



Mizoribine inhibits hepatitis C virus RNA replication: Effect of combination with interferon- α

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Abstract

Interferon (IFN)- α monotherapy, as well as the more effective combination therapy of IFN- α and ribavirin, are currently used for patients with chronic hepatitis C caused by hepatitis C virus (HCV) infection, although the mechanisms of the antiviral effects of these reagents on HCV remain ambiguous, and side effects such as anemia due to the administration of ribavirin present a problem for patients who are advanced in years. Using a recently developed reporter assay system in which genome-length dicistronic HCV RNA encoding *Renilla* luciferase gene was found to replicate efficiently, we found that mizoribine, an imidazole nucleoside, inhibited HCV RNA replication. The anti-HCV activity of mizoribine (IC_{50} : approximately 100 μ M) was similar to that of ribavirin. Using this genome-length HCV RNA replication monitor system, we were the first to demonstrate that the combination of IFN- α and ribavirin exhibited more effective anti-HCV activity than the use of IFN- α alone. Moreover, we found that the anti-HCV activity of mizoribine in co-treatment with IFN- α was at least equivalent to that of ribavirin. This effect was apparent in the presence of at least 5 μ M mizoribine. Since mizoribine is currently used in several clinical applications and has not been associated with severe side effects, mizoribine is considered to be of potential use as a new anti-HCV reagent in combination with IFN- α .
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Hepatitis C virus (HCV) is the major causative agent of chronic hepatitis (CH) [1,2], which progresses to liver cirrhosis and hepatocellular carcinoma [3,4]. Since at least 170 million people are currently infected with HCV worldwide, this infection is a global health problem [5]. HCV is an enveloped RNA virus belonging to *Flaviviridae*, the genome of which consists of a positive-stranded 9.6-kilobase (kb) RNA encoding an approximately 3000 amino acid polyprotein precursor [6,7]. This precursor protein is cleaved by the host and viral proteinases to generate at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NSSA, and NSSB [8–10].

To date, interferon (IFN)- α (majority) and IFN- β (minority) are used as effective anti-HCV reagents in clinical therapy for patients with CH C; however, the effectiveness of IFN is limited to about 30% of the reported cases [11]. In 1998, combined treatment with IFN- α and ribavirin, a nucleoside analogue, has been shown to be more effective (although the effectiveness remains at less than 50%) than treatment with IFN alone [12,13]; nonetheless, it has been shown that ribavirin alone does not induce a decrease in HCV levels in patients with CH C. Furthermore, it has been reported that the combination of pegylated IFN with ribavirin led to significant improvements in terms of a sustained virological response, when compared to standard IFN and ribavirin combination therapy [14]. However, a sustained virological response is still not induced in approximately half of patients treated with these reagents.

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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 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 diarrhoea 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 μ g/ml; Geneticin, 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-carboxamide) 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 μ l of *Renilla* lysis reagent, and the relative luciferase unit value in 10 μ l of lysates was measured by adding 50 μ l 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 μ M for 72 h in the absence of G418. Then, the number of viable cells was counted in an improved Neubauer-type hemocytometer 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 μ M 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,

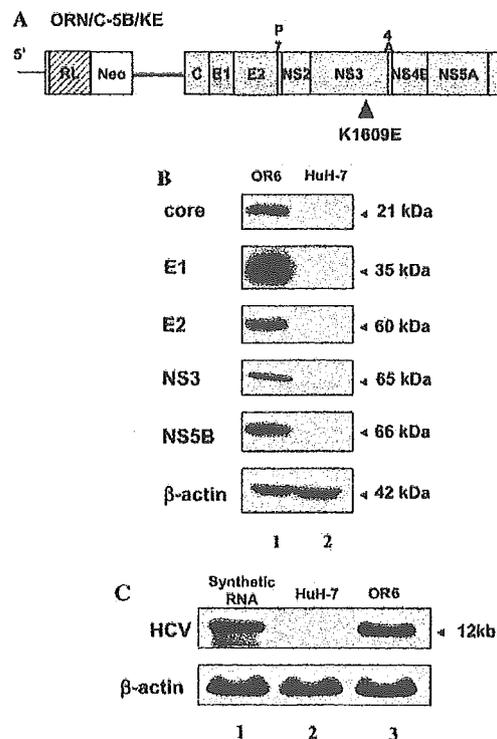


Fig. 1. Characterization of genome-length HCV RNA encoding *Renilla* luciferase gene as a reporter. (A) Schematic gene organization of genome-length HCV RNA encoding *Renilla* luciferase gene. *Renilla* luciferase gene (RL) is depicted as a striped box and is expressed as a fusion protein with Neo. The 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

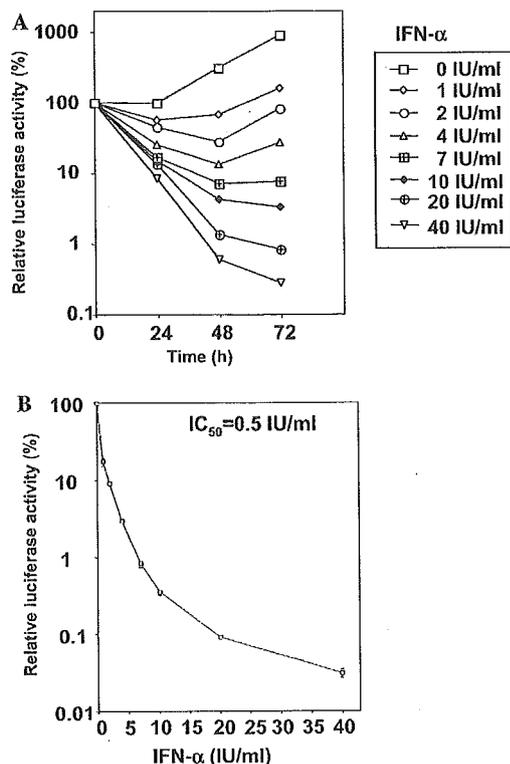


Fig. 2. Inhibition of HCV RNA replication in OR6 cells treated with IFN- α . (A) IFN- α sensitivity of HCV RNA replication in OR6 cells. The cells were treated with IFN- α (0, 1, 2, 4, 7, 10, 20, and 40 IU/ml), and at 24, 48, and 72 h after the treatment, and the *Renilla* luciferase assay was performed 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 \pm 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.

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.

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 μ M). 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 μ M) estimated that the IC_{50} value of ribavirin was 76 μ M (Fig. 3B). We confirmed that ribavirin (up to 200 μ M) 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 ($IC_{50} = 126 \mu$ M) 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 μ M) (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 μ M) estimated that the IC_{50} value of mizoribine was 99 μ M (Fig. 4B). This value was slightly higher than that (76 μ M) of ribavirin, as obtained by our assay system. We confirmed that mizoribine (up to 200 μ M) also did not

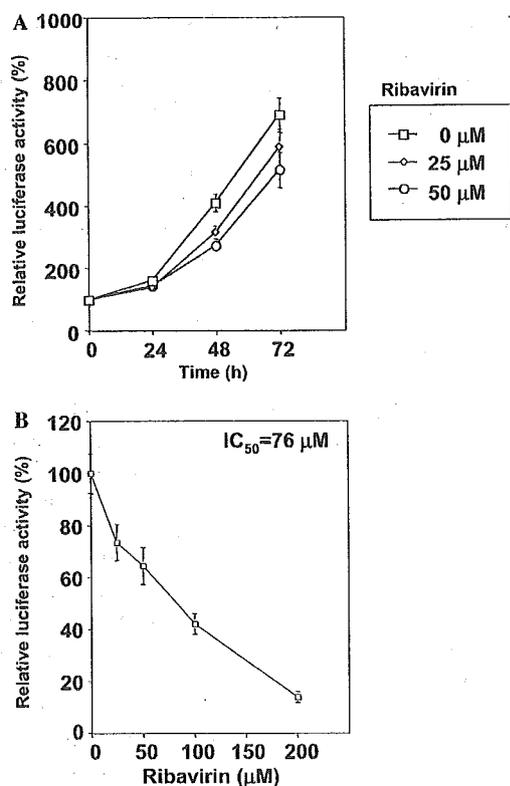


Fig. 3. Ribavirin alone inhibits HCV RNA replication in OR6 cells. (A) Inhibitory effect of ribavirin against HCV RNA replication. OR6 cells were treated with ribavirin (0, 25, and 50 μM), and at 24, 48, and 72 h after treatment, a *Renilla* luciferase assay was performed and the relative luciferase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

