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JOURNAL OF VIROLOGY, May 2006, p. 4510–4520 Vol. 80, No. 9
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.9.4510–4520.2006

Diverse Effects of Cyclosporine on Hepatitis C Virus Strain Replication

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Received 18 December 2005/Accepted 10 February 2006

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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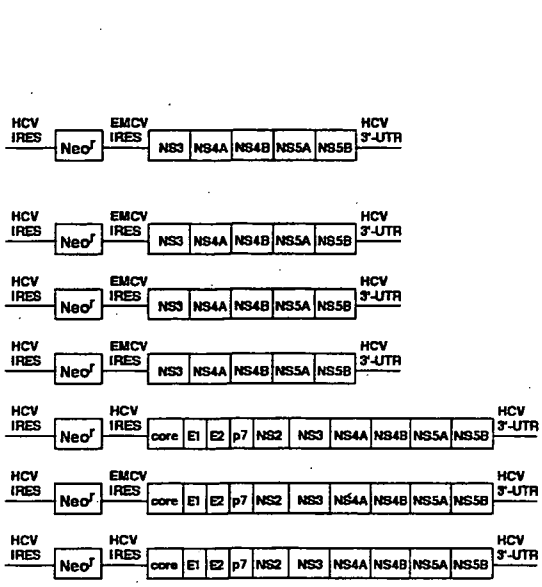

HCV strain (genotype)	replicon cell clone	sensitivity to CsA
NN (1b)	MH-14, #50-1 MH14#W31	highly sensitive
Con1 (1b)	SN1	highly sensitive
O (1b)	sO	highly sensitive
JFH1 (2a)	JFH1#4-1, JFH1#2-3	less sensitive
NN (1b)	NNC#2	highly sensitive
Con1 (1b)	SN1A#2	highly sensitive
Con1 (1b)	SNC#7	highly sensitive

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^r), 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 (7×10^3 to 15×10^3 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 I377/NS3-3' (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

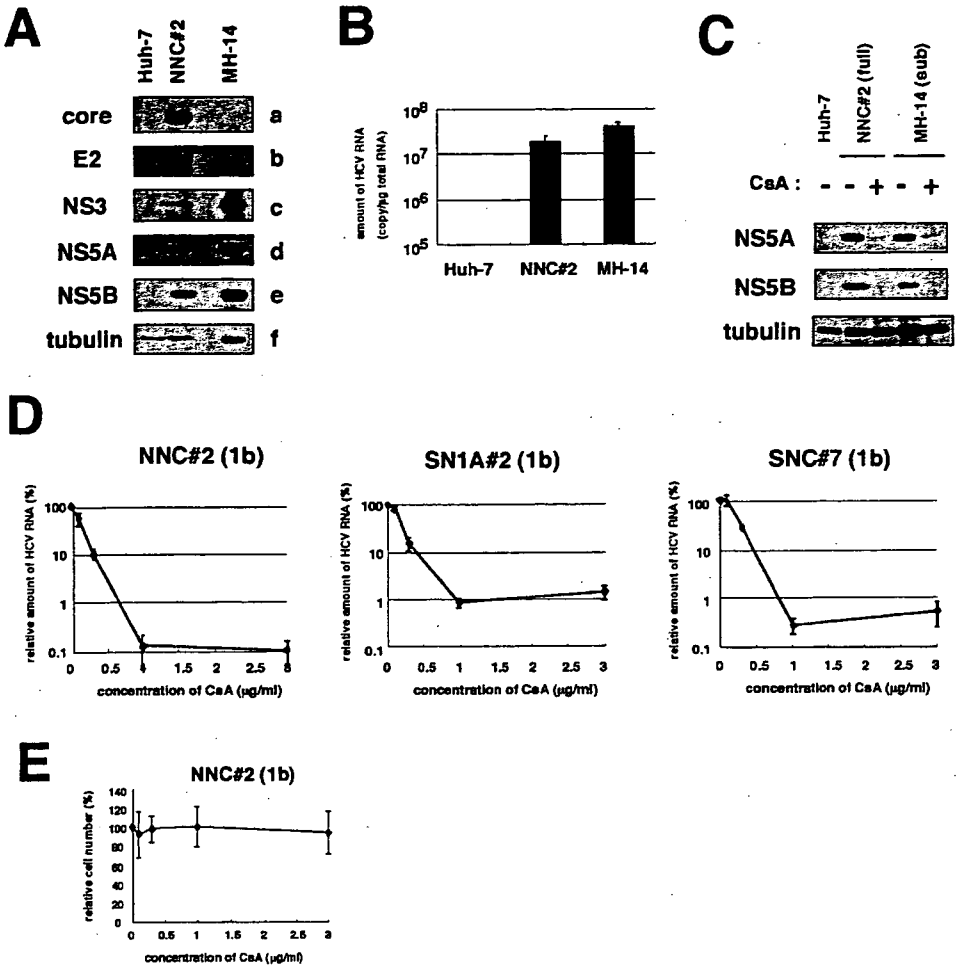

FIG. 2. CsA suppressed the replication of HCV genome, irrespective of the presence of the structural proteins. (A) Detection of HCV proteins from NNC#2 (NN/1b/FL) genome-length replicon. Core (a), E2 (b), NS3 (c), NS5A (d), NS5B (e), and tubulin (f) in Huh-7, NNC#2 (NN/1b/FL), and MH-14 (NN/1b/SG) cells 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-μg/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-β-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 ^{35}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 activity 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

