Materials and Methods

Drugs and Chemicals

CsA was purchased from Sigma Chemical Co (St Louis, MO). FK506 was from Alexis Biochemicals (Lausen, Switzerland). Recombinant human interferon alfa-2b was obtained from Schering-Plough (Kenilworth, NJ). Cyclosporin D (CsD) is a cyclosporin analogue that has valine at position 2 of CsA instead of L-α-aminobutyric acid. CsD was provided by Novartis Pharma Inc (Tokyo, Japan).

Cell Culture

Huh7 and 293 cells were maintained in Dulbecco's modified Eagle medium (Sigma Chemical Co) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing HCV replicon were cultured in medium containing 500 μg/mL G418 (Wako, Osaka, Japan).

HCV Replicons

An HCV subgenomic replicon plasmid, pRep-Feo (Figure 1*A*), was derived from pRep-Neo (originally pHCVIb-neo-delS[12]). The pRep-Feo expresses a fusion gene comprising firefly luciferase (Fluc) and neomycin phosphotransferase, as described elsewhere.[11,13] Another replicon plasmid, pRep-BSD, expresses the blasticidin S (BSD) resistance gene. Replicon RNA was synthesized in vitro by T7-RNA polymerase (Promega, Madison, WI) and transfected into Huh7 cells by electroporation.[11] After culture in the presence of G418 (Wako), cell lines stably expressing the replicons were established (Huh7/Rep-Neo and Huh7/Rep-Feo, respectively).

Retrovirus Vectors Expressing Short Hairpin RNA

Oligodeoxyribonucleotides encoding short hairpin RNA (shRNA) sequences were synthesized and cloned just downstream of human U6 promoter in the plasmid pUC19, and the U6-shRNA cassette was subcloned into a retrovirus plasmid vector, pLNCX2 (Clontech, Palo Alto, CA). Sequences of the shRNAs were as follows: cyclophilin A (CypA) shRNA#3, 5′- GCA ATG TCG AAG AAC ACG GTG GGG TTG ACG GAG CTC GGT CAG CCT CAT CGT GTT CTT CGG CAT TGC TTT TTT -3′; CypA shRNA#441, 5′- GTG ATC TTC TTG CTG GTC TTG CCA TTC CTG GAG CTC GAG GAG TGG CAG GAT CAG CAG GAA GAT CAC TTT TTT -3′; cyclophilin B (CypB) shRNA#294, 5′- GTG AAG TCT CCG CCC TGG ATC ATG AAG TCG GAG CTC GGG CTT CAT GAT CCG GGG CGG AGG CTT CAT TTT TTT -3′; CypB shRNA#467, 5′- GCT TGC CAT CTA GCC AGG CTG TCT TGA CTG GAG CTC GAG TCG AGG CAG CCT GGT TAG ATG GCG AGC TTT TTT -3′; cyclophilin C (CypC) shRNA#0, 5′- GGT AGC AGC AGC CGA GGA CCC GGG CCC ATG GAG CTC GAT GGG CCT GGG TCT TCG GCT GCT GCT GCT TTT TTT -3′; and CypC shRNA#291, 5′- GTG CCA TCT CCA GTG GTG ATG TCA CCT CCG GAG CTC GGG AGG TGG CAT CAT CAT TGG AGG TGG CAC TTT TTT -3′.

Six negative control shRNA vectors were used that had reverse sequences of the respective targets: CypA shRNA#3 rev, CypA shRNA#441 rev, CypB shRNA#294 rev, CypBi#467 rev, CypCi#0 rev, and CypCi#291 rev. Another negative control, shRNA control, directed toward an unrelated target, the Machado–Joseph disease gene. A positive control shRNA vector, HCV shRNA, directed toward the 5′-untranslated region of HCV RNA.[13] The retrovirus plasmids were transfected into a packaging cell line, Retro Pack PT67 (Clontech), and the culture supernatant was applied to Huh7 cells with 4 μg/mL polybrene (Sigma Chemical Co). Huh7 cell lines stably expressing shRNA were established by culture in the presence of 300 μg/mL G418.

Reporter and Expression Plasmids

A plasmid, pISRE-TA-Luc (Invitrogen, Carlsbad, CA), expressed the Fluc gene under the control of the interferon stimulation response element (ISRE).[14] A nuclear factor of activated T cells (NFAT) reporter plasmid, pNFAT-Luc, was purchased from Stratagene (La Jolla, CA). A plasmid, pRL-CMV (Promega), which expresses the *renilla* luciferase (Rluc) gene under the control of the cytomegalovirus early promoter/enhancer, was used to normalize transfection efficiency. A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Figure 1*B*). The plasmid expressed a bicistronic RNA, in which Rluc was translated in a cap-dependent manner and Fluc was translated by HCV IRES-mediated initiation. A plasmid, pcDNA-NS3-5, expressed the HCV nonstructural gene spanning from NS3 to 5 of the HC-J4 clone (Figure 1*C*).[15]

Cyclophilin-Expressing Plasmid Vectors

The coding sequences of human cytoplasmic CypA, CypB, and CypC were amplified by reverse-transcription polymerase chain reaction using the following polymerase chain reaction primers: CypA-S, 5′- CAC CAT GGT CAA CCC CAC CGT GTT CTT CGA -3′; CypA-AS, 5′- TTC GAG TTG TCC ACA GTC AGC AAT GGT GAT -3′; CypB-S, 5′- CAC CAT GAA GGT GCT CCT TGC CGC CGC CCT -3′; CypB-AS, 5′- CTC CTT GGC GAT GGC AAA GGG CTT CTC CAC -3′; CypC-S, 5′- CAC CAT GGG CCC GGG TCC TCG GCT GCT GCT -3′; CypC-AS, 5′- CCA ATC AGC GAT CTC AAC CAC AAA AGG CGT -3′. The amplicons were cloned into pcDNA 3.1 using the Directional TOPO Expression Kit (Invitrogen) to make pcDNA-CypA, -CypB, and -CypC, respectively. The expressed proteins contained a C-terminal polyhistidine (His) tag, which allowed their detection by anti-His antibodies (Invitrogen).

Transfection of Plasmids

Transfection of plasmids was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Luciferase Assays

Luciferase activities were measured using a luminometer (Lumat LB9501; Promega) using the Bright-Glo Luciferase Assay System (Promega) or the Dual-Luciferase Reporter Assay System (Promega). Assays were performed in triplicate.

Northern Blotting

Total cellular RNA was extracted from cells using Isogen (Wako). The RNA was separated by denaturing agarose-formaldehyde gel electrophoresis and transferred to a Hybond-N+ nylon membrane (Amersham Biosciences Corp, Piscataway, NJ). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for β-actin. The signals were detected in a chemiluminescence reaction using the Digoxigenin Luminescent Detection Kit (Roche, Mannheim, Germany) and were visualized using a Fluoro-Imager (Roche).

Western Blotting

Ten micrograms of total cell lysate was separated using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with antibodies against NS5A (BioDesign, Saco, ME), anti-His (C Term; Invitrogen), β-actin (Sigma Chemical Co), and BiP/GRP78 (BD Biosciences, Franklin Lakes, NJ) and then incubated with peroxidase-conjugated secondary antibodies. Immunoreactions were performed using a BM Chemiluminescence Blotting Substrate (Roche) and visualized by Lumi-Imager F1 (Roche) according to the manufacturer's protocol.

Stable Colony Formation Assays

Cells were transfected with a replicon, Rep-BSD, and cultured in the presence of 150 μg/mL BSD (Invitrogen) in the medium. BSD-resistant cell colonies were obtained after ~2 weeks in culture. The colonies were stained with neutral red solution (Invitrogen) and counted.

