Table 3 Clinical and virological characteristics of 239 patients treated with PEG-IFN plus RBV therapy, based on therapeutic response

	SVR ($n = 98$)	Non-SVR ($n = 141$)	P value
Age (years)[a]	56 (27–69)	58 (23–72)	NS
Gender (male/female)	65/33	77/64	0.070
Previous interferon therapy (no/yes)	68/30	99/42	NS
Grade of inflammation (A0–1/2–3)	31/50	37/62	NS
Stage of fibrosis (F0–2/3–4)	68/13	67/33	*0.009*
Body mass index (kg/m^2)[a]	23.3 (15.5-28.1)	23.3 (15.3-31.0)	NS
Pretreatment Hemoglobin (g/dl)[b]	14.6 ± 1.1	14.0 ± 1.4	*<0.001*
Pretreatment ALT (IU/ml)[b]	87 ± 68	86 ± 67	NS
Pretreatment platelet count ($\times 10^3/\mu l$)[b]	178 ± 63	148 ± 51	*<0.001*
Pretreatment LDL cholesterol (mg/dl)[b]	78 ± 21	72 ± 18	NS
Pretreatment serum HCV-RNA level (Log(IU/ml))[b, c]	5.9 ± 0.7	6.2 ± 0.4	*<0.001*
No. of mutations in the ISDR (0–1/2 or more)	66/23	105/11	*0.002*
Type of mutations in the core (dM/non dM)	9/76	21/90	NS
Type of mutations in the core (dW/non dW)	31/54	34/77	NS
PEG-interferon adherence (>80/60–80/<60%)	85/7/6	68/20/53	*<0.001*
Ribavirin adherence (>80/60–80/<60%)	72/19/7	60/28/53	*<0.001*

IFN interferon, *RBV* ribavirin, *SVR* sustained virological response, *NS* not significant, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209\text{-}2248}$, *core* substitution of amino acids 70 and 91, *dM* double mutant: dual substitutions at amino acids 70 and 91, *non dM* non-double mutant: wild type or substitution at either amino acid 70 or 91, *dW* double wild: wild type at amino acids 70 and 91, *non dW* non-double wild: dual or substitution at either amino acid 70 or 91

[a] Median (range) values are shown

[b] Data are mean ± SD

[c] Data are shown as Log(IU/ml)

Table 4 Mutations in the ISDR and core regions analyzed separately for gender based on therapeutic response

	SVR ($n = 98$)	Non-SVR ($n = 141$)	P value
No. of mutations in the ISDR (0–1/2 or more)			
Male	36/21	56/8	*0.002*
Female	30/2	49/3	NS
Type of mutations in the core (dM/non dM)			
Male	8/46	11/48	NS
Female	1/30	10/42	*0.026*
Type of mutations in the core (dW/non dW)			
Male	18/36	16/43	NS
Female	13/18	18/34	NS

likelihood ratio statistic in combination with forward or backward variable selection methods.

Comparison of SVR rates according to the number of mutations in the ISDR sequence

We analyzed first the percentage of patients with more than two mutations in the ISDR among 762 patients who received IFN therapy between December 2000 and April 2008 at Tokyo Medical and Dental University Hospital and associated hospitals. The percentage of patients with more than two mutations in the ISDR was between about 20% and 30% for all ages (Fig. 1a).

Secondly, we analyzed responses to PEG-IFN plus RBV treatment and serum levels of HCV RNA in relation to the number of mutations in the ISDR. In Fig. 1b, patients with SVR are indicated by open circles and those with non-SVR, by closed circles. Although the rate of SVR tended to be higher in patients with increasing numbers of mutations in the ISDR, 5 patients with more than two mutations in the ISDR who experienced drug discontinuation and dose reduction resulted in non-SVR.

We confirmed changes over time in VR rates in patients treated with PEG-IFN plus RBV (Fig. 1c). Patients with more than two mutations in the ISDR are indicated in the figure by open circles and those with none or one mutation in the ISDR, by closed circles. The VR rates tended to be high early in the treatment in patients with more than two mutations in the ISDR.

Finally we compared the PEG-IFN plus RBV treatment efficacy in two groups, divided based on ISDR mutations. Patients with more than two mutations in the ISDR had a significantly higher tendency to achieve SVR in both ITT and per-protocol (PP) analyses ($P < 0.01$) (Fig. 1d), and

Table 5 Clinical and virological characteristics of 239 patients treated with PEG-IFN plus RBV therapy, based on previous interferon therapy

Previous interferon therapy	No (n = 167)	Yes (n = 72)	P value
Sustained response rates	68/167 (41)	30/72 (42)	NS
Age (<65/≥65)	127/40	57/15	NS
Gender (male/female)	93/74	49/23	0.074
Grade of inflammation (A0–1/2–3)	55/72	13/40	*0.018*
Stage of fibrosis (F0–2/3–4)	103/24	32/21	*0.003*
Pretreatment hemoglobin (<14.5/≥14.5)	93/74	41/31	NS
Pretreatment platelet count (<160/≥160 $\times 10^3$)	84/83	50/22	*0.006*
Pretreatment Serum HCV RNA level[a] (<6/≥6)	54/112	25/46	NS
No. of mutations in the ISDR (0–1/2 or more)	116/22	55/12	NS
PEG-interferon adherence (>80/60–80/<60%)	110/18/39	43/9/20	NS
Ribavirin adherence (>80/60–80/<60%)	97/30/40	35/17/20	NS

[a] Data are shown as Log(IU/ml)

Table 6 Multivariate analysis for the clinical and virological factors related to sustained response to PEG-IFN plus RBV therapy in 104 patients who were not intolerant to PEG-IFN plus RBV therapy

Factor	Category	Odds ratio (95% CI)	P value
(a) Five-factor model			
Number of mutations in the ISDR	0 or 1	1	
	2 or more	4.486 (0.922–21.74)	0.063
Pretreatment Hemoglobin (g/dl)		1.250 (0.853–1.833)	NS
Pretreatment Serum HCV RNA level[a]		0.510 (0.224–1.159)	NS
Stage of fibrosis	F 0/1/2	1	
	F 3/4	0.460 (0.153–1.382)	NS
Pretreatment Platelet count ($\times 10^3$/μl)		1.022 (0.949–1.101)	
(b) Step-wise variable selection			
Number of mutations in the ISDR	0 or 1	1	
	2 or more	5.181 (1.129–23.81)	*0.034*

