

Effect of Hepatitis C Virus (HCV) NS5B-Nucleolin Interaction on HCV Replication with HCV Subgenomic Replicon

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We previously reported that nucleolin, a representative nucleolar marker, interacts with nonstructural protein 5B (NS5B) of hepatitis C virus (HCV) through two independent regions of NS5B, amino acids 208 to 214 and 500 to 506. We also showed that truncated nucleolin that harbors the NS5B-binding region inhibited the RNA-dependent RNA polymerase activity of NS5B in vitro, suggesting that nucleolin may be involved in HCV replication. To address this question, we focused on NS5B amino acids 208 to 214. We constructed one alanine-substituted clustered mutant (CM) replicon, in which all the amino acids in this region were changed to alanine, as well as seven different point mutant (PM) replicons, each of which harbored an alanine substitution at one of the amino acids in the region. After transfection into Huh7 cells, the CM replicon and the PM replicon containing NS5B W208A could not replicate, whereas the remaining PM replicons were able to replicate. In vivo immunoprecipitation also showed that the W208 residue of NS5B was essential for its interaction with nucleolin, strongly suggesting that this interaction is essential for HCV replication. To gain further insight into the role of nucleolin in HCV replication, we utilized the small interfering RNA (siRNA) technique to investigate the knockdown effect of nucleolin on HCV replication. Cotransfection of replicon RNA and nucleolin siRNA into Huh7 cells moderately inhibited HCV replication, although suppression of nucleolin did not affect cell proliferation. Taken together, our findings strongly suggest that nucleolin is a host component that interacts with HCV NS5B and is indispensable for HCV replication.

Hepatitis C virus (HCV) is a major cause of chronic hepatitis around the world (1, 7). Chronic infection with HCV results in liver cirrhosis and may lead to hepatocellular carcinoma (53, 54). HCV is an enveloped positive-strand RNA virus belonging to the genus *Hepacivirus* in the family *Flaviviridae*. The HCV RNA genome is ~9.6 kb in length and consists of a 5' nontranslated region (NTR), a large open reading frame, and a 3' NTR. The 5' NTR contains an internal ribosome entry site, which mediates the translation of a single polyprotein of ~3,000 amino acid (aa) residues (61, 64). This polyprotein is cleaved by host and viral proteases into at least 10 different products (33). At the amino terminus of the polyprotein are the core protein, E1, and E2, followed by p7, a hydrophobic peptide with unknown function, and the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The 3' NTR consists of a short variable sequence, a poly(U)-poly(UC) tract, and a highly conserved X region and is critical for HCV RNA replication and HCV infection (13, 29, 69, 71).

HCV is unique among positive-strand RNA viruses in that it causes persistent and chronic infections. In addition, the high mutation rate of the gene encoding the E2 protein allows it to escape host immune surveillance, which is strongly associated with chronic inflammation of the liver (19, 23, 66, 67). As a result, HCV replication has become a target for the treatment of chronically infected individuals. The RNA-dependent RNA

polymerase (RdRp) NS5B is the central catalytic enzyme in HCV RNA replication. Several recombinant and catalytically active forms of NS5B have been expressed and purified from insect cells and *Escherichia coli*, and these proteins have provided insights into the biochemical and catalytic properties of NS5B (2, 12, 34, 68). Studies of HCV replication in vitro have to overcome several difficulties, since replication requires all or most NS proteins and/or host proteins and occurs at the membrane. An understanding of the biology of HCV replication has been facilitated by the development of subgenomic and full-length HCV replicons, which express HCV proteins and replicate their RNA when transfected into human hepatoma-cell-derived Huh7 cells and other cell lines (22, 24, 35).

Nucleolin is a major nucleolar phosphoprotein, and nucleolin-specific antibodies have been used to identify nucleoli (14, 59). Nucleolin has been shown to be an RNA chaperone and/or shuttling protein for various host and viral components in nucleoli, nucleoplasm, cytoplasm, and the plasma membrane (18, 37, 41). We previously reported that the transient expression of NS5B causes the redistribution of endogenous nucleolin from the nucleus to the cytoplasm and that nucleolin and NS5B interact, in vitro and in vivo, through two independent regions of NS5B, aa 208 to 214 and 500 to 506. We also showed that the C-terminal region of nucleolin inhibited NS5B RdRp activity through this interaction in vitro (20). Because full-length nucleolin was not available in that experimental condition (70), we could not determine the exact role of this interaction in vivo.

To further investigate the interaction between nucleolin and NS5B, we focused on NS5B aa 208 to 214. We prepared a

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series of mutant replicons in which each amino acid within this region was altered to alanine(s). Here, we report that the W208 residue is critical for transient HCV replication as well as for binding to nucleolin *in vivo*. HCV replication was considerably inhibited in cells in which endogenous nucleolin was transiently down-regulated by small interfering RNA (siRNA). Our results strongly suggest the involvement of nucleolin in HCV replication through its interaction with NS5B and that nucleolin acts as a positive modulator of HCV replication.

MATERIALS AND METHODS

Construction of plasmids. The plasmid pNNRZ2RU (28), which harbors a subgenomic replicon derived from MT-2C cells infected with HCV (a genotype 1b isolate, M1LE [GenBank accession no. AB080299]) and contains wild-type M1LE replicon (M1LE/wild) cDNA, was digested with MluI and BglII, and the obtained fragment was inserted into the MluI and BglII sites of the vector pGL3Basic (Promega) to create pGL3-*MluI-BglII*. The intermediate vector pGL3-*MluI-BglII-S232I* was constructed by introducing the point mutation S232I of NS5A into the MluI and SacI sites of pGL3-*MluI-BglII* by site-directed mutagenesis using primers carrying the necessary nucleotide changes. Subsequently, mutations were introduced into pGL3-*MluI-BglII-S232I*, which was digested with MluI and BglII. The resulting DNA fragments were subsequently ligated into the MluI and BglII sites of pNNRZ2RU. Plasmids containing the individual NS5B substitutions W208A, K209A, S210A, K211A, K212A, C213A, and P214A and the 7-amino-acid alanine substitution, cm211, were constructed by introducing each mutation into the Eco81I and NdeI sites of pGL3-*MluI-BglII-S232I* by site-directed mutagenesis using primers carrying the necessary nucleotide changes.

