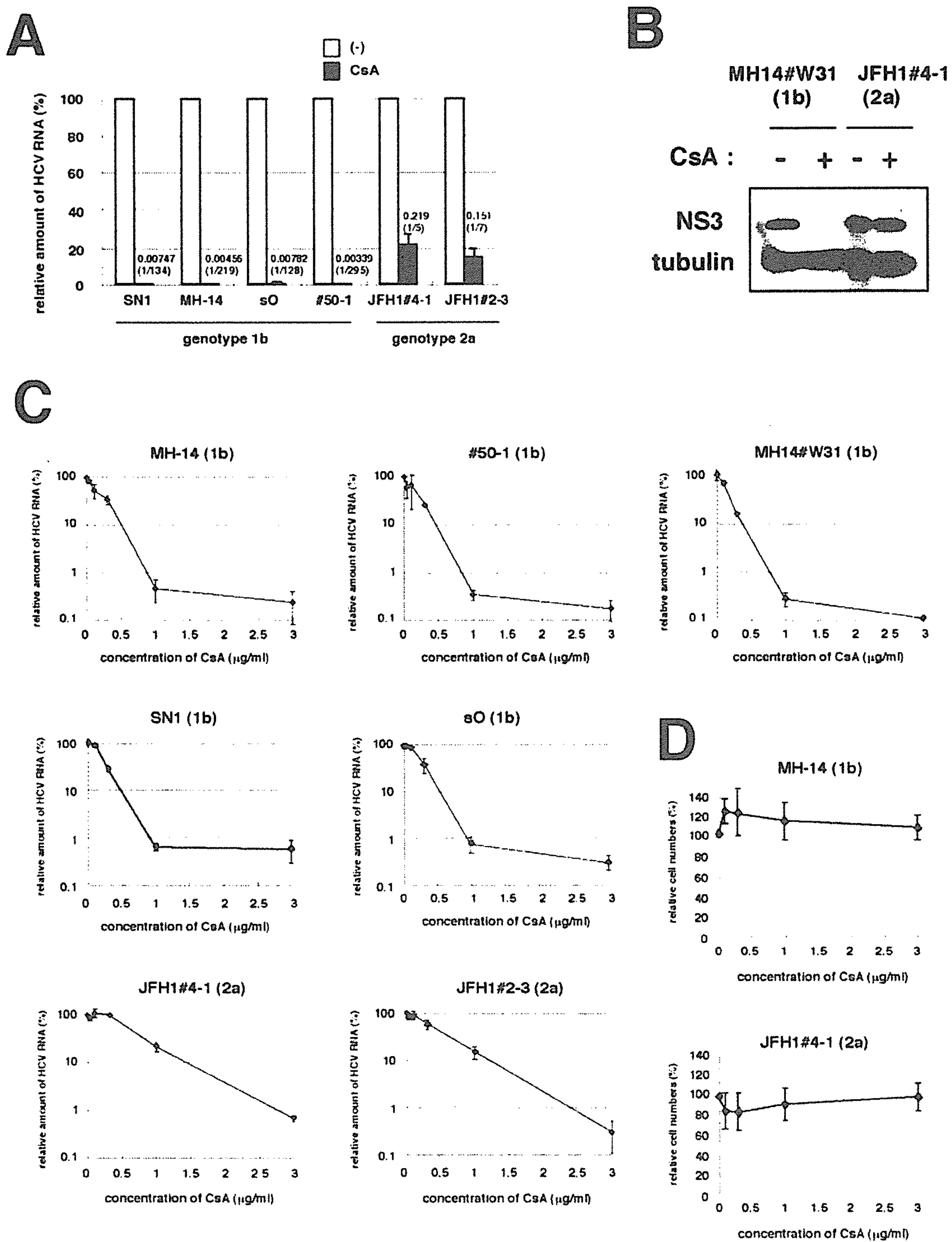

FIG. 3. Replication of a genotype 2a strain, JFH1, was less sensitive to CsA. (A) Sensitivity to CsA of HCV genotype 1b and JFH1 replicons. SN1 (Con1/1b/SG), MH-14 (NN/1b/SG), sO (O/1b/SG), #50-1 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 (JFH1/2a/SG) cells, carrying HCV subgenomic replicon, were treated with 1-μg/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-μg/ml CsA. The numbers above the black bars indicate fold difference of the titer with 1-μg/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-μg/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.

