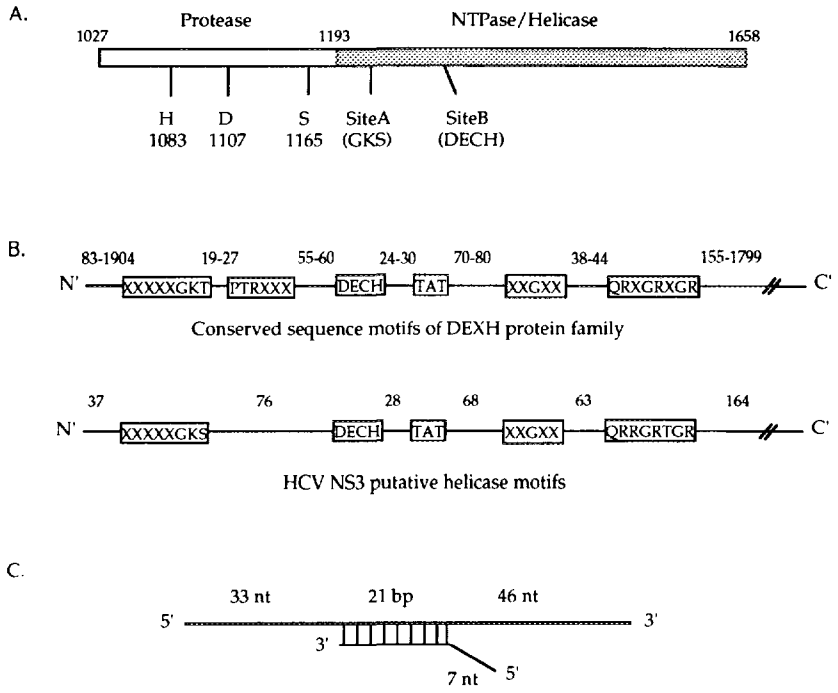


Figure 1. A. Schematic presentation of the HCV NS3 protein. Numbers indicate the amino acid positions of the HCV-1 polyprotein. B. Conserved sequence motif of DEXH box RNA helicase proteins and comparative alignment of the RNA helicase domain of the HCV NS3 protein. Numbers between boxes indicate the distance in amino acid residues. C. Structure of double strand RNA substrate for RNA helicase assay. The upper line indicates the ^{32}P-labeled RNA strand. The lower line indicates the unlabeled RNA strand.

NS4B, NS4B-NS5A, and NS5A-NS5B junctions (6). Two thirds of the NS3 C'-terminal fragment belongs to the DEAD box NTPase/RNA helicase family (2, 7). The DEAD box protein family has eight highly conserved amino acid motifs, one of which is the DEAD region, where it is also known as an ATPase motif. The DEAD protein family consists of three subfamilies, DEAD proteins, DEAH proteins (8) and DEXH proteins(9). Fig. 1B shows the conserved sequence motifs of the DEXH protein family and the corresponding motifs of HCV NS3. The HCV NS3 protein has a sequence motif of DECH which puts it in the DEXH protein subfamily. Suzich *et al.* (10) showed that two thirds of the carboxy terminal fragment of HCV NS3 expressed in *E. coli* had polynucleotide-stimulated NTPase activity. Recently, Warrener and Collett (11) demonstrated that bovine viral diarrhea virus (BVDV) NS3 protein expressed in a baculovirus expression system had an RNA helicase activity. Drawing from these results, we expected that HCV NS3 has helicase activity. We employed a bacterial pET His-Tag expression system to purify the

recombinant HCV NS3 protein and investigated the enzymatic activity of the HCV NS3 protein. This is the first report that the carboxy terminal fragment of the HCV NS3 protein expressed in *E. coli* possesses an RNA helicase activity. It requires ATP and a divalent ion for its activity.