The vector pNKFLAG (49) was used to express amino-terminally FLAG-tagged proteins. The plasmid pNNRZ2RU was subcloned by PCR using the primers 5'-TATCGAGCTCGATGTCAATGCTACTCATGGACAGGT-3' (NS5B For), which contains an artificial initiation codon downstream of the SacI site, and 5'-ATGGATGGATCCGCGGGGTCGGGCGCGAGACAGGCT-3' (NS5Bt Rev), which contains a BamHI site. NS5Bt, containing full-length NS5B truncated by 21 aa at the C terminus, was subcloned into the SacI and BamHI sites of pNKFLAG to create pNKFLAGNS5Bt.

The plasmid pNKGST/Nucleolin (20) was used for the expression of glutathione-S-transferase (GST)-fused nucleolin proteins. FLAG-labeled plasmids containing the individual NS5B substitutions W208A, K209A, S210A, K211A, K212A, C213A, and P214A and the 7-amino-acid alanine substitution cm211 were constructed by introducing fragments of pGL3-*MluI-BglII-S232I* containing each mutation into the EcoRI and SmaI sites of pNKGSTNS5Bt.

The sequences of all the constructs were confirmed using the dideoxy sequence method. The plasmids pLMH14 and pLMH14/GHD (40) were used as templates for replicon RNA LMH14 and LMH14/GHD, respectively.

Cell culture. We used two kinds of Huh7 cells, one derived from our own laboratory's original Huh7 cells, designated Huh7-DMB (56), and the other cured of MH14 gamma interferon, designated cured MH14 (40). Huh7-DMB cells were used for colony-forming assays, and cured MH14 cells were used for luciferase assays. Both types of Huh7 cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, 100 U of penicillin, and 100 µg of streptomycin.

In vitro transcription and purification of RNA. All plasmids harboring replicon RNA were linearized with XbaI and column purified (PCR purification kit; Promega). RNA was synthesized and purified as described previously (56).

RNA transfection and selection of G418-resistant cells. Subconfluent Huh7 cells were trypsinized, washed once with phosphate-buffered saline (PBS) that does not contain Ca and Mg [PBS(-)], and resuspended at 10⁷ cells/ml in OPTI-MEM (Gibco-BRL, Invitrogen Life Technologies). One hundred nanograms of *neo* replicon RNA, with or without 1 µM of each siRNA, was added to 400 µl of each cell suspension in a cuvette with a gap width of 0.4 cm (Bio-Rad). The mixture was immediately transfected into Huh7 cells by electroporation with a GenePulser II system (Bio-Rad) set to 270 V and 975 µF. Following a 10-min incubation at room temperature, the cells were transferred into 10 ml of growth medium and seeded into a 10-cm-diameter cell culture dish. To select G418-resistant cells, the medium was replaced with fresh medium containing 1 mg/ml of G418 (GENETICIN; Gibco-BRL, Invitrogen Life Technologies) 24 h after transfection. After changing the medium twice per week for 4 weeks, the colonies

were stained with Coomassie brilliant blue (0.6 g/liter in 50% methanol–10% acetic acid).

DNA transfection. Using the same electroporation protocol as described above, 500 ng of pCI-Neo (Promega), which encodes a neomycin resistance marker under the control of a cytomegalovirus (CMV) promoter/enhancer, with or without 1 µM of each siRNA, was transfected into Huh7 cells. G418-resistant cells were selected in medium containing 0.5 mg/ml G418. Four weeks after transfection, the colonies were stained with Coomassie brilliant blue.

Using DMRIE-C reagent (Invitrogen Life Technologies), 300 ng of pGL3 control (Promega), encoding luciferase under the control of a CMV promoter/enhancer, was cotransfected with or without 2 µM of each siRNA according to the manufacturer's instructions. Luciferase activity was assayed 48 and 72 h after transfection.

RNA transfection and luciferase assay. We used a luciferase assay to monitor luciferase replicon activity. Briefly, cured MH14 cells seeded onto 48-well plates were transfected with 250 ng of luciferase replicon RNA, with or without 2 µM of each siRNA, using DMRIE-C reagent according to the manufacturer's instructions. Cell proteins were extracted in a lysis buffer supplied in the Dual-Luciferase Reporter Assay system (Promega), and their luciferase activity was measured. Each assay was performed at least in triplicate, and means and standard deviations were determined.

Preparation of cell extracts, coprecipitation with glutathione resin, and Western blot analysis. COS1 cells were transiently transfected using the calcium-phosphate method. The cells were harvested, washed with PBS(-), and sonicated in PBS lysis buffer [PBS(-) containing 150 mM NaCl, 1.0% Triton X-100, 1 mM EDTA, and 1 mM dithiothreitol] containing 10 µg each of aprotinin and leupeptin per ml. Total cell lysates were diluted 10-fold with PBS lysis buffer, mixed with 20 µl of glutathione-Sepharose 4B beads (glutathione resin) (Amersham Biosciences), and incubated for 3 h on a rotator in a cold room. After extensive washing with PBS(-) containing 1.0% Triton X-100, the bound proteins were eluted, fractionated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE), transferred onto nitrocellulose membranes, and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody (Sigma). The proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences). As a loading control, the nitrocellulose membranes used for Western blot analysis with anti-FLAG M2 monoclonal antibody were reprobated with anti-GST monoclonal antibody (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions (Amersham Biosciences).

siRNA. We purchased siRNA for luciferase GL3 duplex (si-Luc), siRNA for nonspecific control RNA duplex (si-Mix), siRNA for nucleolin (si-Nuc) (GGA AGACGGUGAAAUUGAU-deoxyriboylthymine [dT]dT), and siRNA for HCV (CCUCAAGAAAAACCAAAC-dTdT) from B-Bridge International, Inc., and we purchased siRNA for GFP from QIAGEN.

