on the lipid droplets in Hep39 cells. Cellular TIP47 was not up-regulated in Hep39 cells, resulting in a reduction of TIP47 in the cytosolic fraction (Fig. 4). Since TIP47, originally identified as having the ability to interact with the mannose 6-phosphate/IGF-II receptor (*63*), appears to be essential for the endocytic recycling system (*65–67*), the altered distribution of cellular TIP47 in Hep39 cells could affect intracellular membrane trafficking pathways. Consistent with this assumption, our preliminary results showed that the rate of protein secretion from cells was apparently slower for Hep39 cells than Hepswx cells (unpublished data). Patients chronically infected with HCV (*68*) and HCV core–transgenic mice (*69*) exhibit decreased levels of plasma very low density lipoproteins secreted from the liver, also suggesting interference with intracellular membrane trafficking (secretion pathways) by HCV core proteins. We currently speculate that the reduction in cellular ADRP expression mediated by HCV core protein causes the accumulation of TIP47 in lipid droplets as a substitute, and that the resulting depletion of TIP47 in the cytosol could cause the impairment of intracellular membrane trafficking, followed by the cellular accumulation of membrane lipids and consequent lipid droplet formation. Although further studies remain to be done to confirm these possibilities, we suggest that HCV core protein influences not only the biogenesis of lipid droplets but also intracellular membrane trafficking.

Another interesting finding in this study is that Hep39 cells, unlike Hepswx cells, contain DEAD box proteins, DDX1 and DDX3, as major lipid droplet proteins (Figs. 2 and 5). On the basis of the results of studies involving yeast two-hybrid assays, DDX3 has been shown to be able to interact with HCV core protein, and studies involving immunofluorescent microscopy have revealed that DDX3 is distributed in cytosolic spots such as lipid droplets (*27*, *70*, *71*). These results, together with our present findings, suggest that DDX3 is associated with lipid droplets via HCV core proteins located on lipid droplets. In addition to DDX1 and DDX3, which possess ATPase/RNA helicase activities (*27*, *72*, *73*), several other proteins involved in RNA metabolism/binding, including HC56/gemin 4 and IGF-II mRNA–binding protein 3, were also detected in the lipid droplet fraction of HCV core-expressing Hep39 cells (Tables 1 and 2). Recently Dvorak *et al.* reported that RNA itself can be associated with lipid droplets in human mast cells (*74*). Taken together, these data strongly suggest that lipid droplets containing HCV core proteins may participate in the RNA metabolism of the host and/or HCV in HCV-infected cells. Furthermore, the findings that DDX1 is overexpressed in cell lines derived from tumors such as retinoblastomas and neuroblastomas (*75*), and that cellular expression of DDX3 induces anchorage-independent cell growth (*76*) suggest the involvement of DDX1 and DDX3 in carcinogenesis.

Some groups recently reported profiles of mRNAs up- or down-regulated by expression of the HCV core protein (*77–79*), but these mRNAs included no molecules identified as lipid droplet proteins in this study. Since lipid droplets are a minor organelle in cells, it might be difficult to detect changes in the mRNA expression levels of lipid droplet proteins. The merits of targeted proteomic study are that it is possible to focus on minor cellular fractions, and also to detect changes in the intracellular distributions of proteins. Actually, the mRNA expression levels of TIP47, DDX1, and DDX3 did not change in Hep39 cells (data not shown).

We identified many other lipid droplet proteins found in either Hepswx or Hep39 cells, but their biological functions remain mostly unknown (Tables 1 and 2). Elucidation of the biological functions of these proteins will lead to an advanced understanding of the pathogeneses of HCV-derived liver diseases.

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Enhancement of *de Novo* Fatty Acid Biosynthesis in Hepatic Cell Line Huh7 Expressing Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein plays important roles in the pathogeneses of liver steatosis as well as hepatocellular carcinomas due to HCV infection. In this study, we examined *de novo* fatty acid biosynthesis in hepatic cell line Huh7 cells expressing HCV core protein. The rate of metabolic labeling of cellular fatty acids with [^{3}H]acetate in core-expressing (Uc39-6) cells was *ca.* 1.5-fold higher than that in non-expressing (Uc321) cells. The enzyme activities responsible for fatty acid biosynthesis were assayed *in vitro*. Cytosolic acetyl-CoA carboxylase activity in Uc39-6 cells was *ca.* 1.6-fold higher than that in Uc321 cells. On the other hand, cytosolic fatty acid synthase activity in Uc39-6 cells was only slightly higher than that in Uc321 cells. Immunoblot analysis of acetyl-CoA carboxylase 1 (ACC1), which is a rate-limiting enzyme for fatty acid biosynthesis, revealed a higher expression level of the protein in Uc39-6 cells than in Uc321 cells. The ACC1 mRNA content in Uc39-6 cells was 1.4-fold higher than that in Uc321 cells. These results strongly suggest that enhancement of fatty acid biosynthesis in core-expressing cells is caused by increased expression of fatty acid biosynthetic enzymes, especially ACC1. Up-regulation of *de novo* fatty acid biosynthesis by HCV core protein may affect cellular lipid metabolism, resulting in neutral lipid accumulation in HCV-infected cells.

Key words fatty acid biosynthesis; hepatitis C virus; acetyl-CoA carboxylase; fatty acid synthase

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis.[1,2] Persistent HCV infection, which develops in at least 70 to 80% of infected patients, is strongly correlated with the development of severe liver diseases such as cirrhosis and hepatocellular carcinomas (HCC).[3,4] In addition, liver steatosis, which involves the accumulation of intracellular neutral lipids as lipid droplets, is a hallmark of chronic HCV infection,[5] and is suggested to play a central role in the progression of the following liver cirrhosis and HCC in chronic hepatitis C patients.[6] Since more than 170 million people in the world are currently infected with HCV,[1] understanding the mechanisms by which HCV induces serious liver diseases is one of the most important global public health issues.

HCV, belonging to the *Flaviviridae* family, possesses a linear, positive-stranded RNA genome of *ca.* 9600 nucleotides.[7] The HCV genome has a single open reading frame encoding a precursor polyprotein of *ca.* 3000 amino acids that is processed into at least 10 individual proteins by host and viral proteases.[8] HCV core protein, the product of the N-terminal portion of the polyprotein, forms the nucleocapsid of an HCV virion.[9] Besides its function as a viral structural protein, the core protein causes intracellular lipid accumulation as well as malignant transformation in cultured cells.[10—13] Moreover, transgenic mice expressing HCV core protein developed liver steatosis and the following HCC.[14,15] These results strongly suggest that HCV core protein is involved in the pathogeneses of liver diseases including steatosis due to HCV infection.

