

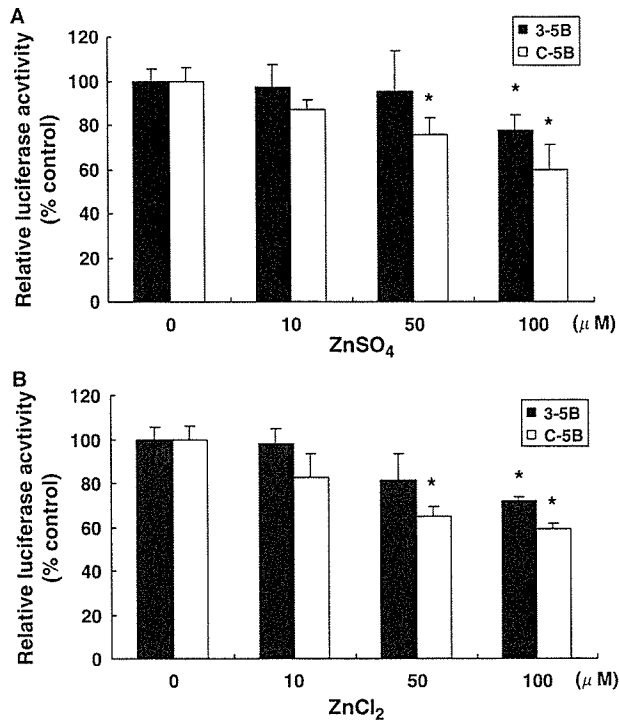
**Fig. 5.** Inhibitory effect of the expression of hepatitis C virus (HCV) proteins. (A) The sO cells were treated for 48 h without reagents (0 μM) as control wells (lane 1), or treated with 100 and 500 μM of iron sulfate (lanes 2 and 3), 100 and 500 μM of iron chloride (lanes 4 and 5), 100 μM zinc sulfate (lane 6), 100 μM zinc chloride (lane 7), or 20 IU/ml interferon-α (IFN-α) (lane 8). The production of NS3 and NS5B protein in sO cells was analyzed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. β-actin was used as an internal control for the amount of protein loaded per lane. (B) The quantification of NS3 and NS5B production by densitometry using Scion Image software in sO normalized for the respective β-actin and the density of non-treated cells was assigned as 100%. These results were based on three separate experiments performed using three separate sets of cells and expressed as the mean ± SD for Western blotting. The asterisk (\*) indicates a significant inhibition of NS3 and NS5B production by reagents ( $P < 0.01$ ). (C) The treatment of O cells with reagents and a Western blot analysis for NS3 and NS5B production were performed as described in (A). (D) The quantification of NS3 and NS5B in O cells was performed as described in (B).

HCV RNA, because sO cells replicate the subgenomic HCV replicon RNA lacking the HCV core to the NS2 region. This hypothesis is supported by the findings that the expression levels of NS3 proteins seemed to decrease more than that of NS5B expression in the O cells-treated zinc salts as shown in Fig. 5C and D.

We showed that iron supplementation inhibited the HCV RNA replication in both systems almost equally. It has recently been reported that iron directly inactivates the RNA-dependent RNA polymerase activity of HCV, which is mediated by the viral NS5B, thus impairing the HCV replication using the subgenomic HCV replicon system (31). The iron compound-induced inhibitory effect of HCV RNA replication on genome-length HCV RNA system may be caused by NS5B, which is a common structure in both systems.

We could not confirm the inhibitory effect of zinc on other genome-length HCV RNA replica-

tion systems because we could not obtain any other cell lines. However, the previous reports that describe iron and not zinc to inhibit significantly the HCV RNA replication in another subgenomic HCV replicon system is consistent with the result of our subgenomic HCV replicon (31), and it is also consistent with our results. In the luciferase reporter system, we confirmed that the inhibitory effect of zinc salts in the genome-length HCV replication system was also observed in a dose-dependent manner. However, 100 μM zinc salts significantly inhibited the luciferase activity in the subgenomic HCV replicon, but less than that in the genome-length HCV RNA replication systems, in contrast to the results of real-time RT-PCR. The luciferase reporter assay system showed reproducible results but the extent of the inhibitory effect between the replication system of subgenomic HCV and that of genome-length HCV RNA was slightly different. In our results of real-time RT-PCR as shown in Fig. 4A



**Fig. 6.** Inhibitory effect of zinc of hepatitis C virus (HCV) RNA replication in ORN/3-5B/KE and ORN/C-5B/KE cells treated with zinc salts on a luciferase reporter assay system. (A) Inhibitory effect of zinc sulfate against HCV RNA replication in ORN/3-5B/KE (black bars) and ORN/C-5B/KE cells (white bars). A *Renilla* Luciferase reporter assay was performed as described in Materials and methods. ORN/3-5B/KE and ORN/C-5B/KE cells were treated for 24 h with zinc sulfate (0, 10, 50, and 100  $\mu$ M), and the relative luciferase activity was calculated. The relative luciferase activity of respective non-treated cells was assigned to be 100%. The data indicate the mean  $\pm$  SD of triplicate findings from three independent experiments. (B) The inhibitory effect of zinc chloride against HCV RNA replication in ORN/3-5B/KE (black bars) and ORN/C-5B/KE cells (white bars). A *Renilla* Luciferase reporter assay was performed as described in (A). The asterisk (\*) indicates a significant inhibition of the *Renilla* luciferase activity by zinc sulfate or zinc chloride ( $P < 0.01$ ).

and B, the HCV replication level in 100  $\mu$ M zinc salts tended to be slightly lower than in either the control or 50  $\mu$ M zinc salts. Therefore, this is probably due to a difference in the detection sensitivity of the real-time RT-PCR and luciferase reporter assay systems.

The subgenomic HCV replicon and the replicable genome-length HCV RNA in this study were highly sensitive to IFN- $\alpha$  as described previously (33, 34). Moreover, clinically, zinc supplementation increased the therapeutic response of IFN- $\alpha$  for intractable chronic hepatitis C (27, 28). However, zinc supplementation did not show the additional or synergistic inhibitory effect of IFN- $\alpha$  in a genome-length HCV RNA replication system (data not shown). The inhibitory effect of zinc on the replication of both replicon cells may be masked with that of IFN- $\alpha$ , because the

inhibitory effect of IFN- $\alpha$  on the replication of both replicon cells is much more effective than that of zinc.

To date, four genome-length HCV RNA replication systems, using N, Con-1, H77, and O strains, have been reported (21–24). Genome-length HCV RNA replication, including the structural region of HCV RNA, closely mimics the *in vivo* situation within an HCV-infected hepatocyte. In this study, different degrees of chemical sensitivity were observed between the subgenomic HCV replicon system and genome-length HCV RNA replication system. This significant difference suggests that a useful investigation may have been overlooked in the subgenomic HCV replicon systems. Previous studies using subgenomic HCV replicon systems should therefore be re-examined using genome-length HCV RNA replication systems.

In conclusion, our study suggests that zinc may play an important role as a negative regulator of HCV replication in genome-length HCV RNA-replicating cells. Zinc supplementation appears to be a novel approach in the development of future strategies for the treatment of chronic hepatitis C. The mechanisms underlying the inhibitory effect of zinc on virus replication are presently being investigated in our laboratory.

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# Different Anti-HCV Profiles of Statins and Their Potential for Combination Therapy With Interferon

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We recently developed a genome-length hepatitis C virus (HCV) RNA replication system (OR6) with luciferase as a reporter. The OR6 assay system has enabled prompt and precise quantification of HCV RNA replication. Pegylated interferon (IFN) and ribavirin combination therapy is the world standard for chronic hepatitis C, but its effectiveness is limited to about 55% of patients. Newer therapeutic approaches are needed. In the present study, we used the OR6 assay system to evaluate the anti-HCV activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, called statins, and their effects in combination with IFN- $\alpha$ . Five types of statins (atorvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin) were examined for their anti-HCV activities. Fluvastatin exhibited the strongest anti-HCV activity (IC<sub>50</sub>: 0.9  $\mu$ mol/L), whereas atorvastatin and simvastatin showed moderate inhibitory effects. However, lovastatin, reported recently as an inhibitor of HCV replication, was shown to exhibit the weakest anti-HCV activity. The anti-HCV activities of statins were reversed by the addition of mevalonate or geranylgeraniol. Surprisingly, however, pravastatin exhibited no anti-HCV activity, although it worked as an inhibitor for HMG-CoA reductase. The combination of IFN and the statins (except for pravastatin) exhibited strong inhibitory effects on HCV RNA replication. In combination with IFN, fluvastatin also exhibited a synergistic inhibitory effect. **In conclusion**, statins, especially fluvastatin, could be potentially useful as new anti-HCV reagents in combination with IFN. (HEPATOLOGY 2006;44:117-125.)

Persistent hepatitis C virus (HCV) infection causes liver fibrosis and hepatocellular carcinoma. Approximately 170 million people worldwide are infected with HCV. The combination of pegylated

interferon (IFN) with ribavirin is the current standard therapy for chronic hepatitis C (CHC) and yields a sustained virological response (SVR) rate of about 55%.<sup>1</sup> This means that about 45% of patients with CHC are still threatened by the progression of the disease to cirrhosis and hepatocellular carcinoma. Until 1999, when Lohmann et al. developed the subgenomic replicon of HCV, it was difficult to screen anti-HCV reagents.<sup>2</sup> Many improvements followed that breakthrough, such as a genome-length HCV RNA replication system<sup>3,4</sup> and a subgenomic replicon with a reporter assay system<sup>5</sup>; more recently, Wakita et al. used a genotype 2a strain, JFH1, to produce the infectious virus in cell culture.<sup>6-8</sup>

Genotype 1 is the major genotype of HCV found in Japan, the United States, and many other countries. Unfortunately, the SVR rate after combination therapy of pegylated IFN with ribavirin is less than 50% for this genotype. To find a more effective therapy especially for CHC patients with genotype 1, we recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6), which has been an effective screening tool.<sup>9,10</sup>

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are in wide use for the treatment of hypercholesterolemia. Recently, it

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*Abbreviations:* HCV, hepatitis C virus; IFN, interferon; CHC, chronic hepatitis C; SVR, sustained virological response; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; 2'-5'-OAS1, 2'-5'-oligoadenylate synthetase 1; LOV, lovastatin; ATV, atorvastatin; PRV, pravastatin; SMV, simvastatin; FLV, fluvastatin; RL, renilla luciferase; LST-1, human liver-specific organic anion transporter-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; AMPH-B, amphotericin B; RT-PCR, reverse-transcription polymerase chain reaction.

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was reported that lovastatin (LOV) inhibited HCV RNA replication.<sup>11,12</sup> These reports suggested the anti-HCV activity of LOV resulted from inhibition of the geranylgeranylation of cellular proteins rather than the inhibition of cholesterol synthesis.<sup>11,12</sup> More recently, FBL2 has been reported to be a host target protein for geranylgeranylation, which is responsible for HCV replication.<sup>13</sup> Although several types of statins are used clinically to lower cholesterol, thus far only LOV has been tested for anti-HCV activity. In the present study, we used the OR6 assay system to test the anti-HCV activity of five statins: atorvastatin (ATV), fluvastatin (FLV), pravastatin (PRV), simvastatin (SMV), and LOV. We found that ATV, FLV, and SMV exhibited stronger anti-HCV activity than that previously reported for LOV and that PRV exhibited no anti-HCV activity, although it worked as an inhibitor for HMG-CoA reductase. Because FLV showed the strongest anti-HCV activity, we also examined the effect of the combination of IFN- $\alpha$  and FLV on HCV RNA replication and found a synergistic inhibitory effect of IFN- $\alpha$  and FLV on HCV RNA replication.