CI confidence interval, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209-2248}$

[a] Data are shown as Log(IU/ml)

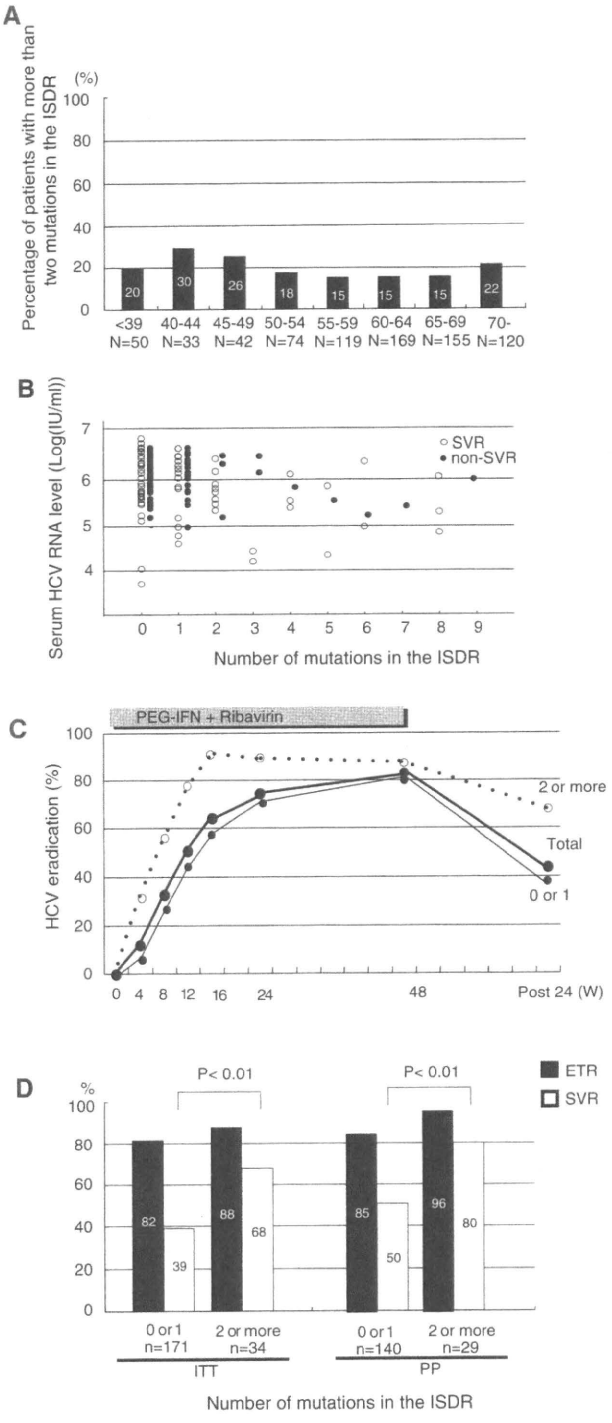
the SVR rates of the patients with good drug adherence was 80%.

Side effects

Side effects leading to treatment discontinuation occurred in 53 patients (22%). Overall, 109 patients (46%) required reduction of the dose of one or both drugs during the treatment regimens (23% required PEG-IFN reduction and 35% required RBV reduction). The most common events leading to drug withdrawal were general fatigue and appetite loss (n = 15), hematologic abnormalities (n = 6), dermatological symptoms (n = 5), retinopathy (n = 5), neuro-psychiatric events (n = 4), and interstitial pneumonia, including severe cough (n = 4).

Discussion

Although the relationship between ISDR mutations and the clinical efficacy of IFN has been conflicting in Western countries [18–24], our results support previous studies reporting a close correlation between the number of mutations in the ISDR and IFN efficacy in patients with chronic HCV-1b infection [11–13]. Because most patients with 4 or more mutations in the ISDR (hereafter classified as the mutant type) experienced SVR with conventional IFN monotherapy, we reported previously that the number of amino acid substitutions in the ISDR was an independent predictor of the response to IFN therapy [12]. In the present study, we demonstrate that ISDR mutations are the most effective predictors of treatment outcome of 48-week



◀ **Fig. 1** **a** The percentages of patients with more than two mutations in the interferon sensitivity determining region in NS5A $_{2209-2248}$ (*ISDR*), according to age (*horizontal axis*) among 762 patients who received interferon (IFN) therapy between December 2000 and April 2008 at Tokyo Medical and Dental University Hospital and associated hospitals. **b** Responses to pegylated (PEG)-IFN plus ribavirin (RBV) treatment and serum levels of hepatitis C virus (HCV) RNA in relation to the number of mutations in the ISDR. Patients with sustained virological response (*SVR*) are indicated by *open circles* and those with non-SVR by *closed circles*. **c** Changes over time in VR rates in patients treated with PEG-IFN plus RBV. Patients with more than two mutations in the ISDR are indicated by *open circles* and those with no or one mutation in the ISDR by *closed circles*, *W* weeks. **d** PEG-IFN plus RBV treatment efficacy divided into two groups based on ISDR mutations. End-of-treatment response (*ETR*) and SVR are shown in both intention-to-treat (ITT) analysis (*left*) and per-protocol (PP) analysis (*right*)

PEG-IFN plus RBV therapy in patients with HCV genotype 1binfection.

In the present study, the SVR rate was not related to gender, age, or previous IFN therapy by univariate analysis. First of all, in regard to gender ($P = 0.07$), as male patients had a higher tendency to achieve SVR than female patients, further validation in larger-scale studies is required to clarify the significance of gender. Secondly, in regard to age, there was no relation to SVR in overall analysis with continuous variables, but younger patients, aged less than 65 years, had a higher rate of response than those aged more than 65 years ($P < 0.05$, data not shown). Actually there are some reports suggesting the relationship of age and SVR [25, 26]. Finally, in regard to previous IFN therapy, as shown in Table 5, treatment was comparably effective in both groups; previous IFN therapy did not affect the SVR rate. The reasons for equivalent response rates in subjects with prior IFN history, which was not expected, are unclear. In our study, the group with prior IFN history had more advanced liver fibrosis and a low platelet count, and stage of fibrosis was one of the factors extracted by univariate analysis as a useful pretreatment marker predicting SVR. We also analyzed the other three parameters extracted by univariate analysis. Although there was no difference in pretreatment hemoglobin, or number of ISDR mutations, the group with prior IFN history tended to have a low serum HCV-RNA level. Further, the group with prior IFN history had a high proportion of male patients. Although the SVR rate was not related to gender, male subjects had a higher tendency to achieve SVR than female subjects.