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

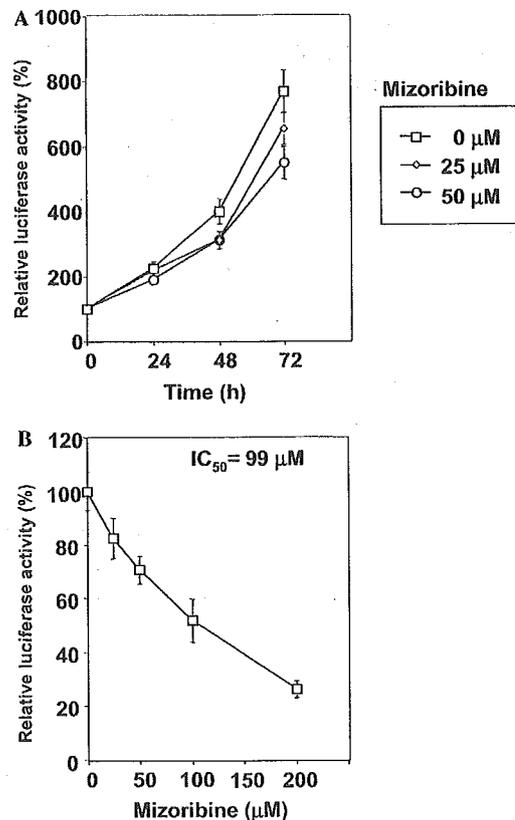


Fig. 4. Mizoribine alone also inhibits HCV RNA replication in OR6 cells. (A) Inhibitory effect of mizoribine against HCV RNA replication. OR6 cells were treated with mizoribine (0, 25, and 50 μM), and at 24, 48, and 72 h after treatment, a *Renilla* luciferase assay was performed and the relative luciferase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

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 $\leq 100 \mu\text{M}$) 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

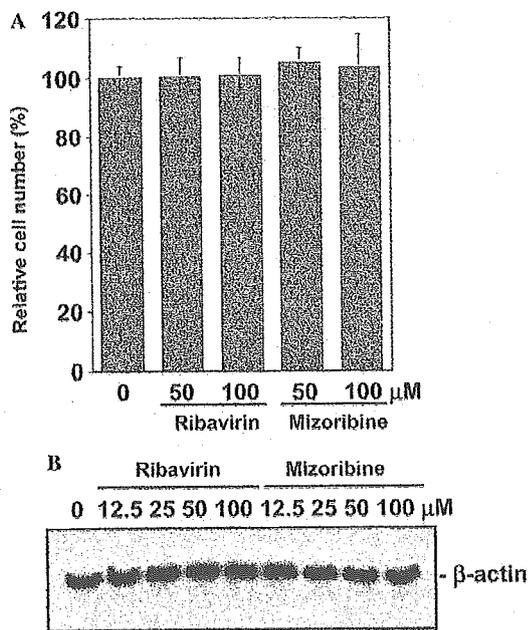


Fig. 5. No cytotoxicity of ribavirin or mizoribine in OR6 cells. (A) Cell viability after treatment with ribavirin or mizoribine. OR6 cells were cultured in the absence or presence of ribavirin or mizoribine (50 and 100 μM each) for 72 h, and then the cell number was determined as described under Materials 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 \pm 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.

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 concen-

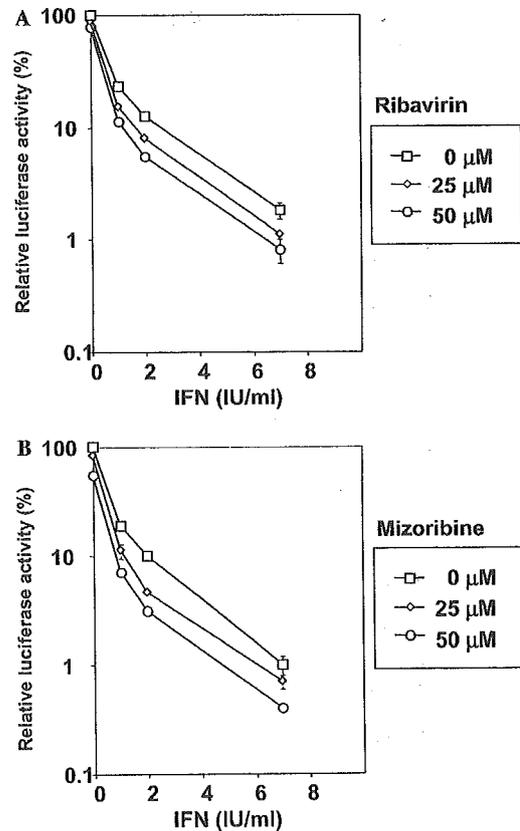


Fig. 6. Both ribavirin and mizoribine enhance the inhibition of HCV RNA replication due to IFN- α . (A) Effect of ribavirin in combination with IFN- α . OR6 cells were co-treated with IFN- α (0, 1, 2, and 7 IU/ml) and ribavirin (0, 25, and 50 μM), and at 72 h after treatment, a *Renilla* luciferase assay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

trations 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).

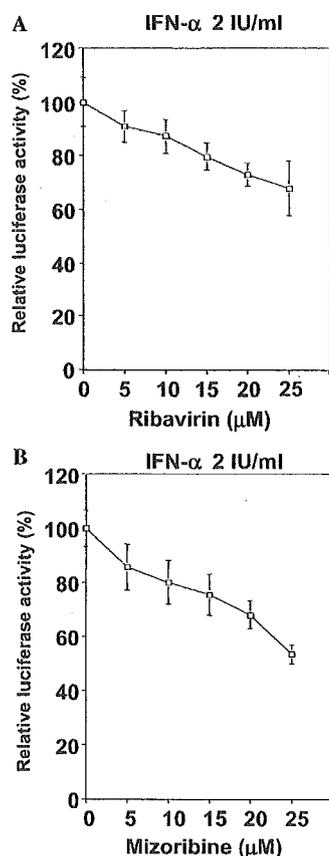


Fig. 7. Low-dose treatment of ribavirin or mizoribine is also effective for increasing the IFN- α inhibition of HCV RNA replication. (A) Effect of ribavirin in combination with IFN- α . OR6 cells were co-treated with ribavirin (0, 5, 10, 15, 20, and 25 μ M) and a fixed concentration (2 IU/ml) of IFN- 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 \pm 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 CHC 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 μ M 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 μ M, and in the latter clinical study [45], the plasma concentration of ribavirin was estimated to be 10–14 μ M [24,40]. In addition, we recently examined the inhibitory effects of mizoribine (25 μ M) 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 μ M did not inhibit the RdRp activity of NS5B purified after expression in insect cells [47]. However, at higher concentrations (several hundreds of μ M) 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 (IC₅₀: 76 and 99 μ M for ribavirin and mizoribine, respectively) were higher than the clinically achievable ribavirin concentration (10–14 μ M) reported previously [24,40]. However, when administered in combination with IFN- α , we demonstrated that a low dose (at least 5 μ M) 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 .

