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Mutational Analysis of the Hepatitis C Virus RNA Helicase

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The carboxyl-terminal three-fourths of the hepatitis C virus (HCV) NS3 protein has been shown to possess an RNA helicase activity, typical of members of the DEAD box family of RNA helicases. In addition, the NS3 protein contains four amino acid motifs conserved in DEAD box proteins. In order to inspect the roles of individual amino acid residues in the four conserved motifs (AXXXXGKS, DECH, TAT, and QRRGRTGR) of the NS3 protein, mutational analysis was used in this study. Thirteen mutant proteins were constructed, and their biochemical activities were examined. Lys1235 in the AXXXXGKS motif was important for basal nucleoside triphosphatase (NTPase) activity in the absence of polynucleotide cofactor. A serine in the X position of the DEXH motif disrupted the NTPase and RNA helicase activities. Alanine substitution at His1318 of the DEXH motif made the protein possess high NTPase activity. In addition, we now report inhibition of NTPase activity of NS3 by polynucleotide cofactor. Gln1486 was indispensable for the enzyme activity, and this residue represents a distinguishing feature between DEAD box and DEXH proteins. There are four Arg residues in the QRRGRTGR motif of the HCV NS3 protein, and the second, Arg1488, was important for RNA binding and enzyme activity, even though it is less well conserved than other Arg residues. Arg1490 and Arg1493 were essential for the enzymatic activity. As the various enzymatic activities were altered by mutation, the enzyme characteristics were also changed.

Hepatitis C virus (HCV) was first cloned in 1989 (6). It is a major causative agent of posttransfusion non-A, non-B hepatitis. A comparative sequence analysis revealed that HCV is a new member of the *Flaviviridae* family along with pestiviruses and flaviviruses (7). HCV has a positive-strand RNA genome about 9.4 kb in size, which displays remarkable sequence diversity among individual isolates. The genome contains a region of 340 nucleotides within the 5′ noncoding region that functions as an internal ribosome entry site (4, 48) and a regulator for translation (55). This 340-nucleotide element is followed by a large single open reading frame encoding a polyprotein of 3,010 amino acids. Upon infection, the genomic RNA of HCV produces a large polyprotein that is processed by cellular and viral proteases to yield at least nine different viral proteins during and after translation (2, 11, 16, 17, 25, 47). The organization of the HCV polyprotein is as follows: NH_2-C-E1-E2(NS1)-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Two virally encoded proteases have been identified in HCV, an NS2/NS3 metalloprotease and an NS3 serine protease (17). The catalytic domain of the NS3 protease has been mapped to the NH_2-terminal region of the NS3 protein. As predicted previously by amino acid analyses, the NS5B protein contains an RNA-dependent RNA polymerase activity (3). Downstream of the open reading frame is a short 3′ untranslated region followed by either a poly(U) homopolymer tract (21) or a poly(A) tract (14, 15). Recently, Tanaka et al. (45) reported that there are about 85 nucleotides after the poly(U) tract.

Amino acid sequence alignments revealed that the three genera of the *Flaviviridae* family (flaviviruses, pestiviruses, and HCV) have conserved sequence motifs of a serine-type proteinase and a nucleoside triphosphatase (NTPase)-RNA helicase in the respective NS3 proteins (7). The HCV NS3 protein belongs to the DEAD (Asp-Glu-Ala-Asp) box RNA helicase family, which contains eight conserved amino acid sequence motifs (10, 26, 28). The DEAD box family consists of three subfamilies of proteins that contain either a DEAD, DEAH (Asp-Glu-Ala-His), or DEXH (Asp-Glu-X-His) motif (26). The DEAD box elements of NS3 proteins from the various types of HCV have the sequence Asp-Glu-Cys-His (DECH), and thus these NS3 proteins are believed to be members of the DEXH protein subfamily (5, 8, 19, 53). A number of DEAD box family proteins have been shown to contain NTPase and RNA helicase activities, such as RNA helicase A from HeLa cells, eIF-4A of mouse, the NPH II protein of vaccinia virus, and the NS3 protein of pestivirus (27, 37, 40, 50). The related yeast protein PRP2 has an NTPase activity but not an RNA helicase activity (24).

The amino acid sequences of four of the eight conserved motifs from several RNA helicases are shown in Fig. 1A. The most NH_2-terminal motif, AXXXXGKT/S, called the A motif of ATP binding proteins, is required for ATP binding and, consequently, for ATP hydrolysis and RNA unwinding (49). According to X-ray crystallographic studies, the A motif forms a phosphate binding loop (P loop) (41). The second conserved motif is the DEAD box element, which was suggested to be a variant of the Walker ATPase B motif. X-ray crystallography showed that the first aspartate residue of the DEAD box is in close proximity to the ATPase A motif and binds to Mg^{2+} through a water molecule (30). The Mg^{2+} is complexed to the β and γ phosphates of the ATP molecule bound at the A motif ATP binding site. In the COOH-terminal region of the DEAD box RNA helicases, the conserved sequences SAT and HRI GRXXR are found. In the DEAH family, SAT and QRI GRXXR are found, while TAT and QRXGRXXR are found in DEXH family. The S/TAT region is thought to be important in RNA unwinding, and the H/QRXGRXXR motif is important in ATP hydrolysis and RNA unwinding. All of the variants of the HCV NS3 protein have the conserved sequences AXXXXGKS, DECH, TAT, and QRRGRTGR.

For *Flaviviridae* viruses, NTPase and RNA helicase activities have been reported. Wengler and Wengler (51) showed that the NS3 protein of the West Nile flavivirus contains an RNA-