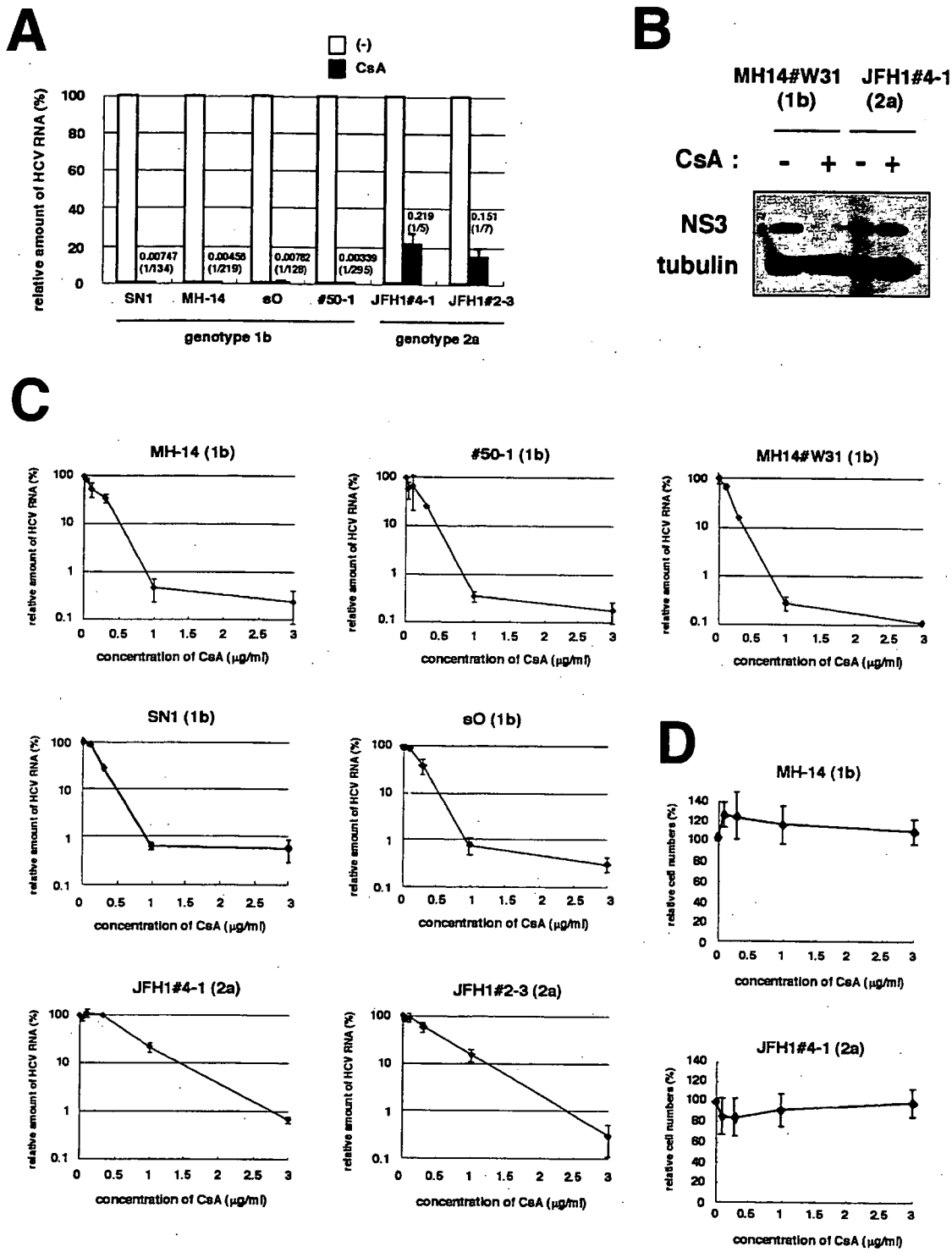

FIG. 3. Replication of a genotype 2a strain, JFH1, was less sensitive to CsA. (A) Sensitivity to CsA of HCV genotype 1b and JFH1 replicons. SN1 (Con1/1b/SG), MH-14 (NN/1b/SG), sO (O/1b/SG), #50-1 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and JFH1#2-3 (JFH1/2a/SG) cells, carrying HCV subgenomic replicon, were treated with 1-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.

