Ubiquitination of eIF4E

Cells respond to stress or apoptotic stimuli through regulating protein synthesis levels (21, 22). Nevertheless, the molecular mechanisms regulating cell reactions to such stimuli remain elusive. Chip, an E3 ligase, and stresses, such as heat shock and cadmium, enhanced the Ub conjugation of eIF4E. Because Chip is involved in the quality control of proteins in cells (34, 35), the ubiquitination/degradation of eIF4E may be, at least in part, controlling protein synthesis in response to stress.

eIF4E plays an important role in translation initiation, and it is important to understand the processes regulating eIF4E protein expression levels, including its degradation. Because eIF4E functions in association with many factors, future studies should examine the role of eIF4E ubiquitination within the context of the entire translation initiation complex.

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Inhibition of hepatitis C virus RNA replication by short hairpin RNA synthesized by T7 RNA polymerase in hepatitis C virus subgenomic replicons

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Abstract

RNA interference (RNAi) is a cellular process that induces gene silencing by which small duplexes of RNA specifically target a homologous sequence for cleavage by cellular ribonucleases. Here, to test the RNAi method for blocking hepatitis C virus (HCV) RNA replication, we created four short hairpin RNAs (shRNAs) targeting the HCV internal ribosome entry site/Core gene transcript using T7 RNA polymerase. shRNA suppressed the replication of HCV RNA in the HCV replicon. On the other hand, short interfering RNAs synthesiz using the T7 RNA polymerase system trigger a potent induction of interferon-α and -β in a variety of cells. We examined whethe e shRNAs synthesized using the T7 RNA polymerase system activated double-stranded RNA-dependent protein kinase, $2'-5'$ oligoadenylate synthetase, or interferon-regulatory factor-3. Our results demonstrated that the T7-transcribed shRNA did not act.vate these proteins in Huh-7 cells and the HCV replicon. These shRNAs are a promising new strategy for anti-HCV gene therapeutics.
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Keywords: Hepatitis C virus; RNAi; Synthesized shRNA; T7 RNA polymerase; IFN; PKR; $2'-5'$OAS; Hepatitis C virus subgenomic replicons

RNA interference (RNAi) occurs in a variety of organisms, including *Caenorhabditis elegans* [1], *Trypanosoma brucei* [2], plants [3], *Drosophila* [4], planaria [5], zebra fish [6], and mouse embryos [7]. In most of these organisms, the injection of a double-stranded RNA (dsRNA) longer than 500 bp specifically suppresses the expression of the gene with the corresponding DNA sequence, but has no effect on genes with unrelated sequences.

RNAi is initiated by the RNase III-like nuclease Dicer, which promotes progressive cleavage of long dsRNAs into 21 to 27 nucleotide (nt) short interfering RNAs (siRNAs)

with two nt 3'-overhangs. Subsequently, the siRNAs are incorporated into an RNA-induced silencing complex (RISC), identified in *Drosophila*, and the protein-RNA effector nuclease complex recognizes and destroys the target mRNAs [8–10].

Hepatitis C virus (HCV) is one of the main causes of liver-related morbidity and mortality [11]. The virus establishes a persistent infection in the liver, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinomas [11]. HCV replication occurs in the cytoplasm and is associated with membranes that appear to be derived from the endoplasmic reticulum. Genomic HCV RNA is translated to produce a 3000-amino acid polypeptide that is processed into at least 10 proteins. The nonstructural proteins 3, 4A, 4B, 5A, and 5B

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form a replicase complex that promotes transcription of a genomic (−) strand intermediate. This serves as a template for the production of (+) strands that are either translated or packaged into virions as genomic RNAs [12,13]. A satisfactory treatment for HCV infection has yet to be developed, however, because studies of HCV have been hampered by the lack of a stable cell-culture system and a small-animal model. One recently reported HCV replicon is a selectable subgenomic HCV RNA, which replicates efficiently and continuously in human hepatoma Huh-7 cells [14,15]. HCV RNA replication is also sensitive to RNAi [16–18].

On the other hand, it was previously reported that dsRNA triggers the production of type I interferon (IFN), and activates dsRNA-dependent protein kinase (PKR) [19] and 2'-5'oligoadenylate synthetase (2'-5'OAS). Furthermore, two recent studies demonstrated that the mechanism of the IFN response might include recognition of the siRNAs by Toll-like receptor-3 (TLR-3) [20]. One simple method for limiting the risk of inducing an IFN response is to use the lowest effective dose of short hairpin RNA (shRNA) vector, as advocated by Bridge et al. [21]. Recently, Kim et al. reported that siRNAs synthesized using the T7 RNA polymerase system can trigger the potent induction of IFN-α and -β in a variety of cells [22].

In the present study, we synthesized four shRNAs targeting the HCV internal ribosome entry site (IRES)/Core gene transcript using T7 RNA polymerase. The greatest inhibitory effects occurred with both HCV 330-349-shRNA and HCV 340-359-shRNA, as the target of the HCV RNA. We also examined whether the shRNAs synthesized using the T7 RNA polymerase system activated PKR, 2'-5'OAS, or IFN-regulatory factor-3 (IRF-3). shRNA synthesized using T7 RNA polymerase did not, however, activate these proteins in Huh-7 cells and HCV replicons.

Materials and methods

shRNA synthesis by T7 RNA polymerase. Desalted DNA oligonucleotides were obtained from Sigma Proligo (Boulder, CO): the T7 promoter 5'-TAATACGACTCACTATAG-3'; EGFP 418-437nt as 5'-CTGGGGC ACAAGCTGGAGTA-3'; HCV 120-139nt as 5'-CCCCCCCTCCCGGG AGAGCC-3', 260-279nt as 5'-AGTGTTGGGTCGCGAAAGGC-3', 330-349nt as 5'-AGA CCGTGCACCATGAGCAC-3', and 340-359nt as 5'-CCATGAGCAC GAATCCTAAA-3'. Loop used CCACACC [23] and overhang used CUU. The oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase was described previously [24]. For each transcription reaction, the oligonucleotide was annealed in sH2O by heating at 95 °C; after 5 min, the heating block was allowed to cool down slowly to obtain the dsDNA. Transcription was performed using AmpliScribe™ T7 High Yield Transcription Kits (EPICENTRE Biotechnologies, Madison, WI) according to the manufacturer's recommended protocol. After incubation at 37 °C for 2 h, 1U RNase free-DNase was added at 37 °C for 15 min. Single-stranded 51nt RNAs were annealed by heating at 95 °C for 5 min followed by 1 h at 37 °C to obtain shRNAs.

Cell culture. Human hepatoma-derived Huh-7 cells and human uterus cancer-derived HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HCV subgenomic replicons were cultured in DMEM-supplemented with

10% FBS, 0.1 mM MEM non-essential amino-acid solution, 2 mM L-glutamine, and penicillin–streptomycin. The growth medium contained the active ingredient G418 (300 µg/ml) [25].

Quantification of HCV RNA by real-time reverse transcription-polymerase chain reaction. HCV replicons were seeded at 1×10^5 cells on 12-well plates for 24 h and transfected with the shRNAs (6, 30 or 60 nM) using Lipofectamine 2000 transfection reagent, according to the manufacturer's recommendation (Invitrogen, Carlsbad, CA). After 48 h, total RNA was isolated from cell cultures using TRIZOL (Invitrogen). HCV RNA was quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) using an ABI 7700 sequence detector (Perkin-Elmer Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed using the following primers and the TaqMan probe located in the five untranslated region (UTR): forward primer (nt 130–146), 5'-CGGGAGA GCCATAGTGG-3'; reverse primer (nt 272–290), 5'-AGTACCACAAG GCCTTTCG-3'; TaqMan probe (nt 148–168), 5'-CTGCGGAACCGG TGAGTACAC-3'. These reagents were purchased from Applied Biosystems. The reporter dye, FAM, was attached to the five end and the quencher dye, TAMRA, was joined to the three end of the probe sequence [26].

RT-PCR. Total cellular RNA was prepared using TRIZOL (Invitrogen). Toll-like receptor (TLR)-3 and IFN-β mRNAs were detected by a RT-PCR High-Plus kit (Toyobo, Kyoto, Japan) with primers specific for these proteins. Primers specific for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used as a loading control in a separate reaction. After 2 min at 94 °C, the reaction parameters were set for 1 min at 94 °C, followed by 1.5 min at 60 °C, for 40 cycles. The sequences of the primers were as follows: TLR-3 sense, 5'-AGCCACCTGAAGTTGACTCAGG-3'; TLR-3 antisense, 5'-CAGTCAAATTCGTGCAGAAGGC-3' [27]; IFN-β sense, 5'-ACCAACAAGTGTCTCCTCCA-3'; and IFN-β antisense, 5'-GAGGTAACCTGTAAGTCTGT-3' [28].