The details of the mechanisms by which HCV core protein causes intracellular neutral lipid accumulation are not well understood. Extensive screening for genes/proteins exhibiting differences in cellular expression involving cDNA microarray or proteome analysis has been performed for HCV core-expressing cultured liver cells or transgenic mice.[16—19] Although various genes/proteins were identified, direct information on the genes/proteins related to lipid metabolism altered by HCV core protein expression has not been obtained yet. Since HCV core protein is distributed mainly in lipid droplets of host cells,[10,13,20—23] the biogenesis and/or functions of lipid droplets might be affected by the core protein. As reported, the core protein appears to inhibit microsomal triglyceride transfer protein activity in the livers of HCV core-transgenic mice, thus interfering with the hepatic assembly and secretion of apo-B-carrying very low density lipoproteins.[24] As a result, triglycerides appear to accumulate within hepatocytes, steatosis developing. HCV core protein also interacts with apoA2, a major component of high-density lipoproteins, in cells.[10,25] These results should be important regarding the pathogenesis of HCV core-derived steatosis, but may not explain all the functions of the core protein causing intracellular neutral lipid accumulation. In this study, we investigated *de novo* fatty acid biosynthesis, which can significantly affect intracellular lipid metabolism, especially neutral lipid accumulation,[26,27] in HCV core-expressing liver cells. We found elevated fatty acid biosynthesis, and higher expression and activities of the enzymes responsible for *de novo* fatty acid biosynthesis in HCV core-expressing cells.

MATERIALS AND METHODS

Cell Lines The human hepatic Huh7 cell line constitutively expressing HCV core protein (Uc39-6) was established by transfection with pcEF39neo.[28] Expression level of HCV core protein in Uc39-6 cells was similar to that in core-expressing Hep39 cells (data not shown), which we established previously.[28,29] Another Huh7 cell line transfected with ex-

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pression vector pcEF321swxneo[28] without the HCV core protein insert (Uc321) was used as a mock control. Both cell lines were plated on collagen-coated dishes (Asahi Techno Glass, Japan) and maintained in normal culture medium (DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 1 mg/ml G418 (Sigma, U.S.A.)) under a 5% CO_2 atmosphere at 37°C. Growth rates under the culture condition we used were comparable between Uc321 and Uc39-6 cells.

Metabolic Labeling of Cellular Lipids Subconfluent cell monolayers in 6-cm dishes were incubated in 2 ml of the normal medium containing 1.3 μM [^{3}H]acetate (Moravek Biochemicals, U.S.A.) for various times. After being washed three times with 3 ml of PBS, the cells were lysed in 1 ml of 0.1% SDS at 4°C, and then 100 and 800 μl aliquots of the resultant cell lysate were used for protein determination and lipid extraction, respectively. Cellular lipids were extracted into the organic solvent[30] and dried up. To determine the total metabolic incorporation of [^{3}H]acetate into the fatty acid moieties of lipids, the extracted lipids were treated with 90% ethanol containing 1 N KOH for 1 h at 70°C and then re-extracted with petroleum ether. Fatty acids were then separated on TLC plates with a solvent system of hexane/diethylether/acetate (70/30/1, vol/vol). The radioactivity of fatty acids was determined with a BAS1800 imaging analysis system (Fuji Film, Japan). The values were normalized as to cell protein.

***In Vitro* Acetyl-CoA Carboxylase (ACC) and Fatty Acid Synthase (FAS) Activity Assays** ACC activity assays were performed using [^{14}C] $KHCO_3$ (American Radiolabeled Chemicals, U.S.A.).[31] FAS activity was measured using [^{14}C]acetyl-CoA (Moravek Biochemicals, U.S.A.).[32] Cytosolic fractions, which contain ACC1 and FAS, were prepared as below. After being washed with PBS, cells were harvested and precipitated by centrifugation (300×*g*, 5 min). The precipitated cells were resuspended in 125 mM potassium phosphate, pH 7.0, and then lysed by sonication. After centrifugation of each lysate at 2500×*g* for 5 min, the cytosolic fraction (supernatant) was separated from the post-nuclear supernatant by centrifugation at 100000×*g* for 60 min. The protein concentrations of the preparations were determined with a BCA protein assay kit (Pierce, U.S.A.).

Immunoblot Analysis Equivalent amounts of proteins from Uc321 and Uc39-6 cells were separated in a 4—12% SDS-polyacrylamide gel and then electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked overnight at 4°C in TBS containing 0.1% Tween 20 and 5% skim milk. The blots were probed with a rabbit polyclonal anti-ACC1 antibody (Upstate, U.S.A.) (1 : 1000), a mouse monoclonal anti-FAS antibody (BD Transduction Laboratories, U.S.A.) (1 : 1000), and a mouse monoclonal anti-HCV core protein antibody (Anogen, Canada) (1 : 2000) for 90 min at room temperature. The blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (BIO-RAD, U.S.A.), or HRP-conjugated goat anti-mouse IgG (GE Healthcare, U.S.A.) at 1 : 2000 dilution for 60 min. Detection of immunoreactive proteins was performed with an ECL system (GE Healthcare, U.S.A.).

Quantitative Real-Time PCR Analysis Cellular total RNAs were prepared with an RNeasy kit (Qiagen, U.S.A.). The total RNA fraction (1 μg) was processed directly to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, U.S.A.). Of the total 20 μl cDNA solution, an aliquot of 0.5 μl was used for each real-time PCR assay. The PCR primers used for human ACC1 were: forward, CTGTTGGCTCAGATACACTC, and reverse, GCCACAGTGAAATCTCGTT. The PCR primers for human FAS were: forward, GTGGGAAGGTGTACCAGTG, and reverse, AGGATGCCCTGGAAATGAG. Quantitative real-time PCR was carried out in a LightCycler (Roche, U.S.A.) using LightCycler-FastStart DNA Master SYBR Green I (Roche, U.S.A.). Specific PCR products amplified against individual genes were used as quantitative standards.

RESULTS

To determine the effect of HCV core protein expression on cellular fatty acid biosynthesis, we established Uc39-6 cells, a human hepatic Huh7 cell line transfected with the pcEF321 mammalian expression vector containing the HCV core protein gene, and Uc321 cells, a control Huh7 cell line transfected with the pcEF321 vector without the core protein gene. Consistent with previous studies involving HCV core-expressing hepatic cell lines,[10,33] HCV core protein was preferentially distributed in the endoplasmic reticulum and lipid droplets in Uc39-6 cells, as determined on immunofluorescence microscopy (data not shown). *De novo* fatty acid biosynthesis in HCV core-expressing and non-expressing cells was examined by metabolic labeling with [^{3}H]acetate. Most biosynthesized [^{3}H]fatty acids were rapidly incorporated into complex lipids such as phospholipids, triglycerides, and cholesteryl esters, but free [^{3}H]fatty acids were not detectable (<1/1000 of total [^{3}H]fatty acids formed) in these cells. Incorporation of radioactivity into the fatty acid moieties of complex lipids was *ca.* 1.5-fold higher in HCV core-expressing Uc39-6 cells than that in non-expressing Uc321 cells during the incubation time at 37°C (Fig. 1). Similar results were obtained with other Huh7 cell lines expressing HCV core protein (data not shown), ruling out the possibility that the increase in radiolabeled fatty acids in HCV core-expressing cells are due to the peculiar cell clones. These results indicate that the rate of *de novo* biosynthesis of cellular fatty acids is enhanced in HCV core-expressing cells.