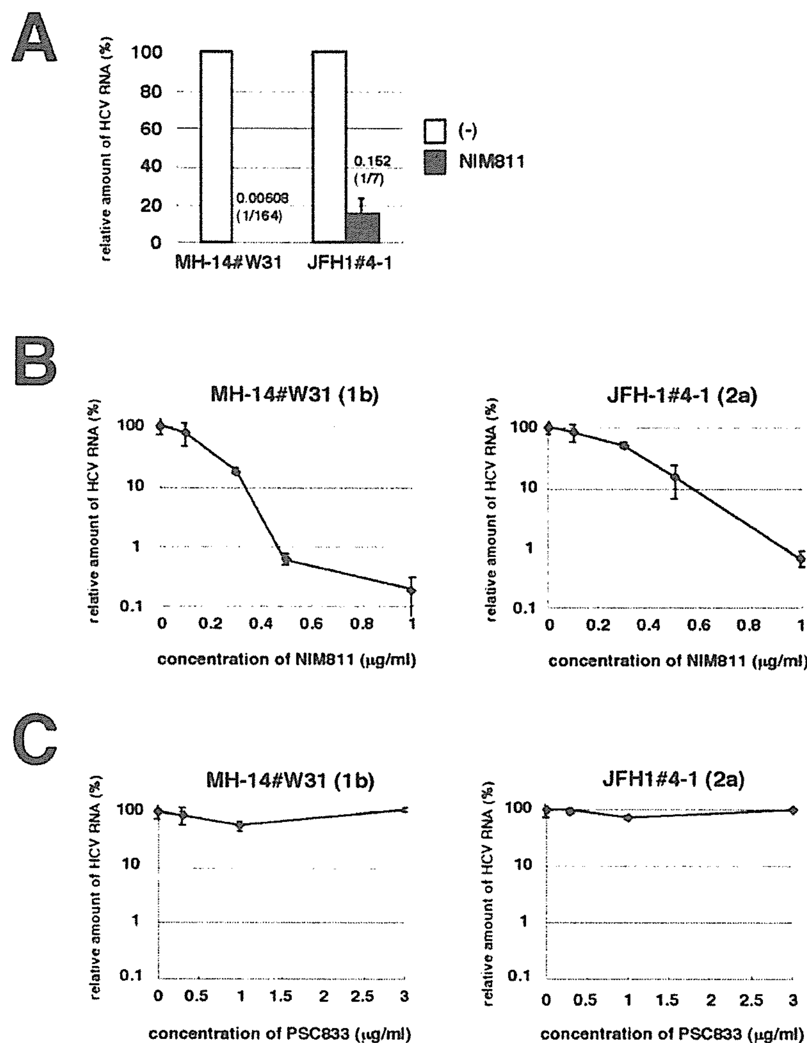

FIG. 4. JFH1 replication was less sensitive to a CsA derivative, NIM811. (A) MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were treated with 0.5-μg/ml NIM811 for 7 days. HCV RNA titers were quantified as described in the legend to Fig. 3A. White bars, no treatment; black bars, 0.5-μg/ml NIM811. (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 awere estimated to be 0.13, 0.19, and 0.24 μg/ml, respectively. The 90% inhibitory concentrations (IC_{90}) of CsA in these cells were 0.68, 0.94, and 0.81 μg/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 μg/ml did not affect the

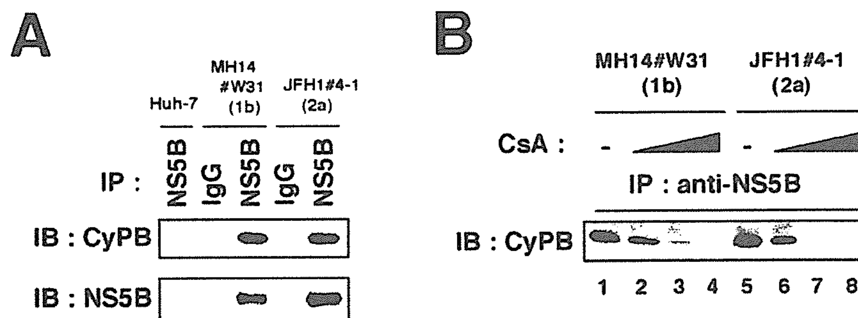

FIG. 5. Interaction of HCV NS5B with CyPB in the JFH1 replicon. (A) Coimmunoprecipitation of endogenous CyPB with NS5B. Lysates from MH14#W31 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells as a negative control were used for immunoprecipitation with normal mouse immunoglobulin G (IgG) or anti-NS5B 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 μg/ml in lanes 2 and 6, 1 μg/ml in lanes 3 and 7, and 3 μg/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-μg/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 μg/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 μg/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 μg/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 μg/ml, respectively. The IC_{90} was 0.86, 0.82, 0.76, 0.88, 0.92, 2.77, and 2.39 μg/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 μg/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 μg/ml, respectively. The IC_{90} were 0.46 and 0.93 μg/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