## Materials and Methods

**Cell Cultures.** OR6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and G418 (300  $\mu$ g/mL; Geneticin, Invitrogen) and passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA was eliminated by IFN- $\alpha$  treatment (500 IU/mL for 2 weeks) without G418, as previously described.<sup>9</sup> HepG2 and PH5CH8 (human immortalized hepatocytes) cells were cultured as previously described.<sup>14,15</sup>

**Luciferase Reporter Assay.** For the renilla luciferase (RL) assay,  $2 \times 10^4$  OR6 cells were plated in 24-well plates at least in triplicate for each assay and were cultured for 24 hours. The cells were treated with statins for 72 hours and were harvested with Renilla lysis reagent (Promega) and subjected to the RL assay according to the manufacturer's protocol.

**Reagents.** FLV, LOV, and PRV were purchased from Calbiochem. ATV and SMV were purchased from Astellas Pharma Inc. and Banyu Pharmaceutical Co. Ltd., respectively. Mevalonate, geranylgeraniol, and geranylgeranyl pyrophosphate were purchased from Sigma.

**Reverse-Transcriptase Polymerase Chain Reaction.** Total RNA from the cultured cells was extracted with the RNeasy Mini Kit (Qiagen). Reverse-transcriptase polymerase chain reaction (RT-PCR) for HMG-CoA reductase, human liver-specific organic anion transporter (LST-1), 2'-5'-oligoadenylate synthetase 1 (2'-5'-

OAS1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by a method described previously.<sup>16</sup> Briefly, using cellular total RNA (2  $\mu$ g), cDNA was synthesized using Superscript II with oligo dT primer. One-tenth of the synthesized cDNA was subjected to polymerase chain reaction (PCR) with the following primer pairs: HMG-CoA reductase, 5'-ATGCCATCCCTGTTG-GAGTG-3' and 5'-TGTTTCATCCCCATGGCATCCC-3'; LST-1, 5'-TGGCACACGTGGGTCATGTAGG-3' and 5'-CACTATCTGCCCCAGCAGAAGG-3'; 2'-5'-OAS1, 5'-AGTACCTGAGAAGGCAGCTCACGA-3' and 5'-ACTGGCATTTCAGAGGATGGTGCCAG-3'; and GAPDH, 5'-GACTCATGACCACAGTCCATGC-3', and 5'-GAGGAGACCACCTGGTGCTCAG-3'.

**Western Blot Analysis.** Preparation of the cell lysate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting were performed as previously described.<sup>17</sup> The antibodies used in this study were those against Core (Institute of Immunology, Tokyo), NS3 (Novocastra Laboratories, Newcastle upon Tyne, UK), NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science),  $\beta$ -actin (Sigma, St. Louis, MO), STAT1 (BD Transduction Laboratories, San Diego, CA), and phospho-STAT1 (Y701; Cell Signaling Technology, Beverly, MA). Immunocomplexes on the membranes were detected by the enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA).

**Plasmid Construction.** The plasmids pORN/C-5B/KE (Fig. 1A) and pHCV-O were described previously.<sup>9</sup> To construct the pEMCV-RL, two fragments, the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) and the RL gene, were amplified by PCR from pORN/C-5B/KE using the following primer pairs: EMCV IRES, 5'-CGGGATCCGCGGGACTCGG-GGGTTCG-3' and 5'-CCGCTCGAGGGTATTAT-CGTGTTTTTCAAAGG-3'; and RL, 5'-CCGCTC-GAGATGGCTTCCAAGGTGTACGACC-3', and 5'-GCTCTAGACTAGACGTTGATCCTGGCGC-3'. The two fragments were ligated into the *Bam*HI and *Xba*I sites in pcDNA 3.1/Zeo (Invitrogen).

**Statistical Analysis.** Differences in anti-HCV activity between statins were tested using the Student *t* test. *P* values < .05 were considered statistically significant.

## Results

**Inhibition of HCV RNA Replication by Statins.** We recently developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as a reporter.<sup>9,10</sup> This OR6 reporter assay system has enabled prompt and precise quantification of HCV

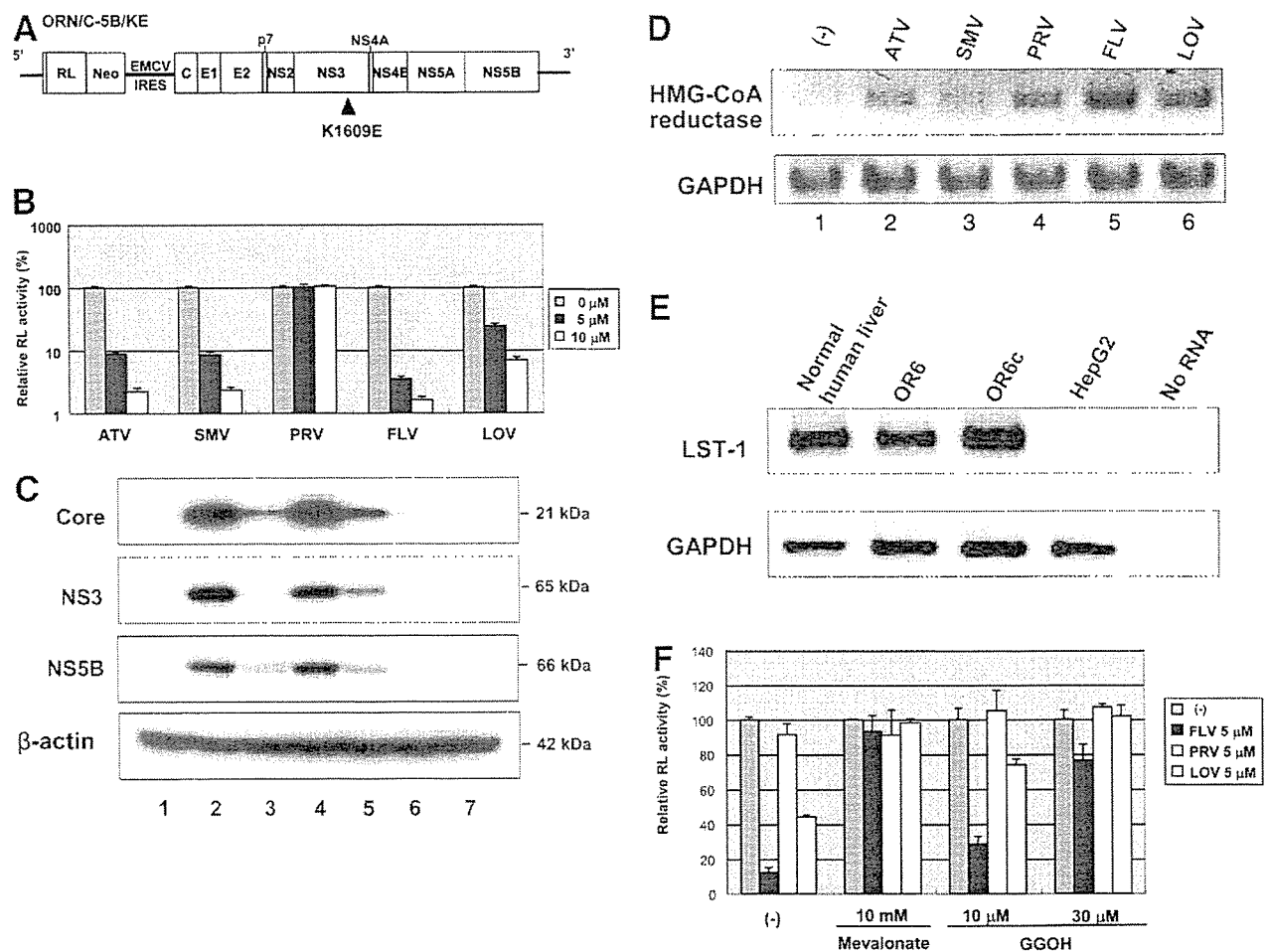


Fig. 1. Different inhibitory effects of various statins on HCV RNA replication in OR6 cells. (A) Schematic gene organization of genome-length HCV RNA encoding the *RL* gene, ORN/C-5B/KE, replicating in OR6 cells. *RL* is expressed as a fusion protein with Neo. The position of an adaptive mutation, K1609E, is indicated by a triangle. (B) Reporter assay on statin sensitivity of HCV RNA replication. OR6 cells were treated with ATV, SMV, PRV, FLV, and LOV (0, 5, and 10  $\mu\text{mol/L}$  each). After 72 hours of treatment, the *RL* assay was performed as described in the Materials and Methods section. Shown here is the relative *RL* activity (%) calculated when the *RL* activity of untreated cells was assigned as 100%. The data indicate means  $\pm$  SDs of triplicates from three independent experiments. (C) Western blot analysis of statin sensitivity of HCV RNA replication: lane 1, cured OR6 cells (OR6c) used as a negative control; lane 2, untreated OR6 cells; lane 3, OR6 cells treated with IFN- $\alpha$  (10 IU/mL); lane 4, OR6 cells treated with PRV (5  $\mu\text{mol/L}$ ); lane 5, OR6 cells treated with LOV (5  $\mu\text{mol/L}$ ); lane 6, OR6 cells treated with FLV (5  $\mu\text{mol/L}$ ); lane 7, OR6 cells treated with FLV (10  $\mu\text{mol/L}$ ). After 96 hours of treatment, the production of Core, NS3, and NS5B was analyzed by immunoblotting using anti-Core, anti-NS3, and anti-NS5B antibodies, respectively.  $\beta$ -actin was used as a control for the amount of protein loaded per lane. (D) Feedback induction of HMG-CoA reductase after treatment of statins: lane 1, untreated OR6c cells; lane 2, OR6c cells treated with ATV (10  $\mu\text{mol/L}$ ); lane 3, OR6c cells treated with SMV (10  $\mu\text{mol/L}$ ); lane 4, OR6c cells treated with PRV (10  $\mu\text{mol/L}$ ); lane 5, OR6c cells treated with FLV (10  $\mu\text{mol/L}$ ); lane 6, OR6c cells treated with LOV (10  $\mu\text{mol/L}$ ). After 24 hours of treatment, RT-PCR for HMG-CoA reductase was performed as described in the Materials and Methods section. RT-PCR for GAPDH was performed as an internal control. RT-PCR products (376 bp for HMG-CoA reductase and 334 bp for GAPDH) were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (E) RT-PCR analysis of LST-1 mRNA. Total RNA was extracted from OR6, OR6c, and HepG2 cells and subjected to RT-PCR analysis with primer sets for LST-1 (241 bp) and GAPDH (334 bp) as described in (D). Normal human liver total RNA (Clontech) was used as a positive control for LST-1. (F) Mevalonate and geranylgeraniol restore HCV RNA replication in statin-treated cells. OR6 cells were treated with FLV, PRV, and LOV (5  $\mu\text{mol/L}$  each) alone or in the presence of mevalonate (10 mmol/L) or geranylgeraniol (GGOH, 10 and 30  $\mu\text{mol/L}$ ). After 72 hours of treatment, the *RL* assay was performed as described in (B).

RNA replication. Therefore, in the present study, we examined whether several types of statins currently used in clinical therapy exhibit anti-HCV activity, as has already been reported for LOV.<sup>11,12</sup> This time, the antiviral activities of five statins—ATV, FLV, LOV, PRV, and SMV—were tested using the OR6 assay system (Fig. 1A). The

results revealed that ATV, FLV, and SMV exhibited stronger anti-HCV activity than did LOV and that FLV exhibited the strongest anti-HCV activity among the statins tested (Fig. 1B). Surprisingly, however, PRV had no inhibitory effect on HCV RNA replication (Fig. 1B). Similar results were obtained from the analysis of the ex-

pression levels of HCV proteins (Fig. 1C). The anti-HCV activity of 5  $\mu\text{mol/L}$  FLV was compatible with that of 10 IU/mL IFN- $\alpha$  (Fig. 1C).