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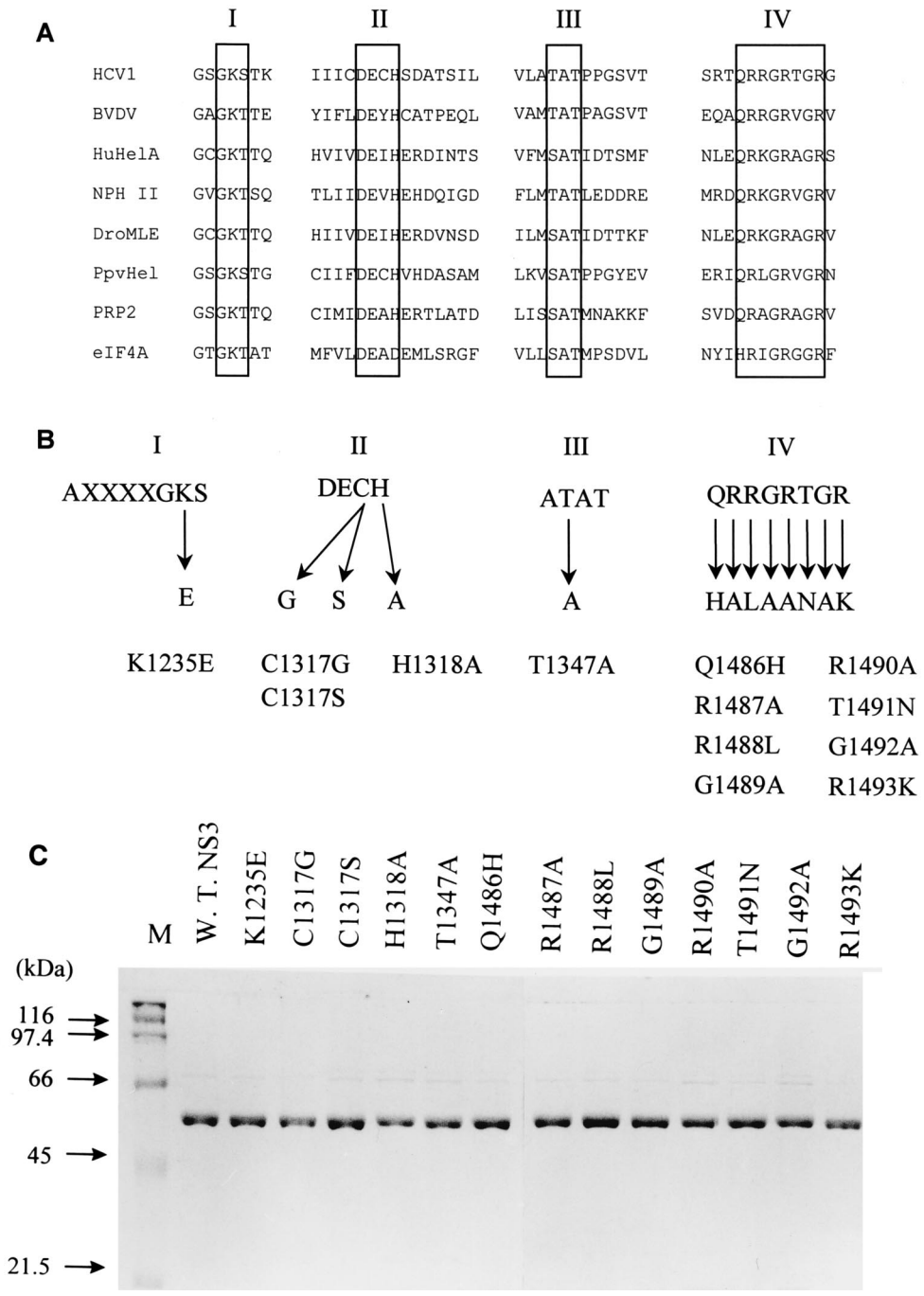

FIG. 1. (A) Comparative sequence alignment of HCV NS3 and several DEAD box RNA helicases. The amino acid sequences of four conserved sequence motifs are shown. Sequence data were from the GenBank database, and accession numbers are as follows: HCV-1, M62321; BVDV, I35851; human RNA helicase A (HuHel), I13848; bovine helicase II (BoHel2), X82829; *Drosophila* maleless protein (DroMLE), M74121; plum pox virus CI protein (PpvHel), M92280; yeast PRP2 protein, X55936; and eIF-4A (eucaryotic translation initiation factor 4A), X03040. (B) Conserved sequences of the HCV NS3 protein and the amino acid residues mutated in this study. (C) SDS-PAGE analysis of the purified NS3H protein and mutated proteins. Proteins were subjected to SDS–10% PAGE and Coomassie blue staining. Each lane contains about 2 μg of protein. The molecular mass of the NS3H proteins was about 54 kDa. Lane M, molecular mass markers; lane W.T.NS3, wild-type NS3H protein.

stimulated NTPase activity. Suzich et al. (42) reported that the NS3 proteins of the yellow fever virus and of HCV expressed in and purified from *Escherichia coli* exhibit an NTPase activity. The p80 protein of bovine viral diarrhea virus (BVDV) expressed in insect cells has an RNA-stimulated NTPase activity (44) and an RNA helicase activity (50). The RNA helicase activity of the HCV NS3 protein has also been demonstrated by our group and other researchers by using a recombinant COOH-terminal fragment of the NS3 protein (18, 22, 43). Like other RNA helicases, the HCV RNA helicase requires ATP and divalent cations such as Mg^{2+} or Mn^{2+} for optimal enzymatic activity.

Point mutational studies of other DEAD box RNA helicases have been reported. Pause et al. (32, 33) performed a mutational analysis on the eIF-4A protein of mouse, which is a prototype RNA helicase. Gross and Shuman (12, 13) studied the effects of several point mutations introduced into the vaccinia virus NPH-II protein. In this study, point mutations were introduced into the conserved sequence motifs of the HCV NS3 protein. Several amino acid residues of the AXXXXGKS/T, DECH, TAT, and QRRGRTGR regions were individually replaced. The CTPase, ATPase, RNA binding, and RNA helicase activities of all forms of the mutated protein were tested and compared to the activities of the wild-type protein. We also compared the effects of each mutation on the various enzymatic activities of NS3 with their corresponding effects on the eIF-4A and NPH II proteins.

MATERIALS AND METHODS

Expression and purification of the HCV NS3 protein. For expression of the COOH-terminal three-fourths of the HCV NS3 protein, PCR was used to amplify a 1.4-kb DNA fragment encompassing amino acids 1193 to 1658 of HCV type 1 (HCV-1) cDNA. The amino acid numbers used in this report are from the HCV-1 polyprotein sequence (Genbank accession no. M62321). The PCR cycle was 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. The cycle was repeated 35 times. The DNA sequence of the sense primer (JCK-1) was 5′-GGGGATC CGGTGGACTTTATCCCT-3′, while that of the antisense primer (JCK-7) was 5′-GGAAGCTTGCTCGTGACGACCTCG-3′. The PCR product was digested with *Bam*HI and *Hin*dIII at 25°C for 3 h and at 37°C for 1 h and inserted into the *Bam*HI and *Hin*dIII sites of pET21b (Novagen Inc., Madison, Wis.). The recombinant plasmid was designated pET21bNS3H. *E. coli* BL21(DE3) cells were transformed with pET21bNS3H, and the inserted NS3 fragment was verified by DNA sequencing. pET21bNS3H produces a 466-amino-acid polypeptide spanning amino acids 1193 to 1658 of the HCV-1 polyprotein, and this protein is designated NS3H.

The NS3H protein contains a His tag (six consecutive histidine residues) at the COOH-terminal end for easier purification. In order to extract the NS3H protein from *E. coli* BL21(DE3) cells harboring pET21bNS3H, expression of NS3H was induced by adding isopropylthio-β-D-galactoside (IPTG) at a final concentration of 1 mM to 200 ml of Luria-Bertani medium with 10 μg of ampicillin per ml. After 3 h of culture at 37°C, the cells were harvested by centrifugation at 6,000 × *g* for 10 min. The *E. coli* cell pellet was resuspended with 20 ml of 1× binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl [pH 7.9]). Resuspended cells were frozen at −70°C for at least 30 min and then thawed in ice-cold water. The cells were then further disrupted by brief sonication with a micro tip (50% duty cycle) for 2 min. The mixture was centrifuged at 27,000 × *g* for 30 min at 4°C. The supernatant containing the recombinant NS3H protein was saved.