Western blot analysis for endogenous nucleolin. Using the electroporation protocol described above, 1 µM of each siRNA was transfected into Huh7-DMB cells. After 48 h, the cells were harvested, washed with PBS(-), and sonicated in PBS lysis buffer. Total cell lysates were fractionated by SDS–10% PAGE, transferred onto nitrocellulose membranes, and subjected to Western blot analysis with rabbit polyclonal anti-nucleolin antibody (103C) (20), mouse monoclonal anti-nucleolin antibody (C23, sc-8031; Santa Cruz Biotechnology, Inc.), and mouse monoclonal anti-β-actin antibody (Sigma). The proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

RESULTS

We previously reported that NS5B from HCV subtype 1b isolate JK-1 and nucleolin interact *in vitro* and *in vivo* and that two regions of NS5B, amino acids 208 to 214 and 500 to 506, are both indispensable for binding to nucleolin. We also reported that the C-terminal region of nucleolin inhibited the RdRp activity of NS5B in a dose-dependent manner (20). Although the effect of full-length nucleolin could not be determined, because we could not obtain recombinant full-length nucleolin, these results strongly suggested that nucleolin may be a component of the HCV replication complex and, through its interaction with NS5B, may modulate HCV replication. To further investigate this question, we determined the biological effect of the interaction between NS5B from HCV subtype 1b

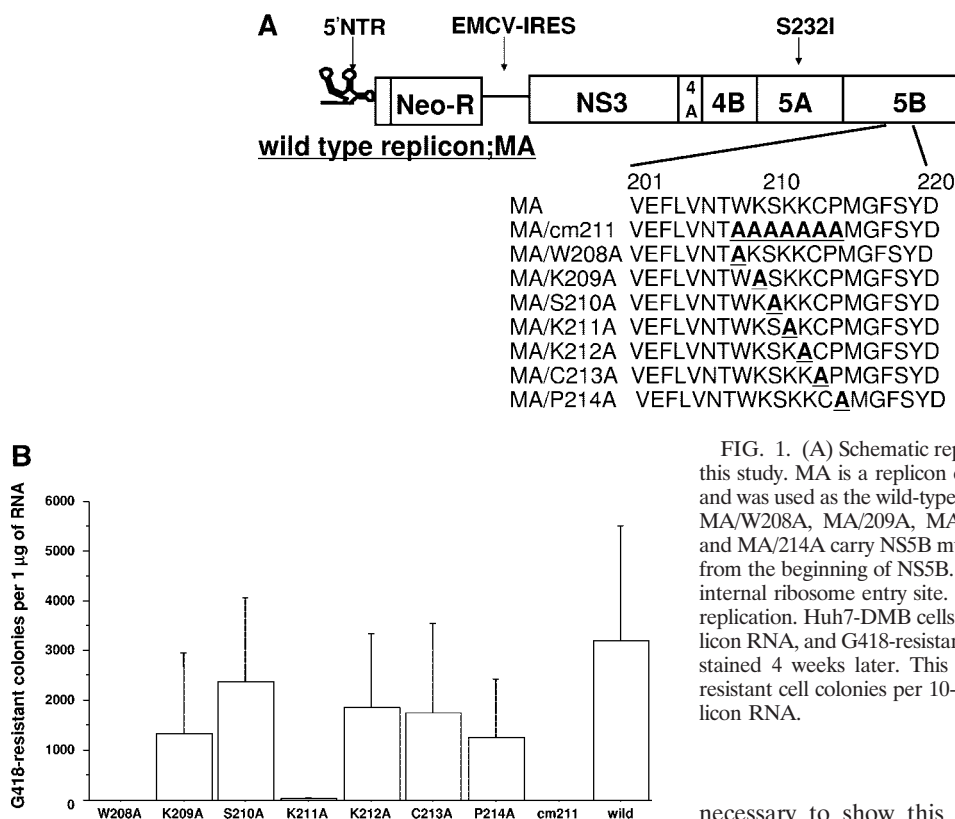


FIG. 1. (A) Schematic representation of the mutant replicons used in this study. MA is a replicon carrying the point mutation S232I in NS5A and was used as the wild-type replicon. The mutant replicons MA/cm211, MA/W208A, MA/209A, MA/S210A, MA/211A, MA/212A, MA/213A, and MA/214A carry NS5B mutations in MA, as shown. Numbering starts from the beginning of NS5B. EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site. (B) Effect of each mutation on HCV RNA replication. Huh7-DMB cells were electroporated with 1 µg of each replicon RNA, and G418-resistant cells were selected with 1 mg/ml G418 and stained 4 weeks later. This figure shows the mean number of G418-resistant cell colonies per 10-cm-diameter cell culture dish per 1 µg replicon RNA.

isolate M1LE and nucleolin on HCV replication using an HCV subgenomic replicon system.

Scanning of aa 208 to 214 in an HCV subgenomic replicon.