MATERIALS AND METHODS

Expression and purification of the HCV NS3 protein. For expressing two thirds of the HCV NS3 protein, polymerase chain reaction (PCR) was used to amplify a 1.4 Kb DNA fragment encompassing amino acids 1193 to 1658 from HCV-1 cDNA. The sense primer used was JCK-1 5'-GGGGATCCGGTGGACTTTATCCCT-3', and the antisense primer JCK-7 5'-GGAAGCTTGCTCGTGACGACCTCG-3'. The PCR product was digested with BamHI and HindIII inserted into BamHI and HindIII sites of pET21b (purchased from Novagen. WI). The recombinant plasmid was designated pET21b-NS3HCV and transformed into *E.coli* BL21 (DE3), and the inserted region was verified by sequencing. pET21b-NS3HCV contains His-Tag (6 histidines) at the C-terminal end for easier purification. pET21b-NS3HCV was induced by adding IPTG at the final concentration of 1 mM to exponentially growing cells in LB medium with 10 μg/ml of ampicillin. After 3 hrs of culturing at 37°C, the cells were harvested and disrupted. Soluble parts of cell extract were loaded onto a metal-binding column. Resin-bound protein was eluted with 1 M imidazole, 0.5 M NaCl, 20 mM Tris-Cl pH 7.9. Eluted fractions were subjected to SDS-PAGE, and protein containing fractions were pooled and dialyzed against 50 mM Tris-Cl pH 7.9 for 4 hrs.

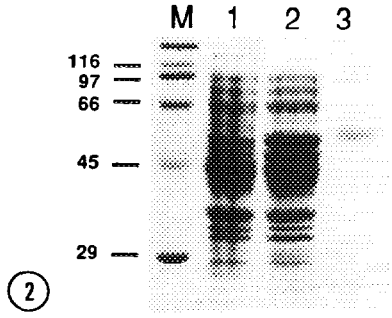
Preparation of substrate for RNA helicase. Fig. 1C. shows the structure of the double strand RNA used as a substrate of an RNA helicase. The long strand was prepared by *in vitro* transcription of pGEM1 that had been cleaved with PvuII, and the short strand was transcribed from the BamHI-digested pSP65. Both strands were transcribed with SP6 RNA polymerase (New England Biolabs) according to the manufacturer's manual. After the transcription reaction, each aliquot was treated with RNase-free DNase (Promega) and extracted with phenol:chloroform, and precipitated with ethanol. Each RNA strand was resuspended with 25 μl of hybridization buffer (20 mM HEPES-KOH pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS), and mixed. The mixture was heated to 100°C for 5 min. and incubated at 65°C for 30 min. and incubated at 25°C overnight. The long strand RNA was labeled with [α-^{32}P]-CTP, and the specific activity of the labeled substrate was 1 - 1.5 x 10^5 cpm/pmol ds RNA substrate. Duplex RNA was electrophoresed on 6% native polyacrylamide gel (30:0.8), and the location of the ds RNA was identified by autoradiography. To recover the RNA substrate, a sliced gel fragment was ground in 400 μl of elution buffer (0.5 M ammonium acetate, 0.1% SDS, 10 mM EDTA) and shaken vigorously at 4°C for 2 hrs. The supernatants were extracted with chloroform and precipitated with ethanol, and the RNA pellet was dissolved in D.W.

RNA helicase assay. RNA helicase assay was performed in 20 μl of reaction mixture : 1 pmol NS3 protein, 0.5 pmol ds RNA substrate, 25 mM MOPS-KOH (pH 6.5), 5 mM ATP, 3 mM $MnCl_2$, 2 mM DTT, 100 μg/ml BSA, and 2.5 U RNasin (Promega). The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 5 μl of 5 x termination buffer [0.1 M Tris-Cl (pH 7.4), 20 mM EDTA, 0.5% SDS,

0.1% NP-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 50% glycerol]. Each aliquot was loaded on 6% native polyacrylamide gel (30:0.8) and electrophoresed at 80 V for 3 hr. The ds RNA substrate and unwound RNA strand were visualized by autoradiography. To investigate the effect of ATP and divalent metal ion on the NS3 helicase activity, the same reactions were carried out with 1, 2, 3, 4, and 6 mM Mn^{2+} or Mg^{2+} in the presence of 1 mM or 5 mM ATP. Strand separation efficiencies were calculated by quantitating the radioactivities of the bands with PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Purification of the HCV NS3 protein. pET21b-NS3HCV consists of 466 amino acid residues from the C' terminus of HCV NS3 and 6 histidine residues and 19 additional residues from the pET expression vector. About 54 kDa of (481 amino acid residues) HCV NS3-His-tag fusion protein was induced by 1 mM IPTG from *E.coli* BL21 (DE3) harboring the recombinant plasmid (Fig. 2, lanes 1 and 2). One or more protein bands of about 50 kDa appeared by IPTG induction, but only the 54 kDa NS3-His fusion protein was purified from the metal binding affinity column (lane 3). Induced protein was soluble and about 400 μg of protein with approximately 95% purity was obtained from 200 ml of the bacterial culture. The NTPase assay on polyethyleneimine cellulose TLC plate (J.T.Baker) was performed as previously