In our present study, the SVR rate was not related to core mutations. As described in previous reports [17, 27, 28], amino acid substitutions in the core region are regarded as predictors of response to PEG-IFN plus RBV therapy in Japanese patients infected with HCV genotype 1b. In the present study, the SVR rate was not related to the pattern of amino acid substitution in the overall analysis. The reasons for these discrepant results are unclear, but females with dual substitutions at amino acids 70 and 91 had a lower tendency to achieve SVR. Further studies are necessary to clarify the mechanism of action for amino acid substitutions in the core region of HCV.

Recent studies suggest that the mutations in the ISDR are associated with response to combination therapy with IFN and RBV [29–32]. Most recently, it has been reported

that amino acid substitutions in the core and mutations in the ISDR are predictive of virological response to the combination therapy in patients with HCV genotype 1b and a high viral load [28]. There are some reports suggesting that the mutations in the ISDR may not serve as a predictor for treatment outcome [33, 34], but as the numbers of subjects in these studies were around 30, a number which is not sufficient to evaluate the results, this factor may explain these discrepant results.

The mechanisms of IFN sensitivity in relation to the sequence of the HCV $NS5A_{2209–2248}$ region are not clear. However the "mutant-type" ISDR correlates with a low viral load, as reported previously [12, 35, 36]; most patients in the present study with two or more mutations in the ISDR had high levels of virus. Furthermore, stepwise multiple logistic regression analysis of the factors, including substitution of the ISDR and the viral load, revealed that both of them were independent predictive variables of SVR, and the odds ratio of the number of mutations in the ISDR was the highest in the pretreatment factors associated with SVR by multivariate analysis. The precise mechanism involved must be elucidated in further in vitro studies.

There have been several reports that suggest biological roles of the ISDR in the response to IFN and in HCV infection. Double-stranded RNA-dependent protein kinase (PKR) is a critical component of the cellular antiviral responses induced by IFN. Gale et al. [37, 38] have reported that mutations within the PKR-binding region of NS5A, including ISDR, can disrupt the NS5A–PKR interaction, possibly rendering HCV sensitive to the antiviral effects of IFN. Toll-like receptor (TLR) has also been reported to play various roles in many viral infections, and it has been reported that NS5A bound MyD88, a major adaptor molecule of TLR-mediated signaling, and inhibited the TLR–MyD88 signaling pathway by a direct interaction with the death domain of MyD88 through the ISDR [39]. Furthermore, it has been reported that the lipid droplet is an important organelle for HCV production, and NS5A is a key protein that recruits replication complexes to lipid droplets for the production of infectious viral particles [40]. While the mechanism of action of the ISDR in the response to IFN or viral replication remains to be proven, these findings suggest new aspects of HCV infections.

In our previous report [12], patients with 4 or more mutations in the ISDR experienced SVR with conventional IFN monotherapy, but in more effective therapy with PEG-IFN plus RBV combination therapy, the number of mutations as a predictor of SVR decreased from 4 to 2. Watanabe et al. [41] have also reported that the number and position of mutations in the ISDR correlated with IFN efficacy in HCV-1b infection. Moreover, it has been reported that patients with viruses mutated at positions 2209, 2216, or 2227 more frequently experienced SVR than did those without these mutations. Another group has also reported regarding statistical analysis, using a database of 675 individual ISDR sequences in HCV-NS5A and the IFN response [42]. They have shown that IFN-sensitive viruses contain a larger and more diverse collection of substitutions than IFN-resistant viruses. While it remains unknown how the numbers of mutations are involved in the biological role of ISDR, or which sites of mutation and changes of amino acid are also important for the response to IFN-based treatment, it is thought that the functional importance of numbers or sites of mutations can be explained in terms of interaction between NS5A and some target molecules such as PKR, MyD88, and lipid droplets.

In vitro studies have shown that the introduction of NS5A mutations enables an HCV replicon to replicate efficiently [10, 43, 44]. In our previous report, site-specific mutation of the ISDR also modulated HCV replication [45]. The ISDR was identified originally as the site that determines the sensitivity of HCV to IFN [12]. This indicates that the ISDR mutations are not lethal in vivo. Furthermore, mutations in the ISDR are closely associated clinically with decreased serum HCV RNA levels [42], whereas ISDR mutations in the HCV replicon enhance replication. While the explanation for this paradox has not become clear, a big difference between the environment of cultured cells and that in the human liver is thought contribute to this phenomenon.

We found that the percentage of patients with more than two mutations in the ISDR was between 20% and 30% for all ages; thus, around one-fifth of patients are thought likely to experience SVR. Indeed, the SVR rate among patients with two or more mutations in the ISDR sequence was 68% (ITT) and 80% (PP) compared to 39% (ITT) and 50% (PP) among those patients with no or one mutation in the present study. Furthermore, predictive factors such as serum HCV RNA level, stage of fibrosis, and hemoglobin also aid in the assessments of treatment, and we can use these parameters to develop a treatment strategy.

Several prospective randomized trials have shown that 72-week extended therapy improves SVR by 7.5%–12% in late viral responders [46, 47]. One cohort study showed that 72-week treatment for late viral responders achieved an even higher SVR, of 67.1%, which was 21% higher than the SVR achieved with 48-week treatment [48]. These reports demonstrate that tailoring of treatment duration by on-treatment viral response can further improve the outcomes of antiviral therapy. In our 48-week based treatment, 90% of patients with more than 2 ISDR mutations cleared the virus within 12 weeks of treatment (early viral response; EVR) and consequently achieved 30% higher SVR than those with 1 or no ISDR mutation. These results

suggest that ISDR mutations will remain a significant predictor of good response to IFN therapies, including 72-week extension.

In conclusion, ISDR mutations are the most effective predictors of treatment outcomes in multivariate analysis. The number of mutations in the ISDR sequence of HCV-1b ($\geq$2) is the most effective parameter which will facilitate further the selection of patients with a high likelihood of response to PEG-IFN plus RBV treatment.