The NS3H protein was purified from the supernatant fraction by nickel affinity column chromatography at 4°C. To prepare the affinity column, a 2-ml bed volume of His-bind resin (Novagen Inc.) was packed in a chromatography column, and the resin was charged with nickel by flushing the column with 5 volumes of 1× charge buffer (50 mM $NiSO_4$). The column was then equilibrated with 3 volumes of 1× binding buffer. The soluble fraction of the cell extract was loaded onto the His-binding column, and the column bed was washed with 10 volumes of 1× binding buffer and 6 volumes of 1× washing buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl [pH 7.9]). Resin-bound protein was eluted with 1× elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-Cl [pH 7.9]). The volume of each fraction was half of the bed volume. Eluted fractions were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) for analysis, and fractions containing protein were pooled and dialyzed against 50 mM Tris-Cl (pH 7.5) at 4°C for at least 4 h. The final protein concentration was determined by the Bradford protein assay (reagents were from Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's instructions. Spectrophotometric measurements were made on a Shimadzu UV-160/2 instrument at 595 nm. To preserve the protein, glycerol was added at a final concentration of 20%, and the protein was stored at −70°C. Mutant proteins were similarly purified.

Construction of NS3H mutants. The positions of point mutations in the conserved sequence motifs of the HCV RNA helicase are shown in Fig. 1B. The Lys residue of the AXXXXGKS motif was changed to Glu (K1235E) to investigate the effect of a positively charged amino acid in the wild type. The DECH motif was mutated to either DEGH or DESH (C1317G and C1317S, respectively). The histidine of the DEXH motif was mutated to an alanine (H1318A). The first threonine residue in the TAT motif was mutated to an alanine (T1347A). In the QRRGRTGR motif, Q1486H, R1487A, R1488L, G1489A, R1490A, T1491N, G1492A, and R1493K were produced. Q1486H was constructed to investigate whether the Gln residue in the QRRGRTGR motif of the DEXH family protein could be replaced by a His residue, as in the HRIGRGGR motif of the DEAD family protein.

In vitro mutagenesis. The *Bam*HI-*Hin*dIII HCV NS3H fragment from pET21bNS3H was ligated into the *Bam*HI and *Hin*dIII sites of pSELECT-1 (Promega, Madison, Wis.). The recombinant plasmid was designated pSEL-NS3. *E. coli* JM109 cells were transformed with pSEL-NS3, and the plasmid-harboring cells were isolated on Luria-Bertani agar–tetracycline plates. Single-stranded DNA was extracted from the transformed *E. coli* strain, and in vitro mutagenesis was performed according to the manufacturer's manual. The plasmid DNA encoding the point-mutated protein was extracted from the transformed JM109 cells, and the mutated sequences were verified by dideoxy DNA sequencing (38). The mutated forms of the *Bam*HI-*Hin*dIII DNA fragments from pSEL-NS3 were inserted into the *Bam*HI and *Hin*dIII sites of expression plasmid pET21b. Aliquots of *E. coli* BL21(DE3) cells were individually transformed with each mutated plasmid. Expression of the mutant proteins was induced with IPTG, and each was purified as described above.

Analysis of the NTPase activities of the various mutated proteins. The NTPase activities of mutated and wild-type NS3H proteins were assessed by measuring the extent of hydrolysis of [α-^{32}P]CTP (400 Ci/mmol) (Amersham Corp., Buckinghamshire, United Kingdom) and [α-^{32}P]ATP to [α-^{32}P]CDP and [α-^{32}P]ADP, respectively, by thin-layer chromatography (TLC). The reaction mixture (10 μl), containing 10 pmol of NS3H protein, 1 μCi of [α-^{32}P]CTP, 50 mM Tris-Cl (pH 7.0), 1 mM NaCl, and 2.5 mM $MgCl_2$, was incubated at 37°C for 30 min. The reaction was stopped by adding 1 μl of 20 mM EDTA to a final concentration of 2 mM. To analyze the products of hydrolysis of CTP, 1 μl of each reaction mixture was spotted onto a polyethyleneimine-cellulose plate (J. T. Baker Inc., Phillipsburg, N.J.), and the plate was then developed with 0.375 M potassium phosphate (pH 3.5) in a TLC chamber. The air-dried plate was exposed to X-ray film. The amount of radioactivity in each spot was measured with a PhosphorImager (Molecular Dynamics).

To investigate the enzymatic characteristics of the proteins, hydrolysis of several different concentrations of CTP or ATP was measured by the TLC method. For CTPase activity, the reaction mixture contained 1 μCi of [α-^{32}P]CTP, 10 pmol of NS3H protein, 50 mM Tris-Cl (pH 7.0), 1 mM NaCl, 2.5 mM $MgCl_2$, and 10, 30, 50, 75, 100, 200, or 500 μM unlabeled CTP. In case of poly(U) stimulation of NTPase activity, 1 μg of poly(U) was added to the reaction mixture. Each reaction mixture was incubated at 37°C for 30 s, and the reaction was stopped by adding EDTA. Because the NTPase activity of the H1318A mutant protein in the absence of poly(U) was higher than those of other mutated forms of the NS3H protein, unlabeled CTP was used at 100, 200, 500, 1,000, and 2,000 μM in this case. For the ATPase activity, the labeled and unlabeled CTP were replaced by labeled and unlabeled ATP, respectively. The NTPase activity was measured as described above.

In vitro transcription. The method of in vitro transcription of the RNA strand from the pGEM 1, pGEM 3, and pSP65 plasmids was described previously (22). Briefly, pGEM 1 (4 μg) and pGEM 3 (4 μg) were cleaved with *Pvu*II, and pSP65 (4 μg) was cleaved with *Bam*HI. The linearized plasmid was then separated on a 1% agarose gel in 1× TAE. The DNA fragment was then eluted from the agarose gel with a JETsorb gel extraction kit (Genomed Inc., Raleigh, N.C.). The eluted DNA fragment was dissolved in 60 μl of double-distilled water (ddH_2O). The in vitro transcription mixture contained 10 μl of 10× transcription mixture (400 mM Tris-Cl [pH 7.5], 60 mM $MgCl_2$, 20 mM spermidine, and 100 mM NaCl), 1 μl of 1 M dithiothreitol (DTT), 50 U of RNasin (Promega), 20 μl of a 2.5 mM nucleoside triphosphate mixture, and 50 U of SP6 RNA polymerase in a final volume of 100 μl. The reaction mixture was incubated at either 37 or 42°C for 90 min. RNase-free DNase (5 U) (Promega) was then added, and the mixture was incubated further at 37°C for 30 min. ddH_2O (300 μl) was added, and the mixture was extracted with 400 μl of a 1:1 mixture of water-saturated phenol (pH 5.5) and chloroform. The supernatant was transferred to a fresh tube, 8 M ammonium acetate (180 μl) and 100% ethanol (1 ml) were added, and the mixture was incubated at −70°C for at least 30 min. The RNA was recovered by microcentrifugation at 12,000 × *g* for 15 min at 4°C, and the nucleic acid pellet was washed with 70% ethanol.