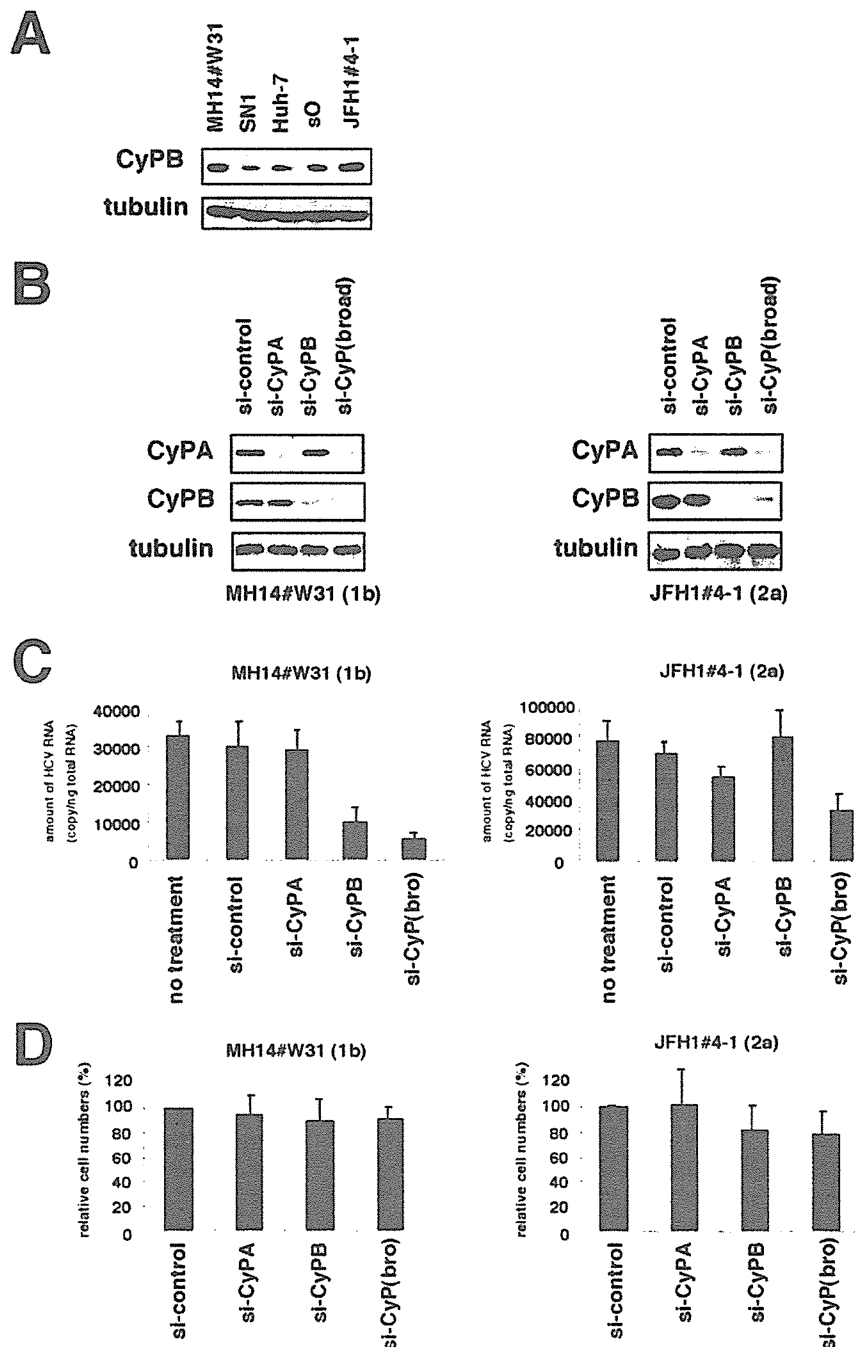

FIG. 6. CyPB in HCV replication of genotype 1b and JFH1. (A) Expression level of endogenous CyPB protein (top) and tubulin as an internal control (bottom) in MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells. (B) Knockdown of endogenous CyP proteins. MH14#W31 (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

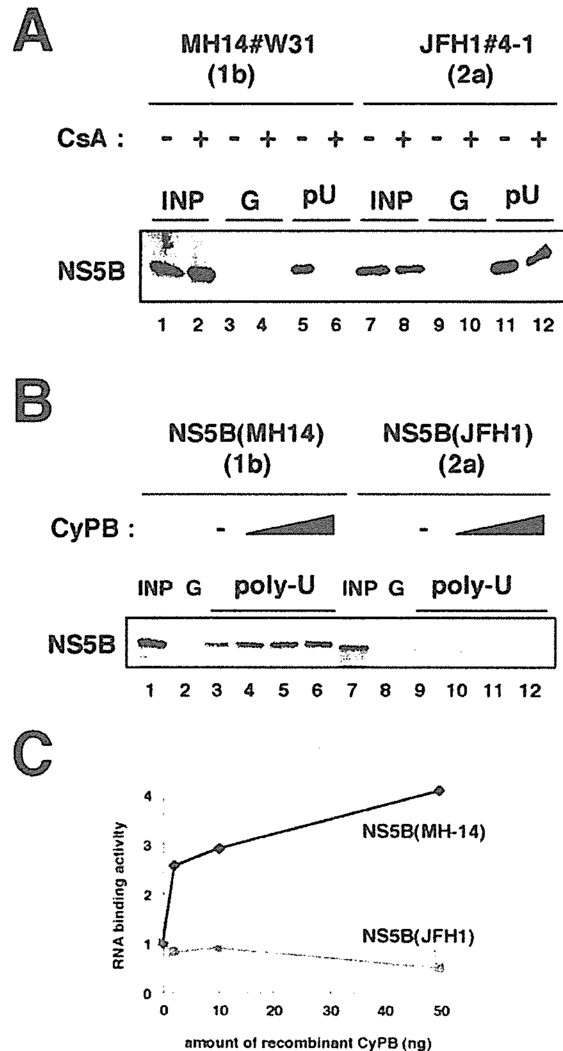

FIG. 7. RNA binding capacity of JFH1 NS5B was independent of CyPB. (A) An RNA-protein binding precipitation assay was performed using MH14#W31 (NN/1b/SG) cells (lanes 1 to 6) and JFH1#4-1 (JFH1/2a/SG) cells (lanes 7 to 12) as described in Materials and Methods. MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) 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 [^{35}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.

```
       1        10        20        30        40        50        60        70
NN     SMSYSWTGALITPCAAEESKLPINPLSNSLLRHHNMVYATTSRSAGLRQKKVTFDRLQVLDDHYRDVLKE
Con1   ----T-------------T-----A----------L---------S------------------------
O      ----T-------------------A--------------------SQ-----------------------
JFH1   --------------SP--E-------------Y--K--C---K--SQ-A-------T----A--DS---D

               80        90       100       110       120       130       140
NN     MKAKASTVKAKLLSIEEACKLTPPHSAKSKYGYGAKDVRNLSSRAVNHIRSVWEDLLEDTETPIDTTIMA
Con1   --------------V------------R--F------------K---------K----------------
O      --------------V------------R--F------------K-----H---K-------------V--
JFH1   I-LA--K-S-R--TL----Q-------R----F---E--S--G------K---K-----PQ---P-----

              150       160       170       180       190       200       210
NN     KSEVFCVQPEKGGRKPARLIVFPDLGVRVCEKMALYDVVSTLPQAVMGSSYGFQYSPKQRVEFLVNTWKS
Con1   -N-------------------------------------------------------G--------A--A
O      -N-------------------------------------------------------G--------A---
JFH1   -N-----D-A---K-------Y---------------ITQK-------A--------A----Y-LKA-AE

              220       230       240       250       260       270       280
NN     KKCPMGFSYDTRCFDSTVTENDIRVEESIYQCCDLAPEAKLAIKSLTERLYVGGPLTNSKGQNCGYRRCR
Con1   -------A-------------------------------RQ--R-------I------------------
O      --T----A-------------------------------RQ--R-------I------------------
JFH1   --D-----------------R---T------A-S-PE--RT--H-----------MF-----T-------

              290       300       310       320       330       340       350
NN     ASGVLTTSCGNTLTCYLKASAACRAAKLQDCTMLVNGDDLVVICESAGTQEDAASLRVFTEAMTRYSAPP
Con1   -------------------A---------------C----------------E----A------------
O      -----------------------------------C----------------------------------
JFH1   --------M---I---V--L---K--GIVAP----C-------S--Q--E--ERN--A------------

              360       370       380       390       400       410       420
NN     GDPPQPEYDLELITSCSSNVSVAHDASGKRVYYLTRDPTTPLARAAWETARHTPVNSWLGNIIMYAPTLW
Con1   ----K-----------------------------------------------------------------
O      ----------------------------------------------------------------------
JFH1   ----R------------------LGPR-R-R------------------V--S-I--------Q----I-

              430       440       450       460       470       480       490
NN     ARMILMTHFFSILLAQEQLEKALDCQIYGACYSIEPLDLPQIIERLHGLSAFSLHSYSPGEINRVASCLR
Con1   -------------------------------------------Q--------------------------
O      ------------------------------T------------Q--------------------------
JFH1   V--V---------MV-DT-DQN-NFEM--SV--VN-----A--------D---M-T--HH-LT----A--

              500       510       520       530       540       550       560
NN     KLGVPPLRVWRHRARSVRAKLLSQGGRAATCGKYLFNWAVKTKLKLTPIPAASRLDLSGWFVAGYSGGDI
Con1   -------------------R--------------------R------------Q----S-----------
O      ----------------------------------------R-----------------------------
JFH1   ---A------KS---A---S-I-R--K--V--R---------------L-E-RL----S--TV-AG----

              570       580       590 591
NN     YHSLSRARPRWFMWCLLLLSVGVGIYLLPNR
Con1   -------------------------------
O      -------------------------------
JFH1   F--V------SLLFG----F----LF---A-
```