Dimethylthiazol Carboxymethoxyphenyl Sulfophenyl Tetrazolium Assays

To evaluate cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were per-

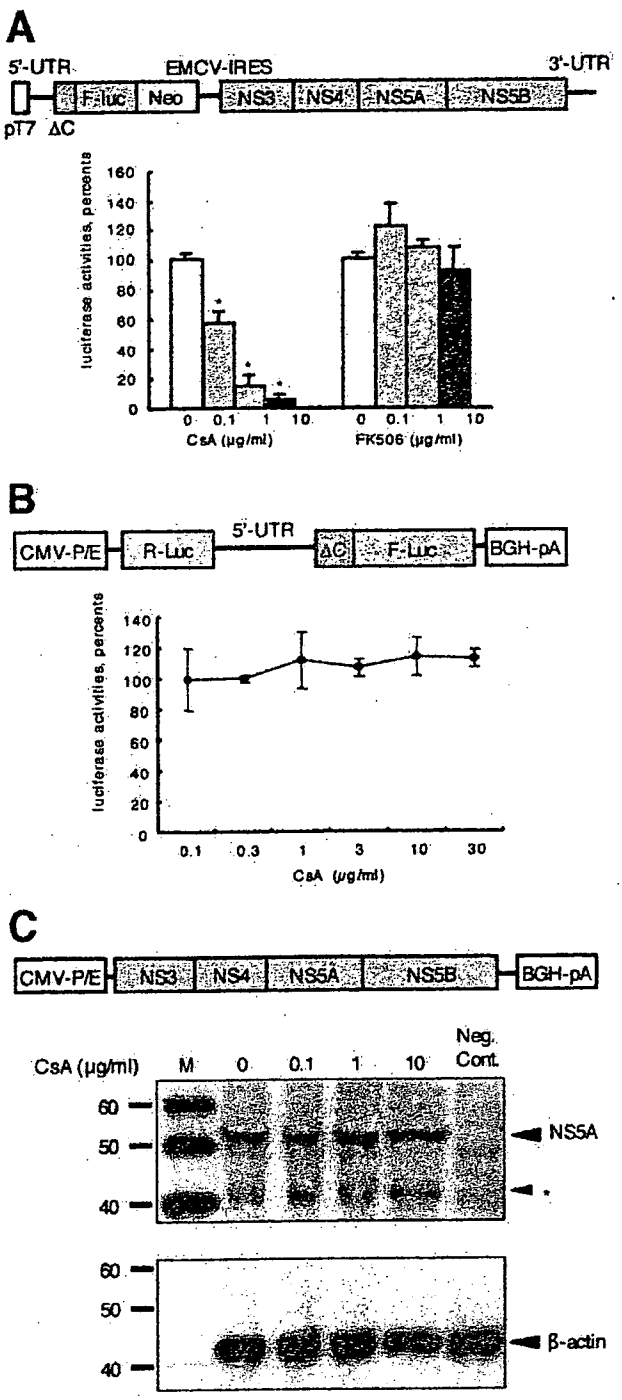


Figure 1. CsA does not influence HCV IRES-mediated translation or the processing of HCV nonstructural proteins. (*A*) Huh7/Rep-Feo cells that constitutively express the HCV Feo replicon (*top*) were cultured in the presence of CsA and FK506 at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured after 48 hours of culture. Assays were performed in triplicate. *Error bars* indicate mean ± 2 SD. **P* values of less than .05. (*B*) A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc (*top*), was stably transfected into Huh7 cells (Huh7/neo-Rluc IRES-Fluc). The cells were cultured with CsA at the concentrations indicated, and dual luciferase activities were measured after 48 hours of treatment. Values are displayed as ratios of Fluc to Rluc. *Error bars* indicate mean ± 2 SD. (*C*) Western blotting. An expression plasmid, pcDNA-NS3-5 (*top*), which expressed HCV nonstructural genes NS3–NS5, was used. The plasmid was transfected into 293 cells. At 24 hours after transfection, the cells were treated with the amounts of CsA indicated (0, 0.1, 1, and 10 μg/mL). The cells were harvested after 48 hours of treatment. The cell lysate was separated on NuPAGE 4%–12% Bis-Tris gels, transferred onto a polyvinylidene difluoride membrane, and incubated with a monoclonal anti-NS5A antibody or an anti–β-actin antibody. *Lane 1*, protein size markers. The pcDNA-NS3-5 was transfected into 293 cells and cultured in the absence (*lane 2*) or the presence of CsA at 0.1, 1 and 10 μg/mL, respectively (*lanes 3–5*). Neg. Cont., untransfected 293 cells. The *asterisk* indicates partially cleaved NS5A protein.

formed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's directions.

Statistical Analyses

Statistical analyses were performed using Student *t* test; *P* values of less than .05 was considered statistically significant.

Results

CsA Does Not Suppress Translation of HCV RNA or Posttranslational Processing of the Viral Protein

Treatment of Huh7/Rep-Feo cells with CsA at concentrations of 0, 0.1, 1, and 10 μg/mL resulted in a dose-dependent decrease in internal luciferase activities as reported (Figure 1*A*). The luciferase activities were 57% and 15% of the CsA-negative control at concentrations of 0.1 and 1 μg/mL, respectively. FK506, in contrast, did not suppress expression of the replicon at concentrations 10–100 times above the clinical range of 5–15 μg/mL.[16] We investigated which stage of the HCV replication cycle is affected by CsA. To determine whether CsA suppresses HCV IRES-dependent translation, we used an Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc (Huh7/neo-Rluc IRES-Fluc; Figure 1*B*). Treatment of these cells with CsA resulted in no significant change of the internal luciferase activities at concentrations of CsA that suppressed expression of the HCV replicon. We next determined whether CsA suppresses posttranslational self-cleavage of the viral nonstructural polyprotein. A plasmid, pcDNA-NS3-5, was transfected into 293 cells and cultured in the presence of CsA. Western blotting of the cell lysate showed that the expression levels of NS5A and other NS proteins did not change significantly (Figure 1*C*). These results suggested that the effect of CsA on HCV replication does not involve suppression of IRES-mediated viral protein synthesis or a blockade of posttranslational cleavage of the nonstructural proteins.

CsA Does Not Activate Interferon-Stimulated Genes or NFAT-Induced Genes in Huh7 Cells

To determine whether the action of CsA and FK506 involves activation of interferon-stimulated gene responses in the host Huh7 cells, the ISRE-luciferase reporter plasmid, pISRE-TA-Luc, was transfected into Huh7/Rep-Neo cells and cultured in the presence of CsA and FK506 at concentrations of 0, 0.1, 1, and 10 μg/mL. In contrast to interferon, which elevated ISRE-promoter activities significantly, CsA and FK506 showed no effects on the luciferase activities at 48 hours after transfection (Figure 2*A*). These results suggest that the action of CsA on the intracellular replication of the HCV rep-

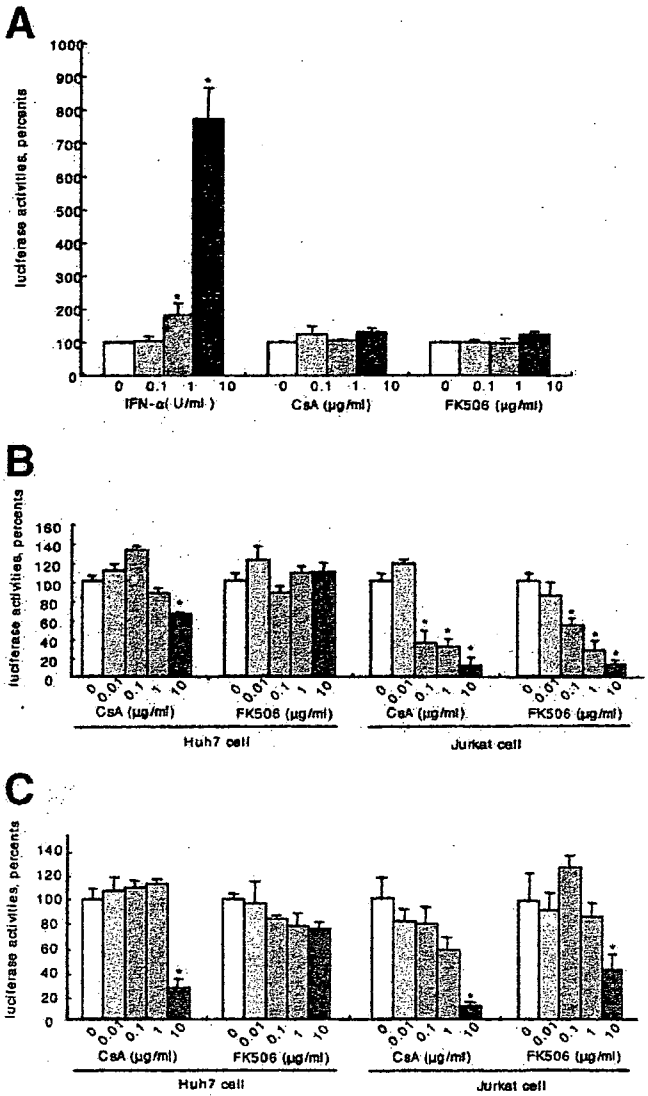


Figure 2. CsA does not affect interferon-stimulated responses and NFAT activities. (*A*) The ISRE-luciferase plasmids were transfected into Huh7/Rep-Neo cells and cultured in the presence of CsA and FK506 at concentrations of 0, 0.1, 1, and 10 μg/mL. As a positive control, cells were treated with interferon alfa at concentrations of 0, 0.1, 1, and 10 U/mL. Luciferase activities were measured at 48 hours after transfection. **P* values of less than .05. (*B*) The NFAT-luciferase plasmid was transfected into Huh7 cells and into Jurkat cells and cultured in the presence of CsA or FK506 at concentrations of 0, 0.01, 0.1, 1, or 10 μg/mL. A plasmid, pRL-CMV, which expresses the *renilla* luciferase gene under the control of the cytomegalovirus early promoter/enhancer, was used to monitor transfection efficiency. Luciferase activities were measured at 48 hours after transfection. Values are displayed as ratios of Fluc to Rluc. *Error bars* indicate mean ± 2 SD. **P* values of less than .05. (*C*) The control *renilla* luciferase activities in each sample. Assays were performed in triplicate. *Error bars* indicate mean ± 2 SD. **P* values of less than .05.

licon does not involve activation of interferon-stimulated genes.

The principal mechanism of the immune suppressive action of CsA and FK506 is to inhibit antigen-stimulated activation of cellular calcineurin, a phosphatase that catalyzes the dephosphorylation and nuclear translocation of a transcription factor, NFAT, to induce expression of inflammatory cytokines and their receptors.[8,17,18] To study the effects of CsA and FK506 on the calcineurin-NFAT pathway in Huh7 cells, the NFAT-luciferase reporter plasmid, pNFAT-luc, was transfected into cells and treated with CsA and FK506 at concentrations of 0, 0.01, 0.1, 1, and 10 μg/mL. CsA or FK506 treatment of T lymphocyte–derived Jurkat cells significantly suppressed NFAT activities. On the contrary, neither CsA nor FK506 showed effects on the NFAT-luciferase activities in Huh7/Rep-Neo cells (Figure 2*B*). Although the NFAT-luciferase activity was apparently decreased at 10 μg/mL of CsA, which was ~20 times higher than the clinically available concentration (0.1–0.5 μg/mL),[19] the control *renilla* luciferase activities expressed from a cotransfected plasmid, pRL-CMV, tended to decrease at this concentration. These results show that CsA does not influence NFAT-mediated signaling pathways in Huh7 cells and that the presence of the anti-HCV effect of CsA in the absence of the NFAT suppression indicates that the action of CsA on HCV is independent of the calcineurin/NFAT-mediated cell signaling pathway.

Knockdown of Cyclophilins Suppresses HCV Replication

Because of the differing effects of CsA and FK506 on HCV replication and the lack of effect of both drugs on the calcineurin/NFAT pathways in Huh7 cells, we speculated that the antiviral action of CsA against HCV may be mediated by cellular cyclophilins, a family of proteins that show specific binding to CsA. Cyclophilins possess a peptidyl prolyl *cis-trans* isomerase (PPIase) activity, which catalyzes *cis-trans* conversion of peptide bonds preceding proline and contributes to the correct protein folding as a molecular cochaperone.[20–22] Cyclophilins comprise at least 15 subtypes and are present in every compartment of the cell, including the cytoplasm, endoplasmic reticulum (ER), and nucleus.[23] Among them, we have investigated CypA, CypB, and CypC, which localize predominantly in the cytoplasm or microsome fraction where the HCV replicase complex is located.[24] To study effects of the cyclophilin expression on the replication of the HCV replicon, we suppressed expression of CypA, CypB, and CypC using plasmid vectors expressing shRNA.