Analysis of RNA binding activity. The RNA binding activities of the wild-type and mutated forms of the NS3H proteins were measured by an electrophoretic gel mobility shift assay. The RNA strand made by in vitro transcription of pGEM3 was denatured by heating at 95°C and cooling on ice. The denatured RNA (1 pmol) and individual proteins (1, 2, 5, and 10 pmol each) were mixed in a 20-μl reaction volume containing 25 mM MOPS (morpholinepropanesulfonic acid)-KOH (pH 6.5), 5 mM ATP, 3 mM $MgCl_2$, and 2 mM DTT. The reaction mixture was incubated at 37°C for 10 min, and glycerol (2 μl) was added to stop the reaction. The RNA-protein mixture was loaded onto a 0.25× Tris-borate-EDTA–8% polyacrylamide gel (79:1) and electrophoresed at a constant current of 12 mA. The separated unbound RNA and the protein-RNA complex were visualized by autoradiography.

Construction of a dsRNA substrate for the RNA helicase assay. The structure of the double-stranded RNA (dsRNA) used in this study was previously described (22). After the in vitro transcription reaction was carried out as described above, each RNA strand was resuspended in 25 μl of hybridization buffer (20 mM HEPES-KOH [pH 7.6], 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS). The RNA mixtures were combined, and the RNA strands were hybridized by boiling for 5 min and then incubating at 65°C for 30 min and at 25°C overnight. The long-strand RNA was labeled with [α-^{32}P]CTP, and the specific activity of the labeled dsRNA substrate was 1×10^5 to 1.5×10^5 cpm/pmol. Duplex RNA was

TABLE 1. ATPase activities of wild-type and mutated forms of NS3H

NS3H protein	Specific ATPase activity[a]		Fold stimulation of ATPase activity by poly(U)
	Without poly(U)	With poly(U)	
Wild type	43	196	4.56
K1235E	5.5	56	10.2
C1317G	57	39	0.68
C1317S	0	0	
H1318A	620	66	0.11
T1347A	39	42	1.1
Q1486H	0	0	
R1487A	52	132	2.54
R1488L	29	12.2	0.42
G1489A	21	95	4.5
R1490A	31	0	
T1491N	29	98	3.4
G1492A	15.5	41	2.6
R1493K	0	0	

[a] Expressed as picomoles of ATP hydrolyzed minute^{-1} picomole of protein^{-1}.

subjected to electrophoresis on an 8% native polyacrylamide gel and localized by autoradiography. To recover the dsRNA substrate, a sliced gel fragment was ground in 500 μl of elution buffer (0.5 M ammonium acetate, 0.1% SDS, 10 mM EDTA) and shaken vigorously at 4°C for 2 h. The supernatant was extracted with chloroform, and the RNA was precipitated with ethanol. The dsRNA was recovered by centrifugation at 12,000 × *g* for 15 min at 4°C, and the RNA pellet was dissolved in ddH_2O.

RNA helicase assay. The RNA helicase assay was performed with a final volume of 20 μl containing NS3 protein (1, 2, 5, and 10 pmol), 0.5 pmol of dsRNA, 25 mM MOPS-KOH (pH 6.5), 5 mM ATP, 3 mM $MgCl_2$ (or $MnCl_2$), 2 mM DTT, 100 μg of bovine serum albumin per ml, and 2.5 U of RNasin. The reaction mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 5 μl of termination buffer (0.1 M Tris-Cl [pH 7.4], 20 mM EDTA, 0.5% SDS, 0.1% Nonidet P-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 50% glycerol), and each aliquot was loaded onto an 8% native polyacrylamide gel. Strand separation was visualized by autoradiography. Strand separation efficiencies were calculated by quantitating the radioactivities of the bands with the use of PhosphorImager.

RESULTS

Protein expression and purification. Figure 1A shows the amino acid sequences conserved among known RNA helicases. The mutated positions and the amino acids substituted are illustrated in Fig. 1B. We used four different sequencing primers to verify mutated sequences in four conserved motifs. To identify the mutated nucleotide sequence, we sequenced about 300 nucleotides around each mutated nucleotide. No unwanted mutation was found; however, there remains the possibility of unwanted mutations in unsequenced regions. All of the proteins were purified as described in Materials and Methods. Figure 1C shows the SDS-PAGE results for 2 μg of each purified NS3H variant protein. The estimated molecular size of NS3H and the other mutated proteins was about 54 kDa, and this molecular size coincides with that estimated by sequence analysis. There were one or two protein bands at about 70 kDa on the SDS-polyacrylamide gel (Fig. 1C), which were always present at the end of the purification. However, these protein bands appeared even in *E. coli* that harbored pET21b without the NS3 fragment, and they did not affect the enzymatic activity of the NS3H proteins. As we showed previously, the enzymatic activities detected in this fraction arose from the recombinant NS3H protein. The identically eluted fraction from *E. coli* harboring no recombinant plasmid has no detectable enzymatic activity, and the enzyme activity of the purified NS3H protein was inhibited by NS3-specific antibodies (22).

ATPase and CTPase activities of the HCV NS3 protein. The ATPase and CTPase activities of the HCV NS3H protein and its mutated forms are shown in Tables 1 and 2. The overall ATPase and CTPase activities of each protein were nearly identical. The NTPase activity of the HCV NS3 RNA helicases is known to be stimulated by nucleic acids. In the absence of poly(U), the ATPase activity of the HCV NS3 protein was about 43 pmol min^{-1} and the CTPase activity was about 52 pmol min^{-1} pmol^{-1} in this study. ATPase and CTPase activities were increased about 4.5-fold by addition of poly(U). Suzich et al. (42) also reported that the ATPase activity of the HCV NS3 protein was stimulated 5.9-fold in the presence of poly(U).

In the absence of poly(U), the NTPase activity of the wild-type NS3H protein was six- to sevenfold greater than that of the K1235E AXXXXGKS motif mutant. However, the NTPase activity of the K1235E mutant was stimulated dramatically (more than 10-fold) by poly(U). The C1317G DECH motif mutant showed an NTPase activity similar to that of wild-type NS3H in the absence of poly(U); however, the NTPase activity of the C1317G mutant was not at all stimulated by poly(U) and, in fact, was slightly inhibited. The C1317S DECH motif mutant showed no NTPase activity at all. This result implies that not all amino acid residues can occupy the third position of the DECH motif, even though the HCV RNA helicase belongs to the DEXH box family.