Huh-7 cells and HCV replicon transfection and reporter gene assay. Huh-7 cells and HCV replicons were seeded at 5×10^4 cells on 24-well plates for 24 h and transfected with the plasmid DNA pIRF-3/Luc (0.5 µg) using Lipofectamine 2000 transfection reagent according to the manufacturer's recommendation (Invitrogen). After 4 h, shRNA (60 or 200 nM) or polyinosinic acid:polycytidylic acid (polyI:C) (0.2 µg/ml) were transfected using Lipofectamine 2000 transfection reagent. Untreated cells were used as a control. After 20 h, luciferase activity was measured in the cell lysates using a luminometer (Berthold, Bad Wildbad, Germany).

Western blot analysis. Transfections of the shRNAs or polyI:C were performed in Huh-7 cells and HCV replicons with Lipofectamine 2000 reagent in accordance with the manufacturer's recommendation (Invitrogen). After 24 h, the cell extracts were prepared with lysis buffer. A sample (80 µg) of the total cell lysate was separated using 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The membrane was incubated with monoclonal anti-PKR (Thr446) (Cell Signaling Tech., Beverly, MA) and detection was performed via a chemiluminescence reaction.

Ribosomal RNA-cleavage assay. Huh-7 cells and HCV replicons were transfected with the shRNAs or polyI:C using Lipofectamine 2000 in accordance with the manufacturer's recommendation (Invitrogen). After 24 h, the cells were harvested, washed in phosphate-buffered saline (PBS), and stored at −80 °C. The cell pellets were lysed in 1.5 pellet volumes of NP-40 lysis buffer containing 10 mM Hepes (pH 7.5), 90 mM KCl, 1.0 mM magnesium acetate, 0.5% (v/v) Nonidet P-40, 2.0 mM fresh 2-mercaptoethanol, and 100 µg/ml fresh leupeptin. The buffer was added to the frozen pellet and left on ice until it had thawed. The cell pellets were then dispersed in the buffer and left on ice for at least 5 min. The crude lysate was centrifuged at 10,000g and 4 °C for 10 min before the supernatant was transferred to a clean tube. The cell lysate containing 200 µg protein was then combined with 2 µl of 10 cleavage buffer [comprising 100 mM Hepes (pH 7.5), 1 M KCl, 50 mM magnesium acetate, 10 mM ATP, and 0.14 M 2-mercaptoethanol], plus 2 µl of a 10 concentration of the desired 2–5A activator and sH2O to a final reaction volume of 20 µl. Immediately after the addition of the 2–5A activator, incubation was initiated at 30 °C [29]. The positive control 2–5A sample was a gift from

Professor Sawai of the Department of Chemistry, Faculty of Engineering, Gunma University, Japan.

Results

Inhibition of HCV RNA replication of synthesized shRNAs using T7 RNA polymerase in the HCV replicon

We synthesized four shRNAs targeting the HCV IRES/ Core gene transcript using T7 RNA polymerase (Fig. 1A) and verified the sequences using 18% polyacrylamide gel electrophoresis (data not shown). The sequences are shown in Fig. 1B. To assess the inhibitory effects of the shRNAs on the intracellular replication of HCV, we used HCV rep- licons. Transfection of the shRNA into the HCV replicons (Fig. 1A), which stably express the HCV subgenome, indi- cated that the 330-349-shRNA and 340-359-shRNA both inhibited HCV RNA replication in a dose-dependent man- ner (Fig. 2). On the other hand, the control, EGFP- shRNA, did not induce efficient inhibition (Fig. 2).

shRNA stimulates TLR-3 in Huh-7 cells but not in the HCV replicons

To date, in mammals, 11 TLRs have been identified that recognize pathogen-associated molecular patterns, such as bacterial cell wall materials, bacterial or viral genomic DNA and RNA, and small molecules. At least four TLRs
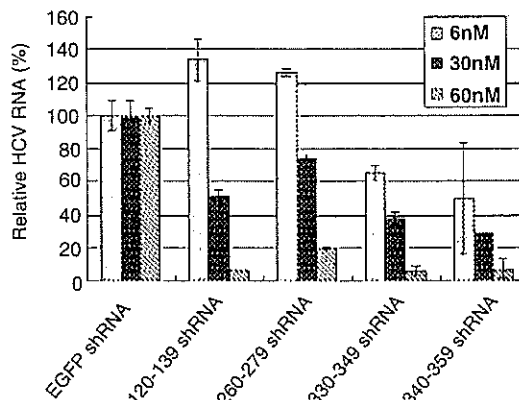

Fig. 2. Inhibition of HCV replication by shRNAs synthesized using the T7 RNA polymerase system in the HCV replicons. The effect of transfection with shRNAs or control EGFP HCV replication was measured by RT-PCR of HCV RNA 48 h after transfection. All values are shown as the percentages of the EGFP negative control.

(TLR-3, 7, 8, and 9) recognize and respond to mono-, oli- go-, and polynucleotides of natural and/or synthetic origin. TLRs 3, 7, and 8 recognize viral and synthetic single- stranded and dsRNAs, such as polyI:C and siRNA [30].

Toll-like receptor-3 recognizes dsRNA, which is com- monly produced during viral replication, and is required for the full induction of IFN-α/β and pro-inflammatory
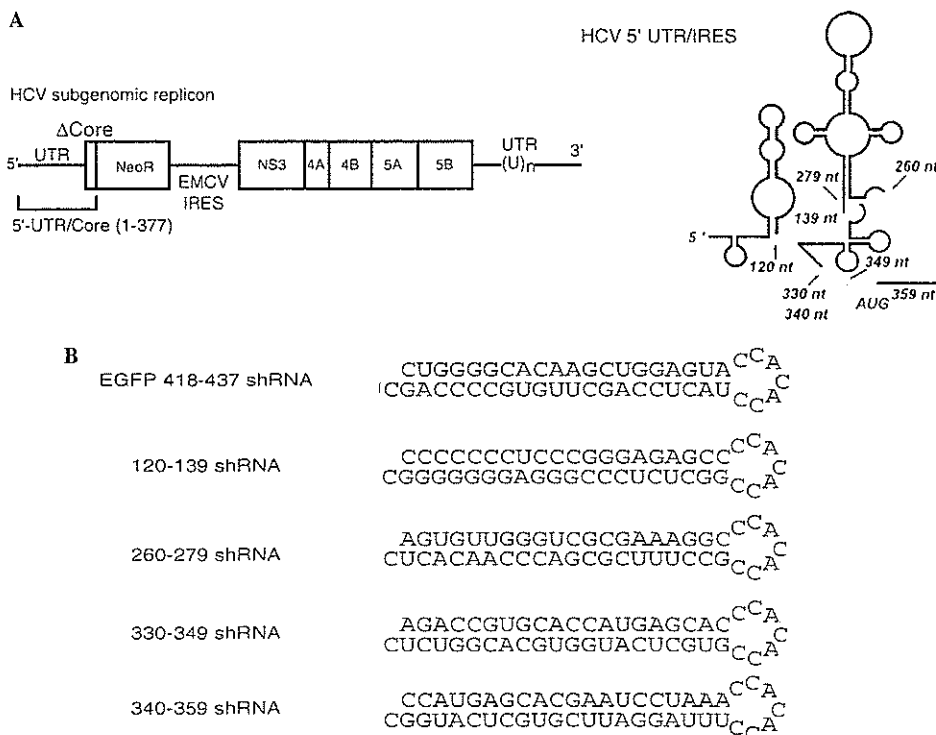

Fig. 1. shRNA-targeted regions of the HCV subgenomic replicons and shRNA sequences. (A) Schematic representation of HCV subgenomic replicons and shRNA targeted regions on HCV 5′-UTR/IRES secondary structure. (B) Sequences of shRNAs synthesized by phage polymerase. The control shRNA:EGFP-shRNA.

cytokines in response to exogenous stimulation by synthetic dsRNA or virus-derived dsRNA. Similar to TLR-4, TLR-3 activation can induce IFN-α/β expression via a MyD88-independent, TRIF-, NAP1-, and TBK1- dependent signaling pathway [31].

Therefore, we examined whether the shRNAs synthesized using T7 RNA polymerase induced type I IFN in Huh-7 cells and HCV replicons. First, we analyzed the expression of TLR-3 mRNA. Total cellular RNA was isolated from the cells and analyzed by RT-PCR using specific TLR-3-detection primers. TLR-3 mRNA was detected in all of the cells (Fig. 3A). Next, we examined whether 340-359-shRNA synthesized using T7 RNA polymerase activated IRF-3 in Huh-7 cells and HCV replicons. We assessed the trigger for IRF-3 phosphorylation by 340-359-shRNA in Huh-7 cells and HCV replicons. For this analysis, we constructed a luciferase reporter gene-expression vector (pIRF-3/Luc reporter) with an IRF-3 binding region (5′-GAAACCGAAACT-3′) in the pGL3-basic vector [32]. The pIRF-3/Luc and 340-359-shRNA were then co-transfected into Huh-7 cells and the HCV replicons using Lipofectamine 2000. IRF-3 activation was monitored using a luciferase assay (Fig. 3B). The internal control, polyI:C, simultaneously induced phosphorylation of IRF-3 and Luc gene expression in the Huh-7 cells. In contrast, the 340-359-shRNA mediated neither the phosphorylation of IRF-3 nor Luc gene expression in these cells. Both polyI:C and the 340-359-shRNA, however, failed to trigger the phosphorylation of IRF-3 and Luc gene expression in HCV replicons (Fig. 3B).