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Hepatitis C virus proteins exhibit conflicting effects on the interferon system in human hepatocyte cells

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Abstract

We previously found that hepatitis C virus (HCV) core protein (Core) activated the interferon (IFN)-inducible 40/46 kDa 2'-5'-oligoadenylate synthetase (2'-5'-OAS) gene through an IFN-stimulated response element (ISRE) in non-neoplastic human hepatocyte PH5CH8 cells. Here, we found that Core and NS5B synergistically enhanced the 2'-5'-OAS gene promoter activity through ISRE. Further analysis revealed that amino acid positions 12 and/or 13 of Core and RNA-dependent RNA polymerase activity of NS5B were essential for the activation of the 2'-5'-OAS gene promoter. Interestingly, we observed that the activation by Core or NS5B was still partially enhanced by even the NS5B or Core mutant lacking the activating ability, respectively, suggesting an indirect interaction between Core and NS5B. Furthermore, we showed that the activation by NS5B could be explained by NS5B's induction of IFN- β , however, IFN- β was not induced by Core. Moreover, we showed that the synergistic effect of Core and NS5B was not invalidated by NS3-4A, although NS3-4A significantly inhibited the activation by combination of Core and NS5B. Taken together, our findings reveal that NS5B/Core and NS3-4A exhibit conflicting effects (activation and inhibition) on the IFN system in PH5CH8 cells, and suggest that such effects may promote the distraction of the host defense system to lead to persistent infection.
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Keywords: Hepatitis C virus; Interferon system; Core; NS5B; 2'-5'-Oligoadenylate synthetase; Interferon- β ; NS3-4A

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis [1,2], which progresses to liver cirrhosis and hepatocellular carcinoma [3,4]. Since at least 170 million people are currently infected with HCV worldwide, this infection is a global health problem [5]. HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the Flaviviridae [6,7]. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues, which is cleaved co- and post-translationally into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and virally encoded

serine proteinase located in the amino-terminal domain of NS3. Activity of NS3 requires NS4A, a protein consisting of 54 aa residues, to form a stable complex with the NS3 domain [8–10]. NS5B possessing an RNA-dependent RNA polymerase (RdRp) activity is the central enzyme in replication of the HCV genome [10].

Interferon (IFN), one important effector of the innate immune response, is induced by different viral or bacterial components through Toll-like receptor (TLR)-dependent and -independent mechanisms. The binding of type I IFNs (IFN- α and IFN- β) to specific cell-surface receptors (IFNAR1 and IFNAR2c) triggers activation of the intracellular IFN signaling pathway (JAK–STAT). The activated JAK–STAT pathway induces the expression of a large number of IFN-stimulated genes (ISG), including cellular antiviral molecules such as 2'-5'-oligoadenylate synthetase (2'-5'-OAS), double stranded RNA (dsRNA)-activated

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protein kinase (PKR), dsRNA-specific adenosine deaminase I (ADARI), and *Mx* genes through the JAK–STAT signaling transduction pathway [11–14].

We previously found that HCV core protein (Core) activated the IFN-inducible 40/46 kDa 2'-5'-OAS gene in non-cancerous human hepatocyte PH5CH8 cells [15], but not in cancerous human hepatocyte HuH-7 cells (H. Dansako and N. Kato, unpublished). Further analysis revealed that Core, irrespective of HCV genotypes and strains, activated the gene (2'-5'-OAS, PKR, ADARI, etc.) promoters possessing an IFN-stimulated response element (ISRE) [16], and that the N-terminal 20 aa region of Core was important to the activation of the promoter, although this N-terminal region did not change the perinuclear localization of Core [16]. These findings suggest that the Core's activation of the 2'-5'-OAS gene contributes to the degradation of HCV RNA. However, it is still difficult to clarify this point, because of the lack of a reproducible and efficient HCV proliferation system using PH5CH8 cells [17].

On the other hand, Foy et al. [18] recently found that an HCV serine protease, NS3-4A, blocks virus-induced activation of IFN regulatory factor 3 (IRF-3), a transcription factor playing a critical role in the induction of type I IFNs (initially IFN- β and subsequently IFN- α). This finding using HuH-7 cells suggests that NS3-4A mediates proteolysis of a cellular protein within an antiviral signaling pathway upstream of IRF-3, leading to the persistent viral infection. The recently identified Toll-IL1 receptor domain-containing adaptor inducing IFN- β (TRIF) is a possible candidate for this cellular protein [19]. However, the activation by Core and the suppression by NS3-4A on the IFN system seem to be contradictory phenomena, although both findings have been obtained by using different human hepatocyte cell lines [15,16,18].

To clarify the mechanism(s) underlying activation or suppression by HCV proteins on the IFN system, we further characterized the effects of Core and NS proteins, including NS3-4A, on IFN signaling using PH5CH8 cells, which have recently been shown to retain robust IFN responses to dsRNA as well as viral infection, suggesting that they more closely resemble normal hepatocytes in vivo [20].

In the present study using PH5CH8 cells, we report that NS5B synergistically enhanced the gene activation by Core through ISRE, and that the activation by Core or NS5B was suppressed by NS3-4A, but the synergistic effect of Core and NS5B was still observed even in the presence of NS3-4A.

Materials and methods

Cell lines. Non-neoplastic human PH5CH8 hepatocytes, which are susceptible to HCV infection and supportive of HCV replication [21], were maintained as described previously [22].

Construction of expression vectors. pCXbsr/NS3-4A, pCXbsr/NS4A, pCXbsr/NS4B, and pCXbsr/NS5B, which contain the resistance gene for blasticidin and encode NS3-4A, NS4A, NS4B, and NS5B derived from the HCV 1B-1 strain belonging to genotype 1b (Accession No. AB0802999)

[23], respectively, were constructed according to the previously described method [15]. pCXpur [24], which contains the resistance gene for puromycin, was also used for the construction of pCXpur/Core and pCXpur/NS3-4A. The DNA fragments encoding Core and NS3-4A derived from HCV 1B-1 strain [23] were also subcloned into the *EcoRI* and *NotI* sites of pCXpur.

pCXbsr/Core Δ (2–6), pCXbsr/Core Δ (2–11), pCXbsr/Core Δ (2–16), and pCXbsr/Core Δ (2–21), which encode 5, 10, 15, and 20 aa N-truncated Core (1b-P) [15], respectively, were constructed according to the previously described method [15]. pCXbsr/Core R9T-K10S- Δ (11–13) was constructed by PCR mutagenesis with primers containing base alterations. pCXbsr/NS5BA C21, pCXbsr/NS5BA C56, and pCXbsr/NS5BA C97, which encode 21, 56, and 97 aa C-truncated NS5B derived from HCV 1B-1 strain [23], respectively, were also constructed according to the previously described method [15]. pCXbsr/NS5B G317V and pCXbsr/NS5B R154T-K155S- Δ (156–158) were constructed by PCR mutagenesis with primers containing base alterations. The nucleotide sequences of these constructed expression vectors were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Luciferase reporter assay. For the dual luciferase assay, we used firefly luciferase reporter vectors, p2'-5'-OAS(-159)-Luci [25] containing the -159 to +82 region of the 2'-5'-OAS gene, pIFN β (-125)-Luc [25] containing the IFN- β gene promoter region (-125 to +19), and pISRE-Luc (Stratagene, La Jolla, CA) containing five repeats of the consensus ISRE sequence (AGTTTCACTTCC). The reporter assay was carried out as previously described [15,16]. Briefly, a total of 1.5×10^5 cells were seeded in a six-well plate 24 h before transfection. Then, 0.5 μ g firefly luciferase reporter plasmid (p2'-5'-OAS(-159)-Luci, pIFN β (-125)-Luc, or pISRE-Luc), 1–2 μ g HCV protein expression effector plasmid (pCXbsr series), and 1 ng pRL-CMV (Promega, Madison, WI) as an internal control reporter were transfected into PH5CH8 cells. To maintain the efficiency of transfection, up to 2 μ g (4 μ g in some cases) of pCXbsr instead of HCV protein expression vectors was used as the effector plasmid DNA. The cells were cultured for 48 h, and then a dual luciferase assay was performed according to the manufacturer's protocol (Promega). In some cases, the cells were cultured for 42 h and then treated with IFN- β (500 IU/ml) for 6 h before the reporter assay. Three independent triplicate transfection experiments were conducted in order to verify the reproducibility of the results. Relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used to detect luciferase activity.