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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-R SPSYGRSRSRSRSRSRSRSNSRSRSY-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

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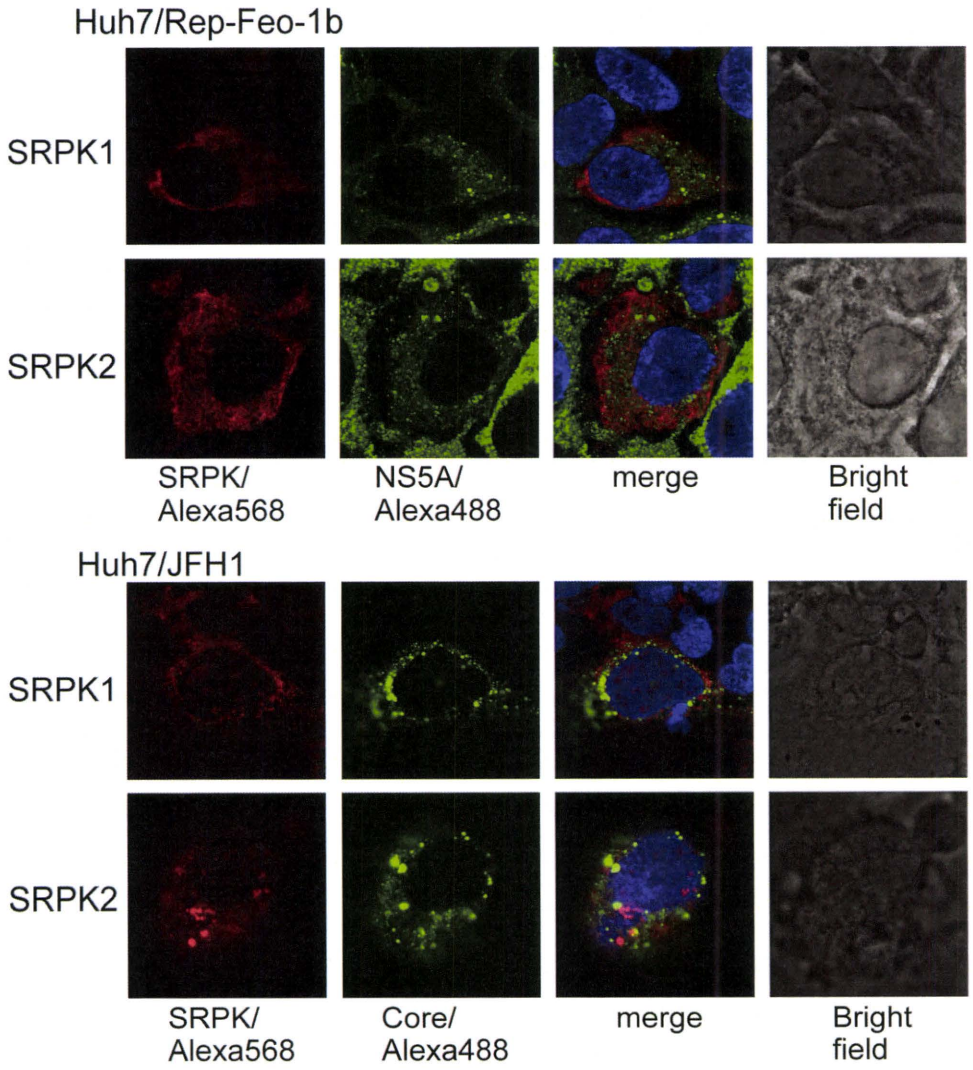

FIG. 1. Immunofluorescence microscopy. Expression plasmids for SRPK1 and SRPK2 were transfected into Huh7/Rep-Feo-1b cells or into HCV-JFH1-infected Huh7.5.1 cells. Forty-eight hours after transfection, cells were fixed and incubated with mouse anti-NS5A or anti-core antibodies and rabbit anti-HA antibody, 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

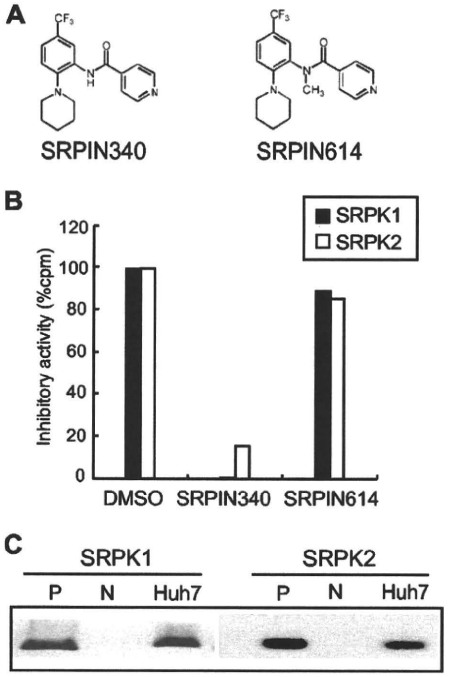

FIG. 2. Chemical structures and activities of SRPIN340 and SRPIN614. (A) Chemical structures of the SRPK inhibitor, SRPIN340, and activity-lacking control, SRPIN614. (B) Relative kinase activities of SRPK1 (black columns) and SRPK2 (white columns) *in vitro*, in the presence of the reagents indicated, 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