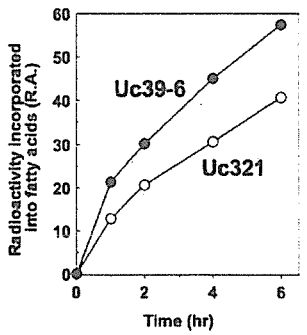

Fig. 1. Fatty Acid Biosynthesis in HCV Core-Expressing Uc39-6 and Control Uc321 Cells

Cells were metabolically labeled with [^{3}H]acetate for the indicated times. The radioactivity incorporated into the fatty acid moieties of lipids was determined as described under Materials and Methods. Open circles, Uc321 cells; closed circles, Uc39-6 cells. R.A., relative radioactivity. Data are representative of four independent experiments.

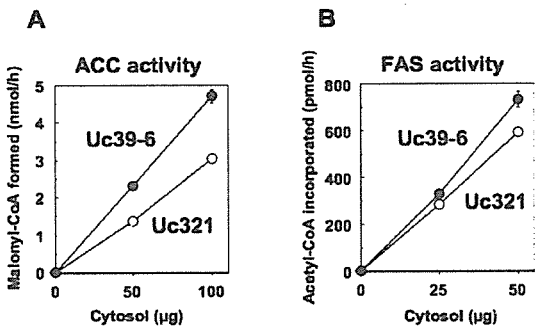

Fig. 2. ACC and FAS Activities in Uc321 and Uc39-6 Cytosolic Fractions

In vitro activity assaying of ACC (A) and FAS (B) was performed as described under Materials and Methods. Open circles, Uc321 cells; closed circles, Uc39-6 cells. Data are expressed as means±S.D. for three determinations.

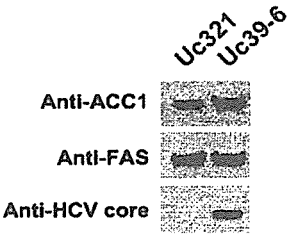

Fig. 3. Immunoblot Analysis of ACC1 and FAS in Uc321 and Uc39-6 Cells

Total cell lysates (30 μg of protein per lane) of Uc321 and Uc39-6 cells were analyzed by immunoblotting with anti-ACC1, anti-FAS, and anti-HCV core antibodies.

Fatty acid biosynthesis is carried out by cytosolic enzymes, *i.e.*, ACC1 and FAS.[34] We assayed these enzyme activities in cytosolic fractions of Uc321 and Uc39-6 cells. HCV core-expressing Uc39-6 cells showed a *ca.* 1.6-fold higher level of ACC activity than Uc321 cells (Fig. 2A). The cytosolic FAS activity in Uc39-6 cells was slightly higher than that in Uc321 cells (Fig. 2B). These results demonstrate that the enzymatic activities responsible for fatty acid biosynthesis, especially ACC activity, are elevated in HCV core-expressing Uc39-6 cells, consistent with the results of metabolic labeling experiments involving [^{3}H]acetate.

Cytosolic ACC and FAS activities are attributed to ACC1 and FAS molecules, respectively.[34] We next performed immunoblot analyses of ACC1 and FAS molecules in lysates of Uc321 and Uc39-6 cells. Uc39-6 cells contained a *ca.* 2-fold higher amount of ACC1 protein than Uc321 cells (Fig. 3), whereas the protein level of FAS in UC39-6 cells was comparable to that in Uc321 cells. These results suggest that the elevated protein level of ACC1, a rate-limiting enzyme for fatty acid biosynthesis, may contribute to the higher ACC activity, leading to enhanced fatty acid biosynthesis, in HCV core-expressing Uc39-6 cells. We also determined the mRNA levels of ACC1 and FAS in Uc321 and Uc39-6 cells by quantitative real-time PCR. Consistent with the protein levels of ACC1, the ACC1 mRNA content in Uc39-6 cells was significantly higher than that in Uc321 cells (Fig. 4A). The FAS mRNA content in Uc39-6 cells was also higher than that in Uc321 cells (Fig. 4B). These results suggest that elevated expression of the ACC1 (and FAS) gene(s) causes a higher rate of *de novo* fatty acid biosynthesis in HCV core-expressing cells.

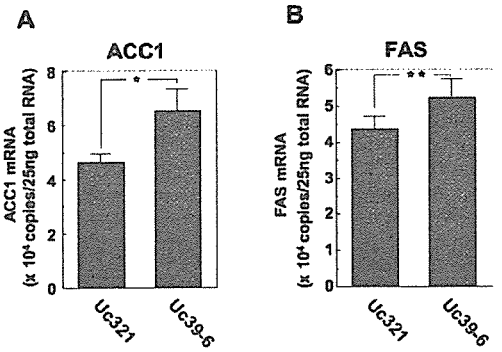

Fig. 4. ACC1 and FAS mRNA Levels in Uc321 and Uc39-6 Cells

Total RNA was isolated from Uc321 and Uc39-6 cells, and the ACC1 (A) and FAS (B) mRNA levels were determined by quantitative real-time PCR. Data are expressed as means±S.D. for three determinations. Statistical significance of differences in mRNA levels between Uc321 and Uc39-6 cells was evaluated using Student's *t* test. $*p<0.01$, $**p<0.025$.

DISCUSSION

In this study we showed that the rate of *de novo* fatty acid biosynthesis was elevated in HCV core-expressing cells (Fig. 1). We also demonstrated that the protein and mRNA expression levels as well as the *in vitro* enzymatic activity of ACC1, which is a rate-limiting key enzyme for fatty acid biosynthesis, were significantly enhanced in HCV core-expressing Uc39-6 cells (Figs. 2—4). These results strongly suggest that the higher expression of ACC1 contributes to the increased fatty acid biosynthesis in HCV core-expressing cells.

Many studies have demonstrated that HCV core protein substantially affects various cellular regulatory processes including gene transcription.[35—37] These biological activities of HCV core protein might be involved in the mechanism by which HCV core protein enhances expression of the ACC1 gene.

HCV core protein is localized mainly in lipid droplets of host cells,[10,13,20—23] and a small portion of ACC1 molecules is also associated with lipid droplets.[38] It is well known that the activity of ACC1 can be regulated posttranslationally through its phosphorylation, and allosteric effectors such as citrate and fatty acids.[39] Thus, we can not exclude the possibility that HCV core protein activates ACC1 directly in lipid droplets, although we have not examined this yet.

