# A Point Mutation Abolishes the Helicase but Not the Nucleoside Triphosphatase Activity of Hepatitis C Virus NS3 Protein

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**The NS3 protein of hepatitis C virus contains a bipartite structure consisting of an N-terminal serine protease and a C-terminal DEAD box helicase. We show that the C-terminal domain has ATPase and panhelicase activities. The integrity of the helicase function is dependent on the conserved DEAD motif and can be abolished by a His-Ala point mutation, leaving a fully functional nucleoside triphosphatase.**

The RNA genome of hepatitis C virus (HCV) encodes a single polyprotein of 3,010 to 3,033 amino acids (1, 2, 9, 16) that is processed into several structural (C, E1, and E2/NS1) and nonstructural (NS2 to NS5) proteins by cellular, as well as viral, proteases (2, 4, 7, 16). The NS3 protein (72 kDa) of HCV harbors multiple enzymatic activities in its two-domain structure. A serine protease with a chymotrypsin-like fold is located in the N-terminal 190 amino acids of NS3 (11, 13), while the C terminus of NS3 shows the conserved motifs found in the DEAD box nucleoside triphosphatase (NTPase)-helicase family (2, 3). In recent publications, both NTPase activity and RNA helicase activity have been demonstrated for HCV NS3 (6, 8, 10, 14, 15). Comparison of the published data, however, revealed differing characteristics of the helicase activity in various protein preparations (6, 8, 10, 15). Although these analyses were not quantitative, the specific activity of the NS3 helicase appeared to vary by over 1,000-fold. Moreover, the lack of any stringent controls makes it difficult to assess the potential contribution from contaminating helicase activities derived from the host cells. In this report, we clarify and extend the characterization of the NS3 helicase activity. We describe the activity of both full-length NS3 and the isolated helicase domain. In addition to the wild-type protein, we analyzed the effects of point mutations in the conserved nucleotide binding and DExH box motifs.

**Expression of HCV helicase.** To characterize the helicase activity of HCV NS3, we subcloned the C-terminal domain of the NS3 protein, containing the conserved helicase motifs (3), into an *Escherichia coli* T7 polymerase overexpression system. The construct pRSETNS3 (Fig. 1A) spans 423 amino acids from V1193 to K1615 of the HCV NS3 gene with an additional N-terminal 31 amino acids derived from the cloning vector, containing a six-His tag. In addition to the wild-type protein, we constructed pRSETDECA, with a His-to-Ala mutation in the DECH motif, and pRSETGQS and pRSETGAS, where GQS and GAS replace GKS in the nucleotide binding motif. These mutant clones were used to analyze the functional interaction between the helicase and ATPase activities and as controls in the helicase and NTPase assays. The DECH-to-DECA mutation has been shown to uncouple helicase from the NTPase activity in vaccinia virus nucleoside triphosphate phosphohydrolase II (NPHII) (5), whereas mutations in the

conserved GKS motif have been shown to abolish nucleotide binding of the yeast Upf1 protein (18).

All of the expression plasmids produced a protein of 54 kDa when transformed into an *E. coli* BL21 *lysS* mutant and induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Ni-agarose affinity chromatography was used to purify the recombinant polypeptides. The purity of the correct product was approximately 80% of the yield after the Ni column purifica-
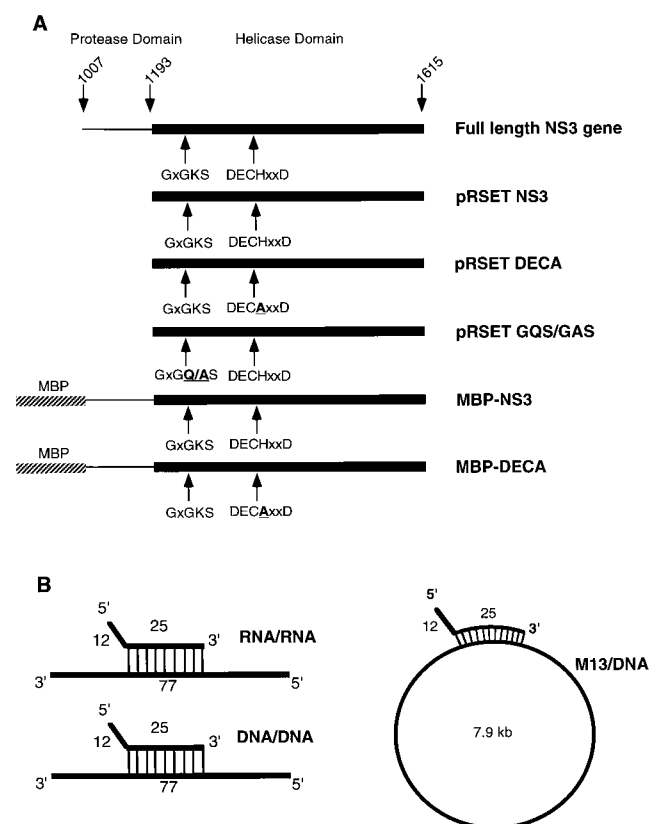


