TABLE I. Comparison of Pretreatment Factors Between Model Building and Validation Patients

	Model (n = 304)	Validation (n = 201)	*P*-value
Age (years)	55.6 (9.4)	56.0 (12.2)	0.80
Male (%)	53 (%)	55 (%)	0.13
Body mass index (kg/m^2)	23.1 (3.1)	23.1 (4.0)	0.99
Albumin (g/dl)	4.0 (0.3)	4.0 (0.3)	0.47
Creatinine (mg/dl)	0.72 (0.15)	0.72 (0.14)	0.62
AST (IU/L)	63.3 (45.6)	58.9 (46.4)	0.91
ALT (IU/L)	78.7 (58.6)	74.5 (67.5)	0.68
GGT (IU/L)	53.2 (49.1)	57.4 (63.5)	0.43
Total cholesterol (mg/dl)	170.9 (32.6)	169.4 (34.1)	0.33
Triglyceride (mg/dl)	107.0 (44.7)	105.7 (48.0)	0.90
LDL-C (mg/dl)	95.5 (28.0)	96.4 (28.8)	0.34
White blood cell count (/μl)	4,902 (1,489)	4,906 (1,319)	0.86
Hemoglobin (g/dl)	14.1 (1.3)	14.3 (1.4)	0.09
Platelets (10^9/L)	164 (56)	172 (55)	0.68
HCVRNA (10^3 IU/ml)	1,859 (1,468)	2,021 (1,393)	0.09
ISDR mutations: ≥2 (%)	15 (%)	20 (%)	0.11
Core70: mutant (%)	36 (%)	29 (%)	0.22
Core91: mutant (%)	40 (%)	36 (%)	0.20
Fibrosis: F2–4 (%)	49 (%)	48 (%)	0.36
Activity: A2–3 (%)	42 (%)	34 (%)	0.10

AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; LDL-C, low-density-lipoprotein-cholesterol; ISDR, interferon sensitivity-determining region.
Data expressed as mean (SD).

0–3: A0 (no activity), A1 (mild activity), A2 (moderate activity), and A3 (severe activity). Sustained virological response was defined as undetectable HCV RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic Systems) at week 24 after the completion of therapy.

Statistical Analysis

A database of pretreatment variables included hematological tests (hemoglobin level, white blood cell count, and platelet count), blood chemistry tests (serum levels of creatinine, albumin, aspartate aminotransferase, alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), total cholesterol, triglyceride, and low-density lipoprotein cholesterol (LDL-C)), viral factors (HCV RNA titer, number of substitutions in ISDR, substitutions in the amino acid positions 70 and 91 of the core region), histological findings (stage of fibrosis and grade of activity) and patient characteristics (age, sex, and body mass index). Based on this database, decision-tree analysis was used to define a predictive model for sustained virological response.

Student's *t*-test was used for the univariable comparison of quantitative variables and Fisher's exact test was used for the comparison of qualitative variables. For the multivariable analysis for factors associated with sustained virological response, logistic regression models with backward selection were used to identify independent predictors of sustained virological response. Variables that showed significant association with sustained virological response by univariable analysis were included in the multivariable analysis. IBM-SPSS software v.15.0 (SPSS, Inc., Chicago, IL) was used for these analyses. For the decision-tree analysis [Segal and Bloch, 1989], the data mining software IBM SPSS Modeler 13 (IBM SPSS, Inc.) was used, as reported previously [Kurosaki et al., 2010a,b]. In brief, the software searched for the optimal split variables to build a decision-tree structure. The entire study population was first evaluated to determine the variables and cut-off points for the most significant division into two subgroups having different probabilities of sustained virological response. Thereafter, analysis was repeated on all subgroups in the same way until either no additional significant variable was detected or the sample size was below 20.

RESULTS

Generation of the Decision-Tree Model

The decision-tree analysis selected five predictive variables to produce six subgroups of patients (Fig. 1). The number of substitutions in ISDR was selected as the best predictor of sustained virological response. The possibility of achieving sustained virological response was 83% for patients with two or more substitutions in ISDR compared with 44% for patients with a single or no substitution. Among patients with a single or no substitution in ISDR, age, with an optimal cut-off of 60 years, was selected as the variable of second split. Patients younger than 60 had the higher probability of sustained virological response (55%) compared with those older than 60 years (31%). Among younger patients, amino acid substitution at Core70 was selected as the third variable of split—wild-type sequence being the predictor of favorable response compared with the mutant type (65% vs. 36%). Among patients with wild-type Core70, the level of serum LDL-C was selected as the fourth variable of split, with an optimal cutoff of

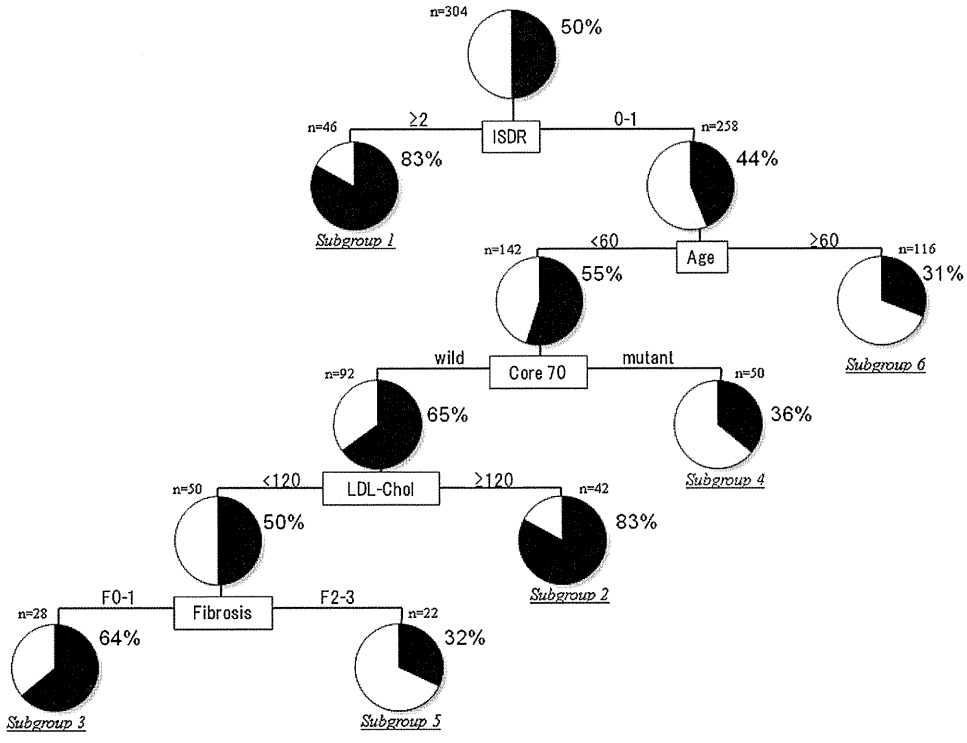

Fig. 1. Decision-tree model. Boxes indicate the factors used for splitting and the cutoff value for the split. Pie charts indicate the rate of sustained virological response for each group of patients after splitting. Terminal subgroups of patients discriminated by the analysis are numbered from 1 to 7. The rate of sustained virological response was >80% in subgroups 1 and 2, 64% in subgroup 3, and 31–36% in subgroups 4, 5, and 6. LDL-C represents low-density lipoprotein cholesterol and Core70 represents amino acid substitution at position 70 of the core region.

120 mg/dl. Patients with higher LDL-C level had the higher probability of sustained virological response (83% vs. 50%). The stage of fibrosis was selected as the final variable of split, with significant fibrosis (F2–4) being the predictor of lower sustained virological response probability (64% vs. 32%).

Among the six subgroups derived by this decision tree, the subgroup of patients with two or more substitutions in ISDR (subgroup 1) or with a single or no substitution in ISDR but younger than 60 years of age, having the wild-type Core70 and high serum level of LDL-C (≥120 mg/dl) (subgroup 2) showed the highest probability of sustained virological response (83%).

Validation of the Decision-Tree Model

The decision-tree model was validated using a validation dataset of 201 cases that were not included the model-building dataset. Each patient in the validation set was allocated to subgroups 1–6 using the flowchart form of the decision tree. The rates of sustained virological response were 75% for subgroup 1, 73% for subgroup 2, 65% for subgroup 3, 41% for subgroup 4, 46% for subgroup 5, and 33% for subgroup 6. The rates of sustained virological response for each subgroup of patients were correlated closely between the model building dataset and the validation dataset ($r^2 = 0.94$) (Fig. 2).

The six subgroups were reconstructed into three groups according to their rate of sustained virological response: the high-probability group consisted of subgroups 1 and 2, the intermediate-probability group consisted of subgroup 3, and the low-probability group consisted of subgroups 4, 5, and 6. The rate of sustained virological response in the high-probability group was high on a consistent basis: 83% for model-building patients and 74% for validation patients. The rate of sustained virological response in the intermediate-probability group was 64% for model building patients and 65% for internal validation patients. The rate of sustained virological response in the low-probability group was low on a consistent basis: 32% for model-building patients and 36% for internal validation patients (Fig. 3). Thirty percent of the patients were classified into the high-probability group and 10% of the patients were classified into intermediate-probability group, which means that about 40% of patients with higher than average probability of achieving sustained virological response were identified.