Western blot analysis. Preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed as described previously [26]. The antibodies used in this study were those against Core (Institute of Immunology, Tokyo), anti-NS3 (Novocastra Laboratories, Newcastle, UK), anti-NS4A (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-NS4B [9], anti-NS5A [9], anti-NS5B (a generous gift from Dr. M. Kohara), and β -actin (Sigma, St. Louis, MO). Immunocomplexes were detected by a Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

Reverse transcription-PCR. Total cellular RNA was extracted using an Isogen extraction kit (Nippon Gene, Toyama, Japan). Before reverse transcription (RT), the RNA was treated with RNase-free DNase I (TaKaRa Bio, Ohtsu, Japan) to completely remove the genomic DNA as described previously [16]. RT-PCR was performed by a method described previously [16]. The sequences of IFN- β (Accession No. V00547), IRF-1 (Accession No. NM_002198), IRF-3 (Accession No. NM_001571), and IRF-7 (Accession No. U73036) were used to design specific primers. The sequences of the sense and antisense primers for IFN- β were 5'-CCCTG AGGAGATTAAGCAGCTGC-3' and 5'-AGTTCCTTAGGATTC CACTCTGAC-3'. The sequences of the sense and antisense primers for IRF-1 were 5'-GCCCTGACTCCAGCACTGTGCG-3' and 5'-ATTG AGTAGGTACCCCTTCCC-3'. The sequences of the sense and antisense primers for IRF-3 were 5'-ACACATACTGGGCGAGTGAGC-3' and 5'-G CAGGTCCACAGTATTCTCC-3'. The sequences of the sense and

antisense primers for IRF-7 were 5'-GTACGGGTGGGCAGTAGA GAC-3' and 5'-CAGCAGTTCCTCCGTGTAGC-3'. RT-PCR was performed using primer sets for IFNAR1 [16], IFNAR2c [16], STAT1 [16], STAT2 [16], and GAPDH [27] used in the previous reports [16]. Real-time LightCycler PCR was performed by a method described previously [27].

Preparation of PH5CH8 cells stably expressing HCV proteins. PH5CH8 cells were infected with retrovirus pCXbsr [24] encoding various HCV proteins, as described previously [28]. pCXbsr/Core (1b-P) [15], pCXbsr/NS3-4A, pCXbsr/NS5A (1b-P) [15], and pCXbsr/NS5B were used to obtain the PH5CH8 cells stably expressing Core, NS3-4A, NS5A, and NS5B, respectively. At 2 days postinfection, the PH5CH8 cells were changed with fresh medium containing blasticidin (20 µg/ml), and the culture was continued for 7 days to select the cells expressing HCV proteins.

Results

Core and NS5B synergistically enhance 2'-5'-OAS gene promoter activity

We previously found that Core activated the IFN-inducible 40/46 kDa 2'-5'-OAS gene through an ISRE in human immortalized hepatocyte PH5CH8 cells [15]. However, in that study, the effect of Core in the presence of other HCV proteins, especially NS proteins, was not examined. Since NS proteins coexist with Core when HCV replicates and proliferates in the infected cells, we examined the effects of the combination of Core and NS proteins (NS3, NS4A, NS4B, NS5A, and NS5B) on the 2'-5'-OAS gene promoter in PH5CH8 cells using a dual luciferase reporter

assay. As shown in Fig. 1A, we found that the combination of Core and NS5B exhibited more effective enhancement (approximately 20-fold) than the core protein alone (approximately 7-fold), whereas NS3, NS4A, NS4B, and NS5A had no effects when used in combination with Core. Since this finding suggested that NS5B per se might be able to activate the 2'-5'-OAS gene promoter, we next examined the effect of NS5B alone on the 2'-5'-OAS gene promoter. The results revealed that NS5B, but not NS3, NS4A, NS4B, or NS5A, could enhance luciferase activity as well as Core—i.e., by approximately 7-fold (Fig. 1B). The effect of NS5B was not further enhanced by the combination with other NS proteins (data not shown). In addition, we confirmed the transient expression of Core and NS proteins from the expression vectors used in these experiments (Fig. 1C). In summary, these results indicated that the combination of Core and NS5B synergistically (approximately 1.5-fold) enhanced the 2'-5'-OAS gene promoter activity in PH5CH8 cells.

Deletion analysis of Core and NS5B to identify the critical region for activation of the 2'-5'-OAS gene promoter

Since we previously showed that the N-terminal 20 aa region of Core was important for activation of the 2'-5'-OAS gene promoter [16], we here speculated that NS5B may also possess an aa sequence similar to the N-terminal 20 aa region of Core. In confirmation of this hypoth-

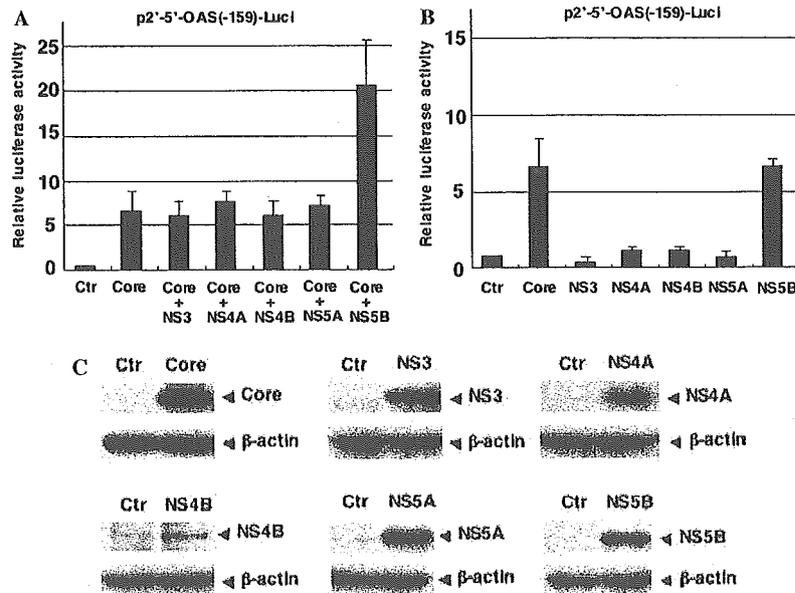


Fig. 1. NS5B synergistically enhanced Core's activation of the human 2'-5'-OAS gene promoter in PH5CH8 cells. (A) NS5B enhanced Core's activation of the 2'-5'-OAS gene promoter. The dual luciferase reporter assay was performed as described in Materials and methods. The relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). The lysate of cells transfected with expression vector pCXbsr was used as a control (Ctrl). One microgram of HCV protein expression effector plasmid was used. (B) NS5B per se activated the 2'-5'-OAS gene promoter. The dual luciferase reporter assay was performed as described in (A). (C) Western blot analysis of HCV proteins. The production of Core, NS3, NS4A, NS4B, NS5A, and NS5B in PH5CH8 cells transfected with HCV protein expression plasmids was analyzed by immunoblotting using anti-Core, anti-NS3, anti-NS4A, anti-NS4B, anti-NS5A, and anti-NS5B antibodies, respectively. PH5CH8 cells transfected with pCXbsr plasmid were used as a control (Ctrl). β-Actin was used as a control for the amount of protein loaded per lane.

esis, our analysis revealed a KxxRKxxR motif in both Core (KPQRKTKR at aa 6–13) and NS5B (KGGRKPAR at 151–158) (Figs. 2A and B). In NS5B, this motif is located in the priming and interrogation sites, which are essential for the RdRp activity of NS5B [29]. Therefore, we examined whether or not this motif is critical for the activation of the 2'-5'-OAS gene promoter by using the Core and NS5B mutants lacking this motif. Several N-truncated forms of Core were also used in order to narrow down the critical region for the promoter activation (Fig. 2A). In addition, one NS5B mutant (G317V in the GDD motif, aa 317–319, located in the catalytic site) and three C-truncated forms (Δ C21, Δ C56, and Δ C97, lacking 21, 56, and 97 aa, respectively) of NS5B were used in order to clarify whether or not RdRp activity and endoplasmic reticulum (ER) membrane anchorage of NS5B are required for the promoter activation (Fig. 2B). It has been known that the last 21 aa are necessary and sufficient to target NS5B to the cytosolic side of the ER membrane [30]. Although Δ C21 and Δ C56, but not Δ C97, possess RdRp activity in vitro, Δ C56 shows higher RdRp activity than Δ C21 [31].

The results of the reporter assay regarding the Core mutants revealed that aa 12 and 13 were critical aa residues for the activation of the 2'-5'-OAS gene promoter, because the activity of Core R9T-K10S- Δ (11–13) was remarkably decreased, whereas core Δ (2–11) lacking aa 2–11 still maintained the activity for the promoter activation (Fig. 2C). It is noteworthy that aa 12 and 13 are located within the KxxRKxxR motif (aa 12 and 13 are underlined). In addition, the results revealed that aa 17–21 was also involved in the promoter activation, because the enhancing activity of core Δ (2–16) (approximately 5-fold) was completely abolished in core Δ (2–21) (Fig. 2C).