FIG. 1. (A) Schematic representation of the constructs used to express HCV (strain H) NS3 in *E. coli*. Constructs in plasmid pRSETA (Invitrogen) contain the helicase domain from amino acid 1193 to amino acid 1615. GxGKS and DECHxxD mark the conserved motifs required for helicase and NTPase activities. pRSETDECA and pRSETGQS/GAS contain mutations in these motifs, as indicated. Also shown is the MBP fusion construct for expression of full-length NS3 and mutant MBP-DECA. (B) Schematic representation of the helicase substrates. Lengths of single- and double-stranded regions are in nucleotides.

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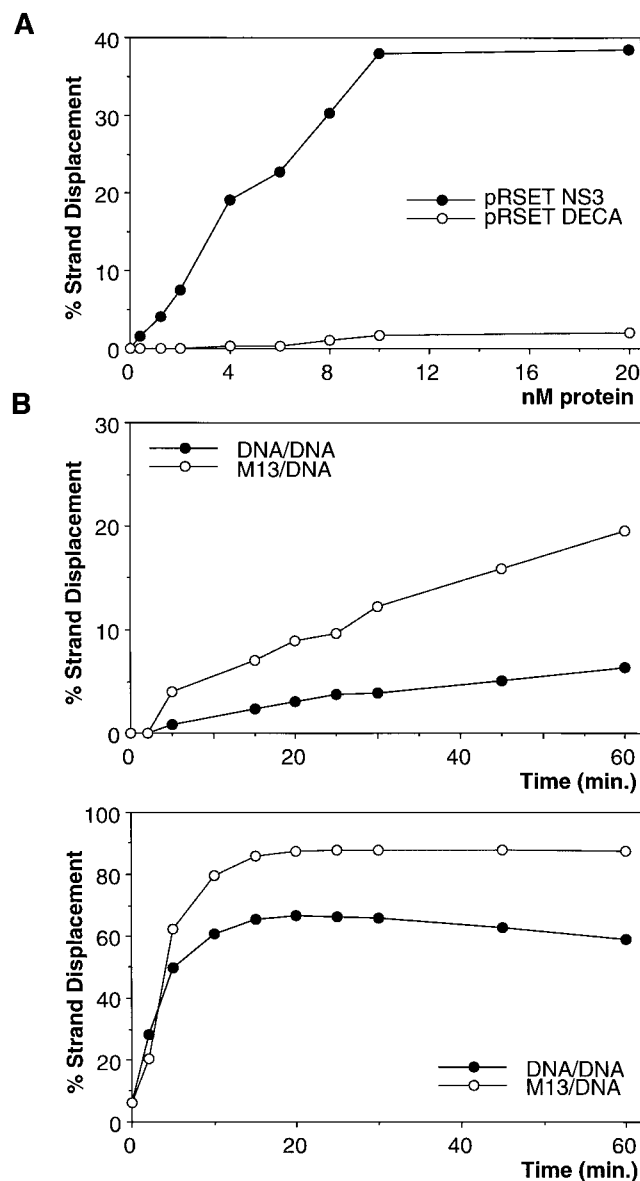
FIG. 2. (A) RNA helicase activity of HCV NS3. Shown are dose-response curves for the NS3 wild-type and DECA mutant helicase domains. The reaction mixtures consisted of 25 mM K-morpholinepropanesulfonic acid (MOPS; pH 6.8), 100-µg/ml bovine serum albumin, 2 mM dithiothreitol, 3 mM $MgCl_2$, 5 mM ATP, 5 U of RNasin (Promega), and 10 pM substrate in a final volume of 20 µl. The reaction was carried out at 37°C for 30 min and stopped by addition of 4 µl of dye (17). Mixtures were electrophoresed on 15% polyacrylamide (acrylamide-bisacrylamide ratio, 30:1)–0.5× Tris-borate-EDTA–0.1% sodium dodecyl sulfate gels at 15 mA of constant current for 2 h, and the gels were dried and exposed to PhosphorImager screens. Helicase activity was quantified by using a Fujix BAS 1000 PhosphorImager with MacBas 1.01 software. (B) Comparison of template strand lengths by using M13 or a DNA 77-mer. Shown are time courses with either 1 nM (top) or 8 nM (bottom) pRSETNS3 protein under standard helicase conditions. The substrate concentration was 50 pM.

