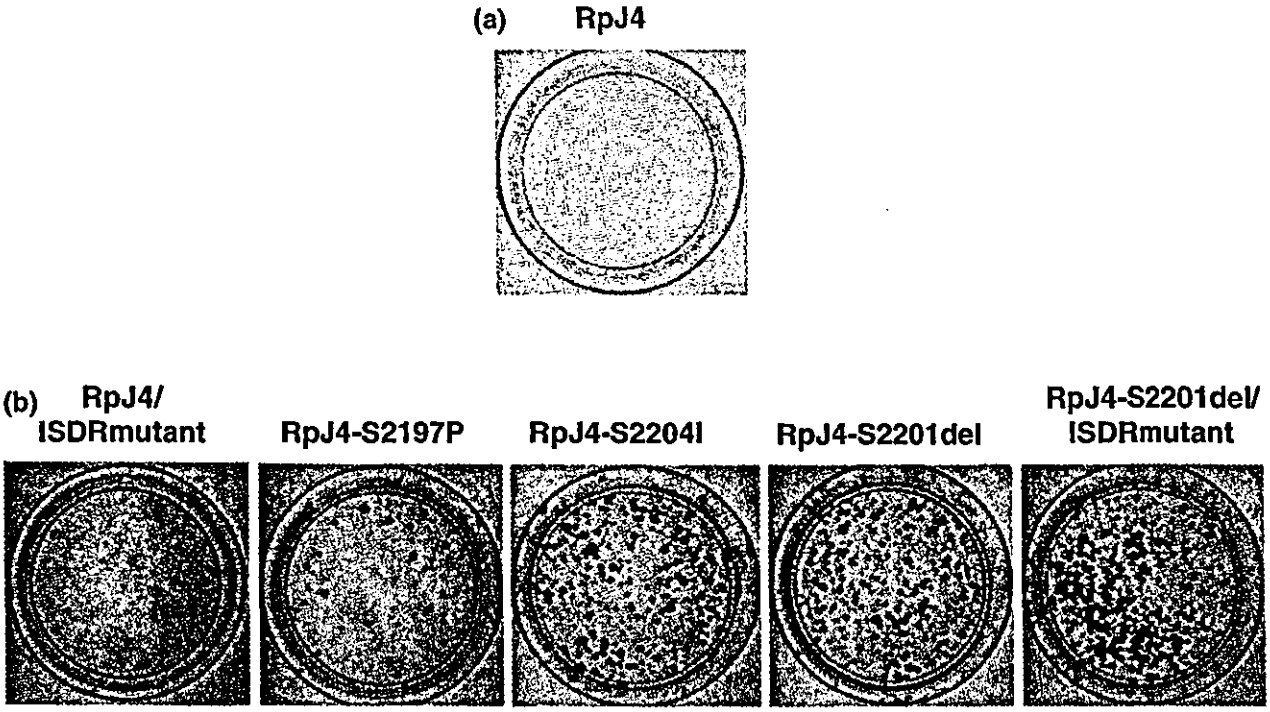

Fig. 3 G418-resistant Huh-7 colonies after transfection of Rp-J4 RNA. (a) Huh-7 cells transfected with RpJ4 RNA did not form a single colony after G418 selection, indicating that RpJ4 RNA is replication incompetent in its native sequence. (b) RpJ4 RNA with three kinds of the serine substitution produced resistant colonies at different efficiencies, (e.g. the S2201deletion mutant was most efficient). Although RpJ4 with six amino acid substitutions in ISDR did not produce any colony, RpJ4 with both ISDR substitutions and mutations in the serine cluster exhibited increased efficiency of colony formation.

Table 1 Comparison of CFU among RpJ4 replicons with different adaptive mutations

Replicon constructs	CFU (/μg RNA) Median (range)
RpJ4	0
RpJ4/ISDR mutant	0
RpJ4-S2197P	60 (54–114)*,**
RpJ4-S2204I	765 (405–837)
RpJ4-S2201del	816 (669–860)***
RpJ4-S2201del/ISDR mutant	1140 (861–1185)***

To determine CFU of a given replicon, G418-resistant colonies were stained and counted with Neutral red 3 weeks after electroporation of RpJ4 with different adaptive mutations. Data were analysed by Mann–Whitney *U*-test.
*$P < 0.05$, RpJ4-S2197P vs. RpJ4-S2204I.
**$P < 0.05$, RpJ4-S2197P vs. RpJ4-S2201del.
***$P < 0.05$, RpJ4-S2201del vs. RpJ4-S2201del/ISDR mutant.

different efficiencies, the highest with RpJ4-S2201del and the lowest with RpJ4-S2197P (Fig. 3b, Table 1). Transfection of RpJ4 with six amino acid substitutions in ISDR without the serine cluster mutation (RpJ4/ISDR mutant) did not produce any colony. However, transfection of RpJ4 replicon with mutations in both regions (RpJ4-S2201del/ISDR mutant) resulted in enhanced colony formation in comparison with RpJ4-S2201del (Fig. 3b, Table 1). From these G418-resistant colonies, two or more different replicon-harbouring cell lines were established in each construct.

Detection of RpJ4 RNA and NS5A protein in G418-resistant Huh-7 cells

In order to confirm RNA-based replication of the RpJ4 replicons in Huh-7 cells, Northern blots for HCV replicon RNA in established cell lines were performed using strand-specific riboprobes. Both positive- and negative-stranded HCV replicon RNA were present, positive strands exhibited much higher levels in these established cell lines. These data strongly suggested that these RpJ4 replicons replicated autonomously (Fig. 4). However, their replication level in the established cell lines did not consistently reflect differences in the effect of NS5A adaptive mutations. In Fig. 4, there were no apparent differences in replicon-RNA levels among RpJ4 replicons except RpJ4-S2201del. Even with RpJ4-S2201del, higher level of replication was observed in another established cell line (data not shown). On the other hand, lower level of replication was observed with RpJ4-S2204I in another cell line (Fig. 6). Western blots

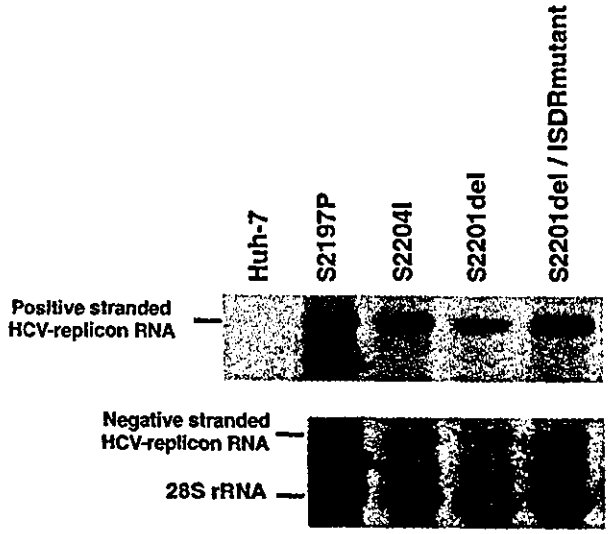

Fig. 4 Northern blots of positive and negative HCV replicon RNA strands in RpJ4 harbouring cell lines. Northern blots for HCV replicon RNA in established cell lines were performed using strand-specific riboprobes. Both positive- and negative-stranded HCV replicon RNA were present, positive strands exhibited much higher levels in these established cell lines. However, their replication level in established cell lines did not consistently reflect differences in colony-forming efficiencies of RpJ4 replicons. In this figure, there was no apparent difference in replicon-RNA level among RpJ4 replicons except RpJ4-S2201del. Even with RpJ4-S2201del, higher level of replication was observed in another established cell line (data not shown).

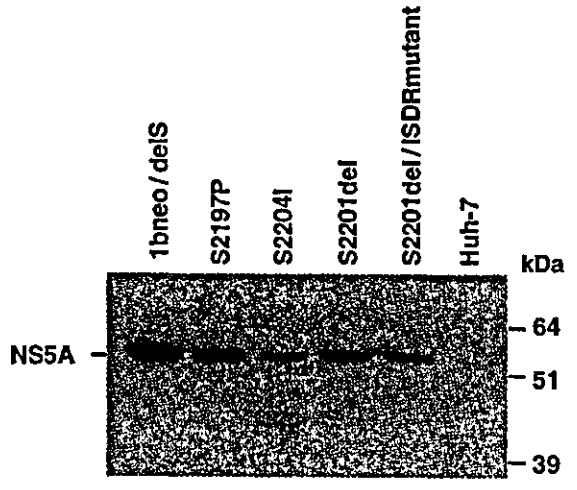

Fig. 5 Western blots of NS5A in HC-J4 replicon harbouring cells. NS5A was detected in all cell lines harbouring RpJ4 replicons. Although level of expression is different in each lane in this figure, repeated experiments disclosed that expression level was dependent on the cell clones even established from RpJ4 with the same genetic structure.

demonstrated that all of these cells harbouring replicons expressed NS5A protein (Fig. 5). These proteins were estimated to be 56 kDa in size, regardless of constitutive adaptive mutations. Although level of expression was different in each lane in this figure, repeated experiments disclosed that expression level was dependent on the cell clones even established from RpJ4 with the same genetic structure, suggesting certain cellular factor(s) play a role in replication of RpJ4 replicons.

No further mutations appeared in cell culture

The entire HCV coding sequence of RpJ4 replicons with different adaptive mutations was determined by direct sequencing in an effort to identify additional mutations in the cell lines. After 10 months in culture, the established replicon-harbouring cell lines expressed no nucleotide or amino acid changes in any of the RpJ4 replicons. These results suggested that the introduction of an adaptive mutation into the serine cluster of the RpJ4 replicon is sufficient and no further mutations are selected in cell culture.

RpJ4 derivatives all respond to interferon

The effect of interferon-alpha on RpJ4 replicons was investigated. HCV-RNA in the RpJ4 replicons with different adaptive mutations disappeared completely 48 h after treatment with interferon (1000 IU/mL) (Fig. 6). RpJ4 replicons were also treated with smaller doses of interferon (10 or 100 IU/mL). With these smaller doses, RNAs of various RpJ4 replicons were still detected 48 h after the treatment in Northern blots despite their decrease. However, the extent of decrease was almost similar among RpJ4 replicons with different NS5A mutations (data not shown). These data suggested that the anti-viral effect of interferon is effective in RpJ4 replicons as reported previously in replicons derived from other HCV isolates [5,11]. Moreover, the sensitivity to interferon did not significantly change regardless of mutation loci, i.e. in the serine cluster region or in ISDR.