Huh7/Rep-Feo cells were transfected with shRNA-expression vectors CypA shRNA#3, CypA shRNA#441, CypB shRNA#294, CypB shRNA#467, CypC shRNA#0, and CypC shRNA#291. Western blotting revealed that expression of the respective cyclophilins was suppressed by the shRNA vectors (Figure 3*B*). Concomitantly, the luciferase activities of Huh7/Rep-Feo cells were suppressed significantly by the transfection of each cyclophilin-directed shRNA. On the contrary, transfection of shRNAs that have reverse sequences of the corresponding shRNA vectors did not show any significant effects of HCV replication (Figure 3*A*). To investigate further the effects of the cyclophilin knockdown on HCV replication, we quantified the colony-forming ability of the HCV replicon in the cell lines in which the cyclophilins were stably knocked down. The 6 cyclophilin-directed shRNA-expression vectors were transfected individually into Huh7 cells by the retroviral transduction, and stable knocked-down cell lines were established for each cyclophilin. A replicon, Rep-BSD, was transfected into these 6 cell lines and into cells transfected with negative and positive control shRNAs and cultured in the presence of BSD. As shown in Figure 4, numbers of the BSD-resistant colonies were ~700 and ~800 in naive Huh7 and Huh7/shRNA negative control, respectively; however, as observed in the transient transfection assays (Figure 3), numbers of the BSD-resistant colonies were markedly reduced in the cells stably transfected with shRNA for CypA, CypB, and CypC (Figure 4).

Inhibition of HCV Replication by CsD

CsD is a cyclosporin analogue that lacks the immunosuppressive activity of CsA (<10%).[25] CsD shows binding to cyclophilin but has attenuated effects on the calcineurin/NFAT pathway. Treatment of Huh7/Rep-Feo with CsD showed that the luciferase activities were inhibited significantly, as much as for CsA (Figure 5*A*). Also, replicon RNA in Huh7/Rep-Feo, which was detectable in CsA-negative control cells by Northern blotting (Figure 5*B*), decreased substantially following treatment with CsD at concentrations of 1 and 3 μg/mL. Similarly, by Western blotting (Figure 5*C*), the expression of HCV NS5A protein was shown to be reduced by corresponding amounts following treatment with CsD and with CsA.

Unfolded Protein Response Agents Suppress HCV Replication

It has been reported that cyclosporins induce a cellular stress response called the unfolded protein response, which is elicited by accumulation of misfolded proteins in the ER.[26–28] Because the replicase complex of

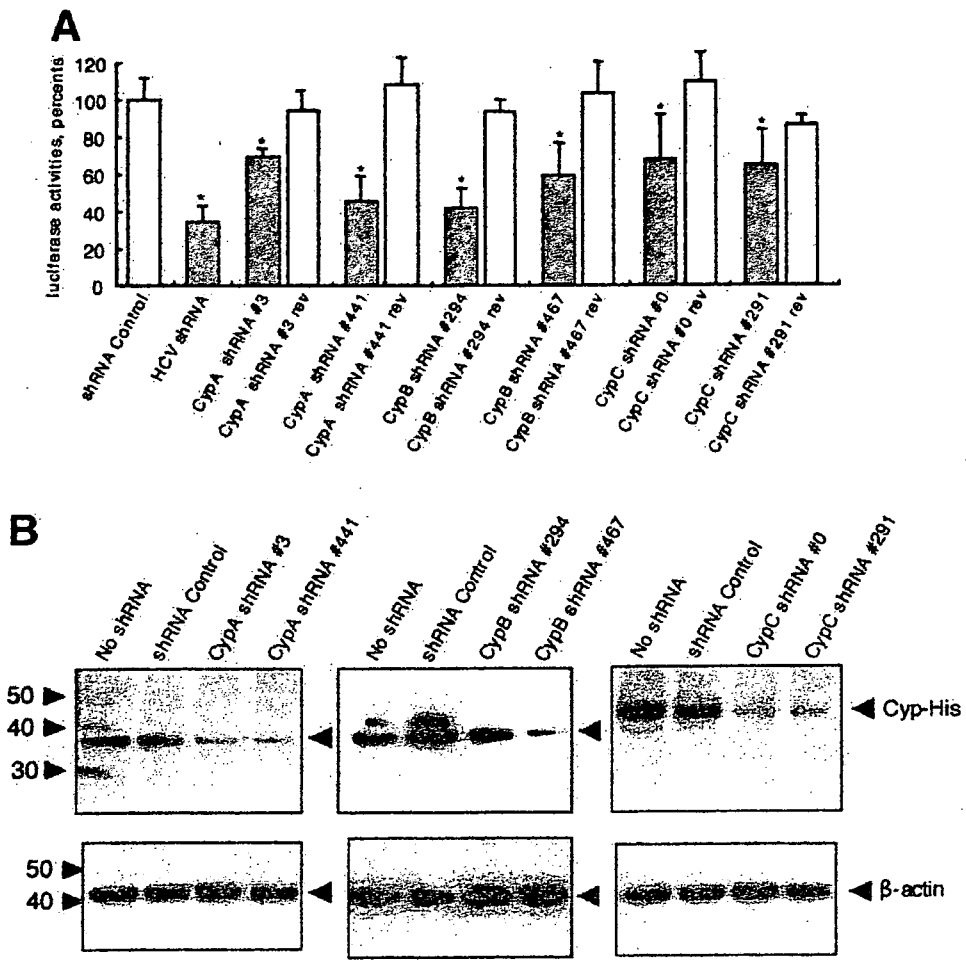


Figure 3. Knockdown of cyclophilins suppresses HCV replication. (*A*) Huh7/Rep-Feo cells were transfected with shRNA-expression plasmid vectors CypA shRNA#3, CypA shRNA#441, CypB shRNA#294, CypB shRNA#467, CypC shRNA#0, and CypC shRNA#291, which are directed against different regions of the respective cyclophilin genes. As negative and positive controls, shRNA control or an HCV shRNA was used as described.[13] We also transfected negative controls shRNA-expression plasmids that have reverse sequences of the corresponding shRNA vectors. Luciferase assays were performed at 48 hours after transfection. *Error bars* indicate mean ± 2 SD. **P* values of less than .05. (*B*) Western blotting. Cotransfection of the indicated shRNA-expression vectors and the cyclophilin-expressing vectors into Huh7 cells was performed. The expression of each cyclophilin protein contained a C-terminal His tag, which allowed its detection by anti-His antibodies. Ten micrograms of total cellular protein was separated by polyacrylamide gel electrophoresis and transferred onto the membrane. The membrane was incubated with anti-His antibodies or anti–β-actin antibodies (Sigma Chemical Co).

HCV RNA is localized in the ER,[24] we explored the possibility that drug-induced disruption of ER function may cause alterations in HCV replication. Western blotting revealed that treatment of Huh7 cells with CsA resulted in increased expression of BiP/GRP78 as reported (Figure 6*A*), while treatment with FK506 did not affect BiP/GRP78 expression. We next treated Huh7/Rep-Feo cells with reagents that induce an unfolded protein response: thapsigargin, mercaptoethanol, and tunicamycin. Treatment of the cells with thapsigargin, which causes depletion of calcium in the ER by inhibition of the Ca^{2+} adenosine triphosphatase transporter irreversibly, and mercaptoethanol, which disrupts disulfide bond formation, resulted in significant decreases in the internal luciferase activities to 79% and 38% of the control at concentrations of 0.001 and 0.003 μg/mL of thapsigargin and to 61% and 17% of the control at concentrations of 0.01 and 0.03 μg/mL of mercaptoethanol, respectively (Figure 6*B*). MTS assays did not show significant cytotoxic effects at those concentrations, suggesting that these effects are not due to cytotoxic cell death (Figure 6*C*). On the contrary, treatment with tunicamycin, which inhibits asparagines (N)-linked glycosylation, did not show significant effects on HCV replication at concentrations below the cytotoxic level.

Discussion

Several recent reports suggest that CsA is clinically effective in the treatment of HCV infection[29] and