To exclude the possibility that only PRV was unable to inhibit HMG-CoA reductase, we examined the expression of HMG-CoA reductase in statin-treated OR6c cells by RT-PCR, because HMG-CoA reductase was known to show positive feedback when statins were active in the cells.<sup>18</sup> OR6c cells were treated with statins in the same way as were the OR6 cells used to measure the anti-HCV activity of statins, except that sampling for RT-PCR was performed after 24 hours of statin treatment. All statins, including PRV, enhanced expression of the HMG-CoA reductase gene (Fig. 1D). Although PRV is hydrophilic and does not cross cellular membranes passively, it has been reported that a human liver-specific organic anion transporter, LST-1, mediates the uptake of PRV in human hepatocytes but not in HepG2 cells, which showed very low PRV uptake.<sup>19,20</sup> Therefore, we examined the expression levels of LST-1 in OR6 and OR6c cells using an RT-PCR method. OR6 and OR6c cells expressed LST-1 at levels equivalent to that in normal human liver, confirming that LST-1 was not expressed in the HepG2 cells (Fig. 1E). These findings suggest that PRV is taken up actively in OR6 and OR6c cells. In summary, these results indicate all statins tested inhibit HMG-CoA reductase and suggest the anti-HCV action of statins is not a result of direct inhibition of HMG-CoA reductase.

Regarding the mechanism underlying the anti-HCV action of statins, it has thus far been reported that the inhibitory effect of LOV can be overcome by the addition of mevalonate (the product of the HMG-CoA reductase reaction) or geranylgeraniol (a donor of prenyl groups for protein geranylgeranyl transferase reaction).<sup>11,12</sup> These observations suggest that some geranylgeranylated proteins are required for HCV RNA replication and that LOV blocks HCV RNA replication by depleting endogenous geranylgeranyl pyrophosphate (the mevalonate-derived donor of protein geranylgeranylation). To evaluate this mechanism, we examined the effects of mevalonate and geranylgeraniol on the anti-HCV activities of the statins used in this study. OR6 cells were treated with 5  $\mu\text{mol/L}$  FLV, PRV, or LOV alone or in the presence of mevalonate (10 mmol/L) or geranylgeraniol (10 or 30  $\mu\text{mol/L}$ ). Mevalonate and geranylgeraniol restored HCV RNA replication in the statin-treated cells, although 10  $\mu\text{mol/L}$  geranylgeraniol exhibited partial restoration (Fig. 1F). In addition, we observed that the anti-HCV activities of the statins could be blocked by the addition of geranylgeranyl pyrophosphate (20  $\mu\text{mol/L}$ ) in the OR6 cells (data not shown), indicating geranylgeranyl pyrophosphate is also taken up in OR6 cells. These findings sup-

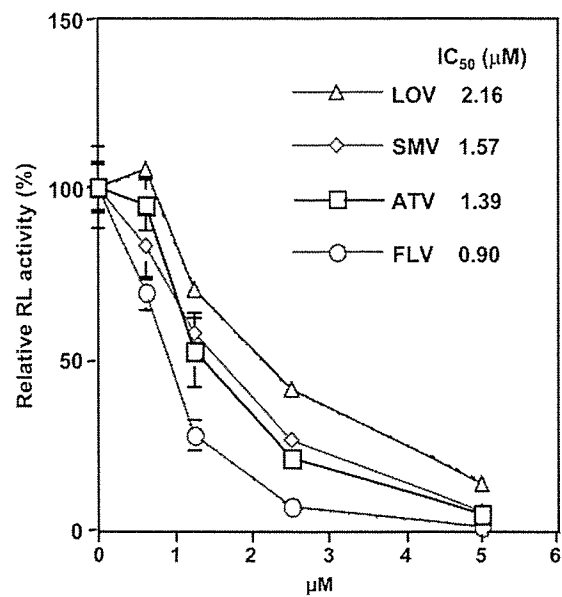


Fig. 2. Dose-dependent inhibition of HCV RNA replication by statins. OR6 cells were treated with LOV, SMV, ATV, and FLV at concentrations of 0.625, 1.25, 2.5, and 5  $\mu\text{mol/L}$ . After 72 hours of treatment, the RL assay was performed, and RL activity was calculated as shown in Fig. 1B.

port two previous reports<sup>11,12</sup> that found the inhibition of HCV RNA replication by statins was not correlated with their cholesterol-lowering activities, although the reason for the lack of anti-HCV activity by PRV remains vague.

**Anti-HCV Activity of FLV Significantly Stronger Than Those of Other Statins.** From the dose-response curves after 72 hours of treatment with the statins, the concentrations of FLV, ATV, SMV, and LOV required for a 50% reduction in RL activity ( $\text{IC}_{50}$ ) were calculated to be 0.90, 1.39, 1.57, and 2.16  $\mu\text{mol/L}$ , respectively (Fig. 2). Consistent with the results shown in Fig. 1, the anti-HCV activity of FLV ( $P < .01$  at 0.625–5  $\mu\text{mol/L}$ ), ATV ( $P < .05$  at 1.25  $\mu\text{mol/L}$ ;  $P < .01$  at 2.5 and 5  $\mu\text{mol/L}$ ), and SMV ( $P < .05$  at 0.625 and 1.25  $\mu\text{mol/L}$ ;  $P < .01$  at 2.5 and 5  $\mu\text{mol/L}$ ) was significantly stronger than that previously reported for LOV. In addition, the anti-HCV activity of FLV was significantly stronger than those of SMV ( $P < .01$  at 1.25–5  $\mu\text{mol/L}$ ) and ATV ( $P < .05$  at 1.25  $\mu\text{mol/L}$ ;  $P < .01$  at 2.5 and 5  $\mu\text{mol/L}$ ).

**Anti-HCV Activity of Statins Not Due to Their Cytotoxicity.** Since it has been reported that the proliferation of the HCV replicon is dependent on host-cell growth,<sup>21</sup> it remained to be clarified whether the inhibitory effects of statins on HCV RNA replication were caused by their cytotoxicity. To examine this possibility, we investigated the cytotoxic effects of statins on OR6 cells. A comparison of cell viability in the untreated cells with that in the cells treated with each statin (5  $\mu\text{mol/L}$  each) showed no significant decrease in the number of

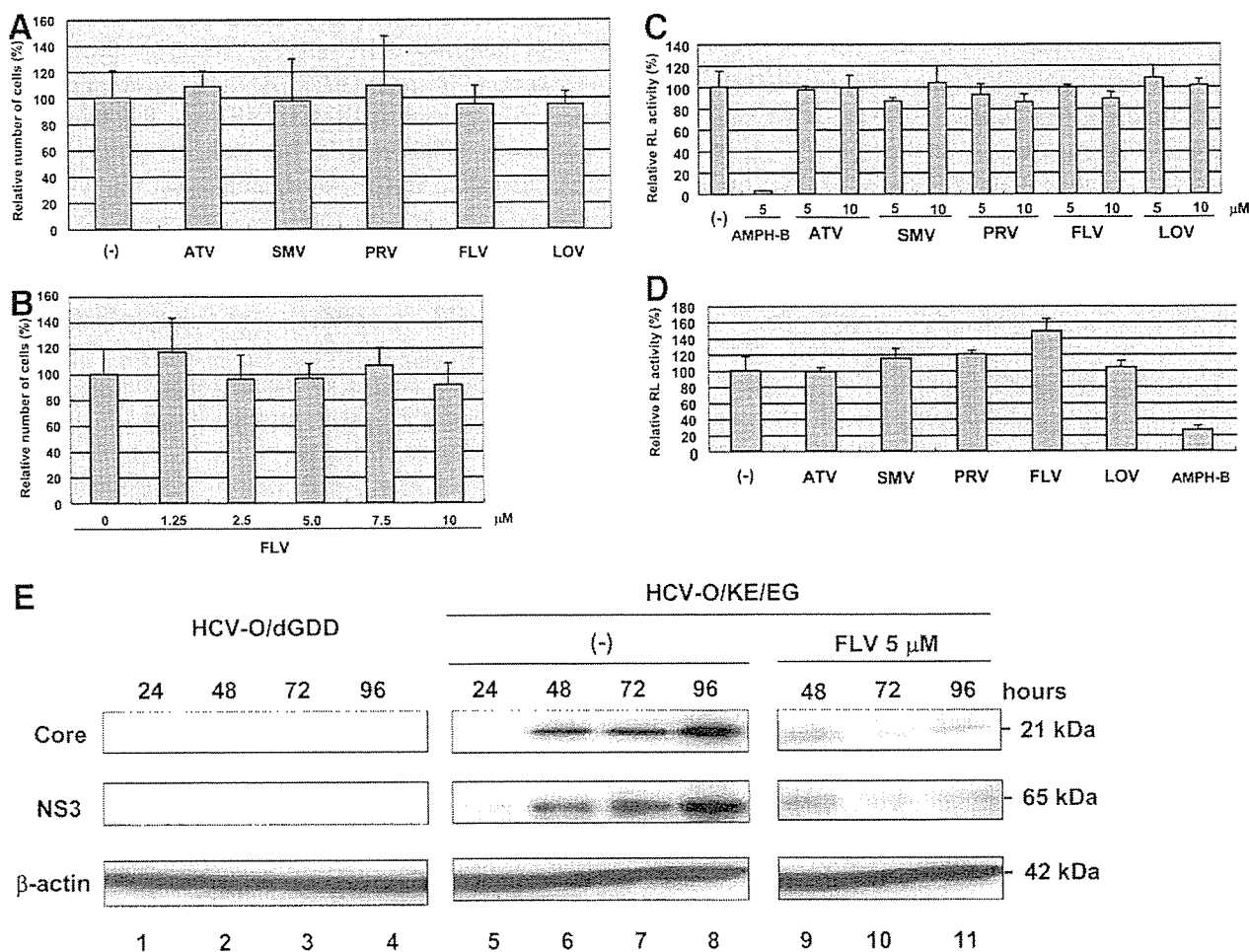


Fig. 3. Anti-HCV activity of the statins was not a result of inhibition of cell growth, RL activity, or EMCV IRES activity. (A) Cell viability after treatment with the statins. OR6 cells were cultured in the absence and in the presence of ATV, SMV, PRV, FLV, and LOV (5  $\mu$ mol/L each), and then the number of viable cells was counted after trypan blue dye treatment. Shown here is the relative cell number (%) calculated when the cell number of untreated cells was assigned as 100%. The data indicate means  $\pm$  SDs of triplicates from two independent experiments. (B) Cell viability after treatment with FLV. OR6 cells were cultured in the absence and in the presence of FLV (1.25, 2.5, 5, 7.5, and 10  $\mu$ mol/L) for 72 hours, and then the number of viable cells was counted as described in (A). (C) No inhibition of RL activity by the statins. ATV, SMV, PRV, FLV, and LOV (5 or 10  $\mu$ mol/L each) were directly added to the cell lysates from OR6 cells, and then the RL assay was performed. The relative RL activity was calculated as shown in Fig. 1B. AMPH-B (5  $\mu$ mol/L) was used as a control reagent, which directly affected RL activity. (D) No inhibition of EMCV IRES activity by the statins. After pEMCV-RL was introduced into the OR6c cells, the cells were treated with ATV, SMV, PRV, FLV, and LOV (5  $\mu$ mol/L each). After 72 hours of treatment, the RL assay was performed. Relative RL activity was calculated as shown in Fig. 1B. AMPH-B (2.5  $\mu$ mol/L) was used as a control reagent, which directly affected RL activity. (E) Replication of authentic HCV RNA prevented by the statins. Authentic HCV-O RNA, HCV-O/KE/EG RNA, was introduced into the OR6c cells by electroporation as described previously.<sup>3</sup> After 24 (lane 5), 48 (lane 6), 72 (lane 7), and 96 (lane 8) hours of electroporation, production of Core and NS3 was analyzed by immunoblotting using anti-Core and anti-NS3 antibodies. Production of Core and NS3 after 24 (lane 1), 48 (lane 2), 72 (lane 3), and 96 (lane 4) hours of electroporation of HCV-O/dGDD RNA (negative control) was also analyzed. After 24 hours of electroporation of HCV-O/KE/EG RNA, the cells were treated with FLV (5  $\mu$ mol/L), and then production of Core and NS3 was analyzed after 48 (lane 9), 72 (lane 10), and 96 (lane 11) hours of electroporation.  $\beta$ -actin was used as a control for the amount of protein loaded per lane.

cells following treatment with statins (Fig. 3A). However, it was recently reported that statins inhibited the proliferation of hepatocellular carcinoma cell lines (HuH-7 and HepG2) by inducing apoptosis and G1/S cell-cycle arrest.<sup>22</sup> Because that study found the IC<sub>50</sub> of FLV in HuH-7 cells to be 10  $\pm$  3  $\mu$ mol/L, we further examined the effects of FLV on the proliferation of OR6 cells by varying the concentration (up to 10  $\mu$ mol/L) of FLV.

