Direct Interaction between Nucleolin and Hepatitis C Virus NS5B*

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Hepatitis C virus (HCV) NS5B is an RNA-dependent RNA polymerase (RdRP), a central catalytic enzyme in HCV replication. While studying the subcellular localization of a NS5B mutant lacking the C-terminal membrane-anchoring domain, NS5Bt, we found that expression of the green fluorescent protein (GFP)-fused form was exclusively nucleolar. Interestingly, the distribution of endogenous nucleolin changed greatly in the cells expressing GFP-NS5B, with nucleolin colocalized with GFP-NS5B in perinuclear regions in addition to the nucleolus, suggesting that NS5B retains the ability to bind nucleolin. The interaction between nucleolin and NS5B was demonstrated by GST pull-down assay. GST pull-down assay results indicated that C-terminal region of nucleolin was important for its binding to NS5B.

Scanning clustered alanine substitution mutants library of NS5B revealed two sites on NS5B that binds nucleolin. NS5B amino acids 208–214 and 500–506 were both found to be indispensable for the nucleolin binding. We reported that the latter sequence is essential for oligomerization of NS5B, which is a prerequisite for the RdRP activity. C-terminal nucleolin inhibited the NS5B RdRP activity in a dose-dependent manner. Taken together, this indicates the binding ability of nucleolin may be involved in NS5B functions.

The hepatitis C virus (HCV) is a major cause of chronic hepatitis around the world (1–3). Chronic infection with HCV results in liver cirrhosis and often hepatocellular carcinoma (4, 5). HCV belongs to the Flaviviridae family and has a positivesense single-stranded RNA genome. The HCV RNA genome is ~9.5 kb in length and consists of a long 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 polyprotein is cleaved by host and viral proteases into three structural proteins and seven nonstructural proteins: NH2-C-E1-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (6, 7). The 5′-untranslated region of 341 nucleotides contains an internal ribosome entry site, which consists of four stem-loop structures followed by an initiation codon for the polypeptide. The 3′-untranslated region of 200–300 nucleotides includes a short variable sequence, a poly(U) region, a polypyrimidine (U/C) tract, and a highly conserved X region, and is believed to be important for HCV RNA replication as reported previously (8).

Studies on HCV replication have been a focus of attention because inhibition of HCV replication not only may have therapeutic significance for chronic hepatitis but also could reduce the incidence of or even prevent hepatocellular carcinoma. Little is known about the mechanism involved, however, because of a lack of efficient cell culture systems (3, 6), although some aspects of the replication process have been recently elucidated with HCV RNA replicon systems (9, 10). HCV NS5B, located at the most C terminus of the polyprotein, is an RNA-dependent RNA polymerase (RdRP), the central catalytic enzyme of HCV replication, as was demonstrated with the recombinant forms of NS5B expressed and purified from insect cells and Escherichia coli (6, 11–14). Crystal models of NS5B clearly confirm that it belongs to the large family of nucleic acid-dependent nucleic acid polymerases sharing the fingers and thumb subdomain structure with conserved motifs (15–17). NS5B also has several unique properties including two loops connecting fingers and a thick thumb conserved only among HCV isolates.