Huh-7 cells and HCV replicons that were transfected with the 340-359-shRNA were also used to evaluate the induction of IFN-β gene expression. The transcribed IFN-β mRNA was detected using RT-PCR analysis with an appropriate primer. IFN-β mRNA expression was detected in polyI:C-transfected Huh-7 cells (Fig. 4, lane 3), but not in cells transfected with the 340-359-shRNA (Fig. 4, lanes 4 and 5). In contrast, IFN-β mRNA expression was not observed when either the 340-359-shRNA (Fig. 4, lanes 4 and 5) or polyI:C (lane 3) was transfected into HCV replicons. These results suggested that the synthesized shRNAs using T7 RNA polymerase did not induce type I IFN production.

Detection of PKR phosphorylation induced by synthesized shRNAs using T7 RNA polymerase

Activation of PKR by viral dsRNA and synthesized dsRNA results in autophosphorylation and subsequent phosphorylation of the eukaryotic initiation factor 2α subunit, causing general inhibition of cellular protein synthesis. In addition to its role as a translational inhibitor, PKR is also a component of signal transduction pathways that regulate events such as cell growth and stress responses [33].

To investigate the association of these transductional pathways in relation to the inhibition of HCV replication, we analyzed the phosphorylated PKR activity induced by the 340-359-shRNA in Huh-7 cells and HCV replicons. The levels of phosphorylated PKR activity were detected with a Western blot assay. Phosphorylated PKR activity
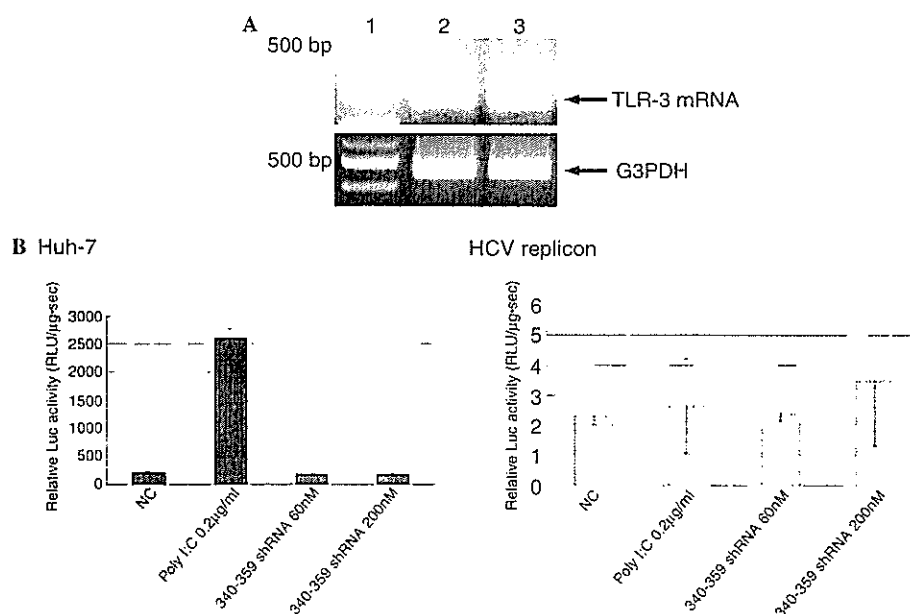

Fig. 3. Activation of IRF-3 after transfection of Huh-7 cells and HCV replicons with shRNAs synthesized using the T7 RNA polymerase system. (A) RT-PCR analysis of TLR-3 mRNA expression in Huh-7 cells and HCV replicons cells. The RT-PCR assay for TLR-3 mRNA was performed using TLR-3 mRNA-specific primers with concurrent amplification of G3PDH mRNA. The RT-PCR-amplified products were fractionated by electrophoresis on a 2.0% agarose gel and stained with ethidium bromide. Lane 1, DNA ladder; lane 2, Huh-7 cells; lane 3, HCV replicons. (B) Huh-7 cells and HCV replicons transfected with either polyI:C (0.2 μg) or the 330-349-shRNA (60 or 200 nM) were treated with 0.5 μg pIRF-3/Luc plasmid. After 24 h, the cell lysates were prepared and assayed for luciferase activity. NC, pIRF-3/Luc.
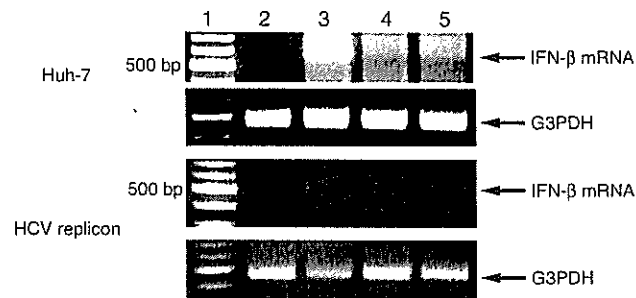
Fig. 4. RT-PCR analysis of IFN-β mRNA expression in Huh-7 cells and HCV replicons. The RT-PCR-amplified products were fractionated by electrophoresis on a 2.0% agarose gel and stained with ethidium bromide. Lane 1, DNA ladder; lane 2, negative control; lane 3, polyI:C (10 μg); lanes 4 and 5, 330-349-shRNA (60 and 200 nM, respectively).

was detected in polyI:C-transfected Huh-7 cells as an internal control (Fig. 5A, lane 3), whereas no such activity was detected in the cells transfected with the 340-359-shRNA (Fig. 5A, lanes 4 and 5). In addition, no phosphorylated PKR activity was detected in HCV replicons transfected with either polyI:C (Fig. 5A, lane 3) or the 340-359-shRNA (Fig. 5A, lanes 4 and 5).

Detection of activated RNase L following transfection with synthesized shRNAs using T7 RNA polymerase

Activation of 2'-5'OAS by viral dsRNA and synthesized dsRNA results in autophosphorylation and subsequent activation of RNase L, causing general inhibition of cellular protein synthesis. Thus, the RNase L induced by the synthesized shRNAs might have degraded the HCV

RNA. Therefore, we examined whether synthesized shRNAs activated RNase L in Huh-7 cells and HCV replicons using a ribosomal RNA-cleavage assay to investigate the induction of RNase L activity in the target cells. The cells were transfected with either 340-359-shRNA or polyI:C using Lipofectamine 2000. The positive control contained the phosphorylated 2'-5'OAS. Total RNA was extracted with TRIzol and analyzed using a ribosomal RNA-cleavage assay with 1.0% agarose gel electrophoresis. RNase L activity was detected after the addition of the positive control (phosphorylated 2'-5'OAS) to both Huh-7 cells and HCV replicons (Fig. 5B, lane 2). In contrast, no RNase L activity was detected in cells that were transfected with the 340-359-shRNA (Fig. 5B, lanes 3 and 4). Notably, RNase L activity was not stimulated in Huh-7 cells and HCV replicons that were transfected with polyI:C as an internal control (Fig. 5B, lane 5). In addition, polyI:C induced negligible RNase L activity in HeLa cells (data not shown). These results demonstrated that the T7-transcribed shRNA did not induce RNase L activity in Huh-7 cells, HeLa cells, or HCV replicons.

Discussion

The present study examined whether HCV RNA replication was inhibited by an RNAi mechanism. We synthesized four shRNAs targeting the HCV IRES/Core gene transcript using T7 RNA polymerase (Figs. 1A and B). The 330-349-shRNA and the 340-359-shRNA both inhibited HCV RNA replication in a dose-dependent manner (Fig. 2). On the other hand, the control, EGFP-shRNA, did not induce efficient inhibition (Fig. 2). These findings
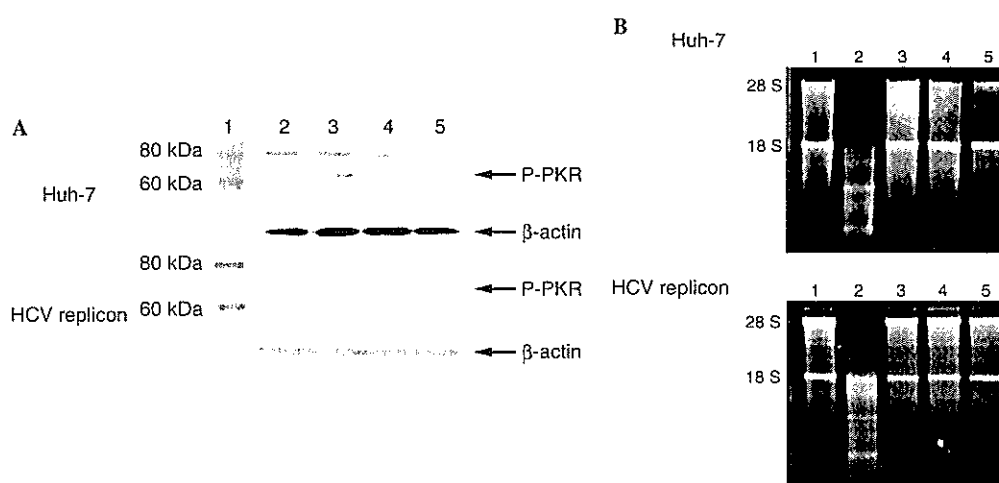

Fig. 5. Activation of PKR and RNase L with shRNAs synthesized using the T7 RNA polymerase system. (A) After 24 h, the Huh-7 cells and HCV replicons were lysed with lysis buffer. The lysates (80 μg protein/lane) were assayed by Western blot analysis with antibodies against phosphorylated PKR or β-actin as a control. The protein bands were visualized using the ECL method (Amersham). Lane 1, biotinylated protein marker; lane 2, negative-control cells; lane 3, cells transfected with 10 μg polyI:C; lanes 4 and 5, cells transfected with 330-349-shRNA (60 and 200 nM, respectively). (B) An RNase L assay was performed for Huh-7 cells and HCV replicons. Lane 1, negative control; lane 2, p5A2 p5 A2 p5 A (1 μM); lanes 3 and 4, 330-349-shRNA (60 and 200 nM, respectively); lane 5, Huh-7 cells and HCV replicons, polyI:C (10 μg).

suggest that targeting the region that includes the AUG of the HCV IRES/Core is effective.

