HCV NS5B is an RNA-dependent RNA polymerase (RdRP), a central catalytic enzyme for HCV replication, which has the “palm and fingers” substructure. We recently identified five novel residues critical for RdRP activity (Qin, W., Yamashita, T., Shirota, Y., Lin, Y., Wei, W., and Murakami, S. (2001) Hepatology 33, 728–737). Among them, GLU-18 and His-502, far from the catalytic center, may be involved in conformational change(s) for RdRP activity as addressed in some palmitic and fingers enzymes. We examined the possibility that NS5B is oligomerized, and we could detect the interaction between two different tagged NS5B proteins in vitro and transiently expressed in mammalian cells. By scanning 27 clustered and then point alanine substitutions in vitro and in vivo, Glu-18 and His-502 were found to be essential for the homomeric interaction in vivo and in vitro, strongly suggesting a close relationship between oligomerization and RdRP activity of NS5B. The two residues, Glu-18 and His-502, with substitutions at these two positions did not exhibit RdRP activity, but a mixture of the two wild-type NS5B proteins did. These results clearly indicate that Glu-18 and His-502 are required for the oligomerization and RdRP activity.

Hepatitis C Virus (HCV) is the causative agent of parenterally transmitted hepatitis type B. HCV infection frequently leads to chronic hepatitis, liver cirrhosis, and eventually hepatocellular carcinoma (4, 5). In the case of HCV-associated hepatocellular carcinoma, there is often prolonged active inflammation manifested by high alanine aminotransferase level generally associated with high virus load. Therefore, eradication of replication of HCV would be expected to reduce or even prevent incidence of hepatocellular carcinoma. HCV has a positive-sense single-stranded RNA genome of approximately 9.6 kb, which contains a large open reading frame encoding a polyprotein of ∼3,000 amino acid residues and two highly conserved untranslated regions flanking the 5' and 3' ends of the genome. The viral encoded polypeptide precursor is cotranslationally or posttranslationally processed by host and viral proteases into at least 10 different products: NH2-E1-E3-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (6 and the references therein). The non-structural proteins NS2-5B are thought to be required for viral genome replication and the non-structural protein NS5A is an RNA-dependent RNA polymerase (RdRP), a core enzyme for HCV replication. RdRPs are unique nucleic acid-dependent nucleic acid polymerases having the palm and fingers substructure. Oligomerized forms of poliovirus 3D (18–20) and hepatitis C virus RNA polymerase (RdRP) were found in the long loop, and His-502 on the outer surface of the thick thumb. The latter two seem to be far from the pocket for catalytic activity (12). We suspected that such a defect in RdRP activity by introducing one or several amino acid substitutions leads to a flattened spherical shape as revealed by the crystal models (15–17). Recently we identified five novel residues located outside of the conserved motifs as critical for RdRP activity by two-step scanning for alanine, clustering, and then point mutation of alanine. These include Tyr-191 near the catalytic center, Cys-274 and Tyr-276 at the fingertips, Glu-18 in the long loop, and His-502 on the outer surface of the thick thumb. The latter two seem to be far from the pocket for catalytic activity (12). We suspected that such a defect in RdRP activity suggests some conformational changes in NS5B. The oligomerization of several enzymes has been reported among the nucleic acid polymerase family with the palm and fingers substructure. Oligomerized forms of poliovirus 3D (18–20) and the heterodimer of HIV reverse transcriptase (RT) (21, 22) have been studied extensively and demonstrated to be critical for catalytic activity. A certain conformation seems to be prerequisite to the catalytic activity of these enzymes described as closed or open and inactive or active forms.