Effect of Dose Reductions of PEG-IFN and RBV

The possible effect of drug reductions was analyzed in the three groups of patients divided by decision tree (low-, intermediate-, and high-probability groups)

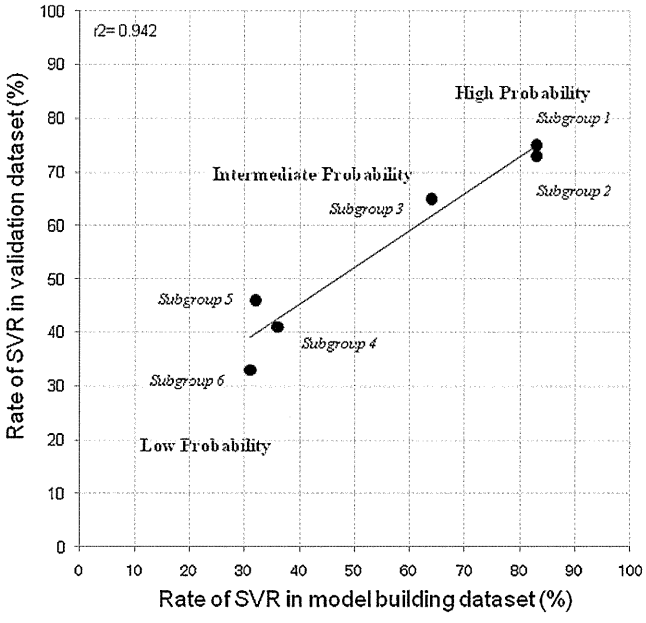

Fig. 2. Validation of the decision-tree analysis: Subgroup-stratified comparison of the rate of sustained virological response. Each patient in the validation set was allocated to subgroups 1–6 by following the flowchart form of the decision tree, and the rates of sustained virological response were then calculated and plotted for each subgroup. The x-axis represents the rate of sustained virological response in the model-building datasets and the y-axis represents the rate of sustained virological response in the validation datasets. The rates of achieving sustained virological response in each subgroup of patients correlated closely between the model-building dataset and the validation dataset (correlation coefficient: $r^2 = 0.94$).

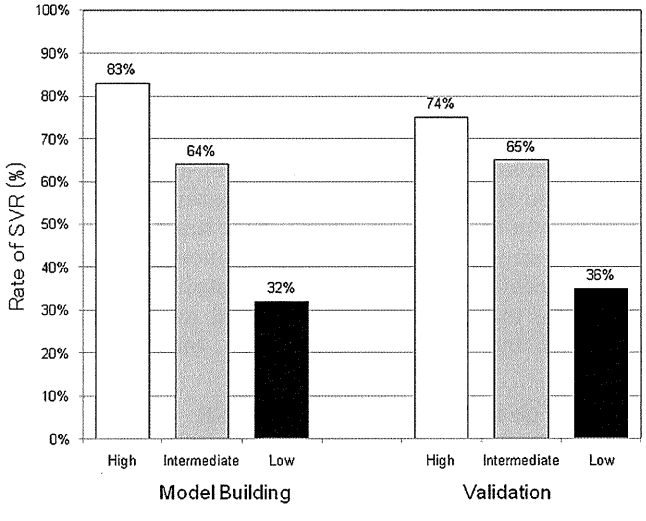

Fig. 3. Comparison of sustained virological response rates between groups divided by the decision tree. The rate of sustained virological response was compared between three groups of patients as divided by the decision-tree analysis. Black, gray, and white boxes indicate the low-probability group (subgroup 4, 5, and 6), intermediate-probability group (subgroup 3), and high-probability group (subgroup 1 and 2), respectively. The rate of sustained virological response showed significant difference between the three groups.

(Fig. 4). Patients were stratified according to the cumulative drug exposure with PEG-IFN and RBV: the good adherence group consisted of patients who took ≥80% planned doses of both PEG-IFN and RBV; the poor adherence group consisted of patients who took <80% of planned doses of both PEG-IFN and RBV. Even after adjustment for drug adherence, the three groups of patients divided by decision-tree analysis still had low, intermediate, and high probability of achieving sustained virological response, respectively, indicating that this model predicts sustained virological response independent of drug exposure.

Multivariable Logistic Regression Analysis

Age, sex, serum levels of creatinine, ALT, GGT, LDL-C, hemoglobin, platelet count, HCV RNA titer, ISDR substitution, substitution at Core70, substitution at Core91, histological stage of fibrosis, and grade of activity were found to be associated with sustained virological response by standard univariable analysis. Multivariable analysis including these factors showed that age, sex, LDL-C levels, GGT levels, platelet count, ISDR substitution, and substitution at Core70 showed independent associations with sustained virological response (Table II). Substitution in ISDR had the highest odds ratio, at 9.92. Fibrosis, which was selected as a significant predictor of response in the decision-tree analysis, was not found to be an independent predictor of response in standard multivariable analysis, indicating that the decision-tree analysis could identify significant predictors that would apply specifically to selected patients.

DISCUSSION

The present study revealed that viral factors such as substitutions in ISDR and Core70 are significant and independent predictors of sustained virological response to PEG-IFN plus RBV in chronic hepatitis C. In a decision-tree model for the pretreatment prediction of sustained virological response, the number of substitutions in ISDR was the best predictor of sustained virological response, followed by younger age, wild-type sequence at Core70, higher level of LDL-C, and absent fibrosis. This decision-tree model could identify patients with high probability of sustained virological response (83%) among difficult-to-treat genotype 1b chronic hepatitis C patients. Using this model, rapid estimates of the response before treatment can be made by allocating patients to specific subgroups with a defined rate of response simply by following the flowchart form. Because more potent therapy, such as a combination of protease inhibitor, PEG-IFN, and RBV, is under clinical trial and may become available in the near future [Hezode et al., 2009; McHutchison et al., 2009], pretreatment prediction of the likelihood of sustained virological response may be useful for both patients and physicians to support clinical decisions whether to start current standard therapy or to wait for emerging new therapies.

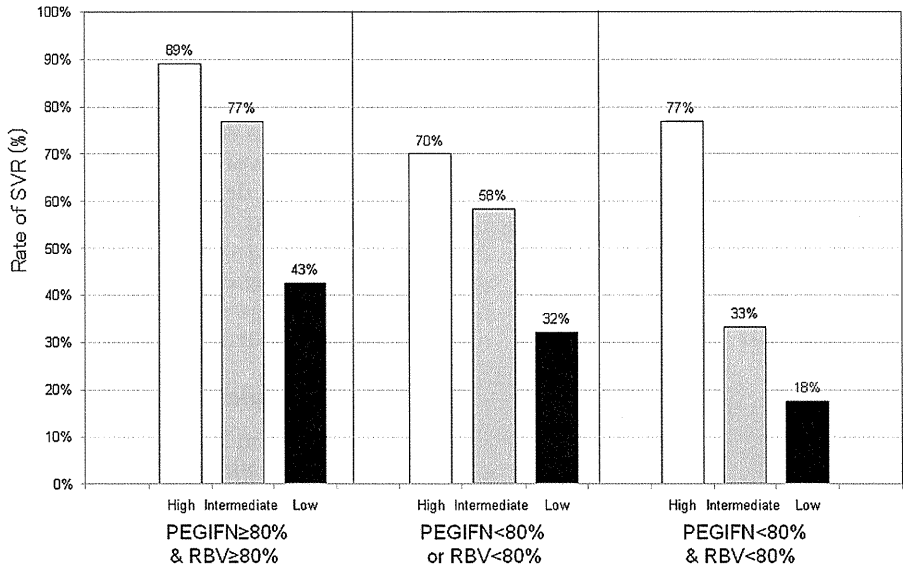

Fig. 4. Comparison of the rate of sustained virological response between the decision-tree groups stratified by drug adherence. The three groups of patients divided by the decision tree (black, gray, and white boxes indicating the low-, intermediate-, and high-probability groups, respectively) were further stratified according to cumulative drug exposure to PEG-IFN and RBV.

Two or more substitutions in ISDR had a strong impact on sustained virological response, because this factor was selected as a top variable in decision-tree analysis and had the highest odds ratio in multivariable analysis. Moreover, even among patients with unfavorable ISDR (0 or 1 mutation), younger patients (<60 years) with the wild-type sequence at Core70 and high level of LDL-C (≥120 mg/dl) had a high rate of sustained virological response. The sustained virological response rate of these two subgroups of patients was 83% in the model-building patients and 75% in the validation patients. Thus, patients with high possibility of sustained virological response could be extracted by the combined analysis of ISDR and Core70. These patients may be the best-suited candidates for treatment with the current combination therapy. Conversely, the following patients with 0–1 mutation in ISDR had a low probability of sustained virological response (32–35%): (1) older (>60 years); or (2) younger (<60 years) patients but having mutant-type sequence at Core70; or (3) younger (<60 years) patients having a wild-type sequence at Core70, but having a low level of LDL-C (<120 mg/dl) and advanced fibrosis. These patients may be advised to wait for a more effective therapy. Decision may be made on a case-by-case basis, taking into account the potential risk of disease progression while waiting.