We also found that the FAS mRNA expression level was slightly enhanced, but the protein level as well as the *in vitro* enzymatic activity of FAS were not significantly elevated in Uc39-6 cells (Figs. 2—4). FAS appears to make a lesser contribution to the enhancement of fatty acid biosynthesis in Uc39-6 cells under our culture conditions, although further investigations are needed.

We think that our findings may provide a new insight into the metabolic pathways for lipids by which HCV core protein causes steatosis, one of the characteristic indications of chronic hepatitis C infection. Although in the future we have to determine whether or not our *in vitro* findings are applicable to an *in vivo* situation on HCV infection, inhibition of ACC1 and/or FAS activity might be effective for preventing the liver steatosis due to HCV infection.

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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-*Mlu*I-*Bgl*II. The intermediate vector pGL3-*Mlu*I-*Bgl*II-S232I was constructed by introducing the point mutation S232I of NS5A into the MluI and SacI sites of pGL3-*Mlu*I-*Bgl*II by site-directed mutagenesis using primers carrying the necessary nucleotide changes. Subsequently, mutations were introduced into pGL3-*Mlu*I-*Bgl*II-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-*Mlu*I-*Bgl*II-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′-TATCGAGCTCGATGTCAATGTCCTACTCATGGACAGGT-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-*Mlu*I-*Bgl*II-S232I containing each mutation into the EcoRI and SmaI sites of pNKFLAGNS5Bt.

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 10^7 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.

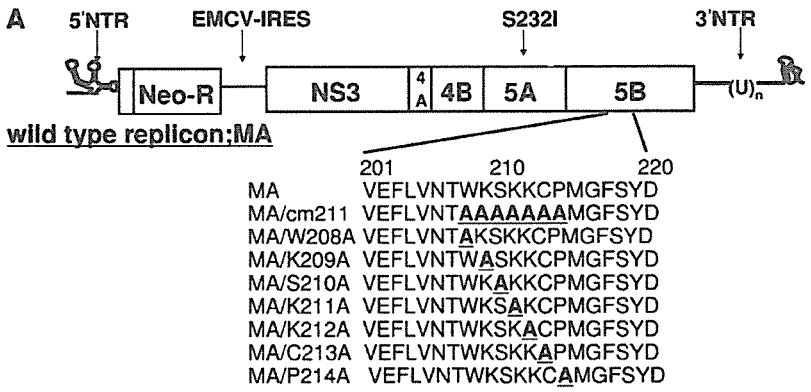
Preparation of cell extracts, coprecipitation with glutathione resin, and Western blot analysis. COS1 cells were transiently transfected using the calcium-phosphate method. The cells were harvested, washed with PBS(−), and sonicated in PBS lysis buffer [PBS(−) containing 150 mM NaCl, 1.0% Triton X-100, 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 reprobed 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-deoxyribosylthymine [dT]dT), and siRNA for HCV (CCUCAAAGAAAAACCAAAC-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



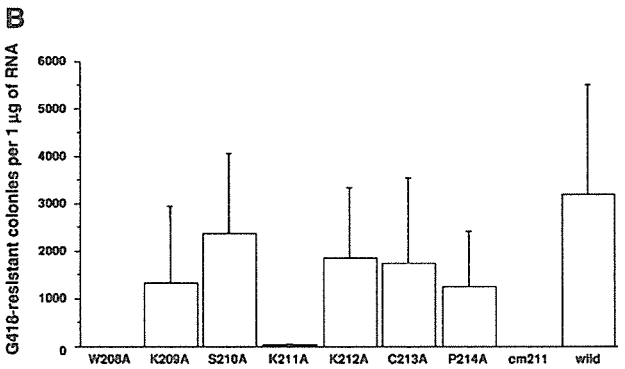


FIG. 1. (A) Schematic representation of the mutant replicons used in this study. MA is a replicon carrying the point mutation S232I in NS5A and was used as the wild-type replicon. The mutant replicons MA/cm211, MA/W208A, MA/209A, MA/S210A, MA/211A, MA/212A, MA/213A, and MA/214A carry NS5B mutations in 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

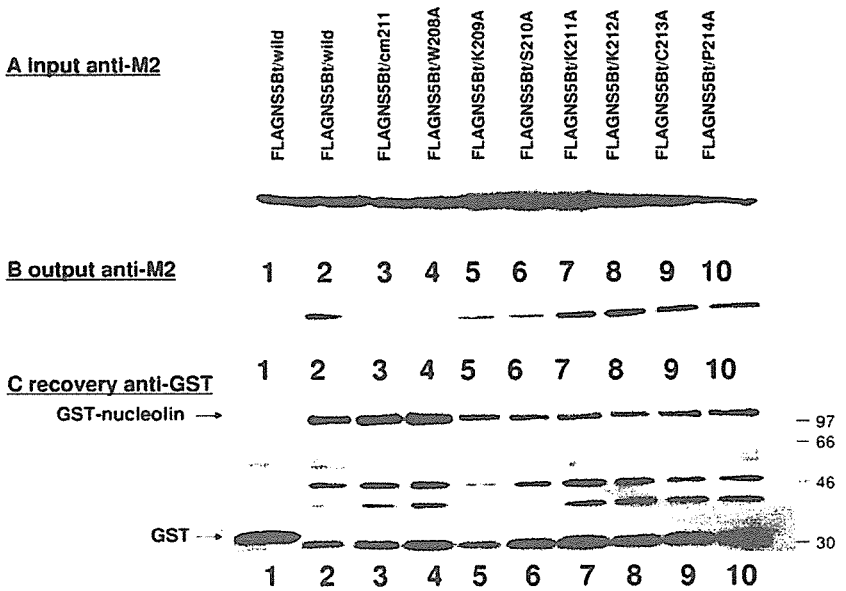


FIG. 2. Interaction between nucleolin and NS5B of HCV isolate M1LE and an essential residue for this interaction. COS1 cells were transiently cotransfected with mammalian expression vectors expressing FLAG-NS5Bt proteins (lanes: 1 and 2, wild type; 3, cm211; 4, W208A; 5, K209A; 6, S210A; 7, K211A; 8, 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 reprobed 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 μM, 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 μM 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.

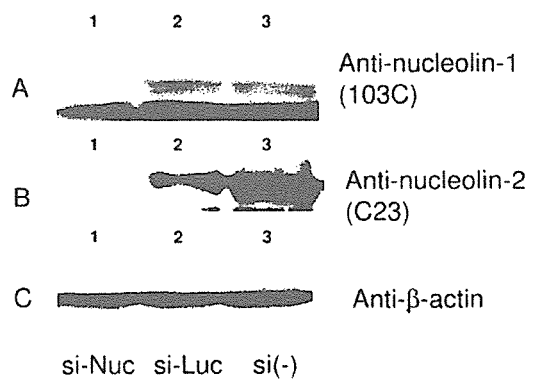


FIG. 3. Knockdown of endogenous nucleolin by siRNA. Huh7-DMB cells were electroporated with 1 μM si-Nuc and si-Luc. After 48 h, total cell lysates were fractionated by SDS–10% PAGE and subjected to Western blot analysis with the anti-nucleolin antibodies anti-nucleolin-1 (103C) in A and anti-nucleolin-2 (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(−)].