tion, and each preparation yielded between 8 and 20 mg/liter (data not shown).

To compare the activity of the helicase domain with that of full-length NS3, we attempted to overexpress and purify the entire protein in a number of systems. The protein was found to be largely insoluble, except as a maltose binding protein (MBP)-His fusion overexpressed by using the T7 system in *E. coli*. Purification involved affinity chromatography of the bac-

terial lysate on amylose resin followed by Ni resin. The final eluate was about 80% pure, and the yield averaged 0.5 mg/liter of induced cells.

**Activity of HCV helicase.** The initial evaluation of the helicase activity of HCV NS3 was performed with a double-stranded RNA (dsRNA) substrate (RNA/RNA in Fig. 1B). The substrate was incubated with the helicase domain of NS3 under conditions previously reported to support helicase activity with the homologous protein from the related pestivirus bovine viral diarrhea virus (17). Titration of wild-type NS3 displayed nearly linear helicase activity up to 10 nM protein, where it reached a plateau at roughly 40% strand displacement. The DECA point mutation showed no helicase activity over this range of protein input, demonstrating that the DECA mutant shows the same inactive phenotype in the context of the NS3 helicase as it does in vaccinia virus NPHII (5) (Fig. 2A).

Variation of reaction parameters, such as pH, divalent metal concentration, and ATP concentration, did not result in any significant improvement of the helicase activity, indicating that the optimal reaction conditions for bovine viral diarrhea virus and HCV helicases are similar (data not shown). HCV NS3 helicase had been reported to function on both RNA/DNA (6) and DNA/DNA (15) templates. We compared the activity of dsRNA with two dsDNA substrates (shown in Fig. 1B). We found a dsDNA substrate consisting of M13 and an annealed oligonucleotide (M13/DNA) to be the optimal substrate in our helicase assays. The displacement of the oligonucleotide from the M13 template proceeded essentially to completion, whereas the DNA/DNA and RNA/RNA templates plateaued at around 60 and 40% displacement, respectively. Moreover, the rate of displacement for the M13/DNA template was higher than for the DNA/DNA and RNA/RNA substrates. This superiority of an M13/DNA template over a DNA/DNA template has been observed for a number of DNA helicases (e.g., *E. coli* DnaB and herpes simplex virus helicase [unpublished data]) and may result from an increase in the local concentration of protein provided by M13 DNA or the increase in the number of binding sites per template.

To assess whether the protease domain might influence the helicase reaction, we also expressed the full-length NS3 protein as an MBP fusion in *E. coli*. Comparison of full-length NS3 and the isolated helicase domain showed that the two enzyme preparations had similar specific activities on an M13/DNA template (Fig. 3), indicating that the N-terminal protease domain
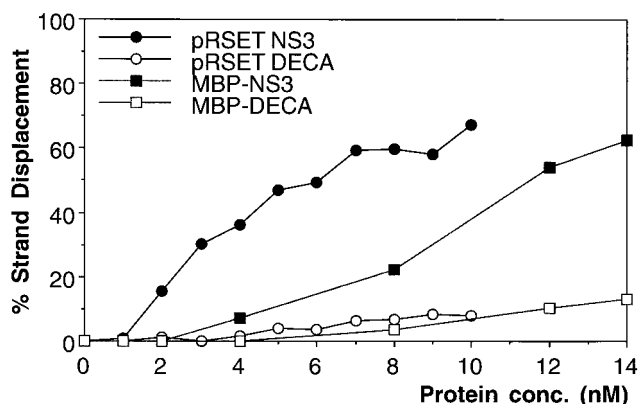