Establishment of RpJ4 replicon without mutation in the serine cluster region by highly replicon permissive cells

Naïve and interferon-cured Huh-7 cells were transfected with luciferase-expressing RpJ4 replicons for the transient assay to determine transduction efficiencies. Following transfection into naïve Huh-7 cells, difference of luciferase activity compared with replication-defective replicon (RplucJ4-NS5Bdel) was not detected in any of the RpJ4 replicons, suggesting replication competency of RpJ4 is quite weak even with adaptive replicons when measured as transient luciferase activities. In contrast, after transfection into interferon-cured Huh-7 cells, luciferase activities of RplucJ4-S2201del/ISDR mutant and RplucJ4-S2201del, highly adaptive RpJ4 replicons, were evident from 24

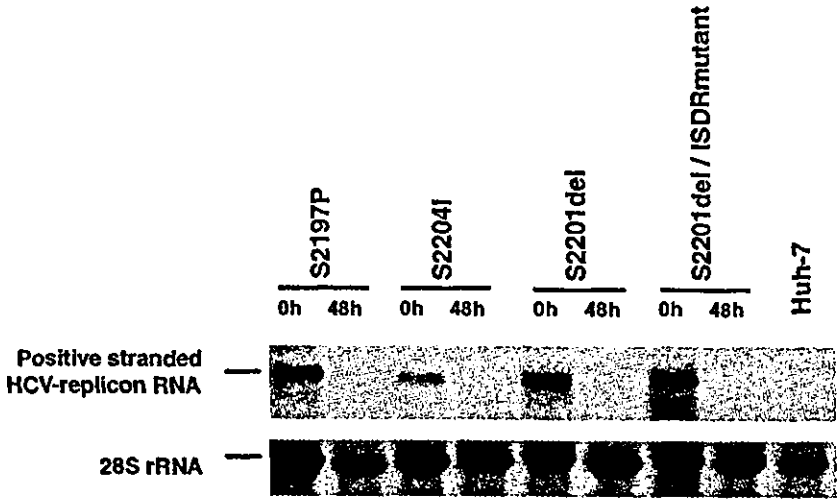

Fig. 6 Responses to interferon treatment in RpJ4 with various NS5A mutations. RpJ4 replicons with various mutations in NS5A all responded to interferon (1000 IU/mL) within 48 h, regardless of mutations in ISDR or in the serine cluster region.

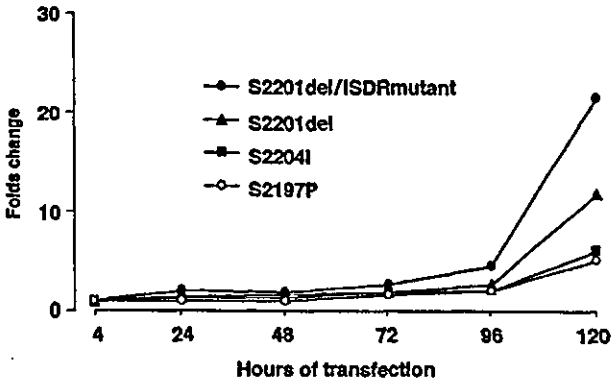

Fig. 7 Transient replication of luciferase-reporter RpJ4 derivatives in cured Huh7 cells. Luciferase activities of RplucJ4-S2201del/ISDR mutant and RplucJ4-S2201del, highly adaptive RpJ4 replicons, were evident 24–120 h after transfection into cured Huh-7 cells. Luciferase activities of RpJ4 replicons were measured by the luminometer and expressed as fold change from the level of luciferase activity of RplucJ4-NS5Bdel.

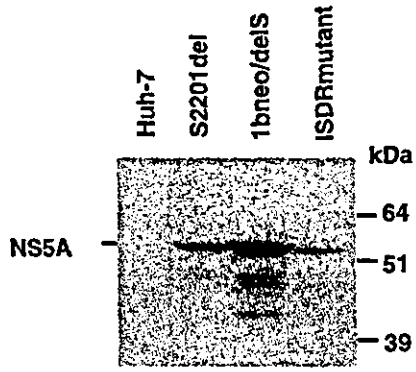

Fig. 8 Western blots using anti-NS5A antibody in RpJ4 replicons without mutations in the serine cluster region (RpJ4/ISDR mutant). RpJ4/ISDR mutant, RpJ4 replicons without mutations in the serine cluster region, also expressed NS5A protein.

to 120 h (Fig. 7). These results supported the contention that interferon-cured Huh-7 cells are highly permissive for RpJ4 replicons.

Subsequently, RpJ4 and RpJ4/ISDR mutant, neither of which have mutations in the serine cluster region and had not produced G418-resistant colonies in naïve Huh-7 cells, were transfected into interferon-cured Huh-7 cells. RpJ4 still did not produce G418-resistant colonies, whereas RpJ4/ISDR mutant produced numerous colonies (>1000 colonies/μg RNA). Stable cell lines were established and HCV replicon RNA was confirmed in these cell lines by Northern blots (data not shown). Sequence analysis of replicating replicons did not reveal additional mutations in the entire NS5A region. Western blots for NS5A protein demonstrated, as in the other RpJ4 replicon, that there was also a single band estimated to be 56 kDa (Fig. 8).

DISCUSSION

In the present study, we demonstrated that a HCV subgenomic replicon derived from a chimpanzee-infectious HCV isolate of HC-J4 replicated continuously in naïve Huh-7 cells if mutations in the serine cluster region of NS5A were introduced. Moreover, the introduction of mutations in ISDR further enhanced the replication competency, permitting replicons devoid of the serine cluster mutation to replicate in interferon-cured highly permissive Huh-7 cells. These results suggest an essential role for the NS5A protein in the regulation of different HCV replicons.

Amino acid mutations in the serine cluster region of NS5A, the same mutations required for Con-1 replicon, were

necessary for replication of RpJ4 in naïve Huh-7 cells. The original RpJ4 without amino acid mutations in this region was replication incompetent. A comparison of the NS3-5 amino acid sequences between HC-J4 and Con-1 yielded 86 amino acid differences among a total of 1986 amino acids. In spite of this large difference in protein structure, the result that the same serine cluster mutations confer competency to RpJ4 strongly suggested that the serine cluster region has an essential role in HCV replication irrespective of HCV isolates. Guo et al. reported that one of the adaptive mutations in the serine cluster region for Con-1 replicon, S2201del, also served as highly adaptive for HCV-N replicon [5]. The absence of additional adaptive mutations in established RpJ4 replicons even after 10 months in culture demonstrated that these mutations are sufficient to promote RpJ4 replication. Considering the high mutation rate of RNA polymerase, it seems rather surprising that no additional mutation occurred in RpJ4. As sequences of the replicons were determined by direct sequencing, the possibility cannot be excluded that RpJ4 acquired additional mutations in its minor subpopulations. In addition, as RpJ4 has three amino acid mutations in the upstream of NS3 due to the design of construct, these mutations may have affected the level of adaptation. However, it is evident that these mutations in NS3 alone cannot enable RpJ4 to self-replicate. Cumulatively, these findings indicate that the NS5A serine cluster mutations are essential for HCV replicons replication in naïve Huh-7 cells.

Colony-forming efficiency was dependent on the location in the serine cluster region, but the level of adaptation induced by each mutation was similar between RpJ4 and HCV-Con1 replicons [11]. For example, S2204I was more adaptive than S2197P in both RpJ4 and Con-1 replicons. Therefore, adaptive mutations may share the same mechanism for replication enhancement regardless of the structural diversities in other regions of HCV. However, as demonstrated in Northern and Western blotting analysis, once cell lines were established by replicons with different levels of adaptation, the differences in replication level and NS5A protein expression per cell did not seem to reflect the degree of transduction efficiency, suggesting that the host cell plays an additional important role in determining the replication in each cell line as noticed in the previous report [13].

NS5A is a phosphoprotein and exists in two phosphorylated forms, i.e. p56 and p58, the basal phosphorylated and hyperphosphorylated forms respectively [32,33]. In previous studies based on HCV-polyprotein expression vector plasmids in cultured cells, p58 was not formed only when one of three serine residues, i.e. S2197, S2201 and S2204, was replaced with alanine [32]. Very interestingly, these amino acid substitutions occupy positions identical to those of the adaptive mutations in the serine cluster region. This may suggest that the adaptive mutations in the serine cluster region might alter the phosphorylation state of NS5A and, thereby, affect p58 formation. The serine cluster mutations were absent in HCV sequences obtained from 338 HCV-1b patients (Enomoto N. *et al.*, unpublished data), as well as in HCV sequences in GenBank. Therefore, these adaptive mutations may be detrimental *in vivo*. Supporting this view, a recent infection study for chimpanzees using a full-length Con1 genome disclosed that a wild-type Con1 genome was infectious, while Con1 with highly adaptive mutations, combination of two adaptive mutations in NS3 (E1202G and T1280I) and a single mutation in NS5A (S2197P) were not infectious. Con1 with a single mutation in NS5A (S2197P) was infectious, but viruses recovered from infected chimpanzees had a reversion back to the original Con1 sequence [34]. It is conceivable that some unidentified cellular factor(s) interacting with HCV NS proteins in different way between *in vitro* and *in vivo*.