We previously reported the expression and purification of a soluble form of NS5B truncated at the C terminus, NS5Bt, which retains RdRP activity in vitro. A 21-amino acid sequence was deduced to be the membrane-anchoring domain, which is dispensable for RdRP activity, but important for perinuclear localization of the full-size NS5B (12, 18). The subcellular localization of NS5Bt was exclusively nuclear and distinct from the perinuclear localization of NS5B in the GFP-fused form transiently overexpressed in mammalian cell lines (14). The membrane anchoring of NS5B might be important for HCV replication because most of the HCV NS proteins seem to be involved in the replication process by forming a dynamic replication complex attached to membranes. All NS proteins except NS3 have the intrinsic ability to interact with membranes, and even NS3 is recruited to membrane through the formation of a complex with NS4A, a cofactor of NS3 protease. In addition, HCV replication requires direct protein-protein interactions among NS proteins in a temporal order. HCV RNA and NS proteins have been reported to interact with many host factors, and some may modulate HCV replication. Recently, we reported that the RdRP activity of NS5B is regulated by homomeric oligomerization of NS5B and heteromeric interaction with NS5A (19, 20). There may be host protein(s), which modulate HCV replication by interacting with RdRP, the catalytic enzyme of HCV replication (21).
The exclusive nuclear localization of NS5Bt, an artificial truncated form missing the membrane-anchoring domain, may reflect a new property of NS5Bt to interact with some nuclear component(s), which may affect NS5B function. Furthermore, the nuclear clusters of NS5Bt seemed to be similar to the altered nucleoli described previously (22). Therefore, we examined whether nuclear NS5Bt is colocalized with nucleolin and the factor(s) involved in the interaction. Here we report that a nucleolar protein, nucleolin, can directly bind to NS5Bt in vivo and in vitro, even when the full-length NS5Bt is localized to perinuclear membranes.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—A plasmid derived from pGENK1 (23, 24), pGENKS, was used to express the recombinant HCV NS5B in E. coli, which contains multiple cloning sites (EcoRI and BamHI) downstream of the sequences encoding GST protein (14, 25). pYFLAG plasmid for expression of FLAG-tagged bacterial recombinant proteins was derived from pFLAGHis (a gift from R. Roeder) by replacing the Ndel-HindIII fragment with an insert containing a multiple cloning site to generate the EcoRI and BamHI digestion sites (25, 26). The bacterial His-tag expression vector, pYUTHis, was derived from pLHis (27) and harbors EcoRI and BamHI digestion sites.

The mammalian expression vectors were derived from pSG5UTPL (27). The pNKFLAG vector, which was constructed by replacement of the NotI-BamHI site of pSG5UTPL with a fragment composed of an artificial initiation codon and sequences encoding FLAG epitope and multiple cloning sites (EcoRI and BamHI), was used to express N-terminally FLAG-tagged proteins (25). The pNKGST vector, a GST-fused protein expression vector, was constructed by replacement of the NotI-BamHI site with a fragment encoding GST protein, threonin bin digestion sites, and multiple cloning sites (EcoRI and BamHI) derived from pGENKS (25). The pGFP vector was prepared by PCR using pGFPES65T (Clontech Co. Ltd.) as a template with a set of primers: GFPNotFor, which has the artificial EcoRV/NotI site, and GFPBamRev, which has the artificial EcoRI site. The DNA fragment with artificial EcoRV and BamHI sites was inserted into the pSG5UTPL blunt and BamHI vector, in which the EcoRI site was blunted with the Klenow DNA fragment before digestion by BamHI. The resulting plasmid, which expresses a GFP-fused protein in mammalian cells under the control of the SV 40 promoter, pGFP, was used for construction of GFP-fused NS5B protein.

HCV JK-1 DNA (28) harboring NS5B was subcloned by PCR using the sets of primers NS5BBeFor and NS5BBeRev, which has an artificial EcoRI and BamHI sites, respectively (14, 25). The truncated mutants and substitution mutants of NS5B were constructed as reported (25). Nucleolin constructs were generated from a human nucleolin cDNA clone (a generous gift from Chung Lee), which we subcloned into the EcoRI-BamHI site of pNKFLAG to create pNK-FLAG-nucleolin. The truncated mutants of nucleolin were subcloned by PCR with primers reported previously (30–32). The sequences of all of the constructions were confirmed by the dideoxy sequence method.

Expression and Purification of Bacterial Recombinant NS5B and Nucleolin Proteins—GST-fused HCV NS5Bt proteins was expressed and purified as described previously (14). Briefly, the wild-type pGENKS-NS5Bt was transformed into E. coli strain BL21 pLYsS (DE3) by treatment with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 °C for 4 h. The cells were harvested by centrifugation and suspended in FBST buffer A (phosphate-buffered saline (−), 1% Triton-X, and 1 mM dithiothreitol (DTT)). After centrifugation of the sonicated cell lysates, the supernatants were passed through DEAE-Sepharose 4B beads, and the cells were harvested by centrifugation and resuspended in buffer B (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 1 mM PMSF, 10 mM leupeptin and aprotinin, and 1 mM DTT). The cell lysate was diluted 3-fold with lysis buffer, mixed with 10 μl of glutathione-Sepharose 4B beads (GST resin) (Amersham Biosciences). The proteins were visualized by enhanced chemiluminescence according to the instructions from the manufacturer (Amersham Biosciences).