First, we tested the importance of NS5B aa 208 to 214, a region essential for nucleolin binding, in HCV RNA replication. For this purpose, we prepared eight mutant replicons (Fig. 1A). The wild-type replicon was represented by MA, in which S232 of NS5A was altered to I, because this mutant replicon can efficiently replicate in Huh7 cells (36, 56). In the replicon MA/cm211, each of the amino acids at positions 208 to 214 of NS5B was changed to alanine, whereas in the replicons MA/W208A, K209A, S210A, K211A, K212A, C213A, and P214A, each individual amino acid residue was changed to alanine. All of these mutant replicons were transfected into Huh7-DMB cells, which were selected with G418, and the number of G418-resistant colonies was used as an indication of HCV RNA replication. In cells transfected with MA/cm211 and MA/W208A, we observed no G418-resistant colonies, whereas in cells transfected with the six other point mutant replicons, as well as in cells transfected with MA/K211, we detected G418-resistant colonies, but they were fewer than those detected with wild-type replicon MA (Fig. 1B). Our negative control, the mutant replicon M1LE/5B-VDD, in which the GDD motif of NS5B was mutated to VDD, yielded no G418-resistant colonies (data not shown). The results of this experiment indicated that the region of NS5B at aa 208 to 214, especially W208, is essential for HCV RNA replication.

Interaction between nucleolin and NS5B. Although we have shown that NS5B from isolate JK-1 binds to nucleolin, it was

necessary to show this in isolate M1LE. Due to the poor recovery of soluble full-length NS5B, we utilized NS5Bt (68), a soluble form of NS5B in which the C-terminal 21 aa were truncated, to dissect the interaction between NS5B and nucleolin. Previously, we confirmed that these 21 deleted amino acids were not essential for this interaction (20). FLAG-NS5Bt and GST-nucleolin were transiently coexpressed in COS1 cells, after which the lysates were subjected to a GST pull-down assay and the bound proteins were immunologically detected with anti-FLAG M2 and anti-GST antibodies. We found that GST-nucleolin could bind FLAG-NS5Bt from the M1LE isolate, whereas GST could not, indicating that nucleolin interacts with NS5B in both JK-1 and M1LE isolates (Fig. 2). To determine the essential region/residues of NS5B required for its binding to nucleolin, we again focused on aa 208 to 214 using the alanine scanning method (3). We prepared FLAG-NS5Bt/cm211, in which aa 208 to 214 were all replaced by alanine residues, and showed that it could not bind to GST-nucleolin in an *in vivo* immunoprecipitation assay (Fig. 2), indicating that aa 208 to 214 of NS5B in both M1LE and JK-1 isolates constitute a critical region for the binding of nucleolin. To identify the exact residue(s) within aa 208 to 214 critical for binding to nucleolin, we prepared seven alanine-substituted point mutants in which each amino acid was replaced by alanine, and we tested the ability of each point mutant to bind to GST-nucleolin. Using an *in vivo* immunoprecipitation assay, we found that of the seven point mutants, only FLAG-NS5Bt/W208A could not bind to GST-nucleolin (Fig. 2), indicating that W208 of NS5B is essential for this binding and may be essential for HCV replication.

Suppression of endogenous nucleolin by siRNA. To identify the siRNA sequence that knocks down the expression of endogenous nucleolin, we used the prediction services of

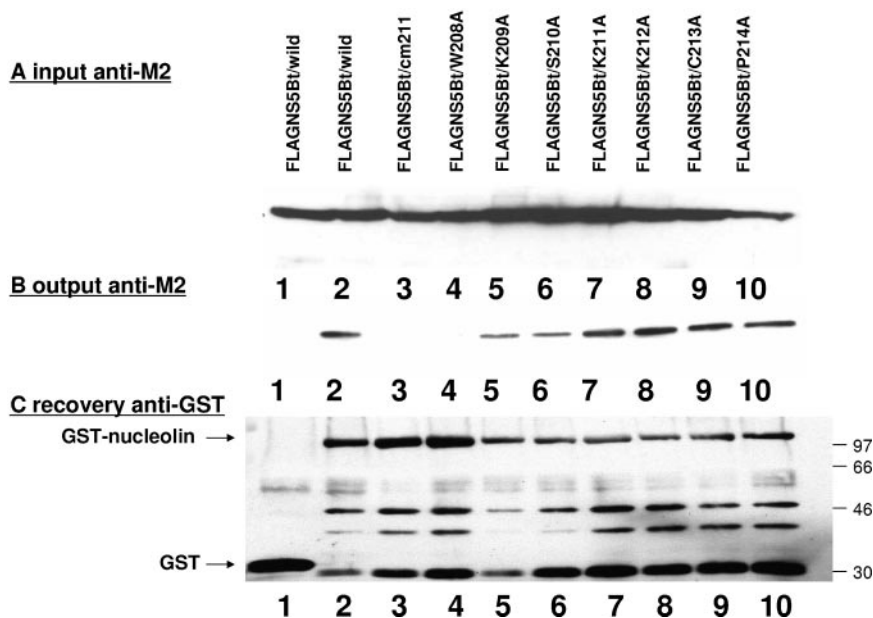


FIG. 2. Interaction between nucleolin and NS5B of HCV isolate M1LE and an essential residue for this interaction. COS1 cells were transiently cotransfected with mammalian expression vectors expressing FLAG-NS5Bt proteins (lanes: 1 and 2, wild type; 3, cm211; 4, W208A; 5, K209A; 6, S210A; 7, K211A; 8, K212A; 9, C213A; 10, P214A) and GST protein alone (lane 1) or GST-nucleolin protein (lanes 2 to 10). (A) Input of FLAG-NS5Bt proteins. Total lysates were fractionated by SDS-10% PAGE and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. (B) Output of FLAG-NS5Bt proteins. Coprecipitants by glutathione resin were washed with PBS(-) containing 1.0% Triton X-100, fractionated by SDS-10% PAGE, and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. (C) Recovery of GST or GST-nucleolin proteins. The nitrocellulose membrane used for Western blot analysis of coprecipitants with anti-FLAG M2 antibody was reprobbed with anti-GST antibody. Molecular masses (kilodaltons) are indicated to the right of the panel.

iGENE (Tsukuba, Japan). We selected one sequence, si-Nuc, and, as a control for siRNA transfection, we utilized siRNA for luciferase (si-Luc) (GL3 luciferase duplex). Forty-eight hours after electroporation of each siRNA, at a concentration of 1 μ M, into Huh7-DMB, the lysates were analyzed by Western blotting analysis with two kinds of antibody to nucleolin. We found that both anti-nucleolin antibodies detected the expression of endogenous nucleolin. Although si-Nuc efficiently knocked down the expression of endogenous nucleolin, si-Luc did not (Fig. 3), showing the specificity of the former. In addition, real-time PCR showed that si-Nuc decreased nucleolin mRNA by about one-third compared with si-Luc (data not shown).