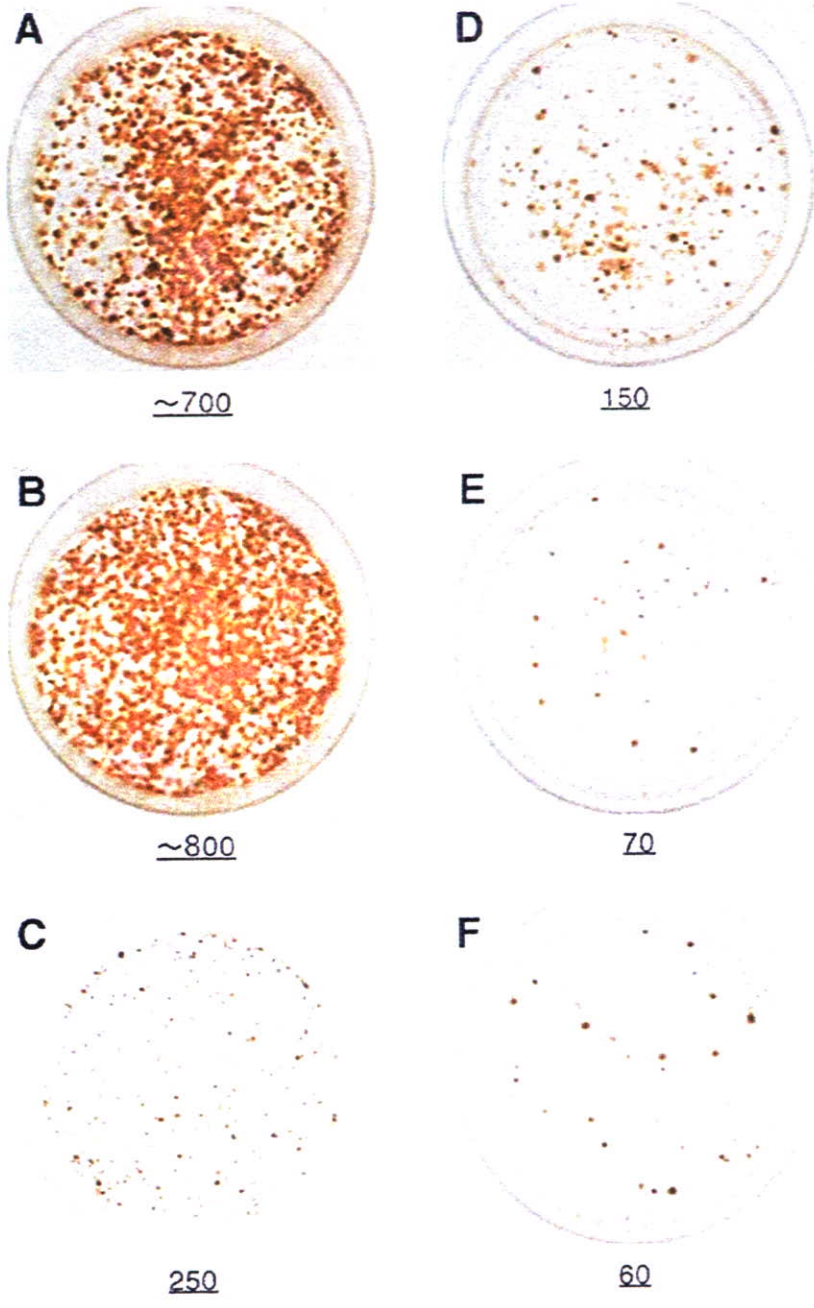
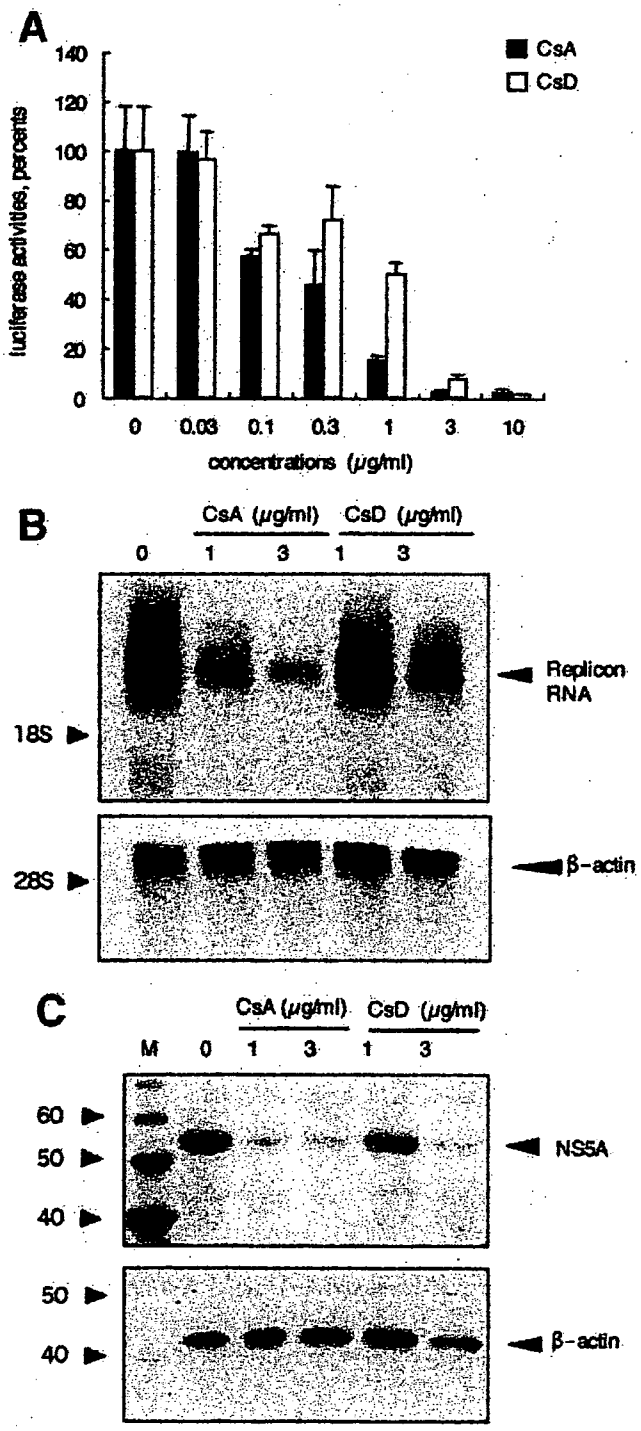


Figure 4. The replicon colony-forming activities of cell lines in which cyclophilins have been stably knocked down by shRNA. Colony-forming activities of HCV replicon were quantified in the cell lines in which cyclophilins were stably knocked down by shRNA-expression vectors. The 6 cyclophilin-directed shRNA-expression vectors were stably transfected into Huh7 cells by retroviral transduction. A replicon, Rep-BSD, was transfected into the cell lines, respectively, and along with cell lines transfected with negative and positive control shRNAs cultured in the presence of BSD. Two weeks after the transfection, the cell colonies were visualized by treating with neutral red solution. Numbers below the plates refer to the approximate numbers of the cell colonies. (*A*) Naive Huh7, (*B*) Huh7/shRNA control, (*C*) Huh7/HCV shRNA#331, (*D*) Huh7/CypA shRNA#3, (*E*) Huh7/CypB shRNA#294, and (*F*) Huh7/CypC shRNA#291.

that CsA has antiviral activity against HCV in vitro.[9,10] Watashi et al reported the in vitro effect of CsA on HCV replication using an HCV replicon system and a cultured cell line that supports HCV replication.[9] However, little is understood about the mechanisms of its action against HCV replication and the mechanism of its clinical efficacy. Our present results show that the action of CsA did not involve the calcineurin/NFAT-mediated pathway that is shared with FK506 (Figure 2*B*), showing that the anti-HCV effect of CsA is not associated with its immunosuppressive activity. On the other hand, knockdown of CypA, CypB, and CypC expression by shRNA suppressed HCV replication substantially (Figures 3 and 4). Furthermore, CsD, an analogue of cyclosporin with weak immunosuppressive activity but that retains the ability to bind cyclophilins, was similarly effective in suppressing HCV replication (Figure 5). Collectively, it was shown that the anti-HCV action of the cyclosporins is through blockade of the activities of cellular cyclophilins.

In our results, both CsA and FK506 suppressed NFAT-mediated luciferase reporter activities in a T lymphocyte–derived cell line, while neither CsA nor FK506 suppressed NFAT activities in hepatoma-derived Huh7 cells (Figure 2*B*). It has been reported that CsA shows divergent effects of NFAT-mediated luciferase activity

among cells of different origins, possibly because NFAT does not participate in autoregulatory activation of its own promoter in CsA-nonresponding cells.[9,30,31] With these findings, although our results may not completely exclude the partial involvement of the NFAT-mediated pathway, the major action of CsA against HCV replication is not through the calcineurin/NFAT pathway but through cyclophilin-mediated mechanisms.



The PPIase activity of cyclophilins has been reported by Takahashi et al and other researchers.[20–22] Through this activity, cyclophilins contribute to the maturation of several proteins, such as carbonic anhydrase[32] and the human immunodeficiency virus *gag* protein.[33,34] Furthermore, the enzymatic activity of cyclophilins may underlie several other functions of these proteins, including cell signaling, mitochondrial function, molecular chaperone activity, RNA splicing, stress response, gene expression, and regulation of kinase activity.[21,35] As for HCV, the viral structural and nonstructural proteins are processed from a single polyprotein of 3000 amino acids by ER membrane-bound signal peptidases and by 2 self-coded serine proteases.[36] It has been reported that folding and assembly of HCV proteins require interaction with ER chaperone proteins such as calreticulin, BiP, and HSP90.[37,38] In this study, the replication levels of the HCV replicon decreased substantially following the knockdown of CypA, CypB, and CypC. Moreover, our preliminary data have shown that overexpression of cyclophilins attenuated the effects of CsA on HCV replication. These results imply that the effects of CsA on HCV replication involve functional blockade of cyclophilins and, more importantly, that constitutive expression of the cytoplasmic cyclophilins such as CypA, CypB, and CypC may be necessary for HCV replication, possibly through assisting functional maturation of the viral proteins as molecular co-chaperone proteins. Although it is still unclear how cyclophilins support the processing of HCV proteins and the replication of the viral genome, these molecules could be potential targets to counteract HCV infection and replication.

One third of nascent proteins are transported to the ER, where they are subjected to posttranslational modifications such as folding, glycosylation, and oligomer-

Figure 5. Suppression of HCV replication by CsD. (*A*) Huh7/Rep-Feo cells were cultured with the concentrations of CsA (*closed boxes*) indicated or an analogue of cyclosporin, CsD (*open boxes*), in the medium. Luciferase assays were performed after 48 hours of culture. Luciferase assays were performed in triplicate. *Error bars* indicate mean ± 2 SD. (*B*) Northern blotting. Huh7/Rep-Neo was cultured with the concentrations of CsA or CsD indicated, and RNA was extracted after 48 hours of culture. Ten micrograms of total cellular RNA was applied to each lane. The upper part of the membrane containing the HCV replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with a β-actin probe. *Lane 1*, replicon alone; *lane 2*, 1 μg/mL CsA; *lane 3*, 3 μg/mL CsA; *lane 4*, 1 μg/mL CsD, and *lane 5*, 3 μg/mLCsD. (*C*) Western blotting. Ten micrograms of total cellular protein was separated by polyacrylamide gel electrophoresis and transferred onto the membrane. The membrane was incubated with a monoclonal anti-NS5A antibody or an anti–β-actin antibody. *Lane 1*, protein size markers; *lane 2*, replicon alone; *lane 3*, 1 μg/mL CsA; *lane 4*, 3 μg/mL CsA; *lane 5*, 1 μg/mL CsD; and *lane 6*, 3 μg/mL CsD.