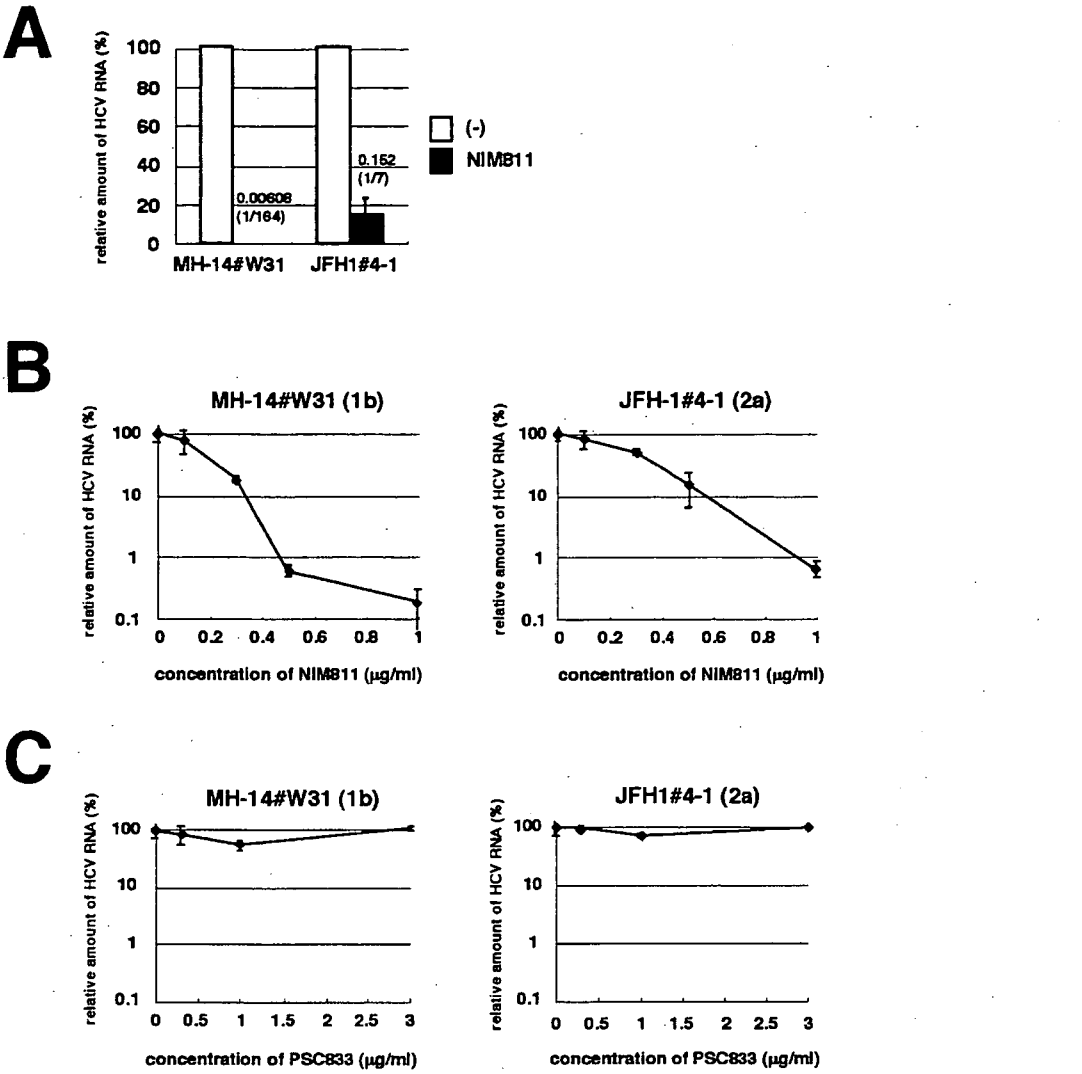

FIG. 4. JFH1 replication was less sensitive to a CsA derivative, NIM811. (A) MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG) cells were treated with 0.5-μg/ml NIM811 for 7 days. HCV RNA titers were quantified as described in the legend to Fig. 3A. White bars, no treatment; black bars, 0.5-μg/ml NIM811. (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

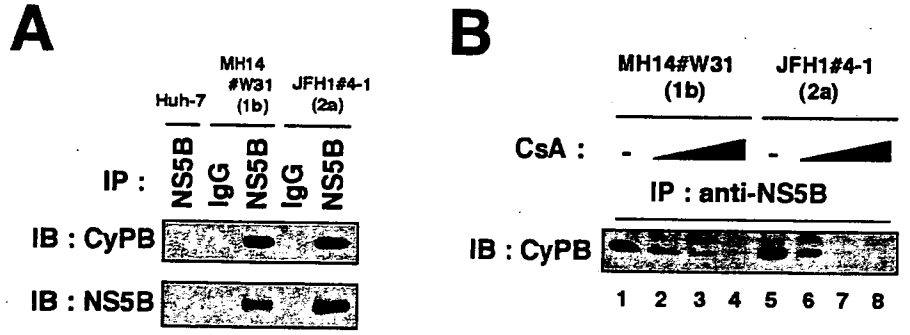

FIG. 5. Interaction of HCV NS5B with CyPB in the JFH1 replicon. (A) Coimmunoprecipitation of endogenous CyPB with NS5B. Lysates from MH14#W31 (NN/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells as a negative control were used for immunoprecipitation with normal mouse immunoglobulin G (IgG) or anti-NS5B 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