Here we report that HCV NS5B has an intrinsic ability to oligomerize or dimerize, and that two residues, Glu-18 and His-502, are critical for the oligomerization, which is prerequisite for RdRP activity. The two exposed residues might be directly involved in the interaction, and the enzyme activity since these residues are exchangeable in a strict sense.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Mutagenesis**—The bacterial expression vectors pGENKS, pYFLAG, and pLHis and the mammalian expression vectors pNKFLAG and pNKGST were reported previously (23–25). An alanine-scanning method was applied to construct NS5B mutations to minimize the effects of substituted amino acid residues by site-directed mutagen-
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![Diagram of HCV NS5B](image)

**Fig. 1. Two residues of HCV NS5B critical for RdRP activity (12) and oligomerization.** The two residues, Glu-18 (E18) and His-502 (H502), are shown (red) in secondary structures according to a crystal model of HCV NS5B (Protein Data Bank accession code 1QUV) reported by Ago et al. (17). Glu-18 and His-502 are located at the long loop and helix T of NS5B (15–17), respectively, which are exposed toward the back side of the right-hand model. Graphics were processed by Cinema 3D version 3.0.

The positions of clustered and point alanine substitutions of HCV NS5B are shown as reported previously (12, 26). The positions of clustered alanine substitutions of HCV NS5B are shown as reported previously (12).

**Expression and Purification of Bacterial HCV NS5B:—**GST-fused NS5B mutant proteins were expressed and purified. The GST-NS5Bt protein was expressed as described by Pong et al. (18) and then incubated with 0.2 μg of Flag-tagged NS5B in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1% Triton X-100, and 10% glycerol. After washing with phosphate-buffered saline containing 1% Triton X-100 for 4 h on a rotator at room temperature. Alternatively, the GST-NS5B and FLAG-NS5B lysates were combined and incubated for 16 h at 4°C and then applied to glutathione-Sepharose 4B resin. After a wash with phosphate-buffered saline containing 1% Triton X-100, the bound proteins were solubilized with SDS loading buffer, fractionated by SDS-10% PAGE, and subjected to Western blot analysis with anti-NS5B IgG antibody.

**Glycerol Gradient and Molecular Sieving Analyses of HCV NS5B—**Two micrograms of purified His-NS5Bt protein in gradient buffer (50 mM Tri-HCl, pH 8.0, 50 mM NaCl, 0.1% Triton X-100, and 10% glycerol) was loaded onto Superose 6 column (PC 3.2/30). Fractionation was carried out using SMART system (Amersham Biosciences, Inc.) at a flow rate 30 cm2/min at 4°C, and the peaks were detected by absorption at 280 nm and SDS-PAGE analysis. The molecular marker proteins (each 200 μg) were loaded onto the manufacturer of 200–500 cm2 were confirmed with transiently coexpressed wild type and FLAG-NS5Bt-bound resin. These results in vitro and in vivo indicate that HCV NS5B is capable of oligomerization or homomeric interaction.

Next we confirmed the homomeric interaction of HCV NS5B in mammalian cells. The mammalian expression vectors pRKGST-5Bt and pNKFLAG-5B were transiently transfected into COS-1 cells, and the proteins were overexpressed. The amounts of protein were adjusted to similar levels as detected by anti-FLAG-M2 and anti-GST antibody (Fig. 2b, upper and bottom). The supernatants of lysates were incubated with GST resin and subjected to pull-down assay and Western blotting using anti-FLAG M2 antibody (Fig. 2b, middle). The homomeric interaction of the N5Bt proteins was observed in COS-1 cells since FLAG-NS5Bt protein was efficiently recovered by GST-NS5Bt, indicating a specific homomeric interaction.

**Delineation of the Homomeric Interaction Residues in HCV NS5B—**A library of the clustered and point substitution mutants of HCV NS5B was used to determine the residues of HCV NS5B important for interaction with itself. The library covers one-third of all residues and two-thirds of aromatic and charged amino acid residues, which are outside of the conserved motif but conserved among HCV-isolated clones (6). All but cm20 covering amino acids 17–23 and cm2 covering amino acids 500–506 of GST-NS5Bt proteins exhibited the ability to recover FLAG-NS5Bt in vitro (Fig. 3a). The defect of cm20 and cm2 was confirmed with transiently coexpressed wild type FLAG-NS5Bt and mutant versions of GST-NS5B in mammalian cells. Point alanine substitutions covering the sequences of
cm20 and cm2 were examined for oligomerization in vitro and in vivo. All mutations with the exception of E18A and H502A exhibited the ability to bind wild type NS5B in vitro and wild type NS5B in vivo (Fig. 3, a and b, data not shown). These results definitely demonstrate that Glu-18 and His-502, indispensable for the RdRP activity as reported previously (6), are the only residues critical for the oligomerization.

