Moreover, the side effects of these reagents are also in some cases severe enough to lead to treatment cessation. Although the development of new effective anti-HCV reagents is urgently needed for the elimination of HCV from the human body, the lack of reproducible and efficient HCV proliferation in a cell culture has been a serious obstacle to the development of anti-HCV reagents [15].

As an efficient replication system of the HCV RNA genome in cell culture, an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions derived from the strain Con-1 was first established in 1999 using a human hepatoma cell line, HuH-7 [16]. Since then, several additional replicon systems derived from the N, H77, 1B-1, and JFH-1 strains have been developed [17–20]. In addition, genome-length HCV RNA replication systems derived from the Con-1, N, and H77 strains have been also developed [17,21,22]. Moreover, in order to easily monitor the replication of HCV subgenomic RNA, several HCV replicons expressing the firefly luciferase reporter [23,24], β-lactamase reporter [25], or secreted alkaline phosphatase [26] have also been developed, although no convenient system of monitoring genome-length HCV RNA replication has been established to date. Therefore, the HCV subgenomic and genome-length RNA replication systems established thus far have been become powerful tools for the screening and evaluation of candidates for new anti-HCV reagents, including IFN and ribavirin [22–25,27].

We also previously established an HCV subgenomic replicon derived from the O strain (an older designation for this strain is 1B-2) [28]; we also recently developed a genome-length HCV RNA replication system derived from the O strain [29]. The characterization of genome-length HCV RNA replicating cells revealed the presence of an adaptive mutation (K1609E) in the NS3 helicase region [29]. Using this adaptive mutation, we established the first cell line (ORN/C-5B/KE) in which genome-length HCV RNA encoding the *Renilla* luciferase reporter gene replicated efficiently [29], and we developed a new convenient reporter assay system using ORN/C-5B/KE cells monitoring the replication of HCV RNA [29]. This reporter assay system demonstrated the usefulness of IFN-α's anti-HCV effect, since the values of *Renilla* luciferase correlated well with the level of HCV RNA after IFN treatment [29]. Therefore, this assay system is expected to become more useful for various studies of HCV than the HCV subgenomic replicon-based reporter assay systems [23–26] developed to date, because the older systems lack the core-NS2 regions containing structural proteins likely to be involved in the events that take place in the HCV-infected human liver.

Mizoribine is an imidazole nucleoside, which is isolated from culture medium of the mold *Eupenicillium brefeldianum* M-2166, and is structurally similar to ribavirin and acts as an immunosuppressant which exerts its effects without severe side effects [30].

In 1984, mizoribine was authorized by the Japanese Government as an immunosuppressive drug for renal transplantation, thereafter lupus nephritis, rheumatoid arthritis, and nephritic syndrome were also added to the list in 1990, 1992, and 1995, respectively [30,31]. Single use or combinatorial use of mizoribine with other immunosuppressive drugs including steroid, azathioprine, methotrexate, or cyclosporin has been accepted in clinical practice, because of good synergistic effects among them without any adverse effects [31]. On the other hand, mizoribine has been known to possess antiviral activities against influenza virus types A and B as in vitro effects [32]. Since it has been recently reported that mizoribine inhibited the replication of bovine viral diarrhea virus that shares a similar structural organization with HCV [33], we speculated that mizoribine possesses similar anti-HCV activity to that of ribavirin, as reported using HCV subgenomic replicon cells [24,34].

To evaluate whether or not mizoribine possesses anti-HCV activity, our monitoring system of genome-length HCV RNA replication was used. Here, we report the findings that not only ribavirin, but also mizoribine, inhibits HCV RNA replication and increases the anti-HCV activity of IFN-α.

Materials and methods

Cell cultures. ORN/C-5B/KE6 cells (designated as OR6 cells; Ikeda et al., in preparation), a cell line cloned from ORN/C-5B/KE cells [29] that supports genome-length HCV RNA encoding the luciferase reporter gene, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in the presence of G418 (300 μg/ml; Geneticine, Invitrogen). The OR6 cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase (Neo) was produced by the efficient intracellular replication of HCV RNA. Therefore, when HCV RNA was excluded from the cells, or when HCV RNA levels decreased, the cells were killed in the presence of G418.

Compounds. Mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) was kindly provided by the Asahi Kasei Pharma (Tokyo, Japan). Ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxiamide) was also kindly provided by the Yamasa (Chiba, Japan). The purities of both reagents exceeded 99%. Human IFN-α was purchased from Sigma–Aldrich (I-2396).

Northern blot analysis. Total RNAs from the cultured cells were extracted with the RNeasy extraction kit (Qiagen) and were quantified by spectrophotometry at 260 nm. Four micrograms of RNA was used for the detection of HCV RNA and β-actin with reagents included in the Northern Max kit (Ambion). Northern blotting and hybridization were performed as described previously [18,29]. An RNA Ladder (Invitrogen) was used to mark molecular length.

Western blot analysis. The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as previously described [9]. The antibodies used to examine the expression levels of HCV proteins were those against the core (Institute of Immunology, Tokyo), E1 (a generous gift from

M. Kohara, Tokyo Metropolitan Institute of Medical Science), E2 [35], NS3 (Novocastra Laboratories, UK), and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science). Anti-β-actin antibody (AC-15, Sigma–Aldrich) was also used to detect β-actin as the internal control. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences, Wellesley, MA).

Antiviral assays. To monitor the antiviral effect of IFN-α, ribavirin, or mizoribine, OR6 cells were plated onto 24-well plates (1.5×10^4 cells per well) and cultured for 24 h. Then, the cells were treated with human IFN-α, ribavirin, or mizoribine at several concentrations for 24, 48, and 72 h, and the cells were also treated with combination of IFN-α and ribavirin or IFN-α and mizoribine at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using the *Renilla* luciferase assay system (Promega). Briefly, after removal of the medium, the cells were washed twice with PBS. The cells were extracted with 100 μl of *Renilla* lysis reagent, and the relative luciferase unit value in 10 μl of lysates was measured by adding 50 μl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used for the detection of luciferase activity.

Cell viability. To examine the cytotoxic effects of ribavirin and mizoribine on OR6 cells, the cells were seeded at a density of 4×10^5 cells per dish onto dishes with a diameter of 95 mm. After 24-h culture, the cells were treated with or without ribavirin or mizoribine at final concentrations of 50 and 100 μM for 72 h in the absence of G418. Then, the number of viable cells was counted in an improved Neubauer-type hemotocytometer after trypan blue dye (Invitrogen) treatment. In addition, in order to examine the β-actin levels in OR6 cells treated with ribavirin or mizoribine, the cells were seeded at a density of 1×10^5 cells per well onto six-well plates. After 24-h culture, the cells were treated with or without ribavirin or mizoribine at final concentrations of 12.5, 25, 50, and 100 μM for 72 h. Then, Western blot analysis was performed using anti-β-actin antibody as described above.

Results

Establishment of cloned cells in which genome-length HCV RNA encoding Renilla luciferase reporter gene replicates efficiently

Recently, we developed a dicistronic genome-length HCV RNA (O strain) replication system that stably expresses *Renilla* luciferase as a reporter in order to facilitate the monitoring of HCV replication [29]. The schematic organization of the genes of genome-length HCV RNA encoding the *Renilla* luciferase gene (ORN/C-5B/KE) is shown in Fig. 1A. Since this replication system consists of a polyclonal cell line in which ORN/C-5B/KE RNA replicates efficiently, we attempted to obtain a cloned cell line which exhibited more efficient and stable replication of ORN/C-5B/KE RNA. We thus obtained several cloned ORN/C-5B/KE cell lines supporting the efficient replication of genome-length HCV RNA, and we characterized these stable cell lines (Ikeda et al., in preparation). In this study, the OR6 cell line, one of the cloned cell lines, was used for the evaluation of the antiviral activity of IFN-α, ribavirin, and mizoribine, as described below. We first confirmed the presence of HCV RNA and HCV proteins in OR6 cells by Northern and Western blot analyses,

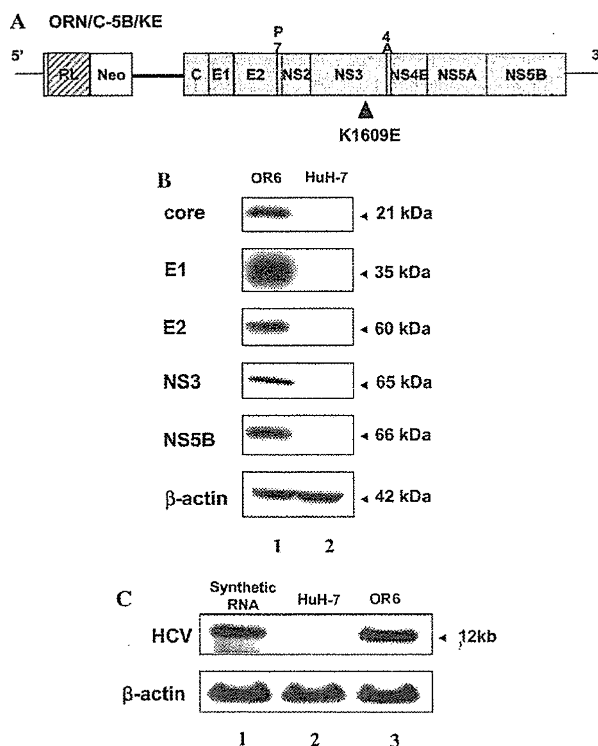

Fig. 1. Characterization of genome-length HCV RNA encoding *Renilla* luciferase gene as a reporter. (A) Schematic gene organization of genome-length HCV RNA encoding *Renilla* luciferase gene. *Renilla* luciferase gene (RL) is depicted as a striped box and is expressed as a fusion protein with Neo. The position of an adaptive mutation, K1609E, is indicated by a triangle. (B) Western blot analysis. Production of core, E1, E2, NS3, and NS5B in OR6 cells (lane 1) and HuH-7 cells (lane 2) were analyzed by immunoblotting using anti-core, anti-E1, anti-E2, anti-NS3, and anti-NS5B antibodies, respectively. β-Actin was used as a control for the amount of protein loaded per lane. (C) Northern blot analysis. Total RNAs from HuH-7 cells (lane 2) and OR6 cells (lane 3) were analyzed by Northern blot analysis using a positive-stranded HCV RNA-specific RNA probe (upper panel) and a β-actin-specific RNA probe (lower panel), respectively. In vitro-synthesized transcript of ORN/C-5B/KE (lane 1; 10^8 genome equivalents spiked into normal cellular RNA) was used for the comparison of expression levels.

respectively. Twelve kilobases of HCV-specific RNA (Fig. 1B), and core, E1, E2, NS3, and NS5B proteins (Fig. 1C) was clearly detected, indicating that genome-length HCV RNA efficiently replicates in OR6 cells.