Another intriguing observation is that the introduction of interferon-sensitive mutations into ISDR further enhanced the replicative competency of RpJ4. When six amino acid substitutions in ISDR, downstream to the serine cluster, were introduced to RpJ4-S2201del, replicative competency was enhanced in comparison with RpJ4-S2201del. Furthermore, when transfected into interferon-cured highly permissive Huh-7 cells [31], the RpJ4/ISDR mutant replicated, whereas the RpJ4 remained incompetent, suggesting that ISDR mutations alone may be adaptive under certain conditions. In fact, Ikeda et al. reported that replication of the HCV-N replicon without adaptive mutations were dependent on four amino acid insertions in ISDR as removing this insertion impaired replication [7]. We also could establish replicon-harbouring cell lines with RpJ4 possessing the ISDR sequence identical to that of HCV-N, demonstrating this particular ISDR structure also worked as adaptive for RpJ4 in highly permissive cells (data not shown). Meanwhile, we could not establish replicon-harbouring cell lines with RpJ4 possessing fewer amino acid substitutions in ISDR (one or three amino acid substitutions obtained from patients' sera) even with highly-permissive cured Huh-7 cells. In contrast to the serine cluster mutations, ISDR mutations are frequently observed in the HCV genome obtained from patients with chronic hepatitis C showing decreased serum viral titres and increased interferon sensitivity [15,16]. This indicates that the ISDR mutations are not lethal *in vivo*, although the relationship between these ISDR mutations and replication efficiency *in vitro* is opposite. ISDR was originally identified as the site that determines the sensitivity of HCV to interferon [15,16]. The mutation of ISDR is closely associated with decreased serum HCV-RNA levels, whereas ISDR mutations in the HCV replicon enhance replication. A number of studies have attempted to identify the mechanism using NS5A protein expression *in vitro* or in transgenic mice. A variety of putative NS5A functions were postulated, such as binding to PKR [26], TRADD [35], Grb2 [36], p21 [37,38] or other proteins that may influence the pathogenesis of hepatitis C by antiviral effects, apoptosis, signal transduction,

or regulating cell cycles [38–42]. Requirement of adaptive mutations around and within ISDR for HCV replicon system strongly suggests that the NS5A protein plays a central role in regulating HCV replication or persistence through unknown mechanisms.

As ISDR mutations may enhance clinical interferon efficacy, it is of interest to determine the interferon sensitivities of HCV replicons between RpJ4 with or without ISDR mutation. We could not detect differences in RpJ4 replicons with different NS5A mutations. However, a more comprehensive and sensitive dose–response study by real-time RT-PCR, luciferase [30] or secreted embryonic alkaline phosphatase (SEAP) [43] is necessary as Northern blots are rather insensitive for detecting slight changes in interferon sensitivity.

In conclusion, we established new replicons derived from HC-J4, and demonstrated that serine cluster and ISDR mutations of NS5A are a general requirement for different HCV replicons. This finding is applicable to the generation of new HCV replicons by introducing NS5A mutants. The opposite effects of these mutations on replication *in vivo* and *in vitro* suggest different roles for cellular factors interacting with NS5A in normal hepatocytes *vs* cultured hepatoma cells. Elucidating these issues will help to clarify the detailed molecular mechanism of HCV replication and contribute to the development of an effective treatment for chronic hepatitis.

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Specific inhibition of hepatitis C virus replication by cyclosporin A

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Abstract

The difficulty in eradicating hepatitis C virus (HCV) infection is attributable to the limited treatment options against the virus. Recently, cyclosporin A (CsA), a widely used immunosuppressive drug, has been reported to be effective against HCV infection [J. Gastroenterol. 38 (2003) 567], although little is understood about the mechanism of its action against HCV. In this study, we investigated the anti-viral effects of CsA using an HCV replicon system. Human hepatoma Huh7 cells were transfected with an HCV replicon expressing a chimeric gene encoding a luciferase reporter and neomycin phosphotransferase (Huh7/Rep-Feo). Treatment of the Huh7/Rep-Feo cells with CsA resulted in suppression of the replication of the HCV replicon in a dose-dependent manner, with an IC50 of ~0.5 μg/ml. There were no changes in the rate of cell growth or viability, suggesting that the effect of CsA against HCV is specific and not due to cytotoxicity. In contrast, FK506, another immunosuppressive drug, did not suppress HCV replication. CsA did not activate interferon-stimulated gene responses, suggesting that its action is independent of that of interferon. In conclusion, CsA inhibits HCV replication in vitro specifically at clinical concentrations. Further defining its mode of action against HCV replication potentially may be important for identifying novel molecular targets to treat HCV infection.

Keywords: Hepatitis C virus; Replicon; Cyclosporin A; FK506; ISRE; Cyclophilins

Hepatitis C virus (HCV), which infects 170 million people worldwide, is characterized by chronic liver inflammation and liver fibrogenesis, leading to end-stage liver failure and hepatocellular malignancy [1,2]. Attempts to control HCV infection have been unsatisfactory because of the limited treatment options against the virus. Present therapies against HCV infection are based on high dose administration of interferon (IFN)-α in combination with ribavirin, a synthetic guanosine analog [3,4]. However, success rates remain at around 30–40% of patients treated. Furthermore, treatment with IFN and ribavirin carries a significant risk of serious side effects. Thus, the development of new therapeutic agents is a high priority goal.

Recently, CsA, the most widely used immunosuppressive drug, was reported to be clinically effective against HCV infection [5]. A subsequent controlled trial showed that a combination of CsA with IFN is more effective than IFN monotherapy, especially in patients with a high viral load [6]. In T cells, which are the major cellular targets of CsA, CsA binds to soluble cytosolic proteins called cyclophilins, and the cyclophilin–CsA complexes block calcineurin, which inhibits stimulation of the NFAT-induced genes which are essential for the activation of T cells [7]. However, despite the clinical effectiveness of CsA, little is understood about its anti-viral mechanisms in patients with chronic hepatitis C. In particular, elucidation of the mechanism of the anti-viral effects of CsA may give new insights into the replication of HCV and elucidate potential targets for anti-HCV therapy.

In the present study, we investigated the effects of CsA on the intracellular replication of the HCV genome in vitro, using an HCV replicon system, reported

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recently in a cultured human hepatoma Huh7 cell line [8]. We demonstrated that CsA inhibits HCV replication in vitro substantially and specifically, and that the mechanism of action is independent of that of IFN.

Materials and methods

Drugs. CsA was purchased from Sigma (St. Louis, MO). FK506 was from Alexis Biochemicals (Lausen, Switzerland). Recombinant human IFNα-2b was obtained from Schering-Plough (Kenilworth, NJ).

Cell culture. A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum at 37 °C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 200 μg/ml G418 (Wako, Osaka, Japan).

HCV replicon constructs. An HCV subgenomic replicon plasmid, pHCVIbneo-delS, was derived from an infectious HCV clone, HCV-N, genotype Ib [9]. The replicon pHCVIbneo-delS was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotransferase, as described elsewhere (pRep-Feo) [10]. RNA was synthesized from pHCVIbneo-delS and pRep-Feo using T7-polymerase (Lumat LB9501; Promega) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/Rep-Neo and Huh7/Rep-Feo, respectively).

Reporter constructs. A plasmid, pISRE-TA-Luc (Invitrogen, Carlsbad, CA), expressed luciferase reporter gene under control of the interferon stimulation response element (ISRE). pRL-CMV (Promega), which expresses *Renilla* luciferase gene under the control of the cytomegalovirus early promoter/enhancer, was used as a control for transfection efficiency of pISRE-TA-Luc. A plasmid, pECMV/IRES-Rluc, was used as a control to analyze translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (ECMV-IRES) which mediates translation of HCV non-structure gene of replicon constructs, pHCVIbneo-delS and pRep-Feo. The pECMV/IRES-Rluc expressed mRNA consisted of ECMV-IRES and *Renilla* luciferase reporter gene under control of cytomegalovirus early promoter/enhancer.

Luciferase assays. Luciferase activities were quantified using a luminometer (Promega) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means ± SD as percentages of the controls.

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–Pharmacia Biotech). 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 a Digoxigenin Luminescent Detection Kit (Roche, Mannheim, Germany) and 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 a monoclonal anti-NS5A antibody (BioDesign, Saco, ME) and detection was carried out in a chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche).

MTS assays. To evaluate cytotoxicity, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

Statistical analyses. Statistical analyses were performed using Student's *t* test; *p* values less than 0.05 were considered as statistically significant.

Results

Suppression of hepatitis C virus replication by cyclosporin A (Figs. 1 and 2)

To assess the effects of CsA on the intracellular replication of the HCV genome, Huh7/Rep-Feo cells were cultured with various concentrations of CsA in the medium. The luciferase activities of the Huh7/Rep-Feo

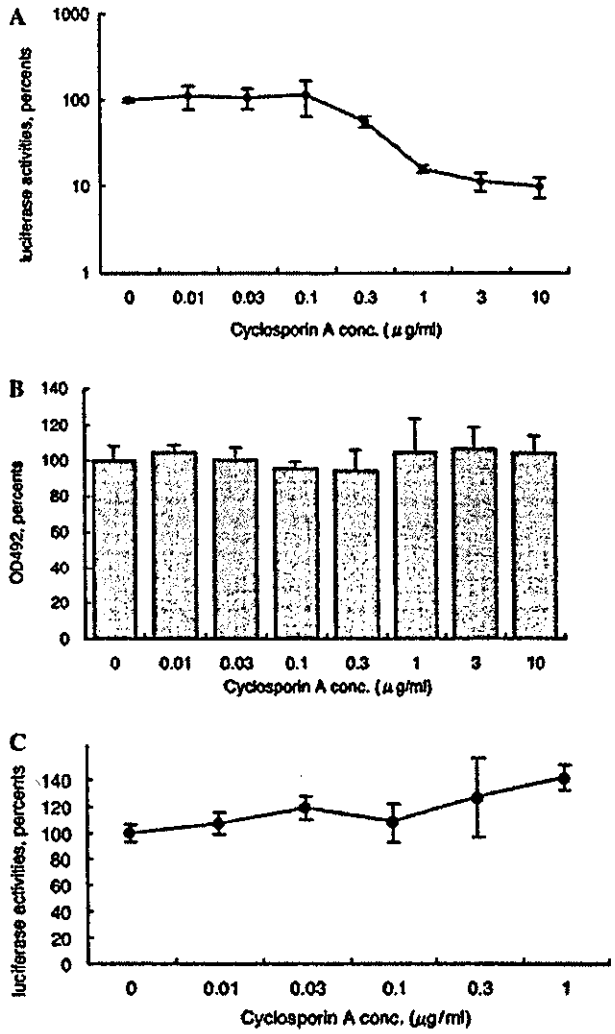

Fig. 1. Suppression of hepatitis C virus replication by cyclosporin A. (A) Huh7/Rep-Feo cells, which constitutively express a hepatitis C virus replicon, enable the quantification of replication levels through the measurement of luciferase activity. Relative log (dose)–response curves in the presence of various concentrations of CsA. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD. (B) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of CsA indicated. (C) A plasmid, pECMV/IRES-Rluc, was used to analyze the translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (ECMV-IRES) which mediates translation of HCV non-structure gene of replicon constructs. The pECMV/IRES-Rluc was transfected into Huh7 cells. The transfected cells were cultured in the presence of indicated concentrations of CsA and luciferase activities were measured at 48 h of transfection. The assays were done in triplicate. Error bars indicate means ± 2SD.