GST Pull-down Assay in Vitro—Approximately 1 μg of GST or GST-NS5Bt bacterial recombinant protein immobilized on 10 μl of glutathione-Sepharose 4B beads (GST resin) (Amersham Biosciences) was incubated with 1 μg of bacterially expressed nucleolin for 4 h. The reaction was stopped by transferring the reaction solution to DE81 filters (Whatman), which were then washed extensively with 0.5 M NaH2PO4, pH 7.0, and briefly rinsed with 70% ethanol. The bound protein was visualized by a scintillation counter. To examine the effect of binding to Ni2+-nitrilotriacetic acid-Sepharose (Qiagen). The resin was washed several times with buffer B and buffer C (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 10% glycerol, 1 mM PMSF, 10 mM leupeptin and aprotinin, and 1 mM DTT). The bound protein was eluted with buffer C containing 0.5 M imidazole. The eluted solution was dialyzed against buffer D (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM DTT, and 0.1% Triton X-100). Next, E. coli strain BL21 pLYsS (DE3) transformed by pYFLAG-nucleolin was grown to an A600 of 0.5 at 37 °C, and protein expression was induced by 1 mM IPTG at 37 °C for 4 h. The cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM PMSF, 10 mM leupeptin and aprotinin, and 1 mM DTT). Incubation of the sonicated supernatant with 10 μl of M2 resin (Kodak Scientific Imaging Systems) was followed by extensive washing with lysis buffer and elution with lysis buffer containing FLAG peptide (0.1 mg/ml). The eluted solution was dialyzed against buffer C.

Cell Lines and DNA Transfection—COS1 cells (a monkey kidney cell line) and HLE cells (a human hepatoma cell line) were cultured at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum plus units of penicillin and 100 μg/ml streptomycin. DNA transfection was performed with the calcium phosphate method as reported (14).

Subcellular Localization of Transiently Expressed NS5B—Subcellular localization of NS5B in mammalian cells was examined with a transient transfection system using HLE cells. Approximately 106 cells were plated on a slide glass in a Quadriperm microscope slide culture well (Heraeus Co. Ltd.) 1 day before transfection with the GFP-fused NS5B expression plasmid. The cells were transfected by the calcium phosphate method. The transfected cells were rinsed with PBS (−) and fixed with 1.5% paraformaldehyde in PBS (−) for 30 min. before post-fixation for 5 min in 100% cold methanol. These slides were air-dried at −25 °C and stored at −80 °C. GFP-fused proteins were detected after counterstaining with 0.0005% Evans-Blue in PBS (−). For immunostaining, the slides were stained with anti-mouse nucleolin antibody (1:3,000) (kindly provided by Y. Hirose, Cancer Research Institute, Kanazawa University) and amplified by horseradish peroxidase-labeled anti-mouse IgG (Dako) and detected with 3,3′-diaminobenzidine and Fast Red. The processed slides were examined using a confocal laser microscope (LSM 510, Carl Zeiss Co. Ltd.), and the images were visualized by digital printing (Pictography 4000, Fuji Co. Ltd.).

Preparation of Cell Extracts, Co-precipitation with GST Resin, and Western Blot Analysis—Transient transfections of COS1 cells were carried out as described previously (26, 33). The transfected cells were harvested and washed with PBS (−) and sonicated in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 10 mM leupeptin and aprotinin, and 1 mM DTT). Total cell lysate was diluted 3-fold with lysis buffer, mixed with 10 μl of glutathione-Sepharose 4B beads, and then incubated for 4 h at 4 °C. The reaction was stopped by transferring the reaction solution to DE81 filters (Whatman), which were then washed extensively with 0.5 M NaH2PO4, pH 7.0, and briefly rinsed with 70% ethanol. The bound protein was visualized by a scintillation counter. To examine the effect of
nucleolin on NS5B RdRP activity, 5, 10, 50, 100, and 250 nM of Histagged nucleolin was added before preincubation.