The H1318A mutant, which has an alanine residue at the histidine position of the DECH motif, showed interesting properties compared with those of other related proteins. For example, the ATPase activity of the vaccinia virus NPH II protein is stimulated 12-fold by nucleic acids (13). In contrast, the ATPase activity of the DEVA mutant of the DEVH domain of NPH II was fivefold higher than that of the wild-type protein in the absence of nucleic acid cofactors. In addition, the DEVA mutant showed almost the same ATPase activity in the presence or absence of nucleic acids cofactors. Gross and Shuman regarded this mutation as a gain-of-function mutation (13). The H1318A DECH motif mutant of the HCV NS3 protein showed a unique feature. This DECA mutant had a very strong NTPase activity in the absence of poly(U); it exhibited 14.4-fold higher ATPase activity and 9.4-fold higher CTPase activity than did the wild-type protein. Also, the CTPase activity of the DECA mutant without poly(U) was about twofold higher than the poly(U)-stimulated activity of wild-type NS3H. The enzymatic parameters could not be determined with less than 500 μM substrate, so the amount of

TABLE 2. CTPase activities of wild-type and mutated forms of NS3H

NS3H protein	Specific CTPase activity[a]		Fold stimulation of CTPase activity by poly(U)
	Without poly(U)	With poly(U)	
Wild type	52	229	4.4
K1235E	7.7	89	11.6
C1317G	60	43	0.72
C1317S	0	0	
H1318A	490	73	0.15
T1347A	42	36	0.86
Q1486H	0	0	
R1487A	61	95	1.6
R1488L	27	14	0.52
G1489A	16	56	3.5
R1490A	24	0	
T1491N	22	73	3.3
G1492A	19.6	36	1.83
R1493K	0	0	

[a] Expressed as picomoles of CTP hydrolyzed minute^{-1} picomole of protein^{-1}.

TABLE 3. ATPase enzymatic parameters of wild-type and mutated forms of NS3H

NS3H protein	Without poly(U)		With poly(U)	
	K_m (μM)	V_{max} (% of wild type)	K_m (μM)	V_{max} (% of wild type)
Wild type	42	11.0	490	100
K1235E	8	2.2	80	15.3
C1317G	16	9.6	30	6.2
C1317S	—[a]	—	—	—
H1318A	720	340	86	14.1
T1347A	45	10.0	25	7.2
Q1486H	—	—	—	—
R1487A	80	19.2	100	27.0
R1488L	16	2.8	19	4.0
G1489A	9	2.4	30	8.0
R1490A	67	8.8	—	—
T1491N	34	6.2	104	21.8
G1492A	12	3.0	21	5.2
R1493K	—	—	—	—

[a] —, ATPase activity was not detectable.

substrate was increased to 1 and 2 mM. Interestingly, the NTPase activity of the H1318A mutant was inhibited substantially by addition of poly(U). In the case of NPH II, the K_m values of the wild-type DECH and mutant DEVA proteins were nearly the same, but the K_m of the DECA mutant (H1318A) differed from that of wild-type HCV NS3H (Table 3). Mutation in TAT region also affected the NTPase activity of the HCV NS3 protein. For the T1347A mutant, the NTPase activity without poly(U) was nearly identical to that of the wild-type protein, but the NTPase activity of the T1347A mutant was decreased to one-fourth in the presence of poly(U).

The Q1486H QRRGRTGR motif mutant completely lost its NTPase activity. In the case of the mouse protein eIF-4A (33), Gln substitution at the His residue of the HRIGRGGR motif yielded 30% NTPase activity compared to that of the wild-type protein. In addition, a mutated form of the vaccinia virus NPH II protein (Ala instead of Gln in the QRKGRVGR motif) showed a decrease in V_{max} to about 5% of that of the wild-type protein. The R1487A mutant showed nearly same NTPase activity as did the wild-type protein. However, poly(U) did not stimulate the NTPase activity of the R1487A mutant. The R1488L mutant showed NTPase activity that was about half that of the wild-type protein, and poly(U) inhibited the NTPase activity of the R1488L mutant. The G1489A mutant showed one-third of the NTPase activity of the wild-type protein, and the NTPase activity of the G1489A mutant was stimulated by poly(U) in a manner similar to that for the wild-type protein. The R1490A mutant displayed an NTPase activity that was about 50% of that of the wild type, and this NTPase activity was completely lost when poly(U) was added. In contrast, the T1491N mutant showed a level of NTPase activity that was 50% of that of the wild type, but in this case poly(U) stimulated this activity threefold. The NTPase activity of the G1492A mutant was similar to that of the T1491N mutant, but the stimulatory effect of poly(U) was less than that for the T1491N mutant. The R1493K mutant completely lost its NTPase activity, in either the presence or absence of poly(U). The NTPase activities of the H1318A, R1488L, and R1490A mutants were inhibited severely by the presence of poly(U). This is the first report of inhibition of NTPase activity by poly(U) for a DEAD box protein.

RNA binding and RNA helicase activities of the mutated forms of NS3H. Figure 2 shows the RNA helicase activities of the mutated HCV NS3 proteins. RNA helicase activity was tested with 1, 2, 5, and 10 pmol of each protein. Among 13 mutant proteins, 7 mutants (the C1317G, H1318A, T1347A, R1487A, G1489A, T1491N, and G1492A mutants) showed dsRNA-unwinding activity. The C1317G, R1487A, G1489A, and R1487A proteins showed activity profiles similar to that of the wild-type protein. The H1318A, T1347A, and G1492A proteins possessed decreased RNA-unwinding activity compared to the wild-type protein.

The K1235E mutant lost its RNA helicase activity and showed very low NTPase activity compared to the wild-type protein. The C1317G mutant had helicase levels that were nearly the same as those for the wild-type protein, although the NTPase activity of the C1317G mutant was not stimulated by poly(U). Serine could not replace the cysteine residue in the DECH motif, as the C1317S mutant lost both NTPase and RNA helicase activities. The T1347A mutant retained about half of its RNA helicase activity compared to the wild-type protein. The Q1486H mutant lost its RNA helicase activity, as expected, because it had lost its NTPase activity. The R1487A mutant displayed RNA helicase activity that was similar to that of the wild type. Mutation at this site in eIF-4A of mouse (33) led to a complete loss of RNA helicase activity, while in the case of the vaccinia virus NPH II mutant (13), RNA helicase activity was decreased to 50% of that of the wild type. The R1488L mutant lost its RNA helicase activity, while its NTPase activity was about half that of the wild-type protein in the absence of poly(U). Poly(U) inhibited the NTPase activity of R1448L by 50%. Mutations at the same site in eIF-4A led to a loss of RNA helicase activity, while NPH II helicase activity dropped to about 50% of that of the wild type as a result of the mutation.

When the first glycine residue in the QRRGRTGR motif of the HCV NS3 protein was changed to an alanine (G1489A), the RNA helicase activity was not affected. In contrast, the R1490A mutant showed a complete loss of RNA helicase activity. These results are not surprising, as the R1490A mutant completely lost its NTPase activity with addition of poly(U). Mutation of the threonine in the QRRGRTGR motif (T1491N) was the most tolerable mutation in the HCV NS3 protein; the RNA helicase activity of this mutant was almost same as that of the wild type. The RNA helicase activity of the G1492A mutant was decreased to one-third of that of wild type. The arginine at amino acid 1493 appears to be very important for enzyme activity of the HCV RNA helicase. Mutation at this site completely destroyed both the NTPase and RNA helicase activities.

