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Inhibition of Hepatitis C Virus Replication by a Specific Inhibitor of Serine-Arginine-Rich Protein Kinase▽

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Splicing of messenger RNAs is regulated by site-specific binding of members of the serine-arginine-rich (SR) protein family, and SR protein kinases (SRPK) 1 and 2 regulate overall activity of the SR proteins by phosphorylation of their RS domains. We have reported that specifically designed SRPK inhibitors suppressed effectively several DNA and RNA viruses *in vitro* and *in vivo*. Here, we show that an SRPK inhibitor, SRPIN340, suppressed in a dose-dependent fashion expression of a hepatitis C virus (HCV) subgenomic replicon and replication of the HCV-JFH1 clone *in vitro*. The inhibitory effects were not associated with antiproliferative or nonspecific cytotoxic effects on the host cells. Overexpression of SRPK1 or SRPK2 resulted in augmentation of HCV replication, while small interfering RNA (siRNA) knockdown of the SRPKs suppressed HCV replication significantly. Immunocytochemistry showed that SRPKs and the HCV core and NS5A proteins colocalized to some extent in the perinuclear area. Our results demonstrate that SRPKs are host factors essential for HCV replication and that functional inhibitors of these kinases may constitute a new class of antiviral agents against HCV infection.

Hepatitis C virus (HCV) infects up to 170 million people worldwide, and these infections frequently are characterized by chronic liver inflammation, leading to decompensated liver cirrhosis and hepatocellular cancers (1). Alpha and beta interferons are the mainstay of HCV therapeutics. However, the most effective pegylated interferon plus ribavirin combination therapies can eliminate HCV from around half of the patients only (6). These difficulties in eradicating HCV are compounded by the limited treatment options. For this reason, the development of safe and effective therapeutic agents against HCV has been a strong motivation in academia and industry (23).

Serine-arginine-rich (SR) proteins are a family of non-small nuclear ribonucleoprotein particle (non-snRNP) splicing factors that are highly conserved throughout the eukaryotes. They harbor one or two RNA recognition motifs and an RS domain at the amino and carboxyl termini, respectively (29). RS domains consist of multiple consecutive Arg-Ser/Ser-Arg dipeptide repeats, in which the Ser residues are extensively phosphorylated by several kinases, including SR protein kinases (SRPKs). SRPK1 was the first SR protein kinase to be cloned, on the basis of its ability to phosphorylate SR proteins *in vitro* (8, 9), and two other structurally related kinases, SRPK2 and SRPK3, also have been shown to phosphorylate SR proteins (16, 31). Although the precise physiological role of this phosphorylation remains unknown, it is expected that phosphorylation of SR proteins affects their protein-protein and protein-RNA interactions, intracellular localization and trafficking, and alternative splicing of pre-mRNA (21).

As SRPK-dependent herpes simplex virus (HSV) splicing and SRPK-mediated phosphorylation of hepatitis B virus (HBV) core protein have been reported (4, 25, 33), it is reasonable to expect that SR proteins and SRPK might be suitable targets for therapeutic modulation of various viral infections. Actually, we found that increased activity of SRPK2 upregulated human immunodeficiency virus (HIV) expression and that an isonicotinamide compound, SRPIN340, which preferentially inhibited SRPK1 and SRPK2, suppressed propagation of Sindbis virus, HIV, and cytomegalovirus (7). In this study, we investigated the effects of SRPIN340 on HCV replication using the HCV subgenomic replicon system (27, 32) and HCV-JFH1 virus cell culture (30, 34). Here, we demonstrate that cellular SRPK is required for HCV replication and suggest that the inhibitor of SRPK could be used therapeutically.

MATERIALS AND METHODS

SRPK inhibitor. SRPIN340, *N*-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl] isonicotinamide, inhibits SRPK1 and SRPK2 kinase activities potently (7). SRPIN340 does not inhibit other classes of SRPKs significantly, including Clk1 and Clk and other classes of SR kinases. SRPIN614, *N*-methyl-*N*-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl]isonicotinamide, is a negative-control compound that has no suppressive effects on SRPK1 or SRPK2. SRPIN340 and SRPIN614 were synthesized in-house (7).

***In vitro* kinase assay.** Kinase activities of SRPKs were assayed as described previously (18). Briefly, His_6-tagged recombinant SRPK1 or SRPK2 was expressed in *Escherichia coli* and purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography. The purified SRPK1 or SRPK2 was incubated in the presence of ATP, [γ-^{32}P]ATP, and a synthetic peptide of the SF2/ASF RS domain (NH_2-RSPSYGRSRSRSRSRSRSRSNSRSRSY-OH) at pH 7.5 and 30°C for 10 min. The reaction mixtures were spotted onto phosphocellulose membranes (What-

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man, Kent, United Kingdom) and washed with 5% phosphoric acid solution, and the radioactivity was measured using a liquid scintillation counter. The net radioactivity was deduced by subtracting the background count from the reaction mixture without kinase, and the data are expressed as the percentage of the control sample containing the solvent.

Cells and cell culture. Huh7 and Huh7.5.1 cell lines (34) were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. To maintain cell lines carrying the HCV replicon (Huh7/Rep-Feo cells), G418 (Nacalai Tesque, Kyoto, Japan) was added to the culture medium to a final concentration of 500 μg/ml.

HCV replicon constructs and transfection. The HCV replicon plasmids, which contain Rep-Feo, were derived from the HCV-N strain (pHC1bneo/delS [Rep-Feo-1b]) and the HCV-JFH1 strain (pSGR-JFH1 [Rep-Feo-2a]) (10, 14). These constructs express a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase. RNA synthesis and transfection of the replicon have been described (Huh7/Rep-Feo-1b, Huh7/Rep-Feo-2a) (27, 32).

HCV cell culture system. A plasmid, pJFH1-full (30, 34), which encodes the full-length HCV-JFH1 sequence, was linearized and used as the template for synthesis of HCV RNA using the RiboMax large-scale RNA production system (Promega, Madison, WI) (26). After DNase I (RQ-1, RNase-free DNase, Promega) treatment, the transcribed HCV RNA was purified using ISOGEN (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh7.5.1 cells were washed twice, and 5×10^6 cells were suspended in Opti-MEM I (Invitrogen, Carlsbad, CA) containing 10 μg of HCV RNA, transferred into a 4-mm electroporation cuvette, and subjected to an electric pulse (1,050 μF and 270 V) using the Easy Ject system (EquiBio, Middlesex, United Kingdom). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish. The transfected cells were split every 3 to 5 days. The culture supernatants were subsequently transferred onto uninfected Huh7 cells.

RT-PCR. SRPK mRNA was detected by reverse transcription-PCR (RT-PCR) as described previously (12). The primers used were SRPK1-S (5′-GCG AAT GCA GGA AAT TGA GG-3′) and SRPK1-AS (5′-CAT AAG CGT TTG ATC CTG GC-3′) and SRPK2-S (5′-CCC TGC GGA CTA CTG CAA AGG-3′) and SRPK2-AS (5′-CAT TGC AAC AAA TCT TTT CCC-3′).

Luciferase assays. Luciferase activity was measured with a Lumat LM9501 luminometer (Promega) using a Bright-Glo luciferase assay system (Promega) or a Dual-Luciferase reporter assay system (Promega), as described previously (22).

MTS assays. To evaluate cell viability, dimethylthiazol carboxymethoxy-phenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega), as described previously (24).

Quantification of HCV core antigen in culture media. Culture media from JFH1-RNA-transfected Huh7 cells were collected, passed through a 0.45-μm filter (MILLEX-HA; Millipore, Bedford, MA), and stored at −80°C. The concentrations of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV antigen; Ortho-Clinical Diagnostics, Tokyo, Japan).

Real-time RT-PCR analysis. The real-time RT-PCR was done as previously described (11). Briefly, total cellular RNA was isolated using ISOGEN (Nippon Gene), reverse transcribed, and subjected to real-time PCR analyses. Expression of mRNA was quantified using the TaqMan universal PCR master mix and the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA).

Western blot analysis. Western blotting was performed as described previously (11). Briefly, 10 μg of total cell lysate was separated by SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with the primary antibodies, followed by a peroxidase-labeled anti-IgG antibody, and visualized by chemiluminescence using the ECL Western blotting analysis system (Amersham Biosciences, Buckinghamshire, United Kingdom). The antibodies used were mouse monoclonal anti-HCV-core antibody (Abcam, Cambridge, MA), mouse monoclonal anti-HCV-NS5A antibody (Biodesign), and mouse anti-beta-actin antibody (Sigma).

Indirect immunofluorescence assay. Cells seeded onto tissue culture chamber slides were fixed with cold acetone. The cells were incubated with anti-hemagglutinin (HA) and anti-core or anti-NS5A antibodies and subsequently with Alexa 488- or Alexa 568-labeled secondary antibodies. Cells were mounted with VECTA SHIELD mounting medium and DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories) and visualized by fluorescence microscopy (BZ-8000; Keyence) and confocal laser microscopy (FLUOVIEW FV10i; Olympus, Tokyo, Japan).

Synthetic siRNA. The small interfering RNAs (siRNAs) were designed to target SRPK1 and SRPK2. Sequences of SRPK1-directed siRNAs were as follows: no. 1, 5′-UUA AUG ACU UCA AUC ACU CCA UUG C-3′; no. 2, 5′-UAA GAA AUC UGU GAA GCC AGC UGC C-3′. Sequences of SRPK2-directed siRNAs were as follows: no. 3, 5′-AAU ACU GCC UAG CAG CUC UAU GAU G-3′; no. 4, 5′-UCA GCU UGG UGA UGU GUC GCA GUU C-3′. The control siRNA has been described previously (32).

Plasmid constructs. Plasmid pEMCV/IRES/Rluc, which is a renilla luciferase expression plasmid that is driven by an encephalomyocarditis virus internal ribosome entry site (EMCV-IRES), has been described (19). Eukaryote expression plasmids for SRPK1 and SRPK2, pME-HA-SRPK1 and pME-HA-SRPK2, have been described (16).

Calculation of EC_{50}. The 50% effective concentration (EC_{50}) was calculated as the concentration of an inhibitor required for 50% reduction in replicon-based luciferase activity. We used probit regression analysis to obtain values.

Statistical analyses. Statistical analyses were performed using Student's *t* test; *P* values of less than 0.05 were considered statistically significant.

RESULTS

Immunofluorescence microscopy of SRPK and HCV proteins. We first studied the subcellular localization of SRPK1 and SRPK2 and their association with HCV proteins. Expression plasmids for SRPK1 or SRPK2 were transfected into HCV replicon-expressing or HCV-JFH1-infected cells. Immunofluorescence analysis was performed 48 h after transfection (Fig. 1). SRPK1 and SRPK2 were distributed diffusely in the cytoplasm, and HCV core and NS5A proteins were localized at the perinuclear rim and also in the cytoplasm. Although most portions of SRPKs and the viral NS5A and core proteins were localized in different cellular compartments, SRPKs and the HCV core and NS5A proteins colocalized to some extent in the perinuclear area.

SRPIN340 inhibits kinase activities of SRPK1 and SRPK2. The *in vitro* kinase assays showed that SRPIN340 (Fig. 2A) inhibited the kinase activities of SRPK1 and SRPK2. Ten μM SRPIN340 inhibited SRPK1- and SRPK2-mediated phosphorylation of synthetic RS-repeat peptide substrate by 99.2% and 85%, respectively (Fig. 2B), which was consistent with the results of our previous study (7). The K_i value for inhibition of SRPK1 kinase activity was 0.89 μM. SRPIN614, which lacked SRPK inhibitory action, did not inhibit SRPK1 or SRPK2 activity significantly.