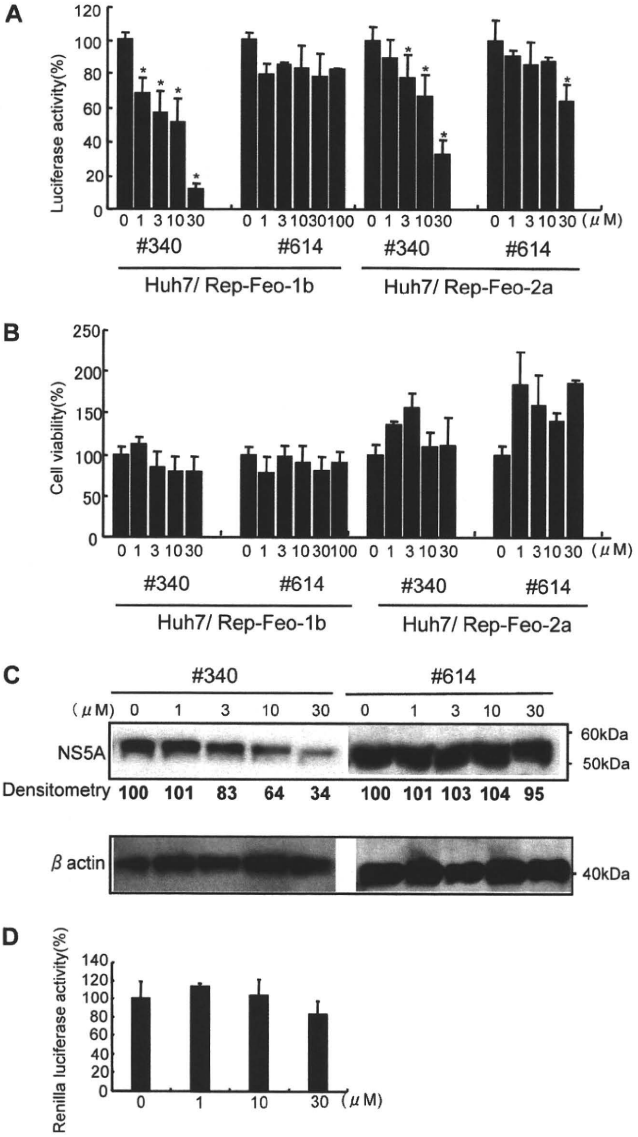

FIG. 3. Effects of SRPIN340 and SRPIN614 on expression of HCV subgenomic replicons. Huh7/Rep-Feo-1b or Huh7/Rep-Feo-2a cells were cultured in the presence of SRPIN340 (no. 340) or SRPIN614 (no. 614) at the concentrations indicated. After 48 h of culture, a luciferase assay (A), a cell viability assay (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.

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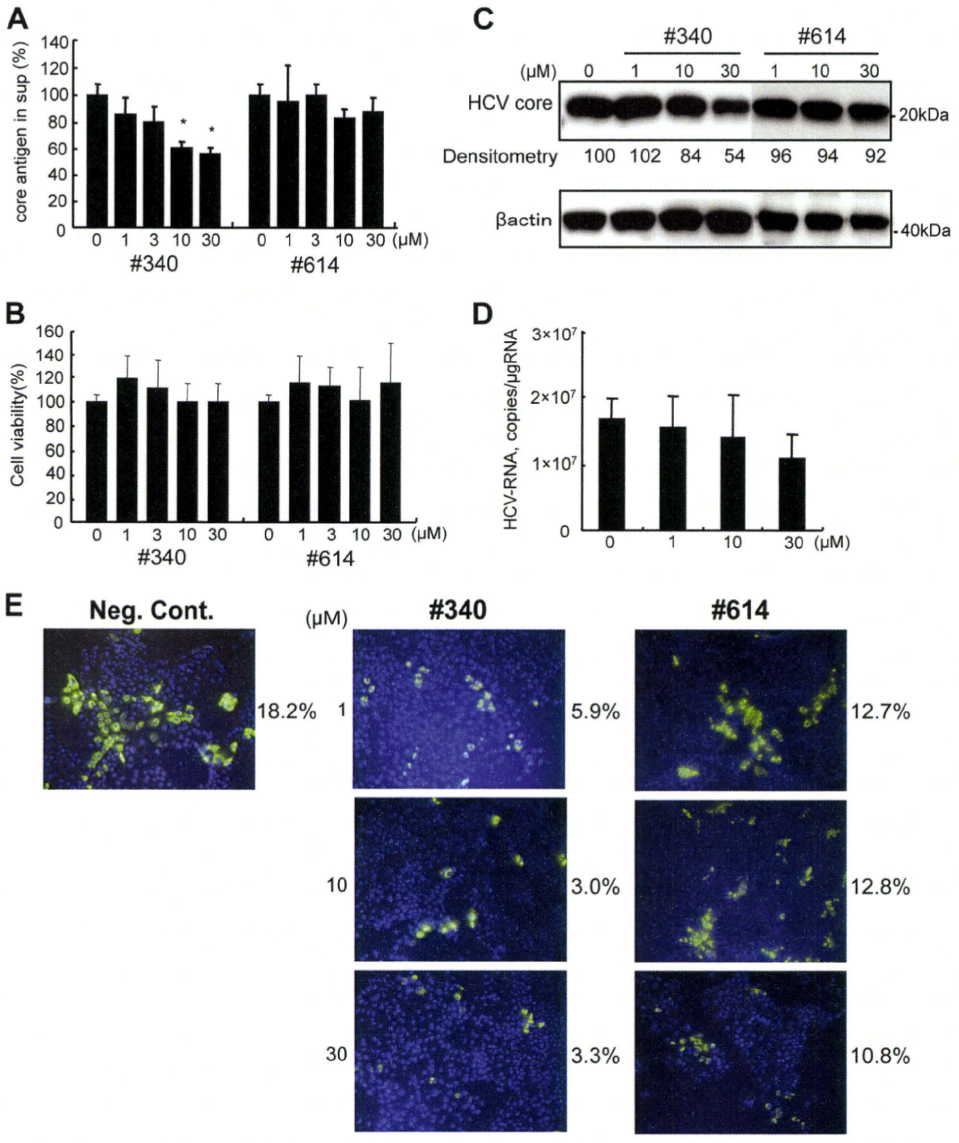

FIG. 4. Effect of SRPIN340 and SRPIN614 on HCV-JFH1 virus replication. HCV-JFH1-stably infected Huh7 cells of ~14 days were cultured in the presence of SRPIN340 or SRPIN614 at the concentrations indicated. After 48 h, cellular and supernatant HCV core antigens were detected. (A) HCV core antigen assays 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

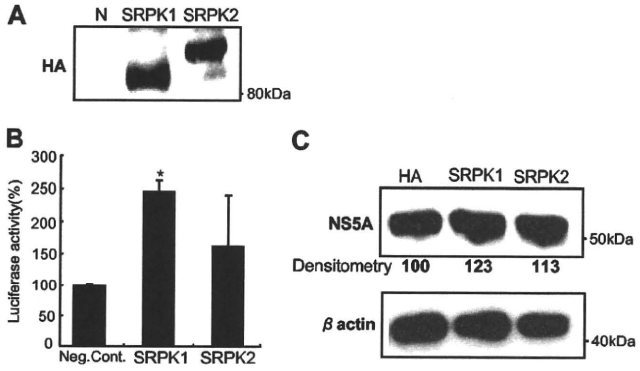

FIG. 5. Effects of overexpression of SRPK1 and SRPK2 on HCV replication. (A) The expression of transfected HA-tagged SRPK1 and SRPK2 was detected by anti-HA antibody. (B) Huh7/Rep-Feo-1b cells seeded on 24-well plates were transfected with 0.2 μg of expression plasmids for SRPK1 or SRPK2 or empty vector. 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.