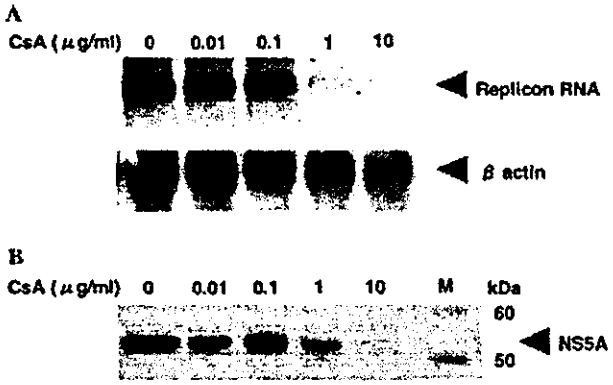

Fig. 2. Suppression of replicon RNA and NS 5A synthesis by CsA. (A) Northern hybridization. The replicon cells described by Seeger were cultured in the presence of the amounts of CsA indicated and harvested at 48 h after exposure. Ten micrograms of total cellular RNA was electrophoresed in each lane. 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, Naïve replicon; lane 2, 0.01 μg/ml CsA; lane 3, 0.1 μg/ml CsA; lane 4, 1 μg/ml CsA; and lane 5, 10 μg/ml CsA. (B) Western blotting. Ten micrograms of total cellular protein was electrophoresed in each lane. Monoclonal anti-NS5A antibody and polyclonal anti-luciferase antibody were used as the primary antibodies. Lane 1, sample without CsA; lanes 2–5, cells cultured with CsA at concentrations of 0.01 (2), 0.1 (3), 1 (4), and 10 μg/ml (5). Lane 5, protein size marker, MagicMark (Invitrogen).

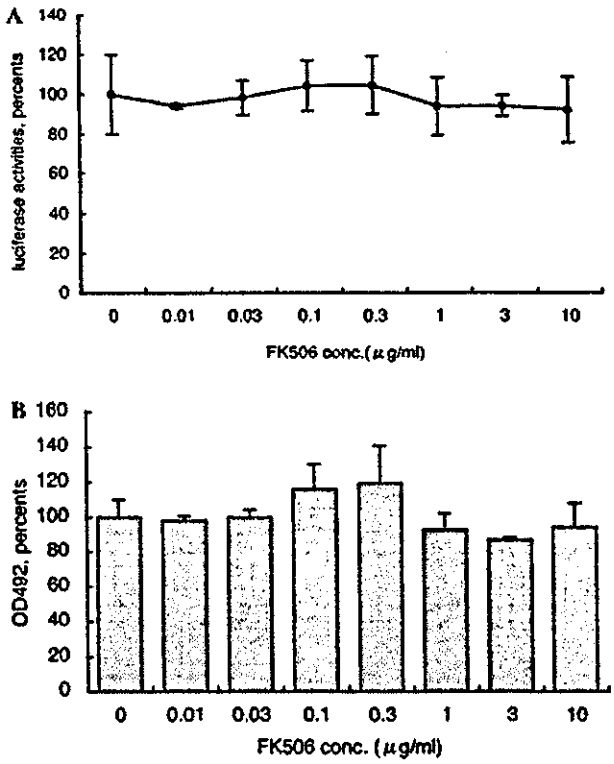

Fig. 3. Absence of an inhibitory effect of FK506. (A) To assess the effects of another immunosuppressive agent, FK506, Huh7/Rep-Feo cells were cultured with various concentrations of FK506 in the medium, and luciferase assays were performed after 48 h of culture. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD. (B) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of FK506 indicated.

cells showed that the replication of the HCV replicon was suppressed by CsA in a dose-dependent manner. The luciferase activities were 56% and 16% of the control at CsA concentrations of 0.3 and 1 μg/ml, respectively (Fig. 1A). The MTS assay did not show any effect on cell viablilty or replication (Fig. 1B). Moreover, efficiency of the EMCV-IRES-mediated translation was not affected by CsA (Fig. 1C). These results suggest that the decrease in HCV replication is due to a specific suppressive effect of CsA on HCV replication, and not due to cytotoxicity of CsA or an artificial effect on the EMCV-IRES which direct translation of HCV non-structure protein of the replicon.

In Northern blot analysis (Fig. 2A), levels of the replicon RNA, which were detectable in CsA-negative control cells, decreased substantially following treatment with CsA at concentrations of 1 and 10 μg/ml. Densitometric analysis of the replicon RNA showed that the intracellular levels of the replicon RNA in Huh7/Rep-Feo correlated well with the luciferase activities (data not shown). Similarly, in Western blotting (Fig. 2B), the HCV non-structural protein, NS5A, translated from the HCV replicon, decreased by corresponding amounts in response to treatment with CsA.

Absence of an inhibitory effect of FK506 (Fig. 3)

FK506 (tacrolimus), another immunosuppressive agent, shares many mechanisms of action with CsA. FK506 is another inhibitor for the calcineurin/NFAT pathway and also blocks the activation of the JNK and p38 pathways in T cells [11]. To assess the potential effect of FK506, Huh7/Rep-Feo cells were cultured with FK506 at 0.01–10 μg/ml. The luciferase activities of the cells did not show a significant effect of FK506 on HCV replication at concentrations covering the range achievable clinically.

Cyclosporin A does not elicit an interferon-stimulated response (Fig. 4)

It has been reported that the HCV replicon is highly sensitive to IFN [12]. To determine whether the action of CsA on HCV subgenomic replication involves activation of IFN-stimulated gene responses, the ISRE-luciferase plasmids were transfected into Huh7/Rep-Feo 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 for the activation of ISRE reporter activity, the ISRE-luciferase-transfected cells were cultured with IFNα-2b at concentrations of 0, 0.1, 1, and 10 U/ml. The luciferase activities at 48 h after transfection showed that there were no significant effects of CsA and FK506 on

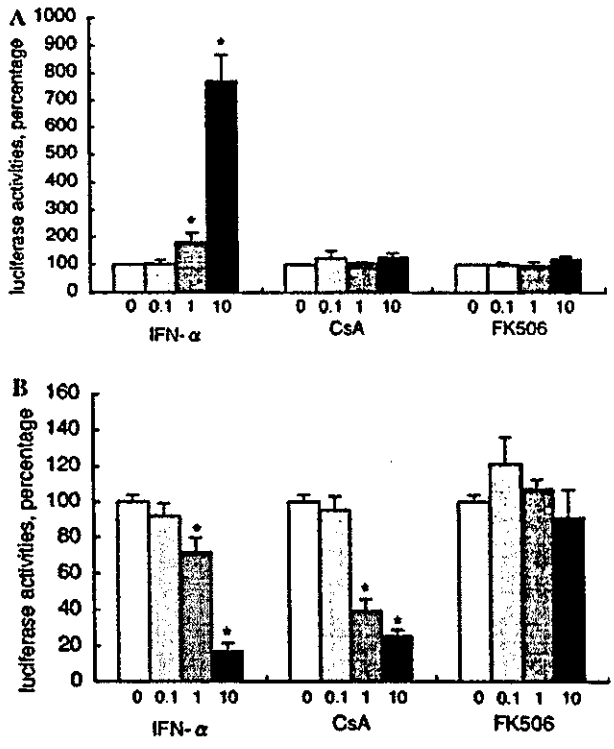

Fig. 4. Cyclosporin A does not elicit an interferon response. (A) ISRE-responsive luciferase reporter plasmids were transfected into cells containing replicon in the presence of the indicated concentrations of IFN (U/ml), CsA, and FK506 (μg/ml) in the culture medium. Luciferase assays were carried out 48 h after transfection. Values are presented as percentages of drug-negative controls. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD. *p value <0.05. (B) Huh7/Rep-Feo cells were cultured with the indicated concentrations of IFN (U/ml), CsA, and FK506 (μg/ml). Luciferase activities of the cell lysates were carried out after 24 h of exposure. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD.

ISRE-promoter activities, while IFNα-2b had significant effects (Fig. 4A). As reported above, the luciferase activities of the Huh7/Rep-Feo cells showed that the replication of the HCV replicon was suppressed by IFNα-2b, as well as CsA, in a dose-dependent manner, but not by FK506 in this condition (Fig. 4B). These results suggest that the action of CsA on the intracellular replication of HCV replicon is independent of the IFN pathway.

Discussion

Our present results demonstrate that CsA inhibits the intracellular replication of an HCV subgenomic replicon at clinically achievable drug concentrations. The Northern and Western blot analyses revealed that both RNA synthesis and its translation were reduced by CsA in a dose-dependent manner. Treatment of Huh7 cells with CsA did not activate IFN-stimulated gene responses, suggesting that the mechanisms of action of CsA are independent of those of IFN. In addition, FK506, another drug used frequently and which shares an immunosuppressive mechanism with CsA, did not show any inhibitory effect on HCV replication, suggesting that the anti-HCV effect of CsA is not associated with immunosuppressive activity.

CsA and FK506, although structurally dissimilar, have a similar mode of immunosuppressive action in preventing induction of inflammatory gene expression in activated T cells. Both CsA and FK506 bind specifically to a family of soluble cytosolic proteins called immunophilins [7]. CsA is bound by cyclophilins and FK506 is bound by the FK506 binding proteins (FKBPs). The cyclophilin–CsA and FKBPs–FK506 complexes inhibit the phosphatase activity of calcineurin, that mediates phosphorylation, and nuclear translocation of the transcription factor, NFAT, critical in the expression of cytokines and their receptors, respectively [7,13,14]. In our present study, contrary to the effect of CsA on HCV replication, FK506, which has an immunosuppressive activity 100-fold greater than that of CsA [15,16], did not show an inhibitory effect on HCV replication. The findings demonstrate that the anti-viral action of CsA against HCV is not through suppression of NFAT-regulated gene responses but through distinct mechanisms that are not shared with FK506.