FLV (at least at concentrations  $\leq$  10  $\mu$ mol/L) did not inhibit the proliferation of OR6 cells (Fig. 3B), suggesting that FLV does not induce apoptosis or G1/S cell-cycle arrest in OR6 cells, although the OR6 cell line is a HuH-7-derived cell line.<sup>10</sup> In summary, these results indicate that none of the statins showed any cytotoxicity to the OR6 cells at the concentrations used in our assay system. This suggests the statins possess the



ability to inhibit replication of HCV RNA via specific antiviral mechanism(s).

**Anti-HCV Activity of Statins Not Due to Inhibition of RL Activity.** We clearly showed that the anti-HCV activities of statins were not due to their cytotoxicity. However, it remained to be clarified whether the statins used in this study would directly inhibit RL activity, because we recently found that two antifungal compounds, amphotericin B (AMPH-B) and nystatin, drastically inhibited RL activity (Ikeda et al., unpublished data). To examine this possibility, we investigated the effects of statins on RL activity. The addition of the statins to cell lysates prepared from OR6 cells revealed that none of the statins (up to 10  $\mu\text{mol/L}$ ) tested exhibited any inhibitory effect on RL activity, although AMPH-B extensively inhibited RL activity (Fig. 3C). This result excludes the possibility that the statins directly inhibit RL activity.

**Anti-HCV Activity of Statins Not Due to Inhibition of EMCV IRES Activity.** To further exclude the possibility that the anti-HCV activities of statins were a result of the artificial assay system, we next tested the possibility that the statins inhibit EMCV IRES activity, because Core-NS5B is translated in an EMCV-IRES-dependent manner in OR6 cells. The plasmid encoding RL driven by EMCV IRES was transfected into the OR6c cells, and 24 hours after transfection the cells were treated with each statin (5  $\mu\text{mol/L}$  each) for 72 hours. The results revealed none of the statins exhibited any inhibitory effect, although AMPH-B drastically inhibited RL activity again (Fig. 3D). These data suggest the statins had no effect on exogenous genes EMCV IRES and RL introduced into HCV RNA.

**Statins Prevent Replication of Authentic HCV RNA.** To obtain further evidence that the statins prevent HCV RNA replication, we prepared authentic HCV-O-derived genome-length HCV RNA possessing two adaptive mutations (HCV-O/KE/EG). One adaptive mutation, K1609E in NS3, was previously described,<sup>9</sup> and the other, E1202G in NS3, was found in OA cells harboring genome-length HCV-O RNA (Abe et al., in preparation). The combination of these two mutations markedly enhanced the efficiency of HCV RNA replication, and HCV proteins were continuously detected for at least 8 weeks (Abe et al., in preparation). HCV-O/dGDD, from which the GDD motif in NS5B polymerase was deleted, was used as a control. HCV-O/KE/EG and HCV-O/dGDD RNAs were transfected into OR6c cells, and the production of HCV proteins was monitored for 96 hours. The Core and NS3 in the OR6c cells transfected with HCV-O/dGDD RNA were not detected even 96 hours after transfection. However, the Core and NS3 in the OR6c cells transfected with HCV-O/KE/EG RNA

were detected 24 hours after transfection, and their expression increased with time (Fig. 3E). This observation suggests that HCV-O/KE/EG RNA, without exogenous genetic factors such as EMCV IRES and RL, efficiently replicates in OR6c cells. Using such a transient HCV RNA replication system, we demonstrated that the production of Core and NS3 was markedly prevented when the OR6c cells transfected with HCV-O/KE/EG RNA were treated with FLV (5  $\mu\text{mol/L}$ ) at 24 hours after transfection (Fig. 3E). In summary, our results indicate that the statins prevent HCV RNA replication and that their inhibitory effects are not a result of the inhibitory effect toward the exogenous genes introduced into ORN/C-5B RNA replicating in OR6 cells.

**Combination of a Statin with IFN Efficiently Enhances Anti-HCV Activity of IFN.** IFN is the world standard of therapy for CHC, and currently its best partner is ribavirin. Because we found the statins efficiently inhibited HCV activity, we expected the statins to be candidates for use in combination with IFN. To address this point, we examined the inhibitory effects of combinations of IFN- $\alpha$  (0, 2, 4, and 8 IU/mL) and the statins (5  $\mu\text{mol/L}$  each) using the OR6 assay system. As expected, ATV, SMV, FLV, and LOV markedly enhanced the anti-HCV effect of IFN- $\alpha$ , although PRV did not (Fig. 4A). In combination with IFN- $\alpha$ , FLV again was the statin that had the strongest effect. The results indicated that cotreatment was more effective than treatment with IFN- $\alpha$  alone. We thought the mechanism underlying this phenomenon might be statin-induced enhancement of the type I IFN signaling pathway. To examine this possibility, we investigated the phosphorylation status of STAT1 after statin treatment. The results revealed that FLV did not cause phosphorylation of STAT1 in OR6c or OR6 cells, although IFN- $\alpha$  did so efficiently in both types of cells (Fig. 4B). In addition, we confirmed that phosphorylation of STAT2 and STAT3 in both cell types was also not induced by FLV treatment (data not shown). Furthermore, we confirmed that FLV did not affect expression of 2'-5'-OAS1 mRNA and that the expression level induced by IFN- $\alpha$  treatment was not affected by treatment with FLV (Fig. 4C). PRV, which showed no anti-HCV activity, also had no effect on the type I IFN signaling pathway. In summary, these results indicate the statin-induced enhancement of the anti-HCV action of IFN- $\alpha$  is not a result of induction of the type I IFN signaling pathway.

**Cotreatment of IFN- $\alpha$  and FLV Exhibits Synergistic Inhibitory Effects on HCV RNA Replication.** We showed that FLV was the statin tested that exhibited the strongest anti-HCV activity, not only alone, but also in combination with IFN- $\alpha$ . Therefore, we focused on the anti-HCV activity of FLV, minutely examining the in-

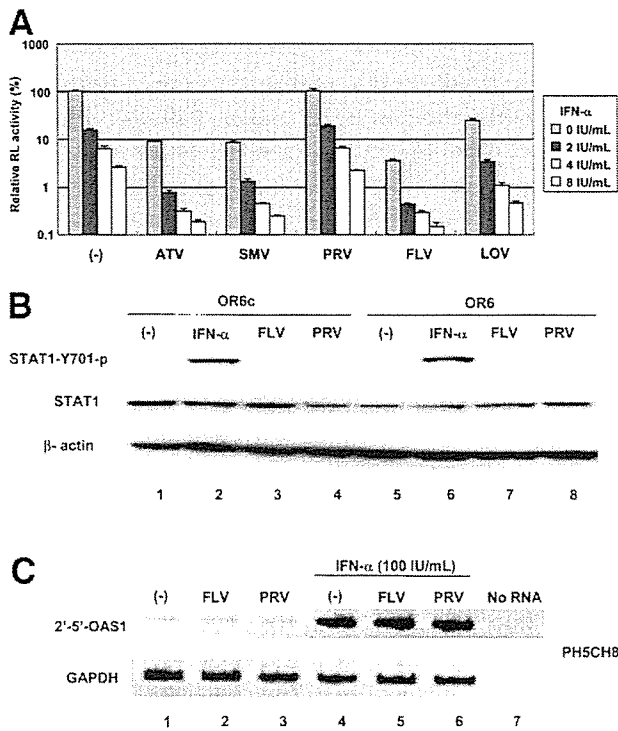


Fig. 4. Statins enhance inhibition of HCV RNA replication because of IFN- $\alpha$ . (A) Effects of statins on the anti-HCV activity of IFN- $\alpha$ . OR6 cells were cotreated with IFN- $\alpha$  (0, 2, 4, and 8 IU/mL) and ATV, SMV, PRV, FLV, or LOV (5  $\mu$ mol/L each). The RL assay was performed, and the relative RL activity was calculated as shown in Fig. 1B. (B) No enhancement of type I IFN signaling by the statins. OR6c or OR6 cells were cultured for 2 hours in the absence and in the presence of IFN- $\alpha$  (100 IU/mL), FLV (10  $\mu$ mol/L), and PRV (10  $\mu$ mol/L), and the cells were subjected to Western blot analysis of STAT1 and its phosphorylation status.  $\beta$ -actin was used as a control for the amount of protein loaded per lane. (C) No induction of the IFN-inducible gene by the statins. PH5CH8 cells were untreated or were treated with FLV (10  $\mu$ mol/L) and PRV (10  $\mu$ mol/L) for 2 hours, and then total RNA extracted from the cells was subjected to RT-PCR for 2'-5'-OAS1 (25 cycles). The PH5CH8 cells were treated for 9 hours with IFN- $\alpha$  (100 IU/mL) alone or in combination with FLV (10  $\mu$ mol/L) or PRV (10  $\mu$ mol/L), and then RT-PCR for 2'-5'-OAS1 was performed. The RT-PCR products (358 bp for 2'-5'-OAS1 and 334 bp for GAPDH) were detected, as shown in Fig. 1D.

hibitory effects of the combination of IFN- $\alpha$  and FLV on genome-length HCV RNA replication. A dose-response curve of FLV was obtained for fixed concentrations of IFN- $\alpha$  of 0, 4, 8, 16, 32, and 64 IU/mL. The results revealed the curves shifted to shift markedly to the bottom as the concentration of IFN- $\alpha$  increased (Fig. 5A), indicating that cotreatment was drastically more effective than treatment with IFN- $\alpha$  alone. Furthermore, we observed that RL activity decreased to almost the background level in the OR6 reporter assay when OR6 cells were cotreated with 64 IU/mL of IFN- $\alpha$  and FLV at concentrations above 1.25  $\mu$ mol/L (Fig. 5A). Because the data in Fig. 5A indicate the possibility of a synergistic effect of the combination of IFN- $\alpha$  and FLV, we exam-

ined whether the effect of this combination is synergistic or additive effect using an isobologram method.<sup>23,24</sup> The anti-HCV activities of IFN- $\alpha$  and FLV in combination were evaluated by the OR6 reporter assay. Dose-response inhibition of HCV RNA replication was evaluated for varying IFN- $\alpha$  concentrations (0-8 IU/mL) in the presence of various doses of FLV (0-7.5  $\mu$ mol/L). The IC<sub>90</sub> values of IFN- $\alpha$  and FLV were 4.0 IU/mL and 6.7  $\mu$ mol/L, respectively. These data were used to generate isoboles, which demonstrated 90% inhibition of HCV RNA replication, and the synergistic anti-HCV action of IFN- $\alpha$  and FLV was revealed by the curvilinear plots of the 90% isoboles (Fig. 5B). In conclusion, we clearly demonstrated that combination treatment of IFN- $\alpha$  and FLV was an overwhelmingly more effective treatment,