It was previously reported that dsRNA triggered production of type I IFN, activation of PKR, and activation of 2'-5'OAS. Recently, Kim et al. demonstrated that siRNAs synthesized using the T7 RNA polymerase system can trigger the potent induction of IFN-α and -β in a variety of cells [22]. In the present study, we investigated whether the dsRNA stimulated various metabolic pathways in HCV replicons in addition to suppressing RNA replication.

First, we examined whether synthesized shRNAs induced IFN-β. It is expected that the induction of IFN-β occurs so that shRNA is recognized by TLR-3. We detected TLR-3 mRNA in Huh-7 cells and HCV replicons. Next, we examined the activation of IRF-3, which is one of the transcription factors. The results suggested that shRNAs did not induce activation of IRF-3 in Huh-7 cells and HCV replicons. Similarly, shRNAs did not induce IFN-β mRNA in Huh-7 cells and HCV replicons. Recent analyses of potential mediators of induction of the IFN response revealed that the initiating 5'-triphosphate is required for IFN induction in HEK 293 and HeLa cells [22]. Our synthesized shRNAs, however, did not induce IFN in Huh-7 cells and HCV replicons. Huh-7 cells might be less sensitive to dsRNA than HEK 293 and HeLa cells.

Next, we examined whether the synthesized shRNA activated PKR and 2'-5'OAS. PKR and 2'-5'OAS are activated by dsRNA and viral infection [34]. PKR activation stops translation. Activation of 2'-5'OAS activates RNase L and degrades RNA [35]. Our synthesized shRNAs, however, did not induce activation of PKR and 2'-5'OAS in Huh-7 cells and HCV replicons. Even polyI:C did not induce activation of these proteins in Huh-7 cells and HCV replicons. These proteins in Huh-7 cells might be insensitive to dsRNA. Therefore, we used HeLa cells, which were sensitive to dsRNA. The synthesized shRNAs also did not induce activation of PKR and 2'-5'OAS in HeLa cells. These results demonstrated an association between the sequence specific-inhibition via the RNAi mechanism without stimulating the TLR-3 signal pathway, PKR [36], or 2'-5'OAS by HCV proteins, because the TLR-3-adaptor protein TRIF is cleaved by HCV nonstructural 3/4A protease [37]. On the other hand, the nonstructural 5A protein might bind with PKR and block dimerization, which inhibits the activation of eukaryotic initiation factor 2α [38,39].

In conclusion, our results demonstrate that shRNAs targeting the HCV IRES/Core gene transcript using T7 RNA polymerase inhibited RNA replication in HCV replicons. In addition, our study revealed that the 330-349-shRNA and 340-359-shRNA inhibit the replication of HCV RNA via an RNAi mechanism without stimulating the TLR-3 signal pathway, PKR, or 2'-5'OAS. This suggests that RNAi might be an effective method for blocking HCV RNA replication in infected cells.

Acknowledgments

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Evaluation of the anti-hepatitis C virus effects of cyclophilin inhibitors, cyclosporin A, and NIM811

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Abstract

Hepatitis C virus (HCV) is a major causative agent of hepatocellular carcinoma. We recently discovered that the immunosuppressant cyclosporin A (CsA) and its analogue lacking immunosuppressive function, NIM811, strongly suppress the replication of HCV in cell culture. Inhibition of a cellular replication cofactor, cyclophilin (CyP) B, is critical for its anti-HCV effects. Here, we explored the potential use of CyP inhibitors for HCV treatment by analyzing the HCV replicon system. Treatment with CsA and NIM811 for 7 days reduced HCV RNA levels by 2–3 logs, and treatment for 3 weeks reduced HCV RNA to undetectable levels. NIM811 exerted higher anti-HCV activity than CsA at lower concentrations. Both CyP inhibitors rapidly reduced HCV RNA levels even further in combination with IFNα without modifying the IFNα signal transduction pathway. In conclusion, CyP inhibitors may provide a novel strategy for anti-HCV treatment.
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Keywords: HCV; Cyclosporin; NIM811; Interferon; Cyclophilin; Cyclosporine; Replication; Replicon; Inhibitor; Therapy

Hepatitis C virus (HCV), which is associated with non-A and non-B hepatitis [1], is a major causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Worldwide, HCV infection constitutes a serious health threat, and is estimated to affect more than 200 million individuals and cause approximately 280,000 deaths per year [2,3]. The current standard therapy for chronic HCV infection is interferon (IFN) or pegylated IFN, either alone or in combination with ribavirin [4,5]. Because treatment with these agents fails to produce sustained virus elimination in about half the total patients [6,7], however, alternative and more effective strategies to treat hepatitis C are needed.

We recently discovered that an immunosuppressant, cyclosporin A (CsA), and its nonimmunosuppressive

analogue, NIM811, suppress HCV genome replication in a cell culture system. The maximum effect of each cyclosporin was comparable to that of IFNα. The anti-HCV effects of the cyclosporins correlated with cyclophilin (CyP) inhibition [8]. We also revealed that CyPB, one of the cellular targets of CsA, regulated HCV replication through its interaction with viral RNA-dependent RNA polymerase NS5B [9]. Cyclosporins suppressed HCV replication by dissociating CyPB from NS5B. These properties recommended the CyP inhibitors as agents for clinical use, especially considering the fact that hepatitis C treatment should preferably suppress the emergence of drug-resistant viruses. Because the CyP inhibitors specifically target a cellular factor, they are expected to exert robust anti-HCV activities with a low risk of developing drug resistance (see Discussion). Therefore, it will assist in the development of new anti-HCV strategies to investigate the effects of cyclosporins on HCV replication in a cell culture system.

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In this report, we examined in detail the anti-HCV effects of CsA and NIM811 and the effects of the cyclosporins in combination with IFNα, using an HCV replicon system [10]. Treatment with CsA and NIM811 reduced HCV RNA in the replicon system. NIM811 was a more potent anti-HCV agent than CsA. We observed further reduction of HCV RNA using a combination of either CsA or NIM811 with IFNα, and detected little to no increase in cytotoxicity. In addition, HCV RNA was reduced to background level after 21 days of treatment with each cyclosporin. Based on these results, CyP inhibitors could potentially serve as a new class of anti-HCV agents.

Materials and methods

Compounds. CsA, IFNα, and ribavirin were purchased from Sigma, Otsuka Pharmaceutical Co., Ltd., and Calbiochem, respectively. NIM811 was generously provided by Novartis (Basel, Switzerland).

Cell Culture. NNC and LMH14 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum, nonessential amino acids (Invitrogen), and L-glutamine (Invitrogen) in the presence of 500 μg/ml G418 (Invitrogen). LucNeo#2 cells were generated by selecting Huh7 cells transfected with LNMH14 RNA in the presence of 800 μg/ml G418.

Plasmid construction. The pLNMH14 plasmid was constructed as follows. The luciferase gene was amplified from pLMH14 [11] by polymerase chain reaction (PCR) with the primers sspfor 5'-AATATTATTG AAGCATTTATCAGGG-3' and lucneorev 5'-GAACCTGCGTGCAAT CCATCTTGCAATTTGGACTTTCCGCCCTTC-3'. The gene for neomycin phosphotransferase (Neo^r) was amplified by PCR from pMH14 using the primers lucneofor 5'-GAAGGGCGGAAAGTCCAAATTGC AAGATGGATTGCACGCAGGTTC-3'and neonotrev 5'-CAATTGTT ACCGCGGCCGCTGGAGGATC-3'. Both cDNA fragments were annealed, followed by PCR amplification using the primers sspfor and neonotrev. The amplified DNA fragment was digested with SspI and AflII and cloned into pMH14.

In vitro RNA synthesis. LNMH14 RNAs were prepared by in vitro transcription using a MEGAscript T7 kit (Ambion), as described previously [12].