Regarding the NS5B mutant forms, the results revealed that the enhancing activities of NS5B R154T-K155S- Δ C (156–158) lacking a KxxRKxxR motif, NS5B Δ C97, and NS5B G317V were almost impaired (Fig. 2D), and that NS5B Δ C56 and NS5B Δ C21 still possessed weak enhancing activities (approximately 6- and 3-fold, respectively). These results suggest that NS5B's activation of the 2'-5'-OAS gene promoter is dependent on the RdRp activity of NS5B.

Characterization of the synergistic effect of Core and NS5B on the 2'-5'-OAS gene promoter

To clarify the mechanism underlying the synergistic effect of Core and NS5B, we further examined the effects of the combinations of Core and NS5B mutants lacking the enhancing activity, or NS5B and Core mutants lacking the enhancing activity. The results showed that the Core's activation of the 2'-5'-OAS gene promoter was no longer enhanced in the combination with NS5B Δ C97 or NS5B R154T-K155S- Δ (156–158) (Fig. 3). Interestingly, however, NS5B G317V lacking the enhancing activity could partially enhance the Core's activation of the 2'-5'-OAS gene promoter (Fig. 3). Similarly, core Δ (2–21) lacking the enhancing activity also could partially enhance the activation by NS5B, whereas the combination of core Δ (2–21) and NS5B mutants such as NS5B Δ C97 exhibited no effect on the promoter activity (Fig. 3). In addition, co-expression of NS5B and NS5B mutants lacking the enhancing activity also had no effect on the promoter activity (Fig. 3), suggesting that these NS5B mutants are not a competitive or dominant-negative inhibitor for the 2'-5'-OAS gene promoter activity. These results also suggest that direct or indirect interaction between Core and NS5B is involved in the

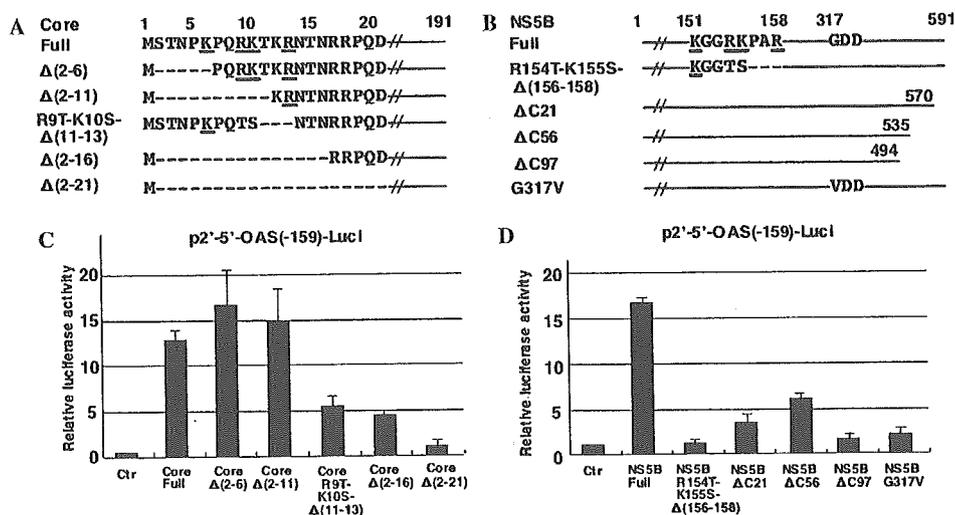


Fig. 2. Deletion analysis of the Core and NS5B. (A) Schematic presentation of the Core mutants used. The aa sequences of aa 1–21 in the Core (1b-P) [15] are indicated. The bars indicate the deleted aa residues, and K and R in the KxxRKxxR motif are underlined. (B) Schematic presentation of the NS5B mutants used. Only the aa sequences in the mutated regions of NS5B are indicated. The bars indicate the deleted aa residues, and K and R in the KxxRKxxR motif are underlined. (C) Effects of the Core mutants on the 2'-5'-OAS gene promoter activity in PH5CH8 cells. The dual luciferase reporter assay was performed as described in Fig. 1A. Two micrograms of the HCV protein expression effector plasmid was used. (D) Effects of the NS5B mutants on the 2'-5'-OAS gene promoter activity in PH5CH8 cells. The dual luciferase reporter assay was performed as described in (C).

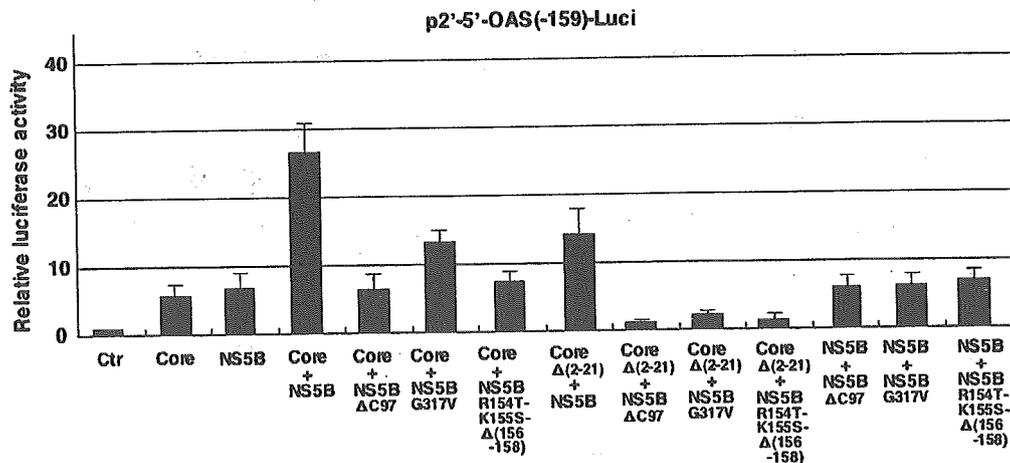


Fig. 3. Characterization of the synergistic effect of Core and NS5B on the 2'-5'-OAS gene promoter. The dual luciferase reporter assay was performed as described in Fig. 1A. One microgram of each HCV protein expression effector plasmid was used.

synergistic effect of both proteins. Although Uchida et al. [32] reported the formation of a complex between Core and NS5B in mammalian cells, we failed to obtain evidence that Core and NS5B could form a complex in PH5CH8 cells using an immunoprecipitation method (data not shown).

The promoter activations by Core and NS5B are differentially suppressed by NS3-4A

Although we showed that Core and NS5B synergistically enhanced the 2'-5'-OAS gene promoter in PH5CH8 cells (Fig. 1), it has recently been reported that the NS3-4A serine protease prevented virus-induced activation of IRF-3, which is a critical factor for the induction of IFN- β , using human hepatoma cell lines including HuH-7 [18]. According to this information, we examined the effect of NS3-4A on the activation of the 2'-5'-OAS gene promoter by Core or NS5B in PH5CH8 cells. We first prepared the NS3-4A expression vector using NS3-4A derived from the 50-1 HCV replicon [33], which could efficiently replicate in HuH-7 cells, suggesting that NS3-4A possessed a powerful serine protease activity. In order to evaluate the effect of NS3-4A, the dose of expression vector for NS3-4A was changed from 0.1 to 1 μ g under the condition of a fixed dose (1 μ g) of expression vector for Core or NS5B. NS3-4A and Core or NS5B were transiently co-expressed in PH5CH8 cells, and a luciferase reporter assay was performed. The results revealed that the NS5B's activation of the 2'-5'-OAS gene promoter was drastically suppressed even when 0.1 μ g NS3-4A expression vector was used (Fig. 4A); however, the activation by Core was only partially suppressed even when 1 μ g NS3-4A expression vector was used (Fig. 4B). These results indicated that NS3-4A had differential suppressive effects toward the activations by Core and NS5B, although the suppressive effect of NS3-4A was consistent with the results reported by Foy et al. [18]. This finding also suggests that the mech-