Effect of nucleolin suppression on HCV replication. To test the effect of nucleolin knockdown on HCV RNA replication, we transfected 1 μ M of si-Nuc or si-Luc along with 100 ng of replicon MA RNA into Huh7-DMB cells and selected the cells with G418. As shown in Fig. 4, we found that cotransfection of si-Nuc reduced the number of G418-resistant colonies, whereas cotransfection of si-Luc did not (Fig. 4). As a control for the efficient transfection of siRNA, we used si-HCV, which targets the HCV internal ribosome entry site and can efficiently suppress HCV replication, as described previously (51). Using this siRNA, we observed no G418-resistant colonies, indicating that siRNA was efficiently transfected under these experimental conditions. To rule out the possibility that suppression of nucleolin may have a detrimental effect on cells and may inhibit HCV RNA replication, we transfected pCI-Neo, which encodes a neomycin resistance gene under the control of a CMV promoter/enhancer, into Huh7-DMB cells,

with or without si-Nuc and si-Luc, and selected the cells with 0.5 mg/dl G418. We found that the suppression of nucleolin expression did not significantly reduce the number of G418-resistant colonies (data not shown). In addition, massive cell death was not observed after the transfection of any siRNA (data not shown). These results indicate that the transient suppression of nucleolin may not affect cell proliferation but that nucleolin may affect the HCV replication complex itself.

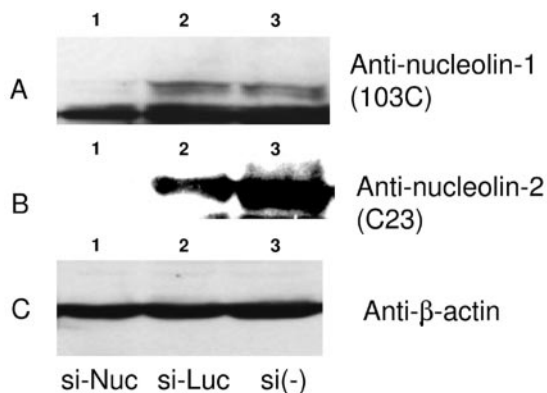


FIG. 3. Knockdown of endogenous nucleolin by siRNA. Huh7-DMB cells were electroporated with 1 μ M si-Nuc and si-Luc. After 48 h, total cell lysates were fractionated by SDS-10% PAGE and subjected to Western blot analysis with the anti-nucleolin antibodies anti-nucleolin-1 (103C) in A and anti-nucleolin-2 (C23) in B and anti- β -actin antibody in C. Lanes: 1, cells transfected with si-Nuc; 2, cells transfected with si-Luc; 3, no siRNA [si(-)].