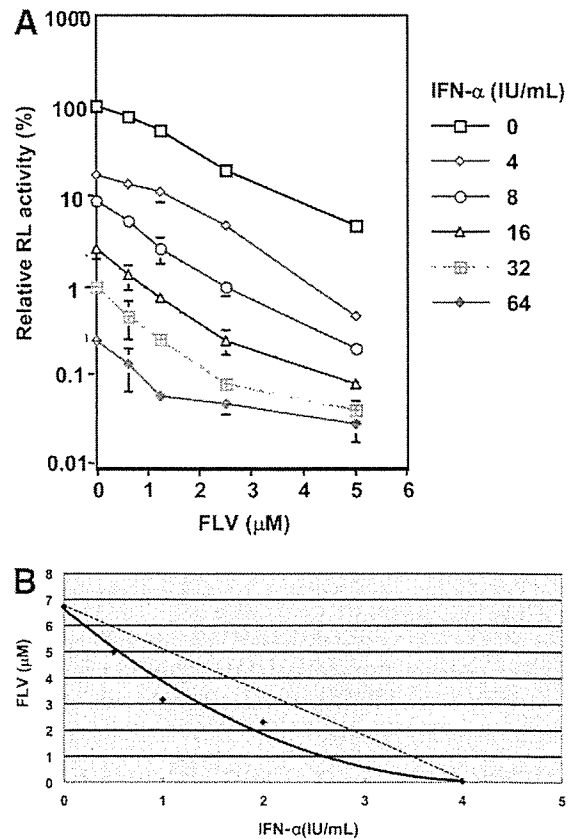


Fig. 5. Synergistic effect of FLV in combination with IFN- $\alpha$  on HCV RNA replication. (A) Effect of FLV in combination with IFN- $\alpha$ . OR6 cells were cotreated with FLV (0, 0.625, 1.25, 2.5, and 5  $\mu$ mol/L) and IFN- $\alpha$  (0, 4, 8, 16, 32, and 64 IU/mL). The RL assay was performed after 72 hours of treatment, and the relative RL activity was calculated as shown in Fig. 1B. (B) Isobole plots of 90% inhibition of HCV RNA replication. OR6 cells were treated with IFN- $\alpha$  (0, 0.5, 1, 2, 4, 6, and 8 IU/mL) in combination with FLV (0, 0.625, 1.25, 2.5, 5, and 7.5  $\mu$ mol/L) for 72 hours, and the RL assay was performed as shown in Fig. 1B to obtain 90% isoboles. The broken line indicates the additive effect in the isobologram method used.<sup>23,24</sup>

compared with the previous results for the combination treatment of IFN- $\alpha$  with ribavirin.<sup>10</sup>

## Discussion

In this study, we found that different statins have different anti-HCV profiles. FLV, ATV, and SMV each exerted a stronger inhibitory effect on HCV RNA replication than did that of LOV reported previously.<sup>11,12</sup> However, PRV exhibited no anti-HCV activity. We also demonstrated that anti-HCV activity was drastically increased when these statins except PRV were used in combination with IFN- $\alpha$ . Because these statins are currently used for the clinical treatment of patients with hypercholesterolemia without inducing severe side effects, our findings suggest that these statins might be useful in combination therapy with IFN- $\alpha$  or IFN- $\alpha$  plus ribavirin.

That PRV exhibited no anti-HCV activity is interesting. From the information on LOV only<sup>10,11</sup> to date, the mechanism underlying statins' inhibition of HCV RNA replication has not been considered their cholesterol-lowering activity but rather their inhibition of geranylgeranylation of cellular proteins. In other words, statins' inhibition of HMG-CoA reductase leads to the reduction of intracellular mevalonate and consequently to a reduction in geranylgeranyl pyrophosphate. In fact, in OR6 cells we observed that mevalonate and geranylgeraniol restored HCV RNA replication in the FLV- or LOV-treated cells. However, we found unexpectedly that PRV did not inhibit HCV RNA replication, whereas PRV inhibited HMG-CoA reductase as effectively as other statins possessing anti-HCV activity. Although PRV is a water-soluble reagent (others are lipophilic), we confirmed PRV did induce expression of HMG-CoA reductase by a positive feedback mechanism<sup>18</sup> and LST-1 was expressed in our cell culture system. These findings suggest the presence of a mechanism in which PRV's inhibition of HMG-CoA reductase does not cause the depletion of geranylgeranyl pyrophosphate. Interestingly, it has been reported that PRV has a unique effect among statins on the induction of p450.<sup>18</sup> Therefore, further studies are needed to explain why PRV exhibits no anti-HCV activity.

We minutely examined the effect of FLV, the statin exhibiting the strongest inhibition of HCV replication of those tested in this study, in combination with IFN- $\alpha$ . We found that a combination treatment of IFN- $\alpha$  and FLV had a synergistic inhibitory effect on HCV RNA replication. Although high doses of IFN- $\alpha$  are more effective than low doses for eliminating HCV from a patient, the side effects increase in a dose-dependent manner. Because ribavirin enhances the effect of IFN- $\alpha$  slightly in a cotreatment, it is the only reagent currently

used with IFN- $\alpha$  to treat patients with CH C. In our previous study of anti-HCV activity using the OR6 assay system, we found the IC<sub>50</sub> of ribavirin to be 76  $\mu$ mol/L.<sup>10</sup> This concentration is much higher than the clinically achievable ribavirin concentration (10-14  $\mu$ mol/L) previously reported.<sup>25</sup> Furthermore, when administered in combination with IFN- $\alpha$  (2 IU/mL) and ribavirin (50  $\mu$ mol/L), HCV RNA replication was reduced by only approximately 50%, compared with the effect of treatment with IFN- $\alpha$  alone.<sup>10</sup> It has been reported that the maximum blood concentration of FLV after 40 mg/day being administered orally for 4 weeks is approximately 0.6  $\mu$ mol/L.<sup>26</sup> This concentration is rather low for the inhibition of HCV replication *in vivo*, because the IC<sub>90</sub> of FLV was assigned as 6.7  $\mu$ mol/L in our assay system (Fig. 5B). In addition, our study showed treatment of OR6 cells with 5  $\mu$ mol/L FLV alone was almost equal to the effect of 10 IU/mL IFN- $\alpha$ . Although statins are known to concentrate in the liver, FLV monotherapy will not be effective for patients with CH C. However, we demonstrated that the combination of IFN- $\alpha$  and FLV exhibited synergistic effects on HCV RNA replication. For example, when administered in combination with IFN- $\alpha$  (2-8 IU/mL) and FLV (5  $\mu$ mol/L), HCV RNA replication fell remarkably, to approximately 3%, compared with the effects of treatment with IFN- $\alpha$  alone (Fig. 4A). From these results, we propose that therapy combining FLV with IFN- $\alpha$  may be effective for the treatment of patients with CH C. Furthermore, additional treatment with reagents in combination (e.g., IFN- $\alpha$ , ribavirin, and FLV) will help to improve the SVR rate.

In conclusion, the results of the present study suggest that statins other than PRV are good reagents for combination therapy with IFN- $\alpha$  in patients with CH C. Although the mechanism by which PRV lacks anti-HCV activity has not been clarified in the present study, a better understanding of this mechanism may lead to the discovery of statin-related anti-HCV reagents possessing no cholesterol-lowering activity. Furthermore, our developed OR6 assay system will be useful for the time-saving screening of new anti-HCV reagents.

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## Diverse Effects of Cyclosporine on Hepatitis C Virus Strain Replication

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

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

Sustained infection of HCV is the major cause of chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (16). Rarely, HCV causes fulminant hepatitis (13). The predominant treatment for HCV-infected patients is interferon (IFN) or polyethylene glycol-conjugated IFN alone or in combination with ribavirin (19, 20). However, alternative anti-HCV therapies are needed because virus is not eliminated in about half of the treated patients (19, 20). Lohmann et al. have developed the HCV subgenomic replicon system, in which an HCV subgenomic replicon autonomously replicates in Huh-7 cells (HCV replicon cells) (18). This replicon comprises the HCV 5' untranslated region (5'UTR) containing an internal ribosomal entry site (IRES), the neomycin phosphotransferase gene, the encephalomyocarditis virus (EMCV) IRES, the coding region for HCV NS3 through NS5B, and the HCV

3'UTR (subgenomic replicon), but it lacks the coding region for the core and envelope proteins, as well as p7 and NS2 (Fig. 1). Subsequently, a genome-length (full-genome) replicon has been developed. This construct contains a full-genome length of HCV, including the coding regions for the core protein through NS2 (Fig. 1) (5, 10). We can evaluate HCV replication using these subgenomic or genome-length replicon systems. Previously, we established HCV subgenomic replicon cells carrying HCV genotype 1b NN strain (15, 29). We demonstrated that an immunosuppressant, cyclosporine (CsA), has anti-HCV activity in these cells (29). In addition, we determined the molecular mechanism of the anti-HCV effect of CsA on this replicon; cyclophilin B (CyPB), one of the cellular targets of CsA, is a cellular replication cofactor of the HCV genome (31). CyPB interacts with NS5B to promote its RNA binding activity (for a detailed description, see reference 31). CsA is suggested to suppress HCV genome replication by inhibiting the functional association of CyPB with NS5B. Another group also reported anti-HCV function of CsA using a subgenomic replicon of other genotype 1b strain, HCV-N (22). In this study, we demonstrate that CsA also has a strong anti-HCV activity in other available genotype 1b replicons carrying the Con1 and O strains (12, 18).