RESULTS

Nucleolar Localization of NS5B Missing the Membrane-anchoring Domain, NS5Bt, and Perinuclear Colocalization of the Full-length NS5B and Nucleolin—We examined whether the nuclear clusters of NS5B lacking the C-terminal 21 amino acid residues, NS5Bt, are colocalized with nucleolin. GFP-fused forms of NS5B and NS5Bt were transiently overexpressed in mammalian cells (HLE) and then analyzed with a confocal microscope after immunological staining with anti-nucleolin antibody. We applied nucleolin as a representative nucleolar marker (Fig. 1). As reported previously, the subcellular localization of NS5Bt was exclusively nuclear with nuclear body-like clusters; however, the full-length NS5B was abundant in perinuclear regions with granular spots (Fig. 1, A and B) (14, 34). The GFP signals in the nucleus completely colocalized with the signals detected by anti-nucleolin antibody, clearly indicating that the nuclear clusters of NS5Bt are nucleoli (Fig. 1B). Interestingly, NS5B and nucleolin did not colocalize in the nucleus, but strong merged signals were observed in perinuclear regions (Fig. 1A). The results demonstrate that GFP-NS5B was colocalized with endogenous nucleolin in perinuclear regions. In the control, nucleolin was exclusively distributed in nucleoli when GFP alone was overexpressed (Fig. 1C), and the GFP signals were spread diffusely in both the nucleus and cytoplasm. The GFP signals and the anti-nucleolin signals were merged in neither the nucleus nor the cytoplasm.

Nucleolar accumulation was observed in the cells expressing NS5B having substitution mutations in the membrane-anchoring domain to disrupt the hydrophobic properties of the domain (data not shown). Therefore, the membrane-anchoring domain is critical to the perinuclear localization of NS5B, but the colocalization of nucleolin with NS5B or NS5Bt was not affected by the domain. These results suggest that NS5B retains the ability to interact directly or indirectly with nucleolin. The strong perinuclear distribution of endogenous nucleolin in the NS5B-expressing cells may imply some role for nucleolin in NS5B function.

HCV NS5B Protein Interacts with Endogenous and Ecotropically Expressed Nucleolin—To examine whether NS5B and NS5Bt can directly interact with nucleolin in vivo, NS5B and NS5Bt in their GST-fused forms were transiently expressed in COS1 cells and the cell lysates were subjected to GST pull-down assay using glutathione-Sepharose 4B resin. The bound proteins were fractionated by SDS-PAGE and immunologically detected by Western blotting with anti-nucleolin antibody. As shown in Fig. 2A, the endogenous nucleolin bound to GST-NS5B and GST-NS5Bt but not to GST alone. Similarly, the specific binding of ecotropically expressed nucleolin and NS5B or NS5Bt was observed when FLAG-tagged nucleolin was transiently coexpressed with GST-NS5B or GST-NS5Bt in COS1 cells (Fig. 2B). The result was confirmed using a different combination, GST-nucleolin and FLAG-NS5B or FLAG-NS5Bt (Fig. 2C). To confirm that the interaction between nucleolin and NS5B in vivo does not require RNA, the lysate of COS1 cells transiently co-transfected with FLAG-nucleolin and GST-NS5Bt was treated with RNase A. The RNase treatment had no effect on interaction between nucleolin and NS5B (data not shown). These results clearly demonstrate that NS5B and NS5Bt specifically bind to both endogenous and ecotropically expressed nucleolin in vivo. Apparently the binding between NS5B and nucleolin is weaker than that between NS5Bt and nucleolin. The result was much exaggerated by the less efficient recovery of nucleolin with NS5B, which is mostly insoluble and difficult to subject to pull-down analyses.

