wide distribution of adaptive mutations throughout NS region of HCV genome, the majority cluster in the central region of NS5A, particularly in the serine cluster region immediately upstream to the interferon sensitivity determining region (ISDR; aa.2209–2248 of HC-J4) or ISDR itself [11–14]. We previously identified ISDR as the sequence element wherein missense mutations are closely related to the clinical efficacy of interferon treatment, as well as to the serum viral loads [15,16]. Although most studies from Japan, reports from Spain and Italy did support the relevance of ISDR [17–21], studies from Europe and North America did not always support its existence [22–24]. Despite the controversy, recent meta-analyses have supported the correlation between ISDR sequence and interferon resistance [25]. Moreover, functions of the ISDR relevant to interferon resistance *in vivo* have been explained *in vitro* by such as inhibition of dsRNA-dependent protein kinase [26].

Therefore, the observation that HCV replicon requires adaptive mutations around ISDR was striking and unexpected. NS5A should have an essential role in HCV replication by as yet unknown mechanisms that requires ISDR participation *in vivo* and *in vitro*. The present study was designed to determine whether or not the HCV replicon system could be applied to other HCV isolates, and particularly if adaptive mutations in NS5A are generally required for this replicon system. In order to achieve this objective, we established a new replicon from HC-J4, one of the proven chimpanzee-infectious HCV isolates.

MATERIALS AND METHODS

Cell culture

Human hepatoma cells (Huh-7) were grown in Dulbecco's modified minimal essential medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 2 mM L-glutamine, nonessential amino acids, 100 IU of penicillin, 100 mg of streptomycin, and 10% foetal bovine serum at 37 °C under 5.0% CO_2. G418 (Wako, Osaka, Japan) was added to the culture medium at a final concentration of 200 μg/mL for cells carrying HCV replicons. The cells were split twice weekly by 1:5 to 1:6, depending on their confluence. Huh-7 cells harbouring different replicons were cultured for 48 h in the presence of interferon-alpha-2b (1000, 100, or 10 IU/mL; Schering-Plough, Kenilworth, NJ, USA) and replication was measured by Northern blots to determine the effect of interferon.

Plasmid constructions

pCV-J4L6S (GenBank accession no. AF054247) was used as a starting material [27] for constructing HC-J4-derived replicon (kindly provided by Jens Bukh, NIH, Bethesda, Washington, DC, USA). pCV-J4L6S is chimpanzee-infectious and most of its structure originates from the HC-J4 isolate of HCV-1b [28] with the exceptions of the 5′ terminus of the HCV-5′ untranslated region (5′UTR) and the HCV-3′ terminus of the 3′ untranslated region (3′UTR) which are derived from pCV-H77C (GenBank accession no. AF011751), a chimpanzee-infectious clone of HCV-1a. In accordance with previous replicon constructs, the HCV structural region of pCV-J4L6S was replaced with the Neo gene and EMCV-IRES by substituting the *AgeI-BsrGI* fragment of pCV-J4L6S with the corresponding fragment of the plasmid 1bneo/delS containing the chimpanzee-infectious clone HCV-N (GenBank accession no. AF139594) derived replicon construct (kindly provided by Christopher Seeger, s Cancer Center, Philadelphia, PA, USA) [5]. We designated this new plasmid harbouring the HC-J4-derived replicon construct as pRpJ4. Due to the design of the construct, pRpJ4 has the three amino acid substitutions derived from the plasmid 1bneo/delS in NS3 upstream to *BsrGI* (aa.14 of HC-J4, valine to leucine; aa.27, lysine to arginine; aa.47, threonine to valine) (Fig. 1).

After an initial failure to establish a self-replicating replicon using pRpJ4, the following mutations were introduced into the NS5A serine cluster region of this plasmid by site-directed mutagenesis (Quick-Change Multi Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA): pRpJ4-S2197P (serine to proline at aa.2197), pRpJ4-S2201del (deletion of serine at aa.2201), and pRpJ4-S2204I (serine to isoleucine at aa.2204). In addition, six amino acid substitutions were introduced into ISDR of pRpJ4 and pRpJ4-S2201del (pRpJ4/ISDR mutant and pRpJ4-S2201del/ISDR mutant), respectively (Fig. 2). This ISDR of HCV is considered to be interferon-sensitive sequence [15,16]. Finally, the Neo gene was replaced with the gene encoding firefly luciferase between the *AscI* and *PmeI* restriction sites in order to generate the corresponding luciferase plasmid for transient-replication assays (pRplucJ4-S2197P from pRpJ4-S2197P, pRplucJ4-S2201del from pRpJ4-S2201del, pRplucJ4-S2204I from pRpJ4-S2204I, and pRplucJ4-S2201del/ISDR mutant from pRpJ4-S2201del/ISDR mutant). As a replication-deficient control for luciferase assay, a replicon was constructed by introducing a NS5B frameshift with *BclI* digestion into the pRplucJ4 (pRplucJ4-NS5Bdel).

In vitro transcription, electroporation and selection of G418-resistant cells

The replicon RNA was synthesized by the RiboMax Large Scale RNA Production System (Promega, Madison, WI, USA) using T7 RNA polymerase after linearizing 1 μg of replicon plasmids by *XbaI* digestion. Transcription was terminated by the addition of 1 U of DNase, the mixture was incubated for 15 min at 37 °C, the transcribed RNA was purified by acid guanidium/phenol/chloroform (AGPC) method (ISOGEN, Wako) to remove trace amounts of residual template DNA completely and resuspended in RNase-free water. Subconfluent Huh-7 cells were trypsinized and 5×10^6 cells were resuspended in 500 μl of serum-free DMEM-F12