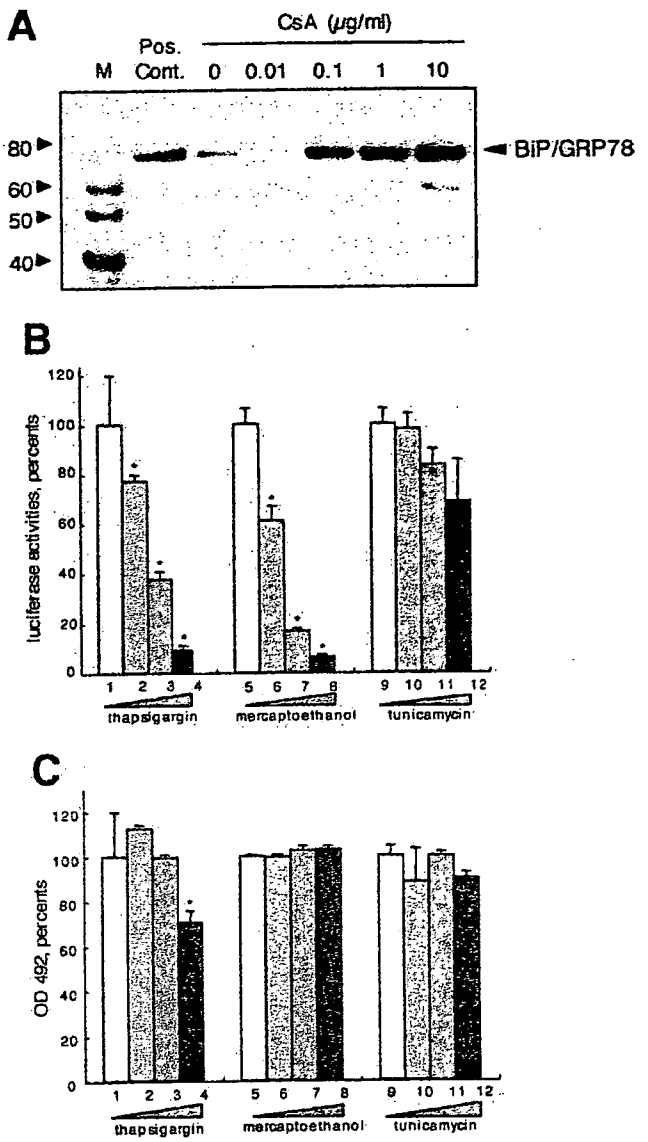


Figure 6. Unfolded protein response following CsA treatment and its effects on HCV replication. (*A*) Huh7 cells were cultured in the presence of CsA at the concentrations indicated, and the cells were harvested at 48 hours after the treatment. Western blotting was performed using anti-Bip/GRP78 antibody. *Lane 1*, protein size markers; *lane 2*, HepG2 lysate as a positive control; *lane 3*, Huh7 alone; *lane 4*, 0.01 μg/mL CsA; *lane 5*, 0.1 μg/mL CsA; *lane 6*, 1 μg/mL CsA; and *lane 7*, 10 μg/mL CsA. (*B*) Huh7/Rep-Feo cells were cultured with various concentrations of thapsigargin, mercaptoethanol, and tunicamycin in the medium. Luciferase assays were performed in triplicate at 48 hours after treatment with each drug. Error bars indicate mean ± 2 SD. Columns 1–4: cells cultured with thapsigargin at concentrations of 0 (column 1), 0.001 (column 2), 0.003 (column 3), and 0.01 μg/mL (column 4). Columns 5–8: cells cultured with mercaptoethanol at concentrations of 0 (column 5), 0.01 (column 6), 0.1 (column 7), and 1 μg/mL (column 8). Columns 9–12: cells cultured with tunicamycin at concentrations of 0 (column 9), 0.01 (column 10), 0.03 (column 11), and 0.1 μg/mL (column 12). **P* values of less than .05. (*C*) MTS assay of Huh7/Rep-Feo cells cultured with the various concentrations of ER stress agents. MTS assays at 48 hours after treatment with each drug were performed in triplicate. *Error bars* indicate mean ± 2 SD. Columns 1–12: cells cultured with the drugs that correspond to those in *B*. **P* values of less than .05.

ization and are exported to various cellular compartments. Various cellular stresses such as heat shock, ischemia, hypoxia, and viral infection and changes in calcium homeostasis prevent protein folding and maturation in the ER and result in the accumulation of misfolded proteins.[39,40] These defective proteins trigger the unfolded protein response and cause induction of molecular chaperone proteins, suppression of translation, and apoptotic cell death.[27,28,41] Paslaru et al reported that treatment of HeLa cells with CsA induced an unfolded protein response that is characterized by synthesis of a stress protein, BiP/GRP78, located inside the ER.[26] Because PPIases support the correct folding of a sufficient number of proteins, their inhibition ought to lead to the accumulation of denatured proteins in various cell compartments. In this study, treatment with thapsigargin, an inhibitor of the Ca^{2+} adenosine triphosphatase (ATPase) transporter, and mercaptoethanol, which disrupts disulfide bond formation, suppressed HCV replication. These results imply that the effects of CsA on HCV replication may involve induction of an unfolded protein response to a level below the cytotoxic range and that the selective alteration of ER function may abrogate HCV replication.

The expanding applications of CsA to infectious diseases such as HCV may cause substantial problems, particularly undesired immunosuppression and possible interference with the effects of anti-infectious agents such as interferon. One solution to overcome these problems is to use cyclosporin analogues.[42,43] Some types of cyclosporin analogues show attenuated effects in terms of blocking T-cell activation but retain activity against the PPIase activity of cyclophilins. These nonimmunosuppressive cyclosporin analogues have equal or even superior effects against in vitro human immunodeficiency virus replication compared with the immunosuppressive CsA.[44] As we have shown, HCV replication was successfully inhibited by CsD, which has weak immunosuppressive activity.[25,45] The inhibition of T-cell activation by cyclosporin analogues is a function of the binding capacities to cyclophilins, whose binding to the residues of cyclosporine is known to be critical for their immunosuppressive activity in the decreasing order of CsA to CsD.[46] However, in this study, the inhibitory effects of CsA and CsD on HCV replication were found to be similar (Figure 5), showing that the anti-HCV effects did not correlate with immunosuppressive activity. Another cyclosporin analogue, NIM811 ([methyl-Ile4]-cyclosporin), has been reported to be a similar inhibitor of CypA-mediated human immunodeficiency virus 1 replication than the parental CsA.[47] Watashi et al confirmed that NIM811 also is effective against HCV replication in

vitro.[9] The availability of nonimmunosuppressive cyclophilin inhibitors that are less toxic than CsA might hold promise of novel antiviral drugs. These drugs should be reevaluated clinically in the light of the new findings presented here.

Given the current status of limited therapy options against HCV infection and the unsatisfactory outcome of therapy, screening of nonimmunosuppressive cyclosporin analogues or agents targeting cellular cyclophilins may be important to develop novel antiviral therapies. In addition, further investigations of the action of cyclophilins on the expression, processing, or maturation of HCV proteins may elucidate new aspects of the viral infection and replication.

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HEPATOLOGY

Mutations in the NS5B region of the hepatitis C virus genome correlate with clinical outcomes of interferon-alpha plus ribavirin combination therapy

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Abstract

Background and Aim: Combination treatments of interferon-alpha (IFN) and ribavirin (RBV) are more effective than those of IFN alone in hepatitis C virus (HCV) infection. However, mechanisms of the action of the combination regimen are not well understood. To elucidate the viral genetic basis of IFN plus RBV combination therapy, genetic variabilities of HCV-1b were analyzed.

Methods: We performed pair-wise comparisons of full-length HCV genomic sequences in three patients' sera before and after initiation of IFN plus RBV treatment. Subsequently, we analyzed amino acid sequences of the NS5B region, which codes for the viral RNA-dependent RNA polymerase, and compared these with the outcomes of the therapy in 81 patients.

Results: Analysis of the entire HCV sequence in patients who received IFN plus RBV therapy did not show consistent amino acid changes between before and after the initiation of the therapy. NS5B sequence analyses revealed that mutations at positions 300–358 of NS5B, including polymerase motif B to E, occurred more frequently in a group of patients exhibiting a sustained viral response (SVR) or an end-of-treatment response (ETR) compared with a group of patients exhibiting a non-response (NR). Closer examination revealed that mutations at aa 309, 333, 338 and 355 of NS5B occurred significantly more frequently in the SVR plus ETR group than in the NR group ($P = 0.0004$). Multivariate analysis showed that the number of mutations at these four sites was an independent predictor of SVR plus ETR *versus* NR.

Conclusions: Particular amino acid changes in the NS5B region of HCV may correlate with outcomes of IFN plus RBV combination therapy.

Key words: amino acid sequence, error catastrophe, RNA-dependent RNA polymerase, transition.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, which can lead to liver cirrhosis and hepatocellular malignancy.[1,2] Interferon (IFN) is the agent of choice for treating HCV infection. However, IFN monotherapy produces sustained virological responses in only 15–20% of patients treated, most of whom relapse after completion of the therapy.[3,4] Several recent studies of combination therapy with IFN alpha 2b and ribavirin (RBV) have shown that the regimen induces higher sustained virological responses than IFN monotherapy. Unfortunately, 50–60% of patients still do not respond to the combination therapy.[5–8]

RBV is a synthetic guanosine analog with broad antiviral actions *in vitro* against various DNA and RNA

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viruses.[9,10] At present, four mechanisms of action have been postulated: (i) immune modulatory effects by a switching of T-cell phenotype from Th2 to Th1 that induces type 1 cytokine responses;[11-13] (ii) inhibition of inosine monophosphate dehydrogenase (IMPDH) leading to depletion of cellular GMP pool;[14] (iii) mutagenic activity against RNA viruses that induces misincorporation of RBV triphosphate into viral RNA leading to error prone replication of viral genome;[15-18] and (iv) inhibition of the activity of HCV NS5B RNA-dependent RNA polymerase (RdRp).[19,20] However, it has not been fully understood which mechanisms of actions of RBV are effective against HCV infection.

Certain genetic structures of viruses may affect the sensitivity to their therapeutic drugs. Nucleoside analogs are widely used against viruses such as human immunodeficiency virus type 1 (HIV) and hepatitis B virus (HBV).[21,22] The antiviral effect of those reagents arises from the inhibition of viral DNA/RNA polymerase activity. However, single or multiple mutation(s) in the viral polymerase confer drug resistance and help the drug resistant strains emerge.[22-30] Also in HCV infection, the INF sensitivity determining region (ISDR) of HCV genome, which we have previously identified, critically determines the virological response to IFN and the treatment outcomes.[31,32] As to RBV, one study of five HCV genotype 1a patients who had undergone RBV monotherapy has reported one mutation in NS5B that may correlate with RBV sensitivity.[33] These findings make us speculate that genetic variability of HCV NS5B region, which codes for RdRp, may correlate with sensitivity to RBV and may influence the outcomes of IFN plus RBV combination therapy.