FIG. 8. Amino acid sequence alignment of NS5B encoded by HCV strains NN, Con1, O, and JFH1. The numbers above the sequence indicate the amino acid numbers. Conserved residues are shown by dashes. The region spanning 521 to 591 aa, which is involved in the interaction with CyPB, is boxed.

recombinant GST-CyPB. The addition of recombinant GST-CyPB increased the binding of genotype 1b NS5B to poly(U) RNA (Fig. 7B and C). However, this augmentation of RNA binding was not observed with NS5B from the JFH1 strain (Fig. 7B and C). From the above results, it is suggested that the RNA binding of JFH1 NS5B is free from regulation by CyPB.

DISCUSSION

Until now, we and another group have utilized subgenomic replicons carrying genotype 1b NN and HCV-N strains to demonstrate that CsA suppresses HCV genome replication (22, 29). This study reveals that CsA is effective on full-genome replicons to almost the same extent. In addition, other available genotype 1b replicons carrying the Con1 and O strains also have a high sensitivity to CsA, consistent with our proposal that HCV genotype 1b is highly sensitive to CsA. However, a fulminant-type genotype 2a replicon, JFH1, was less responsive to CsA, although a high dose of CsA suppressed the replication of this strain.

CyPB interacts with genotype 1b NS5B to stimulate its RNA

binding activity. In contrast, CyPB binds JFH1 NS5B but does not regulate the function of JFH1 NS5B. This is consistent with a previous speculation that genotype 1b and JFH1 replicons utilize the same cellular factors in distinct manners (21). The NS5B sequence of NN strain has 95.0, 95.9, and 70.4% homology to that of Con1, O, and JFH1, respectively (Fig. 8). The region spanning amino acids (aa) 521 to 591 of NS5B, which is involved in the interaction with CyPB (31), is highly conserved among genotype 1b strains NN, Con1, and O while that of JFH1 has 21 substituted residues in this region. The proline at 540 aa, which is important for CyPB binding (31), is conserved but the adjacent residues such as isoleucine at 539 aa and alanine at 541 aa are replaced by leucine and glutamic acid, respectively, in JFH1. Through molecular interactions, CyPB seems to make the conformation of NS5B of genotype 1b strains but not JFH1 suitable for RNA binding (31). The diverse regulation system of NS5B by CyPB among strains may be due to differences in either the sequence or the entire conformation of NS5B. Further study is important for elucidating the regulation mechanism of RNA binding activity of NS5B by CyPB.

Thus, replication in JFH1 replicon is independent of CyPB. Interestingly, human immunodeficiency virus type 1 (HIV-1) strains also have a diversity of CyP dependence on viral proliferation (3, 33). CyPA plays an important role in the life cycle of HIV-1. The interaction of the HIV-1 capsid protein with CyPA that resides within the target cells of infection is critical for HIV-1 replication (7, 24). In peripheral blood mononuclear cells or Jurkat T cells, CsA suppresses the proliferation of HIV-1 group main (M) strain (3). However, certain strains of group outlier (O), such as MVP5180 and MVP9435, are resistant to CsA (3, 33), suggesting the different dependency of the replication on CyPA. Authors have suggested that MVP5180 and MVP9435 clones adapt to replicate independently of CyPA and that this adaptation provides a significant replication advantage for the virus in vivo (3). In vesicular stomatitis virus (VSV) strains, a role for CyPA in virus replication also has been reported (2). CyPA is required for the infection of the VSV-NJ strain but not the VSV-IND strain. These authors proposed that during evolutionary divergence from the ancestral lineages that initially were dependent on CyPA for replication, VSV-IND may have adapted to reduce its dependency on CyPA (2). In the case of HCV, a fulminant type genotype 2a replicon (JFH1) replicates independently of CyPB. It has previously been reported that JFH1 has a much higher competency of replication in the cells than other strains (13). The adaptation to independence from CyPB may contribute to the high capacity of replication of JFH1.

Although the JFH1 replicon is less sensitive to CsA, high concentrations of CsA still suppress replication of the JFH1 replicon. Moreover, the introduction of the siRNA designed to recognize several CyP subtypes [si-CyP(broad)] moderately diminishes HCV RNA in the JFH1 replicon. We suspect that a CyP family member other than CyPB is involved in HCV genome replication. Further analysis is needed on the role of other CyP subtypes.

As there a replicon system for a fulminant-type genotype 1b replicon or chronic-type genotype 2a replicon does not yet exist, we cannot conclude whether chronic-type genotype 2a replicons or fulminant-type replicons are less sensitive to CsA or not. However, there is a clinical report describing cotreatment of patients with chronic hepatitis C with IFN and CsA that resulted in a higher sustained virological rate than with treatment of IFN alone (11). In this report, increase in the sustained virological rate was prominent with patients carrying genotype 1 HCV (51.7% versus 21.9%), while it was relatively weak in patients carrying genotype 2 HCV (66.7% versus 58.3%) (11). Thus, genotype may affect the sensitivity of HCV replication to CsA. However, we cannot exclude the possibility that the diminished sensitivity to CsA is a characteristic only of the fulminant-type genotype 2a strain.

Our results suggest that sensitivity to CsA and replication dependency to CyPB is different among HCV strains. This finding is an important insight into the diversity of the mechanism of HCV genome replication and its sensitivity to antiviral agents.