In a previous decision-tree model using simple and noninvasive standard tests that are available readily worldwide [Kurosaki et al., 2010b], the rate of sustained virological response was at most 65–76% among those in the high-probability group. That model focused on use by general physicians in routine general practice, especially where specialized resources, such as liver biopsy or determination of viral sequences, are not available. In that model, younger age, male sex, higher platelet counts, lower alpha-fetoprotein (AFP) levels, and lower GGT levels were identified as favorable predictive parameters. Higher AFP levels and lower platelet counts that are hallmarks of advanced fibrosis [Shiratori and Omata, 2000; Akuta et al., 2007b] were associated with low probability of sustained virological response in that model. On the other hand, the present analysis aimed to clarify the significance of viral factors for pretreatment prediction of sustained virological response, and to build an advanced model that may be used by specialist physicians engaged in the

TABLE II. Multivariable Logistic Regression Analysis for Factors Associated With SVR

Parameter		Odds	95% CI	*P*-value
Age (years)	<60 vs. ≥60	2.28	1.31–3.94	0.003
Sex	Male vs. female	3.36	1.87–5.99	<0.0001
GGT (IU/L)	<40 vs. ≥40	2.65	1.45–4.85	0.002
LDL-C (mg/dl)	≥120 vs. <120	1.79	0.91–3.53	0.094
Platelets (109/L)	≥120 vs. <120	2.69	1.22–5.90	0.014
ISDR mutations	≥2 vs. 0–1	9.92	3.71–26.54	<0.0001
Core70	Wild vs. mutant	1.92	1.07–3.47	0.030

GGT, gamma-glutamyltransferase; LDL-C, low-density-lipoprotein-cholesterol; ISDR, interferon sensitivity-determining region.

treatment of hepatitis. In the present model, stage of fibrosis was selected as a predictive factor, but at lower level of significance than HCV mutations. The predicted rate of sustained virological response in the high-probability group of the present model is higher than that in the previous model (75–83% vs. 65–76%). These results indicate that substitutions in ISDR and Core70 were important pretreatment predictors of sustained virological response. Determination of these viral factors is not available readily in clinical practice, but is of value for improving the accuracy of pretreatment prediction of sustained virological response.

Substitutions in ISDR and Core70 have been reported previously to be associated with efficacy of IFN therapy. The association between the number of substitutions in ISDR and response to therapy was demonstrated originally in patients treated with IFN mono-therapy [Enomoto et al., 1995, 1996; Kurosaki et al., 1997], but recent studies have reported a positive correlation with PEG-IFN and RBV combination therapy as well [Munoz de Rueda et al., 2008; Shirakawa et al., 2008; Ikeda et al., 2009]. Another important viral factor relevant to treatment response is amino acid substitution in Core70. The sequence of this amino acid was reported originally to be associated with nonresponse to therapy [Akuta et al., 2005], but subsequent studies confirmed the positive correlation of a wild-type Core70 with sustained virological response [Akuta et al., 2009]. The multiple logistic regression analysis showed that ISDR and Core70 were independent factors associated with sustained virological response along with host factors. How these important viral factors and other host factors can be combined to predict response to PEG-IFN plus RBV is an important clinical question. Decision-tree modeling can make the response probability apparent by combining all these factors. Some factors that may be associated with treatment outcome, such as levels of ferritin or homocysteine, were not included. This may be a potential limitation of the present study.

It is of interest that a recent study by Li et al. [2010] has shown that a high serum level of LDL-C is linked to the *IL28B* major allele (CC in rs12979860). In that study, a high serum level of LDL-C was associated with sustained virological response, but it was no longer significant when analyzed together with the *IL28B* genotype in multivariate analysis. Thus, the association between treatment response and LDL cholesterol levels in the present study may reflect the underlining link of LDL cholesterol levels to the *IL28B* genotype. Recent reports indicate that the *IL28B* genotype and HCV substitutions are correlated closely [Akuta et al., 2010; Kurosaki et al., 2010c]. Still, Core70 [Akuta et al., 2010] or ISDR [Kurosaki et al., 2010c] were predictors of response to therapy independent of *IL28B* genotype. Future study is needed to elucidate the possible mechanisms underlying the association between HCV sequences and host genetic factors, and also the role of host and viral factors for the prediction of treatment response.

In conclusion, a data mining analysis emphasized the impact of substitutions in ISDR and Core70 on pretreatment prediction of sustained virological response to PEG-IFN plus RBV therapy. A decision-tree model that includes substitutions in ISDR and Core70 of HCV could identify patients with high probability of sustained virological response, and could thereby improve the predictive accuracy over predictions that are based on standard tests.

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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 SPSYGRSRSRSRSRSRSRSRSNSRSRSY-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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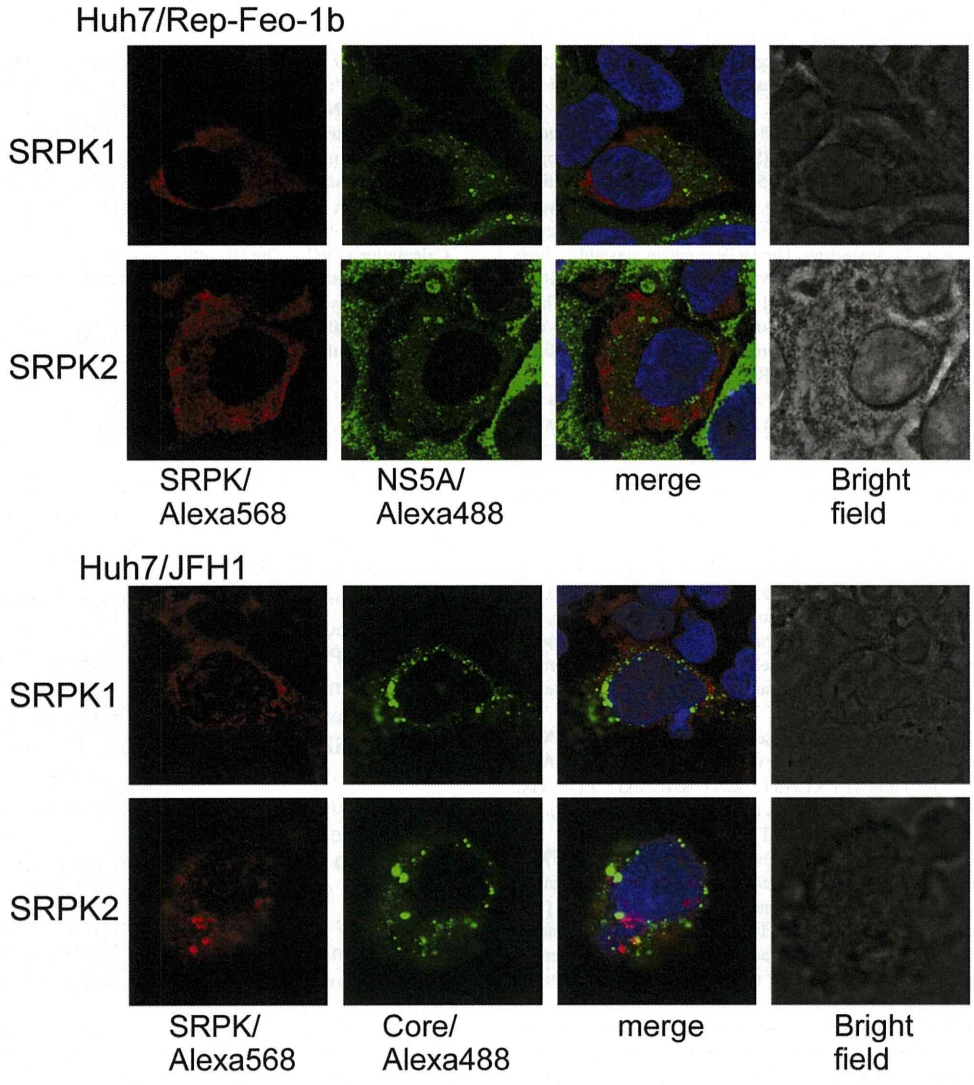

FIG. 1. Immunofluorescence microscopy. Expression plasmids for SRPK1 and SRPK2 were transfected into Huh7/Rep-Feo-1b cells or into HCV-JFH1-infected Huh7.5.1 cells. Forty-eight hours after transfection, cells were fixed and incubated with mouse anti-NS5A or anti-core antibodies and rabbit anti-HA antibody,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