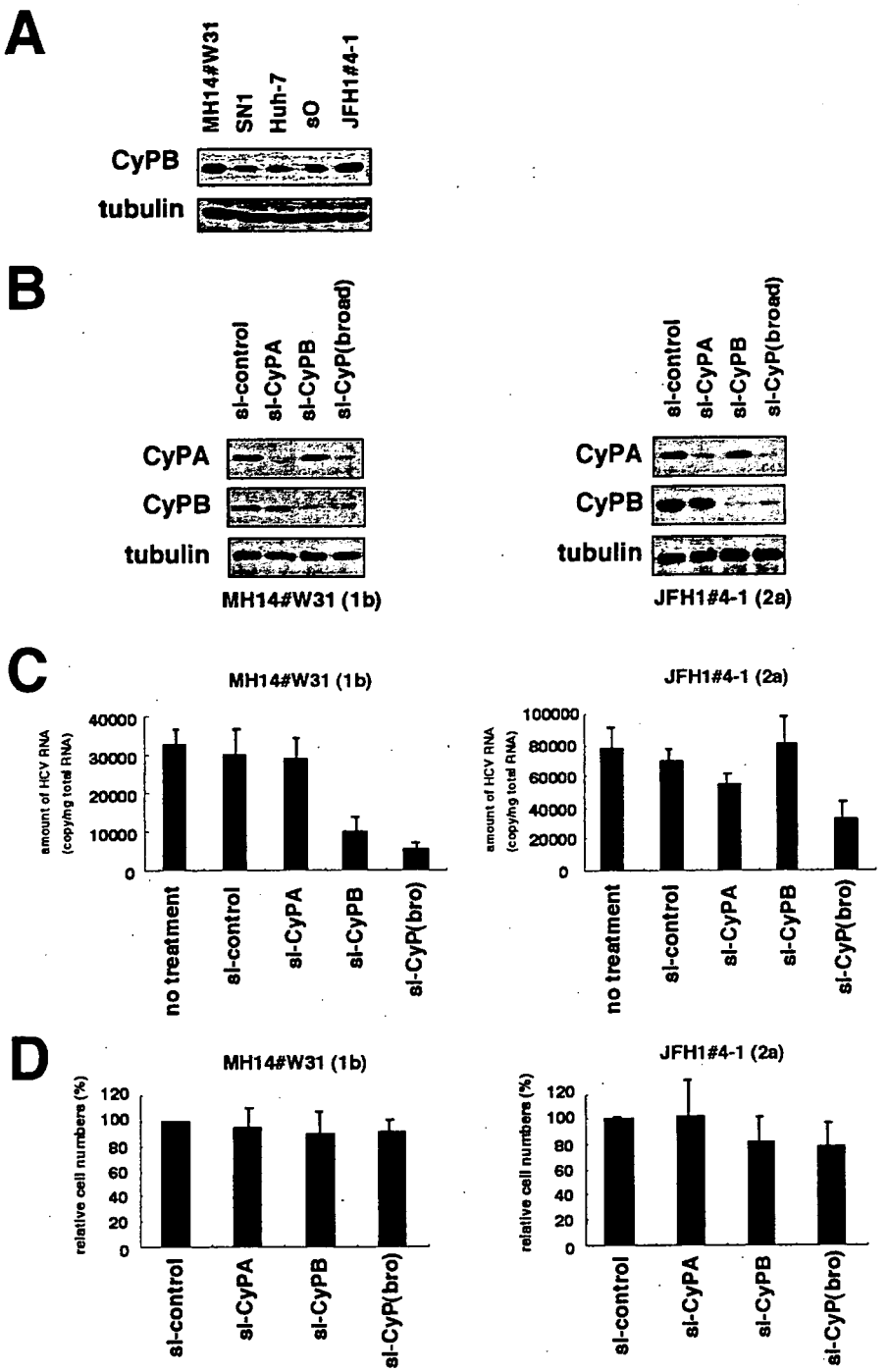

FIG. 6. CyPB in HCV replication of genotype 1b and JFH1. (A) Expression level of endogenous CyPB protein (top) and tubulin as an internal control (bottom) in MH14#W31 (NN/1b/SG), SN1 (Con1/1b/SG), sO (O/1b/SG), JFH1#4-1 (JFH1/2a/SG), and Huh-7 cells. (B) Knockdown of endogenous CyP proteins. MH14#W31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

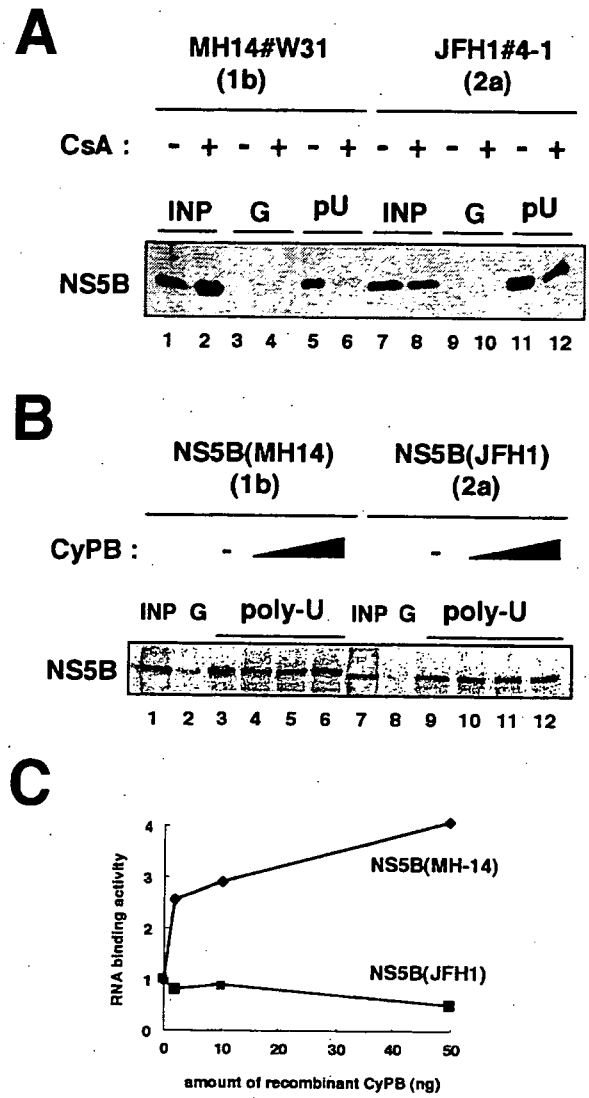

FIG. 7. RNA binding capacity of JFH1 NS5B was independent of CyPB. (A) An RNA-protein binding precipitation assay was performed using MH14#W31 (NN/1b/SG) cells (lanes 1 to 6) and JFH1#4-1 (JFH1/2a/SG) cells (lanes 7 to 12) as described in Materials and Methods. MH14#W31 (NN/1b/SG) and JFH1#4-1 (JFH1/2a/SG)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