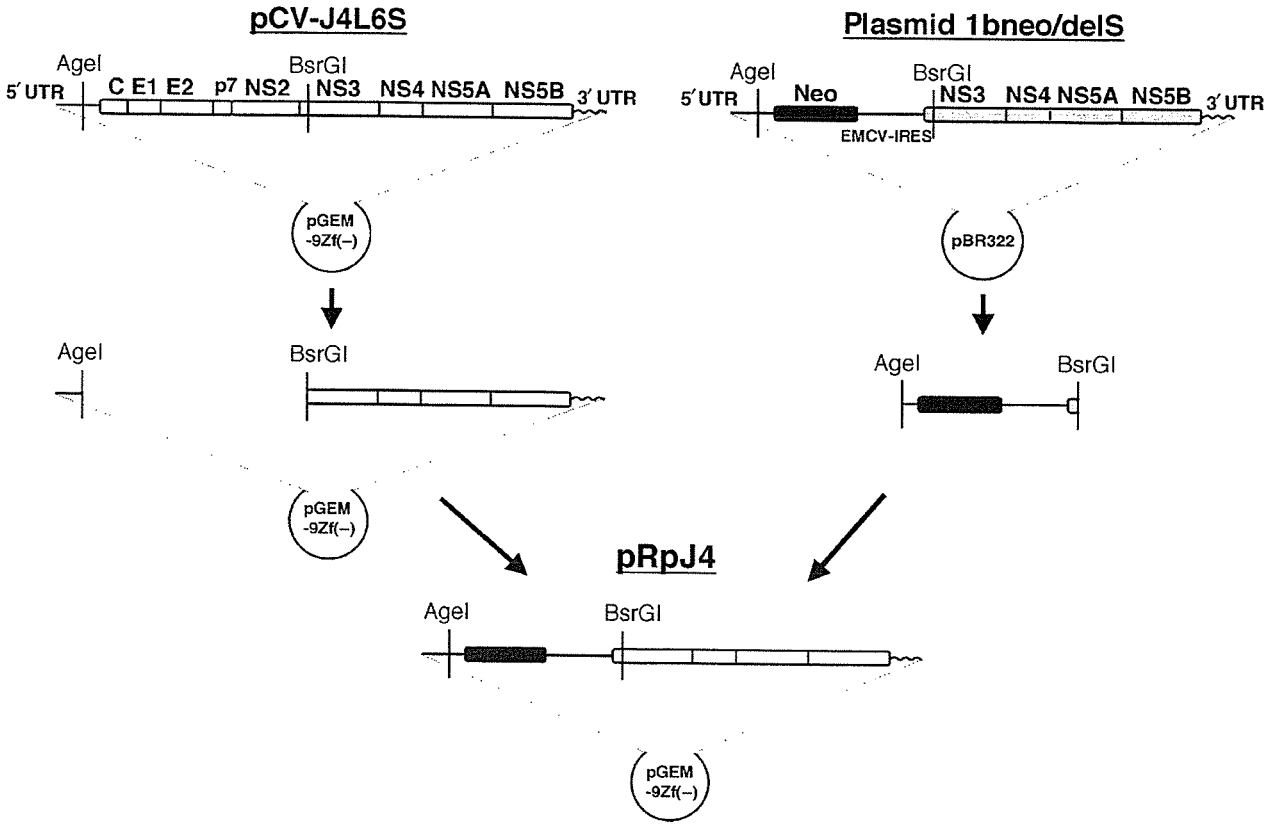

Fig. 1 Strategy for the construction of the HC-J4-derived replicon. pCV-J4L6S containing a full-length HCV genome was used. According to previous replicon constructs, the structural region of the pCV-J4L6S was replaced with the Neo gene and EMCV-IRES by substituting the *Age*I-*Bsr*GI fragment of pCV-J4L6S with the corresponding fragment of the plasmid 1bneo/delS. We designated this new plasmid harbouring the HC-J4-derived replicon construct as pRpJ4. Due to the design of the construct, pRpJ4 has the three amino acid substitutions site derived from plasmid 1neo/delS in NS3 upstream to *Bsr*GI (aa.14 of HC-J4, valine to leucine; aa.27, lysine to arginine and aa.47, threonine to valine).

medium (Sigma) in the presence of 10 μg of replicon RNA, transferred to an electroporation cuvette (4 mm gap width), and subjected to an electric pulse (1050 μF and 270 V) using the EasyJect system (EqiBio, Middlesex, UK). After electroporation, the cell suspension was diluted with DMEM supplemented with 10% foetal bovine serum and plated in 10-cm diameter cell culture dishes. After 24 h, G418 was added to a concentration of 200 μg/mL, and the medium was changed twice weekly. Three weeks after transfection, G418-resistant colonies appeared and cell lines harbouring continuous replicon replication were established. To determine colony-forming units (CFU), G418-resistant colonies obtained after transfection of each replicon were stained with neutral red and counted.

Northern blotting, Western blotting and DNA sequencing

Total cellular RNA for Northern blots was extracted using the AGPC method (ISOGEN, Wako). Ten micrograms of total RNA was electrophoresed through a 1.0% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany). Digoxigenin-labelled plus- and minus-specific riboprobes encompassing the NS3 to NS5 region of HC-J4 were synthesized using a DIG-RNA Labeling Kit (Roche, Mannheim, Germany). Hybridization was conducted with digoxigenin-labelled RNA probes in ULTRAhyb (Ambion, Austin, TX, USA) overnight at 68 °C. Chemiluminescence was detected using the Digoxigenin Luminescent Detection Kit (Roche) according to the manufacturer's protocol.

Ten micrograms of total cellular protein was electrophoresed through a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) and subsequently transferred to an Immobilon polyvinylidene fluoride Membrane (Roche) for Western blots. The membranes were incubated with monoclonal anti-NS5 antibody (BioDesign, Saco, ME, USA) followed by peroxydase (POD)-labelled anti-mouse IgG antibody. Chemiluminescence was detected using a BM Chemiluminescence Blotting Substrate (Roche) according to the manufacturer's protocol.