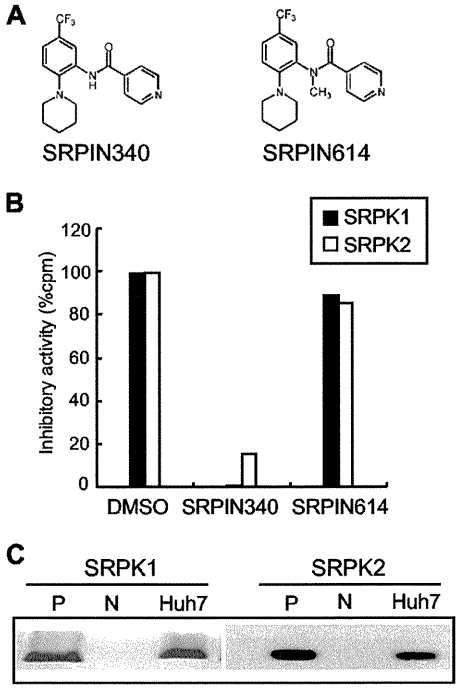

FIG. 2. Chemical structures and activities of SRPIN340 and SRPIN614. (A) Chemical structures of the SRPK inhibitor, SRPIN340, and activity-lacking control, SRPIN614. (B) Relative kinase activities of SRPK1 (black columns) and SRPK2 (white columns) *in vitro*, in the presence of the reagents indicated,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

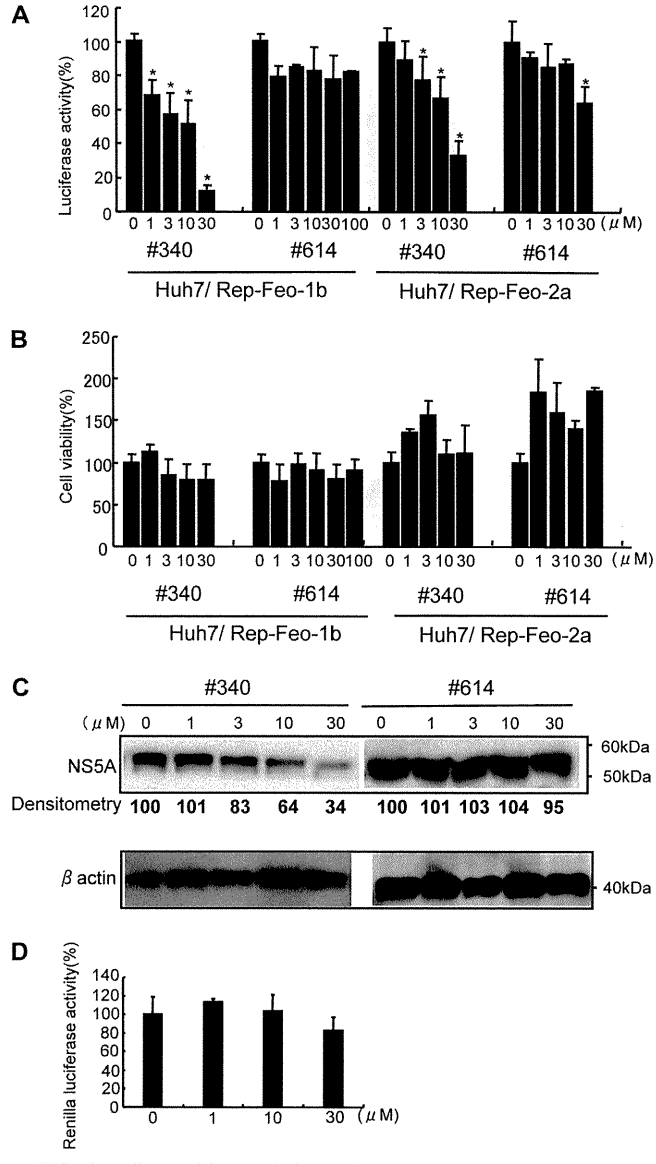

FIG. 3. Effects of SRPIN340 and SRPIN614 on expression of HCV subgenomic replicons. Huh7/Rep-Feo-1b or Huh7/Rep-Feo-2a cells were cultured in the presence of SRPIN340 (no. 340) or SRPIN614 (no. 614) at the concentrations indicated. After 48 h of culture, a luciferase assay (A), a cell viability assay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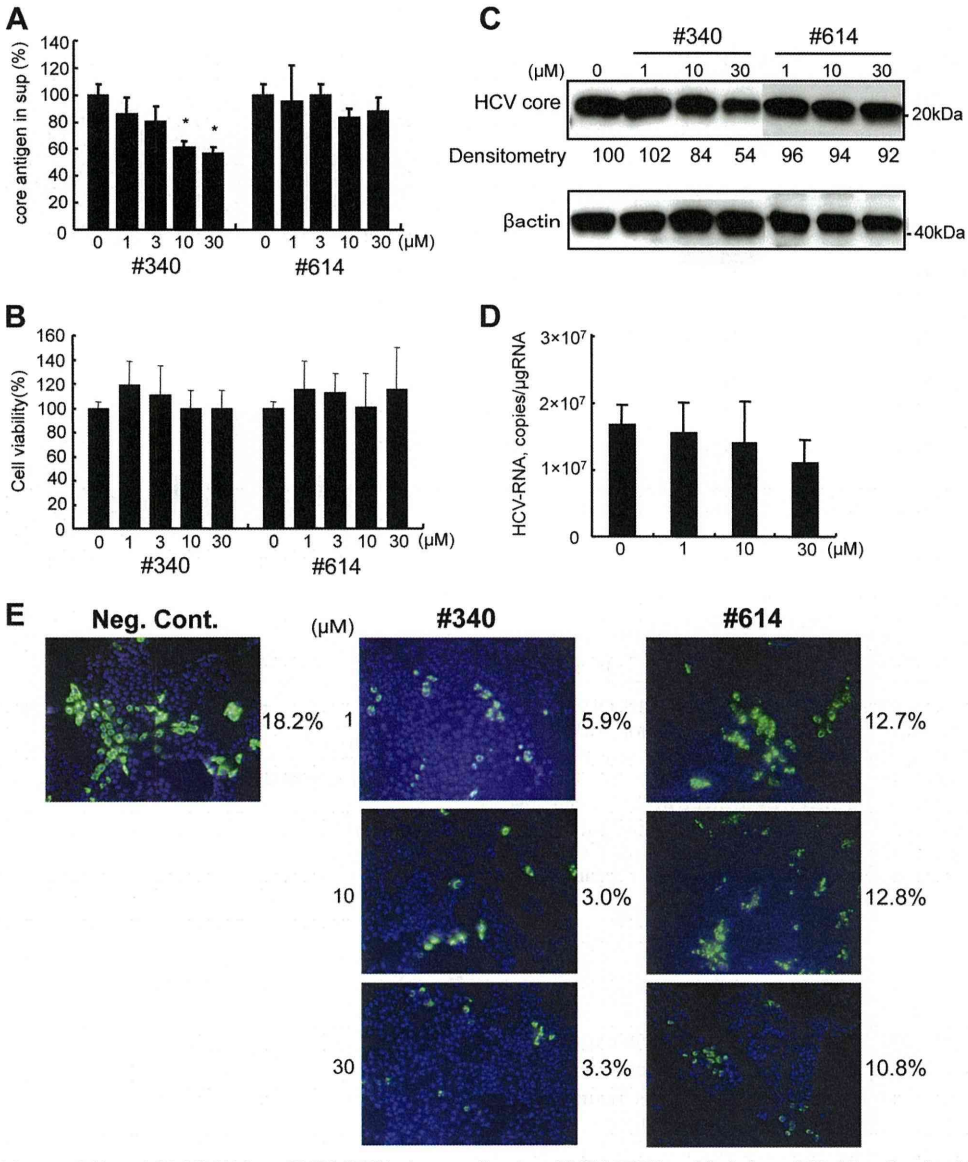

FIG. 4. Effect of SRPIN340 and SRPIN614 on HCV-JFH1 virus replication. HCV-JFH1-stably infected Huh7 cells of ~14 days were cultured in the presence of SRPIN340 or SRPIN614 at the concentrations indicated. After 48 h, cellular and supernatant HCV core antigens were detected. (A) HCV core antigen assays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

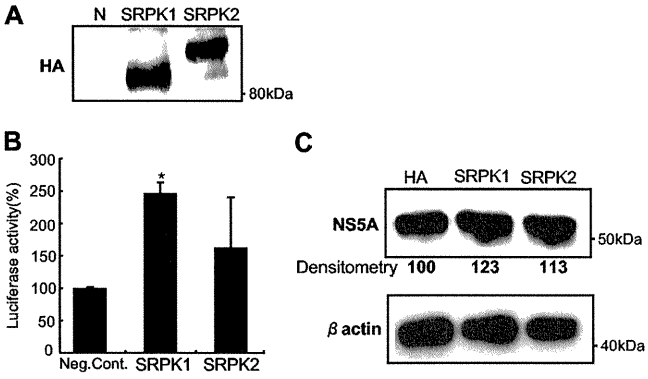

FIG. 5. Effects of overexpression of SRPK1 and SRPK2 on HCV replication. (A) The expression of transfected HA-tagged SRPK1 and SRPK2 was detected by anti-HA antibody. (B) Huh7/Rep-Feo-1b cells seeded on 24-well plates were transfected with 0.2 μg of expression plasmids for SRPK1 or SRPK2 or empty vector.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