SRPK inhibitor effectively suppresses HCV subgenomic replication. Next, we detected expression of SRPK1 and SRPK2 mRNAs in Huh7 cell lines using RT-PCR. As shown in Fig. 2C, both SRPK1 and SRPK2 mRNAs were detectable in Huh7 cells. Next, we assessed the effects of SRPIN340 on replication of the HCV genotype 1b and 2a replicons. SRPIN340 was added to HCV replicon-expressing cells, Huh7/Rep-Feo-1b and Huh7/Rep-Feo-2a. After 48 h of incubation, expression levels of the HCV replicons were measured by luciferase assay. SRPIN340 suppressed HCV 1b and 2a replication in a dose-dependent manner (Fig. 3A). The 50% effective concentrations (EC_{50}) for the HCV 1b and 2a replicons were 4.7 μM and 15.8 μM, respectively. In contrast, SRPIN614, which did not possess SRPK inhibitory activity, did not suppress expression of the replicon even at a concentration of 100 μM. MTS-mediated cell viability assays showed no significant effects of SRPIN340 or SRPIN614 (Fig. 3B). Similarly, we assessed the effect by Western blotting. SRPIN340 suppressed cellular HCV NS5A protein expression levels in a dose-dependent manner (Fig. 3C). SRPIN340 showed no effect on EMCV-IRES-mediated protein expression (Fig. 3D). These results

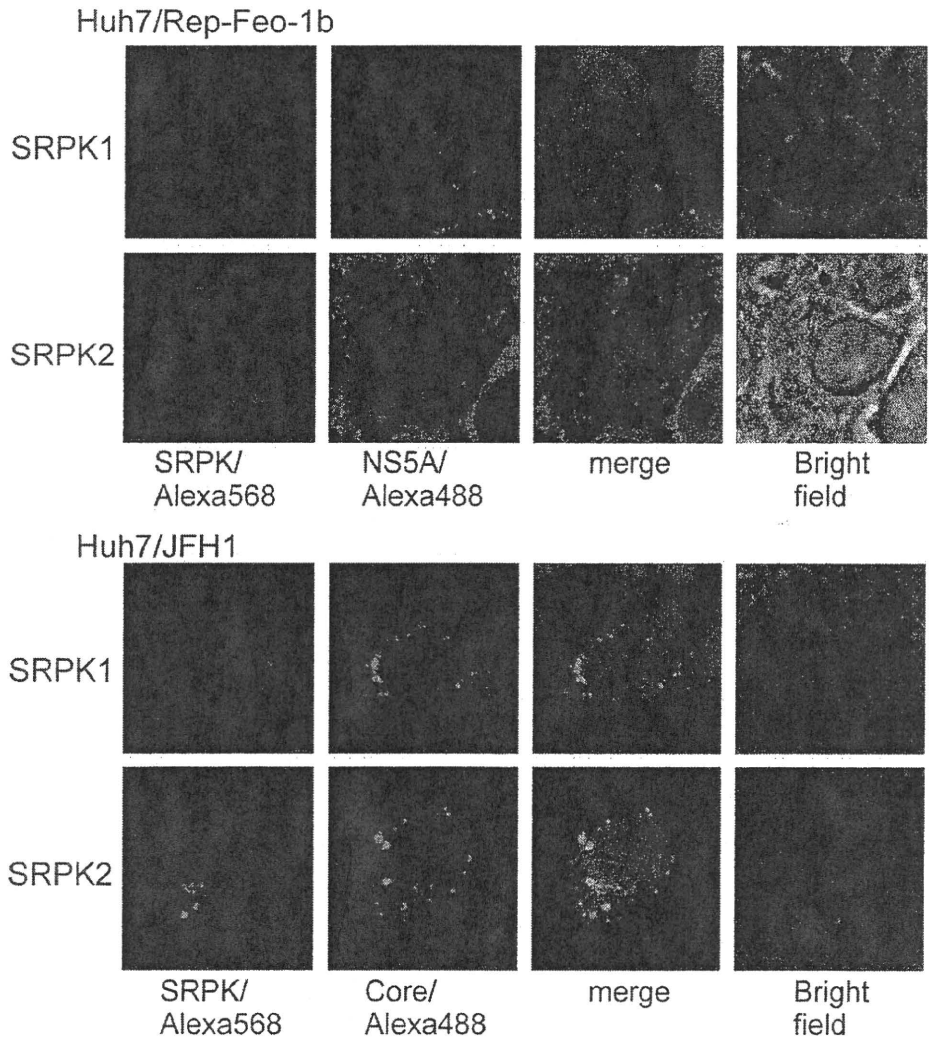

FIG. 1. Immunofluorescence microscopy. Expression plasmids for SRPK1 and SRPK2 were transfected into Huh7/Rep-Feo-1b cells or into HCV-JFH1-infected Huh7.5.1 cells. Forty-eight hours after transfection, cells were fixed and incubated with mouse anti-NS5A or anti-core antibodies and rabbit anti-HA antibody, followed by Alexa Fluor 488-labeled anti-mouse IgG and Alexa Fluor 568-labeled anti-rabbit IgG secondary antibodies. Nuclei were stained with DAPI. Representative immunofluorescence images derived from a number of experiments are shown as three images of a single focal plane of Huh7 cells, showing NS5A and core proteins (green), SRPK1 and SRPK2 (red), DAPI staining (blue), and the superimposed images (merge).

indicated that the SRPK inhibitor had specific suppressive effects on HCV subgenomic replication and that these effects are not due to cytotoxicity.

SRPIN340 suppresses HCV-JFH1 in cell culture. Next, we assessed the effects of the SRPK inhibitor on HCV-JFH1 in cell culture. Various concentrations of SRPIN340 were added to HCV-JFH1-infected Huh7 cells, and core antigen was quantified in the medium after 48 h of incubation. As shown in Fig. 4A, SRPIN340 significantly suppressed HCV core antigen secretion in a dose-dependent manner. An MTS-based cell viability assay did not show significant cytotoxicity from these inhibitors (Fig. 4B). In Western blotting, SRPIN340 suppressed expression of intracellular core protein by HCV-JFH1-infected cells in a dose-dependent manner; incubation with 30 μM SRPIN340 suppressed core protein expression by 54% of the drug-negative control, while SRPIN614 did not suppress core protein expression substantially (Fig. 4C). The effects of SRPIN340 on cellular HCV RNA were confirmed by real-time RT-PCR analyses (Fig. 4D). Similarly, in immunofluorescence microscopy, treatment with SRPIN340 resulted in a dose-dependent decrease in the number of HCV core-positive cells, but no effect was detected following treatment with SRPIN614 (Fig. 4E). These data indicate that SRPK inhibitors have antiviral effects on HCV infection and replication *in vitro*.

Overexpression and knockdown of SRPKs regulated HCV subgenomic replication. Next, we investigated the effects of the cellular expression levels of SRPK on HCV replication by overexpression and knockdown experiments. Expression plasmids for SRPK1 and SRPK2 were transfected individually into Huh7/Rep-Feo-1b cells, and internal luciferase activities were measured 72 h after transfection. The SRPK plasmid-transfected Huh7 cells expressed HA-tagged SRPK1 and SRPK2

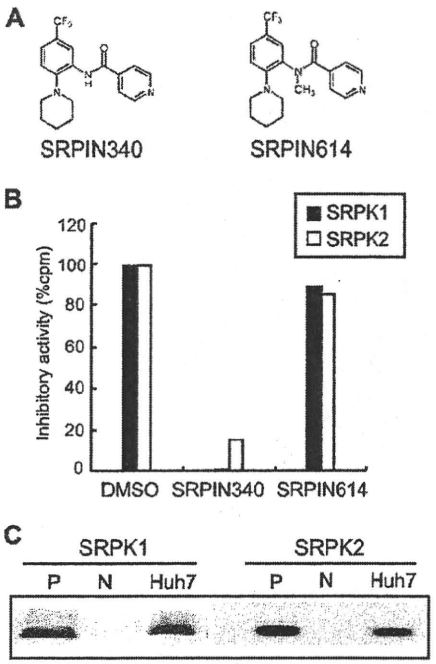

FIG. 2. Chemical structures and activities of SRPIN340 and SRPIN614. (A) Chemical structures of the SRPK inhibitor, SRPIN340, and activity-lacking control, SRPIN614. (B) Relative kinase activities of SRPK1 (black columns) and SRPK2 (white columns) *in vitro*, in the presence of the reagents indicated, SRPIN340, SRPIN614, and dimethyl sulfoxide (DMSO). (C) Expression of SRPK1 and SRPK2 mRNA by RT-PCR. P denotes positive controls, which are 1 ng of the respective SRPK expression plasmids. N denotes the template-lacking negative control.

proteins (Fig. 5A). Transfection efficiencies were ~20% in each experiment and were not different between expression plasmids. As shown in Fig. 5B, the luciferase activities were significantly increased in Huh7/Rep-Feo-1b cells transfected with SRPK1 or SRPK2. Western blotting showed that cellular expression of the HCV NS5A protein was increased in replicon-expressing cells with overexpression of SRPK1 or SRPK2 (Fig. 5C).

Four synthetic siRNAs were used to investigate the effects on HCV replication of suppression of expression of SRPK1 and SRPK2 proteins. These were directed against SRPK1 (siRNA 1 and siRNA 2) and SRPK2 (siRNA 3 and siRNA 4). Transgenic expression of SRPK1 and SRPK2 was specifically suppressed by transfection of the relevant siRNAs into Huh7 cells (Fig. 6A). Next, various amounts of individual siRNA (siRNA 1, 2, 3, or 4) were transfected into Huh7/Rep-Feo-1b cells, and luciferase assays were carried out 48 h after transfection. As shown in Fig. 6B, each siRNA suppressed expression of the HCV replicon. Western blotting also showed suppression of HCV protein expression after transfection of each siRNA (Fig. 6C). These results indicated that expression of SRPK1 and SRPK2 is positively correlated with the efficiency of HCV replication.

Absence of viral or cellular resistance to SRPIN340. In order to assess whether long-term exposure to the antiviral molecule could select a resistant replicon, we compared sensitivity to