HCV NS5B-binding Region Was Mapped near the C Terminal of Nucleolin in Vivo and in Vitro—To map the NS5B-binding region of nucleolin, a series of deletion mutants of nucleolin were constructed (Fig. 3A). NS5Bt was used to dissect the interaction of nucleolin because of the poor recovery of NS5B in soluble form. GST-NS5Bt and a deletion mutant of FLAG-nucleolin were transiently coexpressed in COS1 cells, after which the lysates were subjected to GST pull-down assay, and the bound proteins were immunologically detected by anti-FLAG M2 antibody. As shown in Fig. 3B, the full-size nucleolin and the truncated mutants deleted of the N terminus could bind to NS5Bt, although the smallest construct of the C-terminal region, nucleolin-R, could not. In contrast, the N terminus of nucleolin failed to bind to NS5Bt (Fig. 3B) and nucleolin-1234, -123, -234, and -23 could not bind either. Therefore, the minimum region necessary for binding NS5B is within nucleolin-4R, which harbors RNA-binding domain 4 and the RGG domain. To confirm the direct binding of NS5B and nucleolin, we tried to express the full-length nucleolin in E. coli, but the full-length and the N-terminal region of nucleolin could not be expressed (29). Thus, several FLAG-tagged truncated forms of nucleolin harboring the C-terminal half were bacterially expressed and purified with M2-bound agarose (Fig. 3C). FLAG-nucleolin-1234R and nucleolin-4R were pulled down with GST-NS5Bt, whereas FLAG-nucleolin-1234 was not recovered with GST-NS5Bt. The mapping result in vitro is consistent with that in mammalian cell lysates. Taken together, the data indicate that NS5B can directly bind nucleolin through the 4R region. At least two sequences are important for nucleolin binding.

At Least Two Sequences Are Important for Nucleolin Binding—Next, we addressed the amino acid sequences of NS5B, which are responsible for the binding of nucleolin. Recently, we have constructed a series of clustered alanine substitution mutants of NS5B in which 7 amino acid residues in a row were changed to AAASAAA as reported previously (25). COS1 cells were transiently cotransfected with plasmids expressing wild-
type or mutated FLAG-NS5Bt and GST-nucleolin or GST alone, and then the cell lysates were subjected to GST-pull down assay. The results showed that all FLAG-NS5Bt mutant proteins except NS5Bt-m211 and -m2 could be recovered with GST-nucleolin, but not with GST alone (Fig. 4C). They indicate that two amino acid sequences are critical for the nucleolin-binding. The two sequences, WKSKKNP and WRHRARS in amino acids 500–506. These two sequences are at the bottom of the palm and outer part of the thick thumb, respectively, and

**Fig. 2. Interaction of HCV NS5B and Nucleolin.** A, COS1 cells were transiently transfected with a plasmid expressing GST-NS5B (lane 1), GST-NS5Bt (lane 2), or GST alone (lane 3). Total lysate was separated by 8% SDS-PAGE and subjected to Western blotting with anti-mouse nucleolin antibody (left side, input). Proteins bound to glutathione-Sepharose 4B were washed with washing buffer, fractionated by 8% SDS-PAGE, and detected by Western blotting with anti-mouse nucleolin antibody (right side, bound fraction). B, GST pull-down assay using plasmids expressing GST, GST-NS5B, GST-NS5Bt, and FLAG-human nucleolin. Total lysate and bound protein were separated by 8% SDS-PAGE and detected with anti-FLAG antibody. C, GST pull-down assay using plasmids expressing GST, GST-human nucleolin, and FLAG-NS5B, FLAG-NS5Bt. Total lysate and bound protein were separated by 10% SDS-PAGE and detected with anti-FLAG antibody.

**DISCUSSION**

HCV replication, RNA-dependent RNA synthesis, is distinct from the host macromolecular processes and has been regarded as a target for intervention to block chronic infection and eventual hepatocarcinogenesis (4, 5). HCV replication consists of minus strand replication and plus strand replication. Both processes seem to happen at membranous structures where NS proteins dynamically assemble as a replication complex with HCV RNA. Recent results with HCV RNA replicon systems strongly suggest that HCV RNA replication is under regulation in which NS5A and NS5B are involved (10, 35, 36), although the exact molecular mechanism remains unclear. Recently we proposed that NS5B RdRP activity could be regulated at the level of protein-protein interactions, homomeric oligomerization, and heteromeric interaction between NS5B and NS5A (14, 19, 20).