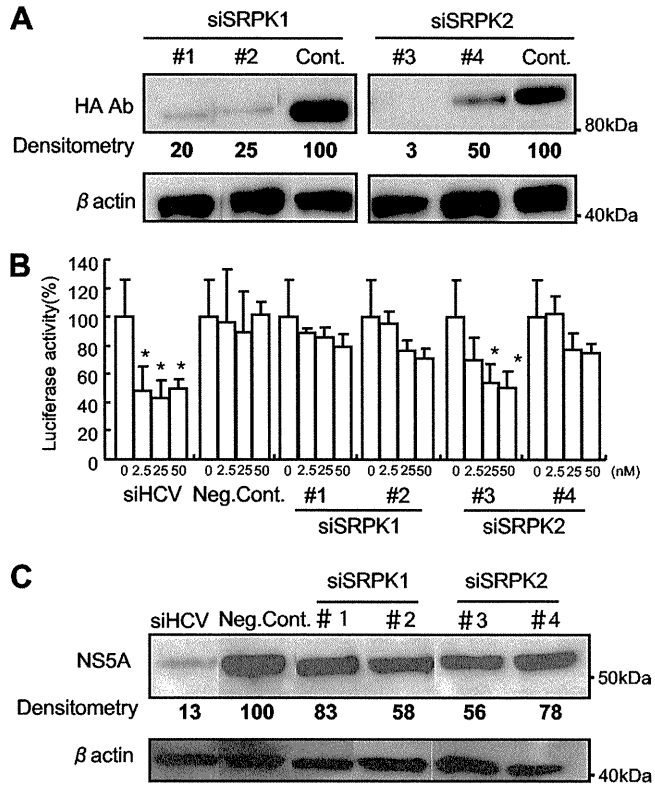

FIG. 6. Effects of siRNA knockdown of SRPK1 and SRPK2. (A) Huh7 cells were transfected with SRPK1 or SRPK2 expression plasmids and siRNA directed against SRPK1 (siSRPK1 no. 1 and siSRPK1 no. 2) or SRPK2 (siSRPK2 no. 3 and siSRPK2 no. 4) or control siRNA (32). Forty-eight hours after transfection, Western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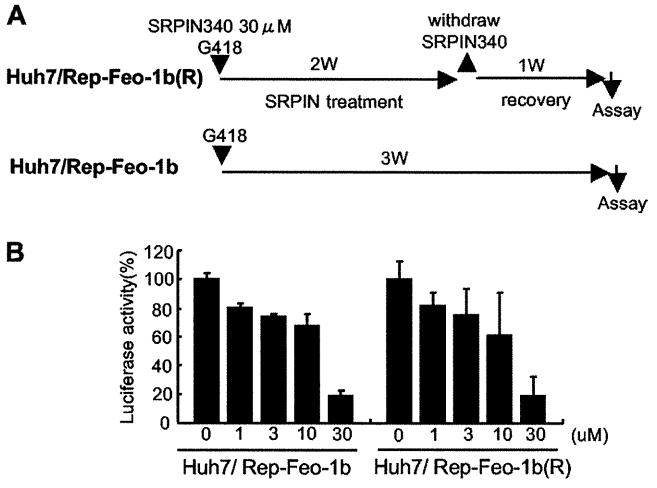

FIG. 7. Drug resistance assay of HCV replicon cells. (A) Schema for the establishment of SRPIN340-resistant cells and the control cells. Huh7/Rep-Feo-1b cells were treated with or without 30 μM SRPIN340 for 2 weeks in the presence of 500 μg/ml of G418. After 1 week of recovery culture without SRPIN340, 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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IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones

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ABSTRACT

Mechanisms of difference in interferon sensitivity between hepatitis C virus (HCV) strains have yet to be clarified. Here, we constructed an infectious genotype2b clone and analyzed differences in interferon-alpha sensitivity between HCV-2b and 2a-JFH1 clones using intergenotypic homologous recombination. The HCV-2b/JFH1 chimeric virus able to infect Huh7.5.1 cells and was significantly more sensitive to IFN than JFH1. IFN-induced expression of MxA and 25-OAS was significantly lower in JFH1 than in 2b/JFH1-infected cells. In JFH1-infected cells, expression of SOCS3 and its inducer, IL-6, was significantly higher than in 2b/JFH1-infected cells. The IFN-resistance of JFH1 cells was negated by siRNA-knock down of SOCS3 expression and by pretreatment with anti-IL6 antibody. In conclusion, intergenotypic differences of IFN sensitivity of HCV may be attributable to the sequences of HCV structural proteins and can be determined by SOCS3 and IL-6 expression levels.
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Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). There is no therapeutic or prophylactic vaccine available for HCV and type I interferons have been the mainstay of HCV therapeutics (Hoofnagle and di Bisceglie, 1997). Antiviral therapeutic options against HCV are limited and yield unsatisfactory responses (Fried et al., 2002). Given these situations, gaining a detailed understanding of the molecular mechanisms of interferon resistance has been a high priority in academia and industry.

Molecular studies of HCV have been hampered by the lack of efficient *in vitro* and *in vivo* models of infection, which has been partly overcome by the development of HCV subgenomic replicons (Blight et al., 2000; Kato et al., 2003; Lohmann et al., 1999) and the HCV-JFH1 cell culture system (Wakita et al., 2005). HCV-JFH1 is an isolate of HCV genotype 2a that was obtained from a patient with fulminant hepatitis C. The full-length JFH1 genome has been shown to produce infectious particles in cell culture. Simultaneously, a robustly replicating intragenotypic chimera has been reported, which consists of the structural region of a genotype 2a, J6-clone and nonstructural region of JFH-1 (Lindenbach et al., 2005).

HCV isolates are classified into seven major genotypes and multiple subtypes (Gottwein et al., 2009). In infected individuals, HCV exists as quasispecies of closely related genomes (Bukh et al., 1995). A number of studies have suggested that the outcome of HCV infection, as well as the response to interferon treatment, depends on the genotype or quasispecies with which the patient is infected. However, it is not clear how these subtle genetic differences of HCV affect viral replication, infectivity and host responses. Thus, it is important to establish multiple cell culture-permissive strains of different genotypes and isolates of the same genotype for their potential value for characterizing the virus life cycle, drug sensitivity and virus-related cell signaling.

Our present work describes the generation of chimeric viruses with their structural regions from genotype 2b and non-structural genes from the HCV-JFH1 strain. The intergenotypic 2b/JFH1 viruses were compared in terms of intracellular replication, infectious virus production and sensitivity to interferon-alpha. Here we show that the differences in sensitivity to interferon are attributable to upregulated expression of the cellular interferon signal attenuator, SOCS3, and that this upregulation is caused by overexpression of interleukin-6 (IL-6).

Abbreviations: HCV, hepatitis C virus; TLR, toll-like receptor; FBS, fetal bovine serum; ISG, interferon-stimulated gene; IFN, interferon; SOCS, suppressor of cytokine signaling; IL, interleukin; ALT, alanine aminotransferase; UTR, untranslated region; CLEIA, chemiluminescence enzyme immunoassay; PVDF, polyvinylidene fluoride; STAT, signal transducer and activator of transcription; IFNAR, interferon alpha/beta receptor.

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Results

In vitro and in vivo infectivity analyses of HCV-2b and 2b/JFH1 intragenotypic chimeras

First, we investigated the infectivity of the full-length genotype 2b clone *in vitro* and *in vivo*. The full-length genotype 2b HCV clone was infectious after direct injection of RNA transcribed *in vitro* into the livers of human hepatocyte engrafted albumin-uPA/SCID mice (*see the* Supplementary Fig. 1). However, transfection of the HCV RNA into Huh7.5.1 cells did not lead to replication or secretion of virions. Knowing that the full-length genotype 2b HCV was not infectious *in vitro*, we constructed genotype2b/JFH1 intergenotypic recombinants. We constructed three recombinant clones of 2b/JFH1 (Fig. 1A), which were joined between E2 and p7 (JE31F), NS2 and NS3 (JE39F), and within NS2 at nt. 2867 (JEC3F). After transfection of these chimeric HCV RNAs and JFH-1 RNAs into Huh7.5.1 cells, all four clones expressed detectable amounts of HCV core protein in the cells (Fig. 1B) and culture fluid (Fig. 1C). Among the four clones, JEC3F produced the highest level of core protein in the cells and culture fluid. Similarly, in the reinfection assays, JEC3F infected naïve cells most efficiently (Figs. 1D and E). We then compared the infectivity of JEC3F with the other chimeric viruses, genotype2a J6/JFH1 and the JFH1 clone (Supplementary Fig. 2). Transfection of the individual clones into Huh7.5.1 cells showed that JEC3F and the 2b/JFH1 chimera secreted core protein into the medium most efficiently (Fig. 1C). We measured HCV core antigen and HCV-RNA levels in culture supernatant of JEC3F and JFH-1 infected cells. As shown in Fig. 1F, the ratio between supernatant HCV core antigen and HCV-RNA between JEC3F and JFH1 was well correlated each other.