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

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Abstract

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

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

RNAi is initiated by the RNase III-like nuclease Dicer, which promotes progressive cleavage of long dsRNAs into 21 to 27 nucleotide (nt) short interfering RNAs (siRNAs) with two nt 3′-overhangs. Subsequently, the siRNAs are incorporated into an RNA-induced silencing complex (RISC), identified in *Drosophila*, and the protein-RNA effector nuclease complex recognizes and destroys the target mRNAs [8–10].

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

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

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

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

Materials and methods

shRNA synthesis by T7 RNA polymerase. Desalted DNA oligonucleotides were obtained from Sigma Proligo (Boulder, CO): the T7 promoter 5′-TAATACGACTCACTATAG-3′; EGFP 418–437nt as 5′-CTGGGGC ACAAGCTGGAGTA-3′; HCV 120–139nt as 5′-CCCCCCCTCCCGGG AGAGCC-3′, 260–279nt as 5′-AGTGTTGGGTCGCGAAAGGC-3′, 330–349nt as 5′-AGA CCGTGCACCATGAGCAC-3′, and 340–359nt as 5′-CCATGAGCAC GAATCCTAAA-3′. Loop used CCACACC [23] and overhang used CUU. The oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase was described previously [24]. For each transcription reaction, the oligonucleotide was annealed in sH_2O by heating at 95 °C; after 5 min, the heating block was allowed to cool down slowly to obtain the dsDNA. Transcription was performed using AmpliScribe™ T7 High Yield Transcription Kits (EPICENTRE Biotechnologies, Madison, WI) according to the manufacturer's recommended protocol. After incubation at 37 °C for 2 h, 1U RNase free-DNase was added at 37 °C for 15 min. Single-stranded 51nt RNAs were annealed by heating at 95 °C for 5 min followed by 1 h at 37 °C to obtain shRNAs.

Cell culture. Human hepatoma-derived Huh-7 cells and human uterus cancer-derived HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HCV subgenomic replicons were cultured in DMEM-supplemented with 10% FBS, 0.1 mM MEM non-essential amino-acid solution, 2 mM L-glutamine, and penicillin–streptomycin. The growth medium contained the active ingredient G418 (300 μg/ml) [25].

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

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

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

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

Ribosomal RNA-cleavage assay. Huh-7 cells and HCV replicons were transfected with the shRNAs or polyI:C using Lipofectamine 2000 in accordance with the manufacturer's recommendation (Invitrogen). After 24 h, the cells were harvested, washed in phosphate-buffered saline (PBS), and stored at −80 °C. The cell pellets were lysed in 1.5 pellet volumes of NP-40 lysis buffer containing 10 mM Hepes (pH 7.5), 90 mM KCl, 1.0 mM magnesium acetate, 0.5% (v/v) Nonidet P-40, 2.0 mM fresh 2-mercaptoethanol, and 100 μg/ml fresh leupeptin. The buffer was added to the frozen pellet and left on ice until it had thawed. The cell pellets were then dispersed in the buffer and left on ice for at least 5 min. The crude lysate was centrifuged at 10,000*g* and 4 °C for 10 min before the supernatant was transferred to a clean tube. The cell lysate containing 200 μg protein was then combined with 2 μl of 10 cleavage buffer [comprising 100 mM Hepes (pH 7.5), 1 M KCl, 50 mM magnesium acetate, 10 mM ATP, and 0.14 M 2-mercaptoethanol], plus 2 μl of a 10 concentration of the desired 2–5A activator and sH_2O to a final reaction volume of 20 μl. Immediately after the addition of the 2–5A activator, incubation was initiated at 30 °C [29]. The positive control 2–5A sample was a gift from

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

Results

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

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

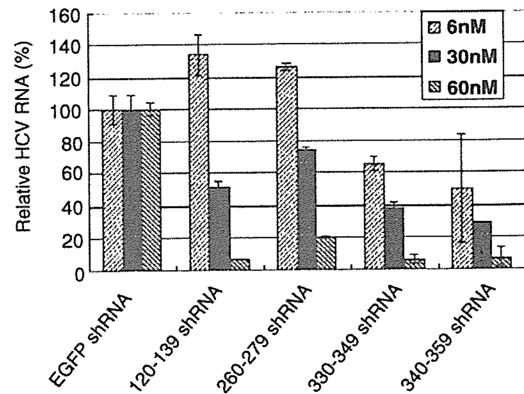

Fig. 2. Inhibition of HCV replication by shRNAs synthesized using the T7 RNA polymerase system in the HCV replicons. The effect of transfection with shRNAs or control EGFP HCV replication was measured by RT-PCR of HCV RNA 48 h after transfection. All values are shown as the percentages of the EGFP negative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

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

To date, in mammals, 11 TLRs have been identified that recognize pathogen-associated molecular patterns, such as bacterial cell wall materials, bacterial or viral genomic DNA and RNA, and small molecules. At least four TLRs (TLR-3, 7, 8, and 9) recognize and respond to mono-, oligo-, and polynucleotides of natural and/or synthetic origin. TLRs 3, 7, and 8 recognize viral and synthetic single-stranded and dsRNAs, such as polyI:C and siRNA [30].