Another function of cyclophilins and FKBPs, which CsA and FK506, respectively, are able to block, is a peptidyl–prolyl *cis–trans* isomerase (PPIase) activity. PPIase catalyzes the *cis–trans* conversion of imide peptide preceding prolines [17], and acts as a molecular chaperone, accelerating the slow steps of correct post-translational folding of some proteins [18]. Cyclophilins are present in every compartment of the cell, including the cytoplasm, endoplasmic reticulum (ER), and nucleus [19]. The maturation steps of HCV proteins include processing of the polyprotein by an ER-membrane-bound signal peptidase and by two viral serine proteases [20]. Recent studies have demonstrated the localization of viral non-structural proteins in the ER forming microscopic intracellular structures, called "membranous webs," which is characterized by convoluted ER membranes [21]. Moreover, folding and assembly of HCV structural and non-structural proteins require interaction with ER chaperone proteins such as calreticulin, BiP, and HSP90 [22,23]. Collectively, it is speculated that certain chaperone activities, such as those of cyclophilins, may be crucial for the processing and maturation of the viral proteins and for viral replication. Thus, one most likely mechanism of action against HCV is blocking of the PPIase activities of cyclophilins. The striking difference between the significant effects observed with CsA and the lack of effect of FK506 may be explained by the different enzymatic properties of cyclophilins and FKBPs [24]. Cyclophilins are non-specific PPIases and are able to isomerize all X-Pro bonds.

On the other hand, FKBPs catalyze the isomerization of a limited set of X-Pro bonds. Therefore, inhibition of FKBPs can be overridden by the action of cyclophilins, whereas inhibition of cyclophilins cannot be substituted by the limited substrate specificity of FKBPs [25].

In addition to the anti-viral activity of CsA against HCV, confirmed by the present results, CsA has been reported to show anti-viral effects against HIV-1 through blocking the activities of cyclophilin A [19]. Cyclophilin A binds to the viral *gag* protein with high affinity, and CsA competes with the *gag* protein for the same binding site on cyclophilin A [26]. Moreover, cyclophilin A is packaged into HIV-1 virions and catalyzes *cis–trans* isomerization of the viral capsid protein as a molecular chaperone [27]. It has been shown that the anti-HIV action of CsA does correlate not with the immunosuppressive potential of the compounds but with their capacity to bind to cyclophilins [28]. These reports demonstrate that the anti-viral action of CsA against HIV-1 is through inhibition of the PPIase activities of cellular cyclophilins, as against HCV suggested in this study.

End-stage HCV liver cirrhosis is a major indication for liver transplantation, accounting for approximately 50% of cases in the US and in Europe [29]. However, viral recurrence occurs in all recipients and HCV-graft hepatitis develops in one-third [30]. Decompensated graft cirrhosis following re-infection with HCV is the main cause of death post-transplantation [31]. Long-term immunosuppression is essential for patients who have undergone transplantation. The two most frequently used drugs are CsA and FK506 and usage of FK506 has increased from 0% before 1996 to nearly 80% after 1999 [31], because the early safety and efficacy of an FK506 regimen after liver transplantation has been shown in two multicenter trials [32,33]. More recently, it has been reported that FK506-based immunosuppression is preferable to cyclosporin A during the first year following liver transplantation [34]. As for transplantation for HCV cirrhosis, however, recent studies from two institutes in Spain and the USA report that disease progression after transplantation has accelerated in recent years [29,31], although the reasons for the worsening outcome are under question. The anti-viral activity of CsA against HCV, demonstrated in this study, should be taken into account when selecting the immunosuppressive regimen for the optimum outcome of HCV-infected recipients.

The expanding applications of CsA, however, may cause substantial problems. Especially, an undesired immunosuppression that may lead to an immunocompromised status and may interfere with the effects of anti-infectious drugs such as IFN. Some cyclosporine analogs that fail to block T cell activation are still able to inhibit the PPIase activity [16,35]. Some of these non-immunosuppressive cyclosporine analogs were, in fact, equal or even superior in anti-HIV activity to immunosuppressive types [36]. Therefore, one solution to overcome the problem due to immunosuppression would be to consider the use of such cyclosporine analogs.

In conclusion, CsA inhibits HCV replication in vitro substantially and specifically. Considering the limited therapy options against HCV infection and the unsatisfactory therapy outcome, with half of the patients unable to eradicate the virus, CsA potentially becomes available as an effective treatment against HCV infection. Further defining the mechanism of action of CsA against HCV replication potentially may be important for identifying novel molecular targets to terminate HCV infection.

Acknowledgments

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Synergistic Inhibition of Intracellular Hepatitis C Virus Replication by Combination of Ribavirin and Interferon-α

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Treatment of hepatitis C virus (HCV) infection with interferon (IFN)-α and ribavirin combination therapy results in superior clinical antiviral responses than does monotherapy with IFN. To explore the virological basis of the effects of combination therapy, we analyzed the effects of IFN-α and ribavirin, singly and in combination, on intracellular HCV replication by use of an HCV replicon system. A new replicon that expressed a selectable chimeric reporter protein comprising firefly luciferase and neomycin phosphotransferase was constructed. The replicon was highly sensitive to IFN-α (50% inhibitory concentration [IC_{50}], 0.5 U/mL). Therapy with ribavirin showed weak suppression of HCV replication at a lower concentration (IC_{50}, 126 μmol/L). The nucleotide sequence diversity of the replicon was increased significantly by therapy with ribavirin, suggesting that error-prone HCV replication was induced by the drug. Importantly, use of a clinically achievable concentration of ribavirin (~10 μmol/L) in combination with IFN showed strong synergistic inhibitory effects on HCV replication. Our results suggest that the direct effects of ribavirin on the genetic stability of the HCV subgenome and its synergistic action combined, with IFN-α, may explain the improved clinical responses to combination therapy.

Hepatitis C virus (HCV) infects 170 million people worldwide and is characterized by chronic liver inflammation and fibrogenesis leading to end-stage liver failure and hepatocellular malignancy [1–3]. The difficulty in eradicating HCV is attributable, in part, to limited treatment options against the virus. Currently available therapies against HCV are based on the administration of high doses of interferon (IFN)-α or IFN-β. However, the results of the IFN monotherapy were unsatisfactory with sustained virus clearance rates of only 15%–20% in treated patients [4–6]. Combination therapy using ribavirin and IFN, which was approved in the United States in 1998, has achieved improved antiviral effects, with sustained viral clearance rates of 30%–40% in treated patients [7–9]. Despite the success of combination therapy with IFN and ribavirin in the clinical setting, little is understood about the antiviral mechanisms of ribavirin against HCV or about the mechanisms of the improved antiviral response to IFN in combination with ribavirin.

Ribavirin is a synthetic guanosine analogue and has actions in vitro against a wide range of RNA and DNA viruses [10]. Possible antiviral mechanisms of ribavirin include immune modulation (by switching the T cell phenotype from type 2 to type 1) [11], antiproliferative effects (by inhibition of cellular GTP synthesis) [10], and direct inhibition of viral replication [12]. However, the antiviral mechanisms of ribavirin on HCV are not completely understood.

Investigations of the molecular mechanisms of intracellular HCV replication have been hampered by the lack of efficient cell culture systems and small animal

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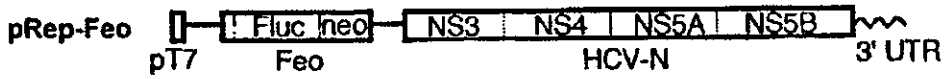

Figure 1. Structure of replicon plasmid constructs. Sequence data have been deposited with GenBank (accession no. AB119282). A hepatitis C virus (HCV) replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS [14] by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene of firefly luciferase (Fluc) and Neo, which we designated as "Feo". NS, nonstructural region; pT7, T7 promoter; 3' UTR, 3' untranslated region [15]

models that support HCV replication. An HCV subgenomic replicon (a synthetic selectable RNA molecule consisting of the neomycin phosphotransferase gene [Neo] and HCV nonstructural genes) replicates efficiently and continuously in the cultured human hepatoma cell line Huh7, as recently described elsewhere [13]. The development of the HCV replicon system has allowed various studies of HCV RNA replication in cell culture.

In the present study, we investigated the mechanisms of action of ribavirin, alone or in combination with IFN, on intracellular HCV replication. To perform quantitative and high-throughput detection of HCV replication levels, we modified the HCV replicon to express a selectable chimeric luciferase reporter protein that allows selection of cells, with continuous expression of the replicon and rapid measurement of the replication levels in stable replicon-expressing cells. Here, we demonstrate that ribavirin has an effect on the genetic stability of the viral subgenome and that the combination of ribavirin and IFN shows strong synergistic effects on viral replication.

MATERIALS AND METHODS

Construction of replicon plasmids expressing chimeric reporter gene. The HCV replicon plasmid, pHCV1bneo-delS, was a gift from Dr. Christoph Seeger (Fox Chase Cancer Center, Philadelphia, PA). The replicon was derived from a chimpanzee infectious clone (strain HCV-N, genotype 1b) [14]. The Neo gene of pHCV1bneo-delS was replaced with a chimeric gene coding for firefly luciferase protein fused in-frame with neomycin phosphotransferase (designated "Feo"), as described elsewhere [15]. A DNA fragment encoding firefly luciferase was generated by polymerase chain reaction (PCR) using the following pair of primers: *Ascl*/Fluc-S, 5'-AAGGGCGCGCCATGGAAGACGCCAAAAACA-3' (sense) and *Ascl*/Fluc-AS, 5'-ATTGGCGCGCCGCAATTTGGACTTTCCGCCCT-3' (antisense). The DNA fragment was digested with *Ascl* (New England Biolabs) and was cloned into the *Ascl* site of the pHCV1bneo-delS, which is located at nt 380, upstream of the Neo gene (pRep-Feo; figure 1).

In vitro transcription and purification of RNA. The replicon RNA synthesis was performed by use of the RiboMax Large Scale RNA Production System (Promega), according to the manufacturer's protocol. In brief, replicon RNA was synthesized by use of T7-RNA polymerase, with linearized pRep-Feo used as the template. After RNA synthesis, the template DNA was digested with RNase-free DNase (Promega), and the replicon RNA was purified by use of Isogen (Wako).