In an attempt to clarify the exclusive nuclear localization of NS5Bt, a form of NS5B deleted of the C-terminal membrane-anchoring domain, here we report that NS5Bt is recruited to nucleoli through the association of nucleolin with NS5B. Despite a report that various forms of NS5B exist in the serum of HCV-infected patients (37), there is no evidence of cleavage at the junction of the anchoring domain in hepatocytes. Therefore, the nucleolar localization of NS5Bt is an artificial phenomenon. Importantly, the full-length NS5B enriched in perinuclear regions; however, endogenous nucleolin was localized in nucleoli. These results clearly indicate that two sequences critical for direct nucleolin binding are indispensable for the nucleolar localization of NS5Bt. The nucleolar targeting of NS5Bt is a result of the direct interaction of NS5B and nucleolin.

**Nucleolin Has an Inhibitory Effect on NS5B RdRP Activity**—The direct binding of nucleolin and NS5B may affect the function of NS5B. First, we addressed whether nucleolin affects the RdRP activity of NS5B in vitro. The purified soluble bacterial recombinant NS5Bt was used for an UMP incorporation assay with poly(A) and oligo(U)14 as template and primer, respectively. We tried but failed to express bacterial or insect recombinant full-length nucleolin. The C-terminal parts of nucleolin with or without the NS5B-binding region (Fig. 3A) were then purified in His-tagged forms, because FLAG-tagged truncated nucleolin proteins were expressed at much lower level for biochemical analyses. His-nucleolin-1234R was difficult to purify in large amounts. As shown in Fig. 6, both His-nucleolin-1234R and -4R, but not His-nucleolin-123 or -23, exhibited an inhibitory effect on the activity of NS5B RdRP in a dose-dependent manner. The nucleolin-1234R retains stronger inhibitory ability on the RdRP activity than nucleolin-4R did, and the NS5B-binding negative constructs (Fig. 6). The result strongly suggests that the truncated nucleolin protein requires the NS5B-binding ability for inhibiting RdRP activity in vitro.

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Fig. 3. A, structures of wild-type and deletion mutant forms of human nucleolin and characteristics of NS5B binding. Boxes represent the structure of different constructs of human nucleolin protein with the boundaries of amino acid residues indicated above. The activity of each nucleolin mutant to interact with NS5B is indicated by a plus or minus sign. NLS, nuclear localization signal; RBD, RNA binding domain. B, GST-NS5Bt and the full-length or deleted FLAG-tagged nucleolin were transiently co-transfected in COS1 cells. GST pull-down assay and Western blot analysis were carried out with anti-FLAG antibody. C, interaction of deletion mutants of FLAG-nucleolin with GST-NS5Bt in vitro by GST pull-down assay. Partially purified deleted FLAG-tagged nucleolin was incubated with GST-NS5Bt or GST protein and pulled down with glutathione-Sepharose 4B resin. The bound proteins were washed with washing buffer, fractionated by 12% SDS-PAGE, and subjected to Western blot analysis with anti-FLAG antibody.
are not close to the pocket for catalytic activity, but rather exposed. There are three basic amino acid residues clustered in both sequences, which are consistent with the proposed nucleolin-binding motifs rich in basic amino acids (38). The anchoring domain at the C terminus plays a role in recruiting the NS5B protein to the cytoplasmic membrane and also functions to redistribute host nucleolar proteins, at least nucleolin.

**FIG. 4.** Interaction of NS5B and nucleolin proteins expressed in mammalian cells. COS1 cells were transiently co-transfected with plasmids expressing FLAG-tagged NS5B and GST-nucleolin. A, total lysate was separated by 10% SDS-PAGE and subjected to Western blot analysis with anti-FLAG antibody. B, proteins bound to glutathione-Sepharose 4B resin were washed with washing buffer, fractionated by 10% SDS-PAGE, and detected by Western blot analysis with anti-FLAG antibody. C, structures of full-size, truncated forms and clustered substitution mutants of NS5B and characteristics of nucleolin binding. NS5Bt-M211 and -m2 have an alanine substitution in the clustered regions of basic residues as described under “Results.”