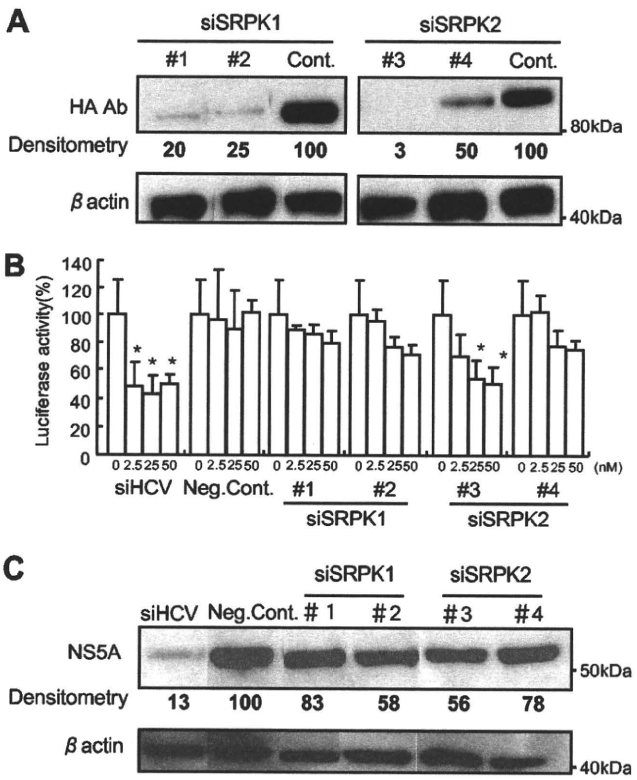

FIG. 6. Effects of siRNA knockdown of SRPK1 and SRPK2. (A) Huh7 cells were transfected with SRPK1 or SRPK2 expression plasmids and siRNA directed against SRPK1 (siSRPK1 no. 1 and siSRPK1 no. 2) or SRPK2 (siSRPK2 no. 3 and siSRPK2 no. 4) or control siRNA (32). Forty-eight hours after transfection, Western 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-

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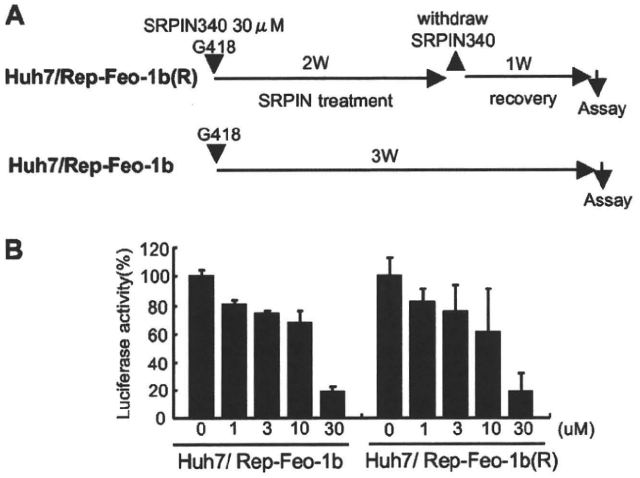

FIG. 7. Drug resistance assay of HCV replicon cells. (A) Schema for the establishment of SRPIN340-resistant cells and the control cells. Huh7/Rep-Feo-1b cells were treated with or without 30 μM SRPIN340 for 2 weeks in the presence of 500 μg/ml of G418. After 1 week of recovery culture without SRPIN340, 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.
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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.

☆ The authors, K.M., N.S., Y.S., M.N., Y.I., S.A., S.K., K.K., A.K., K.T., M.I., N.H., K.C., T.W. and M.W. declare that there is no conflict of interest.

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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

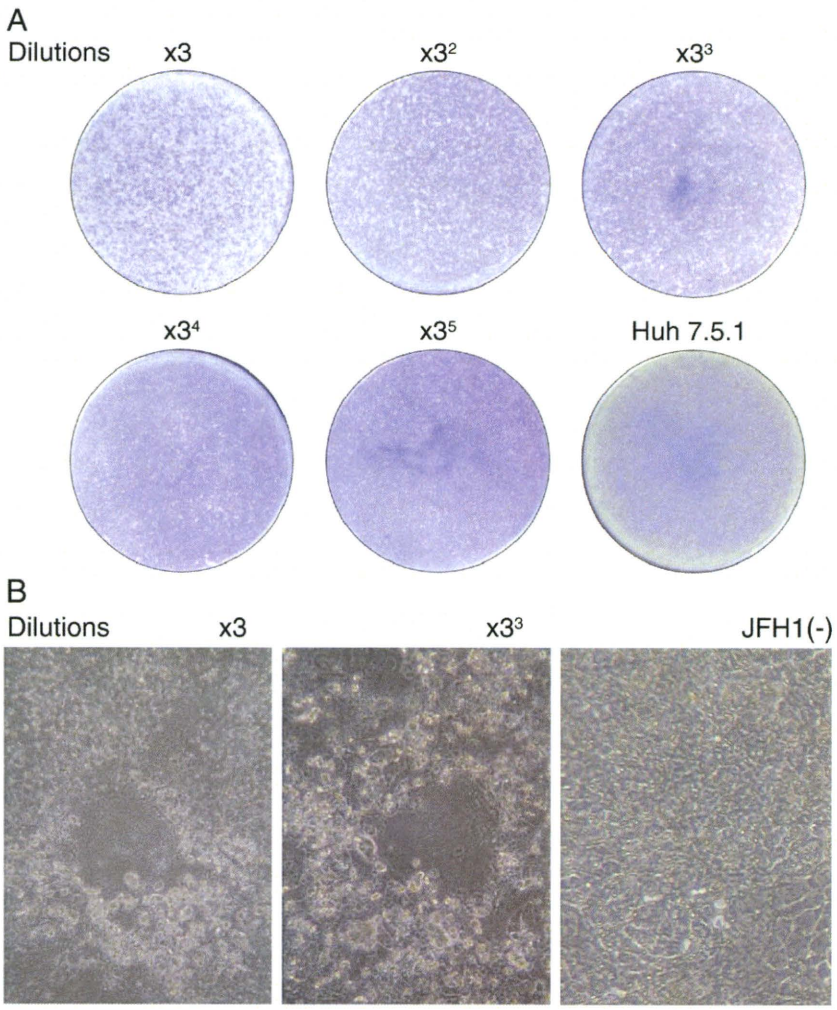

Fig. 1. The cytopathic effects of HCV-JFH1 *in vitro*. A. Plaque assay. Huh-7.5.1 cells were seeded in collagen-coated 60mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO_2 (as described above). After overnight incubation, HCV-infected culture supernatants 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