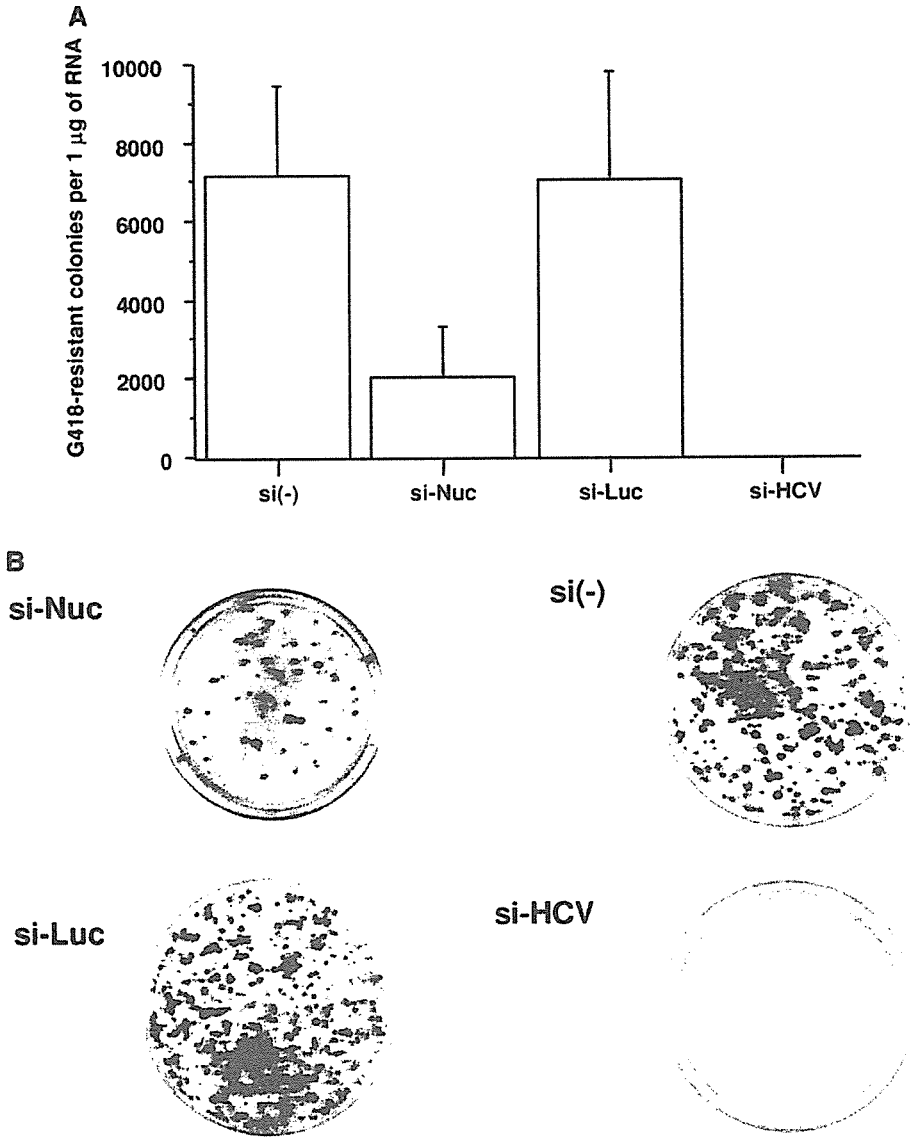


FIG. 4. Effect of suppression of endogenous nucleolin on HCV replication in the MA replicon. Huh7-DMB cells were electroporated with 1 μg of in vitro-transcribed MA RNA plus si-Nuc, si-Luc, si-HCV, or no siRNA [si(−)], and G418-resistant cells were selected with 1 mg/ml G418 and were stained 4 weeks later. (A) Mean number of G418-resistant colonies per 10-cm-diameter cell culture dish per 1 μg 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.

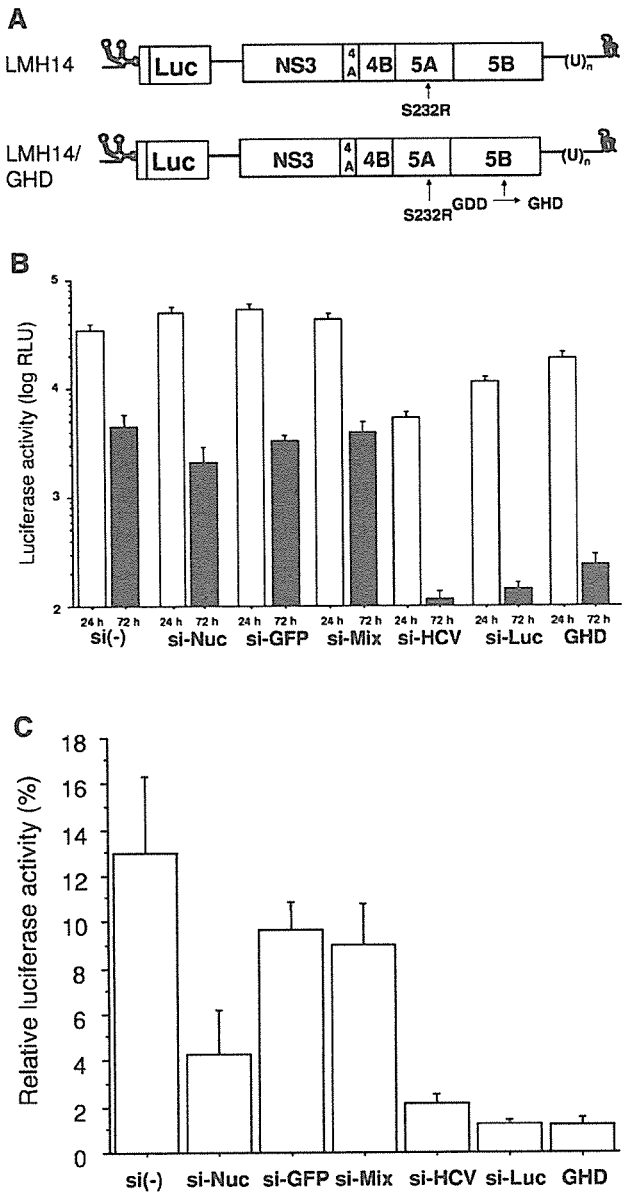


FIG. 5. Effect of suppression of endogenous nucleolin on HCV replication in the LMH14 replicon. (A) Schematic representation of the luciferase replicon. In the LMH14 replicon, the neomycin resistance gene was replaced by a luciferase gene, and S232 of NS5A was replaced by R. In the LMH14/GHD replicon, 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 μM 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 coexpressed 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).