IFN-α efficiently inhibited the replication of genome-length HCV RNA

Since it is well known that the HCV replicon [28,34,36,37] and replicable genome-length HCV RNA [29] are both highly sensitive to IFN-α, the extent of IFN sensitivity of genome-length HCV RNA (O strain) replication was first characterized by a luciferase assay system using OR6 cells. IFN-α treatments of several doses (final concentration: 1–40 IU/ml) were performed

using OR6 cells, and *Renilla* luciferase activity was measured as described under Materials and methods. The results clearly demonstrated that the luciferase activity had decreased in a dose- and time-dependent manner, when the cells were treated with more than 10 IU/ml IFN-α (Fig. 2A). The relative luciferase activity decreased less than 1% at 72 h after treatment with 20 IU/ml IFN-α. These results indicate that the replication of genome-length HCV RNA in the OR6 cells was also highly sensitive to IFN-α. However, interestingly, when the cells were treated with less than 4 IU/ml IFN-α, an IFN-α dose-dependent recovery of luciferase activity was observed at 48 or 72 h after IFN treatment (Fig. 2A), suggesting that such doses of IFN-α were insufficient for the complete abolishment of HCV RNA replication in OR6 cells. This phenomenon may be similar to recurrence in patients with CH C who receive IFN therapy. However, from another perspective, it was of note that this reporter assay was able to distinguish between effects caused by small differences in doses of IFN (e.g., 1 and 2 IU/ml). Based on the dose–response curve at 72 h after treatment with IFN-α, the concentration of IFN-α required for a 50% reduction of luciferase activity (IC_{50}) was calculated to be approximately 0.5 IU/ml (Fig. 2B). This value was almost equal to the previous finding obtained using a HCV subgenomic replicon (N strain)-based luciferase reporter system [24], although the IC_{50} value in that study was obtained at 48 h after IFN treatment.

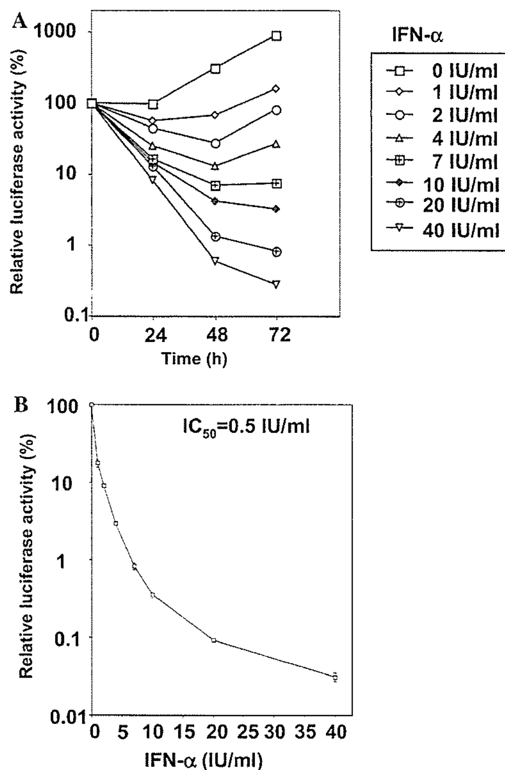

Fig. 2. Inhibition of HCV RNA replication in OR6 cells treated with IFN-α. (A) IFN-α sensitivity of HCV RNA replication in OR6 cells. The cells were treated with IFN-α (0, 1, 2, 4, 7, 10, 20, and 40 IU/ml), and at 24, 48, and 72 h after the treatment, and the *Renilla* luciferase assay was performed as described under Materials and methods. The relative luciferase activity (%) calculated at each point, when the luciferase activity of non-treated cells at 0 h was assigned to be 100%, is presented here. The data indicate means ± SD of triplicates from three independent experiments. (B) Dose–response curve of IFN-α. At 72 h after IFN-α treatment, a *Renilla* luciferase assay was performed.

Ribavirin alone showed inhibitory effects on genome-length HCV RNA replication

Since OR6 cells were considered to be a reliable system for monitoring HCV RNA replication, we then evaluated whether or not ribavirin alone could inhibit the replication of genome-length HCV RNA in OR6 cells. First, luciferase activity was measured over a time course (up to 72 h) following treatment with or without ribavirin (25 and 50 μM). The results revealed a significant decrease in luciferase activity starting 48 h after treatment with ribavirin (Fig. 3A). The dose–response curve measured at 72 h after treatment with ribavirin (up to 200 μM) estimated that the IC_{50} value of ribavirin was 76 μM (Fig. 3B). We confirmed that ribavirin (up to 200 μM) did not inhibit *Renilla* luciferase activity in the reporter assay using HuH-7 cells transfected with pRL-CMV [38], which expresses *Renilla* luciferase under the control of cytomegalovirus promoter (data not shown). These results suggest that ribavirin alone can exert inhibitory effects against the replication of HCV RNA, although its effects were much weaker than those of IFN-α. The IC_{50} value of ribavirin in this study was slightly lower than that ($IC_{50} = 126$ μM) of a previous study using an HCV replicon (N strain)-based luciferase reporter system [24], although the IC_{50} value in that study was obtained at 48 h after treatment.

Mizoribine possessed similar anti-HCV activity to that of ribavirin

Since our assay system using OR6 cells demonstrated the anti-HCV activity of ribavirin alone, we next used our assay system to evaluate the effects of mizoribine, which is an imidazole nucleoside and is currently used to treat several diseases, but has not yet been applied for the treatment of patients with CH C. We found that luciferase activity clearly decreased starting 48 h after treatment with mizoribine (25 and 50 μM) (Fig. 4A). These findings were similar to those observed with ribavirin treatment. The dose–response curve measured at 72 h after treatment with mizoribine (up to 200 μM) estimated that the IC_{50} value of mizoribine was 99 μM (Fig. 4B). This value was slightly higher than that (76 μM) of ribavirin, as obtained by our assay system. We confirmed that mizoribine (up to 200 μM) also did not

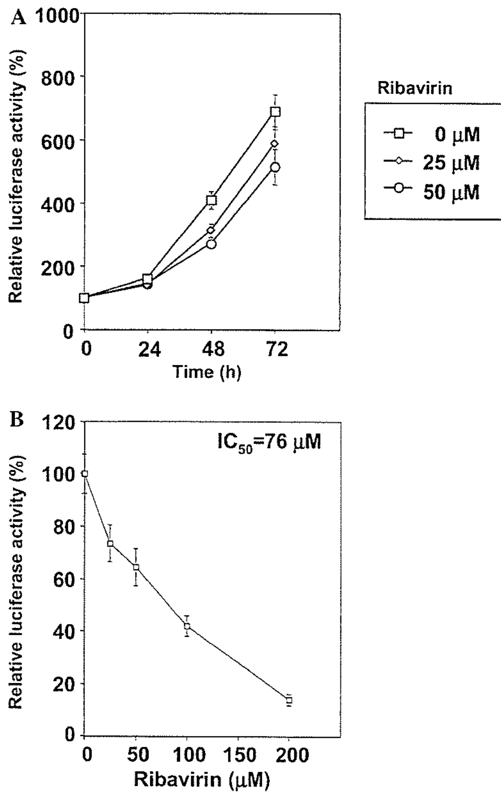

Fig. 3. Ribavirin alone inhibits HCV RNA replication in OR6 cells. (A) Inhibitory effect of ribavirin against HCV RNA replication. OR6 cells were treated with ribavirin (0, 25, and 50 μM), and at 24, 48, and 72 h after treatment, a *Renilla* luciferase assay was performed and the relative luciferase activity was calculated, as shown in Fig. 2. (B) Dose–response curve of ribavirin. At 72 h after ribavirin treatment, a *Renilla* luciferase assay was performed.

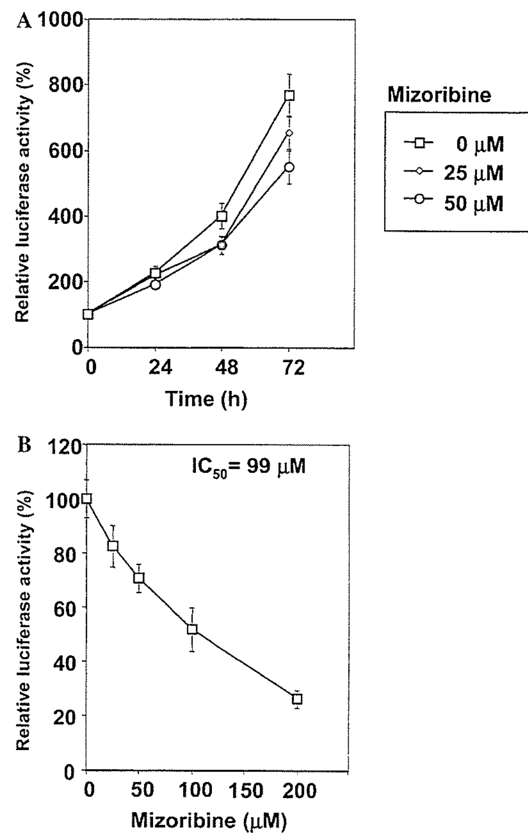

Fig. 4. Mizoribine alone also inhibits HCV RNA replication in OR6 cells. (A) Inhibitory effect of mizoribine against HCV RNA replication. OR6 cells were treated with mizoribine (0, 25, and 50 μM), and at 24, 48, and 72 h after treatment, a *Renilla* luciferase assay was performed and the relative luciferase activity was calculated, as shown in Fig. 2. (B) Dose–response curve of mizoribine. At 72 h after mizoribine treatment, a *Renilla* luciferase assay was performed.

inhibit *Renilla* luciferase activity in the reporter assay using HuH-7 cells transfected with pRL-CMV [38]. In summary, these results suggest that mizoribine alone also possesses the potential to suppress HCV RNA replication.

The anti-HCV activity of ribavirin and mizoribine was not found to be due to cytotoxicity

Since it has been reported that the proliferation of the HCV subgenomic replicon is dependent on host-cell growth [39], it remained to be clarified whether or not the inhibitory effects of ribavirin and mizoribine on HCV RNA replication were caused by their respective cytotoxicities. To examine this possibility, we investigated the cytotoxicities of ribavirin and mizoribine with respect to OR6 cells using two different approaches. First, we examined cell viability at 72 h after treatment with both reagents (50 and 100 μM each). When the number of cells without treatment was compared to that of cells with treatment, no significant decrease in cell number was observed following treatment with ribavirin or mizoribine (Fig. 5A). Second, we examined the amount of β-actin in OR6 cells treated with ribavirin or mizoribine (each up to 100 μM) by Western blot analysis. The results revealed that neither of these reagents led to a decrease in β-actin at concentrations up to 100 μM (Fig. 5B). These results indicated that neither ribavirin nor mizoribine (at least at concentrations ≤100 μM) showed cytotoxicity to the OR6 cells used in our assay system, which suggests that both reagents possess the ability to inhibit the replication of HCV RNA via specific antiviral mechanism(s).