In the present study, we first analyzed effects of RBV on HCV genomic structure and the viral genetic basis of RBV resistance by performing pair-wise comparisons of full-length HCV genomic sequences in patient sera before and after initiation of IFN plus RBV treatment. Subsequently, we have investigated a hypothesis that genomic variability of HCV RdRp may confer resistance or susceptibility to RBV and may correlate with the outcomes of IFN plus RBV combination therapy. Thus, we analyzed amino acid sequences of the NS5B region and the outcomes of IFN plus RBV combination therapy in 81 patients, and found that certain amino acid variations in the NS5B region may associate with the treatment outcomes.

METHODS

Patients of interferon plus ribavirin non-responders

Three patients infected with HCV, genotype 1b, were studied. All patients were non-responders to combination therapy with IFN alfa-2b (Intron A, Schering Plough, Kenilworth, NJ, USA), 6 million units three times per week plus RBV (Rebetoron, Schering Plough), 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. Serum samples were obtained before treatment and at 12 weeks after initiation of the treatment, and pair-wise comparisons of the consensus sequences of full-length HCV genomes were performed. As controls for the IFN plus RBV therapy data, we analyzed our previously published HCV sequence data for three non-responders of IFN monotherapy[32] (deposited with the DDBJ/GenBank/EMBL data libraries under accession number D50483, D50480, D50485, D50481, D50484 and D50482).

RNA extraction, reverse transcription-polymerase chain reaction and direct sequencing

RNA was extracted from patient sera by the modified acid guanidinium thiocyanate-phenol-chloroform (AGPC) method,[34] using ISOGEN reagent (Wako Pure Chemical Industries, Osaka, Japan), and reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described.[32] Full-length HCV genomes were amplified by nested PCR with 21 partially overlapping sets of primers, as previously reported.[32] M13-forward and M13-reverse sequencing primer sequences were attached to the 5′-termini of sense and antisense nested PCR primers. Each PCR product was purified by a spin filtration column (Suprec-02; Takara). Both strands of the PCR products were cycle sequenced with the PRISM dye termination kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions, and consensus nucleotide sequences were determined by an automated DNA sequencer model 373 A (Applied Biosystems).

Sequence analyses

Nucleotide sequencing analysis was performed with a software program (MEGA version 2.1) to calculate values for d_N (non-synonymous substitution), d_S (synonymous substitution), d_N/d_S ratios, and the number of point mutations.

Clinical outcome of combination therapy

Patients were placed into one of three outcome groups.

- Sustained virologic response (SVR): HCV-RNA was not detectable by RT-PCR for 6 months following completion of the therapy.
- End-of-treatment response (ETR): HCV-RNA was not detected at the end of the treatment, but reappeared within 6 months thereafter.
- Non-response (NR): HCV-RNA did not disappear during the treatment.

Nucleoside sequencing analyses of the NS5b region

Amino acid mutations in the conserved motifs (motif A, B, C, D, E, F)[35-38] in NS5B RdRp were retrospectively analyzed in 81 HCV genotype 1b patients who were

treated with IFN alfa-2b, 6 million units three times per week plus RBV, 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. All patients had biopsy-proven chronic hepatitis with positive serum HCV antibodies and serum HCV-RNA. RNA was extracted from sera of the patients before treatment. NS5B region, including motifs A to F, was amplified by RT-PCR and sequences corresponding to nucleotides 7730–8874 of HCV-J were determined.[32] The deduced amino acid sequences of all patients were aligned and compared with consensus sequences for mutations and analyzed for correlation between amino acid mutations of NS5B and the clinical outcome of the combination therapy.

Statistical analyses

Comparisons of differences in categorical data between groups were performed using the χ^2 test and Fishers exact test. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test for two groups and by the Kruskal–Wallis test or Scheffé method for three groups. Multivariate analysis was carried out by multiple logistic regression analysis. *P*-values of less than 0.05 were defined as statistically significant.

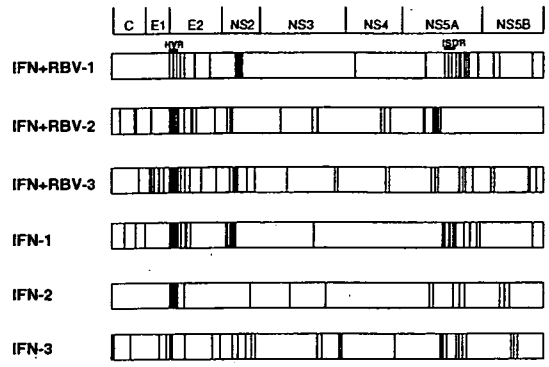

Figure 1 Schematic representation of the distribution of mutations in amino acid residues during the combination therapy and interferon (IFN) monotherapy. Distributions of amino acid changes in the entire hepatitis C virus (HCV) genome in patient serum before treatment and 12 weeks after initiation of treatment are shown. The upper three data are from patients treated with IFN/ribavirin (RBV) combination therapy (IFN + RBV 1–3), and the lower three data are those treated with IFN monotherapy (IFN 1–3). Vertical lines in each HCV polyproteins show position of amino acid differences during the therapy.

RESULTS

Pair-wise comparisons of the full-length HCV genome in three patients before and after initiation of IFN/RBV treatment

HCV genomes from the three study patients comprised 9423 nucleotides and contained an open reading frame of 3010 amino acids. In patient one, 31 amino acid changes were found in the HCV genome. These amino acid changes were clustered in the E2-hypervariable regions (8 of 31) and the NS5A regions (11 of 31). Before treatment, the INF-sensitivity determining lesion (ISDR)[31,32] were 'mutant' type with five amino acid changes compared with consensus sequence, which changed to 'intermediate' type with two amino acid changes after the initiation of treatment. In patient two, 37 amino acid changes were found in the entire HCV genome. The changes were exclusively found in the E2-hypervariable region (16 out of 37 amino acids), while there was no change in the ISDR. In patient three, 56 amino acid changes were found. The changes were exclusively found in the E2 region (24 out of 56 amino acids). Distribution of amino acid changes during the therapy in the three patients treated with combination therapy and three non-responders to IFN monotherapy are illustrated in Figure 1. The numbers of nucleotide changes for the three study patients were 88, 130 and 272, respectively. The d_N/d_S ratios were 0.195, 0.148 and 0.099, respectively. Among the three control subjects who received IFN monotherapy, the numbers of nucleotide changes were 138, 160 and 175, respectively. The d_N/d_S ratios were 0.158, 0.061 and 0.089, respectively. As shown in Figure 2, d_N/d_S ratios tended to be higher in the E2 region than in the other regions during

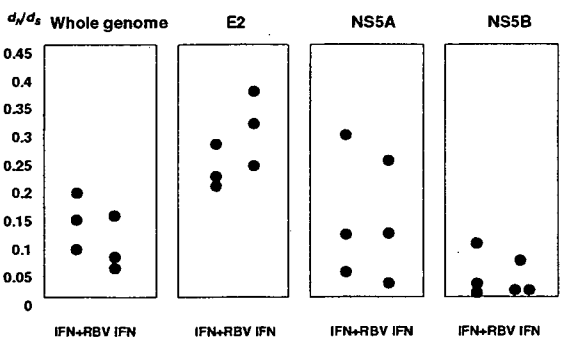

Figure 2 Ratio of non-synonymous to synonymous distances for the E2, NS5A, NS5B and whole hepatitis C virus (HCV) genome. The d_N/d_S ratio in E2 region tended to be higher than other regions during interferon (IFN) monotherapy and during combination therapy. All pairwise d_N/d_S ratios were calculated using MEGA version 2.1 for each subject.

both IFN monotherapy and combination therapy. The numbers of transitional mutations in patients who received the combination therapy had 71 (80.2% of total mutations), 104 (80.0%) and 218 (80.1%) transitional mutations, respectively, and in patients who received IFN monotherapy these were 108 (78.3%), 131 (81.9%) and 130 (75.4%), respectively. The proportion of transitions among IFN monotherapy patients did not differ from the proportion among combination therapy patients.

Two studies have observed two key transitions, C-to-U and G-to-A, in genomic sequences of RBV-treated RNA viruses.[17,18] In the present study, C-to-U and G-to-A mutations comprised 35.5%, 40.6% and 58% of

total mutations, respectively, in the three patients treated with IFN monotherapy, and 43.2%, 38.3% and 37.8%, respectively, in those treated with combination therapy. These results showed no obvious increase in key mutations of C-to-U and G-to-A associated with the combination therapy (Table 1).

Sequence analyses of NS5b region in 81 patients treated with IFN and RBV therapy

To study the correlation between the genetic structures of NS5B and the outcome of IFN plus RBV combination therapy, amino acid sequences of HCV NS5B (aa. 61–407), including motif A-F, were analyzed in 81 patients treated with IFN plus RBV combination therapy. The clinical characteristics of the patients are shown in Table 2. Nineteen (23.5%) patients were SVR, 40 (49.4%) were ETR, and 22 (27.2%) were NR. Clinical variables were analyzed according to the results of the combination therapy. Univariate analysis identified fibrosis stage as significantly lower in the SVR patients than in the other patients. No other clinical parameters were significantly correlated with the responses.

The amino acid sequences of the essential motif B to E of NS5B in these 81 patients are aligned with consensus sequences in Figure 3. Comparison of the NS5B sequences between patients with SVR and patients with non-SVR (ETR and NR) showed no obvious differences. Instead, when we compared the sequences of a patient group of SVR plus ETR with those of patients with NR, the mutations at position NS5B 300–358, including motif B to E between, were more frequent in the SVR plus ETR group than in the NR group. When we analyzed mutations of individual amino acid positions, the frequencies of mutations at aa 309, 333, 338 and 355 of NS5B (the four sites) were found to be more frequent in patients with SVR or ETR than those with NR (Fig. 4). The total number of amino acid changes at these four sites was significantly higher in patients with SVR or ETR than those with NR (0.93 ± 0.89 *vs* 0.27 ± 0.70, $P = 0.0004$). In 19 SVR patients, five patients had no mutations, 10 patients had one mutation, and four patients had two or more mutations at the four sites. In the 40 ETR patients, 18 patients had no mutations, 13 patients had one mutation, and nine patients had two or more mutations at the four sites. In 22 NR patients, 19 patients had no mutations, two patients had one mutation, and one patient had three mutations at the four sites (Fig. 5a). The SVR rates were 11.9% (5 of 42) and 35.9% (14 of 39) in patients who had none and one or more mutations at the four sites, respectively (Fig. 5b). Patients with increased mutations at the four sites tended to be in the SVR or ETR groups. We subsequently analyzed various clinical factors by multivariate analysis among the three response groups to determine the independent predictors for SVR and NR (Table 3). Among these clinical factors, the NS5B mutation described above was independently associated with NR ($P = 0.0185$).