Synergy and antagonism analysis. The effects of drug combinations were evaluated using the Loewe additivity model, in which data were analyzed with CalcuSyn software (Biosoft, Ferguson, Mo.), a computer program based on the method of Chou and Talalay [13]. After converting the dose-effect curves for each drug or drug combination to median-effect plots, the program calculated a combination index (CI) value based on the following equation: $[(D)_1/(D_x)_1] + [(D)_2/(D_x)_2] + [(D)_1(D)_2/(D_x)_1(D_x)_2]$, where $(D_x)_1$ and $(D_x)_2$ are the doses of drugs 1 and 2, respectively, that have the same x effect when used in combination. CI values of <1, 1, and >1 indicate synergy, an additive effect, and antagonism, respectively.

Colony formation assay. NNC cells were treated with drugs (CsA and NIM811 alone or in combination with IFNα) in the presence of 500 μg/ml G418 for 2 weeks, followed by fixation and staining with crystal violet.

Real-time RT-PCR analysis. The 5'-nontranslated region of HCV RNA was quantified using an ABI PRISM 7500 sequence detector (Applied Biosystems), as previously described [8].

RT-PCR analysis. RT-PCR was performed as described previously [8] using the following primer sets: 5'-TGACGCTGACCTGGTTGTCTT-3' and 5'-CAGGCTTCCAGCTGTCTCCTAA-3' to detect mRNA for 2', 5'-oligoadenylate synthetase (2',5'-OAS), 5'-CCGCAGCCAAATTAGC TGTT-3', and 5'-GGCCTATGTAATTCCCCATGG-3' to detect double-strand RNA-dependent protein kinase (PKR), and 5'-TGGAGGGATCT CGCTCCTGG-3' and 5'-ATGGGGAAGGTGAAGGTCGG-3' to detect glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Results

Response of HCV genome replication to treatment with CsA and NIM811

We previously reported that CsA and its nonimmunosuppressive derivative, NIM811, strongly suppress HCV genome replication in the replicon system [8,10]. To characterize the anti-HCV effect profile of cyclosporins, we first examined in detail the cyclosporin responses of HCV replicons. Consistent with previous results, HCV RNA levels in NNC cells, which harbor full-genomic HCV replicons, were decreased by over 2 logs following treatment with 1–3 μg/ml of either CsA or NIM811 for 7 days (Fig. 1A). In this assay, NIM811 tended to decrease HCV RNA more strongly than CsA at lower concentrations; the decreasing effect of NIM811 on HCV RNA at 0.5 μg/ml was about 1 log higher than that of CsA at the same concentration.
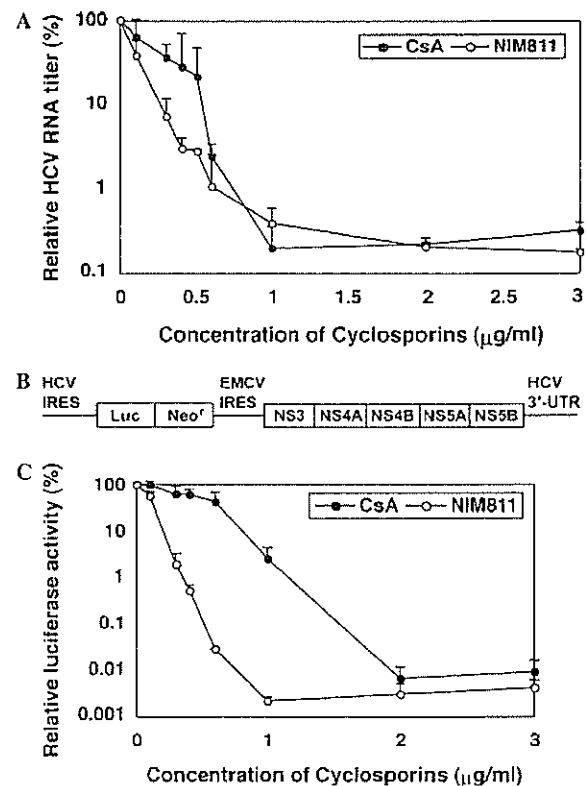

Fig. 1. Response curve of HCV RNA titers vs. the concentration of CsA and its nonimmunosuppressive analogue, NIM811. (A) NNC cells, harboring the HCV full genome replicon, were treated with either CsA or NIM811 for 7 days, and the HCV RNA extracted from these cells was quantified by real-time RT-PCR. The data represent percentages of HCV RNA levels in cells either untreated or treated with CsA or NIM811. (B) Schematic representation of the RNA construct carried in LucNeo#2 cells. LucNeo#2 cells were established as described in the Materials and methods. HCV replication can be monitored by measuring the activity of the resulting luciferase activity. (C) Luciferase activities were measured in the lysates of cells treated with either CsA or NIM811 for 7 days. The data show the means of the results from three independent experiments, with the standard deviation values indicated by error bars.

To confirm this result in another experimental system with higher sensitivity to antiviral agents, we performed a luciferase assay, which quantifies the activity of luciferase driven from a subgenomic HCV replicon construct (Fig. 1B). The maximum effect of treatment with each cyclosporin was a drop in luciferase activity of more than 4 logs (Fig. 1C). The difference in magnitude of suppression between Figs. 1A and C is likely due to differences in the experimental systems, because the response of the full genome replicon to CsA is similar to that of the subgenomic replicon [10]. Also, in this assay, the decreasing effect of NIM811 on HCV RNA at 0.5 μg/ml was approximately 2 logs higher than that of CsA at the same concentration. These results suggest that the anti-HCV effect of NIM811 is more potent than that of CsA, especially at lower concentrations.

Analysis of cotreatment with IFNα and either CsA or NIM811

We examined the effect on HCV replication of cotreatment with both IFNα and a cyclosporin by treating NNC cells for 7 days with varying concentrations of a cyclosporin and IFNα. The combination of each cyclosporin with IFNα showed a greater decrease in HCV RNA levels compared to each compound alone (Figs. 2A and C), with little to no increase in cytotoxicity (Figs. 2B and D). The amplification of the IFNα-induced anti-HCV effects of NIM811 was stronger than that of CsA (Figs. 2A and C). This effect was further demonstrated using a colony formation assay (Fig. 3). Cells treated with IFNα (3 and 10 IU/ml) or each cyclosporin (0.5 and 0.7 μg/ml) survived under G418 selection similarly to untreated control cells,

but a drastic reduction of the colony formation resulting from replicating HCV was apparent following cotreatment with IFNα and either CsA or NIM811. These data suggest that combination treatment of cyclosporins with IFNα exhibits a stronger antiviral effect than single treatments.

The next question is whether the antiviral effect of the combination treatment is synergistic or additive. We therefore analyzed the data in Fig. 2A, obtained by cotreatment with IFNα and CsA, using Loewe additivity models [14] and a computer program, CalcuSyn [13]. Fig. 4 shows the analysis results of the combination effects of IFNα (in units per milliliter) and CsA (in micrograms per milliliter) at a fixed ratio of 100:1. Fig. 4A presents a conservative isobologram, illustrating lines that represent the effective doses (ED_x) of the two compounds that would be required to attain $X\%$ inhibition if the combination were simply additive. The actual experimental doses inducing 50 (filled triangle), 90 (filled square), and 99 (filled circle) % inhibition obtained in the data of Fig. 2A were more than, nearly equal to, and less than, respectively, the expected doses from ED curves which showed the additive interaction between the two compounds. This result indicates antagonistic, nearly additive, and synergistic effects for ED_{50}, ED_{90}, and ED_{99}, respectively, between CsA and IFNα. Combination effect was further examined in Fig. 4B by the calculation of a CI value (In this figure, more than, equal to, and less than 1 of CI value indicate antagonistic, additive, and synergistic effect, respectively). CI values of the combination effects of IFNα and CsA at the fixed ratio of 100:1 in the experiment shown in Fig. 2A were >1 in lower fractional effect and <1 in higher fractional effect, indicating a synergistic effect at high fractional effect levels. A stronger synergistic effect was observed at the dose
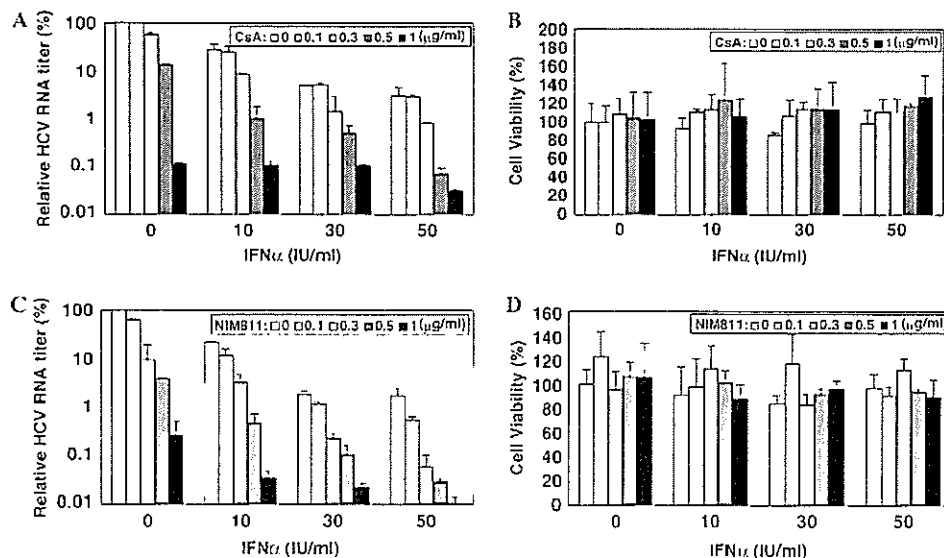