Cells and cell culture. The human hepatoma cell line Huh7 was used as the host for intracellular replication of the HCV replicon and was grown in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mmol/L L-glutamine and 10% fetal calf serum at 37°C under 5.0% CO_2. To select cell lines carrying the HCV replicon, G418 (Wako) was added to the culture medium (final concentration, 250 μg/mL).

RNA transfection and selection of G418-resistant cell lines. Huh7 cells were suspended in ice-cold PBS (Sigma). Cells (5×10^6) were suspended in 10 μg of replicon RNA/Opti-MEM I (Invitrogen) and were subjected to an electric pulse at 1050 μF, 201 Ω, and 270 V by use of the EasyJect Electroporation System (EqiBio). After electroporation, the cell suspension was left for 5 min at room temperature and then was incubated under normal culture conditions in a 10-cm diameter cell culture dish. At 24 h after the transfection, 250 μg/mL G418 was added. At ~2 weeks after the transfection, cells harboring continuous HCV-Feo replicon expression were selected (Huh7/Rep-Feo).

Treatment with IFN and ribavirin. Recombinant human IFN-α-2b (Schering-Plough) and purified ribavirin (Schering-Plough) were used.

Luciferase assays. Luciferase activity was quantified by use of a luminometer (Lumat LB9501; Promega) by use of a Bright-Glo Luciferase Assay System (Promega). Typically, 5×10^3 cells/well, plated onto 48-well plates, were lysed by use of 100 μL of 1× Glo luciferase Buffer (Promega), and the luciferase activity in 30 μL of the lysate was measured by adding an equal volume of Bright-Glo Luciferase Assay Reagent (Promega). Assays were performed in triplicate, and the results were expressed as means ± SD relative light units.

MTS assays. Viable cell growth was determined by 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) reduction assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer's instructions.

Northern-blot hybridization. Total cellular RNA was extracted from the cells by use of Isogen (Wako). Ten micrograms of the total cellular RNA was electrophoresed on a 1.0% agarose gel containing 1× 3-(N-morpholine) propane sulfonic acid and 2.2 mol/L formaldehyde and was transferred to a ny-

lon membrane (Shleichere & Schuell). Hybridization was performed by use of a digoxigenin-labeled DNA probe specific for the full-length replicon sequence (1 ng/mL) in URTRAhyb Hybridization Buffer (Ambion) overnight at 42°C. The replicon RNA was detected by use of a Digoxigenin Luminescent Detection Kit (Roche) and was visualized by use of a Lumi-Imager F1 (Roche).

Analyses of drug synergism. The effects of treatment of Huh7/Rep-Feo cells with ribavirin and IFN, alone and in combination, were analyzed according to classic isobologram analysis [16, 17]. Dose-inhibition curves of IFN and ribavirin, with the 2 drugs used alone or in combination, were drawn. For each drug combination, the IC_{50} values were plotted against the fractional concentration of IFN and ribavirin on the *X*-axis and *Y*-axis, respectively. The combination index (CI) for each combination was calculated by use of the following formula: CI = IC_{50} (IFN combined)/IC_{50} (IFN alone) + IC_{50} (ribavirin combined)/IC_{50} (ribavirin alone). For such plots, the combined effects of 2 drugs can be assessed as either an additive effect (CI, 1), synergy (CI, <1), or antagonism (CI, >1).

Sequence analyses of the HCV replicon. The nonstructural region (NS3-NS5B) of the replicon was amplified by use of nested reverse-transcription (RT)–PCR with 12 partially overlapping primer sets, as reported elsewhere [18]. Consensus nucleotide sequences were determined from both strands by use of Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems) and an automated DNA sequencer (model 373S; Applied Biosystems). To analyze the quasi species of the replicon, the PCR-amplified fragment of the replicon subgenome was cloned into the pGEM-T vector (Promega), and 10 independent clones were sequenced in the 2140–2318 nt region of NS5A, including the interferon sensitivity determining region. Our previous study has shown that the mutation rates in this selected region of the HCV genome are comparable with those of the entire genome [19].

Statistical analysis. Statistical analysis was performed by use of Student's *t* test. $P < .05$ was considered to be statistically significant.

RESULTS

Establishment and characterization of Huh7/Rep-Feo. Transfection of the Rep-Feo replicon RNA onto Huh7 cells and cell culture with 250 μg/mL G418 resulted in numerous drug-resistant colonies (800–1000 colonies/10-cm dish). However, untransfected Huh7 cells and Huh7 cells transfected with a replication-deficient replicon, which carries the neomycin-resistant gene but has a frame-shift mutation in the NS5B polymerase, did not survive in culture with the same concentration of G418. The expression of the HCV nonstructural protein NS5A and the luciferase protein in Huh7/Rep-Feo were confirmed by Western blotting (data not shown). In Northern-blot hybridization, expression of HCV-Feo replicon RNA of the 9.6-kB Feo replicon RNA was detectable in 10 μg of total cellular RNA, indicating high-copy replication of the HCV subgenome in Huh7 cells (figures 2 and 3).

Suppression of HCV RNA replication by IFN. Huh7/Rep-Feo cells were cultured with various concentrations of IFN, and the dose-effect correlation and time courses of replicon expression were measured by use of luciferase assay. IFN caused a marked suppression of HCV RNA replication in a dose-dependent manner (figure 2*A*). The inhibition of HCV RNA replication was detectable with a concentration of IFN as low as 0.01 U/mL. Northern-blot hybridization also demonstrated a reduction of the replicon RNA with IFN (figure 2*B*). In contrast, treatment with IFN had little effect on cellular viability and replication, as shown by the results of the MTS assay, which indicated that the suppression of HCV replication was due to the specific action of IFN and not to nonspecific cytotoxicity (figure 2*C*).

Kinetics of HCV RNA replication and luciferase activities. Huh7/Rep-Feo cells were cultured with various concentrations of IFN, and measurement of internal luciferase activities and Northern-blot hybridization of the replicon RNA were performed simultaneously. The luciferase activities of the Huh7/Rep-Feo cells correlated well with the results of densitometric analyses of the Northern blots (figure 4). In addition, the time kinetics of the luciferase correlated well with the replicon RNA levels. These results demonstrate that the luciferase expression levels of the HCV-Feo replicon convincingly reflect the intracellular expression levels of the replicon RNA.

Effect of ribavirin on HCV replication. Cultures of the Huh7/Rep-Feo cells with ribavirin, at concentrations ranging from 0 to 100 μmol/L, showed dose-dependent repression of the internal luciferase activities (figure 3*A*). Northern-blot hybridization and densitometry of each blot showed essentially identical results to those of the luciferase assay (figure 3*B*). Because the signals on the blot were present only at the position of intact replicon RNA, these findings suggest that the replicon RNA was not degraded by treatment with ribavirin. Levels of β-actin RNA, however, were decreased by treatment with 100 μmol/L ribavirin. The MTS reduction ratios tended to decrease at ribavirin concentrations >75 μmol/L (figure 3*C*).

Ribavirin-induced nucleotide mutations in the replicon. Several studies support the theory that the antiviral action of ribavirin is through the accumulation of mutations in the viral genome following misincorporation of ribavirin-triphosphate—the so-called "error catastrophe" theory [12, 20]. To determine whether the suppressive effects of ribavirin on HCV subgenome replication found in the present study can be attributed to the accumulation of nucleotide mutations, we conducted sequence analysis of the replicon subgenome after treatment with ribavirin. We first compared the consensus sequence

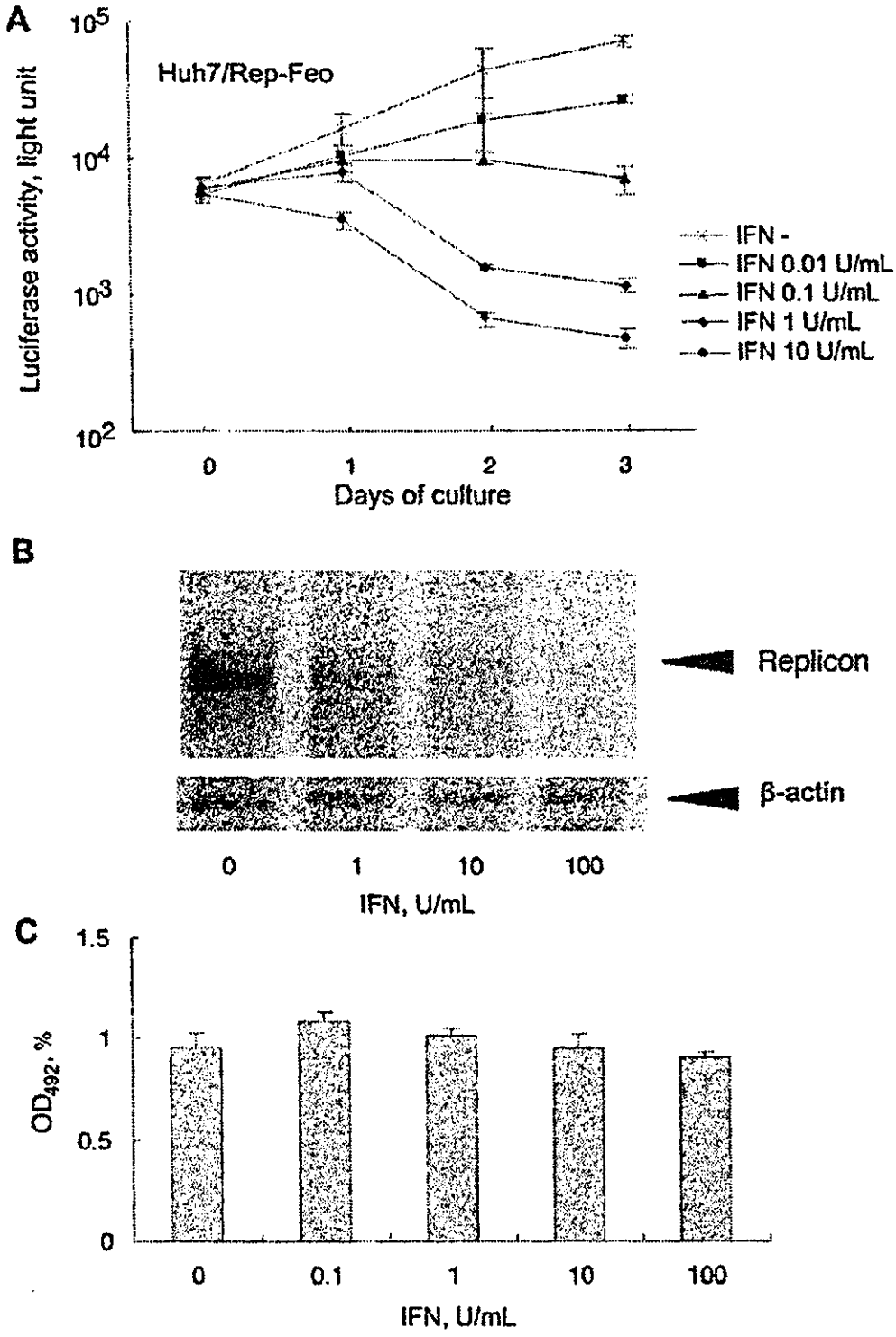

Figure 2. Dose-dependent inhibition of hepatitis C virus (HCV) RNA replications by interferon (IFN). *A*, Huh7/Rep-Feo cells were cultured with the concentrations of IFN indicated in the medium. *B*, Northern-blot hybridization. The upper part of the membrane containing HCV replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with a β-actin probe. *C*, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) assay of Huh7/Rep-Feo cells cultured with the concentrations of IFN indicated.