Mutations of the NS5B region, which codes for the viral RdRp, may alter its enzymatic activities which may influence serum virus load of each patient. In our results, however, there was no obvious correlation between the number of NS5B mutations and serum viral loads in each patient, nor was there a difference in the serum virus loads between the patient groups categorized by the numbers of mutations at aa 309, 333, 338 and 355 of NS5B.

Table 1 Sequence analysis of full genome of hepatitis C virus (HCV) RNA treated with interferon (IFN) plus ribavirin

	G-to-A and C-to-U	Other transition (A-to-G and U-to-C)
IFN plus ribavirin	58.3	72.5
No ribavirin (IFN monotherapy)	60.8	70.4

Mutations per 10 000 nucleotides. A total of 56 538 nucleotides were sequenced.

DISCUSSION

In the present study, we have demonstrated that particular amino acid changes in the NS5B region of HCV

Table 2 Baseline characteristics of the group of 81 patients, segregated according to the clinical outcome of interferon (IFN) plus ribavirin combination therapy

	SVR	ETR	NR	*P*-value
Number of patients	19	40	22	
Age (years)	49.5 ± 12.2	55.9 ± 8.1	57.2 ± 10.6	NS
Sex (male/female)	15/4	27/13	11/11	NS
Baseline ALT (IU/L)	122.2 ± 88.0	80.2 ± 43.2	107.4 ± 73.7	NS
Platelet count (10^3/mm^3)	16.0 ± 5.5	16.3 ± 5.5	14.7 ± 4.5	NS
Fibrosis stage (SD)	1.41 ± 0.71	1.92 ± 0.94	2.10 ± 0.72	0.012†
Serum HCV RNA at baseline (KIU/mL)	480.5 ± 295.7	594.6 ± 239.3	599.9 ± 271.3	NS
Number of ISDR mutations	1.73 ± 2.92	0.80 ± 1.22	1.00 ± 1.80	NS

†Significant differences between SVR and others. Values are expressed as mean ± SD, except where noted. ALT, alanine aminotransferase; ETR, end-of-treatment responder; NR,-non-responder; NS, not significant; SR, sustained responder.

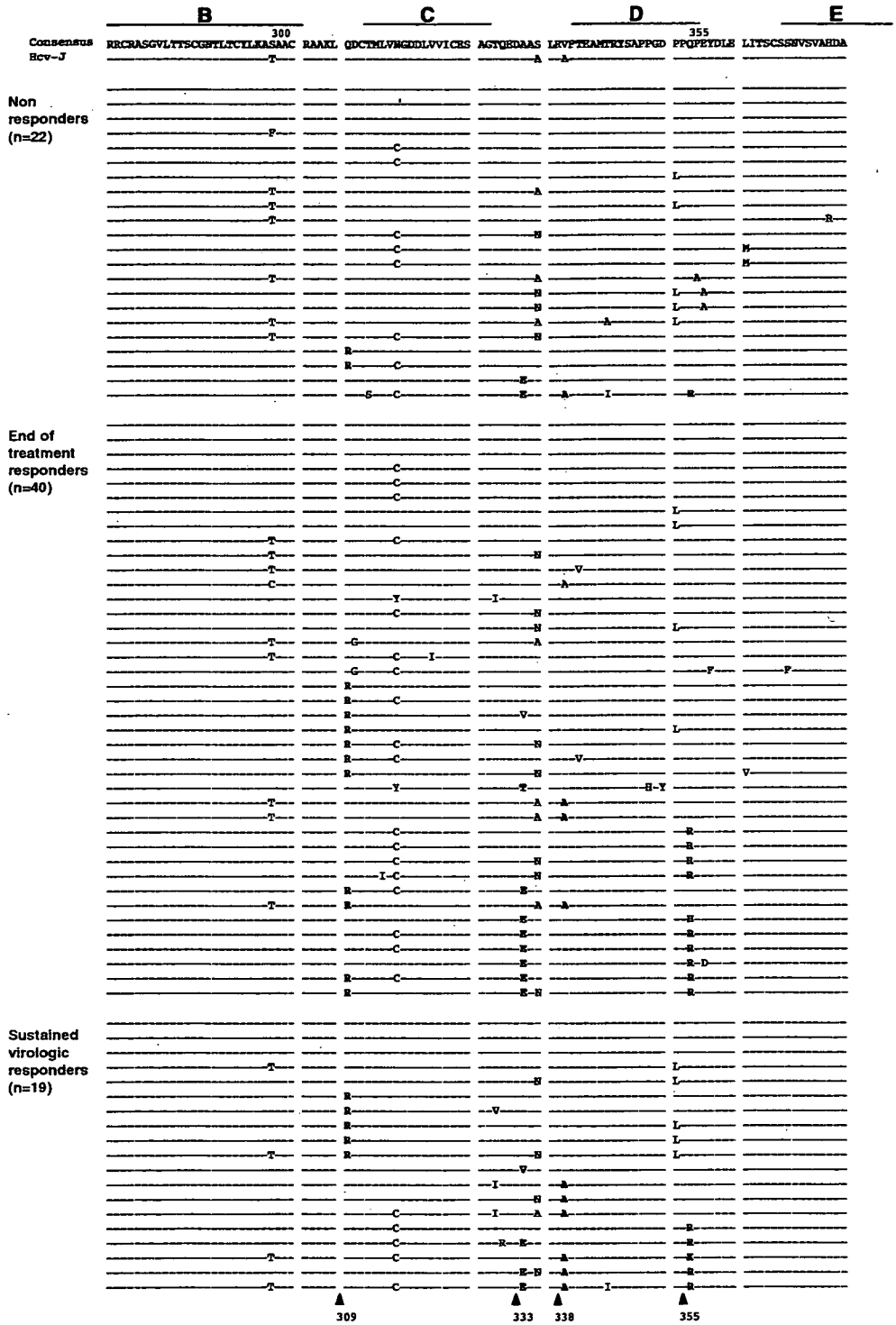

Figure 3 Amino acid sequence alignments of the NS5B motif B-E in 81 patients with chronic hepatitis C virus (HCV)-1b infection and treated with interferon (IFN) plus ribavirin for 24 weeks. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate the identical amino acid residues with consensus sequence and HCV-J shown at the top. At four amino acid positions (NS5B 309, 333, 338 and 355), usages of amino acid residues differ between non-responders (NR) and others. Changes of this part are indicated by bold letter. Outcome of combination therapy is shown on the left side.

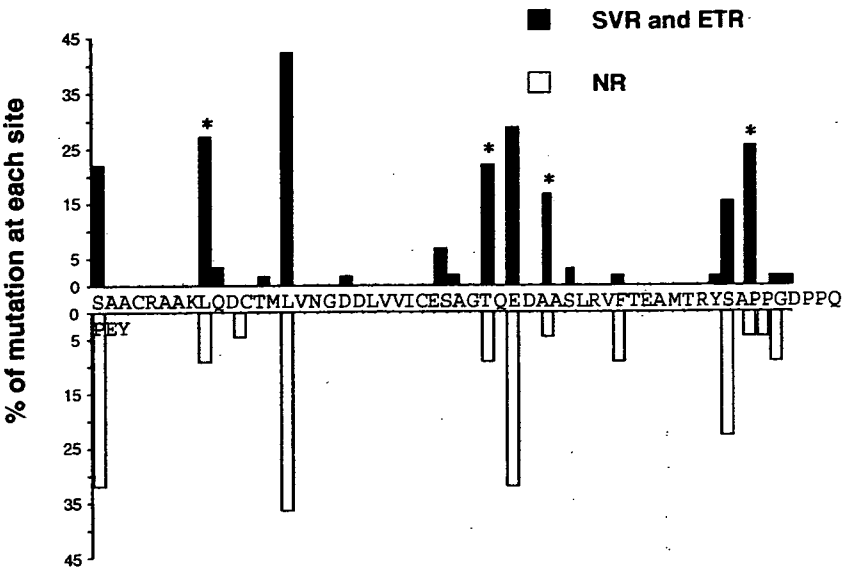

Figure 4 Relationship between frequency of mutations at each site in NS5B 300–358 and the efficacy of interferon (IFN) plus ribavirin treatment. Amino acid residues are indicated by the standard single-letter codes. Among these 59 sites, mutations of aa NS5B 309, 333, 338 and 355 (identified by *) are frequent in sustained virologic response (SVR) and end-of-treatment response (ETR) patients. NR, non-response.

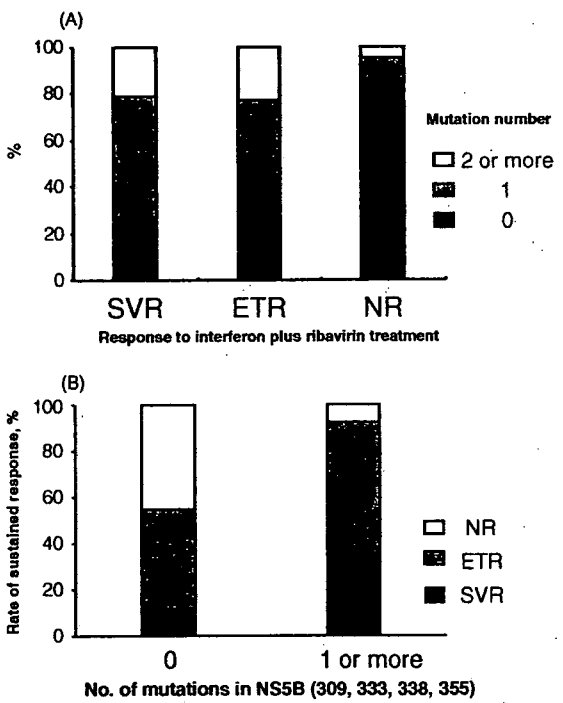

Figure 5 Relationship between number of mutations in NS5B 309, 333, 338, 355 and the outcome of interferon (IFN) plus ribavirin treatment. (a) Distribution of total numbers of mutations at aa. 309, 333, 338 and 355 of NS5B according to sustained virologic response (SVR), end-of-treatment response (ETR) and non-response (NR) patients. (b) Proportion of SVR, ETR and NR patients between groups with or without mutations at aa. 309, 333, 338 and 355 of NS5B.