Co-treatment of IFN-α and mizoribine effectively inhibited HCV RNA replication

Since it has been reported that the combination of IFN-α and ribavirin exhibits synergistic inhibitory effects on the HCV replicon [24], we examined the inhibitory effects of the combination of IFN-α and ribavirin

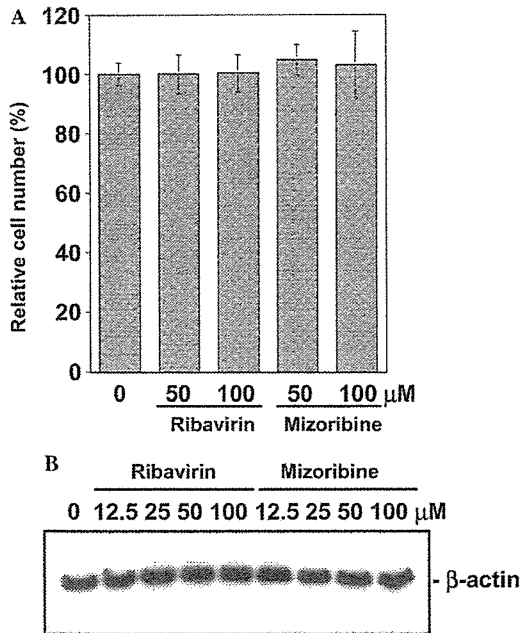

Fig. 5. No cytotoxicity of ribavirin or mizoribine in OR6 cells. (A) Cell viability after treatment with ribavirin or mizoribine. OR6 cells were cultured in the absence or presence of ribavirin or mizoribine (50 and 100 μM each) for 72 h, and then the cell number was determined as described under Materials and methods. The relative cell number (%) calculated at each point, when the cell number of non-treated cells was assigned to be 100%, is presented here. The data indicate means ± SD of triplicates from two independent experiments. (B) Western blot analysis for β-actin. OR6 cells were cultured in the absence or presence of ribavirin or mizoribine (12.5, 25, 50, and 100 μM each), and then the cells were subjected to Western blot analysis using anti-β-actin antibody.

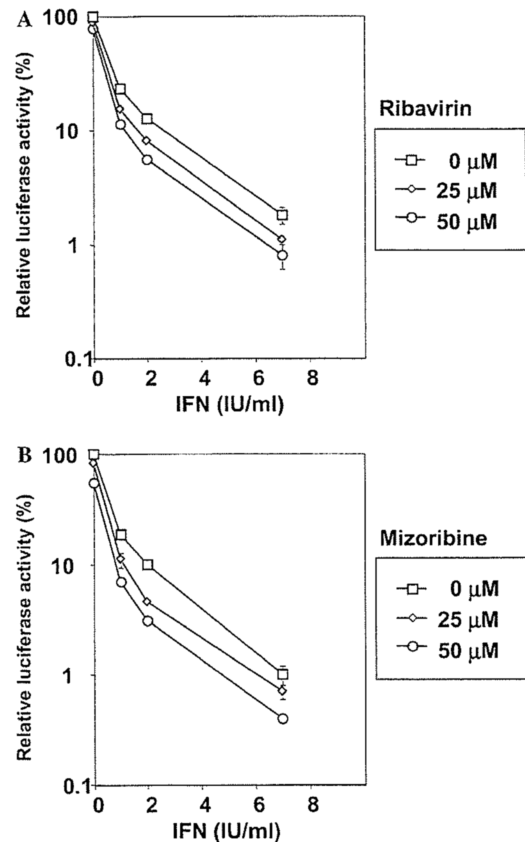

Fig. 6. Both ribavirin and mizoribine enhance the inhibition of HCV RNA replication due to IFN-α. (A) Effect of ribavirin in combination with IFN-α. OR6 cells were co-treated with IFN-α (0, 1, 2, and 7 IU/ml) and ribavirin (0, 25, and 50 μM), and at 72 h after treatment, a *Renilla* luciferase assay was performed and the relative luciferase activity was calculated, as shown in Fig. 2. The data indicate means ± SD of triplicates from two independent experiments. (B) Effect of mizoribine in combination with IFN-α. *Renilla* luciferase assay was performed as described in (A).

or mizoribine on genome-length HCV RNA replication. The dose–response curves of IFN-α (until 7 IU/ml) were obtained under each of the following fixed concentrations of ribavirin or mizoribine: 0, 25, and 50 μM. The results revealed that the curves shifted to the left with increasing concentrations of ribavirin (Fig. 6A) or mizoribine (Fig. 6B) treatment, indicating that co-treatment was more effective than treatment with IFN-α alone. Although the precise mechanism of such a clear effect of co-treatment remains unclear at present, the inhibitory effect of mizoribine (50 μM) appeared to be slightly stronger than that of ribavirin (Fig. 6). These results indicate that anti-HCV activity of mizoribine in co-treatment with IFN-α is at least equivalent to that of ribavirin, suggesting that mizoribine could be useful as a new anti-HCV reagent when in combination therapy with IFN-α.

Although we demonstrated that a 25-μM dose of ribavirin or mizoribine was effective for the inhibition of HCV RNA replication, the clinically achievable concentration of ribavirin has been estimated to be 10–14 μM [24,40]. To examine whether or not concentrations of less than 25 μM of ribavirin or mizoribine exert inhibitory effects on HCV RNA replication, we next obtained a low-dose (5–25 μM) response curve of ribavirin or mizoribine under the condition of a fixed concentration (2 IU/ml) of IFN-α. The results revealed a clear decrease in relative luciferase activity, even in the cells co-treated with 5 μM of ribavirin (Fig. 7A) or mizoribine (Fig. 7B), as compared with that of the cells treated with IFN-α alone. The inhibitory effect of mizoribine at concentration of less than 25 μM also appeared to be slightly stronger than that of ribavirin. It was of note that co-treatment with mizoribine at a dose of 25 μM showed a twofold enhancement of anti-HCV activity, compared with the effects of treatment with IFN-α alone (Fig. 7B); however, it should also be noted that only 20% of the inhibition was observed in the case of solo treatment with mizoribine at a dose of 25 μM (Fig. 4B).

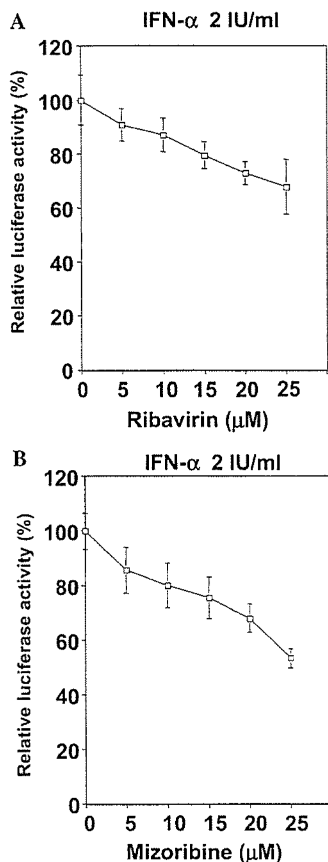

Fig. 7. Low-dose treatment of ribavirin or mizoribine is also effective for increasing the IFN-α inhibition of HCV RNA replication. (A) Effect of ribavirin in combination with IFN-α. OR6 cells were co-treated with ribavirin (0, 5, 10, 15, 20, and 25 μM) and a fixed concentration (2 IU/ml) of IFN-α, and a *Renilla* luciferase assay was performed at 72 h after treatment. The relative luciferase activity calculated at each point, when the luciferase activity of cells treated with IFN-α alone was assigned to be 100%, is presented here. The data indicate means ± SD of triplicates from three independent experiments. (B) Effect of mizoribine in combination with IFN-α. *Renilla* luciferase assay was performed as described in (A).

Discussion

In this study, a newly developed reporter assay system was employed in which genome-length HCV RNA efficiently replicates. Using this system, we demonstrated that ribavirin alone exerts a weak inhibitory effect on HCV RNA replication; however, this inhibitory effect was increased when ribavirin was used in combination with IFN-α. Furthermore, we found that mizoribine inhibited HCV RNA replication at a level equal to that achieved with ribavirin. Since mizoribine is currently used in several clinical treatments without inducing severe side effects, our findings suggest that mizoribine might not only be useful in combination therapy with IFN-α, it might even be used to replace ribavirin in combination therapy with IFN-α.

Since mizoribine, which is structurally similar to ribavirin, showed similar inhibitory effects to those of ribavirin with respect to HCV RNA replication, the anti-HCV activities of ribavirin and mizoribine are expected to be due to similar mechanism(s). However, the mechanism of ribavirin activity in patients with CH C remains poorly understood. To date, four possibilities have been proposed [41]: ribavirin (1) acts as an RNA mutagen that causes mutations of the HCV RNA genome and induces a so-called "error catastrophe"; (2) directly inhibits NS5B-encoded RNA-dependent RNA polymerase (RdRp); (3) enhances host T-cell mediated immunity by switching the T-cell phenotype from type 2 to type 1; and/or (4) inhibits the host enzyme inosine monophosphate dehydrogenase (IMPDH).

As regards the first possibility, several groups have demonstrated that ribavirin was able to induce an "error catastrophe" of the HCV genome [24,42–44]; however, controversial results have been reported to date [45,46]. This discrepancy between results may be due to differences in the concentrations of ribavirin used in these studies, i.e., ribavirin was used at concentrations of more than 100 μM in the former in vitro studies using HCV replicon systems, but in the latter in vitro study using an HCV replicon system [46], ribavirin was used at a concentration of 25 μM, and in the latter clinical study [45], the plasma concentration of ribavirin was estimated to be 10–14 μM [24,40]. In addition, we recently examined the inhibitory effects of mizoribine (25 μM) on the HCV replicon, but no signs of an "error catastrophe" were observed [46]. Taken together, these results suggest that an "error catastrophe" caused by ribavirin or mizoribine may not have contributed to the clearance of HCV RNA following combined treatment with IFN and ribavirin or mizoribine.

The second possibility, namely, that ribavirin or mizoribine directly inhibits the RdRp activity of NS5B, appears to be unlikely, because it has been previously demonstrated that ribavirin triphosphates at concentrations of up to 40 μM did not inhibit the RdRp activity of NS5B purified after expression in insect cells [47]. However, at higher concentrations (several hundreds of μM) of ribavirin triphosphates, NS5B catalyzed the incorporation of ribavirin opposite cytidine or uridine, and substantially the elongation of nascent RNA was blocked [48]. Therefore, ribavirin may weakly affect NS5B RdRp activity, although it is thought that this mechanism does not contribute to the antiviral activity of ribavirin observed in this study.