anism underlying the activation by Core is different from that of the activation by NS5B. Additional similar results were obtained by a luciferase reporter assay using a synthetic promoter possessing five repeats of the consensus ISRE (Figs. 4C and D). It was noteworthy that the enhancement of luciferase activity by NS5B (1 μ g) was impaired when 0.1 μ g NS3-4A expression vector was used for the assay (Fig. 4C). Furthermore, we observed that this suppressive effect of NS3-4A toward the promoter activation by Core or NS5B was clearly impaired when a NS3-4A/S1165A mutant lacking serine protease activity [34] was co-expressed with NS5B or Core (Figs. 4C and D), suggesting that the suppressive effect of NS3-4A is dependent on its serine protease activity. Moreover, we confirmed that NS3 alone (Figs. 5A and B) or NS4A alone (Figs. 5C and D) was not able to suppress the promoter activation by Core or NS5B (each 1 μ g as effector plasmid), even when 1 μ g NS3 or NS4A expression vector was used for the assay. In addition, we confirmed that co-transfection of the NS3 and NS4A expression vectors also showed a similar suppressive effect toward the activation by NS5B (Fig. 5E), indicating that the NS3/4A complex in *trans* [34] is also able to suppress the activation by NS5B. These results suggest that the full protease activity occurring by complex formation between NS3 and NS4A is required for the suppressive effect toward the activation by Core or NS5B. In addition, we observed that NS3/4A complexes in *cis* and in *trans* were no longer able to suppress the signaling occurring after IFN- β treatment (Fig. 5F), suggesting that the target site(s) of NS3-4A is some upstream molecule(s) involved in IFN- β production.

IFN- β is induced by NS5B, but not by Core

As described above, we suggested that NS5B's activation of the 2'-5'-OAS gene promoter was dependent on the RdRp activity of NS5B. Although the 2'-5'-OAS gene

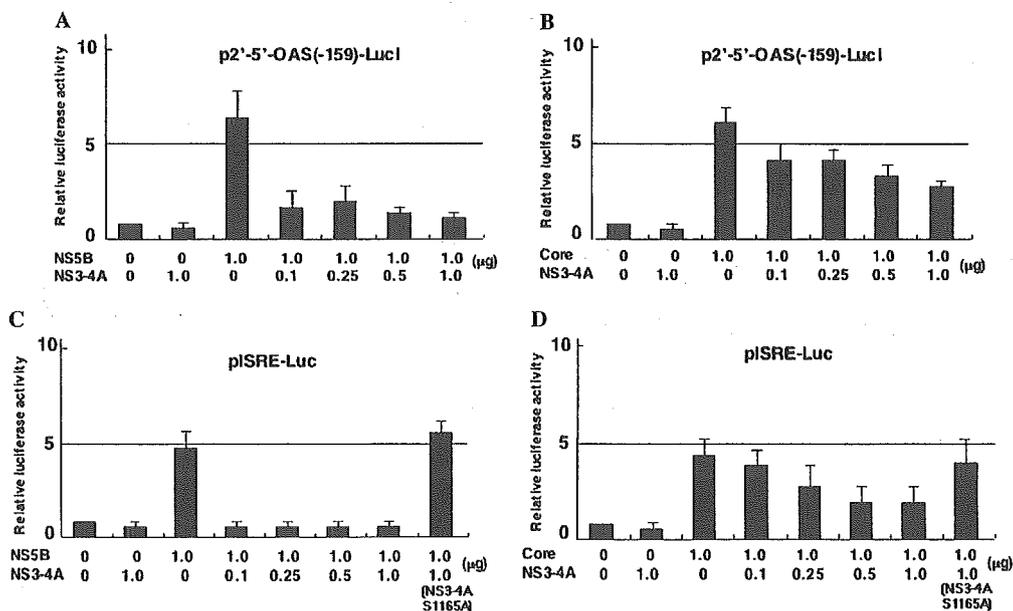


Fig. 4. NS3-4A differentially suppressed the promoter activations by Core and NS5B. The dual luciferase reporter assay was performed as described in Fig. 1A. (A) Effect of NS3-4A on NS5B's activation of the 2'-5'-OAS gene promoter. (B) Effect of NS3-4A on Core's activation of the 2'-5'-OAS gene promoter. (C) Effect of NS3-4A on NS5B's activation of the synthetic ISRE promoter. The expression vector of the NS3-4A/S1165A mutant lacking serine protease activity was also used. (D) Effect of NS3-4A on Core's activation of the synthetic ISRE promoter. The expression vector of the NS3-4A/S1165A mutant lacking serine protease activity was also used.

promoter possesses the ISRE sequence of a variant type (GGTTTCGTTTCCTC), this suggestion is consistent with our recent finding (Naka et al., submitted) that NS5B full form activates the *IFN-β* gene promoter possessing the IRF3 target sequence, which is the same as the consensus ISRE sequence (AGTTTCACTTTCCC). Furthermore, since NS5B could induce the expression of *IFN-β* through the TLR3 signaling pathway in PH5CH8 cells (Naka et al., submitted), we speculated that the activation of the 2'-5'-OAS gene promoter was caused by *IFN-β* induced not only by NS5B but also Core.

To clarify whether or not Core is able to induce the expression of *IFN-β*, RT-PCR analysis of *IFN-β* was performed using PH5CH8 cells stably expressing Core, NS5B, or NS5A (as a control). The expression levels of IRF1, IRF3, IRF7, type I *IFN* receptors (*IFNAR1* and *IFNAR2c*), *STAT1*, and *STAT2*, all of which are involved in *IFN* system, were also examined. The results revealed that Core did not induce *IFN-β*, whereas NS5B induced *IFN-β* and the downstream effector gene *IRF7* (Fig. 6). Neither Core nor NS5A had any effect on the expression levels of the components examined (Fig. 6). We previously showed that Core did not enhance the expression levels or phosphorylation status of the components (*STAT1*, *STAT2*, *Jak1*, and *Tyk2*) of the JAK-*STAT* signaling pathway [16]. This previous finding, taken together with the present results, suggests that the mechanism of activation of the 2'-5'-OAS gene by Core differs from the mechanism of NS5B's induction of *IFN-β*.

The synergistic effect of Core and NS5B toward *IFN-β* gene activation is not invalidated by NS3-4A

Since the synergistic effect of Core and NS5B on the 2'-5'-OAS gene promoter was found in PH5CH8 cells and the activation of the 2'-5'-OAS gene promoter by NS5B could be explained by NS5B's induction of *IFN-β*, we next examined whether or not such a synergistic effect on the *IFN-β* gene promoter is observed in PH5CH8 cells. The effect of NS3-4A was also examined in this experiment. The results revealed that the activity of the *IFN-β* gene promoter was also synergistically (approximately 1.6-fold) enhanced by Core and NS5B, and that NS3-4A drastically suppressed the enhancement by NS5B and partially suppressed the enhancement by Core (Fig. 7A), as observed when the 2'-5'-OAS gene promoter was used (Figs. 1 and 5). In addition, when 0.01, 0.025, and 0.1 μg NS3-4A expression vector were used, the synergistic effects (approximately 3.5-, 3-, and 1.8-fold, respectively) of Core and NS5B were not invalidated (Fig. 7A), although the suppressive effect by NS3-4A was observed. In the assay in which the *IFN-β* gene promoter was also used, the suppressive effect of NS3-4A was clearly impaired when the NS3-4A/S1165A mutant lacking the serine protease activity was expressed, suggesting that the suppressive effect of NS3-4A is dependent on its serine protease activity (Fig. 7A). The expression of Core, NS3, NS3-4A/S1165A, or NS5B in the cells examined was confirmed by Western blot analysis (data not shown).