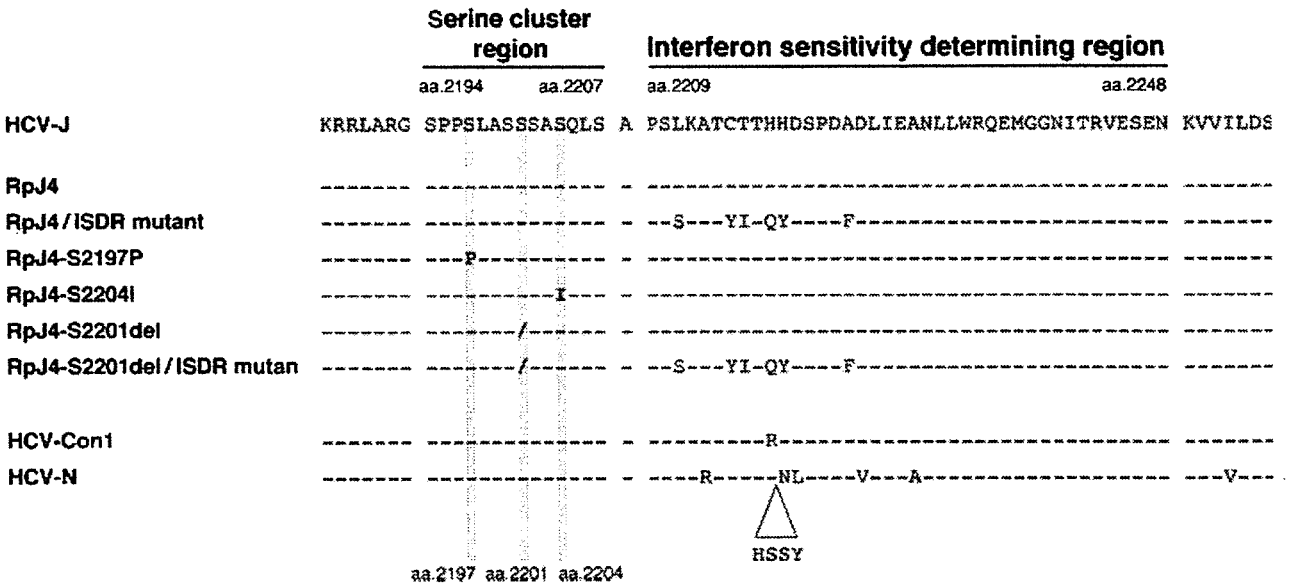

Fig. 2 Introduction of various mutations into the NS5A region for HC-J4-derived replicon constructs. Mutations in the serine cluster region were introduced by site-directed mutagenesis; pRpJ4-S2197P (serine to proline at aa.2197), pRpJ4-S2201del (deletion of serine at aa.2201), and pRpJ4-S2204I (serine to isoleucine at aa.2204). pRpJ4/ISDR mutant, and pRpJ4-S2201del/ISDR mutant were made by introducing six amino acids mutations into ISDR.

The HCV replicon RNAs were amplified from established cell lines by reverse transcriptase-polymerase chain reaction (RT-PCR) for DNA sequencing. Eleven overlapping PCR fragments spanning the entire NS region (nt 1387–7794 of HC-J4) were sequenced directly using the PRISM Dye Termination Kit (Applied Biosystems, Chiba, Japan) as previously reported [29].

Establishing highly permissive cell lines for transient-replication assay

In order to obtain highly permissive cured cell lines for subgenomic replicons, Huh-7 cell lines harbouring 1bneo/delS replicon-derived Feo replicons [30] were cultured in the presence of interferon-alpha-2b (100 IU/mL) without G418 for 2 weeks [31]. Feo replicon has the firefly luciferase-neomycin phosphotransferase fusion gene (Feo) instead of Neo. Cell lines harbouring this replicon can be selected by G418 and the replication levels can be monitored as luciferase activities in the cell lysate [30]. Complete elimination of the replicon RNA from the cells was confirmed by luciferase assay, Northern and Western blots analysis and by the loss of G418 resistance. The efficiency of transduction was assessed in naïve and the cured Huh-7 cells (5×10^6) following electroporation with 10 μg of luciferase-expressing replicon. Cells were harvested 4, 24, 48, 72, 96 and 120 h after electroporation and the luciferase activities of the cell lysates were measured by the luminometer (TD-20/20; Turner Designs, Sunnyvale, CA, USA) with Bright-Glo kit (Promega).

RESULTS

RpJ4 RNA produced G418-resistant Huh-7 colonies after introduction of various mutations in NS5A

For constructing HC-J4-derived replicon, the HCV structural region of pCV-J4L6S was replaced with the Neo gene and EMCV-IRES by substituting the *AgeI-BsrGI* fragment of pCV-J4L6S with the corresponding fragment of the plasmid 1bneo/delS containing the HCV-N-derived replicon construct (Fig. 1). The *in vitro* transcribed RpJ4 replicon RNA was transfected into naïve Huh-7 cells. However, the RpJ4 RNA, with authentic sequence failed to form any colony after G418 selection, suggesting that RpJ4 RNA is replication incompetent in its native sequence (Fig. 3a). Adaptive mutations supporting the HCV-Con1 replicon were reported to be located in the central region of NS5A, particularly in the serine cluster region just upstream to ISDR. ISDR itself served as an adaptive site when the entire ISDR was deleted [11]. On the other hand, the original RpJ4 had no amino acid mutation either in the serine cluster region or in ISDR (Fig. 2). In order to test the possibility that these mutations may enhance RpJ4 replication, three different serine mutations (i.e. S2197P, S2201del and S2204I) were introduced into the serine cluster region of RpJ4. In addition, six amino acid substitutions in ISDR, whose sequence is known to be interferon sensitive[15,16], were also introduced (Fig. 2). Transfection of RpJ4 replicons with these mutations in the serine cluster region into naïve Huh-7 cells yielded G418-resistant colonies at various