As regards the third possibility, i.e., that ribavirin can modulate cellular immunity by switching from type 2 to type 1, the possibility of the induction of IFN-γ in HCV RNA replicating cells treated with ribavirin or mizoribine is reasonable to consider, because the replication of HCV RNA has been reported to be very sensitive to IFN-γ [28,49]. However, since the production of

IFN-γ is well known to be restricted to T-cells and large granular lymphocytes alone [50], it is unlikely that IFN-γ is produced by ribavirin or mizoribine in hepatocyte-based HCV RNA replicating cells.

Since ribavirin and mizoribine are competitive inhibitors of IMPDH, the last possibility considered here would indicate that the inhibition of IMPDH is involved in the suppression of HCV RNA replication. However, it has been reported that other IMPDH inhibitors, namely, mycophenolic acid (MPA) and VX-497, showed only marginal antiviral effects on the HCV replicon system [43], although combination treatment with ribavirin and MPA or VX-497 enhanced anti-HCV replicon activity, and this enhancement was canceled by the addition of guanosine [43]. In addition, an additional observation that a 1,4,5-triazole derivative of ribavirin, an IMPDH inhibitor, was devoid of antiviral activity, has also been reported [41]. Although the present results suggest that IMPDH inhibition plays an important role in the anti-HCV activity of ribavirin, this antiviral activity is not completely accounted for by IMPDH inhibition. Therefore, further analysis will be necessary to clarify this point.

Although ribavirin and mizoribine showed apparent dose-dependent inhibitory effects against HCV RNA replication, their effective concentrations (IC_{50}: 76 and 99 μM for ribavirin and mizoribine, respectively) were higher than the clinically achievable ribavirin concentration (10–14 μM) reported previously [24,40]. However, when administered in combination with IFN-α, we demonstrated that a low dose (at least 5 μM) of mizoribine or ribavirin was able to enhance the anti-HCV activity of IFN-α; however, the precise mechanism of mizoribine or ribavirin activity in combination with IFN-α remains unclear. Therefore, in conclusion, the results of the present study suggest that mizoribine is a good reagent for combination therapy with IFN-α, and that mizoribine could be used to replace ribavirin when used in combination therapy with IFN-α.

Acknowledgments

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Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system ☆

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Abstract

Recently we reported a subgenomic hepatitis C virus (HCV) replicon derived from HCV (HCV-O strain) infected in non-neoplastic human hepatocyte PH5CH8. In this study, we developed a genome-length dicistronic HCV RNA from HCV-O. A cured HuH-7 cell line (sOc) was obtained from a cloned subgenomic replicon cell line (sO) by interferon (IFN) treatment and used for transfection with genome-length HCV RNA. One cloned cell line, O, was successfully selected by G418 treatment following the introduction of genome-length HCV RNA into sOc cells, and the robust expression of HCV RNA and proteins was confirmed. Oc, a cured cell line, was also obtained from the cloned cell line (O) by IFN treatment. The number of colonies increased drastically when genome-length HCV RNA was introduced into Oc cells. However, the cloned cured cell lines, sOc and Oc, differed in their colony formation efficiency despite their common origin. This result suggests that even a cloned cell line can change its characteristics during cell culture. Sequence analysis of HCV RNA from the O cells revealed an amino acid substitution in the NS3 helicase region (K1609E). This substitution worked as an adaptive mutation in transient reporter and colony formation assays. Using the advantages of this adaptive mutation and of Oc cells in colony formation, we established the first cell line in which genome-length dicistronic HCV RNA encoding a luciferase gene replicated efficiently. This culture system is useful tool for the study of HCV replication and mass screening for anti-HCV reagents.

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Keywords: Hepatitis C virus; Genome-length HCV RNA; Replication; Cured cells; Reporter assay

Persistent infection with hepatitis C virus (HCV) causes liver cirrhosis and progresses to hepatocellular carcinoma. The low level of response to interferon therapy by chronic hepatitis C patients remains a worldwide threat to public health. One obstacle to the development of new therapy has been the lack of an efficient HCV replication system. HCV is a positive-stranded RNA virus of the family *Flaviviridae*. The HCV genome encodes a long polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1]. Translation of the HCV open reading frame (ORF) is mediated via the 5′ untranslated region (UTR) and a part of the core coding region carrying the internal ribosomal entry site (IRES). The studies on the mechanism of HCV replication became active after the subgenomic HCV replicon was developed in 1999 [2]. Genetic analysis using this replicon revealed that about 100 nucleotides from the 5′ and 3′ ends are essential RNA elements for replication [3–5]. More recently it was found that conserved nucleotides within NS5B worked as *cis*-acting replication elements (Cre) [6].

☆ The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. AB191333.

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After the first Con-1 replicon, subgenomic HCV replicons derived from N, H77, 1B-1, and JFH-1 strains were developed and tissue, genotype, and host ranges were expanded [7–14]. We also previously reported a subgenomic HCV replicon (1B-2R1) derived from the 1B-2 strain (newly designated as HCV-O in this paper) [15]. The sequence of 1B-2R1 is derived from HCV RNA in non-neoplastic human hepatocyte PH5CH8 inoculated with HCV-O [16]. To facilitate the monitoring of a subgenomic HCV replicon's replication level, several attempts have been made using replicons possessing a reporter gene, such as secreted alkaline phosphatase (SEAP), luciferase, or β-lactamase [17–19]. These subgenomic replicon systems are useful for understanding the mechanism underlying HCV replication and for evaluating the effectiveness of anti-HCV reagents. Subgenomic HCV replicons were used to accumulate information about viral and cellular factors in HCV replication [20–22]. But in attempts to see what happens in HCV-infected human liver, subgenomic HCV replicons were insufficient because they lacked the effects of HCV structural proteins. A genome-length HCV RNA replication system, on the other hand, may reflect the phenomena that HCV-infected human liver undergoes. So far, three genome-length HCV RNA replication systems, using N, Con-1, and H77 strains, have been reported [8,11,23]. We have also tried to develop a genome-length HCV RNA replication system derived from the HCV-O strain. The purpose of this study was to characterize our genome-length HCV RNA replication system and to develop a replication system of genome-length HCV RNA encoding a reporter gene for the simple monitoring of HCV replication levels and for the mass screening of anti-HCV reagents.

Materials and methods

Cell culture system. HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM). Cells supporting subgenomic and genome-length HCV RNAs were maintained in the presence of G418 (300 μg/ml; Geneticin, Invitrogen) and passaged twice a week at a 5:1 split ratio.

Plasmid constructions. The plasmid pON/C-5B contains neomycin phosphotransferase (Neo) downstream of HCV IRES and the full-length HCV-O polyprotein coding sequence downstream of encephalomyocarditis virus (EMCV) IRES. We first constructed an authentic genome-length HCV-O, pHCV-O, using two fragments: the *Eco*RI–*Mlu*I fragment (corresponding to positions 45–2528 of the HCV genome) from pBR322/16-6, which was previously described [24], and the *Mlu*I–*Spe*I fragment (corresponding to positions 2528–3420 of the HCV genome) from the PCR product of serum 1B-2. These two fragments were ligated into the *Eco*RI–*Spe*I fragment of pNSS1RZ2RU with 1B-2R1 sequence (a generous gift from Drs. K. Shimotohno, Kyoto University, and K. Sugiyama, Saitama Medical School), which was previously described [15]. To make a fragment for pON/C-5B, overlapping PCR was used to fuse EMCV IRES to the core protein-coding sequence. The resulting DNA was digested with *Rsr*II and *Cla*I, and then ligated with the *Xba*I–*Rsr*II fragment of pNSS1RZ2RU into the *Cla*I–*Xba*I fragment of pHCV-O.

The plasmids pORN/3-5B/KE and pORN/C-5B/KE were constructed from pON/3-5B/KE and pON/C-5B/KE, respectively, by introducing the PCR product of *Renilla* luciferase (Promega) into the *Asc*I site before the Neo gene.

The K1609E mutation was introduced, and 10 amino acids (MLVNGDDLVV), including the GDD motif, were deleted by QuickChange mutagenesis (Stratagene, La Jolla, CA) as previously described [11].

To construct pOF/3-5B, the Neo gene was replaced with the firefly luciferase gene at the *Asc*I and *Pme*I sites in pON/3-5B.

RNA synthesis. Plasmid DNAs were linearized by *Xba*I and used for RNA synthesis with the T7 MEGAscript Kit (Ambion, Austin, TX). After precipitation with lithium chloride, RNA was washed with 75% ethanol and dissolved in RNase-free water.

RNA transfection and selection of G418-resistant cells. For electroporation, HuH-7 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended at 10^7 cells/ml in PBS. RNA was mixed with 500 μl of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad, Hercules, CA). The mixture was immediately subjected to two pulses of current at 1.2 kV, 25 μF, and maximum resistance. Following 10 min of incubation at room temperature, cells were seeded into 10-cm dishes. Cells were selected in complete DMEM with 300 μg/ml G418.

Northern blot analysis. Total RNAs from the cultured cells were extracted with the RNeasy Mini Kit (Qiagen) and quantified by spectrophotometry at 260 nm. Four micrograms of RNA was used for the detection of HCV RNA and β-actin with reagents included in the Northern Max Kit (Ambion) according to the manufacturer's suggested protocol as described previously [11]. After samples were blotted onto positively charged Hybond-N+ nylon membranes (Amersham-Pharmacia Biotech, Piscataway, NJ), RNAs were immobilized on the membranes by UV cross-linking (Stratagene) and stained with ethidium bromide to locate 28S rRNA on the membrane. The membrane was cut approximately 1 cm below the 28S rRNA band. The upper part of the membrane, containing the HCV RNA, was hybridized with digoxigenin-labeled negative-sense RNA riboprobe complementary to the NS5B region. The lower part of the membrane, containing β-actin mRNA, was hybridized with a digoxigenin-labeled, β-actin-specific riboprobe. For the detection of riboprobe, membranes were incubated with anti-digoxigenin alkaline phosphatase-conjugate, reacted with CSPD (Roche Molecular Biochemical, Indianapolis, IN), and exposed to X-ray film. The synthetic RNAs transcribed from pON/3-5B and pON/C-5B (10^8 genome equivalent spiked into normal cellular RNA) were used to compare the levels of replicon RNA and genome-length HCV RNA.

Western blot analysis. Preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed as described previously [15]. The antibodies used in this study were those against core (Institute of Immunology, Tokyo), anti-E1 (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-E2 [25], anti-NS3 (Novocastra Laboratories, UK), anti-NS4A (a generous gift from Dr. A. Takamizawa, Research Foundation for Microbial Diseases, Osaka University), anti-NS5A [26], anti-NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), and β-actin (Sigma). Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

IFN treatment. The preparation of cured cells (1B-2R1C; designated as sOc in this paper) from subgenomic replicon cells (1B-2R1; designated as sO in this paper) was described previously [15]. To prepare cured cells from genome-length HCV RNA replicating cells (O), O cells were plated onto 6-well dishes for 24 h before IFN treatment. Human IFN-α (Sigma) was added to the cells at a final concentration of 500 IU/ml. The cells were cultured for 2 weeks without

G418 and with the addition of IFN-α (500 IU/ml) at 4-day intervals. The cured cells were named Oc cells.