The RNA binding activities of the wild-type and mutated proteins were measured by gel mobility shift assay (Fig. 3). Most of the mutations did not yield a decrease in RNA binding activity, but the R1488L mutant exhibited about half of the RNA binding activity of the wild-type protein. Moreover, the H1318A and Q1486H mutants showed more than 1.5-fold-higher RNA binding activity than the wild type. From these results, we suggest that amino acid residues other than those in the four conserved motifs also play an important role in RNA binding of the NS3 protein. The ATPase, RNA binding, and RNA helicase activities of each mutated protein compared to those of the wild-type protein are summarized in Table 4.

DISCUSSION

This study presents a mutational analysis of the four conserved sequence motifs of the HCV NS3 protein, an RNA helicase. The biochemical activity of each mutated protein was determined and compared with that of the wild-type HCV

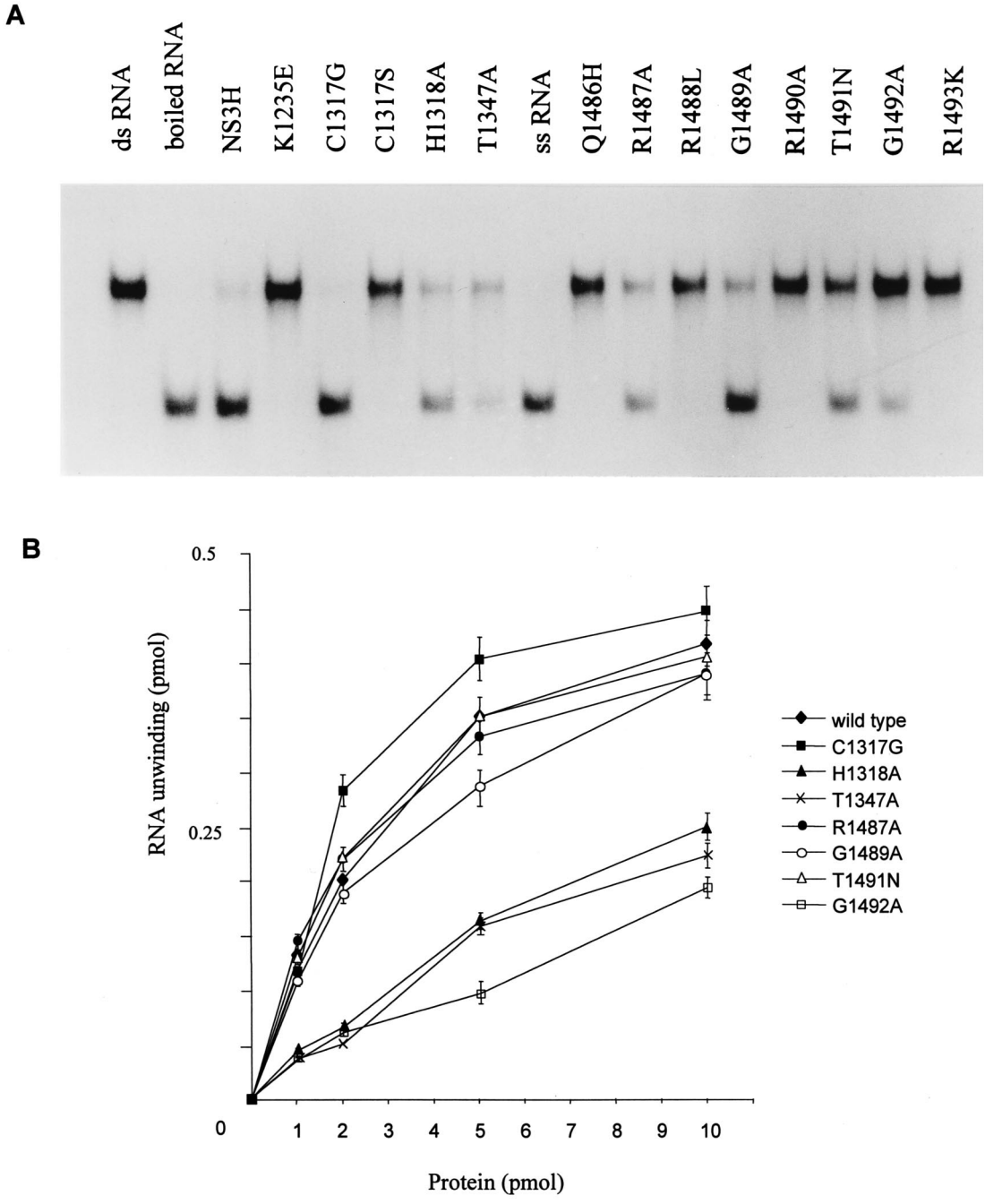

FIG. 2. RNA helicase activities of wild-type HCV NS3 protein and the mutated forms of NS3H. (A) Example of the assay of RNA helicase activity of each point mutant. Ten picomoles of each protein was used. Boiled RNA, dsRNA boiled for 3 min as a positive control; ssRNA: single-stranded RNA in vitro transcribed from *Pvu*II-cleaved pGEM 1. (B) Quantitation of RNA helicase activities of the mutated proteins. The assay was performed as described in Materials and Methods. The amounts of RNA released were plotted as a function of the amount of protein. Each point represents the average and standard error from two experiments; the standard error was less than 10%. Mutant proteins that lost the RNA helicase activity are not represented.

RNA helicase. There have been two reported studies of RNA helicases mutated at the conserved sequence motifs of the DEAD box family of RNA helicases. One is a study conducted with the vaccinia virus NPH II protein, which is a DEXH box family member, and the other report involves the eIF-4A protein of mouse, which is a member of the DEAD box family (12, 13, 32, 33). Several mutated forms of the HCV RNA helicase showed features similar to those of related mutated forms of eIF-4A and NPH II, while others displayed characteristics that were different from those of these proteins. Suzich et al. (42) and Preugschat et al. (35) reported kinetic analyses of the NTPase activity of the NS3 protein. They showed different results, which seemed to have arisen from differences in assay conditions, such as the expression system, the kind of RNA cofactor, and the pH.

For the eIF-4A protein, Pause and Sonenberg (32) replaced the lysine residue of the GKT motif with glutamine. The mutated protein exhibited severely reduced ATP binding activity