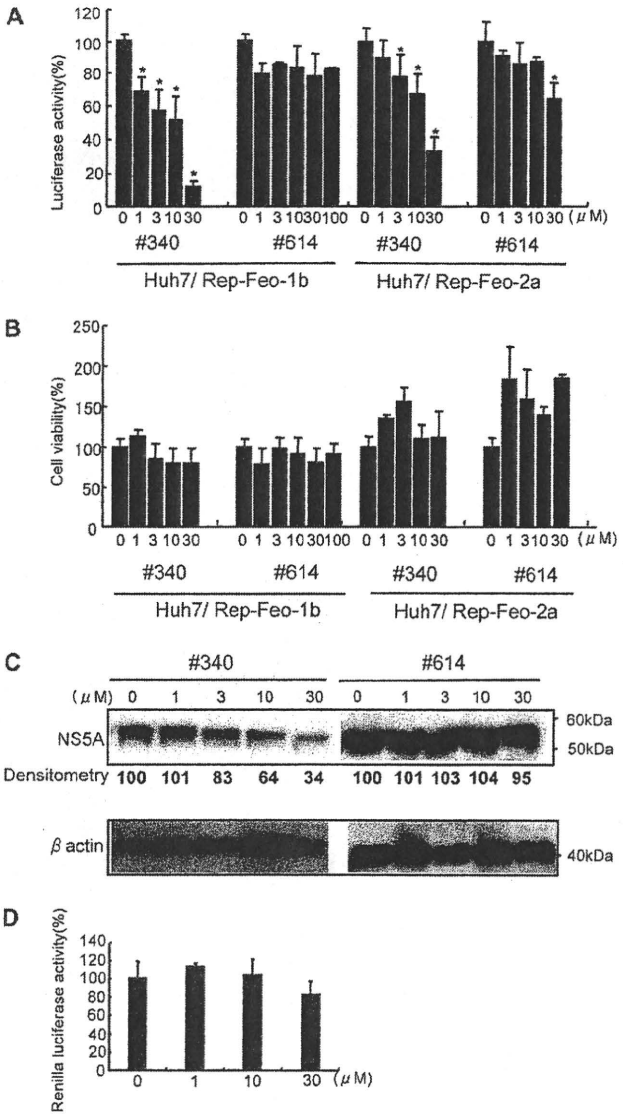

FIG. 3. Effects of SRPIN340 and SRPIN614 on expression of HCV subgenomic replicons. Huh7/Rep-Feo-1b or Huh7/Rep-Feo-2a cells were cultured in the presence of SRPIN340 (no. 340) or SRPIN614 (no. 614) at the concentrations indicated. After 48 h of culture, a luciferase assay (A), a cell viability assay (B), and Western blotting (C) were performed. (A) Effect of SRPIN340 and SRPIN614 on levels of HCV replication represented by replicon-dependent internal luciferase activities. Bars indicate luciferase activities relative to that of the drug-negative control. (B) Effect of SRPIN340 and SRPIN614 on cell viability. MTS assays were performed after culture in the presence of the drugs indicated. Bars indicate values relative to that of the drug-negative control. Asterisks indicate P values of less than 0.05. (C) Western blotting analyses. The expression levels of NS5A and beta-actin were detected by using anti-NS5A and anti-beta-actin antibodies. Densitometry of NS5A protein was performed, and results are indicated as percentages of the drug-negative control. The assay was repeated three times, and a representative result is shown. (D) Effect of SRPIN340 on EMCV-IRES-driven protein expression. Plasmid pECMV/IRES-Rluc was transfected into Huh7 cells. Twenty-four hours after transfection, the cells were incubated in indicated concentrations of SRPIN340. The renilla luciferase assay was performed at 48 h after incubation. In panels A, B, and D, assays were done in quadruplicate, and error bars indicate standard deviations.

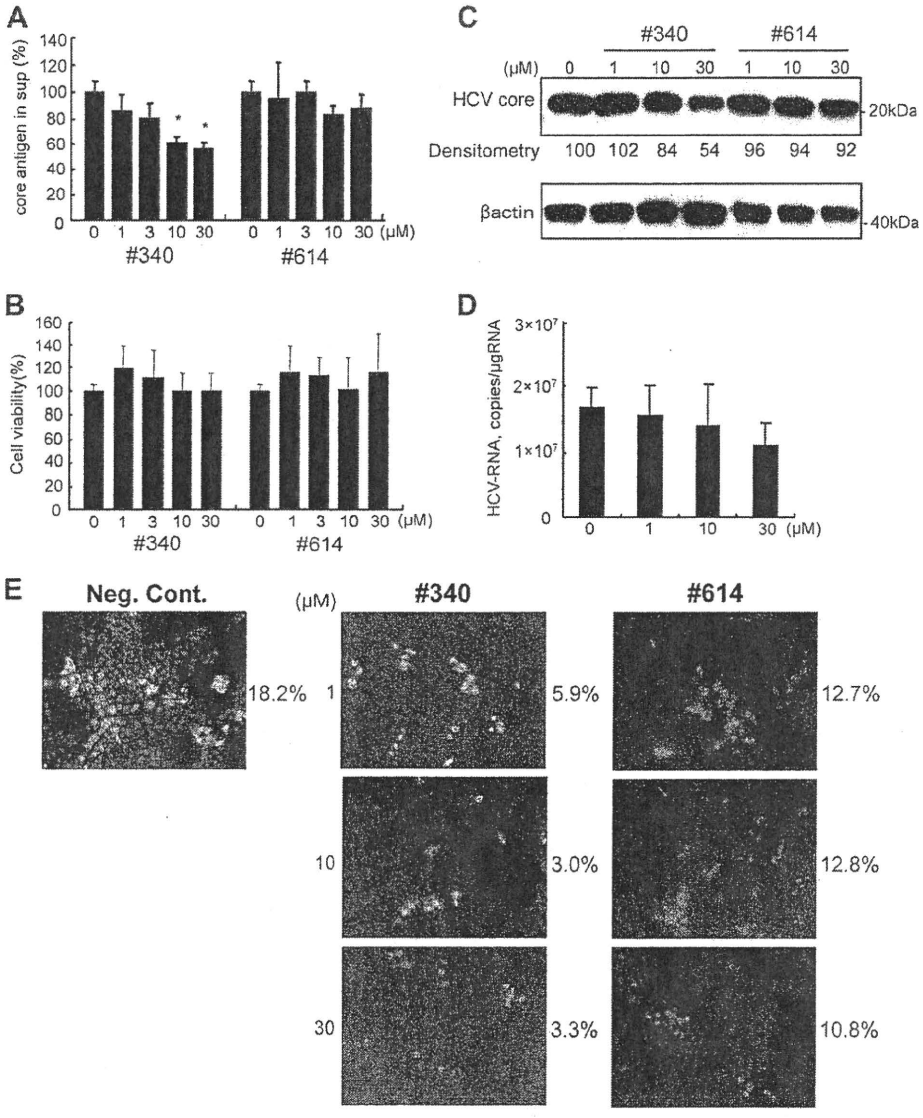

FIG. 4. Effect of SRPIN340 and SRPIN614 on HCV-JFH1 virus replication. HCV-JFH1-stably infected Huh7 cells of ~14 days were cultured in the presence of SRPIN340 or SRPIN614 at the concentrations indicated. After 48 h, cellular and supernatant HCV core antigens were detected. (A) HCV core antigen assays of culture supernatant (sup). Bars indicate values relative to that of the drug-negative control. Asterisks indicate *P* values of less than 0.05. (B) Effect of SRPIN340 and SRPIN614 on cell viability. MTS assays were performed 48 h after culture in the presence of the drugs indicated. Bars indicate values relative to that of the drug-negative control. (C) Western blotting analyses. The expression of HCV core and beta-actin was detected using anti-core and anti-beta-actin antibodies. Densitometry of HCV core protein was performed, and results are indicated as percentages of the drug-negative control. (D) Real-time RT-PCR analyses. Cells were harvested at 48 h after SRPIN340 treatment. (E) Immunofluorescence microscopy. Naïve Huh7.5.1 cells were infected with HCV-JFH1 culture supernatant at a multiplicity of infection of 0.1. Three days after infection, SRPK340 or SRPIN614 was added. After 48 h, cells were incubated with anti-core antibodies followed by Alexa Fluor-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). The percentages of HCV core-positive cells were calculated and are indicated on the right of each view. The assay was repeated three times, and a representative result is shown. Neg. cont., negative control. In panels A, B, and D, assays were done in triplicate, and error bars indicate standard deviations.

SRPIN340 between HCV replicon cells after continuous treatment of the drug and their control cells (Fig. 7A). Huh7/Rep-Feo-1b cells were treated with or without 30 μM SRPIN340 for 2 weeks. After 1 week of recovery culture without SRPIN340, a cell line, designated Huh7/Rep-Feo-1b(R), was established. As shown in Fig. 7B, the suppressive effect of SRPIN340 was not significantly different between Huh/Rep-Feo-1b(R) and its control cell line. These results suggest that SRPIN340 treatment under these conditions may not see the emergence of drug-resistant HCV replicons or cellular hyporesponsiveness to the drug.

DISCUSSION

These results demonstrate that small molecule inhibitors of cellular SRPK1 and SRPK2 (Fig. 2A) efficiently and specifically suppress intracellular replication of HCV subgenomic

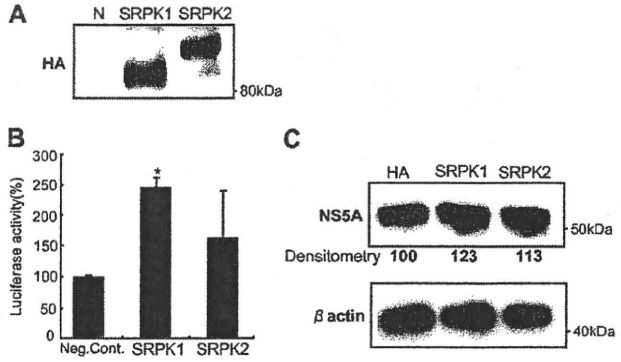

FIG. 5. Effects of overexpression of SRPK1 and SRPK2 on HCV replication. (A) The expression of transfected HA-tagged SRPK1 and SRPK2 was detected by anti-HA antibody. (B) Huh7/Rep-Feo-1b cells seeded on 24-well plates were transfected with 0.2 μg of expression plasmids for SRPK1 or SRPK2 or empty vector. Forty-eight hours after transfection, the levels of HCV replication were measured by luciferase assay. Bars indicate values relative to that of the empty vector-transfected control. Assays were done in triplicate, and error bars indicate standard deviations. Asterisks indicate *P* values of less than 0.05 compared with the control. (C) Expression of HCV NS5A and beta-actin was detected using anti-NS5A and anti-beta-actin antibodies. Densitometry of HCV core protein was performed, and results are indicated as percentages of the control.

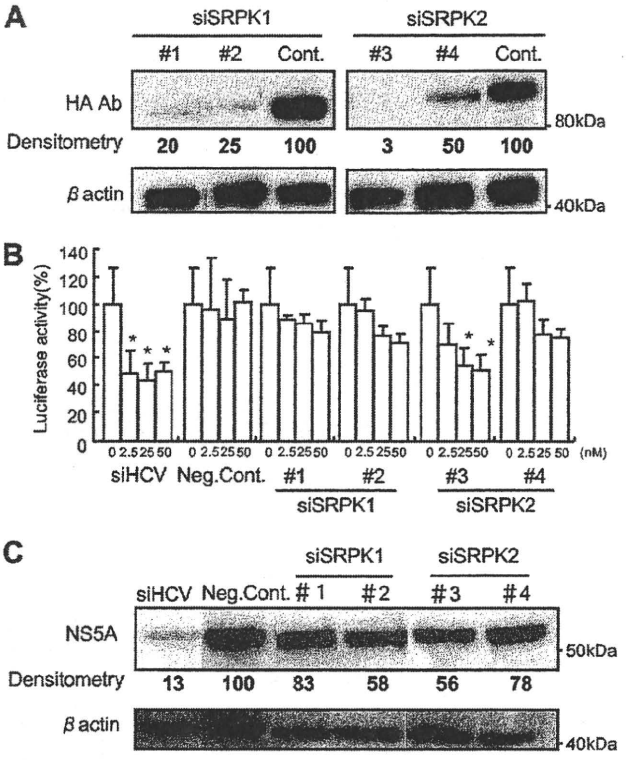

FIG. 6. Effects of siRNA knockdown of SRPK1 and SRPK2. (A) Huh7 cells were transfected with SRPK1 or SRPK2 expression plasmids and siRNA directed against SRPK1 (siSRPK1 no. 1 and siSRPK1 no. 2) or SRPK2 (siSRPK2 no. 3 and siSRPK2 no. 4) or control siRNA (32). Forty-eight hours after transfection, Western blotting was performed using anti-HA and anti-beta-actin antibodies. (B) Effects of siRNAs on HCV replication. The siRNAs indicated were transfected into Huh7/Rep-Feo-1b cells, and luciferase activities were measured 48 h after transfection. siHCV denotes the positive control, siRNA directed against the 5′-untranslated region of the HCV genome, and Neg. Cont. denotes a negative-control siRNA targeting an unrelated gene, which has been described previously (32). Bars indicate values relative to that of the mock-transfected control. Assays were done in triplicate, and error bars indicate standard deviations. Asterisks indicate *P* values of less than 0.05. (C) Western blotting analyses. Fifty micromoles of the siRNAs indicated was transfected into Huh7/Rep-Feo-1b cells. Forty-eight hours after transfection, cells were harvested and subjected to Western blotting. Expression of NS5A and beta-actin was detected with the relevant antibodies. Densitometry of NS5A protein was performed, and results are indicated as percentages of the control.

replicons and HCV-JFH1 viruses in cell culture, in a dose-dependent manner (Fig. 3 and 4). Real-time RT-PCR and Western blot analyses revealed that both RNA synthesis and its translation were reduced by SRPIN340. This inhibition was not associated with antiproliferative or nonspecific cytotoxic effects on the host cells (Fig. 3B and 4B). Transgenic overexpression of SRPK1 or SRPK2 resulted in augmentation of HCV replication and infection (Fig. 5). On the other hand, siRNA-mediated knockdown of these SRPKs suppressed HCV replication significantly (Fig. 6). These results demonstrate the dependence of the virus on the host RNA processing machinery that consists of SR proteins and their regulator, SRPK, and indicate that the inhibition of host SRPKs by small molecules may constitute a novel antiviral treatment against HCV.