Comparisons of sensitivity to IFN between intragenotypic chimeras and JFH1

Next, we investigated the interferon-alpha sensitivity of the three 2b/JFH1 chimeric viruses with different junctions, JE31F, JE39F and JEC3F, as well as JFH1. The four viral RNAs were transfected separately into Huh7.5.1 cells and were treated with 0, 1, 3 or 9 IU/mL of interferon-alpha-2b. Seventy-two hours after addition of interferon, core antigen was measured in the culture fluid. As shown in Fig. 2, all 2b/JFH1 chimeric clones showed significantly higher responses to interferon than JFH1 ($p<0.01$). These results indicate that the relative interferon sensitivity of 2b/JFH1 clones over JFH1 could be attributable to the sequences of HCV-2b-derived structural proteins, especially core, E1 or E2 protein.

Expression of IFN stimulated genes and STAT1 and 2 phosphorylation in HCV-infected cells

Knowing that the 2b/JFH1 chimeric clones are more sensitive to interferon than JFH1, we next analyzed the effects on cellular interferon signaling. We investigated the expression levels of the interferon-stimulated genes (ISGs), 25OAS and MxA mRNAs that mediate antiviral effects (Itsui et al., 2009; Itsui et al., 2006). Induction of 25OAS and MxA by IFN was significantly suppressed in cells infected with HCV-JFH1 and the JEC3F clones. Of note was that the induction of these ISGs was suppressed substantially in JFH1-infected cells compared to JEC3F-infected cells (Figs. 3A and B). We then detected IFN-induced phosphorylation of STAT1 and STAT2 to pSTAT1 and pSTAT2 in uninfected and JFH1- and JEC3F-infected cells. Phosphorylation of STAT1 and STAT2 occurs within minutes after addition of IFN and substantially decreased at time points later than 8 hours {Itsui, 2006 #1025}. Thus, we detected pSTAT1 and pSTAT2 before and at 15 minutes after IFN treatment. As shown in Figs. 3C and D, production of pSTAT1 and pSTAT2 was decreased substantially in JFH1-infected cells, compared with uninfected and JEC3F-infected cells. These finding indicated that the differences in sensitivity to interferon of JFH1 and JEC3F were closely associated with attenuation of the cellular IFN signaling pathway.

SOCS 3 is up-regulated in JFH-infected, IFN-resistant cells

We next investigated the effects of HCV replication on the expression of SOCS1 and SOCS3 that suppress IFN receptor-mediated signaling (Song and Shuai, 1998; Vlotides et al., 2004). While SOCS1 mRNA expression did not differ significantly between uninfected and JFH1- and JEC3F-infected cells, the SOCS3 mRNA expression level was significantly higher in JFH1-infected cells than in uninfected and JEC3F-infected cells (Figs. 4A and B).

Knock down of the SOCS3 gene

To verify that SOCS3 was the key molecule determining the sensitivity to IFN, we performed siRNA knock down of SOCS3 in the virus-infected cells. A SOCS3-directed siRNA was cotransfected with HCV-JFH1 or -JEC3F RNA into Huh7.5.1 cells. Three days after transfection we measured SOCS3 mRNA expression in JFH1 and JEC3F-transfected cells with or without SOCS3-siRNA. Interestingly, SOCS3-knock down in JFH1-transfected cells restored sensitivity of IFN to the same levels as JEC3F-transfected cells (Figs. 5A and B).

Interleukin-6 is involved in SOCS-mediated interferon resistance

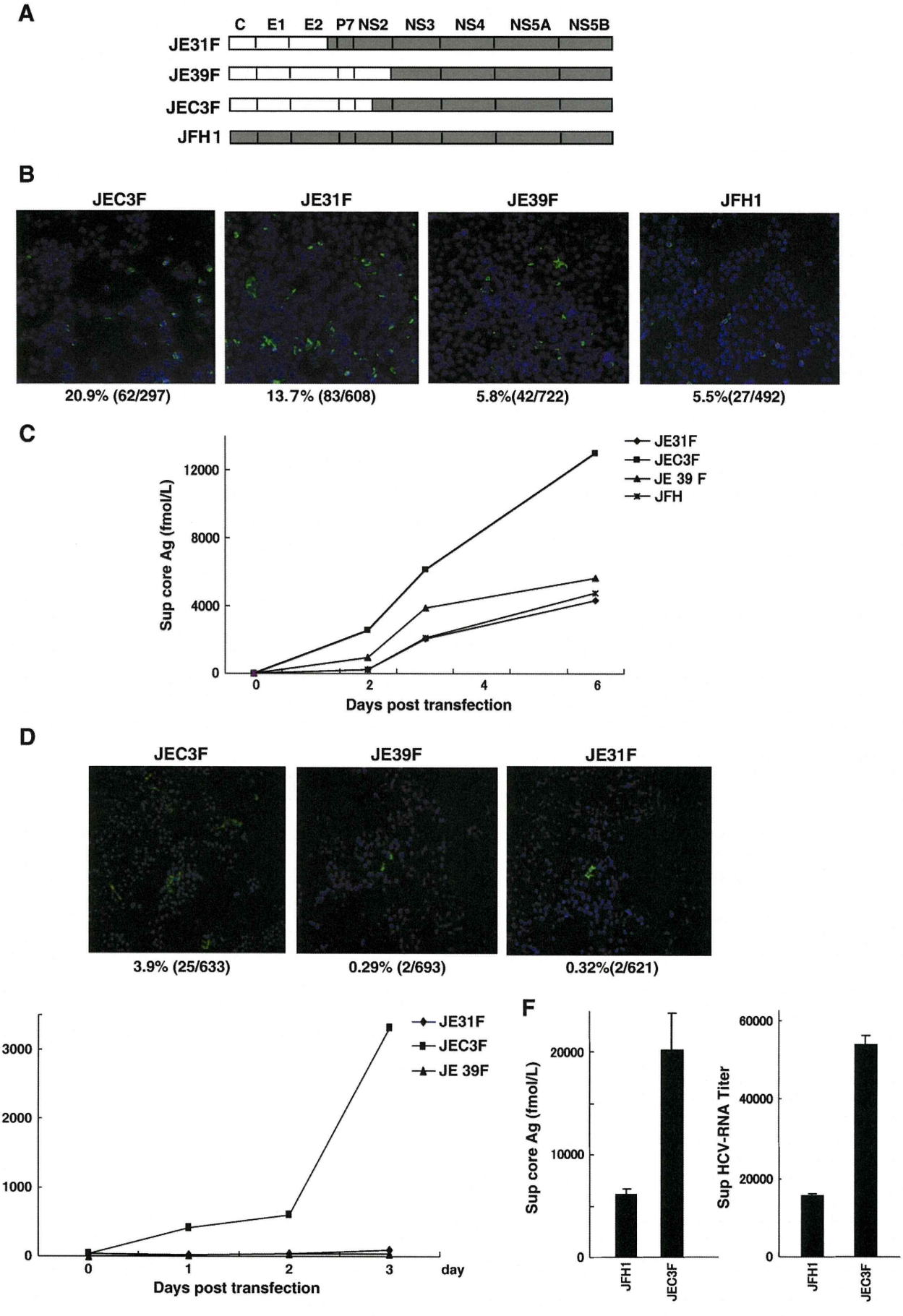
It has been reported that SOCS3 is induced principally by phosphorylated STAT3 (pSTAT3) (Hanada et al., 2003) and that interleukin-6 (IL-6) is a strong inducer of pSTAT3 via receptor-mediated Janus kinase activation in the liver (Ramadori and Christ, 1999). This background led us to investigate whether overexpression of SOCS3 is associated with overproduction of IL-6. We investigated Phosphorylated STAT3 (pSTAT3) expression and IL-6 mRNA expression in JFH1- and JEC3F-transfected Huh7.5.1 cells. Phosphorylated STAT3 level was significantly higher in JFH1-transfected cells than JEC3F-transfected cells and naïve Huh7.5.1cell (Fig. 6A). Moreover IL-6 gene expression level was significantly higher in JFH1-transfected cells than JEC3F-transfected cells (Fig. 6B). Consistent with previous reports, treatment of the Huh7.5.1 cells with IL-6 induced expression of SOCS3 and SOCS1 mRNAs with SOCS3 being much stronger than SOCS1 (Fig. 6C).