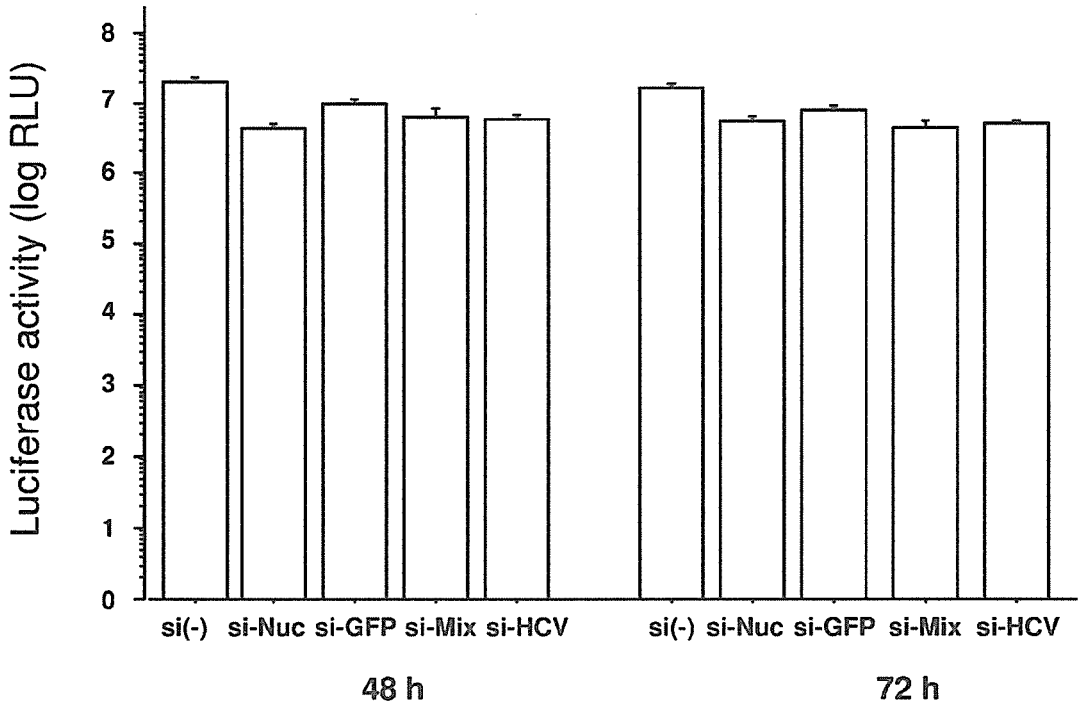


FIG. 6. Effect of suppression of endogenous nucleolin on cell proliferation. The plasmid pGL3 control, encoding the luciferase gene under the control of the CMV promoter/enhancer, was cotransfected with 2 μM of si-Mix, si-GFP, si-Nuc, si-HCV, or no siRNA [si(−)] using DMRIE-C reagent, and luciferase 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 transient 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 NS5Bt 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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Diverse Effects of Cyclosporine on Hepatitis C Virus Strain Replication

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Recently, a production system for infectious particles of hepatitis C virus (HCV) utilizing the genotype 2a JFH1 strain has been developed. This strain has a high capacity for replication in the cells. Cyclosporine (CsA) has a suppressive effect on HCV replication. In this report, we characterize the anti-HCV effect of CsA. We observe that the presence of viral structural proteins does not influence the anti-HCV activity of CsA. Among HCV strains, the replication of genotype 1b replicons was strongly suppressed by treatment with CsA. In contrast, JFH1 replication was less sensitive to CsA and its analog, NIM811. Replication of JFH1 did not require the cellular replication cofactor, cyclophilin B (CyPB). CyPB stimulated the RNA binding activity of NS5B in the genotype 1b replicon but not the genotype 2a JFH1 strain. These findings provide an insight into the mechanisms of diversity governing virus-cell interactions and in the sensitivity of these strains to antiviral agents.

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, has a positive-strand RNA genome (1, 26). The genome encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (6, 8). NS5B is an RNA-dependent RNA polymerase that is crucial for viral genome replication (1, 26). There is genetic heterogeneity within the HCV genome. Currently, these differences are classified into six genotypes that are further segregated into a series of subtypes (4, 23). In Japan, genotype 1b is predominant; roughly 65% of cases of HCV-related chronic hepatitis involve genotype 1b. By comparison, genotype 2a is present in 17% of these patients (13, 23).

Sustained infection of HCV is the major cause of chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (16). Rarely, HCV causes fulminant hepatitis (13). The predominant treatment for HCV-infected patients is interferon (IFN) or polyethylene glycol-conjugated IFN alone or in combination with ribavirin (19, 20). However, alternative anti-HCV therapies are needed because virus is not eliminated in about half of the treated patients (19, 20). Lohmann et al. have developed the HCV subgenomic replicon system, in which an HCV subgenomic replicon autonomously replicates in Huh-7 cells (HCV replicon cells) (18). This replicon comprises the HCV 5′ untranslated region (5′UTR) containing an internal ribosomal entry site (IRES), the neomycin phosphotransferase gene, the encephalomyocarditis virus (EMCV) IRES, the coding region for HCV NS3 through NS5B, and the HCV 3′UTR (subgenomic replicon), but it lacks the coding region for the core and envelope proteins, as well as p7 and NS2 (Fig. 1). Subsequently, a genome-length (full-genome) replicon has been developed. This construct contains a full-genome length of HCV, including the coding regions for the core protein through NS2 (Fig. 1) (5, 10). We can evaluate HCV replication using these subgenomic or genome-length replicon systems. Previously, we established HCV subgenomic replicon cells carrying HCV genotype 1b NN strain (15, 29). We demonstrated that an immunosuppressant, cyclosporine (CsA), has anti-HCV activity in these cells (29). In addition, we determined the molecular mechanism of the anti-HCV effect of CsA on this replicon; cyclophilin B (CyPB), one of the cellular targets of CsA, is a cellular replication cofactor of the HCV genome (31). CyPB interacts with NS5B to promote its RNA binding activity (for a detailed description, see reference 31). CsA is suggested to suppress HCV genome replication by inhibiting the functional association of CyPB with NS5B. Another group also reported anti-HCV function of CsA using a subgenomic replicon of other genotype 1b strain, HCV-N (22). In this study, we demonstrate that CsA also has a strong anti-HCV activity in other available genotype 1b replicons carrying the Con1 and O strains (12, 18).