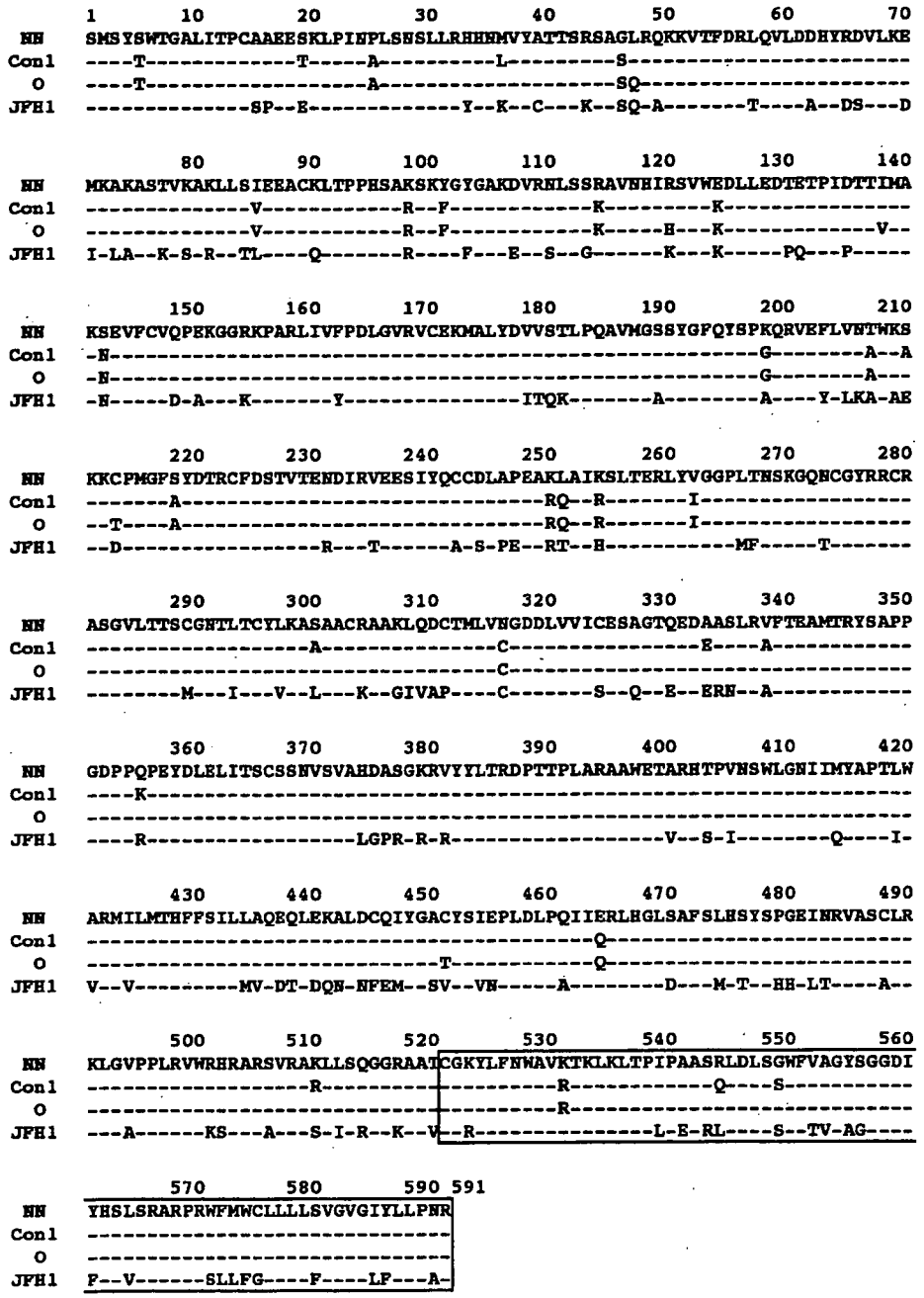

FIG. 8. Amino acid sequence alignment of NS5B encoded by HCV strains NN, Con1, O, and JFH1. The numbers above the sequence indicate the amino acid numbers. Conserved residues are shown by dashes. The region spanning 521 to 591 aa, which is involved in the interaction with CyPB, is boxed.

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.

ACKNOWLEDGMENTS

We thank H. Takahashi and M. Hosaka for preparing replicon cells and generating plasmids. We are grateful to A. Takamizawa at Osaka University, I. Fukuya at Osaka University, and M. Kohara at Tokyo Metropolitan Institute of Medical Science for antibodies and R. Bartenschlager at Heiderberg University for the I377/NS3-3′ sequence. We also appreciate Novartis (Basel, Switzerland) for providing the CsA derivatives NIM811 and PSC833.

This work was supported by grants-in-aid for cancer research and for the second-term comprehensive 10-year strategy for cancer control from the Ministry of Health, Labor, and Welfare; grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology; and grants-in-aid from the Research for the Future from the Japanese Society for the Promotion of Science, the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan, and Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation.

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

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Received 1 March 2006

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.

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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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.03.059

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 *Aff*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.

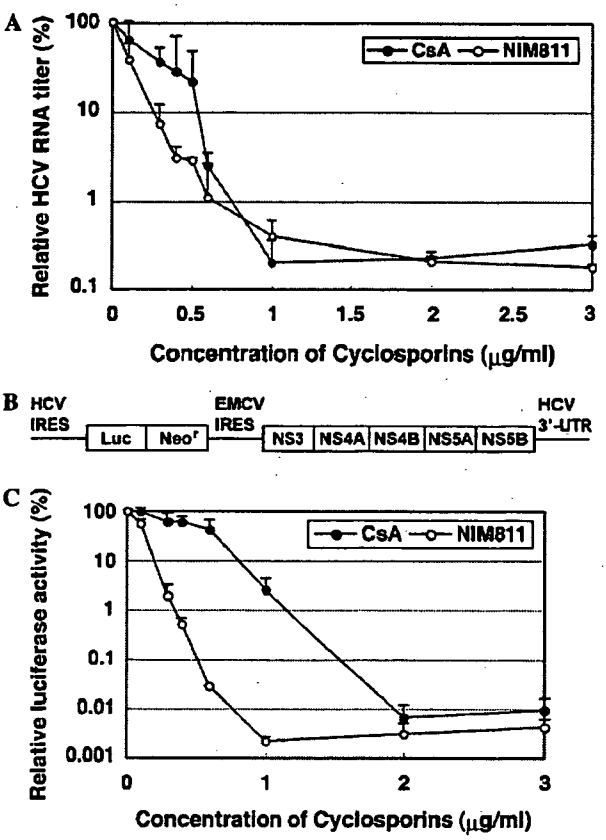

Fig. 1. Response curve of HCV RNA titers vs. the concentration of CsA and its nonimmunosuppressive analogue, NIM811. (A) NNC cells, harboring the HCV full genome replicon, were treated with either CsA or NIM811 for 7 days, and the HCV RNA extracted from these cells was quantified by real-time RT-PCR. 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