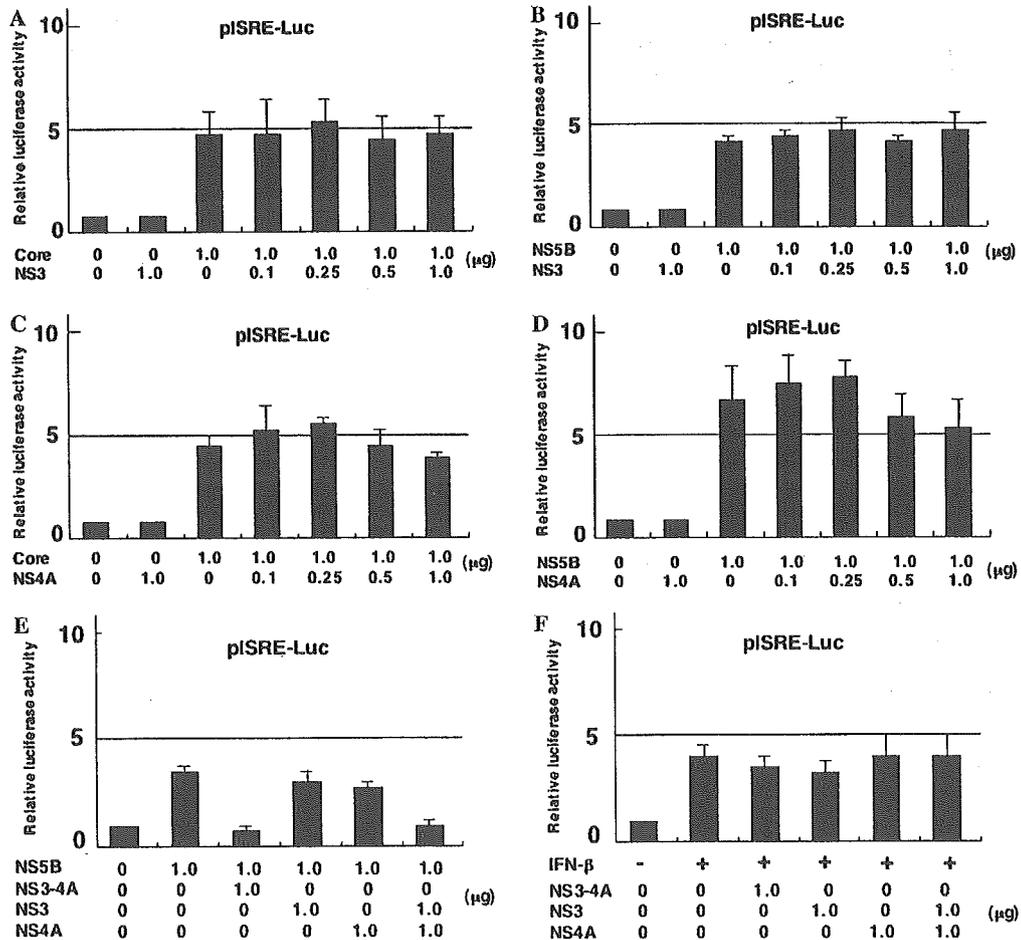


Fig. 5. The synthetic ISRE promoter activation by Core or NS5B is not suppressed by only NS3 or NS4A. Dual luciferase reporter assay was performed as described in Fig. 1A. (A) Effect of NS3 on Core's activation of the synthetic ISRE promoter. (B) Effect of NS3 on NS5B's activation of the synthetic ISRE promoter. (C) Effect of NS4A on Core's activation of the synthetic ISRE promoter. (D) Effect of NS4A on NS5B's activation of the synthetic ISRE promoter. (E) The NS3/4A complex in *trans* as well as in *cis* can suppress NS5B's activation of the synthetic ISRE promoter. (F) The NS3/4A complex in *trans* and in *cis* is not able to suppress the signaling after IFN- β treatment. PH5CH8 cells were treated with IFN- β (500 IU/ml) for 6 h before the reporter assay.

Next, we examined the effect of NS3-4A toward the expression level of IFN- β mRNA in PH5CH8 cells stably expressing Core and/or NS5B. In order to obtain the actual ratios of IFN- β mRNA expression, real-time Light-Cycler PCR was performed. The results revealed that the expression level of IFN- β mRNA in the cells co-expressing Core and NS5B became approximately 8-fold higher than that in the cells expressing NS5B alone (Fig. 7B). Furthermore, we observed that the elevation of IFN- β mRNA in the cells co-expressing Core and NS5B was partially suppressed by NS3-4A expression, although NS3-4A expression in the cells expressing NS5B alone led to complete impairment of the expression of IFN- β mRNA (Fig. 7B). These results are consistent with the results of the reporter assay using the IFN- β gene promoter, as described above. The expression of Core, NS3, or NS5B in the cell lines examined was also confirmed by Western blot analysis (Fig. 7C).

Discussion

In the present study, we found that NS5B as well as Core activated the 2'-5'-*OAS* gene promoter in PH5CH8 cells, that the activity of NS5B was synergistically enhanced in combination with Core, and that this gene activation was dependent on the RdRp activity of NS5B and on aa 12 and 13 of Core. We obtained some data, suggesting that an indirect interaction between Core and NS5B was involved in the synergistic effect for activation of the 2'-5'-*OAS* gene promoter. The activation of the 2'-5'-*OAS* gene promoter by NS5B could be explained by our recent finding that NS5B induces IFN- β (Naka et al., submitted). On the other hand, we observed that NS3-4A extensively suppressed NS5B's activation of the 2'-5'-*OAS* and IFN- β gene promoters in a manner that was dependent on its own protease activity. However, the activation of these gene promoters by Core was only partially

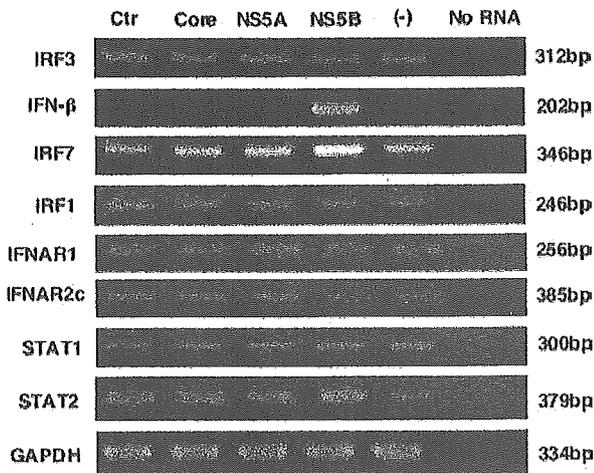


Fig. 6. RT-PCR analysis of IFN- β , interferon regulatory factors (IRF1, IRF3, and IRF7), type I IFN receptors (IFNAR1 and IFNAR2c), transcriptional factors (STAT1 and STAT2), and GAPDH in PH5CH8 cells infected with pCXbsr retrovirus encoding Core, NS5A, or NS5B. The pCXbsr retrovirus was used as a control infection (Ctr). At 48 h postinfection, total RNA was extracted and subjected to RT-PCR analysis using the primer set for IRF3 (312 bp), IFN- β (202 bp), IRF7 (346 bp), IRF1 (246 bp), IFNAR1 (256 bp), IFNAR2c (385 bp), STAT1 (300 bp), STAT2 (379 bp), or GAPDH (334 bp). The PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. Lane (-), PH5CH8 cells without retrovirus infection.

suppressed by NS3-4A, and the synergistic effect of Core and NS5B was not invalidated by NS3-4A.

Since Core and NS5B equivalently activate the 2'-5'-OAS and IFN- β gene promoters and a synthetic ISRE promoter, it is of interest that IFN- β was induced by NS5B, but not by Core. Furthermore, the synergistic effect of Core on the activation of these gene promoters by NS5B is also interesting. Recently, we found that NS5B, but not Core, delayed the cell cycle progression through the S phase in PH5CH8 cells, and we considered that this phenomenon may have been caused by IFN- β induced by NS5B through the activation of TLR3 (Naka et al., submitted). In the present study, we observed that Core enhanced NS5B's induction of IFN- β mRNA and that Core might indirectly interact with NS5B. Therefore, our findings suggest not only that Core's activation of gene promoters through ISRE is synergistically enhanced by NS5B, but also that Core positively modifies the activation of the TLR3 signaling pathway by NS5B, although Core does not activate this pathway directly.