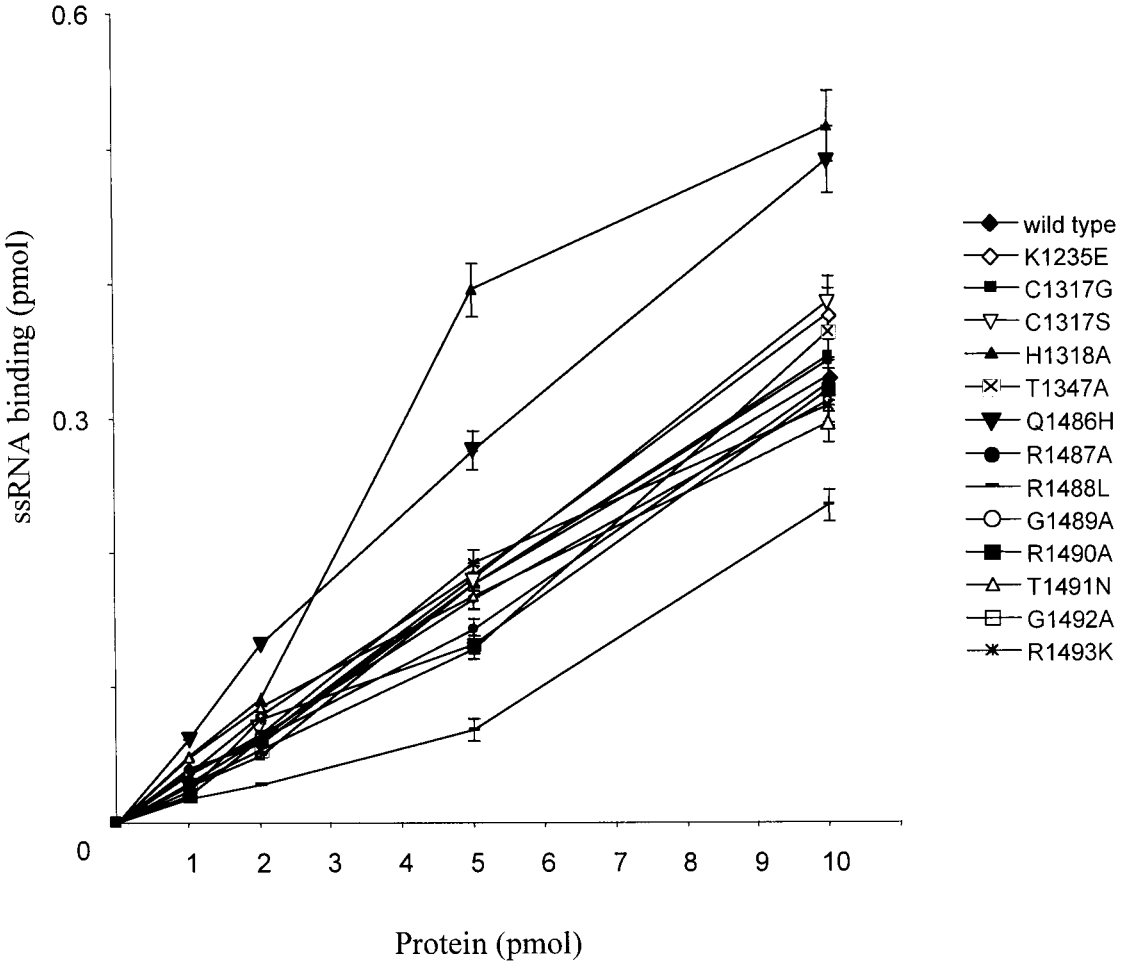

FIG. 3. RNA binding activities of the mutated forms of the NS3H protein. Each point represents the average and standard error from two independent determinants, which varied by less than 10%. The amount of bound RNA is plotted as a function of the protein. ssRNA, single-stranded RNA.

and completely lost ATP hydrolysis and RNA helicase activities. They suggested that because the positively charged lysine was replaced by glutamine, the mutated protein could not interact with the β and γ phosphates of ATP. Gross and Shuman (12) reported that an alanine substitution at the lysine residue of the GKT motif of NPH II decreased its NTPase activity to 1/20 of that of the wild-type protein in the presence of RNA or DNA cofactors. However, this particular mutated protein possessed RNA helicase activity that was one-fourth to one-fifth of that of the wild-type enzyme. The difference in the mutational effects at the same site in eIF-4A and NPH II was a result of the nature of each protein and the sensitivity of the specific experiments. Mutation at the same site of the HCV NS3 protein showed an effect different from those of mutation of eIF-4A and NPH II. We mutated the positively charged lysine at amino acid 1235 of the GKS motif to a negatively charged glutamic acid. The K1235E mutant showed less than 5% of the NTPase activity of the wild-type protein in the absence of poly(U) and about 50% of the NTPase activity of the wild-type protein in the presence of poly(U). The unique feature of the K1235E mutant is that the NTPase activity was stimulated to 13-fold by poly(U). In addition, although the NTPase activity of the K1235E mutant was quite high in the presence of poly(U), the lysine substitution completely destroyed the RNA helicase activity.

The DEXH motif of RNA helicases is also known as the ATP binding B motif, and experiments have shown that any residue may occupy the X position. The BVBD NS3 protein has a tyrosine residue at the X site (34), while the plum pox virus CI protein has a cysteine (31) and *E. coli* UvrB has serine (1). In this study, the cysteine of the DECH motif in the HCV NS3 protein was changed to either glycine or serine. The C1317G mutant had an NTPase activity that was weaker than that of the wild-type protein, while its RNA helicase activity was nearly identical to that of the wild-type protein. However, the C1317S mutant completely lost its enzymatic activities. These results suggest that various factors affect the enzymatic activities of NS3H, including the charge of the amino acid side chains of the conserved motifs and the interactions of these side chains with other amino acid residues.

Mutation of the histidine within the DECH motif also yielded results that differed from those of studies of the eIF-4A and NPH II proteins. The eIF-4A protein has glutamic acid at this site (DEAD). When this residue was changed to histidine, the mutant showed nearly threefold elevation in NTPase activity and the RNA helicase activity was reduced to 10% of that of the wild type. Vaccinia virus NPH II has a histidine residue at this site (DEVH). An alanine substitution yielded a variant of NPH II with an NTPase activity that, regardless of the presence or absence of poly(U), was equivalent to the poly(U)-

TABLE 4. Effects of mutations at four conserved sequence motifs of the HCV NS3 RNA helicase

NS3H protein	Motif sequence[a]	Activity[b] (%)			
		ATPase		RNA binding	RNA helicase
		Without poly(U)	With poly(U)		
Wild type	AXXXXGKS	23	100	100	100
K1235E	AXXXXGES	3	39	114	0
Wild type	DECH	23	100	100	100
C1317G	DEGH	26	20	104	107
C1317S	DESH	0	0	117	0
H1318A	DECA	210	32	157	60
Wild type	TAT	23	100	100	100
T1347A	AAT	20	21	110	53
Wild type	QRRGRTGR	23	100	100	100
Q1486H	HRRGRTGR	0	0	149	0
R1487A	QARGRTGR	27	42	98	93
R1488L	QRLGRTGR	12	6	61	10
G1489A	QRRARTGR	7	25	95	93
R1490A	QRRGATGR	10	0	97	0
T1491N	QRRGRNGR	10	32	90	97
G1492A	QRRGRTAR	7	16	110	46
R1493K	QRRGRTGK	0	0	95	0

[a] Mutated residues are underlined.
[b] The enzymatic activity of 10 pmol of wild-type NS3H [with poly(U) for ATPase activity] was taken as 100%.

stimulated NTPase activity of the wild-type protein. The RNA helicase activity of this mutated protein was reduced to less than 10% of that of the wild type. For the HCV NS3 protein, the H1318A mutant had a very high NTPase activity in the absence of poly(U), and its NTPase activity was inhibited by the addition of poly(U) to the reaction mixture. The H1318A mutant displayed an RNA helicase activity that was about 60% of that of the wild type. Recently, Yao et al. (54) reported the crystal structure of the HCV RNA helicase domain. They showed that His1318 is involved in a network of H bonds between GKT and DEXH motifs. The RNA helicase activity of NPH II was abolished by alanine substitution of the His residue in DEVH (13). However, the RNA helicase activity of the H1318A mutant was not completely abolished in our study. This may imply a difference in the nature of the two proteins. Another possibility is a mutation at an unwanted position in the H118A mutant, because we sequenced only 300 nucleotides around the mutated position.