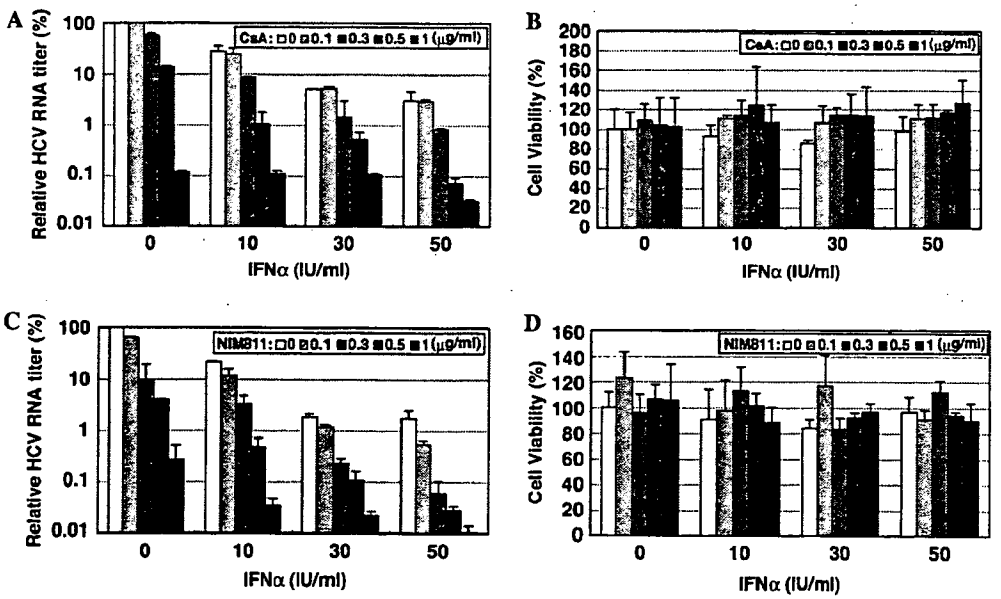

Fig. 2. The effects on HCV replication of cotreatment with both IFNα and cyclosporins. (A,C) NNC cells were treated with varying concentrations of either CsA (A) or NIM811 (C) in combination with various concentrations of IFNα for 7 days. HCV RNA levels were determined by real-time RT-PCR and are shown 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.

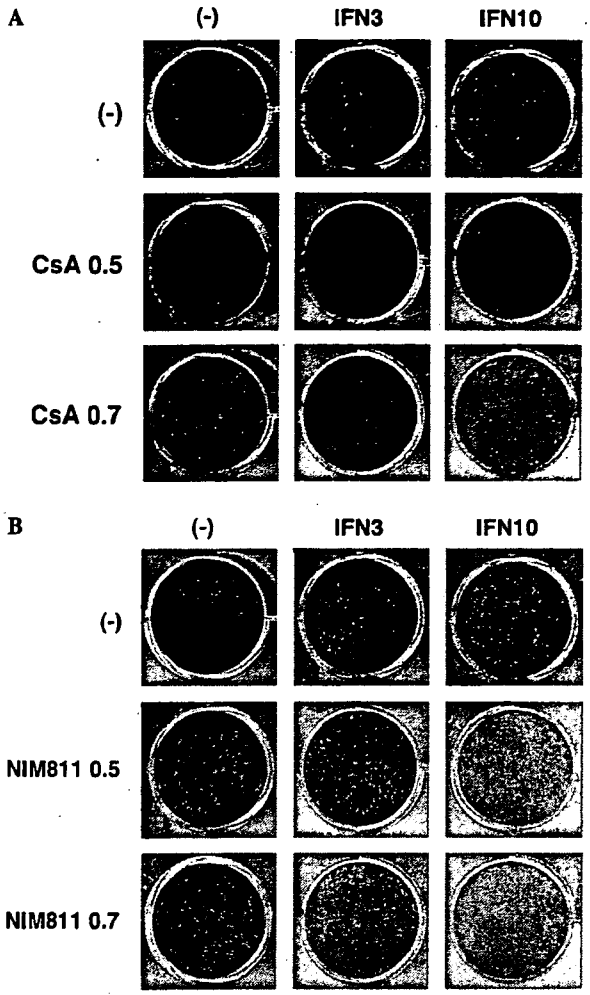

Fig. 3. Colony formation assay showing the effects of cotreatment with IFNα and cyclosporins, CsA (A) or NIM811 (B). NNC cells were treated with cyclosporins in combination with IFNα at the indicated doses in the presence of 500 μg/ml G418. After 2 weeks in culture, cells were fixed and stained with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

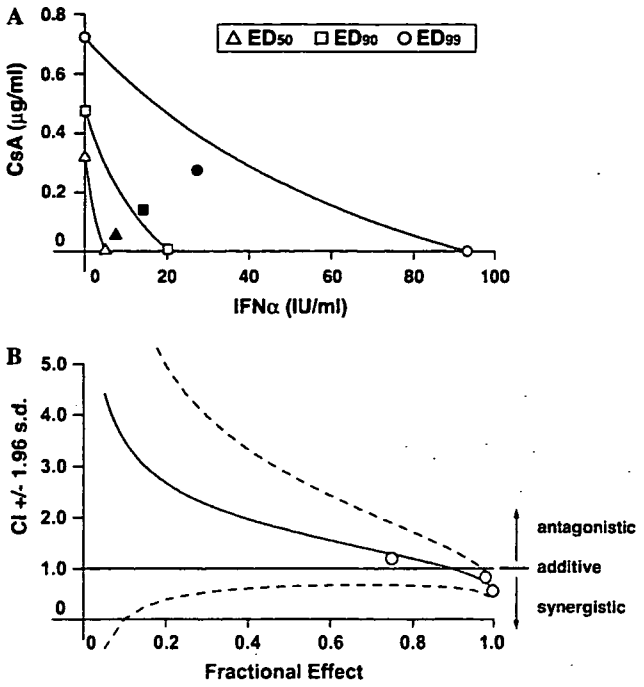

Fig. 4. Analysis of the combination treatment of CsA and IFNα using a Loewe additivity model. (A) Conservative isobologram determined by analyzing the data shown in Fig. 2A using the CalcuSyn program. The lines represent the effective doses (ED_x) of two drugs that would be required to attain *X*% inhibition 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