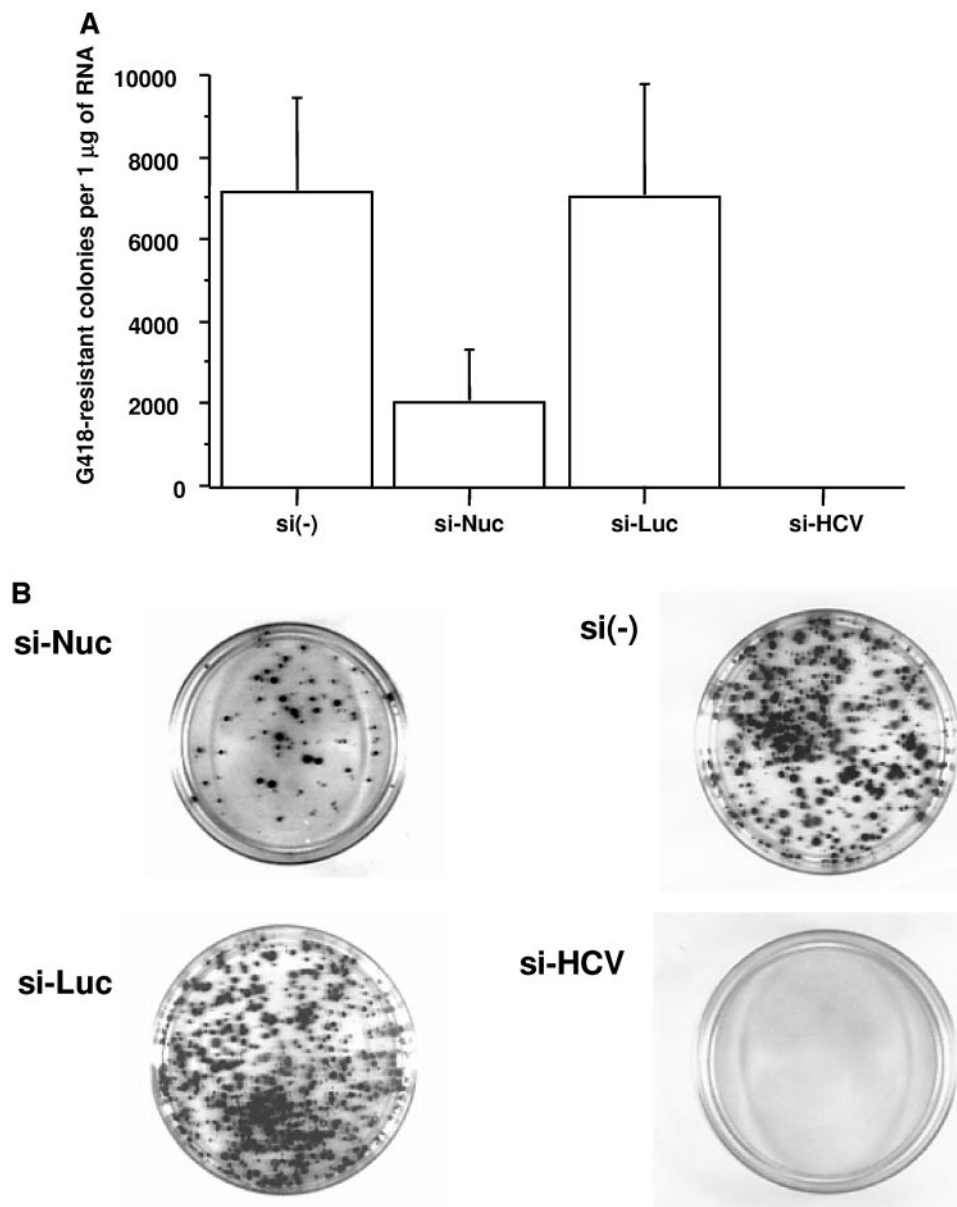


FIG. 4. Effect of suppression of endogenous nucleolin on HCV replication in the MA replicon. Huh7-DMB cells were electroporated with 1 μ g of in vitro-transcribed MA RNA plus si-Nuc, si-Luc, si-HCV, or no siRNA [si(-)], and G418-resistant cells were selected with 1 mg/ml G418 and were stained 4 weeks later. (A) Mean number of G418-resistant colonies per 10-cm-diameter cell culture dish per 1 μ g replicon RNA. Error bars indicate the standard deviations of the results from at least three independent experiments. (B) Visualization of G418-resistant colonies, as described in Materials and Methods.

Because the knockdown effect of siRNA does not continue for more than 3 weeks after transient transfection, the number of G418-resistant colonies may not be a good indicator of HCV RNA replication. We therefore performed a transient replication assay using a replicon in which the neomycin resistance gene was replaced by a luciferase gene, and luciferase activity was used as a marker of HCV RNA replication. Transfection of MH14 RNA, which was used as the wild-type replicon, into a subline of Huh7 cells resulted in highly efficient luciferase activity, whereas a polymerase-defective RNA replicon of MH14, MH4GHD, in which the catalytic GDD motif of NS5B polymerase was replaced by an inactive GHD motif, was used

as a negative control (Fig. 5A). si-HCV and si-Luc suppressed the luciferase activity even at 24 h after transfection, but other siRNAs did not affect the luciferase activity, and luciferase activities in these siRNAs were similar to that of the control (no siRNA) at this point (Fig. 5B). We found that cotransfection of si-Nuc moderately suppressed both luciferase activity at 72 h after transfection and relative luciferase activity, whereas cotransfection of si-GFP and si-Mix did not (Fig. 5B and C). Cotransfection of si-HCV and si-Luc almost completely suppressed luciferase activity at 72 h after transfection. In a transient replication assay, the suppression of endogenous nucleolin also inhibited HCV replication.