Fig. 2. The effects on HCV replication of cotreatment with both IFNα and cyclosporins. (A,C) NNC cells were treated with varying concentrations of either CsA (A) or NIM811 (C) in combination with various concentrations of IFNα for 7 days. HCV RNA levels were determined by real-time RT-PCR and are shown as percentages of the level in cells untreated (control). (B,D) The numbers of NNC cells treated with either CsA (B) or NIM811 (D) in combination with IFNα for 2 days were determined to show the cytotoxicity of the drugs. The data represent means of the results from three independent experiments, with the standard deviation values indicated by error bars.
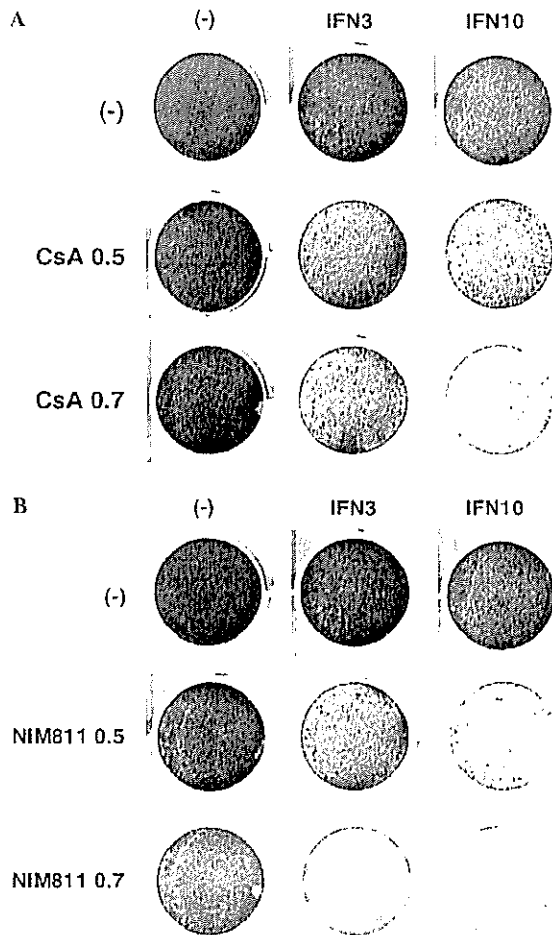
Fig. 3. Colony formation assay showing the effects of cotreatment with IFNα and cyclosporins, CsA (A) or NIM811 (B). NNC cells were treated with cyclosporins in combination with IFNα at the indicated doses in the presence of 500 μg/ml G418. After 2 weeks in culture, cells were fixed and stained with crystal violet.

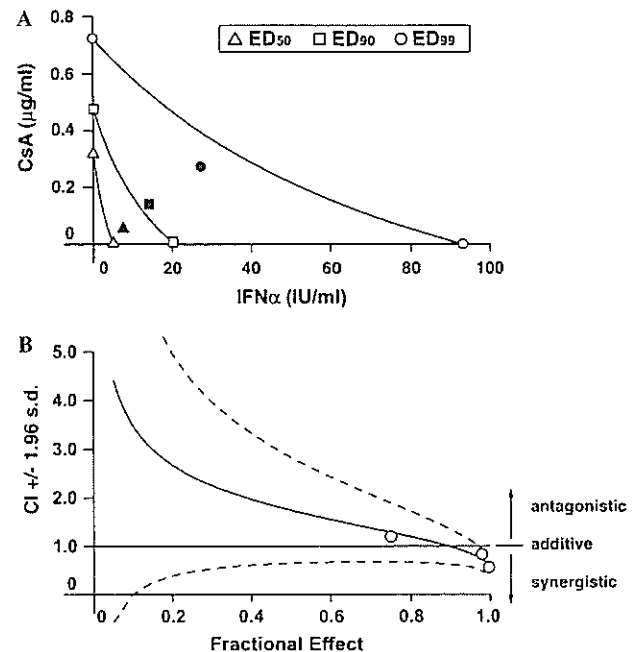Fig. 4. Analysis of the combination treatment of CsA and IFNα using a Loewe additivity model. (A) Conservative isobologram determined by analyzing the data shown in Fig. 2A using the CalcuSyn program. The lines represent the effective doses (ED_x) of two drugs that would be required to attain $X\%$ inhibition if the effects of combination were simply additive, and the filled dots (filled triangles, squares, and circles for $ED_{50, 90}$, and $_{99}$, respectively) are the actual doses used to accomplish these inhibition effects obtained from the data of Fig. 2A. (B) The combination index (CI) was calculated and plotted as the solid curve versus the percent inhibition (i.e., the fractional effect). Two dotted curves represent the 95% confidence intervals (1.96 standard deviations) of the CI.

region providing higher antiviral effects than ED_{99} (data not shown). The data clearly show that the stronger the antiviral effect, the more synergistic the effect of cotreatment becomes, though the cotreatment shows additive to antagonistic interactions at relatively low concentrations. A similar result was obtained by analyzing cotreatment of IFNα and NIM811 (data not shown). Based on our computational analysis, to induce a synergistic antiviral effect by cotreating with IFNα and cyclosporins, it is important to use doses representing more than 90% inhibition. Because the ED_{90} of NIM811 is less than that of CsA, NIM811 more strongly potentiates the antiviral effects of IFNα than does CsA at the same cotreatment dose.

The antiviral effects of cyclosporins alone or in combination with IFNα were sustained for over 10 days

To analyze the anti-HCV kinetics of the cyclosporins and cotreatment with a cyclosporin and IFNα, we treated cells with either cyclosporin, IFNα, or ribavirin alone, or IFNα

in combination with either a cyclosporin or ribavirin for 3, 5, 7, and 10 days and measured the quantity of HCV replicon RNA (Fig. 5). CsA and NIM811, at 0.5 μg/ml, both decreased HCV replicon RNA in a time-dependent manner, resulting in about 2 and 2 logs reduction, respectively, of HCV RNA titers after 10 days of treatment, similar to 10 IU/ml IFNα. On the other hand, the combination of 10 IU/ml IFNα with 0.5 μg/ml CsA or NIM811 led to greater than 3 and around 4 logs reduction, respectively, of HCV RNA after 10 days of treatment. These effects were greater than that of cotreatment with IFNα and ribavirin (200 μM, which was the highest dose without significant cytotoxicity). Three weeks of treatment with CsA or NIM811 reduced HCV RNA to below detectable levels as assayed by real-time RT-PCR (data not shown). These results indicate that the strong antiviral effects of cyclosporin and NIM811 alones or in combination with IFNα were sustained over time and that viruses were eventually eliminated.

Cotreatment with CsA augmented the anti-HCV effects of IFNα without enhancing the IFNα signal transduction pathway

To investigate the mechanisms of action for the enhancing effects of cyclosporins on the anti-HCV activity of
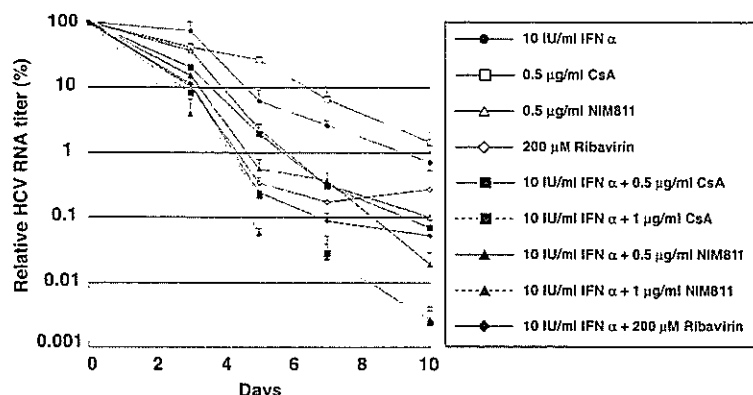
Fig. 5. Time course for the reduction of HCV RNA levels in NNC cells treated with CsA, NIM811, IFNα, or ribavirin. The levels of HCV RNA in the cells treated with the compounds for 3, 5, 7, and 10 days were determined by real-time RT-PCR and plotted vs. the days of treatment. The data represent the means of the results of three independent experiments.
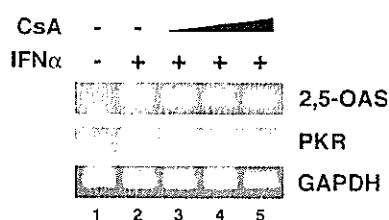

Fig. 6. Effects of the addition of CsA on the IFNα signal transduction pathway. NNC cells were treated either without (lane 1) or with 100 IU/ml IFNα (lanes 2–5) in combination with 0.5 μg/ml (lane 3), 1 μg/ml (lane 4), and 3 μg/ml (lane 5) CsA for 2 days. The mRNAs of 2′,5′-oligoadenylate synthetase (2′,5′-OAS) (upper panel), double-strand RNA-dependent protein kinase (PKR) (middle panel), and glyceraldehydes-3-phophate dehydrogenase (GAPDH) as an internal control (lower panels) were detected by RT-PCR.