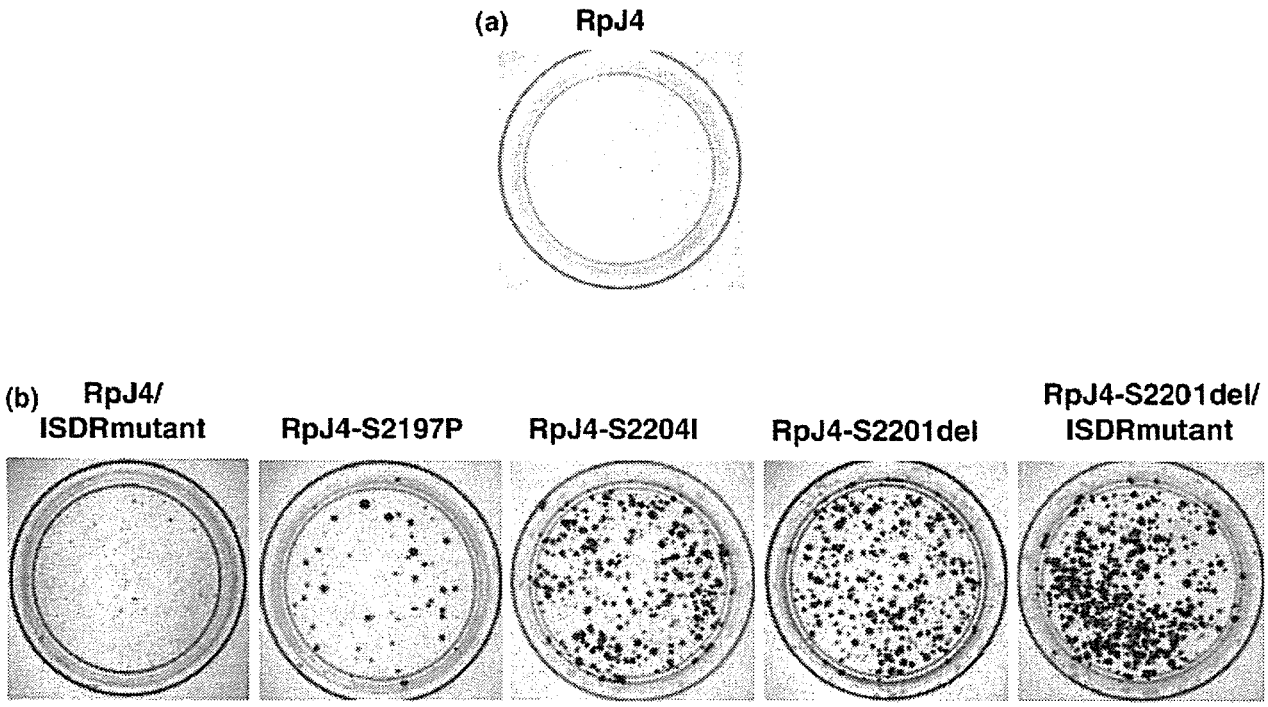

Fig. 3 G418-resistant Huh-7 colonies after transfection of Rp-J4 RNA. (a) Huh-7 cells transfected with RpJ4 RNA did not form a single colony after G418 selection, indicating that RpJ4 RNA is replication incompetent in its native sequence. (b) RpJ4 RNA with three kinds of the serine substitution produced 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

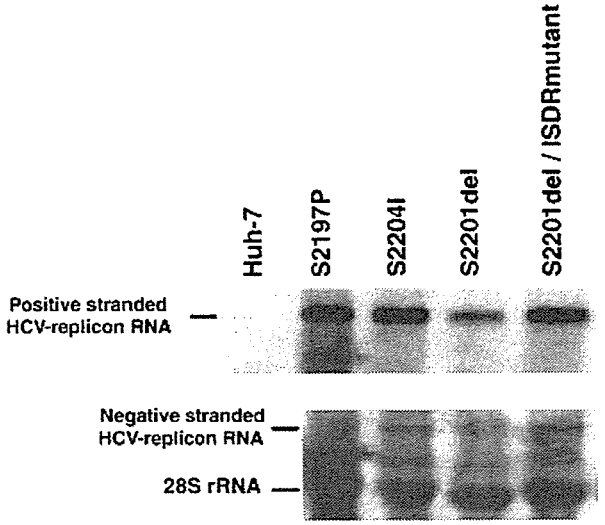

Fig. 4 Northern blots of positive and negative HCV replicon RNA strands in RpJ4 harbouring cell lines. Northern blots for HCV replicon RNA in established cell lines were performed using strand-specific riboprobes. Both positive- and negative-stranded HCV replicon RNA were present, positive strands exhibited 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).

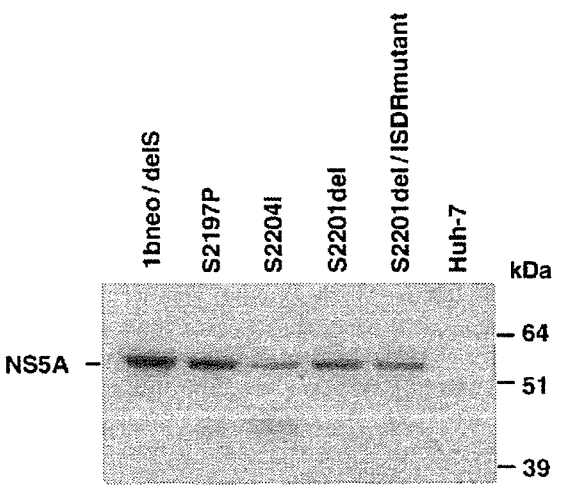

Fig. 5 Western blots of NS5A in HC-J4 replicon harbouring cells. NS5A was detected in all cell lines harbouring RpJ4 replicons. Although level of expression is different in each lane in this figure, repeated experiments disclosed that expression level was dependent on the cell clones even established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

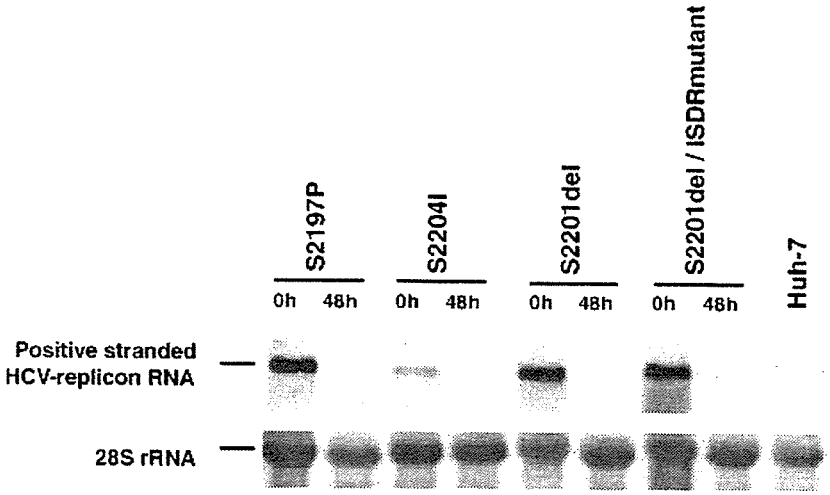

Fig. 6 Responses to interferon treatment in RpJ4 with various NS5A mutations. RpJ4 replicons with various mutations in NS5A all responded to interferon (1000 IU/mL) within 48 h, regardless of mutations in ISDR or in the serine cluster region.