SRPK1 and SRPK2 belong to the serine/threonine protein kinases. The two SRPKs efficiently phosphorylate SR proteins, such as the splicing factors ASF/SF2 and SC35, at their RS domains (3, 31). Overexpression of either SRPK1 or SRPK2 induces the phosphorylation-dependent shift of SR proteins from nuclear speckles to the nucleoplasm (8). Because SR proteins regulate splice site selection and spliceosome assembly, SRPK-mediated phosphorylation and cellular redistribution of SR proteins have been implicated in the control of mRNA maturation and alternative RNA splicing (31).

It remains to be clarified how the SRPK and SR proteins are involved in HCV replication and how the SRPIN340-directed suppression of such proteins leads to suppression of replication. There are several possibilities: that SRPIN340 may suppress processing of mRNAs that encode essential host proteins for HCV replication, that it suppresses alternative processing of the viral genomic RNA, and that certain viral proteins are substrates of host SRPK. Li et al. screened host factors required for HCV propagation through genome-wide siRNA targeting (17). They did not identify SRPKs as essential host proteins for HCV infection. Because our SRPIN340 inhibits both SRPK1 and SRPK2 and may target other family members of SRPK that possess the same target domain, it is still possible that the maintenance of overall SRPK activity may be essential for cellular HCV replication.

Several lines of evidence suggest that the viral life cycle may be partly governed by the regulation of SR protein phosphorylation as part of the RNA-processing machinery. It has been reported that virus infection induces dephosphorylation and functional inactivation of SR proteins. As a possible mechanism, Kanj et al. (13) have reported that adenoviral infection caused cellular accumulation of ceramide, which induces dephosphorylation of SR proteins by activation of the host pro-

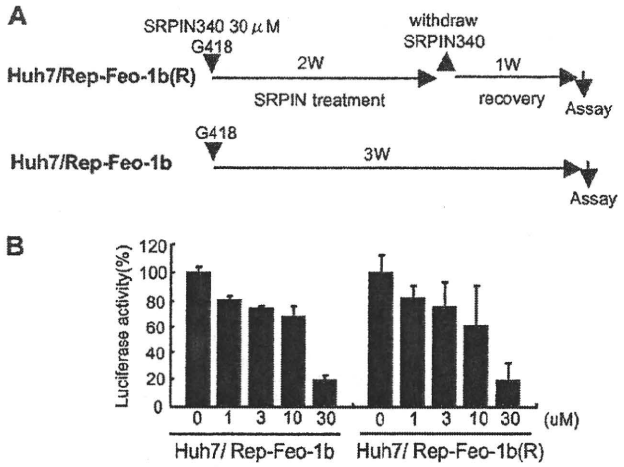

FIG. 7. Drug resistance assay of HCV replicon cells. (A) Schema for the establishment of SRPIN340-resistant cells and the control cells. Huh7/Rep-Feo-1b cells were treated with or without 30 μM SRPIN340 for 2 weeks in the presence of 500 μg/ml of G418. After 1 week of recovery culture without SRPIN340, a cell line, Huh7/Rep-Feo-1b(R), was established. (B) Huh/Rep-Feo-1b and Huh7/Rep-Feo-1b(R) cell lines were cultured in the presence of indicated concentrations of SRPIN340. Forty-eight hours after culture, internal luciferase assays were performed. Bars indicate luciferase activities relative to that of the drug-negative control. Assays were done in quadruplicate, and error bars indicate standard deviations.

tein phosphatase (PP)1 and consequently suppresses viral replication (2). At an early stage of adenoviral infection, the viral E4-ORF4 protein binds to the host PP2A and SR proteins, resulting in dephosphorylation of the SR proteins and consequent activation of IIIa splicing of the viral precursor mRNA that is the dominant transcript of the late phase of infection (5). In HIV infection, the role of SR proteins in the splicing of the proviral RNA has been demonstrated by a report that overexpression of SRp40, SRp55, or SRp75 caused overproduction of HIV (7). HIV Tat controls subcellular localization of SR proteins and establishes efficient HIV replication. These findings suggest that the levels of SR protein phosphorylation are positively correlated with early viral replication in host cells and that SRPIN340 treatment suppresses viral replication at an early stage.

It has been reported that HBV core protein is a substrate of SRPK1- and SRPK2-mediated phosphorylation (4). Phosphorylation of RS domains in HBV core prevents nonspecific RNA binding, which facilitates specific interaction of HBV core with the pregenomic RNA and formation of immature capsids. A functional similarity between HBV core protein and SR proteins has been reported. Our preliminary results showed that SRPIN340 suppressed expression of the viral proteins and secretion of HBe and HBs antigens. While we have not demonstrated SRPK-mediated phosphorylation of HCV proteins, our immunofluorescence microscopy has demonstrated partial colocalization of SRPKs and HCV NS5A and core proteins. These findings may suggest a possible direct interaction between SRPKs and HCV proteins, and those interactions may be the targets of SRPIN340.

Given the current situation of limited therapeutic options against HCV, searching for more potent and less toxic antiviral drugs is needed to improve clinical anti-HCV chemotherapeutics. Several direct antiviral agents against HCV are currently undergoing clinical trials; these include NS3 protease inhibitors and NS5B polymerase inhibitors (28). However, the frequent emergence of drug-resistant mutant viruses is a major weakness of such agents (15). Because our compound, SRPIN340, targets host proteins, it may be effective against multiple HCV genotypes and it is less likely that drug-resistant viruses will emerge (20). Furthermore, the toxicity data available for SRPIN340 are promising (7). No adverse effects were observed when SRPIN340 was administered orally to rats, even at the highest dose (2,000 mg/kg of body weight) for 2 weeks (data not shown). These data support the feasibility of long-term *in vivo* use of this compound to suppress HCV replication. On the other hand, the fact that this inhibitor acts through cellular components still raises concerns regarding its safety in the case of human use. We should not be reassured by the cytotoxicity data and the small-animal data, and further preclinical studies should be planned to address this issue. Overall, our results indicate that SRPIN340, which suppresses a wide range of DNA and RNA viruses, also is effective at suppressing HCV infection and replication. Future studies with SRPIN340, its derivatives, and other chemicals that target SRPKs could be directed toward developing a new class of antiviral treatment regimens and drugs.

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Cell culture and in vivo analyses of cytopathic hepatitis C virus mutants☆,☆☆

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ABSTRACT

HCV-JFH1 yields subclones that develop cytopathic plaques (Sekine-Osajima Y, et al., Virology 2008; 371:71). Here, we investigated viral amino acid substitutions in cytopathic mutant HCV-JFH1 clones and their characteristics in vitro and in vivo. The mutant viruses with individual C2441S, P2938S or R2985P signature substitutions, and with all three substitutions, showed significantly higher intracellular replication efficiencies and greater cytopathic effects than the parental JFH1 in vitro. The mutant HCV-inoculated mice showed significantly higher serum HCV RNA and higher level of expression of ER stress-related proteins in early period of infection. At 8 weeks post inoculation, these signature mutations had reverted to the wild type sequences. HCV-induced cytopathogenicity is associated with the level of intracellular viral replication and is determined by certain amino acid substitutions in HCV-NS5A and NS5B regions. The cytopathic HCV clones exhibit high replication competence in vivo but may be eliminated during the early stages of infection.

Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). Antiviral therapeutic options against HCV have been limited to type I interferons and ribavirin and have yielded unsatisfactory responses (Fried et al., 2002). Given this situation, a precise understanding of the molecular mechanisms of interferon resistance has been a high priority of research in academia and industry.

Molecular analyses of the HCV life cycle, virus–host interactions, and mechanisms of liver cell damage by the virus are not understood completely, mainly because of the lack of cell culture systems. These problems have been overcome to some extent by the development of the HCV subgenomic replicon (Lohmann et al., 1999) and HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCV-JFH1 strain, which is a genotype 2a clone derived from a Japanese fulminant hepatitis patient and can replicate efficiently in Huh7 cells (Kato, 2001; Kato et al., 2003), has contributed to the establishment of the HCV cell culture system. Furthermore, the Huh7-derived cell lines, Huh-7.5 and Huh-7.5.1 cells, allow production of higher viral titers and have a greater permissivity for HCV (Koutsoudakis et al., 2007; Lindenbach et al., 2005; Zhong et al., 2005). The HCV-JFH1 cell culture system now allows us to study the complete HCV life cycle: virus–cell entry, translation, protein processing, RNA replication, virion assembly and virus release.

HCV belongs to the family *Flaviviridae*. One of the characteristics of the *Flaviviridae* is that they cause cytopathic effects (CPE). The viruses have positive strand RNA genomes of ~10 kilo-bases that encode polyproteins of ~3000 amino acids. These proteins are processed post-translationally by cellular and viral proteases into at least 10 mature proteins (Sakamoto and Watanabe, 2009). The viral non-structural proteins accumulate in the ER and direct genomic replication and viral protein synthesis (Bartenschlager and Lohmann, 2000; Jordan et al., 2002; Mottola et al., 2002). It has been recently

Abbreviations: HCV, hepatitis C virus; CPE, cytopathic effect; ER, endoplasmic reticulum; RdRp, RNA dependent RNA polymerase.

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reported that HCV-JFH1 transfected Huh-7.5.1 cells die when all of the cells are infected and intracellular HCV RNA reaches maximum levels (Zhong et al., 2006). These findings suggest HCV-induced cytopathogenicity. However, the mechanisms have not been well documented.

In a previous study, we investigated the cellular effects of HCV infection and replication using the HCV-JFH1 cell culture system and we reported that HCV-JFH1 transfected and infected cells show substantial CPE that are characterized by massive apoptotic cell death with expression of several ER stress-induced proteins. Taking advantage of the CPE, we developed a plaque assay for HCV in cell culture and isolated subclones of HCV that showed enhanced replication and cytopathogenicity (Sekine-Osajima et al., 2008). We have demonstrated that these viral characters were determined by mutations at certain positions in the structural and nonstructural regions of the HCV genome, especially the NS5A and NS5B regions.

In this study, we investigated the mechanisms and viral nucleotide sequences involved in HCV-induced cytopathic effects using HCV-JFH1 cell culture and a newly developed cytopathic plaque-forming assay. We demonstrated that introduction of NS5A and NS5B mutations into the JFH1 clone resulted in a higher replication efficiency, although introduction of these mutations into the JFH1 subgenomic replicon has no effect on viral replication. These mutations do not affect virion entry or release of viral particles but regulate virus replication, and high levels of virus replication result in cytopathogenicity.

Results

Development of cytopathic plaques by HCV infection of Huh-7.5.1 cells

A plaque assay was performed to investigate the morphological CPE following HCV-JFH1 infection (see Materials and methods). Culture supernatants from JFH1-transfected cells were diluted serially and inoculated onto uninfected Huh-7.5.1 cells. The cells were subsequently cultured in medium containing agarose. On 9 days after the inoculation, viable cells were stained and plaques were visualized (Fig. 1A). HCV-inoculated cell cultures developed plaques as unstained areas, accompanied by rounded cells in the periphery (Fig. 1B). The formation of cytopathic plaques was not observed in a parental Huh7 cell line (data not shown). Those results were consistent with our previous study (Sekine-Osajima et al., 2008).