Recently, Wakita and colleagues reported that a replicon of HCV genotype 2a JFH-1 strain, which was isolated from a case of type-C fulminant hepatitis, has a much stronger level of replication activity than genotype 1b replicons in Huh-7 cells (13, 27). A production system of infectious viral particles was recently established with this high-replication-competent strain (17, 27, 34). This viral strain may acquire a growth advantage compared with many other strains, although the underlying mechanism is unknown. In this study, we described a characteristic difference in the replication of JFH1 compared to that of genotype 1b replicons.

Here, we report that JFH1 replication is less sensitive to CsA than genotype 1b strains, although the interaction of

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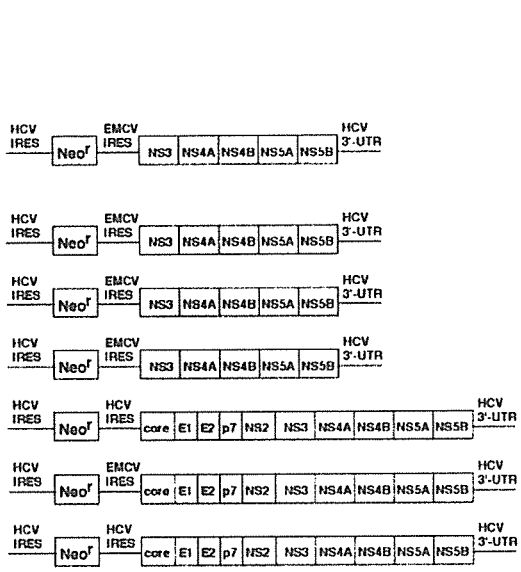

HCV strain (genotype)	replicon cell clone	sensitivity to CsA
NN (1b)	MH-14, #50-1 MH14#W31	highly sensitive
Con1 (1b)	SN1	highly sensitive
O (1b)	sO	highly sensitive
JFH1 (2a)	JFH1#4-1, JFH1#2-3	less sensitive
NN (1b)	NNC#2	highly sensitive
Con1 (1b)	SN1A#2	highly sensitive
Con1 (1b)	SNC#7	highly sensitive

FIG. 1. Schematic representation of the constructs of HCV subgenomic and genome-length replicon RNA. On the left, the constructs of each replicon RNA are shown. HCV strains, as well as genotypes from which the replicon RNA sequences are derived, are indicated in the second column. The names of replicon cell clones established with each replicon RNA are in the third column. The sensitivity to CsA of each replicon RNA revealed in this study is summarized in the fourth column. The replicon RNAs comprise the HCV 5′UTR, including HCV IRES, the neomycin phosphotransferase gene (Neo^r), EMCV IRES, or HCV IRES, the coding region for HCV proteins NS3 to NS5B (subgenomic) or core to NS5B (genome length or full genome), and HCV 3′UTR. MH-14 (NN/1b/SG), #50-1 (NN/1b/SG), MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 (JFH1/2a/SG) cells carry subgenomic replicons, while NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 (Con1/1b/FL) cells have genome-length replicons. NNC#2 (NN/1b/FL) and SNC#7 (Con1/1b/FL) cells contain the replicon RNA without EMCV IRES.

CyPB with NS5B is observed with this replicon. However, genome replication and RNA binding activity of NS5B are independent of CyPB. We have exploited a chemical compound to demonstrate how strain diversity can be generated by underlying differences in the mechanisms of the virus-cell interaction. These findings provide important insight into the mechanisms that mediate the efficacy of antiviral agents.

MATERIALS AND METHODS

Cell culture. Huh-7 cells were cultured in Dulbecco's modified Eagle medium (Invitrogen) with 10% fetal bovine serum, nonessential amino acids (Invitrogen), and L-glutamine (Invitrogen). MH-14, #50-1, MH14#W31, SN1, sO (formerly named 1B2R1), JFH1#4-1, and JFH1#2-3 cells (12, 13, 15, 18, 29), carrying subgenomic replicons, and NNC#2, SN1A#2, and SNC#7 cells, carrying full-genome replicons, were cultured in the above medium supplemented with 300- to 500-μg/ml G418 (Invitrogen). In the assay measuring the response to CsA, NIM811, or PSC833 (Fig. 2, 3, and 4), we seeded small numbers of each replicon cells (7×10^3 to 15×10^3 cells/12-well plate) and treated with each drug. Culture medium was changed every 3 days (CsA, NIM811, or PSC833 was supplemented in the fresh medium for the treatment groups). We did not perform any passages in the assay period. At day 7, the cells were 70 to 90% confluent. A schematic representation of the constructs of HCV replicon RNAs, the name of HCV strains from which the replicon RNA sequences are derived, and the name of replicon cell clones used in this study are summarized in Fig. 1. Since many replicon clones were used in this study, we list "strain/genotype/length of the replicon construct" in parentheses after the names of each cell clone in Results and in the figure legends to avoid confusion between names: for example, MH-14 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and SN1A#2 (Con1/1b/FL) cells. The designations SG and FL indicate subgenomic and full-genome replicons, respectively.

Establishment of replicon cells. MH-14, #50-1, sO, JFH1#4-1, and JFH1#2-3 cells were described previously (12, 13, 15, 29). The replicon RNAs were produced using a MEGAscript T7 kit (Ambion) from pMH14, pSN1, pNNC, pSN1A, and pSNC plasmids for the establishment of the MH14#W31, SN1, NNC#2, SN1A#2, and SNC#7 replicon cells, respectively. For the establishment of MH14#W31, we transfected RNA into the Huh-7 cell strain which was identical to the parental cells of JFH1#4-1 and JFH1#2-3. Each replicon RNA was transfected into Huh-7 cells, following the selection with the medium in the presence of 500- to 1,000-μg/ml G418 for around 4 weeks. The resultant cell colonies were isolated and expanded. The HCV RNA titers in cell clones carrying JFH1 replicons were not significantly different from those in established cell clones carrying genotype 1b replicons.

Plasmid construction. pSN1, the sequence of which is derived from I377NS3-3′ (18), was prepared essentially as described previously (15). pSN1A was generated by inserting the region from the core to NS2 of pM1LE (15) into the upstream coding region for NS3 in pSN1. To obtain pSNC, the EMCV IRES of pSN1A was replaced by the HCV IRES. pNNC was produced by inserting the coding region from NS3 to NS5B of pM1LE into pSNC.

Real-time reverse transcription-PCR (RT-PCR) analysis. The 5′UTR of HCV genome RNA was quantified using the ABI PRISM 7700 sequence detector (Applied Biosystems) as described previously (29).

Immunoblot analysis. Immunoblot analysis was performed as described previously (30). The primary antibodies used in this study were anti-core, anti-E2 (kindly provided by M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-NS3, anti-NS5A (a generous gift from A. Takamizawa, Osaka University), anti-NS5B (NS5B-6; kindly provided by I. Fukuya, Osaka University), anti-CyPA (Upstate Cell Signaling), anti-CyPB (Affinity BioReagents), and anti-tubulin (Oncogene).