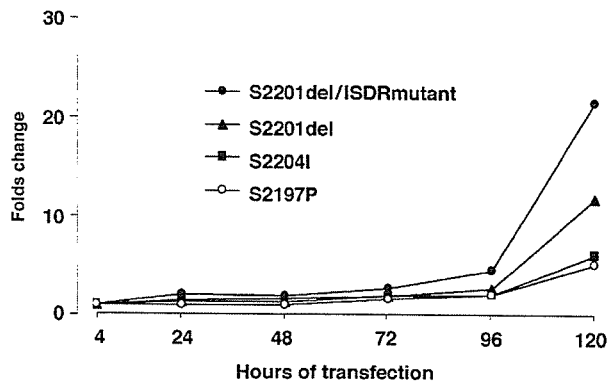

Fig. 7 Transient replication of luciferase-reporter RpJ4 derivatives in cured Huh7 cells. Luciferase activities of RplucJ4-S2201del/ISDR mutant and RplucJ4-S2201del, highly adaptive RpJ4 replicons, were evident 24–120 h after transfection into cured Huh-7 cells. Luciferase activities of RpJ4 replicons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

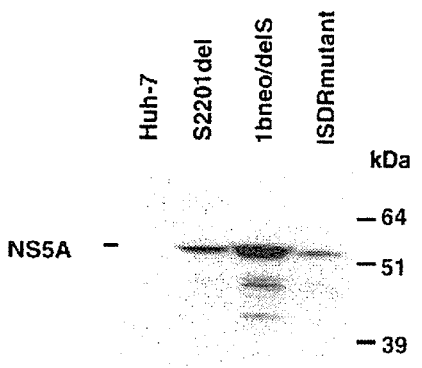

Fig. 8 Western blots using anti-NS5A antibody in RpJ4 replicons without mutations in the serine cluster region (RpJ4/ISDR mutant). RpJ4/ISDR mutant, RpJ4 replicons without mutations in the serine cluster region, also expressed NS5A protein.

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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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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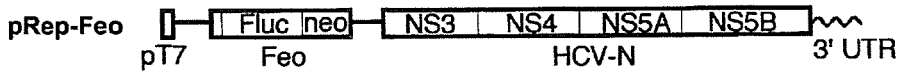

Figure 1. Structure of replicon plasmid constructs. Sequence data have been deposited with GenBank (accession no. AB119282). A hepatitis C virus (HCV) replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS [14] by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene of firefly 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: *Asc*I/Fluc-S, 5′-AAGGGCGCGCCATGGAAGACGCCAAAAACA-3′ (sense) and *Asc*I/Fluc-AS, 5′-ATTGGCGCGCCGCAATTTGGACTTTCCGCCCT-3′ (antisense). The DNA fragment was digested with *Asc*I (New England Biolabs) and was cloned into the *Asc*I 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