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

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

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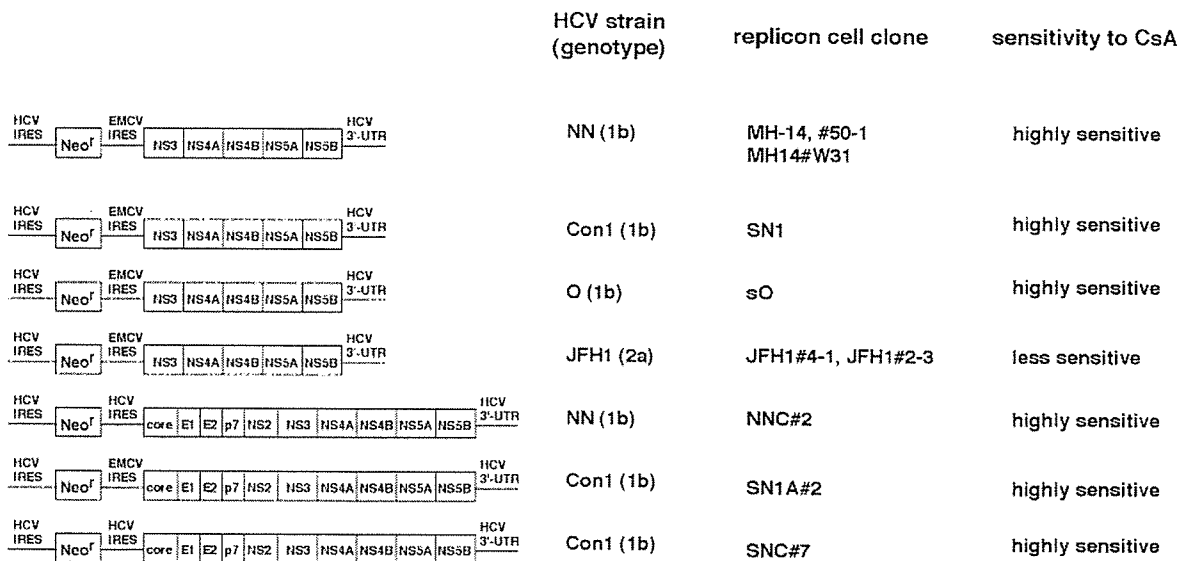


FIG. 1. Schematic representation of the constructs of HCV subgenomic and genome-length replicon RNA. On the left, the constructs of each replicon RNA are shown. HCV strains, as well as genotypes from which the replicon RNA sequences are derived, are indicated in the second column. The names of replicon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

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

MATERIALS AND METHODS

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

**Establishment of replicon cells.** MH-14, #50-1, sO, JFH1#4-1, and JFH1#2-3 cells were described previously (12, 13, 15, 29). The replicon RNAs were produced using a MEGAscript T7 kit (Ambion) from pMH14, pSN1, pNNC, pSN1A, and pSNC plasmids for the establishment of the MH14#W31, SN1,

NNC#2, SN1A#2, and SNC#7 replicon cells, respectively. For the establishment of MH14#W31, we transfected RNA into the Huh-7 cell strain which was identical to the parental cells of JFH1#4-1 and JFH1#2-3. Each replicon RNA was transfected into Huh-7 cells, following the selection with the medium in the presence of 500- to 1,000-µg/ml G418 for around 4 weeks. The resultant cell colonies were isolated and expanded. The HCV RNA titers in cell clones carrying JFH1 replicons were not significantly different from those in established cell clones carrying genotype 1b replicons.

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

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

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

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

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

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

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

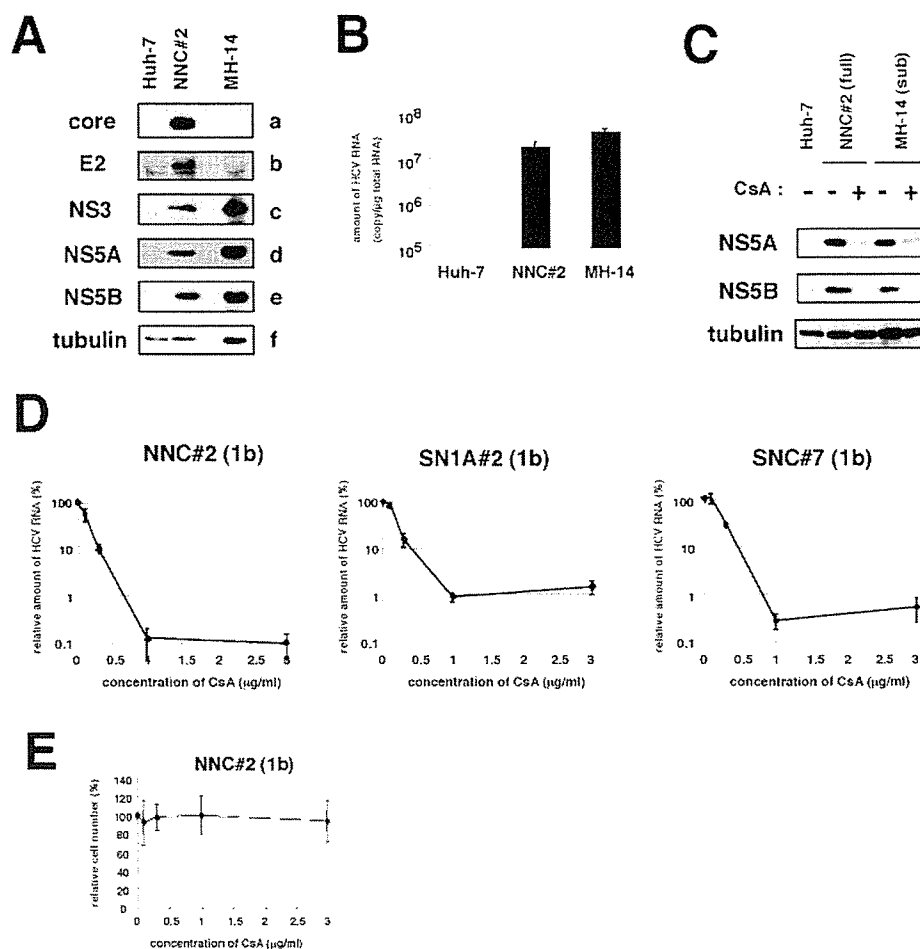


FIG. 2. CsA suppressed the replication of HCV genome, irrespective of the presence of the structural proteins. (A) Detection of HCV proteins from NNC#2 (NN/1b/FL) genome-length replicon. Core (a), E2 (b), NS3 (c), NS5A (d), NS5B (e), and tubulin (f) in Huh-7, NNC#2 (NN/1b/FL), and MH-14 (NN/1b/SG) cells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

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

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

## RESULTS

**CsA suppressed the replication of HCV full-genome replicon.** We and another group have reported an anti-HCV activ-

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

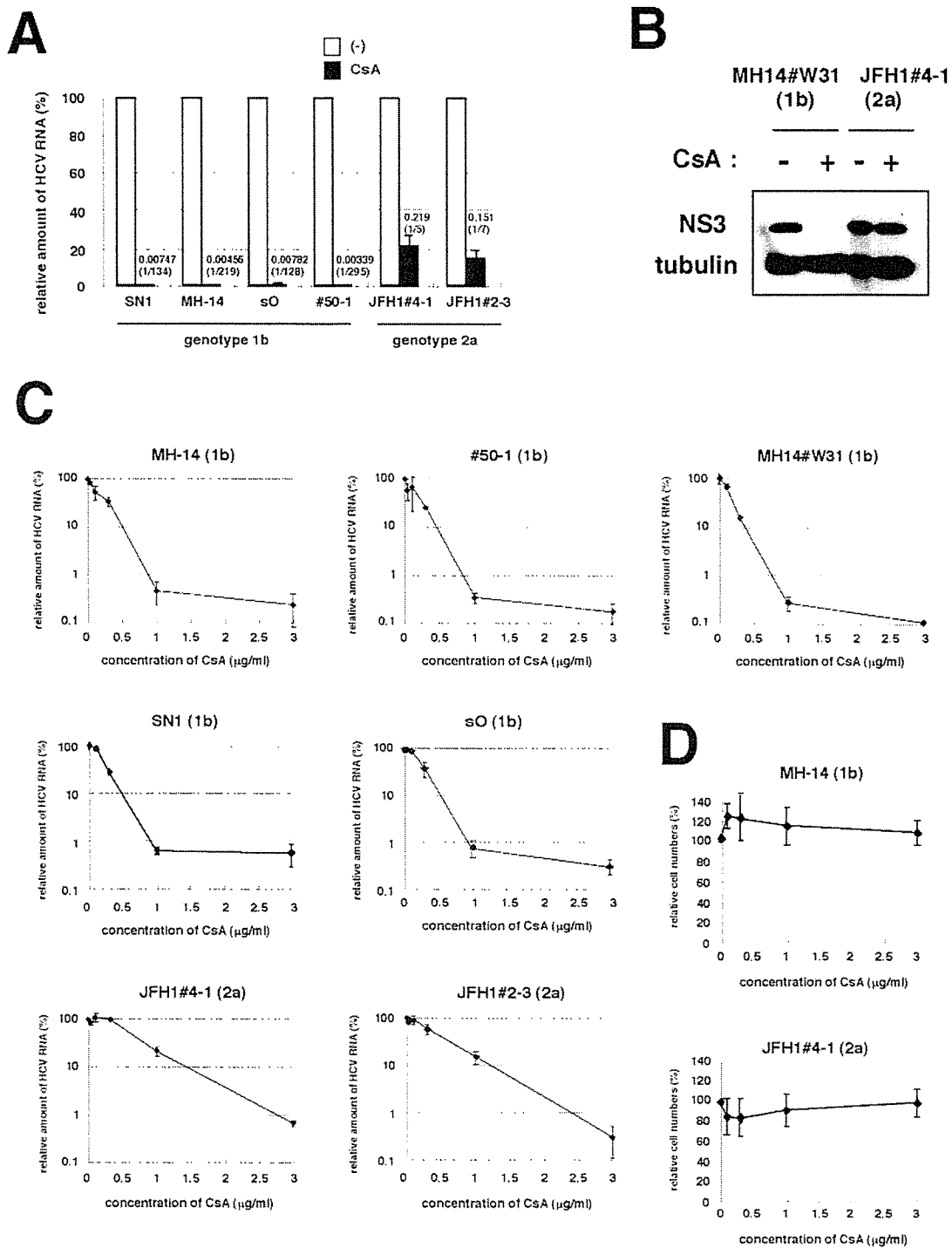


FIG. 3. Replication of a genotype 2a strain, JFH1, was less sensitive to CsA. (A) Sensitivity to CsA of HCV genotype 1b and JFH1 replicons. SN1 (Con1/1b/SG), MH-14 (NN/1b/SG), sO (O/1b/SG), #50-1 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 (JFH1/2a/SG) cells, carrying HCV subgenomic replicon, were treated with 1- $\mu$ g/ml CsA for 7 days. HCV RNA titers were quantified by real-time RT-PCR analysis, and the relative amounts are shown. The bars represent the means of three independent experiments. White bars, no treatment; black bars, 1- $\mu$ g/ml CsA. The numbers above the black bars indicate fold difference of the titer with 1- $\mu$ g/ml CsA treatment compared to no treatment. (B) Levels of NS3 and tubulin as an internal control in MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells without (-) or with (+) 1- $\mu$ g/ml CsA treatment for 5 days were detected by immunoblot analysis. (C) HCV RNA was quantified and plotted as described in the legend to Fig. 2D with genotype 1b replicon cells such as MH-14 (NN/1b/SG), #50-1 (NN/1b/SG), MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), and sO (O/1b/SG) cells and JFH1-carrying replicon cells such as JFH1#4-1 (JFH1/2a/SG) and JFH1#2-3 (JFH1/2a/SG) cells. (D) Effect of CsA on cell proliferation. The growth of MH-14 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were examined as described in the legend for Fig. 2E.