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

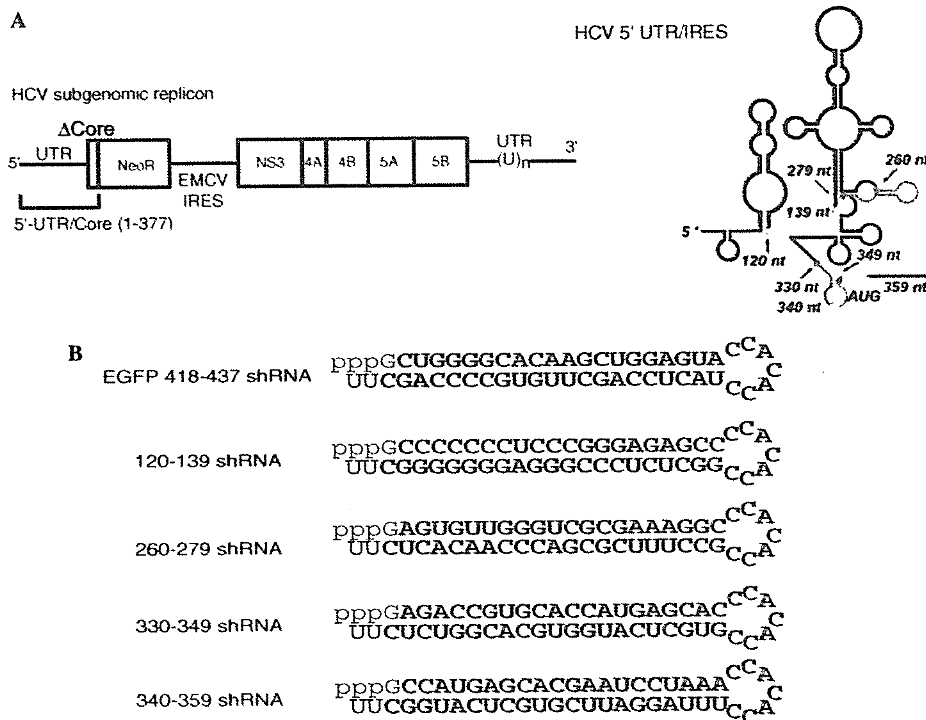

Fig. 1. shRNA-targeted regions of the HCV subgenomic replicons and shRNA sequences. (A) Schematic representation of HCV subgenomic replicons and shRNA targeted regions on HCV 5′-UTR/IRES secondary structure. (B) Sequences of shRNAs synthesized by phage polymerase. The control shRNA:EGFP-shRNA.

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

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

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

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

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

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

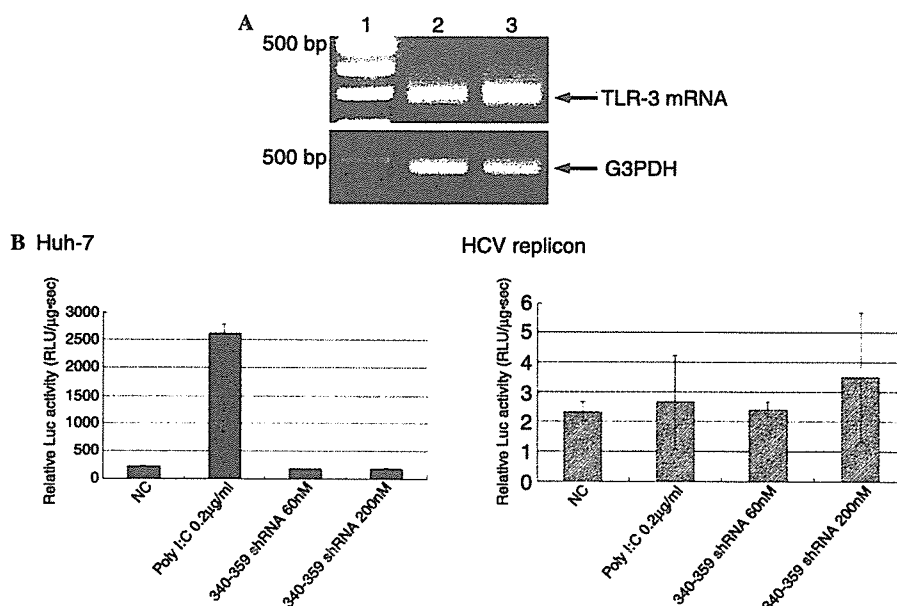

Fig. 3. Activation of IRF-3 after transfection of Huh-7 cells and HCV replicons with shRNAs synthesized using the T7 RNA polymerase system. (A) RT-PCR analysis of TLR-3 mRNA expression in Huh-7 cells and HCV replicons cells. The RT-PCR assay for TLR-3 mRNA was performed using TLR-3 mRNA-specific primers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

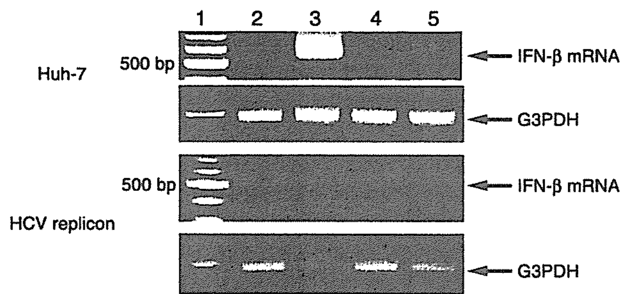

Fig. 4. RT-PCR analysis of IFN-β mRNA expression in Huh-7 cells and HCV replicons. The RT-PCR-amplified products were fractionated by electrophoresis on a 2.0% agarose gel and stained with ethidium bromide. Lane 1, DNA ladder; lane 2, negative control; lane 3, polyI:C (10 μg); lanes 4 and 5, 330-349-shRNA (60 and 200 nM, respectively).

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

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

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

Discussion

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

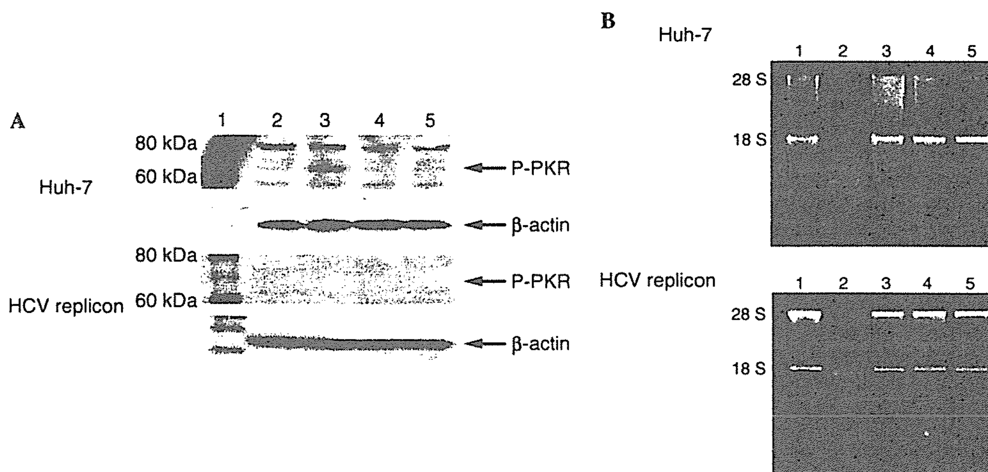

Fig. 5. Activation of PKR and RNase L with shRNAs synthesized using the T7 RNA polymerase system. (A) After 24 h, the Huh-7 cells and HCV replicons were lysed with lysis buffer. The lysates (80 μg protein/lane) were assayed by Western blot analysis with antibodies against phosphorylated PKR or β-actin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