Pause and Sonenberg (32) reported that the SAT motif in eIF-4A played a crucial role in the RNA-unwinding reaction. HCV NS3 has a TAT motif, as do other DEXH RNA helicases. When the first threonine of the TAT motif was changed to alanine (T1347A), the NTPase activity without poly(U) was almost equivalent to that of the wild-type protein, but this activity was not stimulated by poly(U). The RNA helicase activity was about 50% of that of the wild type. Therefore, the TAT motif in the HCV NS3 protein may not be as crucial as is the SAT domain for RNA unwinding by the eIF-4A protein.

Replacement of histidine with glutamine in the HRIGRG GR motif of eIF-4A decreased its NTPase activity and abolished its RNA helicase activity. The replacement of glutamine with alanine in the QRKGRVGR motif of NPH II caused a moderate decrease in NTPase activity and a dramatic decrease in RNA helicase activity of the mutated protein. For the HCV NS3 protein, a glutamine-to-histidine substitution in the QRR GRTGR motif severely affected the enzymatic activities. The NTPase and RNA helicase activities were completely abolished, while the RNA binding activity was increased. These results imply that glutamine 1486 of the HCV NS3 RNA helicase is indispensable for enzymatic activity.

RNA binding activity was completely abolished in all eIF-4A variants mutated within the HRIGRGGR motif. However, the RNA binding activity of NPH II was unaffected by mutations in the QRKGRVGR motif, except for replacement of the first arginine with alanine. Gross and Shuman (13) suggested that the different effects on RNA binding activity of the two proteins were caused by the natures of the two proteins and the difference in the experimental methods employed for measuring RNA binding. eIF-4A has a dissociation constant for RNA of $>10^{-6}$, while that of NPH II is $<10^{-9}$; thus, NPH II can form a much more stable complex with RNA than can eIF-4A. Pause and Sonenberg (33) used a UV cross-linking method to measure RNA binding activity by eIF-4A, while Gross and Shuman used an electrophoretic gel mobility shift assay to assess RNA binding by NPH II. In this study, we measured RNA binding activity by a gel shift mobility assay, and our results show that HCV NS3 resembles NPH II in its RNA binding. To further understand the nature of NS3 protein, it would be useful to test the RNA binding characteristics of each mutated protein by the spectrofluorometric method, as was done by Preugschat et al. (35). Kanai et al. (20) and Kim et al. (23) constructed several truncated forms of the HCV NS3 protein and showed that all of the mutated proteins bound to RNA. Therefore, the RNA binding domain of the HCV NS3 protein seems to be dispersed over the protein. Yao et al. (54) suggested that Lys1386, Arg1369, Lys1397, Lys1398, and Lys1399 play a role in binding to RNA. Mutational analysis of those positions should be performed.

The R1487A substitution in the QRRGRTGR motif had little effect on the enzymatic activities of the NS3 protein. However, when the arginine at amino acid 1488 was replaced with leucine, the NTPase activity was reduced to less than 10% of that of the wild type, the RNA binding activity was reduced about twofold, and the RNA helicase activity was almost abolished. Yao et al. (54) showed that Arg1487, Arg1490, and Arg1493 are located within a 7-Å interval, so they can bind to the phosphates of the RNA backbone. From our results, we suggest that Arg1487 is not a very important residue for the enzymatic activity of the NS3 protein and that Arg1488 is important to maintain the conformation of the protein and can complement the function of Arg1487 in other RNA helicases. Glycine at position 1489 and threonine at position 1491 appear to be less important for the enzymatic activity of NS3. In fact, these positions are less conserved among members of this RNA helicase family. In contrast, arginine at position 1490 is important for the enzymatic activity of the HCV NS3 protein. Substitution of alanine in R1490A mutant abolished NTPase activity in the presence of poly(U), while the mutated protein showed little activity in the absence of poly(U), and the RNA helicase activity was also abolished. For eIF-4A and NPH II, the conserved arginine residues in the HRIGRGGR and QRK GRVGR motifs were very important for enzymatic activity. A glycine at position 1492 is less important for RNA helicase activity than for NTPase activity, as this substitution has a mild effect on RNA helicase activity (reduced to 46%), while the NTPase activity was reduced to less than 10% of that of the wild type. An arginine at position 1493 is crucial for both NTPase and RNA helicase activities, as replacement by lysine completely abolished both enzymatic activities. These results suggest that the strictly conserved amino acid positions within

the sequence motifs are more important for appropriate enzymatic activity than are the less conserved positions.

Much remains to be deciphered regarding the functions of the HCV NS3 protein. The simian virus 40 T antigen, which is known to be an ATP-dependent DNA helicase (36), also unwinds RNA (39). A DNA helicase activity for the HCV NS3 protein has been reported recently (43). This finding implies that there may be a common mechanism for nucleic acid unwinding as well as a common sequence motif within the active sites of DNA and RNA helicases. Studies of the nature of NS3 protein function may offer insight into the nature of DEAD and DEXH box family helicases.

Recent work from several laboratories has revealed that the HCV NS4A protein is a cofactor for the NS3 protease, because it is absolutely required for cleavage at the NS4B/5A junction by the NS3 protease (9, 46). A similar requirement for a cofactor has been reported for pestivirus proteases (52). The domain for physical interaction between the HCV NS3 and NS4A proteins has been mapped to the NH_2-terminal 22 amino acids of the NS3 protein and to the central region of NS4A (46). It has been postulated that the NS4A protein stabilizes the active conformation of the NS3 protease domain and recruits the NS3 protease to the membrane, where proteolytic processing may take place. The RNA helicase activity of the HCV NS3 protein may also be affected by interaction with HCV NS4A. This possibility remains to be tested, as expression of the full-length NS3 protein has not yet been accomplished.

Replication intermediates of the HCV RNA are believed to be present as duplex RNA consisting of positive- and negative-strand RNA molecules (56). O'Reilly et al. (29) showed that the helicase-like and polymerase-like proteins of brome mosaic virus interact with each other. Therefore, the possible interaction and its effect on the functions of the RNA helicase and the RNA polymerase of HCV should be studied.

It was instructive to compare the mutagenesis results for HCV RNA helicase with those for two previously studied RNA helicases, eIF-4A and NPH II, as these three enzymes have common sequence motifs and similar activities. Although our results show evidence of the roles of individual amino acid residues in the overall function of the HCV NS3 RNA helicase, these data cannot be applied to other RNA helicases. Many RNA helicases remain to be investigated; some of them have been shown to have the helicase activity, and others are putative RNA helicases. The nature of the RNA helicase will be understood by studying their characteristics. This report could provide a useful tool for these studies.

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