To monitor the anti-HCV effect of IFN-α on ORN/3-5B/KE and ORN/C-5B/KE cells, 2×10^4 cells were plated onto 24-well plates and cultured for 24 h. Then the cells were treated with IFN-α at a final concentration of 0, 1, 10, and 100 IU/ml for 24 h, and subjected to luciferase and reverse transcription (RT)-PCR assay.

Reverse transcription and PCR. RT-PCR was performed separately in two parts; one part covered from HCV 5′UTR to NS3, with a final product of approximately 5.1 kb. The other part covered from NS2 to most of HCV 3′UTR, with a final product of about 6.1 kb. These fragments overlapped at the NS2 and NS3 regions, and were used for sequence analysis for HCV ORF following subcloning into pBR322. For RT of both parts, the antisense primers 290ROK, 5′-ATTAT TCTAGATCGACCTGGTTCCTGTCCCG-3′ and 386R, 5′-AATG GCCTATTGGCCTGGAG-3′ were used, respectively. The primer pair of 21X, 5′-ATTATTCTAGAGCCAGCCCCCGATTGGGGG CG-3′ and NS3RXOK 5′-ATTATTCTAGAGGCCTGTGAGACT AGTGATGATGC-3′ was used for the PCR of the first part. The primer pair of NS2XOK 5′-ATTATTCTAGACGTGTGGGGACAT CATCTTGGGTC-3′ and 9388RX 5′-ATTATTCTAGAATGGCCT ATTGGCCTGGAGTG-3′ was used for PCR of the second part. KOD-plus DNA polymerase was used for PCR (45 cycles), and each PCR cycle consisted of annealing at 64 °C for 30 s, primer extension at 68 °C for 7 min, and denaturation at 94 °C for 15 s.

cDNA cloning and sequencing. Two PCR products (5.1 and 6.1 kb) were digested with *Xba*I and then subcloned into the *Xba*I site of pBR322MC as previously described [13]. Plasmid insertions were sequenced in both the sense and antisense directions using the Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer Life Sciences) on an ABI PRISM 310 genetic analyzer (Applied Biosystems).

Quantification of HCV RNA. The RNAs were prepared from HCV RNA replicating cell lines, and 2 μg of each total RNA was used for RT with SuperScript II using primer 319R as previously described [27]. One-twentieth of the synthesized cDNA was subjected to real-time LightCycler PCR using primer pairs 104 and 197R as described previously [27].

Luciferase reporter assay. ORN/3-5B/KE and ORN/C-5B/KE cells were prepared as shown above in IFN treatment. After 24 h of this treatment, the cells were harvested with *Renilla* lysis reagent (Promega) and subjected to luciferase assay according to the manufacturer's protocol.

Results

Replication of genome-length HCV-O RNA in G418-resistant cells

Recently we reported the subgenomic replicon derived from genotype 1b virus, HCV-O (previously described as 1B-2) [15]. The source of the replicon RNA was HCV-O infected in the human hepatocyte PH5CH8 cell line. The RNA was prepared from PH5CH8 cells at 8 days post-infection of HCV-O. The sO (previously described as 1B-2R1), one of the cloned replicon cells, was obtained after 3 weeks of G418 selection. Based on the subgenomic replicon (ON/3-5B in Fig. 1) in sO cells, we tried to develop a genome-length HCV-O RNA (ON/C-5B in Fig. 1). To construct it, a structural region was synthesized by RT-PCR using RNA from HCV-O-infected PH5CH8 cells as described previously [24]. EMCV IRES and the core-encoding region were fused by overlapping PCR (see Materials and methods). The gene organization of the subgenomic replicon (ON/3-5B) was the same as that of the dicistronic genome-length HCV-O (ON/C-5B), except that only ON/C-5B contained the structural protein-encoding region (Fig. 1).

In the initial experiment, we used cured subgenomic replicon cells (sOc), because cured cells enhanced the colony formation of the subgenomic replicon more than did parental HuH-7 cells (data not shown). Ten micrograms of ON/C-5B transcripts was electroporated into sOc cells. After 3 weeks of G418 selection, only one colony was obtained. In repeated experiments, the number of G418-resistant colonies reproducibly was one or zero, so in this condition the efficiency of colony formation (ECF) was estimated at less than 0.1 colonies/μg RNA. We designated this cell line as 'O.'

To examine the replication level of ON/C-5B in O cells, total RNA extracted from O cells was subjected to Northern blot analysis for the detection of HCV RNA. As shown in Fig. 2A, the presence of a substantial abundance of HCV-specific RNA with a length of approximately 11 kb was detected in the extracts of total cellular RNA prepared from O cells. The cells contained more ON/C-5B RNA than subgenomic replicon RNA. To determine the level of HCV proteins produced from O cells, Western blot analysis was performed. Abundant structural proteins, core, E1, and E2 were detected in O cells (Fig. 2B). The detection of the nonstructural proteins—NS3, NS4A, NS5A, and NS5B—was also demonstrated in O and sO cells at almost uniform levels (Fig. 2B). These results revealed that the expression levels of HCV RNAs and HCV proteins differed somewhat between O and sO cells, suggesting that the stabilities of

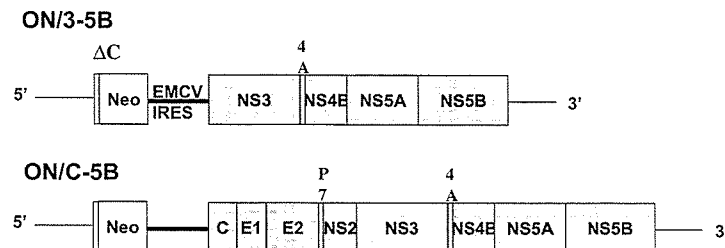

Fig. 1. Organization of subgenomic HCV replicon and genome-length HCV RNA derived from HCV-O. Open reading frames, untranslated regions, EMCV IRES, and Neo genes are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES.

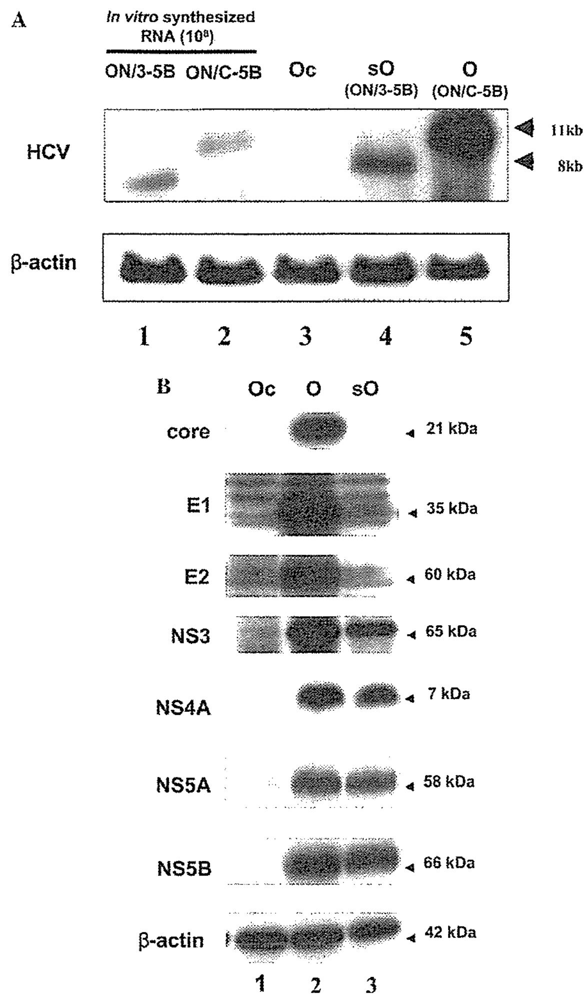

Fig. 2. Detection of HCV RNA and proteins in G418-resistant cell line. (A, top panel) Northern blot analysis of HCV-specific RNA in G418-resistant genome-length HCV RNA-replicating cell line. Lanes 1 and 2, synthetic RNA transcribed from ON/3-5B and ON/C-5B, respectively; lane 3, cured HuH-7 cells (Oc); and lanes 4 and 5, clonally isolated subgenomic HCV replicon and genome-length HCV RNA replicating cell lines by G418 selection, sO (ON/3-5B) and O (ON/C-5B), respectively. (Bottom panel) Northern blot analysis of β-actin mRNA using β-actin-specific RNA probe. (B) Western blot analysis of HCV proteins. Production of core, E1, E2, NS3, NS4A, NS5A, and NS5B in Oc cells (lane 1), O cells (lane 2), and sO cells (lane 3) was analyzed by immunoblotting using anti-core, anti-E1, anti-E2, anti-NS3, anti-NS4A, anti-NS5A, and anti-NS5B antibodies, respectively. β-actin was used as a control for the amount of protein loaded per lane.

genome-length HCV RNA and subgenomic replicon RNA or the efficiency of translation differs between O and sO cells. In summary, we showed the efficient replication of genome-length HCV-O RNA in O cells.

A cloned cell line changed characteristics during cell culture with G418 selection

The O cells were expected to possess the same cellular background as the sO and sOc cells. To examine this, we first obtained the cured cells (Oc) by treating O cells with IFN-α (500 IU/ml) for 2 weeks, and then confirmed that ON/C-5B RNA in Oc cells was not detected by RT-PCR (Figs. 3A and B). In addition, we confirmed that ON/3-5B RNA in sOc cells was also not detected by RT-PCR (Fig. 3B).

We tested the ECFs of sOc and Oc cells by reintroducing ON/C-5B RNA and ON/3-5B RNA into them. Unexpectedly, G418-resistant colonies were produced from ON/C-5B RNA-introduced Oc cells even with 0.02 μg RNA, and the number of colonies increased in an RNA-dose-dependent manner (Fig. 3C). The ECF of ON/C-5B in Oc cells is estimated to be about 50 colonies/μg RNA. In contrast, the ECF of ON/C-5B in sOc cells was less than 0.1 colonies/μg of RNA, although a number of G418-resistant colonies were produced from ON/3-5B RNA-introduced sOc and Oc cells (Fig. 3C). These results suggest that Oc cells possess overwhelming advantages in the replication of genome-length HCV RNA.