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

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

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

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

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

Acknowledgments

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

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Abstract

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

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Keywords: HCV; Cyclosporin; NIM811; Interferon; Cyclophilin; Cyclosporine; Replication; Replicon; Inhibitor; Therapy

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

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

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

Materials and methods

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

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

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

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

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

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

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

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

Results

Response of HCV genome replication to treatment with CsA and NIM811

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

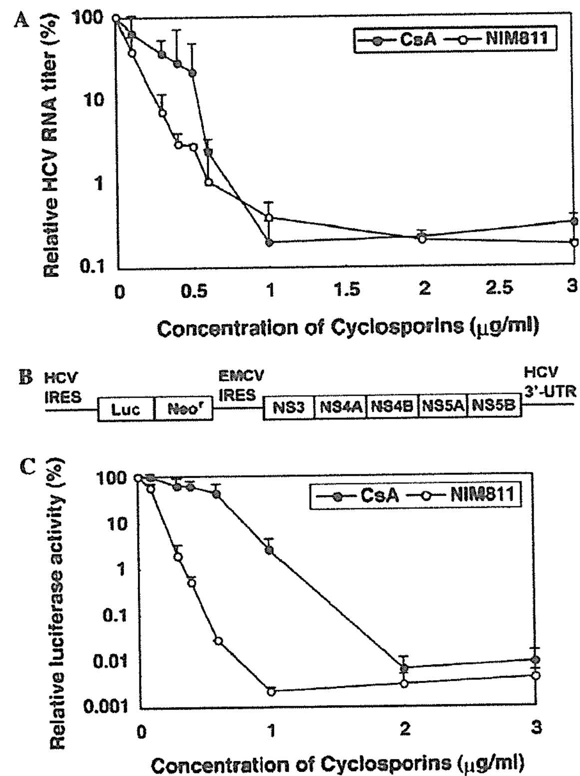

Fig. 1. Response curve of HCV RNA titers vs. the concentration of CsA and its nonimmunosuppressive analogue, NIM811. (A) NNC cells, harboring the HCV full genome replicon, were treated with either CsA or NIM811 for 7 days, and the HCV RNA extracted from these cells was quantified by real-time RT-PCR.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

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

Analysis of cotreatment with IFNα and either CsA or NIM811

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

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

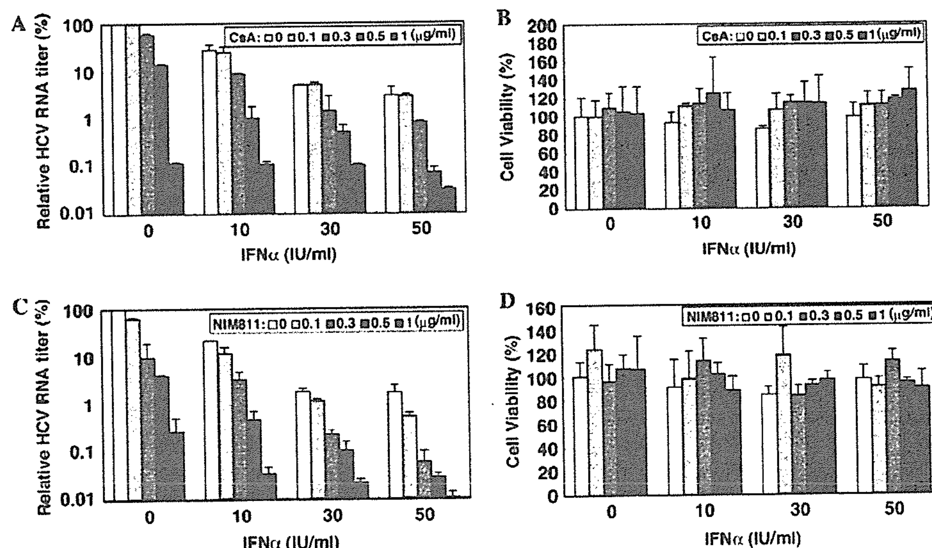

Fig. 2. The effects on HCV replication of cotreatment with both IFNα and cyclosporins. (A,C) NNC cells were treated with varying concentrations of either CsA (A) or NIM811 (C) in combination with various concentrations of IFNα for 7 days. HCV RNA levels were determined by real-time RT-PCR and are shown as percentages of the level in cells untreated (control). (B,D) The numbers of NNC cells treated with either CsA (B) or NIIM811 (D) in combination with IFNα for 2 days were determined to show the cytotoxicity of the drugs. The data represent means of the results from three independent experiments, with the standard deviation values indicated by error bars.

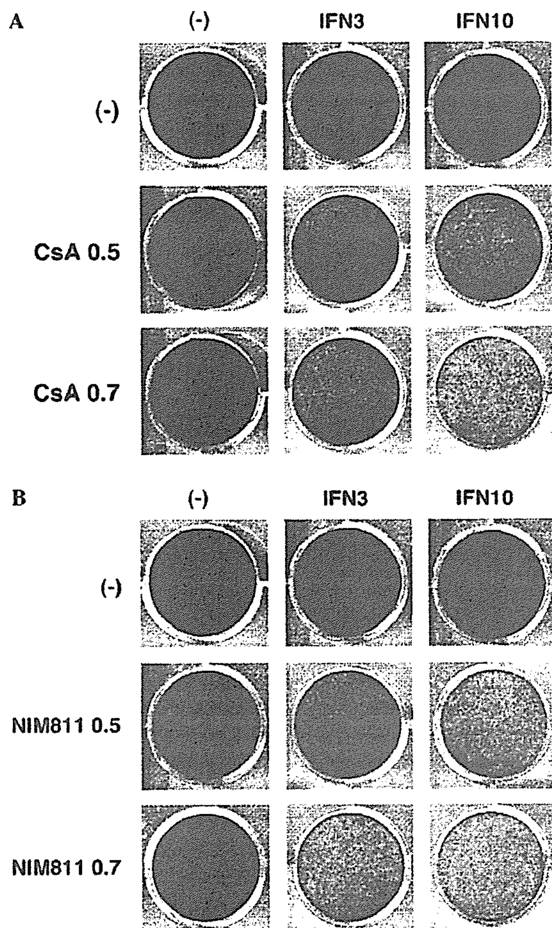

Fig. 3. Colony formation assay showing the effects of cotreatment with IFNα and cyclosporins, CsA (A) or NIM811 (B). NNC cells were treated with cyclosporins in combination with IFNα at the indicated doses in the presence of 500 μg/ml G418. After 2 weeks in culture, cells were fixed and stained with crystal violet.