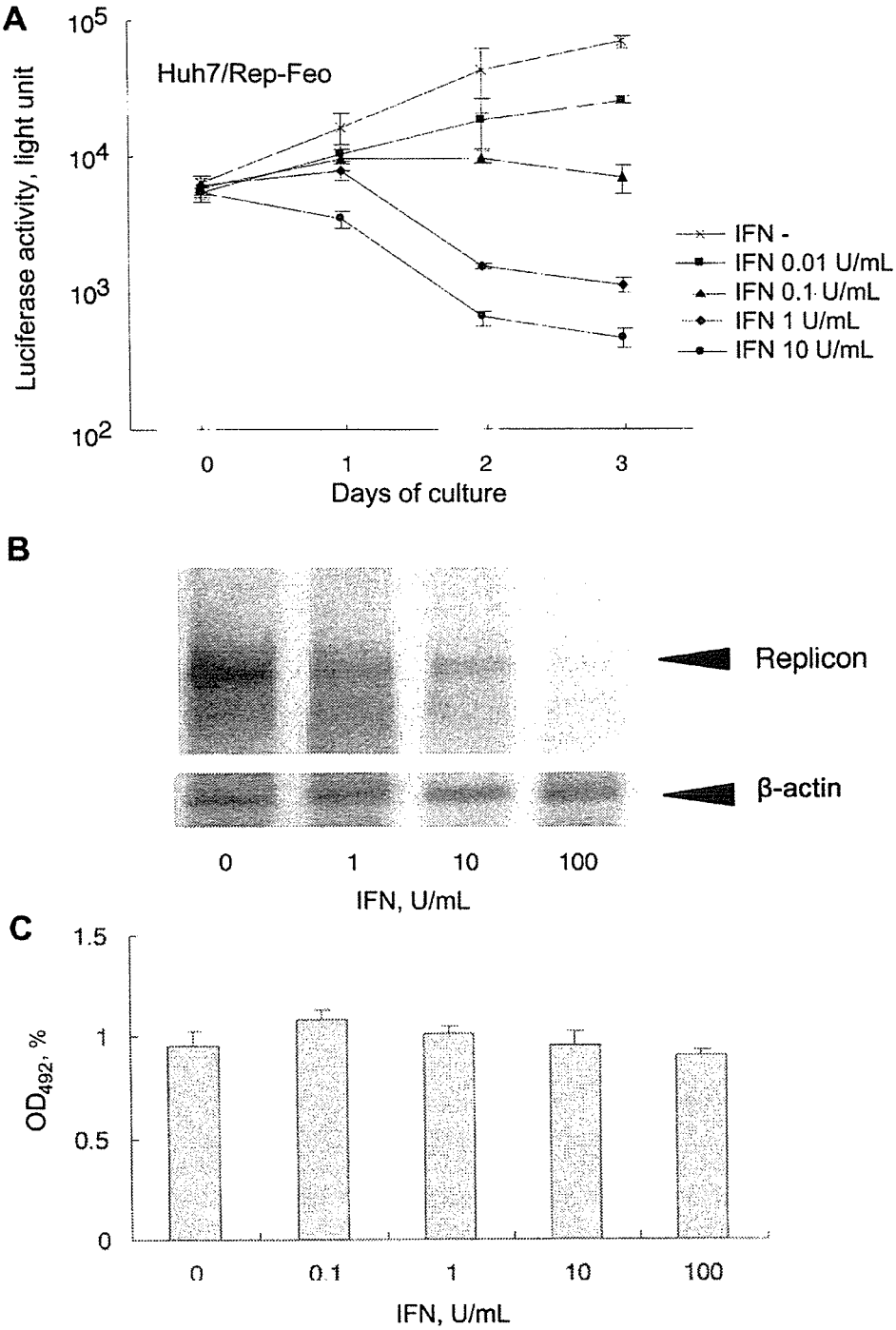

Figure 2. Dose-dependent inhibition of hepatitis C virus (HCV) RNA replications by interferon (IFN). *A*, Huh7/Rep-Feo cells were cultured with the concentrations of IFN indicated in the medium. *B*, Northern-blot hybridization. The upper part of the membrane containing HCV replicon RNA was hybridized 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-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) assay of Huh7/Rep-Feo cells cultured with the concentrations of IFN indicated.

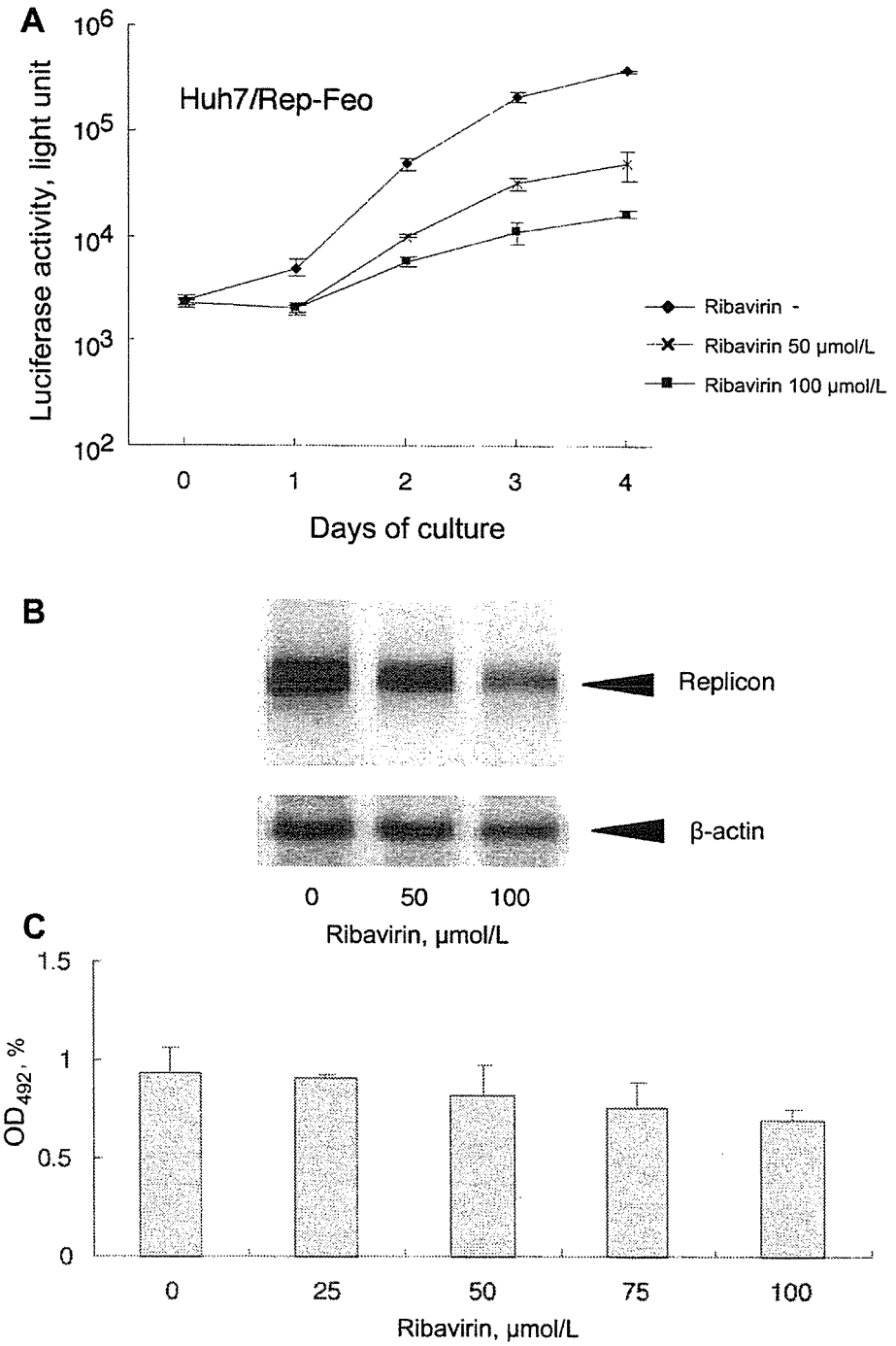

Figure 3. Effects of ribavirin on hepatitis C virus (HCV) RNA replication. *A*, Huh7/Rep-Feo cells were cultured with the concentrations of ribavirin indicated in the medium. *B*, Northern-blot hybridization. The cells were cultured in the presence of the amounts of ribavirin indicated. The cells were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

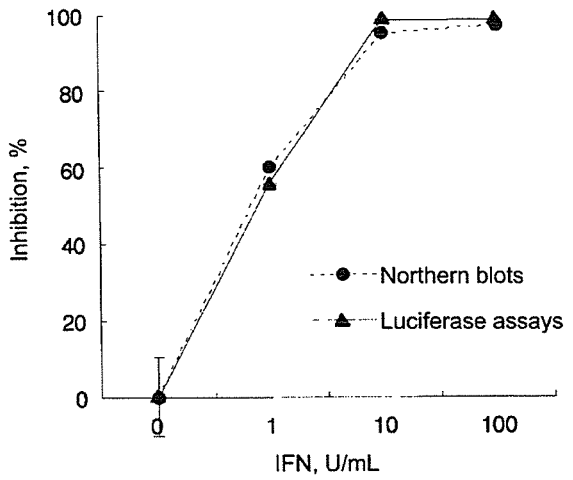

Figure 4. Kinetics of hepatitis C virus (HCV) RNA replication and luciferase activities. Huh7/Rep-Feo cells were cultured with various concentrations of interferon (IFN). Dose-inhibition curves of IFN analysis by the luciferase activities of the Huh7/Rep-Feo cells and densitometric values of the Northern-blot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