IFNα, we examined the IFNα signal transduction pathway following the addition of a cyclosporin in NNC cells. The IFNα-induced upregulation of mRNA coding for 2′,5′-oligoadenylate synthetase (2′,5′-OAS) and double-strand RNA-dependent protein kinase (PKR), which are antiviral proteins downstream of IFNα, were not augmented by the cyclosporin cotreatment (Fig. 6). This result suggests that the IFNα-induced transcriptional activation was not altered by the cyclosporin treatment.

Discussion

We previously reported [8] that CsA and NIM811 suppress HCV replication. In the present study, we evaluated the anti-HCV effects of CsA and NIM811 in detail and revealed that these compounds achieve multiple-log reduction of HCV RNA levels in a cell culture system. NIM811 exhibited a more potent anti-HCV activity than did CsA, especially at relatively low concentrations. We previously demonstrated [9] that CyPB is a cellular replication cofactor that regulates the function of NS5B. CsA suppressed HCV replication via the dissociation of CyPB from NS5B [9]. In addition, NIM811 is reported to bind CyP with higher affinity (about 2-fold) than does CsA [15]. Taken

together, the stronger anti-HCV activity of NIM811 over CsA at low concentrations may be due to NIM811's higher binding affinity to CyPB. In actuality, the strength of suppression of cyclosporins against vaccinia virus correlates with their inhibition/binding activities to CyP [16], in agreement with the above explanation. The higher anti-HCV activity of NIM811 at relatively low concentrations may be important for anti-HCV therapies in vivo because the trough level of CsA in the peripheral blood during the employment of CsA as an immunosuppressive agent during liver transplantation is 0.2–0.3 μg/ml (Peak cyclosporin levels are 0.8–2.3 μg/ml) [17–19]. Thus, NIM811 may eliminate HCV at the concentrations that are permissive in vivo, although other factors, such as pharmacodynamics and side effects, must be validated. Moreover, CsA might exert some pro-viral effects due to its immunosuppressive activity against T lymphocytes [20–22] in addition to its antiviral effects in hepatocytes. Thus, NIM811, which has little immunosuppressive function [15,23], is expected to be preferable to CsA for eliminating HCV in vivo.

Combining antiviral compounds that have different targets is effective in suppressing the emergence of drug-resistant viruses, as illustrated by the example of human immunodeficiency virus. Highly active antiretroviral combination therapy, which consists of a nucleoside backbone plus either a nonnucleoside reverse transcriptase inhibitor or a protease inhibitor, has dramatically decreased the mortality rate of AIDS patients [24]. Combining anti-HCV drugs might be one therapeutic approach to eradicate HCV, in addition to conventional therapy using IFNα, PegIFNα, or either compound in combination with ribavirin. In this study, we showed that both CsA and NIM811 exhibited enhanced anti-HCV effects in combination with IFNα. Importantly, a recent clinical study reported that the combination use of IFNα with CsA achieved a more sustained virological response than did CsA monotherapy [25]. This elevated antiviral effect with CsA cotreatment did not modify the IFNα signal transduction pathway (Fig. 6). Past candidates with anti-HCV potential, such

as protease inhibitors or polymerase inhibitors, which are now undergoing clinical trials, directly target viral proteins and inhibit their enzymatic activity. Because cyclosporins such as CsA and NIM811 target a cellular factor, CyPB, as described above, these compounds could serve as an additional type of anti-HCV agent. Moreover, viruses resistant to cyclosporins are less likely to occur, since anti-viral compounds that target cellular factors generally induce less drug resistance than those inhibiting viral proteins; this difference is due to the high mutation rates of RNA viruses [26–30]. Thus, this novel anti-HCV candidate could provide an alternative strategy to combat HCV.

Acknowledgments

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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, MILE [GenBank accession no. AB080299]) and contains wild-type MILE 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-Bgl*II. The intermediate vector pGL3-*MluI-Bgl*II-S232I was constructed by introducing the point mutation S232I of NS5A into the MluI and SacI sites of pGL3-*MluI-Bgl*II by site-directed mutagenesis using primers carrying the necessary nucleotide changes. Subsequently, mutations were introduced into pGL3-*MluI-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-*MluI-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-*MluI-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 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 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