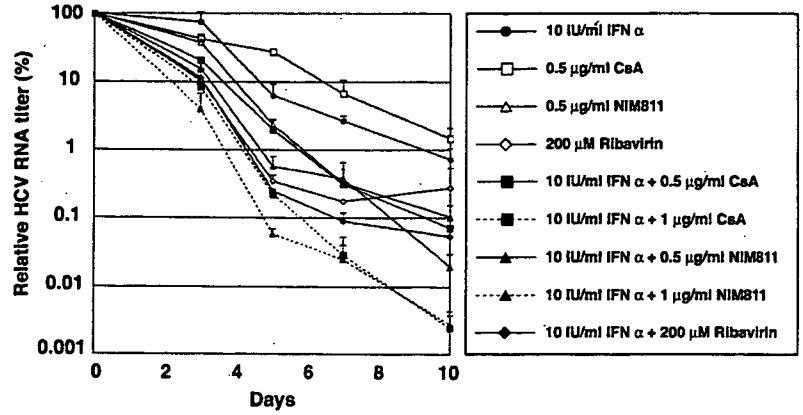

Fig. 5. Time course for the reduction of HCV RNA levels in NNC cells treated with CsA, NIM811, IFNα, or ribavirin. The levels of HCV RNA in the cells treated with the compounds for 3, 5, 7, and 10 days were determined by real-time RT-PCR and plotted vs. the days of treatment. The data represent the means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

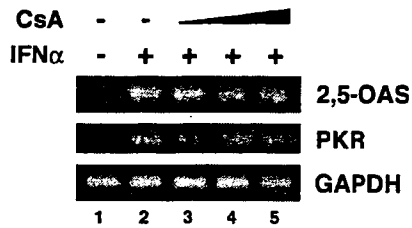

Fig. 6. Effects of the addition of CsA on the IFNα signal transduction pathway. NNC cells were treated either without (lane 1) or with 100 IU/ml IFNα (lanes 2–5) in combination with 0.5 μg/ml (lane 3), 1 μg/ml (lane 4), and 3 μg/ml (lane 5) CsA for 2 days. The mRNAs of 2′,5′-oligoadenylate synthetase (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

as protease inhibitors or polymerase inhibitors, which are now undergoing clinical trials, directly target viral proteins and inhibit their enzymatic activity. Because cyclosporins such as CsA and NIM811 target a cellular factor, CyPB, as described above, these compounds could serve as an additional type of anti-HCV agent. Moreover, viruses resistant to cyclosporins are less likely to occur, since antiviral compounds that target cellular factors generally induce less drug resistance than those inhibiting viral proteins; this difference is due to the high mutation rates of RNA viruses [26–30]. Thus, this novel anti-HCV candidate could provide an alternative strategy to combat HCV.

Acknowledgments

We thank Novartis (Basel, Swizerland) for providing the CsA derivative, NIM811. This work was supported by Grants-in-Aid for cancer research and for the second-term comprehensive 10-year strategies for cancer control from the Ministry of Health, Labor and Welfare. We were also supported by Grants-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, by Grants-in-Aid for the Research for the Future program from the Japanese Society for the Promotion of Science, and by Grants-in-Aid for the Program for Promotion of Fundamental Studies in Health Science from the Organization for Pharmaceutical Safety.

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Journal of Hepatology 46 (2007) 26–36

Journal of Hepatology

www.elsevier.com/locate/jhep

Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes

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See Editorial, pages 1–5

***Background/Aims*: The development of an efficient in vitro infection system for HCV is important in order to develop new anti-HCV strategy. Only Huh7 hepatocyte cell lines were shown to be infected with JFH-1 fulminant HCV-2a strain and its chimeras. Here we aimed to establish a primary hepatocyte cell line that could be infected by HCV particles from patients' sera.**

***Methods*: We transduced primary human hepatocytes with human telomerase reverse transcriptase together with human papilloma virus 18/E6E7 (HPV18/E6E7) genes or simian virus large T gene (SV40 T) to immortalize cells. We also established the HPV18/E6E7-immortalized hepatocytes in which interferon regulatory factor-7 was inactivated. Finally we analyzed HCV infectivity in these cells.**

***Results*: Even after prolonged culture HPV18/E6E7-immortalized hepatocytes exhibited hepatocyte functions and marker expression and were more prone to HCV infection than SV40 T-immortalized hepatocytes. The susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infection was further improved, in particular, by impairing signaling through interferon regulatory factor-7.**

***Conclusions*: HPV18/E6E7-immortalized hepatocytes are useful for the analysis of HCV infection, anti-HCV innate immune response, and screening of antiviral agents with a variety of HCV strains.**

***Keywords*: Immortalization; Primary hepatocytes; HCV infection; IRF-7; IRF-3; HPV18/E6E7; Innate immune response**

1. Introduction

Infection with Hepatitis C virus (HCV) is a serious problem worldwide since 3% of the world's population is chronically infected [1]. Chronic HCV may lead to liver cirrhosis and hepatocellular carcinoma. Current standard therapy utilizes the combination of pegylated interferon-α and ribavirin, which results in a sustained response in only 30–60% of patients [2–5]. Many patients, however, do not qualify for or tolerate standard therapy [6]. Thus, it is important to develop an efficient in vitro infection system for HCV to facilitate the discovery of new anti-HCV strategies. Only Huh7 cell line is permissive for replication, infection and release of the fulminant hepatitis-derived HCV-2a (JFH-1) *strain* and its chimeric derivatives [7–9]. No other hepatocyte cell lines are able to support HCV replication efficiently.