Anti-IL-6 antibody restored IFN-resistance to HCV-infected cells

To investigate whether IL-6 is responsible for HCV infection-induced upregulation of SOCS and for resistance to interferon, JFH1 and JEC3F-infected Huh7.5.1 cells were pretreated with antibodies directed against IL-6 and subsequently treated with interferon. Interestingly, anti-IL-6-treated HCV-infected cells became significantly more susceptible to IFN treatment (Fig. 6D) without affecting viral expression levels in the absence of interferon (Fig. 6E). Cellular levels of SOCS3 mRNA were significantly lower in anti-IL-6-treated cells than untreated cells (Fig. 6F). These results strongly suggested that the interferon resistance of HCV-infected cells and the difference between the two viral strains are partly mediated by internal overproduction of IL-6 and subsequent upregulation of SOCS3.

Determination of the HCV structural region that induced SOCS3 and IL6

We studied further which part of HCV structural polyprotein is responsible for the difference in interferon-sensitivity. We constructed two additional chimeric clones between HCV-2b and JFH1. The 2bCoreJFH1 had the 2b-core region followed by the JFH1-structural and nonstructural regions. JCoreC3F was derived from JEC3F by exchanging the 2b-core with the JFH1-core (Fig. 7A). As


A
C E1 E2 P7 NS2 NS3 NS4 NS5A NS5B
JE31F
JE39F
JEC3F
JFH1
B
JEC3F
JE31F
JE39F
JFH1
20.9% (62/297)
13.7% (83/608)
5.8%(42/722)
5.5%(27/492)
C
Sup core Ag (fmol/L)
12000
8000
4000
0
0
2
4
6
Days post transfection
JE31F
JEC3F
JE 39 F
JFH
D
JEC3F
JE39F
JE31F
3.9% (25/633)
0.29% (2/693)
0.32%(2/621)
E
Sup core Ag fmol/L
3000
2000
1000
0
0
1
2
3
day
Days post transfection
JE31F
JEC3F
JE 39F
F
Sup core Ag (fmol/L)
20000
10000
0
JFH1
JEC3F
Sup HCV-RNA Titer
60000
40000
20000
0
JFH1
JEC3F

shown in Fig. 7B JFH1 and JCoreC3F, which had a JFH1-derived core region, were significantly more resistant to IFN than JEC3F and 2bCoreJFH1, with a 2b-derived core (Fig. 7B). Consistent with the interferon sensitivity results, JFH1 and JcoreC3F-infected cells expressed SOCS3 and IL6 mRNAs at significantly higher levels than JEC3F and 2bCoreJFH1-infected cells (Figs. 7C and D). These differences in gene expression were inversely associated with the cellular expression levels of each HCV chimeric clone (Fig. 7E). These results indicate that the amino acid sequence of the core protein is responsible for IL-6 and SOCS3-medited interferon resistance.

Discussion

In this study, we succeeded in establishing a new genotype 2b infectious HCV clone and genotype 2b/JFH1 cell culture-competent intragenotypic chimeric viruses (Fig. 1). Relative interferon sensitivities of 2b/JFH1 chimeras, compared with HCV-JFH1 virus (Fig. 2), led us to conduct a series of assays to investigate the molecular mechanisms of IFN-related response pathways. We found that IFN-alpha receptor-mediated cellular responses were more attenuated in HCV-JFH1- and 2b/JFH1 chimera-infected than in uninfected Huh7.5.1 cells, but more potently for HCV-JFH1. Precise intragenotypic recombination analyses showed that the amino acid sequence of the HCV core protein is responsible for the differences in interferon sensitivity (Figs. 2, 7). The differences in the interferon-mediated antiviral effects were demonstrated further by the different rates of induction of interferon-inducible MxA and 25-OAS mRNAs (Figs. 3A and B) and IFN induced phosphorylation of STAT1 and STAT2 (Figs. 3D and E). We have demonstrated further that the expression of an interferon signal attenuator, SOCS3, was significantly higher in JFH1 than in 2b/JFH1-infected cells (Song and Shuai, 1998; Vlotides et al., 2004). Indeed, the siRNA-knock down of SOCS3 in JFH1 and 2b/JFH1-infected cells resulted in responsiveness to IFN (Fig. 5). Moreover, cellular expression of IL-6, which increases cytoplasmic phospho-STAT3 (Fig. 6A) and induces SOCS3 expression (Ramadori and Christ, 1999) was significantly higher in JFH1 transfected cells (Fig. 6B). Furthermore, by pre-treatment with anti-IL-6 antibody, JFH1- and 2b/JFH1-infected cells partially recovered elevation of SOCS3 expression and unresponsiveness to IFN (Fig. 6D). Taking all these things together, it is strongly suggested that the differences in IFN sensitivity between genotypes or isolates could be explained by SOCS3-mediated attenuation of interferon responses and, more importantly, IL-6 may constitute a molecular target to reverse such cellular interferon resistance.

Vast numbers of studies have failed to construct infectious HCV clones, other than HCV-JFH1. Murayama, et al. have conducted intragenotypic homologous recombination analyses between HCV-J6 and –JFH1 and have reported that that the NS3 protease and NS5B polymerase are essential for replication of the recombinant virus (Murayama et al., 2007). Up to now, several JFH1-based chimeric viruses have been reported, which include genotypes 4a (Scheel et al., 2008), genotype 1a, 1b, 2a (Pietschmann et al., 2006), genotype 3a (Gottwein et al., 2007), genotype 5 a (Jensen et al., 2008) and, genotype 2b, 6a, 7a (Gottwein et al., 2009). Gottwein, et al. constructed intergenotypic chimeric HCV from JFH1 and genotypes 1 through 7 and analyzed differences in sensitivity to antiviral drugs (Gottwein et al., 2009). However, intergenotypic differences in sensitivity to IFN-alpha and the molecular mechanisms involved have not been well characterized. In this study, we constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), which showed variable sensitivity to IFN and confirmed that the core region is responsible for such IFN sensitivity. This study may support the feasibility of such inter and intragenotypic homologous recombination approaches to characterize differences in viral kinetics and drug responses.

Type I IFNs and their responsive ISGs are the principal mediators of host defense against virus infections, including HCV (Chang et al., 1991; Kalvakolanu, 2003; Ronni et al., 1998). On binding of IFNs to their receptors, IFNAR1 and IFNAR2, Janus kinases-1 and -2 phosphorylate STAT1 and STAT2 to form ISGF-3, which translocates to the nucleus and activates transcription of ISGs (Samuel, 2001; Taniguchi et al., 2001; Taniguchi and Takaoka, 2002). Members of the SOCS family are potent inhibitors of type I and type III IFN-induced activation of the Jak–STAT pathway and subsequent expression of ISGs (Vlotides et al., 2004). In HCV subgenomic replicon-expressing cells, expression levels of SOCS3 were inversely correlated with sensitivity to IFN to suppress viral RNA replication (Zhu et al., 2005).

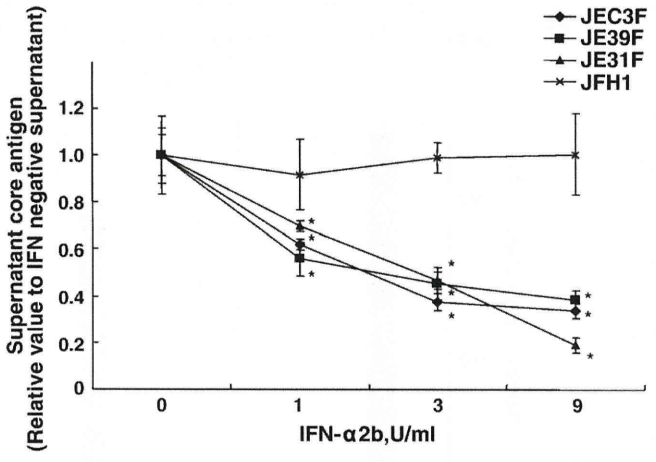


Fig. 2. Comparison IFN-alpha sensitivity among 2b/JFH1 chimeric viruses and JFH1. Ten μg of JE31F, JE39F, JEC3F, JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells. The transfected cells were divided into 12 wells. Forty eight hours after transfection, cells were washed twice with PBS and treated with 0, 1, 3 and, 9 U/ml IFN-alpha-2b. Seventy-two hours after IFN-alpha 2b addition, quantification of HCV core antigen in culture fluids was conducted. The experiments were conducted twice by using Huh 751 cells of different passage, and a representative data was shown. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05.