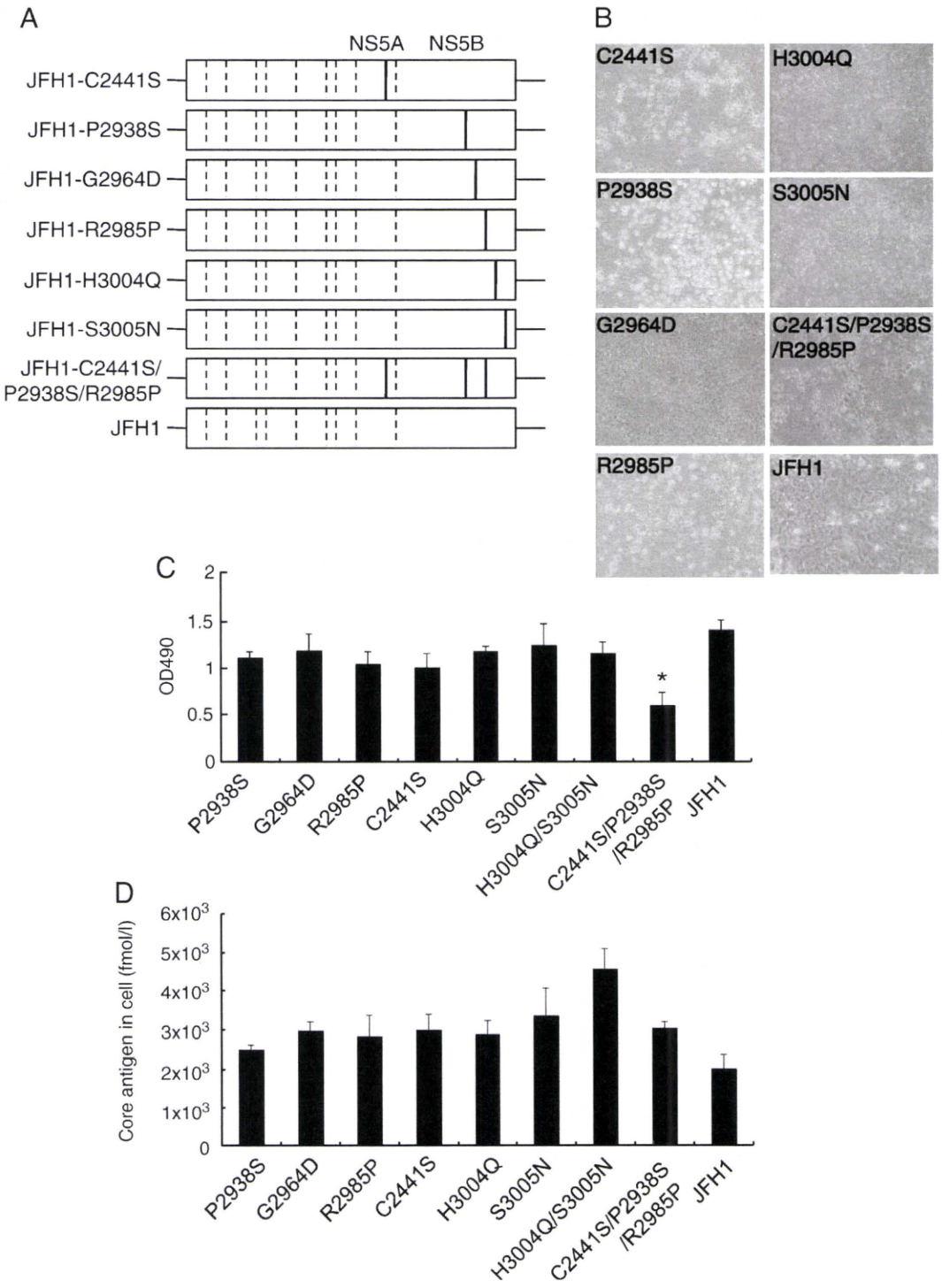

Fig. 2. Introduction of mutations into the NS5A and NS5B regions of JFH1. A. The mutations identified in the cytopathic plaque were introduced individually into the parental JFH1. Each JFH1 mutant was transfected into Huh-7.5.1 cells by electroporation. B. Huh-7.5.1 cells transfected with JFH1-mutants 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 re-infection 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,