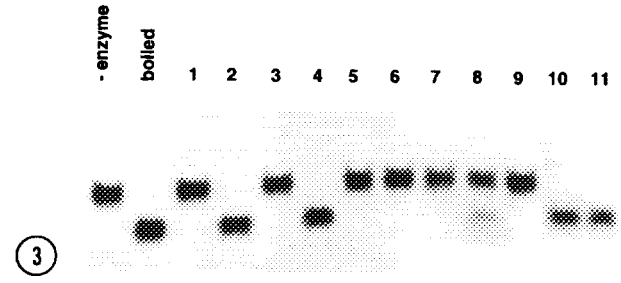


Fig. 2. Expression and purification of HCV NS3 from *E.coli*. M: protein size markers; Lane 1: Total protein from uninduced cells, Lane 2: Total protein from 3 hr IPTG induced cells, Lane 3: HCV NS3:His-tag fusion protein purified by nickel binding chromatography.

Fig. 3. RNA helicase assay of the HCV NS3 protein. -enzyme: reaction without the HCV NS3 protein; boiled: ds RNA strand was boiled for 3 mins. Lane 1: Fraction from negative control cell (pET vector only), Lane 2: 3 mM Mn^{2+} , Lane 3: no Mn^{2+}, Lane 4: 3 mM Mg^{2+}, Lane 5: no Mg^{2+}, Lane 6: 3 mM KCl, Lane 7: no ATP, Lane 8: 1 mM ATP, Lane 9: preincubation of the NS3 protein with NS3-specific monoclonal antibody, Lanes 10, 11: preincubation of the NS3 protein with anticonnexin monoclonal antibody at 0.5 μg, 1.0 μg per 20μl, respectively. Monoclonal antibodies were preincubated with the NS3 protein at room temperature for 5 min.

described (10) to confirm that the final purified protein had active conformation. The purified protein showed an NTPase activity (data not shown).

Properties of the HCV NS3 protein. Double strand RNA was unwound by purified HCV NS3 protein (Fig. 3). Strand displacement was observed by a faster migration of the radio-labeled long strand on 6% polyacrylamide gel. As a negative control, pET21b plasmid without the insert was transformed to *E.coli* BL21 (DE3) and induced with 1 mM IPTG. The negative control cell culture was processed with the same purification step as pET21b-NS3HCV. This fraction showed no enzymatic activity (Fig. 3, lane 1). Most of the known RNA helicases have shown that a divalent metal ion and ATP are required(11, 12). The HCV NS3 RNA helicase also requires divalent ions such as Mg^{2+} and Mn^{2+} (Fig. 3, lane 2 to 5). Strand displacement was observed only when Mg^{2+} or Mn^{2+} ions were present (Fig. 3, lanes 2 and 4). When these divalent cations or ATP were deleted, ds RNA was not unwound (Fig. 3, lanes 3, 5, and 7). Monovalent potassium ion did not substitute divalent ion in activating HCV NS3 helicase activity under these conditions (lane 6). At 1 mM ATP, helicase activity was lower than at 5 mM (lane 8). The enzymatic activity of NS3 completely abolished the treatment of monoclonal antibody specific to HCV NS3 protein (lane 9), and was not blocked by a non-specific antibody at two different concentrations (lanes 10 and 11).

RNA helicase activity of HCV NS3 was dependent on divalent cation and ATP. So, we investigated the activity change of HCV NS3 at various concentrations of the two cofactors (Fig. 4). At a low concentration of ATP (1 mM), helicase activity of NS3 was highest at a low concentration (1 mM) of either divalent cations, and the activity decreased by increasing the concentration of the cations. But, at a high