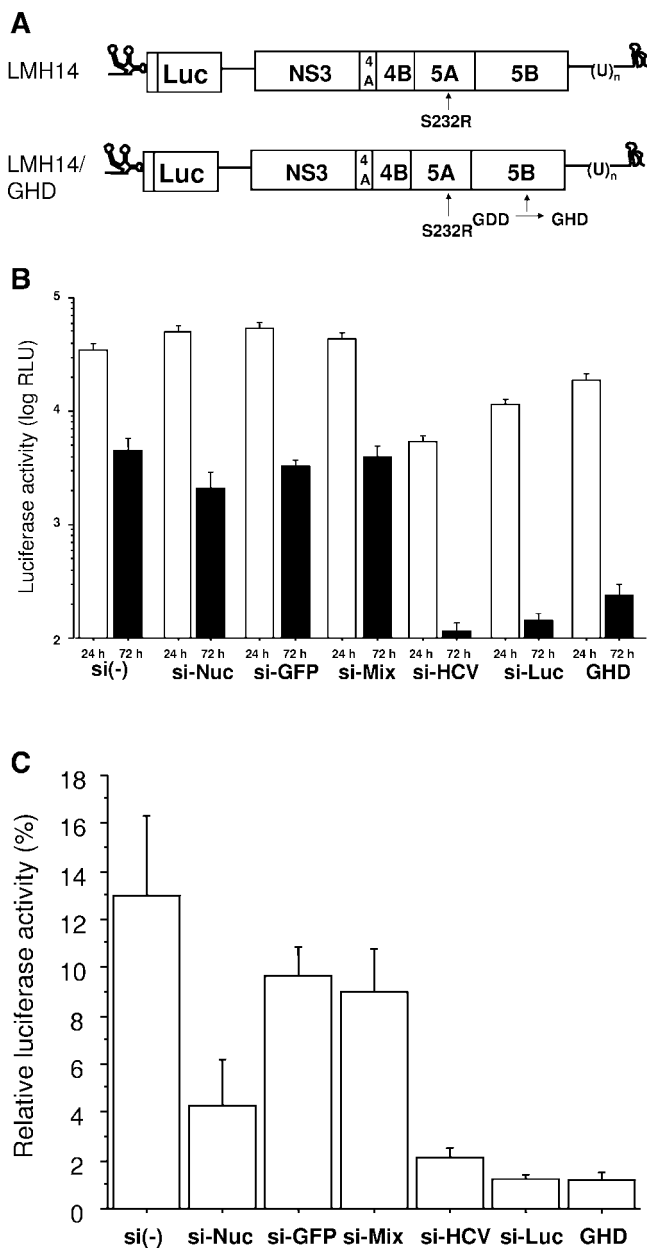


FIG. 5. Effect of suppression of endogenous nucleolin on HCV replication in the LMH14 replicon. (A) Schematic representation of the luciferase replicon. In the LMH14 replicon, the neomycin resistance gene was replaced by a luciferase gene, and S232 of NS5A was replaced by R. In the LMH14/GHD replicon, the NS5B GDD motif in LMH14 was changed to GHD and used as a negative control. (B) Cells were transfected with in vitro-transcribed LHH14 or LMH14/GHD RNA along with 2 μ M of si-Mix, si-GFP, si-Nuc, si-Luc, si-HCV, or no siRNA [si(-)] using the DMRIE-C reagent, and luciferase activity (relative light units [RLU]) was measured 24 and 72 h after transfection. Shown are the activities at 24 and 72 h. Error bars indicate the standard deviations of the results from at least three independent experiments. (C) Activity at 24 h was used as an indication of each transfection. Shown are the ratios of activity (percent) at 72 h relative to that at 24 h. Error bars indicate the standard deviations of the results from at least three independent experiments.

To rule out the cytotoxic effects of the suppression of endogenous nucleolin, we transfected pGL3 control, with or without each siRNA, and measured luciferase activity 48 and 72 h after transfection. We found that cotransfection of each siRNA did not inhibit luciferase activity at both 48 and 72 h (Fig. 6), indicating that both suppression of nucleolin and transfection of siRNA did not have detrimental effects on transfected cells.

DISCUSSION

HCV replication has been found to take place in a distinctly altered membrane structure, or membranous web, of the endoplasmic reticulum (11). When HCV NS proteins are co-expressed in stable cell lines harboring replicons, they colocalize to these membrane structures, indicating that they might form a complex (16, 39, 47). These nonstructural proteins, together with host factors, form the viral replicase, the complex in which viral replication is thought to take place. The in vitro level of the RdRp activity of NS5B is low (12), indicating that cofactors, whether viral and/or host proteins and/or the appropriate cellular environment, are necessary for optimal activity of HCV RdRp. HCV NS5B has been reported to interact with NS3, NS4A, NS4B, NS5A, and NS5B itself (9, 48, 57, 65). Using an HCV subgenomic replicon, we previously reported the critical role of the interaction between NS5A and NS5B and the oligomerization of NS5B itself in HCV replication (36, 56). NS3 and NS4B have been shown to be positive and negative regulators, respectively, of NS5B in the replication complex (46).

In addition to interacting with HCV nonstructural proteins, NS5B has been reported to interact with many host proteins, including a SNARE-like protein (62); eIF4AII, an RNA-dependent ATPase/helicase; a component of the translation initiation complex (30), protein kinase C-related kinase 2, which specifically phosphorylates NS5B (27); and p68, a human RNA helicase I (15). The suppression of protein kinase C-related kinase 2 has been reported to reduce the phosphorylation of NS5B and to inhibit HCV RNA replication (27), and the suppression of p68 has been reported to inhibit the synthesis of negative-strand HCV RNA from the positive strand (15).

Several host proteins have been shown to interact with RdRp of other RNA viruses. For example, in poliovirus, an RdRp and an RdRp precursor interact with human Sam68 (38) and heterogeneous nuclear ribonucleoprotein C1/C2 (5), respectively, and modulate RdRp activity directly or indirectly. Bromo mosaic virus RdRp and tobacco mosaic virus RdRp interact with eukaryotic initiation factor 3 and eukaryotic initiation factor 3-related factor, altering RdRp activity (45, 50).

Here and in a previous report, we identified and characterized the interaction between nucleolin and HCV NS5B (20). Nucleolin was originally identified as a common phosphoprotein of growing eukaryotic cells, although its function is not completely understood. Nucleolin is a multifunctional protein that shuttles between the nucleus and cytoplasm. In addition, it is expressed on the surface of various cells, acting as a receptor for various ligands, including lipoproteins (55), cytokines, growth factors (6, 52, 60), the extracellular matrix (10, 18, 25), bacteria (58), and viruses (4, 8, 21, 41-44).