**Amino Acids Essential for the Homomeric Interaction of HCV NS5B Are Residual-specific and Exchangeable**—To further evaluate the close relationship between the oligomerization and RdRP activity of NS5B, we introduced several substitutions at residues Glu-18 and His-502 and examined their effect on oligomerization in vitro and in vitro and RdRP activity in vitro. All the substitution mutants of NS5B including those in which the residue was replaced with a similarly charged group (E18D, H502R, and H502K) failed to exhibit RdRP activity (Table I) and bind the wild type FLAG-NS5Bt in vitro and FLAG-NS5B in vivo (Fig. 4, a and b). Therefore, the oligomerization of NS5B requires glutamic acid at amino acid 18 and histidine at amino acid 502. The electrostatic force alone cannot explain such a residue-specific result.

Whether or not the oligomerization stringently requires the interaction of two residues, it may be possible to replace these residues without affecting the substructures. FLAG-NS5Bt proteins with H502E were actually pulled down by GST-NS5Bt with E18H in vitro (Fig. 4a), and the result was confirmed in vivo (Fig. 4b). In contrast, no interaction was observed between wild type NS5Bt and that with E18H or with H502E. The effect on RdRP activity of changing the two residues at amino acid 18 and amino acid 502 was examined in vitro. The activity
was recovered by 70% only when the two mutated proteins were mixed (Table I). These results clearly indicate that oligomerization of NS5B is prerequisite for RdRP activity and that Glu-18 and His-502 are critical for the homomeric interaction, which probably occurs via direct interaction of the residues, although there is no direct proof at present.

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**TABLE I**

| Proteins | UMP \(^a\) \(n = 3\) | Relative \(^b\) percentage |
|----------|-----------------|-------------------|
| WT \(^c\) | 39,200 ± 3800 | 100 |
| Nonfused NS5Bt | 36,000 ± 3200 | 91 |
| E18A | 1100 ± 100 | 2.5 |
| E18D | 800 ± 100 | 2 |
| H502A | 900 ± 110 | 2.5 |
| H502K | 1200 ± 110 | 3 |
| H502R | 900 ± 110 | 2.5 |
| H502E | 1800 ± 200 | 4 |
| E18H + H502E | 28,000 ± 1000 | 70 |
| GST | 1200 ± 200 | 5 |
| GST + WT | 32,200 ± 5900 | 100 |
| Y276A + WT | 37,480 ± 3800 | 95 |
| GST | 280 ± 40 | 0.5 |
| GST + WT | 28,000 ± 4000 | 100 |

\(^a\) GST-NS5Bt proteins were used for RdRP assay with the exception of nonfused NS5Bt and GST alone (see “Experimental Procedures”).

\(^b\) Incorporation of \(^32\)PUMP into acid precipitates (see “Experimental Procedures”).

\(^c\) The UMP incorporation by wild type was standardized as 100%.

**FIG. 4.** Amino acids essential for homomeric interaction via Glu-18 and His-502 are residual-specific and exchangeable. a, approximately 0.2 \(\mu\)g of the purified FLAG-NS5Bt protein was incubated with 1 \(\mu\)g of each GST-NS5Bt mutant protein prebound to glutathione-Sepharose 4B resin (see “Experimental Procedures”). The resin-bound proteins were washed with phosphate-buffered saline containing 1% Triton X-100, fractionated by 10% SDS-PAGE, and subjected to Western blot analysis with anti-FLAG antibody. b, COS-1 cells were transfected with mammalian expression plasmids pMRFLAG-NS5Bt and pMRGST-NS5Bt wild type or mutants with a single amino acid substitution as indicated on the top. Pull-down assay and Western blot analysis were carried out with anti-FLAG antibody and anti-GST antibody as described in Fig. 16.