Immunoprecipitation assay and RNA-protein binding precipitation assay. Immunoprecipitation and RNA-protein binding precipitation were performed as described previously (30, 31).

RNA interference technique. The condition of small interfering RNA (siRNA) used in this study was described previously (31). Transfection was performed using siLentFect (Bio-Rad), according to the manufacturer's protocol.

Isolation of replication complex. The HCV replication complex was isolated from cells by treatment with 50-μg/ml digitonin at 27°C for 5 min, following treatment with 0.3-μg/ml proteinase K at 37°C for 5 min as described previously (31).

Purification of recombinant GST-fused CyPB protein. Glutathione *S*-transferase (GST) and GST-fused CyPB (GST-CyPB) protein expression was induced

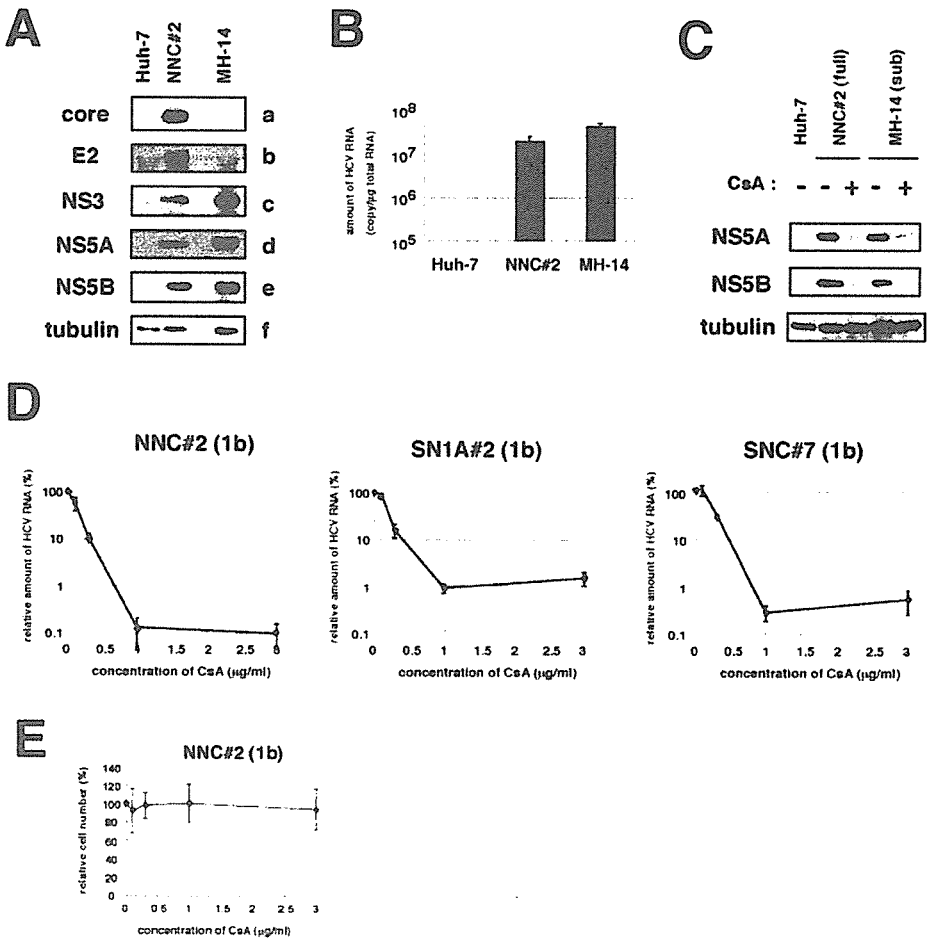

FIG. 2. CsA suppressed the replication of HCV genome, irrespective of the presence of the structural proteins. (A) Detection of HCV proteins from NNC#2 (NN/1b/FL) genome-length replicon. Core (a), E2 (b), NS3 (c), NS5A (d), NS5B (e), and tubulin (f) in Huh-7, NNC#2 (NN/1b/FL), and MH-14 (NN/1b/SG) cells analyzed by immunoblot analysis are shown. (B) HCV RNA in Huh-7, NNC#2 (NN/1b/FL), and MH-14 (NN/1b/SG) cells quantified by real-time RT-PCR analysis. The data represent the means of three independent experiments. (C) CsA decreased the production of HCV proteins in NNC#2 (NN/1b/FL), as well as in MH-14 (NN/1b/SG) cells. After treatment with 1-μg/ml CsA (+) for 5 days or without treatment (−), total-cell lysates of NNC#2 (NN/1b/FL) and MH-14 (NN/1b/SG) cells, together with Huh-7 cells as a negative control, were recovered to examine the production of HCV NS5A (top), NS5B (middle), and tubulin as an internal control (bottom) by immunoblot analysis. The same result was obtained at day 7 after treatment. (D) The sensitivity to CsA of HCV genome-length replicon was almost the same as that of the subgenomic replicon. HCV RNA was quantified by real-time RT-PCR analysis using total RNA from NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 (Con1/1b/FL) cells treated with various concentrations of CsA for 7 days. The relative amount of HCV RNA was plotted against the concentration of CsA (in micrograms per milliliter). (E) Effect of CsA on cell proliferation. NNC#2 (NN/1b/FL) cells were treated with various amount of CsA for 7 days. Cell numbers were counted, and cell numbers relative to those of cells without treatment were plotted against the concentration of CsA.

in transformed BL21 cells (Amersham) with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). The cell lysate was incubated with glutathione-Sepharose resin (Amersham) and washed extensively. The recombinant protein was eluted by glutathione (pH 8.0) and subsequently dialyzed.

In vitro RNA binding assay. In vitro-translated ^{35}S-labeled NS5B proteins and poly(U)-Sepharose (Amersham) or protein G-Sepharose (Amersham) resin as a negative control were incubated in the presence of recombinant GST-CyPB protein at 4°C for 1 h. After being washed, precipitates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by imaging analyzer.

RESULTS

CsA suppressed the replication of HCV full-genome replicon. We and another group have reported an anti-HCV activity of CsA using subgenomic replicons (22, 29). HCV structural proteins, especially the core protein, have multiple functions. These proteins interact with many cellular factors and modulate a variety of cellular functions (32). Potentially, these viral proteins could diminish or circumvent the suppression of HCV genome replication by CsA. Core protein and E2 reportedly modulate the activity of IFN signaling (9, 25). To test this possibility, we established a full-genome HCV replicon system with cells transfected with the NN strain (NNC#2 cells [NN/1b/FL]) (Fig. 1). HCV RNA and protein productions were confirmed by real-time RT-PCR and immunoblot analysis (Fig. 2A and B). In addition, we confirmed that this replication was not due to the integration of the replicon construct into the