A


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         5'NTR        EMCV-IRES                S232I              3'NTR
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wild type replicon;MA

```
              201            210           220
        MA    VEFLVNTWKSKKCPMGFSYD
        MA/cm211   VEFLVNTAAAAAAAMGFSYD
        MA/W208A   VEFLVNTAKSKKCPMGFSYD
        MA/K209A   VEFLVNTWASKKCPMGFSYD
        MA/S210A   VEFLVNTWKAKKCPMGFSYD
        MA/K211A   VEFLVNTWKSAKCPMGFSYD
        MA/K212A   VEFLVNTWKSKACPMGFSYD
        MA/C213A   VEFLVNTWKSKKAPMGFSYD
        MA/P214A   VEFLVNTWKSKKCAMGFSYD
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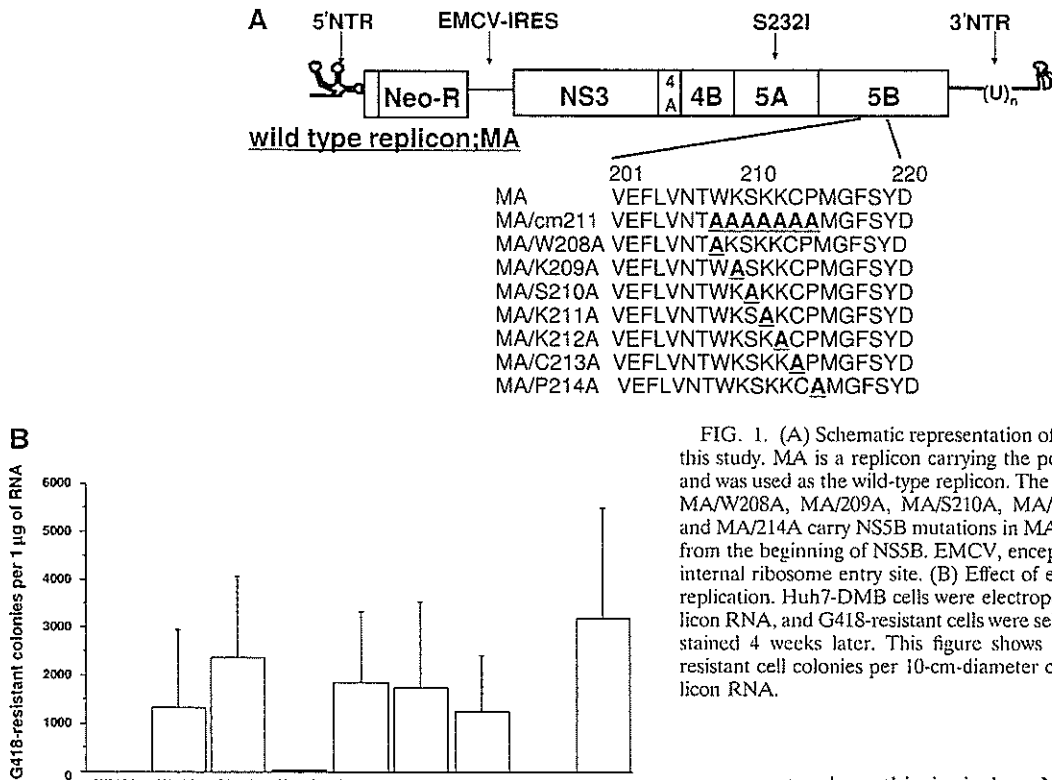
B

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

A input anti-M2

| | FLAGNS5Bt:wild | FLAGNS5Bt:wild | FLAGNS5Bt:cm211 | FLAGNS5Bt:W208A | FLAGNS5Bt:K209A | FLAGNS5Bt:S210A | FLAGNS5Bt:K211A | FLAGNS5Bt:K212A | FLAGNS5Bt:C213A | FLAGNS5Bt:P214A |

B output anti-M2

1 2 3 4 5 6 7 8 9 10

C recovery anti-GST

1 2 3 4 5 6 7 8 9 10

GST-nucleolin →

— 97
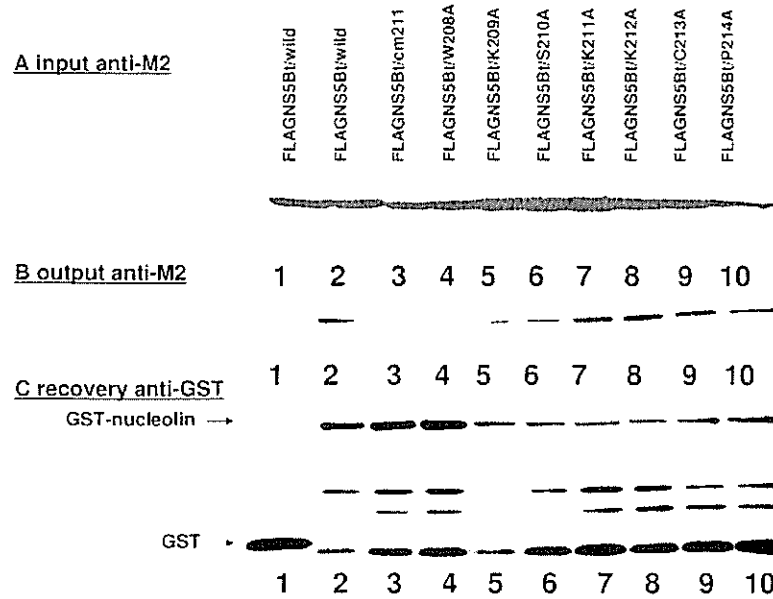— 66

46

GST ►

— 30

1 2 3 4 5 6 7 8 9 10

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.
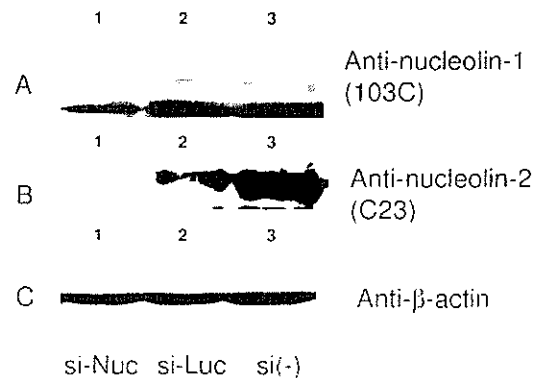
1   2   3

A ▬▬▬▬▬▬▬ Anti-nucleolin-1 (103C)

1   2   3

B ▬▬▬▬▬▬▬ Anti-nucleolin-2 (C23)

1   2   3

C ▬▬▬▬▬▬▬ Anti-β-actin

si-Nuc si-Luc si(-)

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(−)].
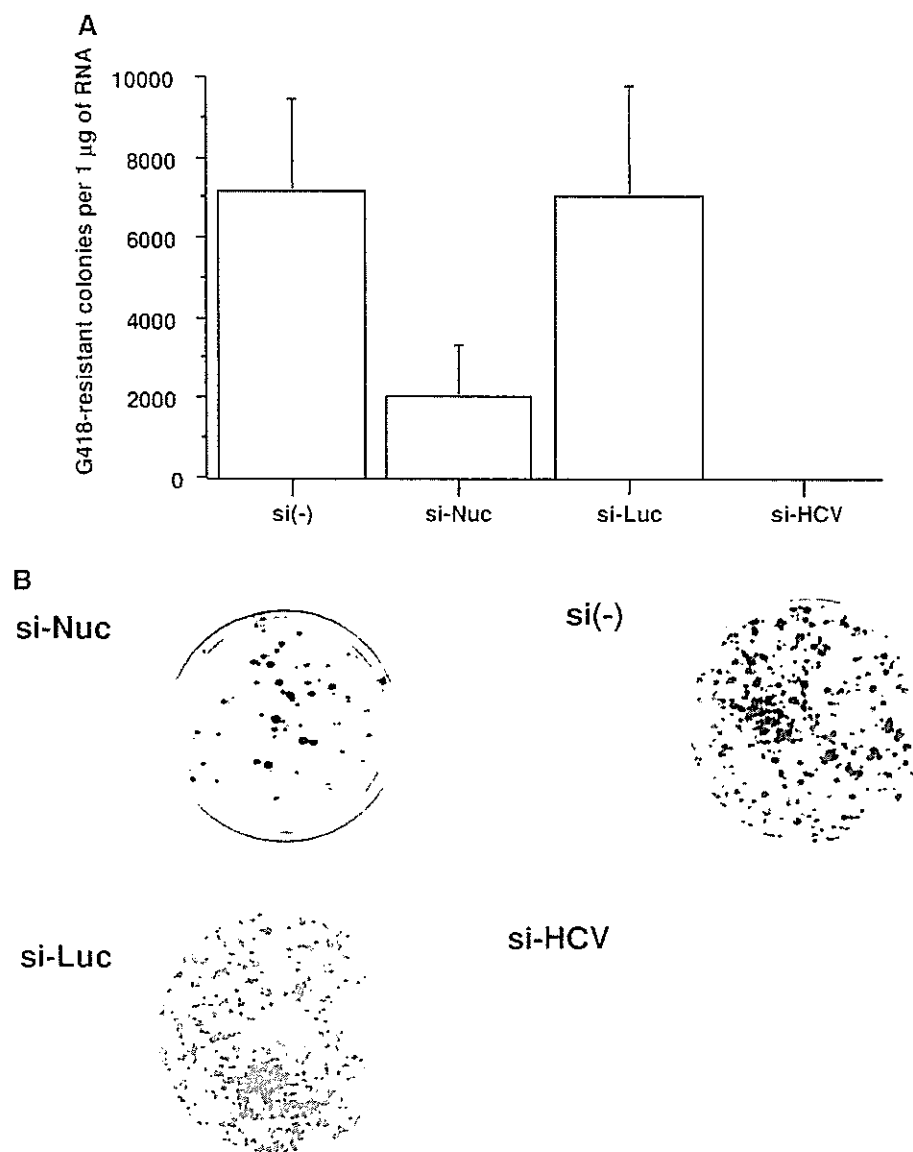
FIG. 4. Effect of suppression of endogenous nucleolin on HCV replication in the MA replicon. Huh7-DMB cells were electroporated with 1 µg of in vitro-transcribed MA RNA plus si-Nuc, si-Luc, si-HCV, or no siRNA [si(−)], and G418-resistant cells were selected with 1 mg/ml G418 and were stained 4 weeks 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.
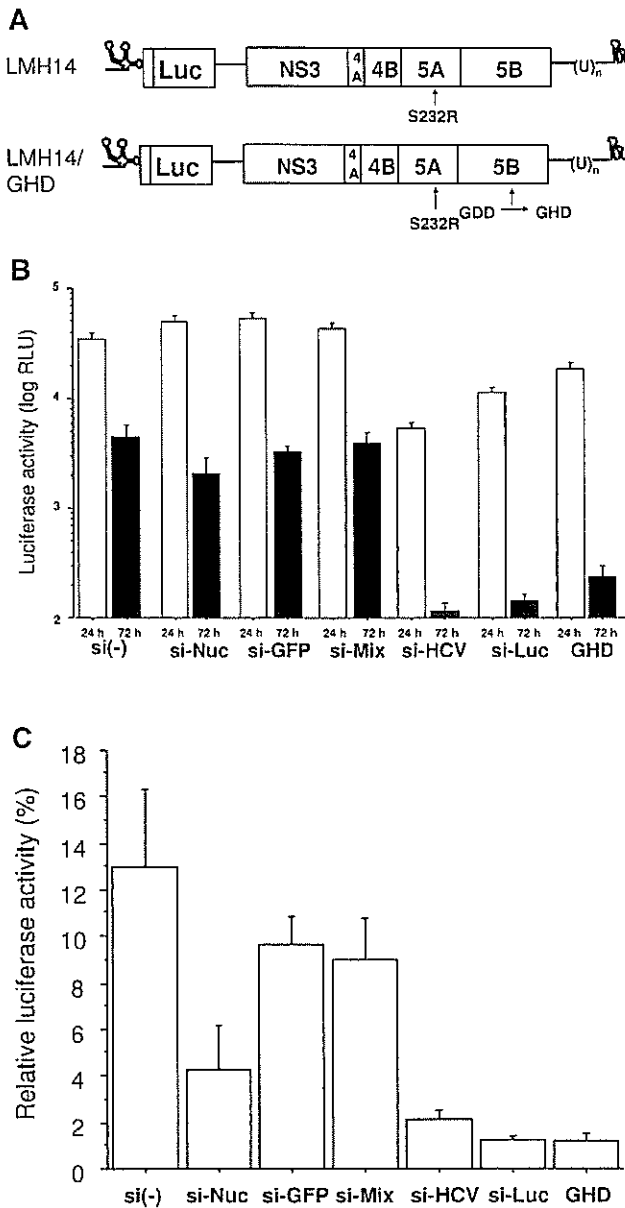
FIG. 5. Effect of suppression of endogenous nucleolin on HCV replication in the LMH14 replicon. (A) Schematic representation of the luciferase replicon. In the LMH14 replicon, the neomycin resistance gene was replaced by a luciferase gene, and S232 of NS5A was replaced by R. In the LMH14/GHD replicon, 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 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).