In the course of studies on the mechanism of Core's activation of gene promoters containing ISRE, we previously showed that Core had no effects on the gene expression levels or phosphorylation status of the major components involved in the JAK-STAT signaling pathway, such as STAT1 and STAT2 [16]. Recently, Imanaka et al. [35] reported that IFN- α -induced STAT-1 phosphorylation and the expression of antiviral genes were inhibited in the suppressor of cytokine signaling (SOCS)-1-expressing cells. Since SOCS-1 is referred to as the STAT-induced STAT

inhibitor or the JAK-binding protein, we speculated that Core may be able to suppress the SOCS-1 expression in PH5CH8 cells, resulting in activation of the JAK-STAT signaling pathway. However, RT-PCR analysis revealed that the level of SOCS-1 mRNA was not changed regardless of the expression of Core in PH5CH8 cells. The other possibility is that Core may modify the affinity of IFN-stimulated gene factor 3 (ISGF3) to the ISRE sequence, since it has recently been reported that Core expression is associated with increased ISGF3-binding activity to the ISRE sequence [36]. Further analysis will be necessary to clarify whether or not the same phenomenon is observed in PH5CH8 cells.

Our observations that NS3-4A drastically suppresses NS5B's activation of the IFN system, especially induction of IFN- β , are consistent with the recent findings [18] that NS3-4A effectively blocks the phosphorylation of IRF3 (a key molecule in innate immunity) that normally occurs in response to virus infection. Recent studies [19,37,38] have indicated that NS3-4A inhibits both the TLR3 signaling pathway (TRIF-dependent pathway) and RIG-I signaling pathway (TRIF-independent pathway). The present study showed that NS3-4A in a serine protease activity-dependent manner inhibited the TLR3 signaling pathway in PH5CH8 cells, based on our recent finding that the induction of IFN- β by NS5B was mediated through the TLR3 but not the RIG-I signaling pathway (Naka et al., submitted). It is likely that TRIF, which was identified recently [19] as a target molecule of cellular components upstream of IRF3, is cleaved by NS3-4A in PH5CH8 cells. However, the induction of IFN- β in PH5CH8 cells co-expressing Core and NS5B was only partially suppressed by NS3-4A, whereas the induction of IFN- β by NS5B only was completely suppressed by NS3-4A. As one of the biological implications of this phenomenon, we speculate that HCV proteins contribute to the maintenance of a low steady state of virus by controlling the expression level of IFN- β in the infected cells, thereby enabling HCV to escape from the host immuno-surveillance system, and facilitating persistent viral infection.

To evaluate this hypothesis, it is important to clarify whether or not the activation of the IFN system by Core and/or NS5B or suppression of the IFN system by NS3-4A occur during the HCV life cycle. Although four kinds of genome-length HCV RNA-replicating cells [39–42] and a reproducible HCV proliferation system in cell culture [43] have been established to date using HuH-7 cells, these HuH-7-based cells would not be suitable to prove our hypothesis, because the TLR3 and/or RIG-I signaling pathway does not function in these cells [38,44]. Therefore, a new HCV RNA-replicating or HCV proliferation cell system needs to be developed using other hepatocyte cell lines possessing intact TLR3 and RIG-I signaling pathways, such as PH5CH8 cells [20]. We are currently conducting a trial to establish genome-length HCV RNA-replicating PH5CH8 cells.

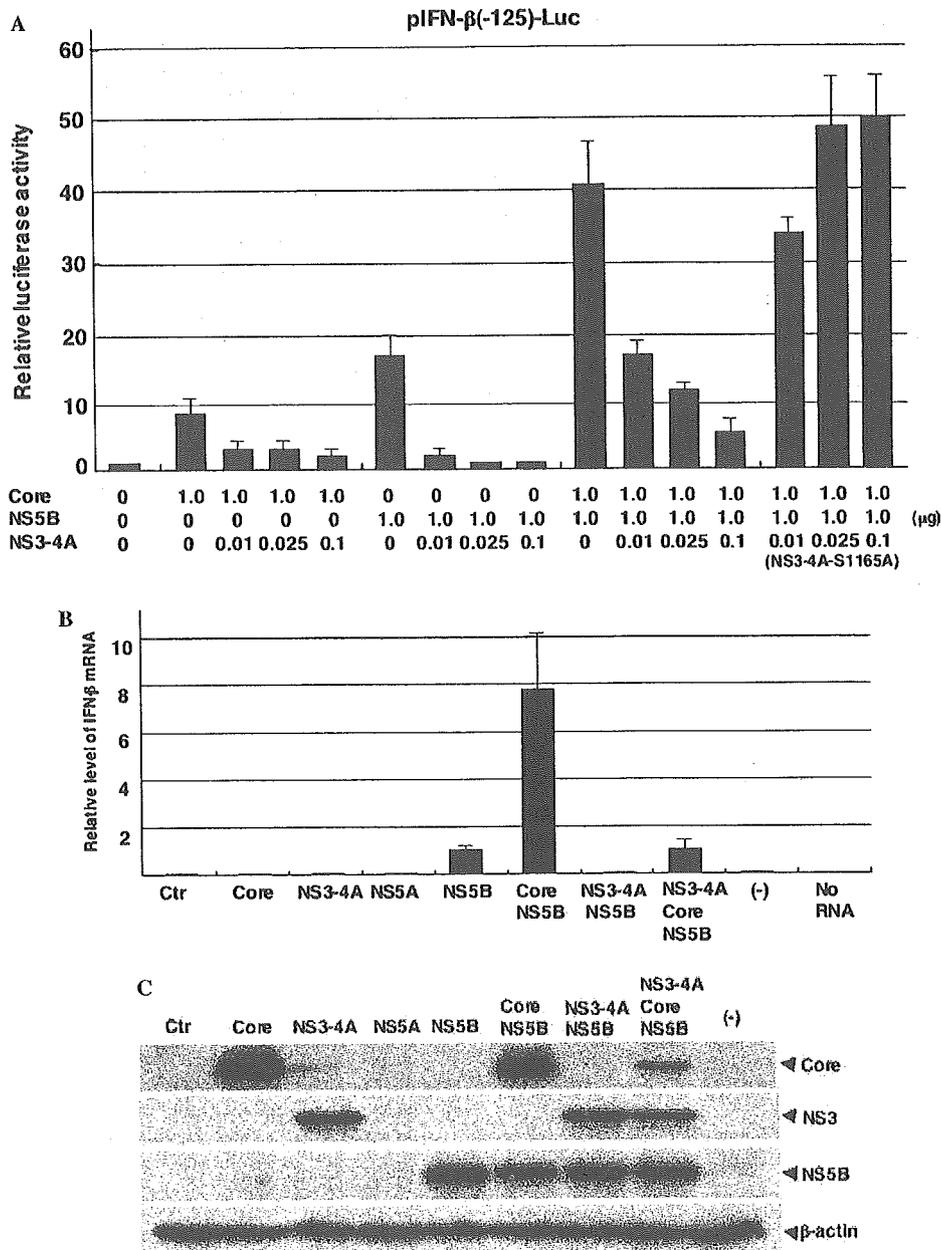


Fig. 7. NS3-4A did not suppress the synergistic effect of Core and NS5B on IFN- β gene expression. (A) Effect of NS3-4A on the activation of the IFN- β gene promoter by Core, by NS5B, and by the synergistic effect of Core and NS5B. The dual luciferase reporter assay was performed as described in Fig. 1A. The expression vector of the NS3-4A/S1165A mutant lacking serine protease activity was also used. (B) Real-time LightCycler PCR analysis of IFN- β in PH5CH8 cells stably expressing HCV protein(s). PH5CH8 cells were infected with retrovirus pCXbsr encoding various HCV proteins, and PH5CH8 cells stably expressing HCV protein(s) were obtained as described in Materials and methods. The pCXbsr retrovirus was used as a control infection (Ctr). Total RNA was extracted from the cells and was subjected to real-time LightCycler PCR analysis using the primer set for IFN- β (202 bp). The experiments were performed in at least triplicate. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of IFN- β mRNA concentration to that of GAPDH. The relative level of IFN- β mRNA calculated, when the level of IFN- β mRNA of PH5CH8 cells expressing NS5B alone was assigned to be 1.0, is presented here. (C) Western blot analysis of HCV proteins. Production of Core, NS3, and NS5B in PH5CH8 cells indicated in (B) was analyzed by immunoblotting using anti-Core, anti-NS3, and anti-NS5B antibodies, respectively. The PH5CH8 cells transfected with pCXbsr plasmid were used as control (Ctr). β -Actin was used as a control for the amount of protein loaded per lane.

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