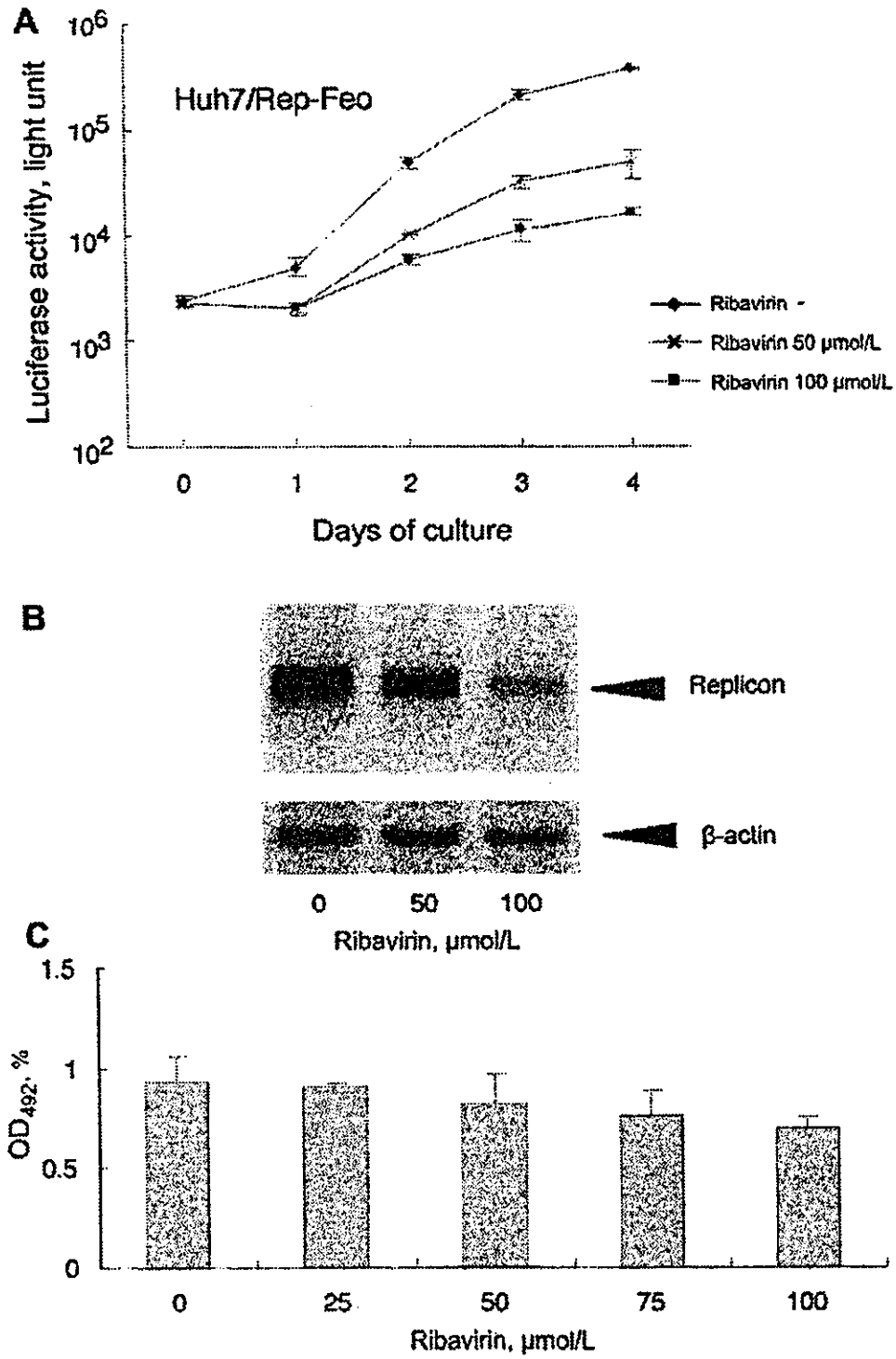

Figure 3. *Effects of ribavirin on hepatitis C virus (HCV) RNA replication. A,* Huh7/Rep-Feo cells were cultured with the concentrations of ribavirin indicated in the medium. *B,* Northern-blot hybridization. The cells were cultured in the presence of the amounts of ribavirin indicated. The cells were harvested after 48 h of exposure. 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. *C,* 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) assay of Huh7/Rep-Feo cells cultured with the concentrations of ribavirin indicated.

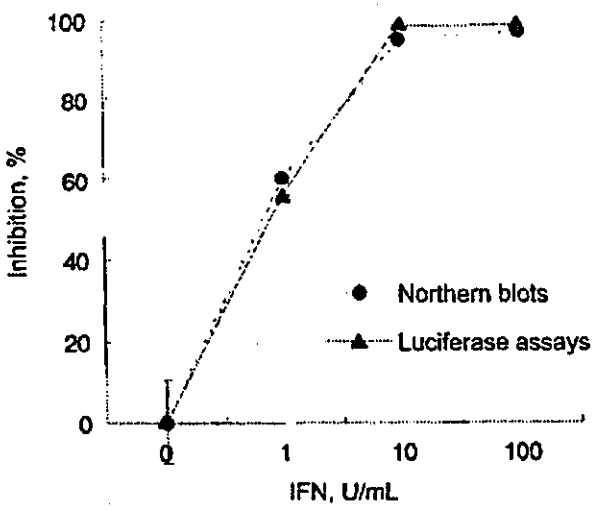

Figure 4. Kinetics of hepatitis C virus (HCV) RNA replication and luciferase activities. Huh7/Rep-Feo cells were cultured with various concentrations of interferon (IFN). Dose-inhibition curves of IFN analysis by the luciferase activities of the Huh7/Rep-Feo cells and densitometric values of the Northern-blot hybridization are shown.

of the HCV replicon treated with 250 μg/mL G418 and 100 μmol/L ribavirin for 8 weeks with that of the replicon cultured with 250 μg/mL G418, but without ribavirin. No nucleotide mutations were found in the consensus sequences of the entire HCV nonstructural region of the replicon.

As has been reported elsewhere, quasi species of HCV are present in patients' serum samples and may be attributed to the lack of proofreading activity of the HCV NS5B RNA–dependent RNA polymerase [21, 22]. We therefore analyzed the quasi species of the HCV replicon sequences treated with 0, 50, and 100 μmol/L ribavirin. RNA was extracted from the cells after 48 h of exposure to ribavirin. After cloning of the RT-PCR DNA fragment into the pGEM-T vector, 10 independent clones were sequenced from each sample. In the replicon cultured without ribavirin, 6 nonsynonymous and 7 synonymous mutations were found in 541 nt of the 10 independent clones from the replicon RNA (figure 5). As shown in table 1, overall sequence diversity increased in the samples treated with ribavirin. In the replicon treated with 100 μmol/L ribavirin, both synonymous and nonsynonymous mutations were significantly greater in number (2.77×10^{-3} mutations/nucleotide and 3.33×10^{-3} mutations/nucleotide, respectively) than those of the replicon cultured without ribavirin (0.37×10^{-3} mutations/nucleotide and 1.48×10^{-3} mutations/nucleotide, respectively; table 1).

Synergistic inhibitory effects of ribavirin and IFN on the replicon. The dose-effect correlations of ribavirin and IFN on the replicon were titrated by treating Huh7/Rep-Feo cells with various concentrations of either drug, and the luciferase activities were plotted against the drug concentrations (figure 6*A* and 6*B*). Both drugs showed strong linear regression of effect on the logarithms of doses ($R^2 = 0.97$ for ribavirin; $R^2 = 0.99$ for IFN). The IC_{50} values were 125.8 ± 3.7 μmol/L for ribavirin and 0.473 ± 0.005 U/mL for IFN.

Next, we conducted the following assay to determine whether ribavirin and IFN have a synergistic inhibitory effect on the replicon. Huh7/Rep-Feo cells were treated with combinations of IFN and ribavirin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentration of ribavirin of 0, 25, 50, and 100 μmol/L, respectively (figure 6*C*). The curves shifted to the left with increasing concentrations of ribavirin, demonstrating synergy of the 2 drugs against the HCV replicon.

The synergy of IFN and ribavirin was evaluated further by use of classic isobologram analysis [16, 17]. The 2 drugs were administered in combination with fixed ratios adjusted by the IC_{50} of each drug (FIC ratio) at 1:0, 100:1, 10:1, 1:1, 1:10, 1:100, and 0:1, respectively, and the dose-effect plots were drawn (figure 6*D*). Each FIC for IFN and for ribavirin, at 50% inhibition, were plotted on the *X*- and *Y*-axis, respectively, to generate an isobologram (figure 6*E*). Each plot showing the FIC ratio of each drug fell far below the line showing additivity, indicating that the effects of the drug combination on intracellular HCV RNA replication is strongly synergistic. The MTS reduction values at drug concentrations used in this isobologram analysis did not show any significant decreases (data not shown), suggesting that the synergistic action of IFN and ribavirin on HCV replication is through their pharmacological effects and is not due to augmentation of cytotoxicity.