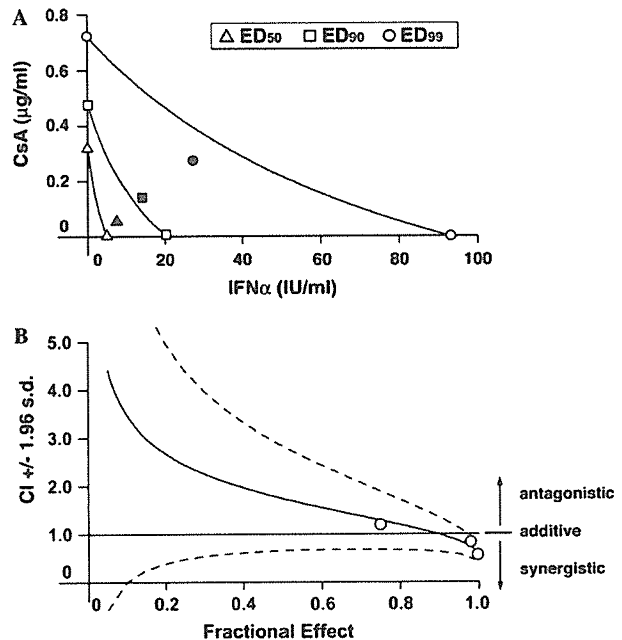

Fig. 4. Analysis of the combination treatment of CsA and IFNα using a Loewe additivity model. (A) Conservative isobologram determined by analyzing the data shown in Fig. 2A using the CalcuSyn program. The lines represent the effective doses (ED_x) of two drugs that would be required to attain X% inhibition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

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

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

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

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

To investigate the mechanisms of action for the enhancing effects of cyclosporins on the anti-HCV activity of

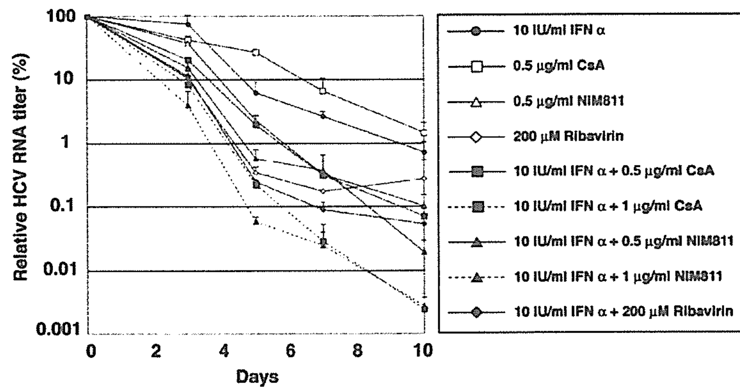

Fig. 5. Time course for the reduction of HCV RNA levels in NNC cells treated with CsA, NIM811, IFNα, or ribavirin. The levels of HCV RNA in the cells treated with the compounds for 3, 5, 7, and 10 days were determined by real-time RT-PCR and plotted vs. the days of treatment. The data represent the means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

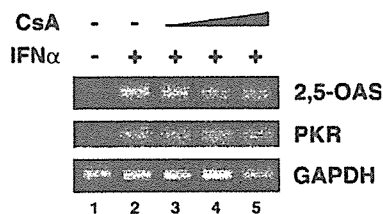

Fig. 6. Effects of the addition of CsA on the IFNα signal transduction pathway. NNC cells were treated either without (lane 1) or with 100 IU/ml IFNα (lanes 2–5) in combination with 0.5 μg/ml (lane 3), 1 μg/ml (lane 4), and 3 μg/ml (lane 5) CsA for 2 days. The mRNAs of 2′,5′-oligoadenylate synthetase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

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

Discussion

We previously reported [8] that CsA and NIM811 suppress HCV replication. In the present study, we evaluated the anti-HCV effects of CsA and NIM811 in detail and revealed that these compounds achieve multiple-log reduction of HCV RNA levels in a cell culture system. NIM811 exhibited a more potent anti-HCV activity than did CsA, especially at relatively low concentrations. We previously demonstrated [9] that CyPB is a cellular replication cofactor that regulates the function of NS5B. CsA suppressed HCV replication via the dissociation of CyPB from NS5B [9]. In addition, NIM811 is reported to bind CyP with higher affinity (about 2-fold) than does CsA [15]. Taken together, the stronger anti-HCV activity of NIM811 over CsA at low concentrations may be due to NIM811's higher binding affinity to CyPB. In actuality, the strength of suppression of cyclosporins against vaccinia virus correlates with their inhibition/binding activities to CyP [16], in agreement with the above explanation. The higher anti-HCV activity of NIM811 at relatively low concentrations may be important for anti-HCV therapies in vivo because the trough level of CsA in the peripheral blood during the employment of CsA as an immunosuppressive agent during liver transplantation is 0.2–0.3 μg/ml (Peak cyclosporin levels are 0.8–2.3 μg/ml) [17–19]. Thus, NIM811 may eliminate HCV at the concentrations that are permissive in vivo, although other factors, such as pharmacodynamics and side effects, must be validated. Moreover, CsA might exert some pro-viral effects due to its immunosuppressive activity against T lymphocytes [20–22] in addition to its antiviral effects in hepatocytes. Thus, NIM811, which has little immunosuppressive function [15,23], is expected to be preferable to CsA for eliminating HCV in vivo.

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