Introduction of mutations in the NS5A and NS5B regions of the JFH1 clone augmented its cytopathic effects

Among the amino acid substitutions that developed in the plaque-derived HCV-JFH1 strains, 6 of the 9 amino acid changes appeared redundantly among 5 independently isolated plaques, and clustered in the C terminal part of the NS5A and NS5B regions. To investigate the phenotype of each amino acid substitution, we constructed mutant JFH1

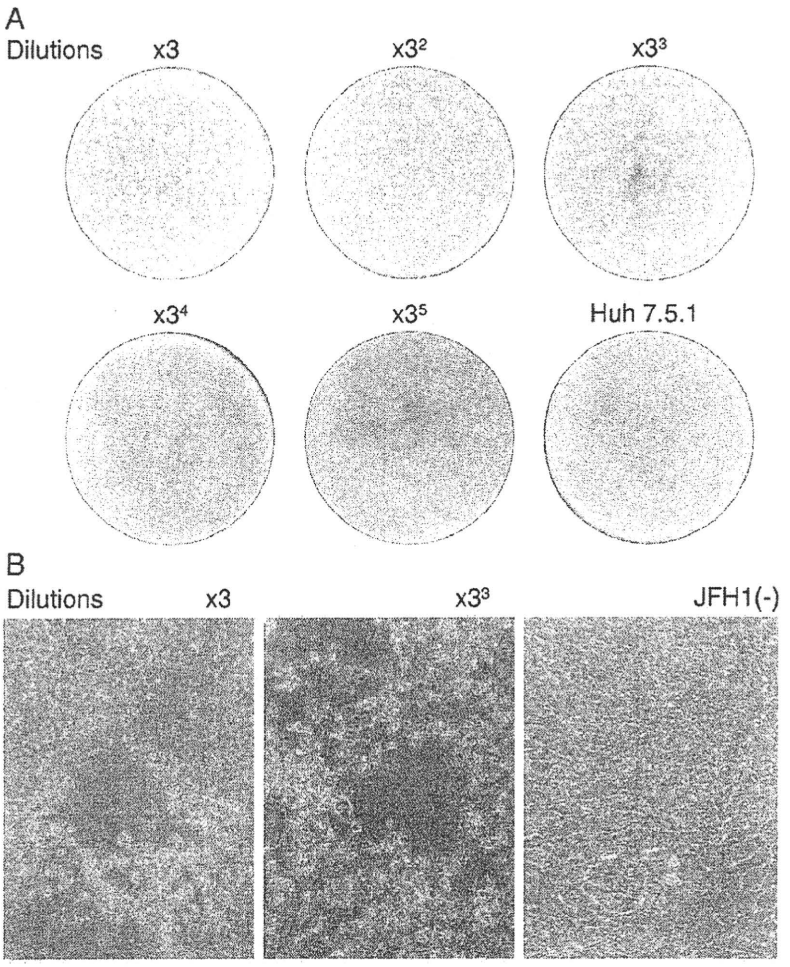

Fig. 1. The cytopathic effects of HCV-JFH1 *in vitro*. A. Plaque assay. Huh-7.5.1 cells were seeded in collagen-coated 60mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO_2 (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methyl-cellulose and incubated under normal conditions. After 7 days culture, formation of cytopathic plaque was visualized by staining with 0.08% crystal violet solution. B. The cytopathic plaques were observed by phase-contrast microscopy at day 7 after HCV-JFH1 infection.

clones in which we introduced separately one amino acid substitution in NS5A and five substitutions in NS5B (Fig. 2A) and transfected the mutant HCV RNAs into Huh-7.5.1 cells. To compare the electroporation efficiencies of viral RNAs, Huh-7.5.1 cells were harvested 8 h after transfection and the levels of intracellular core antigen were measured. There was no difference in the efficiencies of electroporation (Fig. 2D). The substitutions G2964D, H3004Q, and S3005N did not lead to cytopathic effects but three mutant subclones (C2441S, P2938S and R2985P) produced much more cell death compared to the wild type JFH1 (Fig. 2B). To assess the quantitative cytopathic effect seen in host cells for each of the mutants, we also performed MTS assay at 6 days post transfection. It showed that Huh-7.5.1 cells transfected with the triple mutants (C2441S, R2938S, or R2985P) induced apparently much more cytopathic effect compared to the parental JFH1 and other mutant clones, although the three mutant clones encoding the substitutions C2441S, P2938S, or R2985P did not show significant difference but

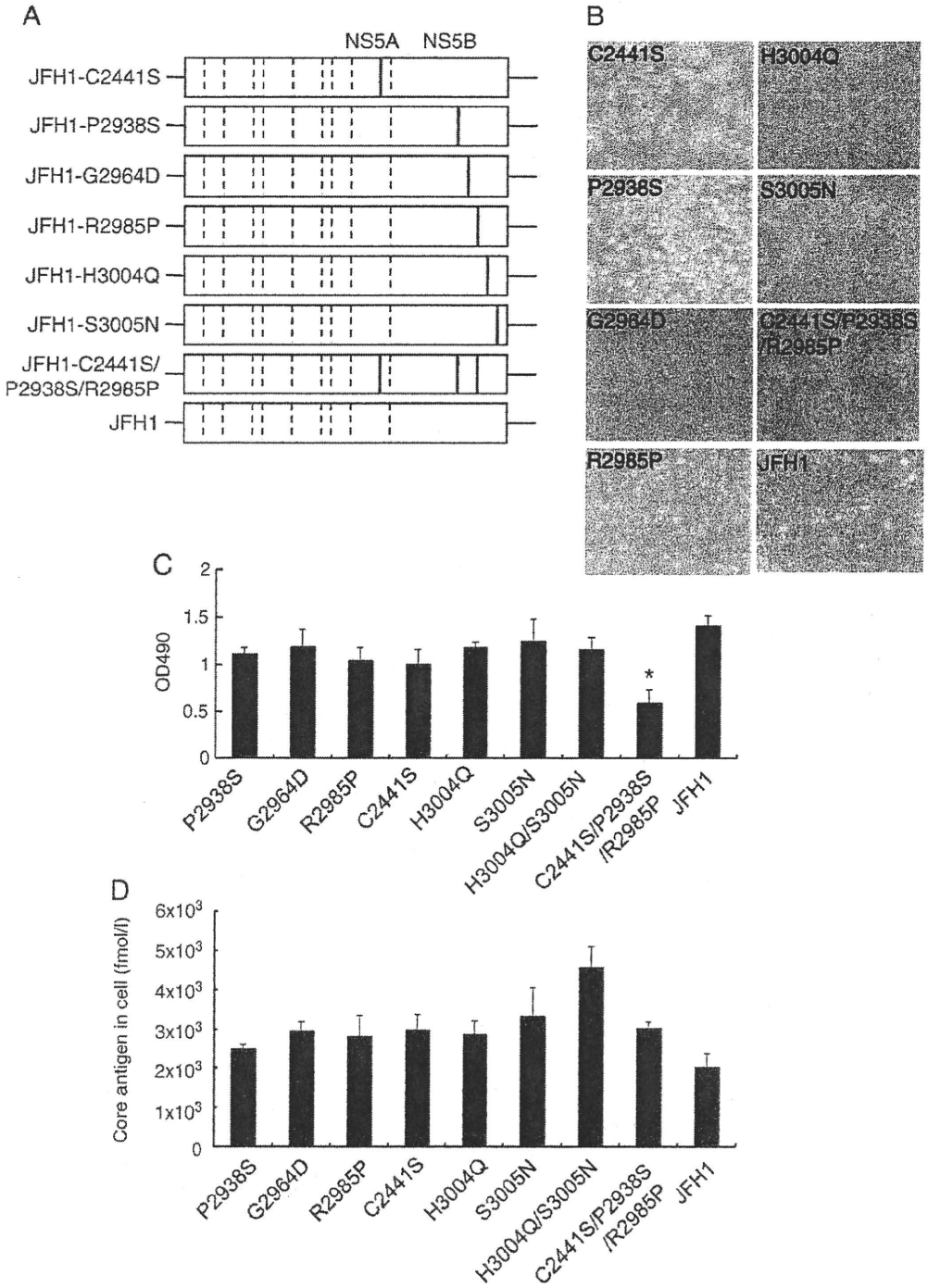

Fig. 2. Introduction of mutations into the NS5A and NS5B regions of JFH1. A. The mutations identified in the cytopathic plaque were introduced individually into the parental JFH1. Each JFH1 mutant was transfected into Huh-7.5.1 cells by electroporation. B. Huh-7.5.1 cells transfected with JFH1-mutants were observed by phase-contrast microscopy at day10 after transfection. C. MTS assay was performed to assess the quantitative cytopathic effect seen in Huh-7.5.1 cells for each of the mutants 6 days post transfection. Asterisks indicate p-values of less than 0.05 as compared with JFH1. D. Huh-7.5.1 cells were harvested at 8 h after transfection and the levels of intracellular core antigen were measured.

showed tendency to introduce more cytopathic effect than the parental JFH1 and the mutant clones encoding the substitutions G2964D, H3004Q, and S3005N (Fig. 2C).

Introduction of NS5A and NS5B mutations into the JFH1 clone led to a greater replication efficiency

To compare the expression levels of each mutant subclone, each HCV RNA was transfected and core antigen was detected subsequently in the culture medium. Similar to Fig. 2B, HCV clones with individual substitutions G2964D, H3004Q and S3005N produced significantly less core antigen or did not replicate at all. In contrast, the C2441S, P2938S and R2985P mutants produced significantly more core antigen than the wild type JFH1. In addition, an HCV clone with all 3 adaptive substitutions (C2441S, P2938S and R2985P) produced more core antigen than any other clone (Fig. 3A).

Next, we harvested the infected cells at 5 days after electroporation and performed western blotting. As shown in Fig. 3B, the three clones encoding the substitutions C2441S, P2938S, or R2985P, and the clone with all three mutations, expressed far more core protein than the parental JFH1, although the clones encoding the substitutions G2964D, H3004Q and S3005N did not express core protein. We also transferred culture media from the mutant clones onto uninfected Huh-7.5.1 cells and performed western blotting and the cells infected with the same mutant subclones as Fig. 3B expressed more core protein (Fig. 3C).

Introduction of NS5A and NS5B mutations into the JFH1 subgenomic replicon

To investigate the primary phase of replication of JFH1 mutants, we constructed JFH1 subgenomic replicons by introducing individually the six mutations in NS5A and NS5B. We transfected each replicon RNA into Huh7 cells and compared their replication levels according to the luciferase activities. Consistently with the mutant viruses, the subgenomic replicon encoding the changes C2441S, P2938S or R2985P, which produced higher amounts of core antigen, did replicate at higher levels than the other subgenomic replicons with single mutation, G2964D, H3004Q and S3005N. However, none of these mutants replicated at higher than the parental JFH1 subgenomic replicon. Furthermore, replicon with triple mutations of C2241S, P2938S and R2985P did not replicate (Fig. 4).