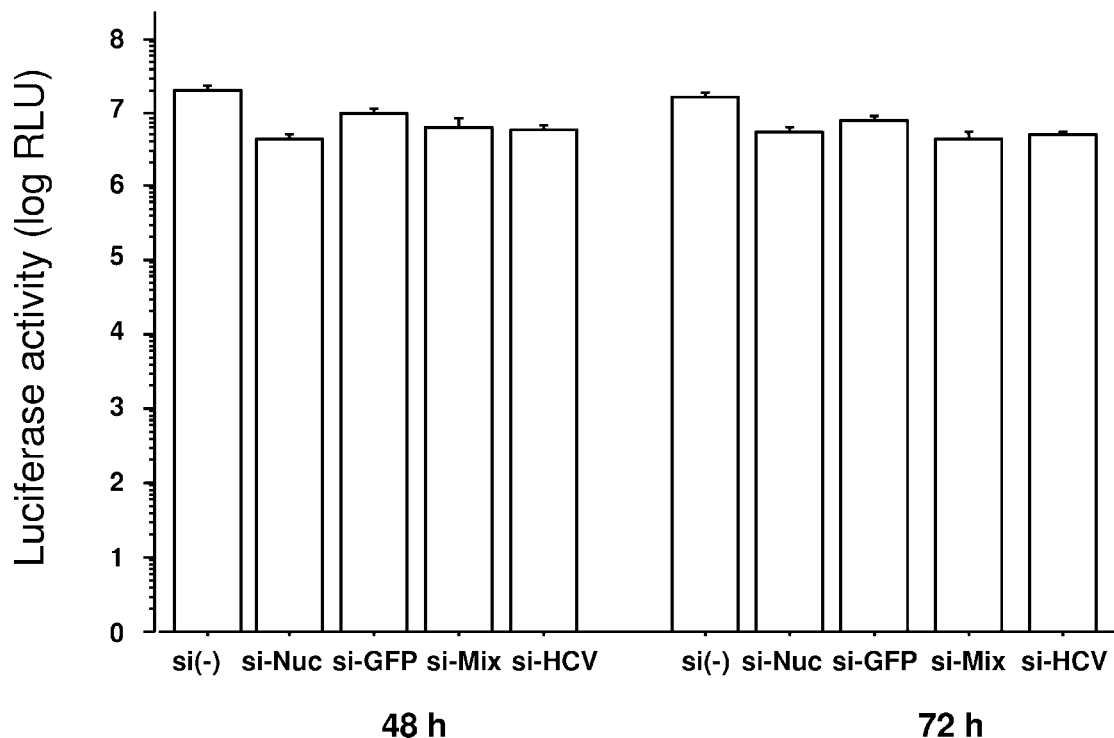


FIG. 6. Effect of suppression of endogenous nucleolin on cell proliferation. The plasmid pGL3 control, encoding the luciferase gene under the control of the CMV promoter/enhancer, was cotransfected with 2 μ M of si-Mix, si-GFP, si-Nuc, si-HCV, or no siRNA [si(-)] using DMRIE-C reagent, and luciferase activity was measured 48 and 72 h after transfection. The error bars indicate the standard deviations of the results from at least three independent experiments.

We found that recombinant C-terminal nucleolin proteins can bind NS5B and inhibit its RdRp activity in a dose-dependent manner (20), suggesting that nucleolin may affect HCV replication by interacting with NS5B. The direct interaction of nucleolin with HCV NS5B *in vivo* and *in vitro* was shown to require two critical stretches of NS5B. Here, we showed that within one of these regions, aa 208 to 214, the W208 residue was critical for both binding of nucleolin and HCV replication. Transient down-regulation of endogenous nucleolin by siRNA considerably inhibited HCV replication in Huh7 cells. These results strongly indicate that nucleolin has an important role in HCV replication through its direct interaction with NS5B.

Our finding of an important positive role for nucleolin in HCV replication is apparently inconsistent with previous findings of an inhibitory role for nucleolin. It was previously reported that purified C-terminal nucleolin proteins inhibited the RdRp activity of NS5B *in vitro*. The latter result, however, may have been due to the use of recombinant truncated nucleolin proteins, because recombinant full-length nucleolin was not available (70). Taken together, however, these results indicate that N-terminal nucleolin may be important for the positive function of nucleolin in HCV replication, although the NS5B-binding region is within the RGG domain and RNA-binding domain 4 is at the C terminus.

Transfection of the mutant replicon containing NS5B W208A, which could not bind nucleolin, led to almost no HCV replication. By contrast, the suppression of nucleolin by siRNA moderately inhibited HCV replication, a result also observed with the tran-

sient assay using luciferase reporter replicon and G418-resistant colony formation. While HCV replication was completely inhibited by MA/W208A, replication was only partially inhibited by si-Nuc, indicating that si-Nuc can transiently suppress, but cannot eliminate, expression of endogenous nucleolin. Recently, nucleolin was reported to inhibit cell cycle progression after heat shock and genotoxic stress by increasing complex formation with human replication protein A (26). When pGL3 control or pCI-Neo was cotransfected with si-Nuc, the luciferase activity or the number of G418-resistant colonies was not reduced, strongly suggesting that the moderate inhibition of nucleolin expression did not have severe cytotoxic effects on siRNA-transfected cells. More efficient suppression of nucleolin may result in more severe inhibition of HCV RNA replication. It is therefore important to determine whether nucleolin is dispensable in mammalian cells as it is in *Saccharomyces pombe* (17) and *Saccharomyces cerevisiae* (31), since nucleolin may constitute a putative therapeutic target to inhibit HCV replication.

Using a clustered alanine substitution mutant library (CM) of NS5B, we previously showed that two stretches of NS5B amino acids, aa 208 to 214 and 500 to 506, were critical for nucleolin binding. According to the crystal models of NS5B, the former stretch is in the palm and the latter stretch is in the bottom of the thumb domain. We focused on identifying residues in aa 208 to 214 that are essential for nucleolin binding and HCV replication, as the CM mutant of aa 500 to 506 was defective in RdRp activity *in vitro* and HCV replication *in vivo* (36, 48, 49). We found that the W208 residue was critical for

both nucleolin binding and HCV replication. This residue is exposed to solvent at the edge of the palm and is not close to the catalytic pocket.

Nucleolin may stabilize monomeric NS5B, making it ready for oligomerization to NS5B, or it may facilitate the formation of a complex between NS5B and template RNA. In both cases, a substoichiometric amount of nucleolin may be required transiently at a step prior to the catalytic RdRp reaction of NS5B. Efforts to determine the contribution of amino acid residues 500 to 508 to nucleolin binding and HCV replication *in vivo* are ongoing and may reveal further correlations. We found that another mutant replicon, MA/K211A, reduced the number of G418-resistant colonies compared with the wild type and the other mutants. Because K211A of NS5B is close to the pocket of catalytic activity and did not affect binding to nucleolin, K211 may contribute to the structural integrity of the pocket or the heat-stable property of RdRp as reported previously (36).

Efficient HCV replication and infection in tissue-cultured cells by using full-length HCV RNA replicons have been reported previously (32, 63, 72). HCV replication occurs in differentiated subcellular fractions and involves dynamic complexes of structural proteins, nonstructural proteins, and HCV RNA demarcated by membrane structures. It is therefore of great interest to determine whether nucleolin is involved in such HCV-replicating intermediates in compartmented subcellular structures.

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