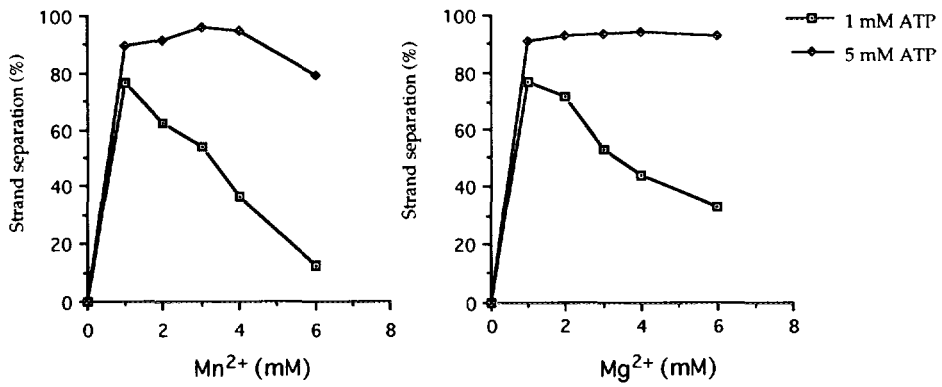


Fig. 4. Activity profiles of the HCV NS3 RNA helicase with different ATP and divalent cation concentrations. The effects of cations were tested at two different ATP concentrations (1 mM and 5 mM).

concentration of ATP (5 mM), most of the substrates were unwound at all tested concentrations of the divalent cations. At 3 mM or 4 mM of cation concentration, either Mg^{2+} or Mn^{2+}, the activity was highest. Warrener and Collett (11) reported that the BVDV NS3 RNA helicase showed some preferences to Mn^{2+} rather than to Mg^{2+}, but HCV NS3 showed a slight bias for Mg^{2+} rather than to Mn^{2+}.

DISCUSSION

DEAD box putative RNA helicases are divided into three subfamilies by amino acid sequence similarity - the DEAD protein family, DEAH protein family and DEXH protein family (9). Several groups reported *in vitro* RNA unwinding activities of viral RNA helicases which were members of the DEAD or DEXH protein families (11, 13). Because HCV contains a positive strand RNA genome, RNA helicase activity is essential for its replication. HCV NS3 protein has similar sequence motifs as other known viral RNA helicases, and it has RNA-stimulated NTPase activity which is a characteristic of DEAD box proteins (10). In this study, we tried to demonstrate the RNA helicase activity of HCV NS3, and showed that it had an RNA helicase activity. The HCV NS3 protein showed RNA helicase activities only in the presence of divalent cations (Mn^{2+} or Mg^{2+}) and ATP. At a lower level of ATP (1 mM), an increasing amount of either cations inhibited the enzymatic activity of NS3. When the ATP concentration was high (5 mM), the helicase activity remained at a high level even when Mg^{2+} or Mn^{2+} cations were present at high concentrations. RNA helicase A purfied from HeLa cells needed only Mg^{2+} for its cofactor, and Mn^{2+} did not substitute for Mg^{2+} (12). Pestivirus NS3 and Vaccinia virus RNA helicase have shown that they could use both cations. In addition to these two viral enzymes, HCV NS3 RNA helicase can use both metal ions. All of our experiments were carried out at pH 6.5. However, without changing other components, when the pH was increased to 7.6, HCV NS3 showed not more than 10% strand separation (data not shown). These characteristics of HCV NS3 RNA helicase imply that it has a similar nature to pestivirus NS3 RNA helicase, which is known to be pH sensitive.

We confirmed that the RNA helicase activity was not derived from *E.coli* contaminants by two methods. First, pET21b plasmid without the insert was used as a negative control. When we tested the enzymatic activity of the same eluted fraction from the negative control cell culture, there was no detectable level of NTPase or RNA helicase activity. Second, NS3 helicase activity was abolished by a NS3-specific monoclonal antibody, but, an unrelated antibody did not affect the activity. From these results we could concluded that the helicase activity was derived not from *E.coli* contaminants, but from the HCV NS3 protein.

Most of the investigated RNA helicases bound to a single strand region and then they unwound double strand RNA moving unidirectionaly (11, 12, 13) or bidirectionaly (14, 15). We used the substrate with the single strand region on both

the 3' and 5' ends. The directionality of HCV NS3 helicase is currently being investigated, and the substrate specificities are also being studied. Suzich *et al.* (10) showed that two thirds of the C'-terminal of HCV NS3 could hydrolyze all NTPs and dNTPs , and we also observed this activity (data not shown). The possibilities of other (d)NTPs being used as energy sources, and other double strand polynucleotides - RNA:DNA, DNA:RNA, or DNA: DNA hybrids - as substrates are now being tested. Our results that truncated NS3 has biochemical activity in spite of deleted N'-terminal proteinase domain suggest that these two domains may act independently. There is no evidence that these two domains are cleaved *in vivo.* yet. The relationship between these two domains would be an interesting subject, and revealing the role of HCV NS3 *in vivo* would be a final goal of these studies. RNA helicase activity of the HCV NS3 protein may be used for developing antiviral agents of HCV.

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