Introduction of NS5A and NS5B mutations into the JFH1 clone had no effect on the production of infectious virions

We sought to investigate the effects of the NS5A and NS5B mutations on virus replication and virion secretion independent of reinfection and spread of the viruses produced. Therefore, we used the S29-subclone of Huh7 cells, which cannot be infected by HCV because of a defect in CD81 expression but does support viral genomic replication and releases infectious HCV particles after transfection (Russell et al., 2008). The Huh7-S29 cells enabled us to evaluate a single cycle of infection and production of virions. Those cell lines did not show apparent cytopathic effects after transfection with HCV RNAs (data not shown). To analyze HCV particle production from cells transfected with the viral genomic RNAs transcribed in vitro, we harvested culture media and cells at 72 h post transfection and measured the core antigen levels in culture media and intracellular HCV RNA by real-time RT-PCR. The C2441S, P2938S, and R2985P mutants produced significantly greater amounts of core antigen in the culture medium than the wild type JFH1. The HCV clone carrying all three mutations produced the greatest amount of core antigen (Fig. 5A, top). Consistent with the core antigen levels in the culture media, intracellular HCV RNA levels were also higher in the cells transfected with the mutated genomes encoding separately C2441S,

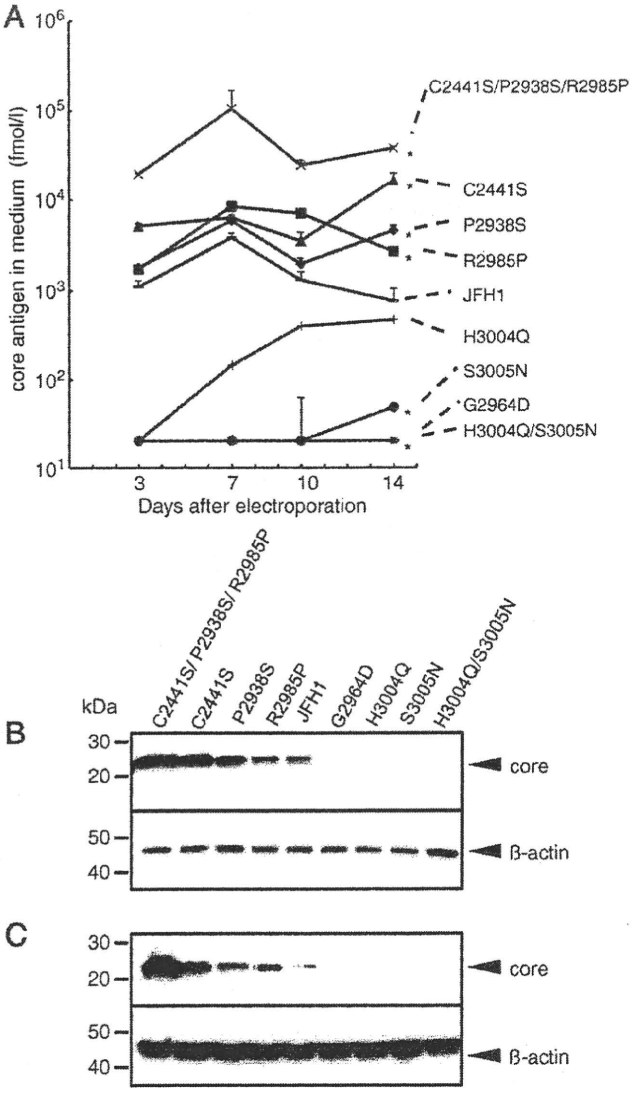

Fig. 3. Replication competences of HCV subclones with NS5A and NS5B mutations. A. Levels of core antigen in the culture medium. The culture media from transfected cells were collected on the days indicated and the levels of core antigen were measured. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Huh-7.5.1 cells transfected with JFH1 mutants were harvested at 5 days after transfection and western blotting was performed. C. The culture media from Huh-7.5.1 cells transfected with JFH1 mutants were transferred onto uninfected Huh-7.5.1 cells. The cells were harvested at 3 days after infection. Western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

P2938S, and R2985P, and that with all three mutations (Fig. 5A, middle), indicating that these mutations affected virus replication. Fig. 5A bottom shows the efficiency of infectious viral particle release from each transfectant, this being expressed as the core antigen level in the culture medium adjusted by dividing by the levels of intracellular HCV RNA. There was no difference in the efficiency of release of virions by the wild type JFH1 and the genomes carrying the C2441S, P2938S or R2985P changes. These results indicated that these three mutations in NS5A and NS5B did not affect virion entry or viral particle release but did regulate virus replication, and a high level of viral replication induces cytopathogenicity. Similarly, as shown in Fig. 3B, the three clones with C2441S, P2938S or R2985P, or all three mutations expressed much higher levels of core protein than the parental JFH1, while clones with G2964D, H3004Q or S3005N mutations did not express detectable amounts of core protein (Fig. 5B).

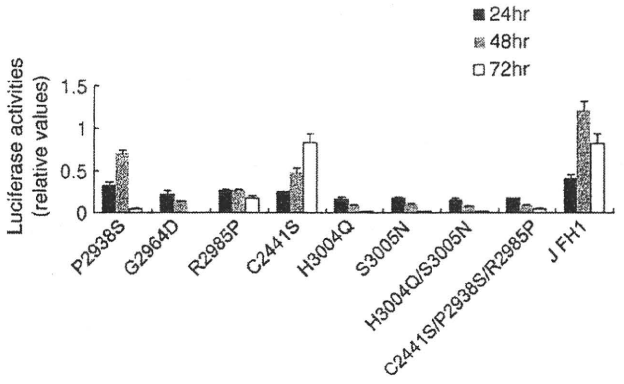

Fig. 4. Luciferase assay of the cytopathic JFH1-subgenomic replicon. Mutations were introduced into 2a-Feo subgenomic replicon and transcribed RNA for each replicon was transfected into Huh7 cells by electroporation. The cells were harvested at 24 h, 48 h and 72 h after electroporation and were used for Luciferase assay. Values are relative values to those of 8 h.

Mutations of NS5A and NS5B are associated with replication competence at earlier stages in vivo

We next used human hepatocyte chimeric mice to investigate the infectivity of the triple mutant of NS5A and NS5B. We confirmed the mouse liver chimerism greater than 70% by immunohistochemical analysis (data not shown). Culture media of the parental JFH1 and the mutant subclone with three mutations (C2441S, P2938S, and R2985P), were collected following transfection of Huh-7.5.1 cells, concentrated, and inoculated intravenously into human hepatocyte chimeric mice. We confirmed that the three mutations in NS5A and NS5B were conserved in the virus genome sequence of cell culture supernatants that were used for inoculation (data not shown). Two mice were inoculated with JFH1 and three were inoculated with the mutant virus. HCV RNA and human albumin in the sera of the mice were detected sequentially.

We repeated the same exam twice and confirmed consistency of the results. In the early phase post inoculation, the concentration of HCV RNA in serum was significantly higher in mice inoculated with the culture medium from the mutant subclone (Fig. 6A), suggesting that the mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated with virus replication in vivo. However, there was no difference in the level of HCV RNA in later period. The disparity of viral production at early time point could be influenced by the disparate numbers of infectious virus between the 2 initial inoculums. However, the sharp elevation of serum HCV RNA at day 5 after dropping at day 3 indicates that the mutants (C2441S, P2938S plus R2985P) are more replication competent at early stages in vivo. Serum levels of human albumin remained constant throughout the observed periods and showed no significant differences between wild and mutant-infected mice (Fig. 6B).

We also investigated expression of ER stress-related proteins, the glucose regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP), in liver of chimeric mice infected with JFH1 or the mutant in the early phase post inoculation. Human hepatocyte chimeric mice were inoculated in the same way as described above, and we verified that the level of virus titer in serum of each mouse was same as presented in Fig. 6A (data not shown). We sacrificed one each mouse that was infected with wild type or mutant JFH1 at 5 day of infection and investigated hepatic expression of GRP78 and CHOP. Liver histology showed no sign of inflammation or cytopathic cell death. However, as shown in Fig. 7, the expression level of both GRP78 and CHOP was higher in mice inoculated with the mutant viruses than the parental JFH1. There was no apparent difference in percents of hepatic chimerism between each mouse. These finding suggested that ER stress-related proteins were upregulated in the liver of HCV-infected mouse and that these responses were more strongly induced in the liver of mutant-infected mouse.

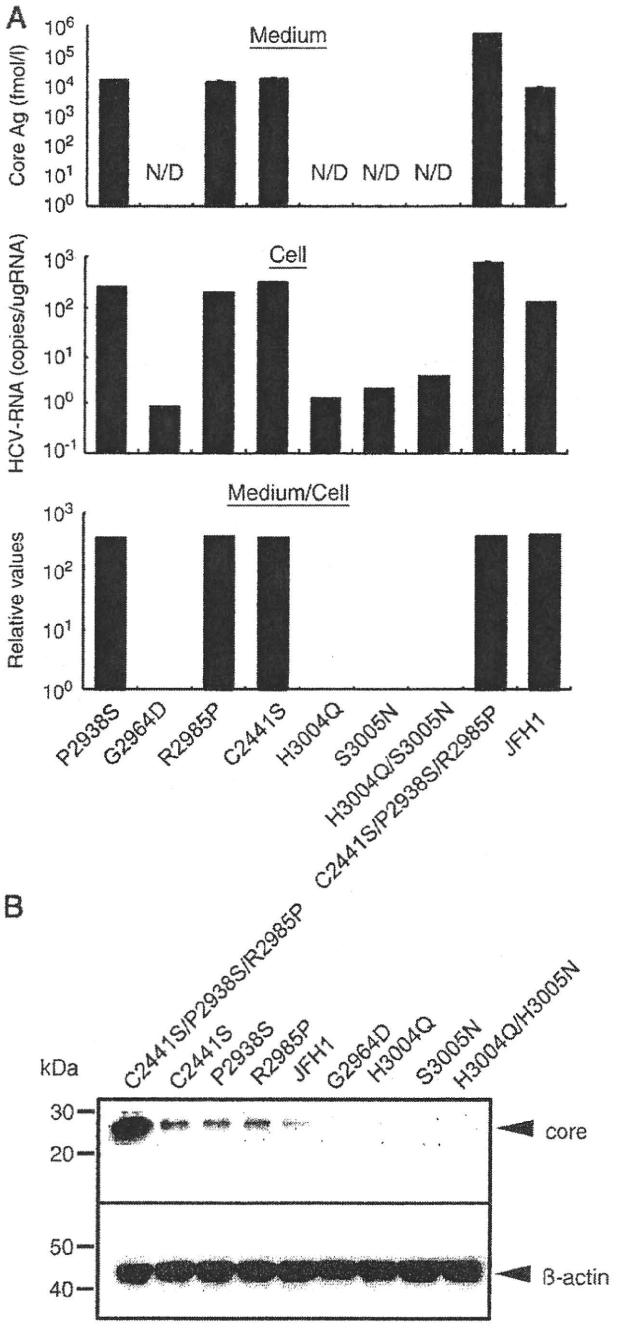

Fig. 5. Analysis of viral replication and production of viral particles using a single-cycle assay. A. Levels of core antigen in the culture media 3 days after transfection of JFH1 mutants into CD81-deficient Huh7-S29 cells (top). Levels of intracellular HCV RNA were quantified by real-time RT-PCR 3 days after transfection of JFH1 mutants into Huh7-S29 cells (middle). To determine the efficiency of infectious viral particle release from Huh7-S29 cells transfected with JFH1 mutants, the levels of core antigen in the culture media were adjusted by dividing by the levels of intracellular HCV RNA (bottom). Core Ag: Core antigen, N/D: not detectable. B. Huh7-S29 cells were harvested at 3 days after transfection of JFH1 mutants and western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

Highly adapted cytopathic mutations reverted to wild type in vivo

Finally, we analyzed the serum viral sequence at the specified time points. On days 1 and 5, the HCV genomic sequences of the mice

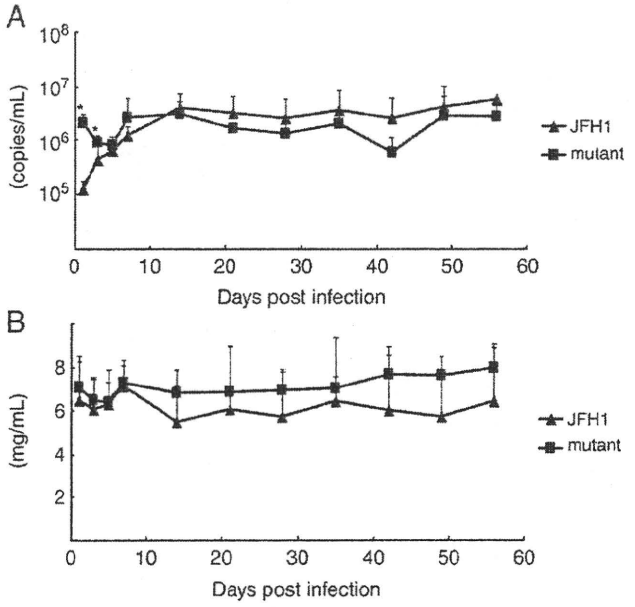

Fig. 6. In vivo analysis of cytopathic JFH1 mutants using human hepatocyte chimeric mice. A. Serial changes in HCV RNA in the sera of mice inoculated with the culture media from JFH1 mutants. The data shows the average of 2 mice for JFH1, and 3 mice for the mutant. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Levels of human albumin in the sera of mice inoculated with the culture media from JFH1 mutants.

inoculated with the cytopathic mutant virus showed conservation of the mutations in codons 2441, 2938 and 2985. However, on days 21 and later, the mutation at codon 2985 had reverted to the wild type JFH1 sequence in all the mutant-injected mice and the mutation at codon 2938 had reverted to the wild type JFH1 sequence in two of the three mice. The C2441S mutation was more stable in the mutant-injected mice, but one mouse had lost it at day 56 (Fig. 8).