Combination of adaptive mutation in NS3 and cured cells enhances the efficiency of colony formation

Information on adaptive mutation has been accumulated so far by using subgenomic replicons [20,22,28], but there has been no systematic analysis of mutations in a genome-length HCV RNA replication system. We therefore performed a sequence analysis of HCV RNA replicating in O cells. RNAs extracted from O cells were subjected to RT-PCR, and then two fragments (5.1 and 6.1 kb) amplified for ORF were subcloned into plasmid for sequence analysis, as described in Materials and methods. The sequences of three independent clones were determined and compared with each other to avoid PCR error and to find conserved mutations. Only one common mutation with an amino acid substitution was detected, and it was in the NS3 helicase region at amino acid position 1609 (Fig. 4). This mutation, from lysine to glutamic acid (K1609E), was seen in previously reported Con1 and 1B-1 replicons, in which the mutation seemed to have little impact on ECF [22]. We examined the effect of this mutation in ON/C-5B on ECF. In the initial experiment, we introduced the ON/C-5B/KE transcript into sOc cells. As shown in Figs. 5C and D, ON/C-5B/KE RNA-introduced sOc cells produce G418-resistant colonies (the ECF is estimated to be about 75 colonies/μg RNA), although no G418-resistant colonies were obtained in ON/C-5B/wt RNA–introduced sOc cells (Figs. 5A and B). These results indicated that the K1609E mutation worked as an adaptive mutation. Furthermore, when ON/C-5B/KE RNA was introduced into Oc cells, the ECF was significantly enhanced (Figs. 5G and H). The estimated ECF of K1609E with Oc was about 1500 colo-

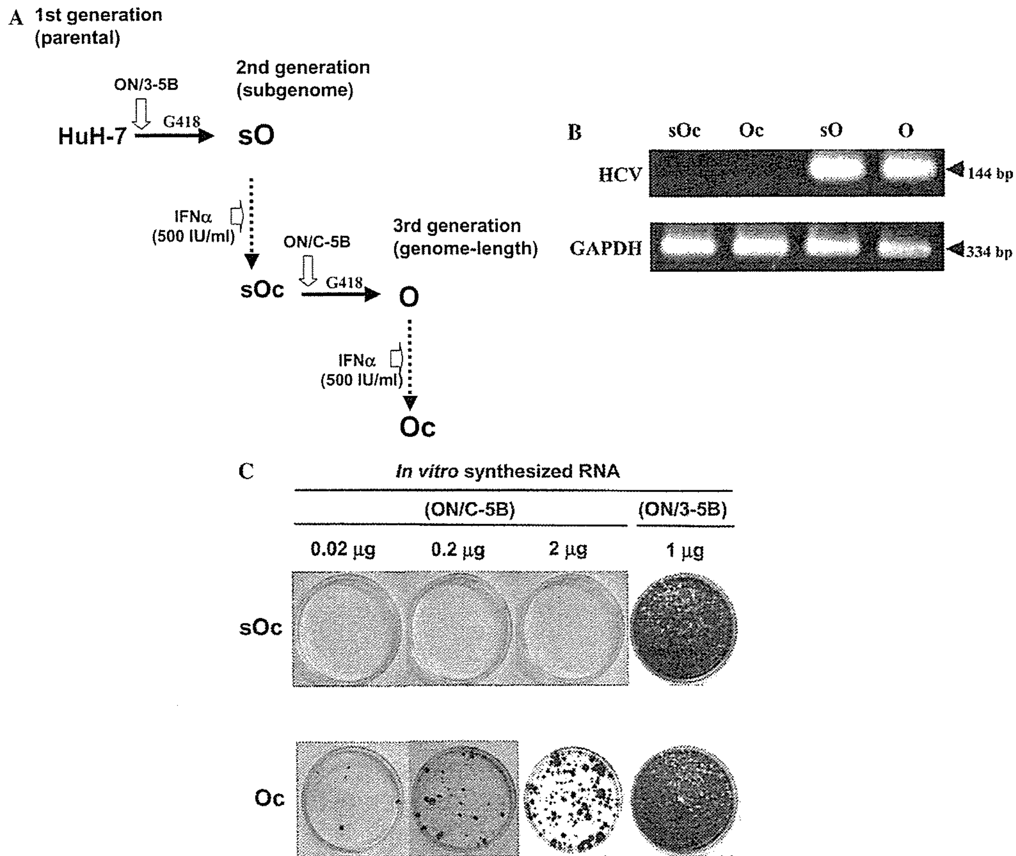

Fig. 3. Characterization of cured cells. (A) Lineage of cured cells. The procedures of G418 and IFN-α treatments are schematically shown. The solid line is the G418 (300 μg/ml) treatment for 4 weeks, and the dotted line is the IFN-α (500 IU/ml) treatment for 2 weeks. The sOc, Oc, sO, and O cells used in this study were obtained in order by the treatment of the indicated reagents, respectively. (B) RT-PCR analysis for the detection of subgenomic and genome-length HCV RNAs. Total RNAs were extracted from sO, sOc, O, and Oc cells, and then RT-PCR was performed as previously described [27]. RT-PCR products (144 bp for HCV and 344 bp for GAPDH) were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (C) Different ECF between sOc and Oc cells. ON/C-5B RNA (0.02, 0.2, and 2 μg per 10-cm dish) and ON/3-5B RNA (1 μg per 10-cm dish) were transfected into sOc cells (top panel) and Oc cells (bottom panel) as described in Materials and methods. The panels show G418-resistant colonies that were stained with Coomassie brilliant blue at 3 weeks after transfection of RNAs [22].

nies/μg RNA. Moreover, a number of G418-resistant colonies were also obtained in ON/C-5B/wt RNA—introduced Oc cells (Figs. 5E and F). These results suggest that Oc cells are superior to sOc cells regarding the intracellular replication of genome-length HCV RNA.

To examine how this effect of K1609E on ECF correlates with early events (i.e., those immediately after electroporation), we constructed subgenomic HCV replicons with the firefly luciferase gene for transient assay (Fig. 6A). The subgenomic replicon with K1609E showed better replicability than the wild-type replicon in Oc cells (Fig. 6B). This indicates that the ECF reflects the effect of adaptive mutation in early events, so the transient assay for adaptive mutations might be suitable for evaluating the establishment of a persistent HCV RNA replication system.

Genome-length dicistronic HCV-O RNA encoding luciferase gene facilitates the monitoring of HCV replication in HuH-7 cells

Thus far, HCV RNA replication systems possessing persistently expressing reporter genes have been limited to studies of subgenomic HCV replicons, and there is no report of a genome-length HCV RNA replication system possessing a reporter gene. The combination of a K1609E adaptive mutation and Oc cells demonstrated a great impact on ECF (Fig. 5). This result encouraged us to construct a convenient genome-length HCV RNA replication system in which a *Renilla* luciferase gene is introduced to facilitate the monitoring of HCV replication.

Ten micrograms of in vitro transcripts from pORN/3-5B/KE and from pORN/C-5B/KE (Fig. 7A) was elec-

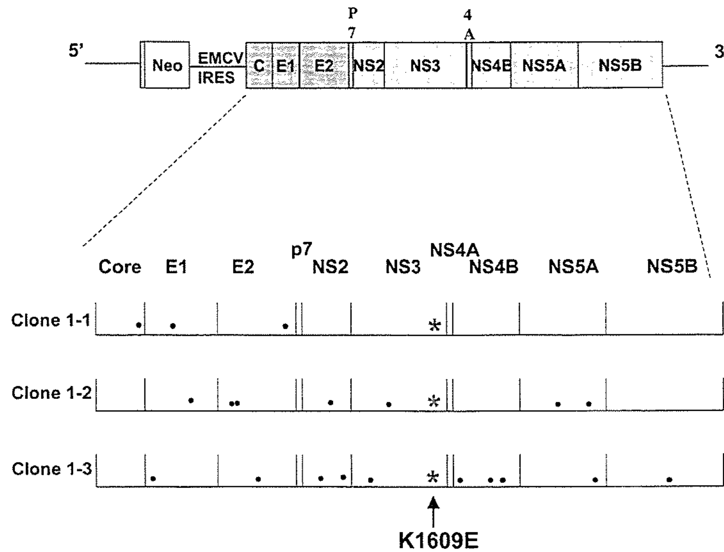

Fig. 4. Amino acid substitutions detected in the genome-length HCV RNA derived from O cells. HCV ORF derived from O cells was amplified by RT-PCR using HCV-specific primer sets. After subcloning, three independent clones were subjected to sequence analysis. A common amino acid substitution (indicated by the asterisk) from lysine to glutamic acid was found at amino acid position 1609 in the NS3 helicase region. The dots indicate clone-specific amino acid substitutions.

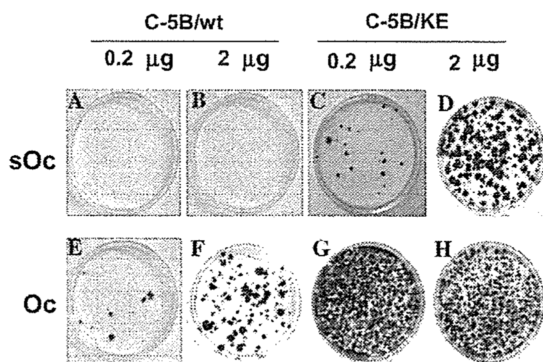

Fig. 5. Effect of K1609E on ECF. ECF was examined using cured cell lines, sOc and Oc. G418-resistant colonies were stained with Coomassie brilliant blue at 3 weeks after electroporation of RNA transcripts without (C-5B/wt; A, B, E, and F) or with (C-5B/KE; C, D, G, and H) the K1609E mutation (0.2 and 2 µg per 10-cm dish) into sOc cells (top panel) or into Oc cells (bottom panel) [22].

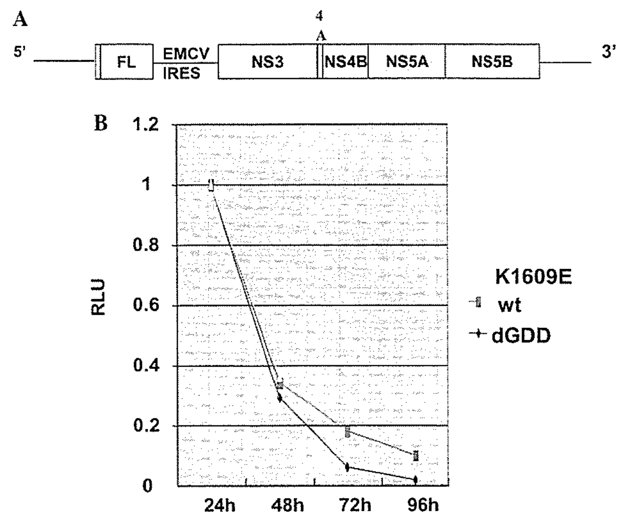

Fig. 6. Effect of K1609E in NS3 on transient replication. (A) The construct of reporter subgenomic HCV replicon carries firefly luciferase instead of Neo. (B) The reporter subgenomic HCV replicon with K1609E was compared with wild-type for transient replication in Oc cells. dGDD indicates the deletion of the GDD motif in the NS5B polymerase, and the subgenomic HCV replicon with the deletion of GDD was used as a negative control.

troporated into Oc cells, and ORN/3-5B/KE and ORN/C-5B/KE cells were selected as polyclonal cell lines by G418 (300 µg/ml) for 4 weeks. The ECF of ORN/C-5B/KE in Oc cells was about 7 colonies/µg RNA. To confirm the presence of HCV RNA in ORN/3-5B/KE and ORN/C-5B/KE cells, Northern blot analysis was performed with total RNA from these cells. As shown in Fig. 7B, 9 and 12 kb of HCV-specific RNA were detected for RNAs from ORN/3-5B/KE and ORN/C-5B/KE cells, respectively. The production of HCV proteins was also detected for the ORN/3-5B/KE cell line with anti-NS3 and anti-NS5B antibodies, and for ORN/C-5B with anti-core, anti-NS3, and anti-NS5B antibodies (Fig. 7C).