**FIG. 5.** Glycerol gradient fractionation of HCV NS5B. The Histagged HCV NS5Bt was expressed in E. coli cells, solubilized in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, and 1 mM DTT and applied to a gradient of 10–35% glycerol in gradient buffer (see “Experimental Procedures”). Gradients were centrifuged with a Ti-55 rotor (Beckmann) at 20 °C and 10,800 \(\times\) g for 20 h, after which 24 fractions (each 0.24 ml) were collected from the gradient top to bottom as indicated. Fractions were separated by SDS-PAGE and detected by Coo-massie Brilliant Blue staining. After detection, protein levels were quantitated densitometrically by NIH Image 1.61 and are displayed as a percentage of the highest detected protein level across all gradient fractions for a given protein. The S values of three marker proteins (albumin, aldolase, and catalase from Amersham Biosciences, Inc.) fractionated separately are also shown. Values are means ± S.D. and are indicated.

**DISCUSSION**

We report here the oligomerization of HCV NS5B in vivo and in vitro. By scanning clustered and point alanine substitution mutants, Glu-18 and His-502 were identified as critical for the homomeric interaction. Because these two residues were also found to be critical for RdRP activity in vitro, the homomeric interaction is indispensable for RdRP activity.

According to crystal models of NS5B, Glu-18 is located in the middle of a long loop connecting the fingers and thumb, which is a unique feature among RdRPs (Fig. 1). Template RNA seems to be in a position close to the connecting part of this loop. His-502 is in helix T that pairs with helix U in the thumb subdomain. The paired helices are one component of the armadillo repeats that are unique among RdRPs and contribute to the thick thumb of HCV NS5B (12, 15–17). The relative position of helix T seems to be the part of the thumb most distal to...
the catalytic center. The two residues (Fig. 1) and are on the specific substructures of NS5B. Importantly, the combination of E18H and H502E resulted in RdRP activity when two mutant NS5B proteins were mixed. These results strongly suggest that the oligomerization occurs through the direct interaction of Glu-18 and His-502, although no direct evidence of this is available at present. The possibility still remains that another residue(s) may also be critical for the oligomerization, because our clustered alanine substitutions covered only around one-third of all residues and two-thirds of the aromatic and charged residues of NS5B. However, the oligomerization of NS5B occurs through a long loop and thick thumb, not between homologous substructures. In that sense, both the oligomerization of NS5B and polio 3D require regions located outside of the conserved motifs (18–20). Because a single mutation of the two residues completely eliminated the oligomerization, the dimerized protein seems to be the catalytically active form of NS5B. This notion is supported by the finding that dimerized NS5Bt was predominant among oligomerized forms in the fractionation profile of NS5B obtained by glycerol density gradient centrifugation. It is possible that the dimerization through Glu-18 and His-502 facilitates or affords higher ordered interactions of NS5B through interaction(s) between undefined residues, which might not be enough for NS5B to dimerize.

Substitutions of the two residues of NS5B, even with residues having same kind of the charge, disrupted the RdRP activity and binding to wild type NS5B. Furthermore, only the combination of E18H and H502E resulted in RdRP activity when two mutant NS5B proteins were mixed. These results strongly suggest that the oligomerization occurs through the direct interaction of Glu-18 and His-502, although no direct evidence of this is available at present. The possibility still remains that another residue(s) may also be critical for the oligomerization, because our clustered alanine substitutions covered only around one-third of all residues and two-thirds of the aromatic and charged residues of NS5B. However, the oligomerization of NS5B occurs through a long loop and thick thumb, not between homologous substructures. In that sense, both the oligomerization of NS5B and polio 3D require regions located outside of the conserved motifs (18–20). Because a single mutation of the two residues completely eliminated the oligomerization, the dimerized protein seems to be the catalytically active form of NS5B. This notion is supported by the finding that dimerized NS5Bt was predominant among oligomerized forms in the fractionation profile of NS5B obtained by glycerol density gradient centrifugation. It is possible that the dimerization through Glu-18 and His-502 facilitates or affords higher ordered interactions of NS5B through interaction(s) between undefined residues, which might not be enough for NS5B to dimerize.
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WITHDRAWN
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RNA: STRUCTURE METABOLISM AND CATALYSIS:
Oligomeric Interaction of Hepatitis C Virus NS5B Is Critical for Catalytic Activity of RNA-dependent RNA Polymerase

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