Discussion

In this study, we investigated the significance of genetic mutations in plaque-purified, cytopathic HCV-JFH1 subclones. Genetically engineered JFH1-mutants encoding C2441S, P2938S, and R2985P led to much more cell death than the wild type JFH1, and also produced significantly higher amounts of core antigen in the culture medium and inside the cells than the parental JFH1 clone. In the single-cycle production assay, which exploited a receptor-deficient Huh7 cell line, the three JFH1-mutants, JFH1-C2441S, P2938S, and R2985P produced significantly more core antigen in the culture medium and expressed equivalently higher amounts of viral genomic RNA in the cells. These data suggest that the three mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated directly with enhanced intracellular replication and resultant virion formation, which correlated with the extent of the cytopathic effects. Interestingly, inoculation of a cytopathogenic mutant, JFH1-C2441S/P2938S/R2985P, into human hepatocyte chimeric mice produced significantly higher plasma HCV RNA concentrations than JFH1 at ~7 days post inoculation. At a later phase of infection, however, the mutations in this mutant HCV reverted partially to the wild type sequences. Taking all things together, it is suggested that in vitro-isolated, genetically modified cytopathic HCV subclones replicate robustly in the acute phase of in vivo infection but are eliminated rapidly and substituted by in vivo adapted clones.

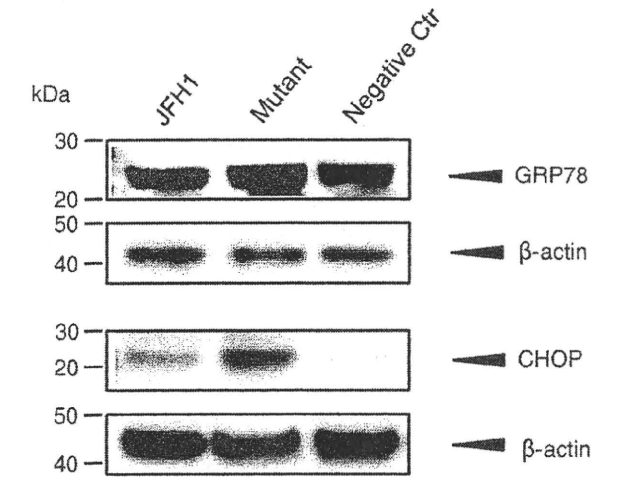

Fig. 7. Expression of ER stress-related proteins in human hepatocytes of chimeric mice infected with JFH1 or the mutant in the early phase. Western-blot analysis of the liver tissues of infected chimeric mice using anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody and anti-beta-actin. Liver samples were obtained at 5 days after inoculation. The negative control liver samples for this study was from uninfected human hepatocyte chimeric mouse.

Four of the five NS5B mutations appeared independently in several isolated subclones. This made us speculate that these amino acid substitutions may affect the enzymatic activity of RdRp. Mapping of the amino acid substitutions in the RdRp tertiary structure revealed that amino acid 2441 is located on the finger domain, and three amino acids, 2938, 2964, and 2985, are on the outer surface of the thumb domain, which corresponds to the opposite side of the nucleotide tunnel. The other substitutions, 3004 and 3005, are within the domain of the polypeptide linking the polymerase to the membrane anchor (Lesburg et al., 1999). Our preliminary study has shown that the NS5B mutations, P2938S and R2985P, did not affect cell-free enzymatic activities of the RNA polymerase. Thus, it is speculated that these mutations may affect the stability of the HCV replicase complex by altering surface affinity to other nonstructural proteins.

There are several reports on cell culture adaptive mutations in the HCV-JFH1 genome that gave more vigorous and consistent virus expression. Most studies involved prolonged cell culture of HCV-JFH1 or multiple rounds of successive passage onto naïve cells. Zhong et al. detected the E2-G451R mutation after culture for more than 60 days. The mutation led to more efficient production of infectious viral particles than wild type JFH1 (Zhong et al., 2006). Delgrange et al. conducted successive virus infections of naïve cells and identified the E2-N534K mutation that facilitated virus-CD81 attachment, and core-F172C and -P173S that increased secretion of virions (Delgrange et al., 2007). Using a similar method, Russell et al. identified E2-N417S that improved virus–cell attachment, and p7-N765D and NS2-Q1012R that increased virion production (Russell et al., 2008). Kaul et al. reported the NS5A-V2440L mutation, that was close to the C terminus and increased virion production (Kaul et al., 2007). Yi et al. used a chimeric virus of genotype 1a and JFH1 and identified the NS3-Q1251L mutation that resulted in enhanced virus production, possibly through improved interactions between NS2 and NS3 that were required for virion formation (Yi et al., 2002). Han et al. used EGFP-tagged virus and identified the mutually dependent mutations, NS3-M1290K and NS5A-T2438I, which improved virus production synergistically (Han et al., 2009).

Of note is that all of the mutations reported above promoted virion secretion or virus–cell surface interaction and none of them showed any effect on intracellular replication of viral RNA or translation of virus proteins. None of the adaptive mutations reported above overlapped with our cytopathogenic mutations. The mutations that we have identified conferred enhanced virus replication and protein expression in the early/acute stages of infection and subsequently led to massive cell death. Our data and the reports of other groups suggest that the HCV genome evolves to adapt to the host cell environment. Mutations that optimize virus secretion or virus–cell entry may be

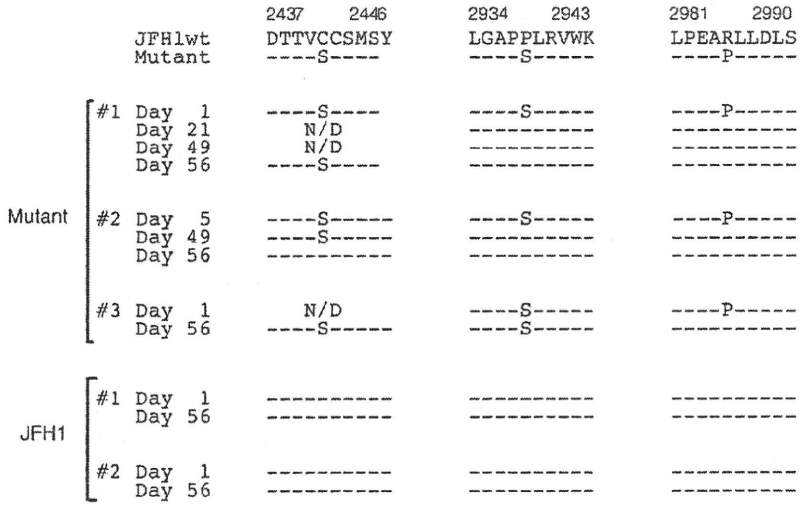

Fig. 8. Nucleotide sequence analysis of virus genomes circulating in the sera of infected mice. We extracted RNA from the sera of mice inoculated with culture media from JFH1 or JFH1-mutants and analyzed the viral sequence at the specified time points. N/D is not detectable. Wt: Wild type.

required for persistent infection in vitro, while those that affect cellular viral RNA replication may possibly promote viral genetic evolution and host cell damage.

The results of in vivo experiments using human hepatocyte chimeric mice were consistent with those of virus cell culture (Figs. 5, 6 and 7). The mutant JFH1 clones showed markedly higher levels of replication than the parental JFH1 in the acute phases. However, the serum HCV titers subsequently leveled out after two weeks of infection, concomitant with reversal of some cytopathic mutations to wild type sequences. Bukh et al. reported that inoculation of the HCV-1b genome into chimpanzee liver resulted in persistent infection, although the mutation reverted rapidly to wild type (Bukh et al., 2002). In this study, the NS5A-C2441S mutation was preserved in 2 of 3 mice, while NS5B-P2938S reverted to the wild type sequences in 2 of 3 mice and NS5B-R2985P reverted to wild type sequences in all 3 mice. These results suggest that the highly adapted JFH1 genome is infectious and viable in vivo, but is not as fit in vitro.

It is not clear why the subgenomic replicons with C2441S, P2938S or R2985P mutations did not show differences in replication levels compared to the wild type JFH1 subgenomic replicon. One may speculate that this discrepancy between the results using full-length HCV genomes and replicons might be the presence or absence of the HCV structural proteins. In addition, three individual substitutions G2964D, H3004Q and S3005N did not enhance viral replication as compared with the parental JFH1 nor did express detectable amounts of core protein. It is speculated that these mutants exist in host cells through co-infection with replication-competent viral clones resulting in enhanced replication.

There is clinical evidence that suggests the pathological outcomes of hepatitis C result from the immune response of the host rather than the direct cytopathic effects of the virus (Cerny and Chisari, 1999). However, several clinical studies have shown that fulminant hepatic failure (FHF, the HCV-JFH1 strain was isolated from such a case) featured massive hepatocyte apoptosis, as characterized by caspase activation and Fas–FasL expression (Leifeld et al., 2006; Mita et al., 2005; Ryo et al., 2000). The ER stress markers, GRP78 and ATF6 are upregulated in HCV-infected liver tissue as the histological grade advances (Shuda et al., 2003). This background and our results in vitro and in vivo suggest that HCV strains with highly infectious and cytopathic gene signatures may replicate aggressively in the acute phase of infection and that certain defects in innate or adaptive immune responses against the virus could lead to severe and persistent liver damage due to cytopathic effects induced directly by HCV. Such mechanisms might explain some rare clinical features of HCV infection, such as fulminant hepatic failure and post-transplantation severe fibrosing cholestatic hepatitis (Delladetsima et al., 1999; Dixon and Crawford, 2007).

In conclusion, we identified three substitutions in cytopathic HCV-JFH1 subclones derived from plaque assay. These substitutions directly enhanced virus replication in the early phases of virus infection in vitro and in vivo. This highly enhanced replication induced ER stress-mediated apoptosis and resulted in cytopathogenicity. Further analyses of cellular effects on HCV replication may elucidate the pathogenesis of HCV infection and may define novel host factors as targets of antiviral chemotherapeutics.

Materials and methods

Cells and cell culture

Huh-7.5.1 cells (Zhong et al., 2005) (kindly provided by Dr Francis V. Chisari) and CD81 deficient Huh7-S29 cells (Russell et al., 2008) (kindly provided by Dr Rodney S. Russell and Dr Robert H. Purcell) were maintained in Dulbecco's modified minimal essential medium (DMEM, Sigma, St. Louis, MO) supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum at 37 °C under 5.0% CO_2.