To demonstrate the correlation between levels of luciferase activity and HCV RNA, a luciferase reporter assay and real-time LightCycler PCR were performed. At 24 h after IFN-α treatment, the *Renilla* luciferase

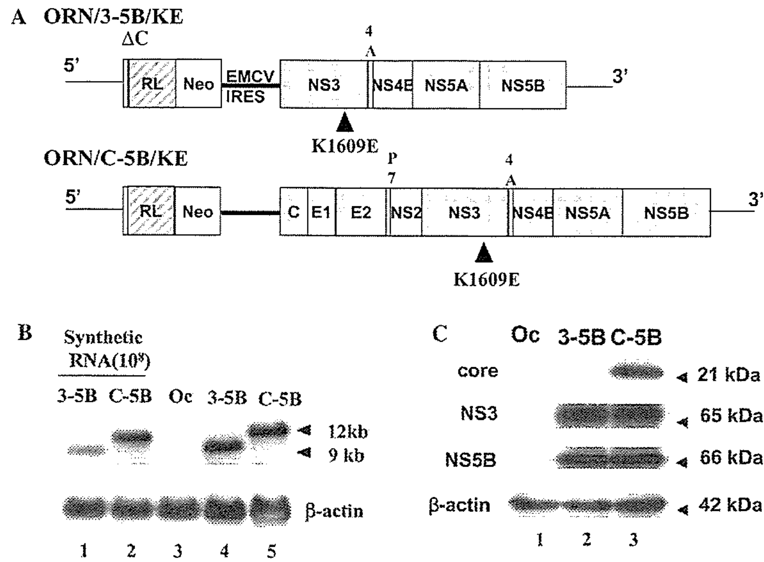

Fig. 7. Characterization of genome-length HCV RNA encoding the *Renilla* luciferase gene as a reporter. (A) Schematic gene organization of subgenomic and genome-length HCV RNA. The basic constructs are described in Fig. 1; the *Renilla* luciferase gene (RL) is depicted as a striped box and is expressed as a fusion protein with Neo. (B) Northern blot analysis was performed for Oc (lane 3), ORN/3-5B/KE cells (lane 4), and ORN/C-5B/KE cells (lane 5) using the HCV RNA-specific probe as shown in Fig. 2. In vitro transcripts of ORN/3-5B/KE (lane 1) and ORN/C-5B/KE (lane 2) were used as size markers. (C) Western blot analysis was performed for Oc cells (lane 1), ORN/3-5B/KE cells (lane 2), and ORN/C-5B/KE cells (lane 3) with anti-core, anti-NS3, and anti-NS5B antibodies as shown in Fig. 2.

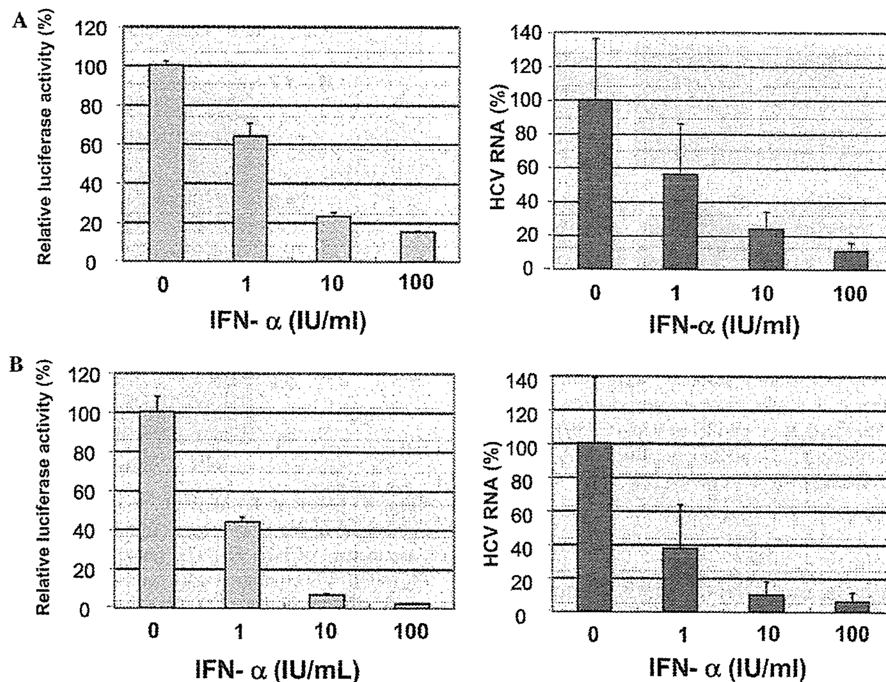

Fig. 8. The effect of IFN-α on the subgenomic HCV replicon and the genome-length HCV RNA replication system possessing the *Renilla* luciferase reporter. (A) The replication level of the subgenomic HCV replicon encoding the *Renilla* luciferase gene was monitored by luciferase reporter assay (left panel) and real-time LightCycler PCR (right panel) at 24 h after IFN-α treatment. (B) The replication level of genome-length HCV RNA encoding the *Renilla* luciferase gene was monitored by luciferase reporter assay (left panel) and real-time LightCycler PCR (right panel).

activity and HCV RNA concentration were examined. As shown in Fig. 8, luciferase activity correlated well with HCV RNA concentration, and IC_{50} of IFN-α was less than 10 IU/ml. The concentration of IC_{50} was compatible with the findings of a previous study [29].

Discussion

In this study, we established a dicistronic genome-length HCV RNA replication system using HCV RNA from HCV-O infected in non-neoplastic human hepatocyte PH5CH8 cells. The characterization of O cells supporting ON/C-5B RNA replication revealed the presence of an adaptive mutation (K1609E) in the NS3 helicase region and a high ECF in cured Oc cells. The combination of the adaptive mutation and cured cells led us to develop a genome-length HCV RNA replication system that stably expresses luciferase as a reporter to facilitate HCV replication monitoring.

For the cells into which genome-length HCV RNA was to be introduced, we chose the cloned cell line sOc, prepared by IFN treatment from subgenomic HCV replicon-supporting cells, since sOc had a higher ECF than its parental HuH-7 cells in a study of subgenomic HCV replicons [30]. In our initial study, only one colony (O cells) was produced after 3 weeks of G418 selection. Oc cells were obtained in 2 weeks of IFN treatment for O cells. We expected that sOc and Oc cells would have similar backgrounds. However, our colony formation experiments using these two cell lines showed unexpected results. The ECF of Oc cells was at least 500 times higher than that of the parental sOc cells. There are several possible reasons for such differences: (1) G418 used for selection affected the cellular factors, (2) IFN treatment for the cured cells altered something in the cellular background, (3) HuH-7 cells lacked a mismatch repair function and accumulated mutations in the genes required for HCV replication, and (4) HCV proteins inhibited the mismatch repair. Concerning possibilities (1) and (2), there are no reliable reports to date that these two reagents (G418 and IFN) worked as mutagens. Concerning (3), many cancer-derived cell lines were reported to lack a mismatch repair function [31,32]. As for (4), we recently reported that the HCV core protein promoted microsatellite instability [33]. However, further comparative experiments using subgenomic HCV replicons would be required to clarify whether the core protein causes the changes in the cellular background. Although we are able to clarify whether or not this alteration occurred by one of the factors described above or through more than one factor synergistically, the results in this study have indicated that even cloned HuH-7 cells have the potential to change their cellular background during culture. On the other hand, it might lead to the chance for us to select the cell line with the stronger ability to support virus replication from heterogeneous cell populations.

Information on adaptive mutations in subgenomic HCV replicons has been accumulated, but the present study is the first to examine precisely the adaptive mutation in genome-length HCV RNA. In this study, we analyzed mutations in genome-length HCV RNA in O cells and found that K1609E in the NS3 helicase region worked as an adaptive mutation. The adaptive mutation of K1609E was also reported by Lohmann et al. [22] but its impact on colony formation was only about 4 times that of the wild-type replicon [22]. We also reported the same mutation in our previous study of the 1B-1 replicon, although we did not assess ECF at that time [13]. In the present study, we found that the ECF of ON/C-5B/KE was significantly enhanced, to about 500 times that of the wild-type ON/C-5B. Also, in the transient reporter assay of the subgenomic HCV replicon, the introduction of K1609E enhanced the efficiency of replication. It was noteworthy that the introduction of S2204I in NS5A, which was reported as an adaptive mutation in HCV-N and Con1 replicons, had little impact on the transient reporter assay of the HCV-O replicon (data not shown). The differences in the effects of adaptive mutation might be due to the differences in HCV strains. To further improve the conditions of colony formation, the ECF in the combination of K1609E mutation and cured Oc cells was tested using ON/C-5B. This combination drastically enhanced the ECF of ON/C-5B. These results suggested that not only viral but also cellular factors were selected during culture with G418 for robust replication of HCV, and that the combination of these factors synergistically enhanced the ECF.