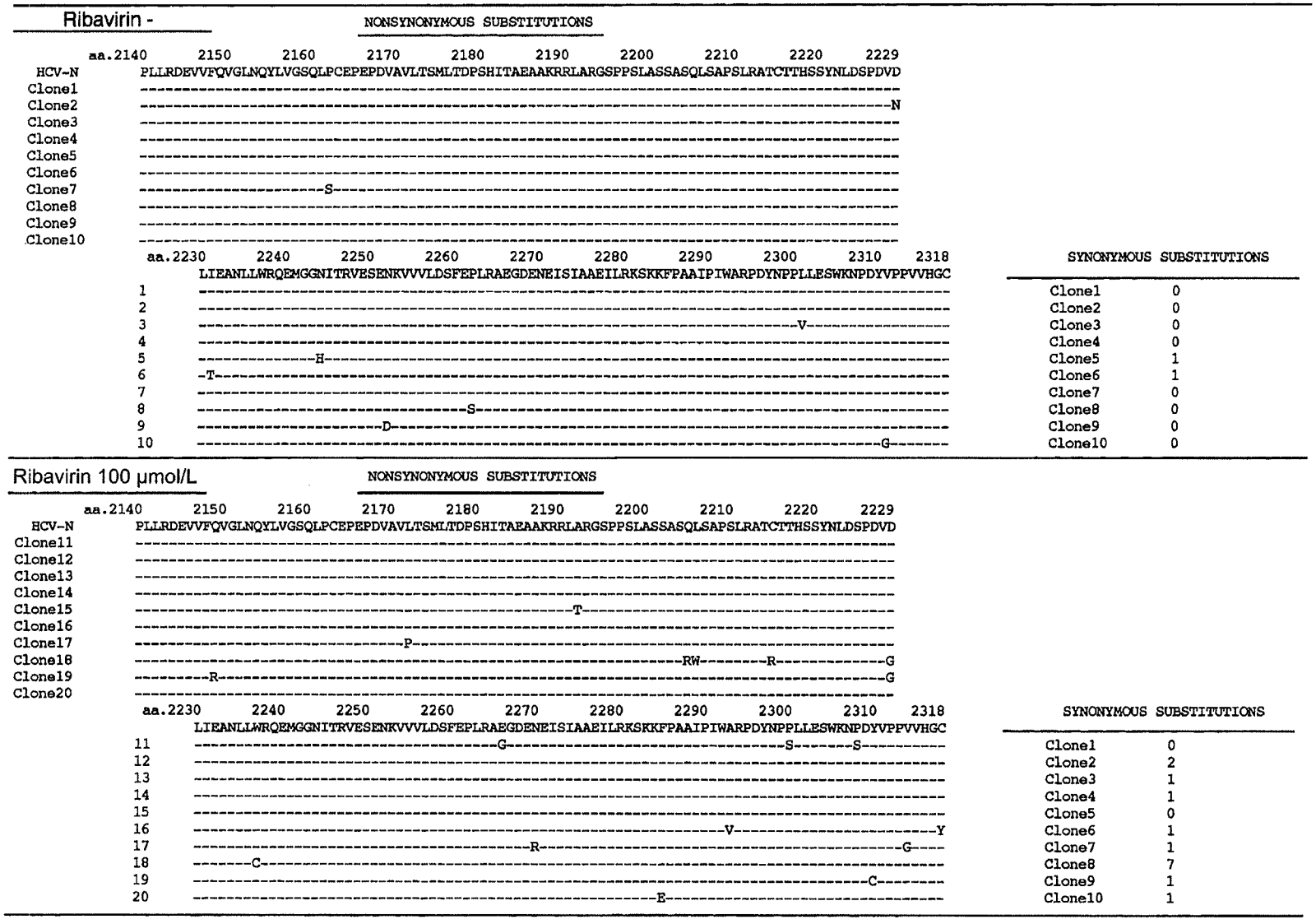

Figure 5. Generation of quasi species by the hepatitis C virus (HCV) replicon system. Sequence alignments were made of 10 independent clones of untreated replicon RNA and replicons treated with 100 μmol/L ribavirin. The alignments were made with HCV-N as the prototype sequence [39]. Amino acid position 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