Sequence analysis

The cDNA from the isolated JFH1-plaque was amplified from cytopathic virus-infected Huh-7.5.1 cells by RT-PCR and subjected to direct sequencing.

In vitro RNA synthesis and transfection

A plasmid, pJFH1full (Wakita et al., 2005), which encodes full-length HCV-JFH1 sequence, was used. In vitro RNA synthesis and transfection were conducted as previously described (Sekine-Osajima et al., 2008). Briefly, HCV RNA was synthesized from linearized pJFH1 plasmid as template and transfected into Huh-7.5.1 cells by electroporation. The transfected cells were split every 3 to 5 days. The culture media were subsequently transferred onto uninfected Huh-7.5.1 cells and Huh7-S29 cells. The levels of HCV replication and viral protein expression were detected by real-time PCR and western blotting.

Plaque assay

HCV plaque assays were performed as reported previously (Sekine-Osajima et al., 2008). Huh-7.5.1 cells were seeded in collagen-coated 60 mm-diameter plates. After overnight incubation, HCV-infected culture media were serially diluted in a final volume of 2 ml per plate and transferred onto the cell monolayer. After ~5 h of incubation, the inocula were removed and the cell monolayer was overlaid with 8 ml of culture medium containing 0.8% methylcellulose (Sigma). After 7 to 12 days culture, cytopathic plaques were visualized by staining with 0.08% crystal violet solution (Sigma). The levels of cytotoxicity were evaluated by counting the plaques and calculating the titer (plaque-forming unit/ml).

Establishment of mutant JFH1 clones

In order to introduce various mutations into the NS5A and NS5B region of JFH1, plasmid pJFH1 was digested with HindIII and the DNA fragment encompassing nt. 8231 to 9731 was subcloned into the pBluescript II SK+ phagemid vector (Stratagene, La Jolla, CA). Mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-Changell Site-Directed Mutagenesis Kit, Stratagene) to generate the following codon changes: P2938S, G2964D, R2985P, H3004Q and S3005N. Finally, the HindIII–HindIII fragments were subcloned back into the parental plasmid, pJFH1. A PCR fragment (nt. 7421–7839) was subcloned into the pGEM-T Easy plasmid vector (Promega, Madison, WI) and digested with RsrII and BsrGI. Finally, after introducing the codon change C2441S, the RsrII–BsrGI fragment was reinserted into the parental plasmid.

Quantification of HCV core antigen in the culture medium

The culture media from JFH1-RNA transfected Huh-7.5.1 cells and Huh7-S29 cells were collected on the days indicated, passed through a 0.45 μm filter (MILLEX-HA, Millipore, Bedford, MA), and stored at −80 °C. The levels of core antigen in the culture media were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Western blotting

Western blotting was carried out as described previously (Itsui et al., 2009). Briefly, 10 μg of total cell lysate were separated by SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) Western Blotting membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Bioscience, Buckinghamshire, UK). The antibodies used were anti-core mouse monoclonal antibody (Abcam, Cambridge, MA), anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-beta-actin antibody (Sigma).

HCV subgenomic replicon constructs

The HCV subgenomic replicon plasmid, pRep-Feo, was derived from the HCV-N strain, pHCV1bneo-delS (Tanabe et al., 2004; Yokota et al., 2003). The replicon RNA was synthesized from pRep-Feo and transfected into Huh7 cells.

Luciferase reporter assay

Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX, Perkin Elmer, Waltham, MA) with a Bright-Glo Luciferase Assay System (Promega) (Tasaka et al., 2007). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assays

To evaluate cell viability, dimethylthiazol carboxymethoxy-phenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega), as described previously (Sakamoto et al., 2007).

Real-time RT-PCR analysis

Total cellular RNA was isolated using an RNeasy Mini Kit (QIAGEN, Valencia, CA). Two micro-grams of total cellular RNA were used to generate cDNA from each sample using SuperScript II (Invitrogen) reverse transcriptase. Expression of mRNA was quantified using TaqMan Universal PCR Master Mix (Applied Biosystems) and the ABI 7500 Real-Time PCR System (Applied Biosystems). The primers used were as follows: HCV-JFH1 sense (positions 285 to 307; 5′-GGT-ACTGCCTGATAGGGTGCTT-3′), HCV-JFH1 antisense (positions 349 to 375; 5′-TGGTTTTTCTTTGAGGTTTAGGATTC-3′), GAPDH sense (5′-CCTCCCGCTTCGCTCTCT-3′), and GAPDH antisense (5′-GCTGGCGACG-CAAAAGA-3′).

HCV RNA inoculation into human hepatocyte chimeric mice

Housing, maintenance, and care of the mice used in this study conformed to the requirement for the humane use of animals in scientific research as defined by Animal Care and Use Committee of our institute. The culture media of Huh-7.5.1 cells transfected with parental JFH1 and JFH1 mutants were collected 10 days after transfection and passed through a 0.45 μm filter. The three mutations introduced in NS5A and NS5B were confirmed to conserve by the sequence analysis of virus genome of cell culture supernatants before inoculation. Filtrated culture medium was then pooled and concentrated using Amicon Ultra-15 (100,000 molecular weight cutoff, Millipore). 100 μl of each culture medium was injected intravenously into human hepatocyte chimeric mice (PXB mice, Phenix Bio, Hiroshima, Japan) (Mercer et al., 2001). The rate of liver chimerism of these human hepatocyte chimeric mice was confirmed more than 70% by immunohistochemical analysis. After infection, blood samples were taken serially and levels for HCV RNA and human albumin were quantified using real-time RT-PCR and an enzyme immunoassay, respectively. RNA was extracted from serum samples and subjected to direct sequence determination.

Protein extraction from human hepatocyte chimeric mice and expression of ER stress-related proteins

5 days post inoculation, mice were sacrificed and proteins were extracted from liver samples with complete Lysis-M Reagent Kit (Roche Applied Science, Indianapolis, IN). One Mini Protease Inhibitor Cocktail Tablet was dissolved into 10 ml of Lysis-M Reagent and 500 μl of this fluid was added to 50 μg of each liver sample and homogenized. The lysate was transferred to a microcentrifuge tube and centrifuged at 14,000×*g* for 5 min. The supernatant containing soluble protein was transferred to a new reaction tube and 20 μg of each protein was used for western blotting to detect ER stress-related proteins.

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Mutations in the interferon sensitivity determining region and virological response to combination therapy with pegylated-interferon alpha 2b plus ribavirin in patients with chronic hepatitis C-1b infection

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Abstract
Background Pegylated-interferon-alpha 2b (PEG-IFN) plus ribavirin (RBV) therapy is currently the de-facto standard treatment for hepatitis C virus (HCV) infection. The aims of this study were to analyze the clinical and virological factors associated with a higher rate of response in patients with HCV genotype 1b infection treated with combination therapy.
Methods We analyzed, retrospectively, 239 patients with chronic hepatitis C-1b infection who received 48 weeks of combination therapy. We assessed clinical and laboratory parameters, including age, gender, pretreatment hemoglobin, platelet counts, HCV RNA titer, liver histology, the number of interferon sensitivity determining region (ISDR) mutations and substitutions of the core amino acids 70 and 91. Drug adherence was monitored in each patient. We carried out univariate and multivariate statistical analyses of these parameters and clinical responses.
Results On an intention-to-treat (ITT) analysis, 98 of the 239 patients (41%) had sustained virological responses (SVRs). Patients with more than two mutations in the ISDR had significantly higher SVR rates (P \ 0.01). Univariate analyses showed that stage of fibrosis, hemoglobin, platelet counts, ISDR mutations, serum HCV RNA level, and adherence to PEG-IFN plus RBV were significantly correlated with SVR rates. Multivariate analysis in subjects with good drug adherence extracted the number of ISDR mutations (two or more: odds ratio [OR] 5.181).
Conclusions The number of mutations in the ISDR sequence of HCV-1b (≥2) is the most effective parameter predicting a favorable clinical outcome of 48-week PEG-IFN plus RBV therapy in patients with HCV genotype 1b infection.

Keywords Hepatitis C virus (HCV) · Chronic hepatitis C · PEG-IFN plus RBV therapy · Combination therapy · Interferon sensitivity determining region (ISDR)

Abbreviations

HCV	Hepatitis C virus
IFN	Interferon
PEG	Polyethylene glycol
PEG-IFN	Pegylated-interferon-alpha 2b
RBV	Ribavirin
ISDR	Interferon sensitivity determining region
BMI	Body mass index

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ALT	Alanine transaminase
dM	Double mutant
ITT analysis	Intention-to-treat analysis
PP analysis	Per protocol analysis
SVR	Sustained virological response
ETR	End of treatment response
PKR	Double stranded RNA-dependent protein kinase
TLR	Toll-like receptor
MyD88	Myeloid differentiation primary response gene 88

Introduction

Hepatitis C virus (HCV) is one of the major pathogens causing chronic hepatitis [1, 2] and eradication of the virus by the host occurs infrequently during the natural course of infection once it becomes chronic. Interferon (IFN) has been used widely as the most effective antiviral agent for chronic hepatitis C. Although ribavirin (RBV), a synthetic guanosine analog, alone does not decrease the serum HCV RNA level [3–5], it has been shown that combination therapy with IFN-alpha (given 3 times weekly) and daily RBV gives a higher sustained response rate than IFN monotherapy [6–8]. Pegylation is the process by which an inert molecule of polyethylene glycol (PEG) is covalently attached to a protein, and the addition of PEG to IFN produces a biologically active molecule with a longer half-life and more favorable pharmacokinetics than the natural molecule. These characteristics allow more convenient, once-weekly dosing [9]. Pegylated (PEG)-IFN plus RBV is significantly more effective than IFN plus RBV or PEG-IFN alone for the treatment of chronic hepatitis C, with sustained virological response rates of ~50% in patients infected with HCV genotype 1b [10].

We reported previously a close correlation between the number of mutations in the nonstructural 5A (NS5A) region of the HCV genome encoding amino acids (aa) at positions 2209–2248 [the IFN sensitivity determining region (ISDR)] and IFN efficacy in patients with HCV genotype 1b infection [11–13]. The aims of this study were to analyze clinical and virological factors associated with a higher rate of response by patients with HCV genotype 1b infection who were treated with combination therapy with pegylated-IFN-alpha 2b (PEG-IFN) plus RBV, and to clarify the relationship between ISDR mutations and virological response to the combination therapy.

Methods

Patients and methods

We analyzed, retrospectively, 239 patients with chronic HCV-1b infection who received combination therapy with PEG-IFN plus RBV between December 2004 and April 2008 at Tokyo Medical and Dental University Hospital (Tokyo, Japan) and associated hospitals participating in the Ochanomizu-Liver Conference Study Group. All patients had histologically or clinically proven chronic active hepatitis and were positive for anti-HCV antibodies and serum HCV RNA by reverse transcription polymerase chain reaction (RT-PCR). Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age; gender; body mass index (BMI); previous IFN therapy; grade of inflammation and stage of fibrosis on liver biopsy; pretreatment biochemical parameters, such as hemoglobin, alanine transaminase (ALT) level, platelet count, low density lipoprotein (LDL) cholesterol, serum HCV RNA level (Log IU/ml); and the amino acid sequence of the IFN sensitivity determining region (aa 2209–2248, ISDR). Liver biopsy specimens were evaluated according to the grade of inflammation and the stage of fibrosis; this was done blindly by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity, and A3 shows severe activity. Fibrosis was staged on a scale of 0–4: F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis, and F4 shows cirrhosis.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of our hospital, and informed written consent was obtained from each patient.

Nucleotide sequencing of the *NS5A* gene

The serum samples were frozen at −80°C until use. Extraction of RNA from serum and RT-PCR were performed as described previously [14]. The PCR and sequencing primers were synthesized with a DNA synthesizer (model 391; Applied Biosystems Japan, Chiba, Japan).