FIG. 3. Helicase activity of the MBP-NS3 full-length fusion compared to that of the helicase domain. Shown are results obtained with the wild-type and DECA mutant forms of each expressed protein. The assay was carried out with 50 pM M13/DNA substrate in 3 mM $MgCl_2$–5 mM ATP–2 mM dithiothreitol–100-µg/ml bovine serum albumin–25 mM MOPS buffer at pH 6.8 for 30 min at 37°C.
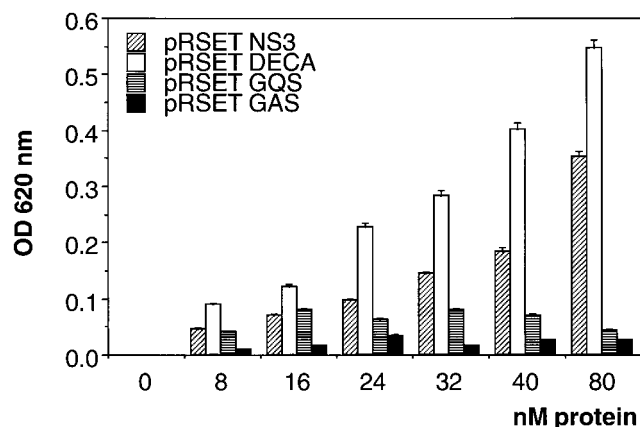
FIG. 4. Comparison of ATPase activities in wild-type and mutant forms of the helicase domain of HCV NS3. Standard ATPase assays were conducted in a total volume of 50 μl containing 20 mM K-HEPES (pH 7.4), 3 mM MgCl$_2$, 2.5 mM ATP, 0.5 μg of single-stranded M13 DNA, 2 mM dithiothreitol, and 100-μg/ml bovine serum albumin incubated at 37°C for 30 min. A dye mixture consisting of 9 parts acid molybdate solution (Sigma) and 1 part 0.1125% malachite green solution (in water) was prepared. The dye mixture was added to the reaction mixture, and the colorimetric assay was allowed to develop for at least 30 min at room temperature. Color development was stopped with 100 μl of 34% sodium citrate. Represented in units of optical density at 620 nm are the relative ATPase activities of the different polypeptides assayed by using increasing total protein amounts. All points are averages of duplicate measurements, and the variation of the data (observed variation, ±5%) is indicated by error bars.

has little, if any, effect on helicase activity. Both the full-length and the helicase domain DECA mutant showed very little helicase activity.

All recombinant proteins were tested for ATPase activity by use of a colorimetric assay (12). The DECA mutant had the same enzymatic activity as the wild-type protein, indicating that this mutant distinguishes between the ATPase and helicase activities. Similar observations have been reported for the NPHII protein of vaccinia virus (5). The Lys-Ala change in the GKS motif completely abolished ATPase activity, and the Lys-Gln change resulted in little residual activity (Fig. 4). As expected, these ATPase mutants lacked detectable helicase activity (Table 1).

The helicase and NTPase activities of NS3 appeared to be separable enzymatic functions connected by the intact DExH motif. A single amino acid change in DExA completely abolished NS3 helicase activity yet did not affect NTPase function. NTPase function was eliminated by a single amino acid change in the conserved nucleotide binding motif (GKS to GAS or

GQS) with a concomitant inactivation of helicase function. It has been postulated that the NS3 protein is absolutely required for HCV replication. Recombination of these point mutations into the viral genome will allow the relative importance of the different enzymatic functions of NS3 to be assessed in the context of viral replication. Moreover, such studies may reveal the precise role of NS3 helicase activity in the viral life cycle.

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TABLE 1. Comparison of wild-type and mutant NS3 proteins[a]

| Polypeptide | % Strand displacement |
| --- | --- |
| None | 0 |
| pRSETNS3 | 84.6 |
| pRSETDECA | 2.1 |
| pRSETGAS | 1.2 |
| pRSETGQS | 0.1 |

[a] Helicase assays were carried out with 8 nM protein and 50 pM M13/DNA substrate in 3 mM MgCl$_2$–5 mM ATP–2 mM dithiothreitol–100-μg/ml bovine serum albumin–25 mM MOPS buffer at pH 6.8 for 30 min at 37°C.