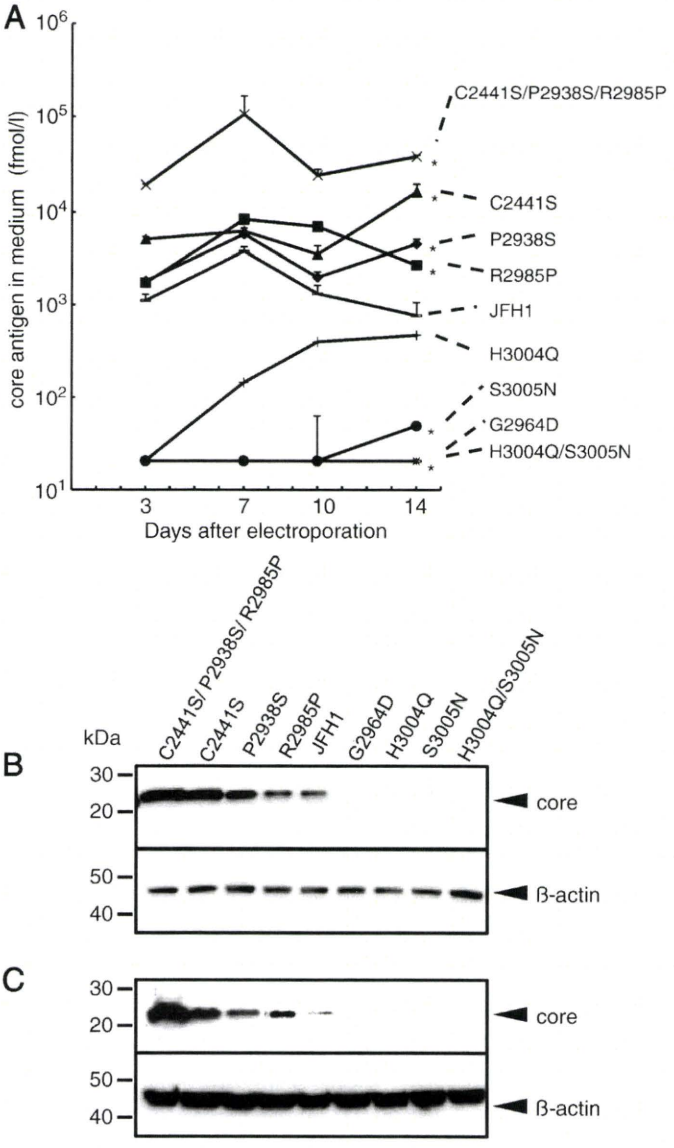

Fig. 3. Replication competences of HCV subclones with NS5A and NS5B mutations. A. Levels of core antigen in the culture medium. The culture media from transfected cells were collected on the days indicated and the levels of core antigen were measured. Asterisks indicate p-values of less than 0.05 as 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).

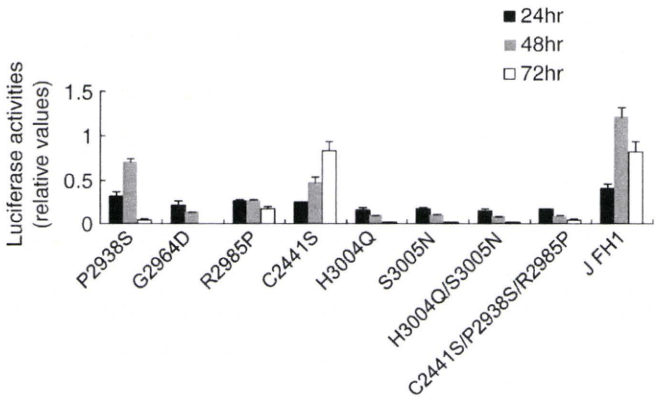

Fig. 4. Luciferase assay of the cytopathic JFH1-subgenomic replicon. Mutations were introduced into 2a-Feo subgenomic replicon and transcribed RNA for each replicon was transfected into Huh7 cells by electroporation. The cells were harvested at 24 h, 48 h and 72 h after electroporation and were used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

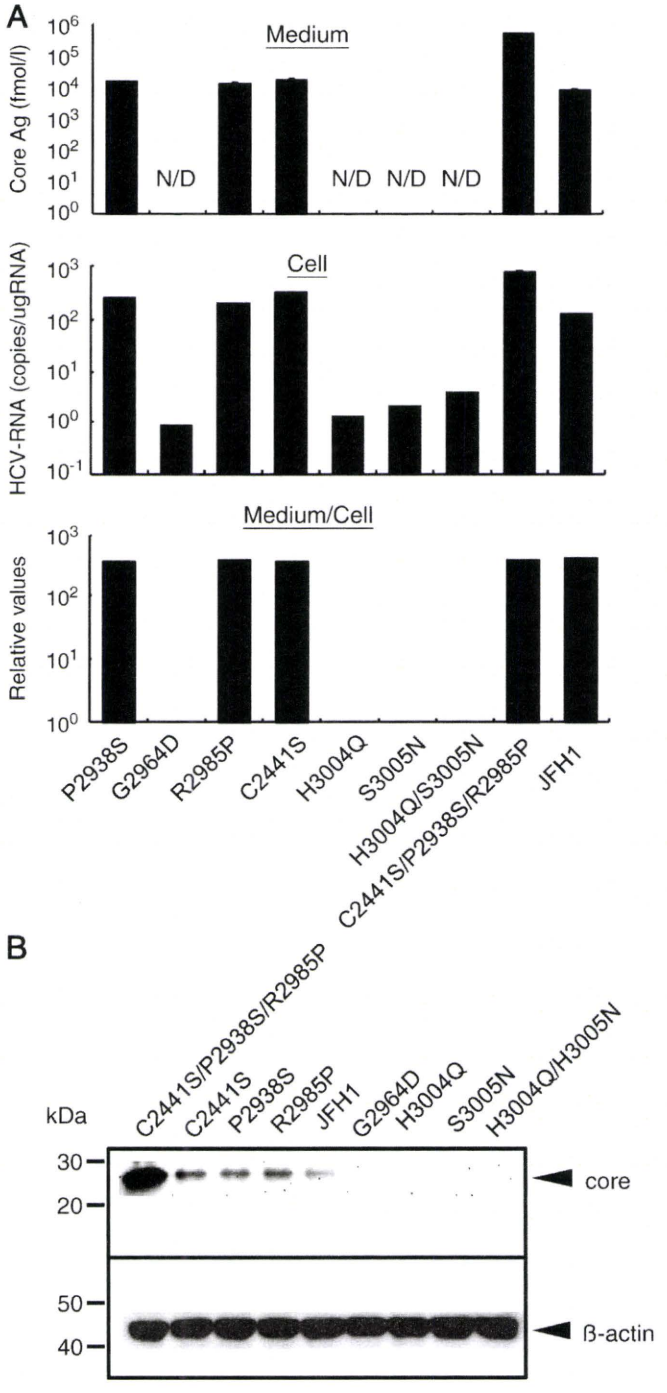

Fig. 5. Analysis of viral replication and production of viral particles using a single-cycle assay. A. Levels of core antigen in the culture media 3 days after transfection of JFH1 mutants into CD81-deficient Huh7-S29 cells (top). Levels of intracellular HCV RNA were quantified by real-time RT-PCR 3 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.

mouse and that these responses were more strongly induced in the liver of mutant-infected mouse.

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