Table 3 Multivariate analysis for the clinical and virological factors affecting virological responses (SVR and NR) to interferon (IFN) plus ribavirin combination therapy in the group of 81 patients

	Patient with SVR *P*-value	Patient with NR *P*-value
Age (years)	0.572	0.598
Sex (male/female)	0.814	0.158
Baseline ALT (IU/L)	0.022	0.981
Platelet count (10^3/mm^3)	0.749	0.627
Mean fibrosis stage (SD)	0.037	0.330
Serum HCV RNA at baseline	0.227	0.890
No. of ISDR mutations	0.491	0.754
No. of NS5B mutations (309,333,338,355)	0.057	0.019

ALT, alanine aminotransferase; ETR, end-of-treatment response; ISDR, interferon sensitivity determining region; NR, non-response; SR, sustained response.

correlate with the clinical outcome of combination therapy. Pair-wise comparisons of the full-length HCV genome in three patient sera obtained before and 12 weeks after the start of IFN plus RBV therapy did not show consistent amino acid changes. The results suggest negative evidence against the presence of treatment-resistant viral sub-populations. On the contrary, subsequent analyses of mutation patterns in the NS5B region in 81 patients showed a significant correlation between particular amino acid mutations of NS5B and the outcome of the combination therapy. Mutations of aa. 309, 333, 338 and 355 of the NS5B were significantly more frequent in SVR and ETR patients, in which the virus has been persistently or at least tempo-

rarily eliminated. Total numbers of mutations at the four amino acid positions were significantly more in SVR and ETR patients compared to NR patients (0.93 ± 0.89 *vs* 0.27 ± 0.70; $P = 0.0004$). These data suggest that particular amino acid mutations of NS5B-RdRp protein may confer sensitivity to combination therapy.

Recently, several studies on mutational analyses of HCV NS5B have identified several key residues responsible for its RdRp activity. Lohmann *et al.* noted that one single amino acid substitution in NS5B increased the efficacy of colony formation by 500-fold in HCV subgenomic replicon.[39] Cheney *et al.* noted that several amino acid substitutions (K155A, R168A, D225N and R386Q) were detrimental to both *in vitro* polymerase activity and replicon RNA replication in Huh-7 cells.[40] Recently, Young *et al.* suggested that NS5B F415Y mutation in HCV-1a was a key resistant variant for RBV monotherapy.[33] However, Y415 is the consensus residue for all genotypes except for 1a and 6a. In the present study of three non-responders, there was no difference at NS5B Y415 between sera collected before treatment and sera collected 12 weeks after the start of treatment with combination therapy.

The locations of the four mutations within the calculated tertiary structure of NS5B RdRp are illustrated in Figure 6a,b. The mutations in NS5B, which were more frequently found in the SVR and the ETR patients, were clustered in motif B to E of RdRp. The amino acid 309 and 355 are both located on the enzyme surface of the substrate entry site. NS5B 333 and 338 are adjacent to the NTP tunnel (Fig. 6b). Because mutations found in HBV and HIV DNA polymerase/reverse transcriptase are known to be located on the surface of the catalytic domain, the mutations in HCV RdRp that were found in the present study may considerably affect their enzymatic activity. Our preliminary data have shown that the HCV subgenomic replicon carrying point mutations in aa. 141 in NS5B less efficiently than the original sequences. Further studies are needed to clarify the role of these point mutations in NS5B in determining the activity of RdRp.

A recent study by Crotty *et al.* has shown that direct antireplicative effects of RBV on viruses include 'error catastrophe' theory in which misincorporations of RBV triphosphate into the viral genome lead to accumulation of mutations in the viral genome and yield defective virus genome. Characteristic pattern of nucleotide mutations by RBV are an increase of G-to-A and C-to-U transition mutations.[17,18] In our present study, although the majority of the mutations were transitions, there was no significant difference in the ratios of the G-to-A and C-to-U mutations between IFN monotherapy and combination therapy (Table 1). One explanation for the discrepancy is that the concentration of RBV in clinical use is too low to act as a mutagen. The clinically achievable blood concentration of RBV is 10–30 μM.[41] On the contrary, an *in vitro* study of polio virus has shown that RBV concentration of 100 μM is required to increase the mutation frequency by at least 1.2-fold.[17] Highly mutated HCV can be excluded or escape detection by RT-PCR and minor clone of HCV quasi-species are excluded by direct sequence of nested PCR products. Therefore, although it is not clear whether RBV is a mutagen against viral genome, our results suggest other mechanisms of RBV contribute to suppress HCV replication, such as inhibition of enzymatic activities of viral RNA polymerase.

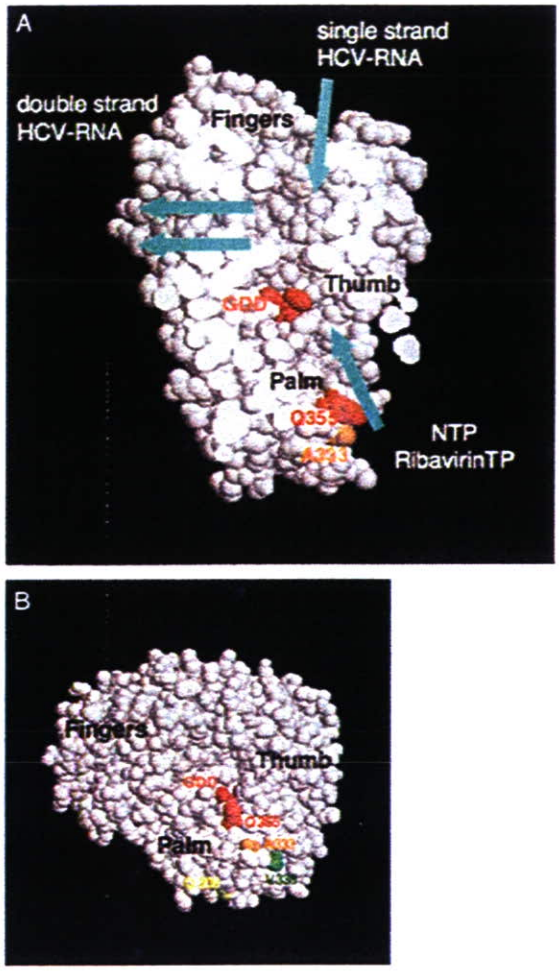

Figure 6 Crystal structure of the hepatitis C virus (HCV) NS5B-RNA dependent RNA polymerase (RdRp). The molecular model of NS5B was constructed using 1QUV from Protein Data Bank (PDB). A space-filling representation of each atom is shown. Graphics were generated using Rasmol 2.7.2.1. (a) Cross-section of the RdRp at level of nucleotide tunnels. The single stranded HCV RNA enters the enzyme through a groove at the top of the finger domain, and the NTP or ribavirin enters the enzyme through the right lower dNTP tunnel (between β fingers and thumb). The essential GDD motif is shown in pink. NS5B 309, 333, 338 and 355 are shown in yellow, orange, green and red, respectively. (b) View from the dNTP entry site.

Many studies have endeavored to identify factors predictive of the outcome of IFN plus RBV combination therapy. Factors that have been examined include pretreatment clinical parameters such as baseline viral load, degree of fibrosis, and gender.[12] One study has

found early viral response (two-log decline of HCV RNA) to be predictive of SVR.[43] Another study showed that ISDR mutations were correlated with the SVR in chronic HCV 1b infection in Taiwan.[44] In the present study, multivariate analysis identified baseline ALT and the degree of fibrosis as independent factors for SVR. Further multivariate analysis showed that the number of mutations at positions NS5B 309, 333, 338 and 355 were independently associated with NR ($P = 0.0185$). The possible implications of our results are that the number of the above-described NS5B mutations is an independent predictive factor and that the parameter predicts NR patients exclusively from SVR or ETR patients. Our results which may enable prediction of NR before initiation of therapy might be of value when we consider indication for IFN plus RBV antiviral therapy or when making a decision about early cessation of the therapy, which may avoid possible side-effects and therapy costs. Although further studies of a larger population of patients are needed, the mutation number might be used to tailor therapy and is a useful factor for clinicians in making a clinical decision to stop treating HCV infection with combination therapy.

Given the absence of proven anti-HCV agents other than IFN and RBV, these combinations will continue to dominate therapy against HCV. Our present results provide evidence of a significant correlation between the response to IFN plus RBV combination therapy in patients with chronic HCV-1b infection and the amino acid changes that were present before therapy in conserved regions of NS5B. Certain amino acid changes in the HCV NS5B-RdRp domain may correlate with the clinical outcome of combination therapy and could thus be an initial predictor for response to IFN plus RBV combination therapy.

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SPECIAL ARTICLE

Consensus Proposals for a Unified System of Nomenclature of Hepatitis C Virus Genotypes

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International standardization and coordination of the nomenclature of variants of hepatitis C virus (HCV) is increasingly needed as more is discovered about the scale of HCV-related liver disease and important biological and antigenic differences that exist between variants. A group of scientists expert in the field of HCV genetic variability, and those involved in development of HCV sequence databases, the Hepatitis Virus Database (Japan), euHCVdb (France), and Los Alamos (United States), met to re-examine the status of HCV genotype nomenclature, resolve conflicting genotype or subtype names among described variants of HCV, and draw up revised criteria for the assignment of new genotypes as they are discovered in the future. A comprehensive listing of all currently classified variants of HCV incorporates a number of agreed genotype and subtype name reassignments to create consistency in nomenclature. The paper also contains consensus proposals for the classification of new variants into genotypes and subtypes, which recognizes and incorporates new knowledge of HCV genetic diversity and epidemiology. A proposal was made that HCV variants be classified into 6 genotypes (representing the 6 genetic groups defined by phylogenetic analysis). Subtype name assignment will be either confirmed or provisional, depending on the availability of complete or partial nucleotide sequence data, or remain unassigned where fewer than 3 examples of a new subtype have been described. In conclusion, these proposals provide the framework by which the HCV databases store and provide access to data on HCV, which will internationally coordinate the assignment of new genotypes and subtypes in the future. (HEPATOLOGY 2005;42:962-973.)

Abbreviations: HCV, hepatitis C virus; IDU, injection drug user; RF, recombinant form; ICTV, International Committee for the Taxonomy of Viruses.

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