Received 5 June 2006; received in revised form 24 July 2006; accepted 1 August 2006; available online 30 October 2006

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0168-8278/$32.00
doi:10.1016/j.jhep.2006.08.018

Normal human hepatocytes are the ideal system in which to study HCV infectivity. When cultured in vitro, however, they proliferate poorly and divide only a few times [10]. Continuous proliferation could be achieved however by introducing oncogenes, such as Simian virus large tumor antigen (SV40 T) [11]. This often resulted in tumor development [12] together with numerical (aneuploidy) and structural (aberrations) chromosome abnormalities [13]. The human papilloma virus E6E7 genes (HPV/E6E7) immortalized multiple cell types that were phenotypically and functionally similar to the parental cells [14–20]. As yet, no human hepatocytes have been immortalized with HPV18/E6E7.

We established a human primary non-neoplastic hepatocyte cell line transduced with the HPV18/E6E7 that retained primary hepatocyte characteristics even after prolonged culture, and were more prone to HCV infection than those cells immortalized with SV40 T antigen. We further improved the susceptibility of HPV18/E6E7-immortalized hepatocytes to HCV infectivity by impairing interferon regulatory factor-7 (IRF-7) expression. These cells are useful to assay infectivity of HCV strains other than JFH-1, HCV replication, innate immune system engagement of HCV, and screening of anti-HCV agents. This infection system using non-neoplastic cells also suggested that IRF-7 plays an important role in eliminating HCV infection.

2. Materials and methods

2.1. Cell cultures

We obtained the approval of the Ethical Committee of Kyoto University for the use of human hepatocytes and sera obtained from HCV-positive patients. Informed consent was obtained from both the hepatocyte donor and HCV-positive patients. Primary hepatocytes (P.H.) were cultured as described [21]. HeLa, 293, Huh-7.5, and PH5CH8 cells were cultured as previously described [22]. For three-dimensional (3D) cultures, Mebiol Gel (Mebiol Inc.) was prepared according to the manufacturer's instructions.

2.2. Plasmids construction

The SV40 T, hTERT and HPV/E6E7 fragments from pAct-SVT, PCX4neo/hTERT, and pLXSN-E6E7 plasmids were inserted into pCSII-EF-RFA plasmid creating the pCSII-EF-SVT, pCSII-EF-hTERT, and pCSII-EF-E6E7 plasmids, respectively. The full-length IRF-3 and IRF-7 genes were cloned by RT-PCR using total RNA isolated from 293 cells as a template and were inserted into pcDNA3 vector. Dominant-negative forms of IRF-3 (DNIRF-3) and IRF-7 (DNIRF-7) were constructed by PCR amplification of the coding region for amino acid residues 108–427 of IRF-3 and 237-514 of IRF-7, respectively. The amplified IRF-3 fragment was cloned into pcDNA3 in frame with a FLAG epitope tag generating pcFLAG-DNIRF-3. The amplified IRF-7 fragment was cloned into pLXSH in frame with HA epitope tag generating pLXSH-HA-DNIRF-7. The pIFNβ promoter-luc and pIFNα promoter-luc plasmids were gifts from Dr. Taniguchi of the Tokyo University. The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were purchased from Invivogen (USA).

2.3. Immunoblot analysis

Immunoblot analysis was performed as described previously [22]. We used anti-SV40 T (Santa Cruz), anti-HPV18/E7 (Santa Cruz), anti-tubulin (Sigma), anti-FLAG (Sigma), and anti-HA (Sigma) antibodies.

2.4. Transfection, small interfering RNA silencing and luciferase assays

Transfection of plasmid DNA was performed using Effectene transfection reagent (Qiagen) as recommended by the manufacturer. The pLXSH-HA-DNIRF-7 plasmid was transfected into the HuS-E/2 clone; transfectants were selected in 100 μg/ml hygromycin B (Gibco). The psiRNA-hIRF-3 and psiRNA-hIRF-7 plasmids were separately transfected into HuS-E/2 cells followed by Zeocin (250 μg/ml) selection. After two weeks of continuous selection, cells were infected with HCV. Luciferase assays were conducted as previously described [22]. The results are presented as relative light units (RLU) normalized to the total content of protein in the cell lysates.

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR

Using 250 ng of total RNA as a template, we performed RT-PCR with a one-step RNA PCR kit (Takara) according to the manufacturer's instructions. The primer sets and reaction conditions used are detailed in Table 1. To measure HCV-RNA titers by real-time RT-PCR, we collected RNA from infected wells. Five hundred nanograms of total cellular RNA was analyzed for the quantity of HCV-RNA as previously described [23].

2.6. HCV infection experiment

HCV infection experiment from serum was done as mentioned before [22]. HCV-infected-serums were titrated and 1×10^5 HCV-RNA copies/ml were used for each infection experiment. Concentrated culture medium for HCV/JFH1-producing cells was prepared as previously described [7]. HCV titer in the concentrated medium was measured, adjusted and added to the cells as mentioned above.

2.7. Blocking of HCV infectivity by anti-CD81

Inhibition of HCV infectivity was performed by blocking CD81 as previously described [7].

3. Results

3.1. Establishment of immortalized primary human hepatocytes

Primary hepatocytes were isolated from liver tissue obtained from a 9-year-old male patient with Primary Hyperoxaluria who had undergone liver transplantation. Hepatocytes were left unmanipulated or transduced with CSII-EF-hTERT alone or in combination with CSII-EF-SVT or CSII-EF-E6E7 to enhance the efficiency of immortalization. After six weeks only cells transduced by the combination of hTERT and either LT or HPV18/E6E7 continued to proliferate. Initially appearing colonies with a growth advantage were picked up and expanded. SV40 T-immortalized cell clones were named HuS-T cells and given numbers from 1 to 7,