To facilitate the monitoring of the replication of a subgenomic HCV replicon, several groups have developed subgenomic HCV replicons with reporter genes such as luciferase, SEAP, or β-lactamase [17–19]. These persistent replication systems could save time and facilitate the mass screening of anti-HCV reagents. However, until now there has been no genome-length HCV RNA replication system with a reporter gene. One of the obstacles to the development of such a system may be low ECF, depending on the size of HCV RNA, or the ability of replicase complexes, including NS5B, to replicate HCV RNA. Our preliminary data showed that a 14-kb HCV RNA, which contained Core-E1-E2-p7-NS2-NS3-NS4A-NS4B fused to Neo at the first cistron instead of Neo in the subgenomic HCV-N replicon, produced colonies containing smaller HCV RNA, i.e., less than 12 kb, with a deletion at the first cistron (M. Ikeda and S.M. Lemon, unpublished data). In our trial to develop a genome-length HCV RNA replication system with a reporter gene, genome-length HCV RNA encoding the firefly luciferase gene (about 12.6 kb in total) failed to produce a G418-resistant colony, although we did obtain colonies containing subgenomic HCV replicon RNA encoding the firefly luciferase gene (about 9.6 kb in total) (data not shown). However, both subgenomic HCV replicon RNA and genome-length HCV RNA encoding the *Renilla* luciferase gene (about 9 and 12 kb in total, respectively) successfully produced

G418-resistant colonies. These results suggest that the NS5B polymerase of HCV-O possesses a limited elongation ability (probably up to a total length of 12 kb). We established a genome-length HCV RNA replication system with *Renilla* luciferase as a reporter using a newly discovered advantage in the combination of the K1609E adaptive mutation and cured Oc cells. The cell line supporting ORN/C-5B/KE derived from Oc cells demonstrated the usefulness of IFN-α's anti-HCV effect, since the values of *Renilla* luciferase correlated well with the level of HCV RNA at 24 h after IFN treatment. One of the most striking advantages of this system is that it allows us to investigate the effect of structural proteins on viral replication. In addition, anti-HCV activity in this system is reflected in the inhibitory level of HCV replication.

We developed a genome-length HCV RNA replication system from HCV-O infected in the non-neoplastic human hepatocyte line PH5CH8. Adaptive mutation was selected among a heterogeneous viral pool during replication, and a cell clone supporting robust HCV replication was selected from a heterogeneous pool of cells during culture. These viral and cellular factors contributed to the enhancement of colony formation and led to the establishment of a genome-length HCV RNA replication system with a reporter. This system has the longest RNA construct reported so far. In conclusion, this genome-length HCV replication system with a reporter gene, developed through the characterization of ON/C-5B, should be a useful tool for the study of HCV replication and for the mass screening of anti-HCV reagents.

Acknowledgments

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Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture

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Hepatitis C virus (HCV) genomic sequences are known to vary widely among HCV strains, but to date there have been few reports on the genetic variations and dynamics of HCV in an experimental system of HCV replication. In this study, a genetic analysis of HCV replicons obtained in long-term culture of two HCV replicon cells (50-1 and 1B-2R1), which were established from two HCV strains, 1B-1 and 1B-2, respectively, was performed. One person cultured 50-1 cells for 18 months, and two people independently cultured 50-1 cells for 12 months. 1B-2R1 cells were also cultured for 12 months. The whole nucleotide sequences of the three independent replicon RNA clones obtained at several time points were determined. It was observed that genetic mutations in both replicons accumulated in a time-dependent manner, and that the mutation rates of both replicons were approximately $3 \cdot 0 \times 10^{-3}$ base substitutions/site/year. The genetic diversity of both replicons was also enlarged in a time-dependent manner. The colony formation assay by transfection of total RNAs isolated from both replicon cells at different time points into naïve HuH-7 cells revealed that the genetic mutations accumulating with time in both replicons apparently improved colony formation efficiency. Taken together, these results suggest that the HCV replicon system is useful for the analysis of evolutionary dynamics and variations of HCV. Using this replicon cell culture system, it was demonstrated further that neither ribavirin nor its derivative mizoribine accelerated the mutation rate or the increase in the genetic diversity of HCV replicon.

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INTRODUCTION

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989), which progresses to liver cirrhosis and hepatocellular carcinoma (Ohkoshi *et al.*, 1990; Saito *et al.*, 1990). HCV belongs to the family *Flaviviridae*, whose genome consists of a positive-stranded RNA molecule of 9·6 kb and encodes a large polyprotein precursor of about 3000 aa residues (Kato *et al.*, 1990a; Tanaka *et al.*, 1995). This polyprotein is processed by a combination of the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, and non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Grakoui *et al.*, 1993; Hijikata *et al.*, 1991, 1993; Mizushima *et al.*, 1994). These HCV proteins not only function in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction and apoptosis (Bartenschlager & Lohmann, 2000; Kato, 2001).

The most characteristic feature of the HCV genome is its remarkable genetic diversity and variation. To date, more than 50 HCV genotypes have been identified worldwide (Bukh *et al.*, 1995; Simmonds, 1995; Tokita *et al.*, 1996). Each of these genotypes shows more than 20 % difference at the nucleotide level and more than 15 % difference at the amino acid level compared with any of the other genotypes, although the 5′ untranslated regions (5′ UTRs) and core protein-encoding regions are highly homologous among the 50 genotypes (homology of >90 %). Comparisons of HCV genomes that belong to a single genotype have revealed 5–8 % diversity in nucleotide sequences and 4–5 % diversity in amino acid sequences (Kato *et al.*, 1990b; Kato, 2001). An analysis of the genetic diversity among the HCV genomes in an individual revealed that the diversity in nucleotide sequences averaged 0·9 %, and distributed throughout

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the genome except in the 5′ UTR (Tanaka *et al.*, 1992). This so-called 'quasispecies' nature of the HCV genome has generally been observed in a single patient with chronic hepatitis C (Kato *et al.*, 1992; Martell *et al.*, 1992). This remarkable genetic diversity of the HCV genome suggests that HCV frequently causes mutations of the viral genome.

To date, two groups have estimated the mutation rate of the HCV genome using specimens from a chimpanzee (interval of 8 years) and a patient (interval of 13 years) infected with HCV (Ogata *et al.*, 1991; Okamoto *et al.*, 1992). They estimated that the mutation rate of the HCV genome was $1{\cdot}4$–$1{\cdot}9 \times 10^{-3}$ base substitutions/site/year; however, it is not clear whether this value indicates the actual mutation rate of the HCV genome, because complicated quasispecies are generally observed in patients or chimpanzees infected with HCV *in vivo*. On the other hand, Major *et al.* (1999) used chimpanzees that received intrahepatic inoculation with a full-length HCV RNA, and they estimated that the mutation rate of the HCV genome was $1{\cdot}5 \times 10^{-3}$ base substitutions/site/year. However, such experiments on HCV replication in humans are ethically problematic. Thus, there have been few reports on the genetic variations of HCV in an experimental system of HCV replication because of the lack of reproducible and efficient HCV proliferation in cell culture (Kato & Shimotohno, 2000).

In 1999, an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions derived from the strain Con-1 was first established by using a human hepatoma cell line, HuH-7 (Lohmann *et al.*, 1999). Since then, several additional replicon systems have been established (Ali *et al.*, 2004; Blight *et al.*, 2000, 2003; Ikeda *et al.*, 2002; Kato *et al.*, 2003a; Pietschmann *et al.*, 2002; Zhu *et al.*, 2003). In these systems, replicated HCV RNAs were detected by Northern blot analysis and the HCV proteins, which were produced, were detected by Western blot analysis. Therefore, HCV replicon systems are thought to be useful for the analysis of genetic variations and dynamics of HCV.

Recently, we also established two HCV replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively, using HuH-7 cells (Kato *et al.*, 2003b; Kishine *et al.*, 2002). The nucleotide sequences of the NS3-NS5B regions in the 50-1 replicon showed differences of 8·1 % from those in the 1B-2R1 replicon (Kato *et al.*, 2003b), although both HCV strains belonged to genotype 1b. In order to understand the genetic variations and dynamics of HCV, we performed genetic analysis of HCV replicons obtained in long-term culture of 50-1 and 1B-2R1 replicon cells (termed 50-1 and 1B-2R1 cells, respectively). Here, we show that the accumulation of genetic mutations and the acquisition of the genetic diversity among HCV replicons are time dependent. In addition, we evaluated the effect of ribavirin and mizoribine on the genetic variations and dynamics of HCV replicons.

METHODS

Cell cultures. 50-1 and 1B-2R1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum and 300 μg G418 (Geneticine; Invitrogen) ml^{-1}. The HCV replicon cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase (Neo^R) was produced by the efficient replication of HCV replicon in the cells. Therefore, when an HCV replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418. 50-1 cells were also cultured in the presence of 5 or 25 μM ribavirin (Sigma) or 25 μM mizoribine (Sigma). In general, these replicon cells were passaged every 4 days.

Northern blot analysis. Total RNA from the cultured cells were prepared using an RNeasy extraction kit (Qiagen). Total RNA (3 μg) was used to detect the HCV replicon RNA and β-actin mRNA. Northern blotting and hybridization were performed as described previously (Ikeda *et al.*, 2002; Kato *et al.*, 2003b). A digoxigenin-labelled, negative-sense RNA probe complementary to the NS5B region (positions 8935–9374 of the HCV genome) was used for the detection of the replicon RNA. A β-actin specific digoxigenin-labelled antisense RNA probe was used to check the amount of RNA. The synthetic RNA transcribed from pNSS1RZ2RU (Kato *et al.*, 2003b) (10^8 and 10^7 genome equivalents spiked into normal cellular RNA) was used to compare the level of replicon RNA. An RNA ladder (Invitrogen) was also used to mark the molecular length.

Western blot analysis. The preparation of cell lysates, SDS-PAGE and immunoblotting analysis with a PVDF membrane were performed as described previously (Hijikata *et al.*, 1993; Naganuma *et al.*, 2000). The antibodies used to examine the expression levels of HCV proteins were those against NS3 (Novocastra Laboratories) and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). Anti-β-actin antibody (AC-15; Sigma) was also used to detect β-actin as an internal control. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences).

RT-PCR. To amplify HCV RNA RT-PCR was performed as described previously (Kato *et al.*, 2003b). Briefly, the total RNA (2 μg) obtained from the replicon cells was used as a template for reverse transcriptase using SuperScript II (Invitrogen). PCR using proofreading KOD-plus DNA polymerase (Toyobo) was performed separately in two parts; one part covered the 5′ UTR to the amino terminal of the NS3 region, and the other part covered the NS3 region to the NS5B region. The PCR yielded a 2033 bp fragment for the former part and a 6107 bp fragment for the latter part.

cDNA cloning and sequencing. The PCR products were subcloned into the *Xba*I site of pBR322MC (Kishine *et al.*, 2002), which was derived from pBR322 and contained the multiple cloning site of pUC19, as described previously (Kato *et al.*, 2003b). Plasmid inserts were sequenced in both the sense and antisense directions by using Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyser (Applied Biosystems).

Molecular evolutionary analysis. Nucleotide sequences of the clones obtained by RT-PCRs from 50-1 and 1B-2R1 cells were analysed by the neighbour-joining analysis using the program GENETYX-MAC (Software Development).

RNA transfection and selection of G418-resistant cells. RNA transfection into Huh-7 cells was performed by electroporation as described previously (Lohmann *et al.*, 1999). Briefly, total RNA (80 μg) isolated from the replicon cells was electroporated into 5×10^6 HuH-7 cells, and then 1×10^5 or 3×10^5 cells were seeded into a 10 cm diameter dish. After 48 h, G418 was added to