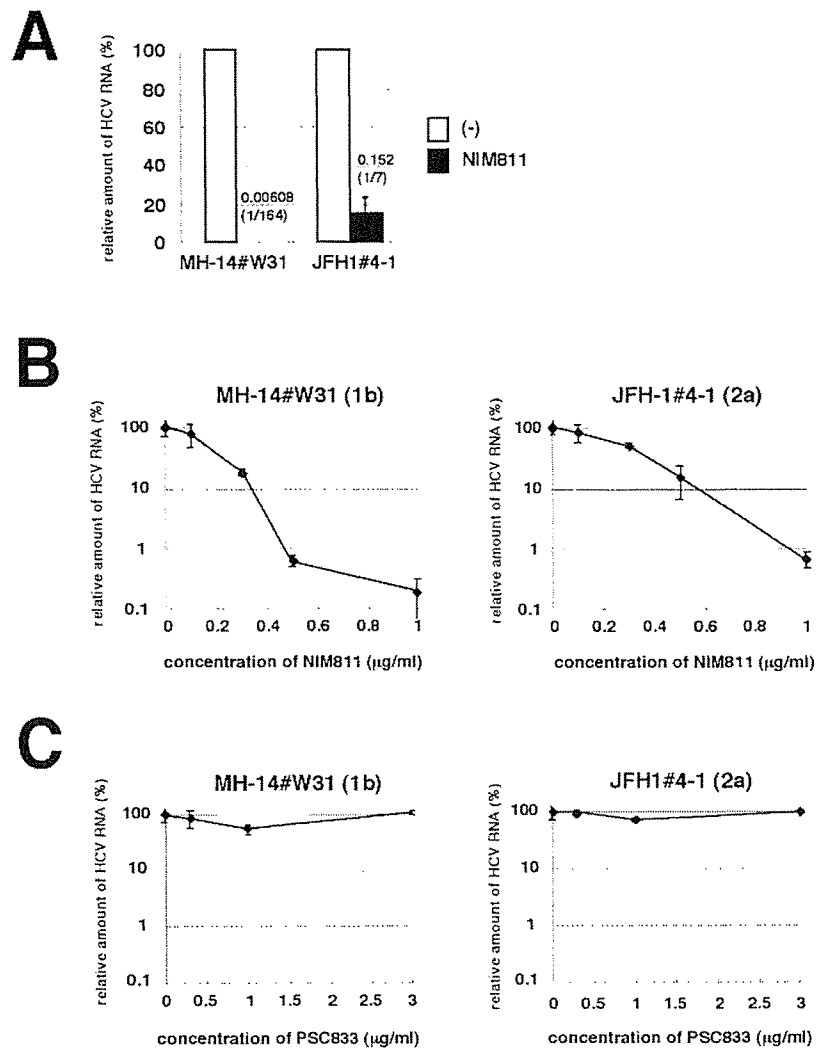


FIG. 4. JFH1 replication was less sensitive to a CsA derivative, NIM811. (A) MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were treated with 0.5- $\mu$ g/ml NIM811 for 7 days. HCV RNA titers were quantified as described in the legend to Fig. 3A. White bars, no treatment; black bars, 0.5- $\mu$ g/ml NIM811. (B and C) HCV RNA in replicon cells treated with various concentrations of NIM811 (B) or PSC833 (C) for 7 days was quantified and plotted against the concentration of NIM811 (B) or PSC833 (C) (in micrograms per milliliter) as described in the legend to Fig. 3C.

cellular genome (data not shown). Similarly, we generated other full-genome replicon cells carrying sequences from the Con1 strain at the nonstructural coding region of the replicon RNA (SN1A#2 [Con1/1b/FL]) and SNC#7 (Con1/1b/FL) cells (Fig. 1). The replicon of SN1A#2 (Con1/1b/FL) cells possessed the EMCV IRES upstream of the open reading frame for HCV proteins, while that of SNC#7 (Con1/1b/FL) cells contained the HCV IRES (Fig. 1). SNC#7 (Con1/1b/FL) cells exhibited almost the same response as that of SN1A#2 (Con1/1b/FL) cells to CsA treatment (Fig. 2D). Consistent with a previous report (22), the EMCV IRES was not responsible for the anti-HCV activity of CsA. We compared the sensitivity to CsA of full-genome replicons with that of subgenomic replicons. CsA strongly decreased the production of HCV proteins in both the full-genome replicon, NNC#2 (NN/1b/FL) cells and the subgenomic replicon, MH-14 (NN/1b/SG)

cells (Fig. 2C). Real-time RT-PCR analysis also revealed a dramatic reduction of the RNA level of full-genome replicons in NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 (Con1/1b/FL) cells (Fig. 2D). The 50% inhibitory concentrations ( $IC_{50}$ ) of CsA in NNC#2 (NN/1b/FL), SN1A#2 (Con1/1b/FL), and SNC#7 (Con1/1b/FL) cells were estimated to be 0.13, 0.19, and 0.24  $\mu$ g/ml, respectively. The 90% inhibitory concentrations ( $IC_{90}$ ) of CsA in these cells were 0.68, 0.94, and 0.81  $\mu$ g/ml, respectively. The CsA dose-response curves of full-genome replicons and subgenomic replicons were similar (i.e., compare SN1A#2 or SNC#7 [Con1/1b/FL] versus SN1 [Con1/1b/SG], NNC#2 [NN/1b/FL] versus MH-14, #50-1, or MH14#W31 [NN/1b/SG]) (Fig. 3C). These results demonstrate that CsA suppresses the replication of full-genome replicons and subgenomic replicons to almost the same extent. Since CsA concentrations of up to 3  $\mu$ g/ml did not affect the

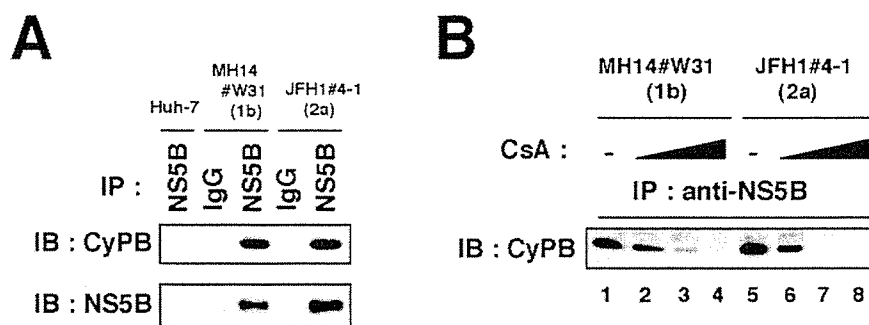


FIG. 5. Interaction of HCV NS5B with CyPB in the JFH1 replicon. (A) Coimmunoprecipitation of endogenous CyPB with NS5B. Lysates from MH14#W31 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells as a negative control were used for immunoprecipitation with normal mouse immunoglobulin G (IgG) or anti-NS5B antibody (NS5B), followed by immunoblot analysis with either anti-CyPB (top) or anti-NS5B antibodies (bottom). IP, antibodies used for immunoprecipitation. (B) The interaction of CyPB with NS5B in JFH1 replicon was disrupted by CsA treatment. Coimmunoprecipitation between CyPB and NS5B was analyzed with MH14#W31 (NN/1b/SG) or JFH1#4-1 (JFH1/2a/SG) cells treated without CsA (lanes 1 and 5) or with CsA (0.3  $\mu$ g/ml in lanes 2 and 6, 1  $\mu$ g/ml in lanes 3 and 7, and 3  $\mu$ g/ml in lanes 4 and 8).

proliferation of any replicon cells (Fig. 2E and data not shown), the effect of CsA on replication is not due to the cytotoxic effect. In addition, we observed the reduction of production of infectious viral particles in the presence of 3- $\mu$ g/ml CsA (data not shown) using the viral production system with full-genome JFH1 RNA (27).

**The JFH1 replicon was less sensitive to CsA than were genotype 1b replicons.** We compared the sensitivity of HCV replication to CsA in several subgenomic replicon cells. We used MH-14 (NN/1b/SG) and #50-1 (NN/1b/SG) cells carrying subgenomic replicons with HCV NN strain (15, 29), SN1 (Con1/1b/SG) cells carrying the Con1 subgenomic replicon (18), and sO (O/1b/SG) cells bearing the subgenomic O strain (12) as genotype 1b replicon-containing cells. We also employed JFH1#4-1 (JFH1/2a/SG) and JFH1#2-3 (JFH1/2a/SG) cell clones carrying the JFH1 subgenomic replicon (13). Treatment of CsA (1  $\mu$ g/ml; 7 days) drastically decreased HCV RNA in all the subgenomic replicon cells carrying the HCV genotype 1b strain. HCV RNA levels in SN1 (Con1/1b/SG), MH-14 (NN/1b/SG), sO (O/1b/SG), and #50-1 (NN/1b/SG) cells decreased to 1/134, 1/219, 1/128, and 1/295, respectively (Fig. 3A). Genotype 1b replicon cells appeared highly sensitive to CsA. In contrast, the effect of CsA on HCV RNA levels in replicon cells containing sequences from the JFH1 strain was limited to 1/5 to 1/7 (Fig. 3A). These results of the response to CsA were reproduced in further additional cell clones.

The cellular characteristics of Huh-7 cell strains differ among laboratories. To exclude the possibility that differences between Huh-7 cell strains influence the sensitivity to CsA, we established genotype 1b replicon cells based on the identical Huh-7 cell strain, which were used as parental cells of JFH1#4-1 (JFH1/2a/SG) and JFH1#2-3 (JFH1/2a/SG) cells. The response of the corresponding replicon cells, MH14#W31 (NN/1b/SG), to CsA was almost the same as that of SN1 (Con1/1b/SG), MH-14 (NN/1b/SG), sO (O/1b/SG), and #50-1 (NN/1b/SG) cells (Fig. 3C). Thus, the difference in sensitivity of JFH1 and genotype 1b strains to CsA can be attributed to the characteristic differences of the HCV strains, not to the parental Huh-7 cell strain. In addition, the reduction of NS3 protein in JFH1#4-1 (JFH1/2a/SG) cells following treatment

with CsA was less prominent than that in MH14#W31 (NN/1b/SG) cells (Fig. 3B).

We examined the dose-response curve of HCV RNA against the concentration of CsA (Fig. 3C). The effect of CsA in genotype 1b replicons plateaued at around 1  $\mu$ g/ml, while in the dose-response curve in JFH1 replicon, the inhibition was not yet saturated (Fig. 3C). As concentrations of CsA up to 3  $\mu$ g/ml did not affect the proliferation rate of any replicon cells (Fig. 3D and data not shown), the effect of CsA on replication was not due to the cytotoxic effect. The  $IC_{50}$  of CsA in MH-14 (NN/1b/SG), #50-1 (NN/1b/SG), MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 (JFH1/2a/SG) cells were estimated to be 0.15, 0.18, 0.16, 0.20, 0.25, 0.67, and 0.43  $\mu$ g/ml, respectively. The  $IC_{90}$  was 0.86, 0.82, 0.76, 0.88, 0.92, 2.77, and 2.39  $\mu$ g/ml, respectively. A similar dose-response curve in the JFH1 replicon was obtained by a transient replication assay with the luciferase reporter driven from a JFH1 replicon construct (data not shown) (14).

**JFH1 replicon was less sensitive to a CsA derivative, NIM811.** Analysis of several CsA derivatives has revealed that the anti-HCV effect of CsA on the genotype 1b replicon is mediated by the inhibition of CyP (31). We examined the sensitivity of JFH1 replicon to CsA derivatives. CsA is known to have three major cellular targets: CyP, calcineurin (CN)/NF-AT, and P glycoprotein (P-gp) (28, 31). A CsA derivative, NIM811, inhibits CyP and P-gp but not CN/NF-AT, while another derivative, PSC833, inhibits P-gp but neither CyP nor CN/NF-AT (31). The decrease of HCV RNA in MH14#W31 (NN/1b/SG) cells with NIM811 treatment (0.5  $\mu$ g/ml; 7 days) was more than an order of magnitude greater than that in JFH1#4-1 (JFH1/2a/SG) cells (Fig. 4A). The slope of the dose-response curve of NIM811 treatment of the JFH1 replicon was gentler than that of genotype 1b (Fig. 4B). The  $IC_{50}$  of NIM811 in MH14W#31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were 0.17 and 0.30  $\mu$ g/ml, respectively. The  $IC_{90}$  were 0.46 and 0.93  $\mu$ g/ml, respectively. In contrast, PSC833, which does not inhibit CyP, did not alter HCV RNA level in either genotype 1b or the JFH1 replicon (Fig. 4C). Thus, a CyP