Fig. 1. Replication and infection competency of HCV-2b/JFH1 chimeric viruses A. Genomic structures of HCV-JFH1, HCV-2b and 2b/JFH1 chimeric viruses. Intergenotypic homologous recombination was conducted between the HCV-2b and JFH1 (2a) clones and three chimeric clones were constructed that were joined between NS2-NS3 (JE39F), and within E2 at nt2541 (JE31F) and NS2 at nt. 2867 (JEC3F). B. Immunocytochemistry of HCV core. HCV RNA-transfected Huh7.5.1 cells were plated onto 22 mm-round micro cover glasses. Immunocytochemistry was performed 4 days after transfection using mouse-anti-core antibody(*green*) and DAPI(*blue*). C. Time courses of 2b/JFH1- and JFH1-transfected cells. *In vitro* transcribed HCV RNAs were transfected into Huh7.5.1 cells by electroporation and HCV core levels of culture fluids were sampled at the time points indicated and core antigen levels were measured. The experiment was done three times with similar results independently. Panel C shows representative date. D. Immunocytochemistry of HCV core. HCV RNA-infected Huh7.5.1 cells using Panel B supernatant that have same amount of HCV core antigen were plated onto 22 mm-round micro cover glasses. Immunocytochemistry was performed 4 days after infection using mouse-anti-core antibody(*green*) and DAPI(*blue*). Numbers at the bottom denote percentages of HCV core-positive cells. E. Time courses of 2b/JFH1 infected cells. JE31F, JE39F, JEC3F RNA-transfected cell culture fluids were used to infect naïve Huh7.5.1 cells in 60 mm-diameter plates at density of 3×10^5 cells per plate. Quantification of HCV core antigen in culture supernatants was carried out at 24 hours, 48 hours, 72 hours and 144 hours after inoculation. The experiment was done three times with similar results independently. Panel E shows representative date. F. Comparison between JFH1 and JEC3F supernatant HCV-RNA titer and core antigen. Four days after JFH1 and JEC3F RNA transfection, culture supernatant was harvested and subjected to both HCV core antigen assay and realtime RT-PCR of HCV-RNA. Assays were done in triplicate and the data are shown as mean ± sd.

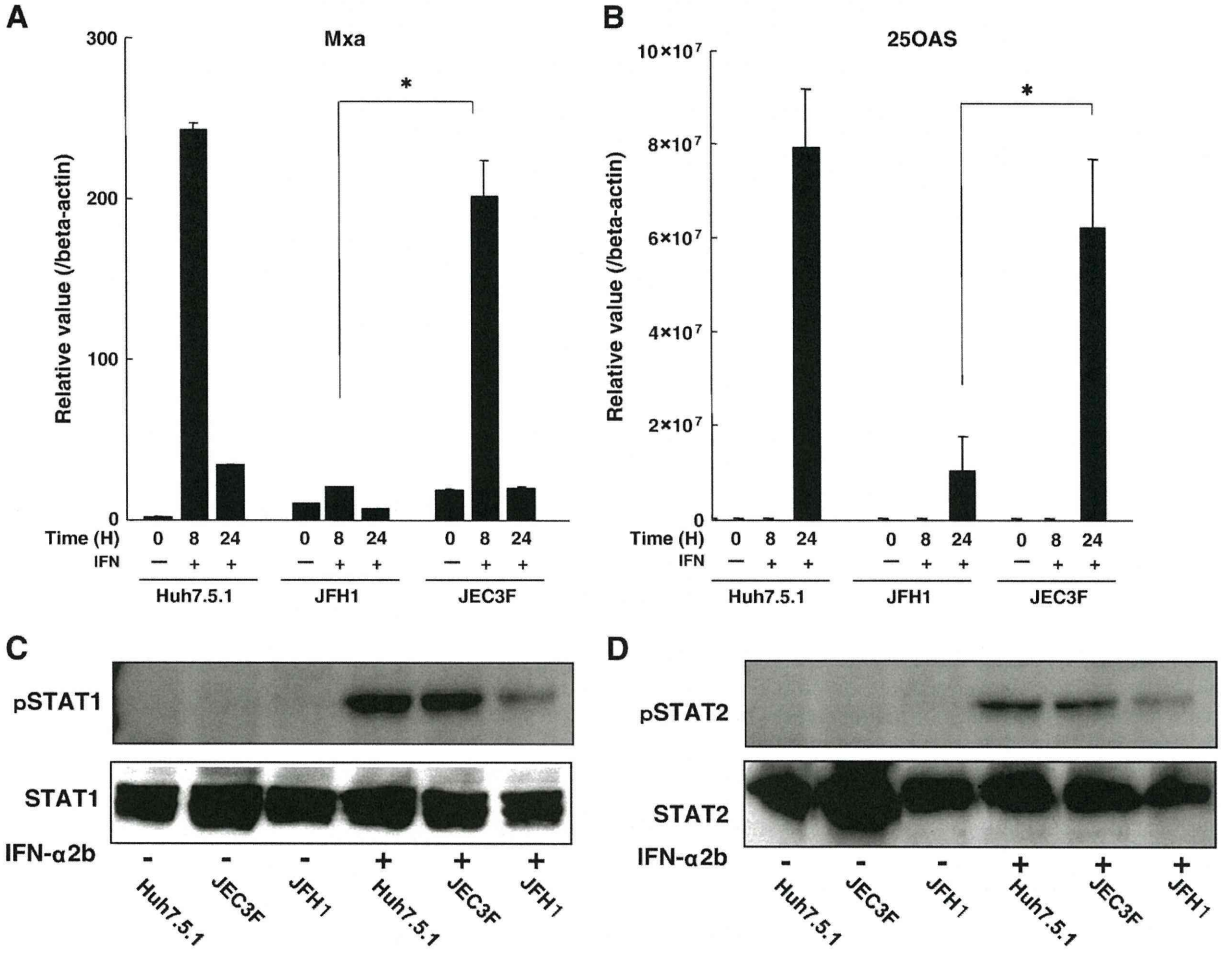


Fig. 3. Induction by interferon of the interferon-inducible genes, MxA (panel A), 25-OAS (panel B) and phosphorylated STAT1 (panel C) and STAT2 (panel D). JEC3F and JFH1 10 μg RNA was transfected into Huh7.5.1 cells. Forty-eight hours after transfection, the cells were treated with 25 U/ml IFN-alpha 2b. Total cellular RNA was isolated before and 8 and 24 hours after IFN treatment. Relative gene expression levels of MxA (panel A) and 25-OAS (panel B) were determined by real-time PCR at the time points indicated. JEC3F and JFH1 RNA and MOCK was transfected into Huh7.5.1 cells. Forty eight hours after transfection, the cells were treated with 25 U/ml IFN-alpha 2b. Total cellular protein was isolated before and 15 minutes after IFN treatment. Ten μg of extracted protein were used for analysis of phosphorylated STAT1, STAT2 protein and STAT1, STAT2 protein as controls. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05.

HCV, on the other hand, counteracts such IFN-mediated antiviral pathways. The NS5A and E2 proteins interfere with the action of IFN by inhibiting the activity of PKR (He and Katze, 2002; Taylor et al., 1999). NS5A also induced expression of IL-8 and attenuated expression of ISGs (Polyak et al., 2001). HCV core protein has been reported to bind the STAT1-SH domain (Lin et al., 2006) or destabilize STAT1 (Lin et al., 2005) to block IFN signaling. It also has been reported that overexpression of core protein upregulated SOCS3 expression (Bode et al., 2003). In this study, we used full-length HCV cell culture and found, *for the first time*, that SOCS3 expression is upregulated differently depending on the genetic sequences of HCV strains and that these differences in SOCS3 expression are associated with sensitivity to IFN. Moreover, overexpression and knock down of SOCS3 expression were closely associated with the IFN sensitivity of the HCV-infected cells. These results indicate that interferon-resistance of HCV-infected cells is directed by overexpression of SOCS3, which may be upregulated by HCV proteins as reported (Bode et al., 2003) (Kawaguchi et al., 2004). A sequence comparison of our HCV2b and JFH1 clones has found 16 amino acid differences. These structural differences of the core protein might affect cellular responses to interferon (*see the* Supplementary Fig. 4).

It has been reported that IL-6 is the principal activator of STAT3 in hepatocytes through binding its receptor (Hanada et al., 2003; Ramadori and Christ, 1999). Furthermore, plasma IL-6 levels are elevated in

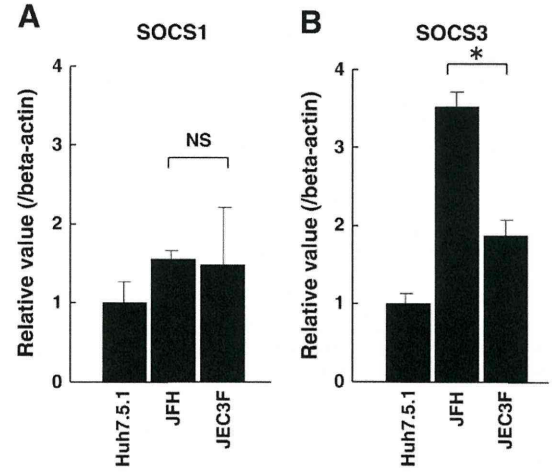


Fig. 4. Expression of SOCS1 mRNA (panel A), SOCS3 mRNA (panel B). Forty-eight hours after transfection of JEC3F, JFH1 10 μg RNA or mock transfection into Huh7.5.1 cells, total RNA and total protein were isolated. Relative gene expression levels of SOCS1 (panel A) and SOCS3 (panel B) and were determined by real time PCR. Values are shown as relative to those of uninfected Huh 751 cells. Assays were done in triplicate and the data are shown as mean ± sd. Asterisks indicate p-values of less than 0.05.