**FIG. 5.** Subcellular localization of NS5Bt-m211 and NS5Bt-m2. HLE cells were transfected with plasmid expressing GFP-NS5Bt-m211 (panel A) and GFP-NS5Bt-m2 (panel B). Transfected cells were fixed and stained as described in the legend to Fig. 1.

**FIG. 6.** His-nucleolin modulates NS5B RdRP activity. Purified GST-NS5Bt (10 nM) in the presence of 0, 5, 10, 50, 100, and 250 nM different deletion mutants of His-nucleolin were examined by poly(A)-dependent UMP incorporation assay as described under “Experimental Procedures.” Bar, S.E. of three independent experiments.
outcome of this redistribution of host protein(s) remains to be understood, but it may seem to modulate HCV replication or/and host functions. Preliminary experiments demonstrated that the redistribution of nucleolin seen in GFP-NS5B expressing cells did not affect the cell cycle by flow cytometric analyses as were not seen with GFP- or GFP-NS5B-expressing cells (data not shown).

We applied nucleolin as a representative nucleolar marker, although nucleolin has been also well documented to act as a shutting protein of RNA, or RNA chaperon, RNA trafficking (39–41). Nucleolin is involved in shuttling between not only the cytoplasm and nucleus, but also the cell surface and cytoplasm. Nucleolin on cell membrane has some role in cell cell interaction and in viral entry (42, 43). Here we demonstrated the direct binding of nucleolin and NS5B in vitro with the bacterial recombiant purified proteins, indicating that the direct binding does not require the presence of RNA. The NS5B-binding abilities of the nucleolin truncated constructs are well correlated to their inhibitory effects on the RdRP activities, strongly suggesting that the direct binding to NS5B is the reason of the inhibition. Nucleolin is also involved in the reproduction of several viruses (38, 44). In poliovirus, nucleolin relocates to the cytoplasm after infection occurs and increases the efficiency of viral gene expression by enhancing viral RNA translation or RNA replication (44). One report recently demonstrated that nucleolin stimulated viral internal ribosome entry site-mediated translation both in vitro and in vivo (45). This ability to bind RNA seems to be important for the switching from replication to translation as the two processes cannot occur at the same time during the life cycle of RNA viruses. In this context, our results of an inhibitory function on RdRP activity may have a relevance to the switching mechanism. It remains highly possible that the outcome of the direct interaction between nucleolin and NS5B in vivo would be opposite to the inhibitory effect we observed because we could address its role only with the truncated constructs in vitro. However, nucleolin-1234R covers almost all functional roles of nucleolin reported so far.

We and other groups have reported that NS5B can be oligomerized, which is a prerequisite for RdRP activity. We identified residue Glu-18 in the long loop and His-502 in the thick thumb, as critical for the oligomerization (19, 46). His-502 is a conserved residue Glu-18 in the long loop and His-502 in the thick thumb, as critical for the oligomerization (19, 46). His-502 is strongly suggesting that the direct binding to NS5B are well correlated to their inhibitory effects on the RdRP activities, strongly suggesting that the direct binding to NS5B is the reason of the inhibition. Nucleolin is also involved in the reproduction of several viruses (38, 44). In poliovirus, nucleolin relocates to the cytoplasm after infection occurs and increases the efficiency of viral gene expression by enhancing viral RNA translation or RNA replication (44). One report recently demonstrated that nucleolin stimulated viral internal ribosome entry site-mediated translation both in vitro and in vivo (45). This ability to bind RNA seems to be important for the switching from replication to translation as the two processes cannot occur at the same time during the life cycle of RNA viruses. In this context, our results of an inhibitory function on RdRP activity may have a relevance to the switching mechanism. It remains highly possible that the outcome of the direct interaction between nucleolin and NS5B in vivo would be opposite to the inhibitory effect we observed because we could address its role only with the truncated constructs in vitro. However, nucleolin-1234R covers almost all functional roles of nucleolin reported so far.

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