In clinical usage, the maximum plasma concentration (C_{max}) of ribavirin ranges from 9.0 to 11.3 μmol/L, which is ~10 times lower than the IC_{50} calculated in the present study. However, the C_{max} of IFN is reported to be ~50 U/mL [23], which is ~50 times greater than the IC_{50}.

Therefore, we assessed the drug synergy within clinically relevant concentration ranges (figure 6*F*). Dose-inhibition analyses were conducted at 5 different IFN:ribavirin ratios (1:0, 1000:1, 300:1, 100:1, and 30:1), and the IC_{50} values were calculated for each combination. For a better graphic representation of the drug synergy, the CI of each combination was plotted against the proportion of ribavirin in the combination. The CI plot for all combinations shifted below the line of additivity (CI, 1), demonstrating that ribavirin and IFN had synergistic inhibitory effects on HCV replication, even at concentrations achievable clinically in plasma.

DISCUSSION

In the present study, we have constructed a new HCV replicon expressing a selectable chimeric reporter Feo protein. Using the HCV-Feo replicon, we have demonstrated that ribavirin showed weak suppressive effect on intracellular HCV replication, that

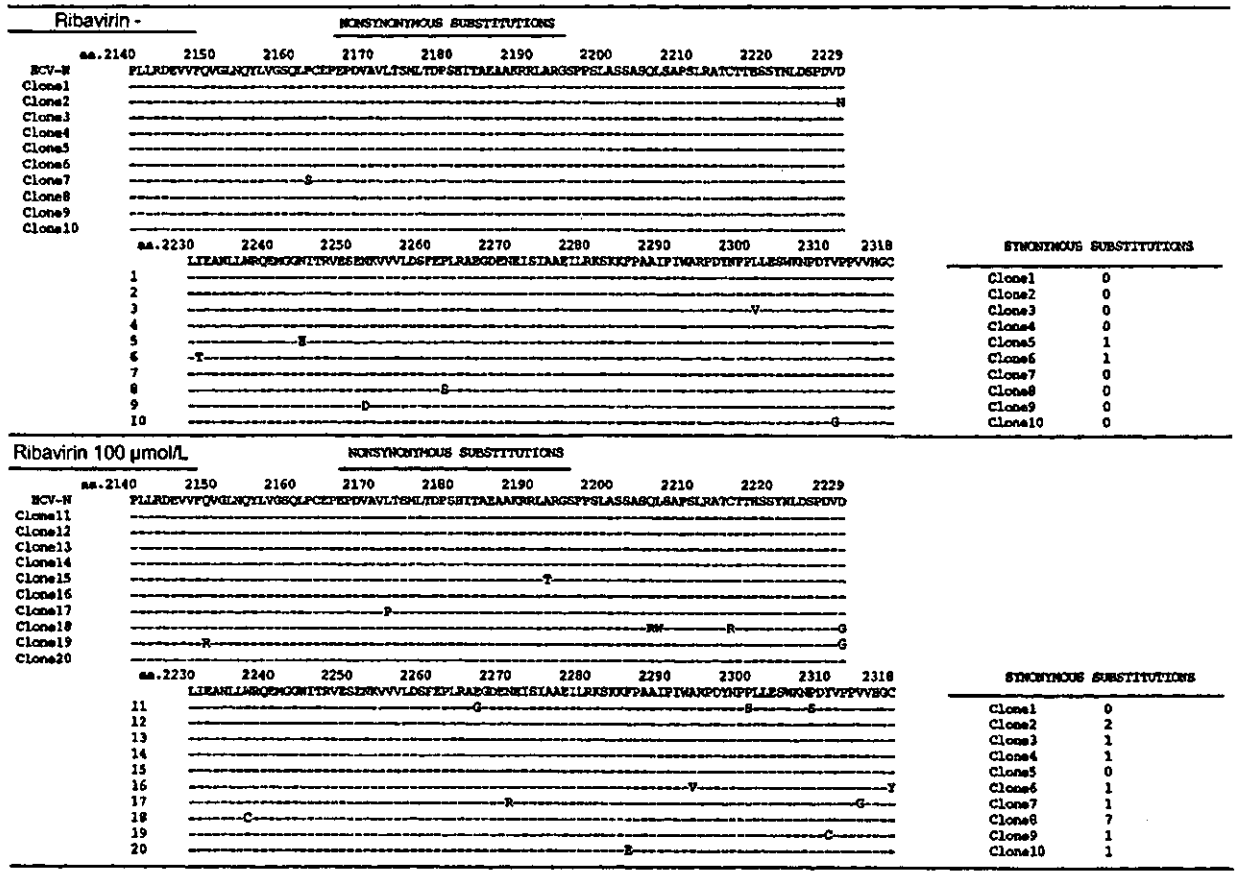

Figure 5. Generation of quasi species by the hepatitis C virus (HCV) replicon system. Sequence alignments were made of 10 independent clones of untreated replicon RNA and replicons treated with 100 µmol/L ribavirin. The alignments were made with HCV-N as the prototype sequence [39]. Amino acid position nos. (aa 2140–2318) correspond to those of full-length sequences of the HCV-N strain. Nonsynonymous substitutions are listed on the left, and synonymous substitutions are listed on the right.

Table 1. Summary of mutations detected in sequence analysis of the hepatitis C virus replicon after treatment with ribavirin.

Ribavirin concentration, μmol/L	10^{-3} nucleotides/site		
	Synonymous mutation	Nonsynonymous mutation	Total
0	0.37	1.48	1.85
50	0.56	0.93	1.48
100	2.77	3.33	6.10

this action possibly involved accumulation of nucleotide mutations (error catastrophe) [12, 20], and, more important, that combination therapy with ribavirin and IFN elicited strong synergy on suppression of the replication of the HCV subgenome.

The HCV replicon system, which supports efficient and continuous replication of the viral subgenome in cell lines, is currently the most suitable system with which to perform molecular studies on HCV replication [13]. To detect the HCV replicon in the cells, several methods have been performed, including Northern-blot hybridization, Western blotting, and real-time PCR analysis [13, 24]. Each of the detection methods, however, has its own shortcomings, such as low sensitivity, difficulty in precise quantification, and time- and resource-consuming multistep procedures. To enable a better understanding of HCV replication and its regulation, more-efficient methods for detailed analysis of the levels of viral replication are required. Our replicon system expressed a selectable chimeric luciferase reporter Feo gene and enabled detection of intracellular replication levels of the HCV subgenome accurately and reproducibly in cells harboring stable expression of the replicon. In fact, suppression of HCV replication by IFN was readily detectable at concentrations as low as 0.01 U/mL. The HCV-Feo replicon has enabled us to performed high-throughput assays so that we can conduct detailed quantitative evaluations of drug synergy through combination of IFN and ribavirin, which requires repetitive collection of data and substantial experimental time. The HCV-Feo replicon system is useful not only for high-throughput quantification of viral replication but also for screening antiviral agents with high efficiency.

Although the use of ribavirin is clinically effective against chronic hepatitis C disease, the virological mechanisms of the action are not understood completely. In clinical practice, monotherapy with ribavirin had a minimal effect on virus load and almost no effect on viral clearance, although serum alanine aminotransferase levels decreased substantially during treatment [25–28]. These findings suggest that the anti-HCV action of ribavirin may occur, in part, through immune modulation, as shown in recent studies of enhancement of HCV-specific Th1 responses by ribavirin [11]. The results of the present study have demonstrated that ribavirin also has a weak inhibitory effect on intracellular HCV replication, which is consistent with the results of another study reporting semiquantitative analyses of the effect of ribavirin on an HCV replicon system [29]. Moreover, we have titrated the time kinetics and dose-effect function of ribavirin precisely by use of the highly quantitative HCV-Feo replicon system and have demonstrated that the effects show linear regression of activities on the logarithms of concentrations of ribavirin, as often observed for various drugs [30].

In contrast to the results of the present study, a previous study that used a T7-promoter/polymerase–based HCV RNA expression system did not show an inhibitory effect of ribavirin on HCV expression [31]. That system, although efficient, featured T7 promoter–driven forced synthesis of plus-strand HCV RNA, which may occur concurrently with self-replication of the HCV RNA by the viral RNA polymerase. Therefore, that system may not reflect solely autonomous viral replication as being subject to treatment with reagents such as ribavirin.

Several studies have reported that a potential mechanism for a direct antiviral action by ribavirin is acting as an RNA mutagen, which may cause error-prone replication, leading to self-destruction of the viral genome [12]. A study by Lanford et al. [32] showed that transfection of replicon-containing RNA extracted from ribavirin-treated cells showed decreased colony-forming activity, implicating some deleterious effect on HCV replicon competency by ribavirin. The present study has demonstrated directly that treatment with ribavirin increases the diversity of the nucleotide sequence of the HCV subgenome. These results suggest that the antiviral action of ribavirin on HCV also involves the induction of error-prone replication, as has been reported for other viruses, such as poliovirus and GB virus B [12, 33]. In the present study, the increase of genetic diversity is most prominent at a ribavirin concentration of 100 μmol/L. Similarly, in the T7-HCV RNA expression/replication model [34], error-generation rates increased significantly across all of the HCV genome, with ribavirin treatment, at a concentration of 50 μmol/L, and decreased at concentrations of ≥400 μmol/L. Moreover, in the present study, the HCV replicon that had survived prolonged treatment with 100 μmol/L ribavirin for 8 weeks had no nucleotide mutations in the consensus sequences, suggesting that only replicons without mutations could replicate continuously and be selected by ribavirin.

The use of ribavirin and IFN in combination elicited strong synergistic effects on HCV replication. The precise mechanisms of the important synergistic antiviral action with IFN and ribavirin are unclear at present and need to be investigated further. Although ribavirin showed substantial dose-dependent inhibition of HCV replication, it was effective only at ranges (IC_{50}, 126 μmol/L; figure 6*A*) far above the concentrations achieved in plasma after administration of standard doses. However, the synergy was present even at the clinically achievable ribavirin concentration of ~10 μmol/L [23]. As shown in the present study, the HCV replicon is highly susceptible to IFN (IC_{50}, 0.5