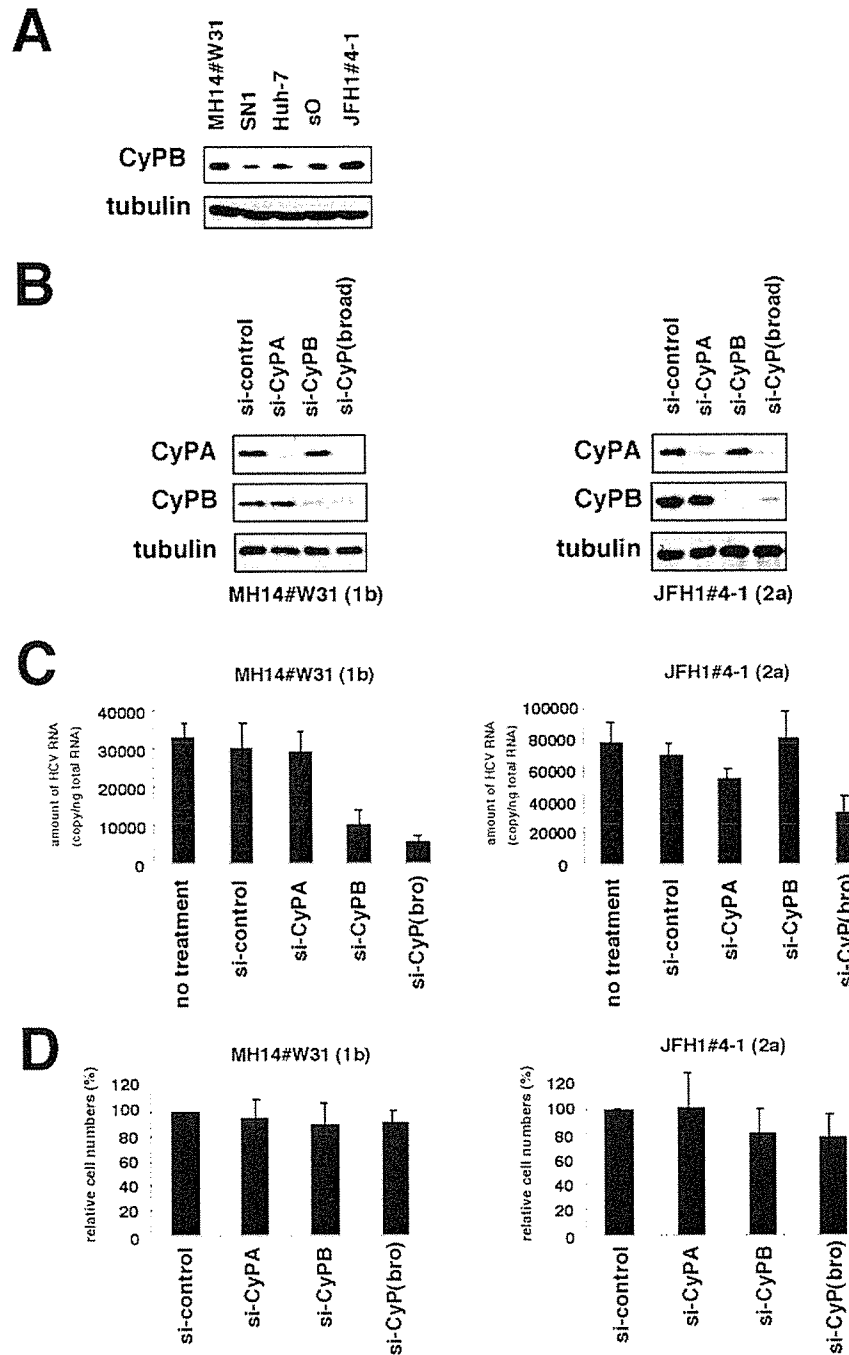


FIG. 6. CyPB in HCV replication of genotype 1b and JFH1. (A) Expression level of endogenous CyPB protein (top) and tubulin as an internal control (bottom) in MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells. (B) Knockdown of endogenous CyP proteins. MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were transfected with siRNA specific for CyPA (si-CyPA), CyPB (si-CyP), a broad range of CyP subtypes [si-CyP(broad)], or a randomized siRNA (si-control). At 72 h posttransfection, CyPA (top), CyPB (middle) and tubulin as an internal control (bottom) were detected in total cell lysates of MH14#W31 (NN/1b/SG) (left) and JFH1#4-1 (JFH1/2a/SG) (right) cells by immunoblot analysis. (C) Depletion of CyPB did not affect HCV replication of JFH1 replicon. At 5 days posttransfection, HCV RNA titers in MH14#W31 (NN/1b/SG) (left) and JFH1#4-1 (JFH1/2a/SG) (right) cells were quantified by real-time RT-PCR analysis. no treatment, treatment with only the transfection reagent in the absence of siRNA. (D) Effect of siRNA on cell proliferation. Cell numbers of MH14W#31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells treated with siRNA for 5 days were counted. Relative cell numbers were indicated.

inhibitor was less effective at suppressing the replication of the JFH1 replicon than genotype 1b replicons.

**Interactions between CyPB and JFH1 NS5B.** Previously, we have shown that CyPB interacts with NS5B to promote HCV genome replication and that CsA inhibits this binding in a genotype 1b replicon (31). Here, we examined the association between CyPB and NS5B in a JFH1 replicon. Immunoprecipitation analysis revealed an interaction of CyPB with NS5B in JFH1#4-1 (JFH1/2a/SG) cells (Fig. 5A). This interaction was dissociated following the treatment of CsA, as observed with the genotype 1b replicon (Fig. 5B).

**Role of CyPB in replication of the JFH1 replicon.** Although we observed some differences of expression levels of endogenous CyPB among the replicon cells in the immunoblot analysis (Fig. 6A), there was no particular correlation between endogenous CyPB expression levels and replication sensitivity to CsA among cells. CyPB reportedly regulates HCV genome replication of the genotype 1b replicon (31). We then explored the requirement of CyPB for the replication of JFH1 replicon with RNA interference. Transfecting siRNAs designed to recognize several CyP subtypes [si-CyP(broad)] (Fig. 6B) reduced HCV RNA to <1/5 in MH14#W31 (NN/1b/SG) cells (Fig. 6C). Specific knockdown of CyPB but not CyPA (Fig. 6B) decreased HCV RNA in MH14#W31 (NN/1b/SG) cells, consistent with a previous report (Fig. 6C) (31). In contrast, HCV RNA in JFH1#4-1 (JFH1/2a/SG) cells was not altered following the suppression of either endogenous CyPA or CyPB (Fig. 6B and C). We observed a weak decrease of HCV RNA levels (around one-half) with si-CyP(broad) (Fig. 6C). These data suggests the possibility that the replication of the JFH1 replicon is independent of CyPB, in contrast to the genotype 1b replicon. In the previous study, it was reported that the doubling time, saturation density, and response to cell confluence of the replicon cells carrying JFH1 were different from those in cells carrying a genotype 1b replicon, suggesting the possibility that the coupling relationship between the replication and cell growth was different between genotype 1b and the JFH1 replicon (21). The introduction of either si-CyPB or si-CyP(broad), however, had little effect on cell growth in MH14#W31 (NN/1b/SG) or JFH1#4-1 (JFH1/2a/SG) cells (Fig. 6D). And we did not observe cells being confluent in the experiment period. The above results suggest that the different response to si-CyPB in the two lines is independent of the conditions of cell growth.

**The role of CyPB in the RNA binding activity of JFH1 NS5B.** CyPB regulates HCV genome replication of a genotype 1b replicon by promoting the RNA binding activity of NS5B (31). We examined the effect of CyPB on the RNA binding activity of NS5B in JFH1. NS5B in the replication complex was isolated from cells by treatment with digitonin-proteinase K, as described previously (31). This fraction was incubated with poly(U) RNA-Sepharose or protein G-Sepharose as a negative control for the detection of RNA binding NS5B in the replication complex. RNA-bound NS5B in this fraction from MH14#W31 (NN/1b/SG) cells was decreased drastically following treatment with CsA (Fig. 7A, lanes 5 and 6). However, the reduction of RNA binding of NS5B in the replication complex of JFH1#4-1 (JFH1/2a/SG) cells was not as prominent (Fig. 7A, lanes 11 and 12). We confirmed this result by an in vitro RNA binding assay, in which in vitro-synthesized NS5B was incubated with poly(U) RNA-Sepharose, together with

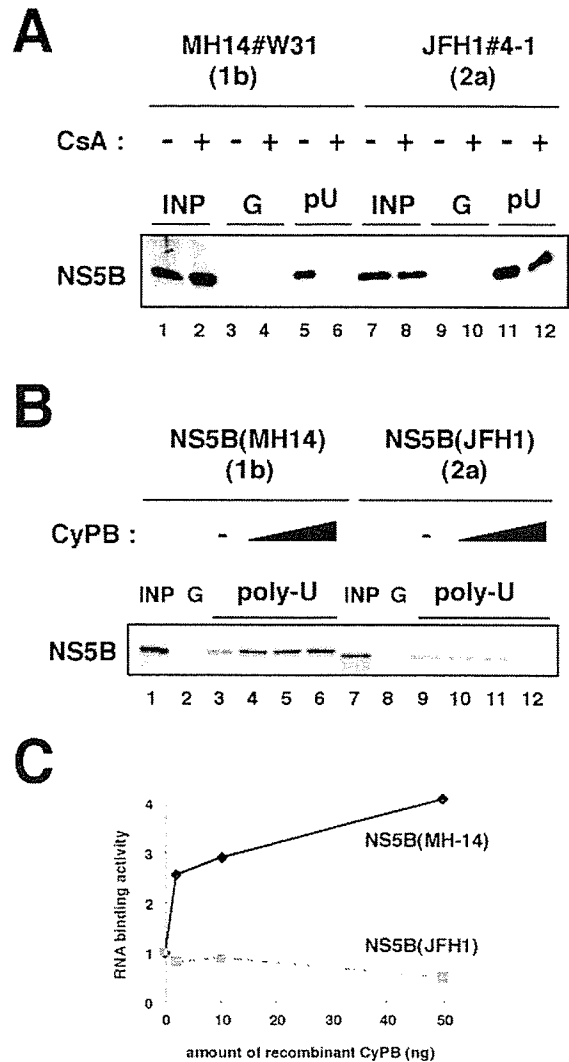


FIG. 7. RNA binding capacity of JFH1 NS5B was independent of CyPB. (A) An RNA-protein binding precipitation assay was performed using MH14#W31 (NN/1b/SG) cells (lanes 1 to 6) and JFH1#4-1 (JFH1/2a/SG) cells (lanes 7 to 12) as described in Materials and Methods. MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells preincubated without (lanes 1, 3, 5, 7, 9, and 11) or with (lanes 2, 4, 6, 8, 10, and 12) CsA were treated with digitonin, followed by digestion with proteinase K to isolate the replication complex. This fraction was then incubated with poly(U) RNA-Sepharose (lanes 5, 6, 11, and 12) or protein G-Sepharose as a negative control (lanes 3, 4, 9, and 10). Precipitates were detected by immunoblot analysis with anti-NS5B antibody. INP, one-sixth of the amount of cell lysate used in the precipitation assay; G and pU, samples with protein G-Sepharose and poly(U)-Sepharose, respectively. (B) An in vitro RNA binding assay was performed as described in Materials and Methods. In vitro-synthesized NS5B of MH-14 (lanes 1 to 6) or JFH1 (lanes 7 to 12) with the rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine was incubated with protein G-Sepharose (lanes 2 and 8) or poly(U)-Sepharose in the absence (lanes 3 and 9) or presence of various amounts of purified recombinant GST-CyPB (2 ng in panels 4 and 10, 10 ng in panels 5 and 11, and 50 ng in panels 6 and 12). The resultant precipitates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by the detection of radiolabeled protein. (C) The density of the bands of NS5B in the RNA binding fraction was quantified and plotted against the amount of the recombinant GST-CyPB (in nanograms). Solid line, NS5B of MH-14; faint line, NS5B of JFH1.