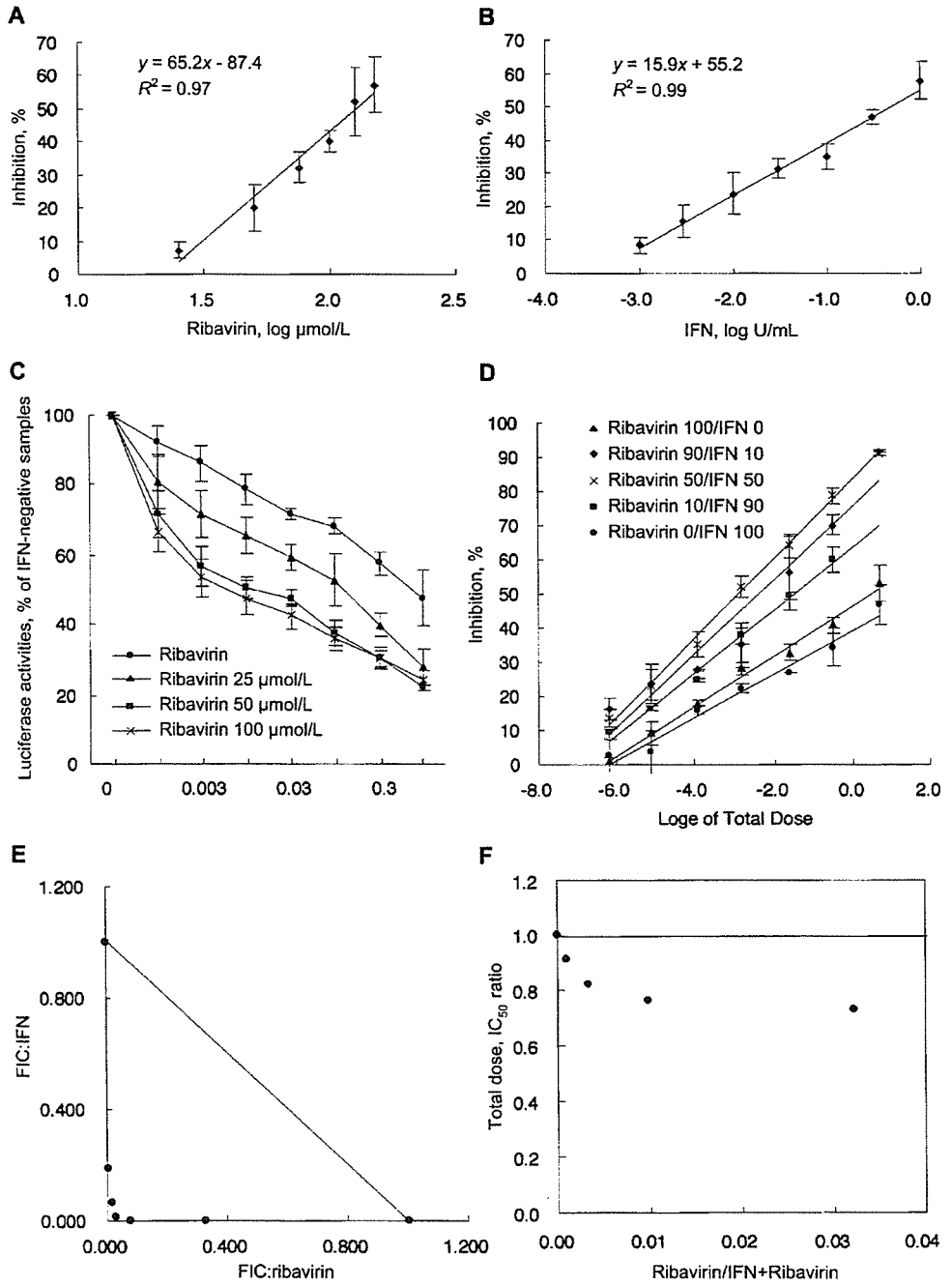

Figure 6. Effect of combination of interferon (IFN) and ribavirin on intracellular hepatitis C virus (HCV) replication. Huh7/Rep-Feo cells were cultured with various concentrations of ribavirin and IFN. Luciferase activities of the cell lysates were measured after 2 days of exposure. Error bars indicate mean ± 2 SD. *A*, Relative log dose-response curves of ribavirin. *B*, Relative log dose-response curves of IFN. *C*, Dose-inhibition curves of IFN combined with the concentrations of ribavirin indicated. The luciferase activities are displayed as percentages of the IFN-negative samples. *D*, Dose-effect curves of IFN and ribavirin combined in proportions indicated, adjusted by the IC_{50} of each drug. *E*, Graphic representation of the isobologram analysis. The fixed ratios adjusted by the IC_{50} (FICs) at 50% inhibition were calculated from the plots in panel *D*. FICs for IFN and ribavirin were plotted on the *X*-axis and *Y*-axis, respectively. A theoretical line of additivity is drawn between the FIC ratio of 1 for each drug that indicates additive effects. All of the FIC plots for the ribavirin-IFN combinations of 1:0, 100:1, 10:1, 1:1, 1:10, 1:100, and 0:1 fell below the line of unity, indicating strong synergy. *F*, The synergy of IFN and ribavirin within clinically relevant ranges of concentration were studied. The dose-inhibition analyses were conducted at 5 different IFN-ribavirin combinations of 1:0, 1000:1, 300:1, 100:1, and 30:1, and the IC_{50} values were calculated for each combination. For better graphic representation, the combination index (CI; see Materials and Methods for definition) was plotted against the proportions of ribavirin in the total combination. The horizontal bar at a level of 1.0 indicates additivity (CI, 1).

U/mL; figure 6*B*). The detailed mechanism by which IFN so effectively suppresses HCV replication is unknown, but a variety of IFN-stimulated genes (ISGs) could be involved—protein kinase PKR, which inhibits viral protein translation; 2′5′-oligoadenyl synthetase, which activates RNase L to degrade viral RNA; and other as-yet-uncharacterized genes [35]. In contrast, HCV proteins, E2 and NS5A in particular, have been shown to counteract the effects of IFN through interaction with the ISGs or with IFN signal transduction, which may contribute to establishment of continuous intracellular replication against IFN treatment, not only clinically but also for the replicon [36–38]. Thus, even slight increases in substitutions in these viral proteins induced by a suboptimal dose of ribavirin could drastically attenuate viral fitness to IFN and further enhance susceptibility of the HCV replicon to IFN, leading to the synergistic effect observed clinically, as well as experimentally, in the present study. Alternatively, ribavirin might directly enhance the expression or activity of the ISGs. In any case, the strong synergistic effects of IFN and ribavirin may explain the virological basis of improved clinical antiviral effects of combination therapy over monotherapy with IFN. However, even with combination therapy, approximately one-half of the patients treated were unable to eradicate HCV. Elucidation of the mechanism of the synergistic effect of ribavirin and IFN is needed urgently to improve the prognosis for HCV-infected patients.

Given the absence of singly effective, proven antiviral agents against HCV, other than IFN, combinations of IFN with agents that possess potential antiviral effects will continue to dominate therapy. The present study has shown strong synergistic effects of IFN and ribavirin on intracellular HCV replication and has demonstrated that the synergistic effects are attributable to direct and specific inhibition of viral replication. These results suggest that the antiviral effects of treatment with IFN may be improved by combination with other, ribavirin-derived nucleoside analogues. Continuation of the search for more-potent and less-toxic antiviral drugs based on ribavirin is mandatory for improving clinical anti-HCV chemotherapeutics.

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