

Establishment of hepatitis C virus replicon cell lines possessing interferon-resistant phenotype

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Abstract

To clarify the mechanism underlying resistance to interferon (IFN) by the hepatitis C virus (HCV) in patients with chronic hepatitis, we attempted to develop an IFN-resistant HCV replicon from the IFN-sensitive 50-1 replicon established previously. By treating 50-1 replicon cells with a prolonged low-dose treatment of IFN- α and then transfecting the total RNA derived from the IFN- α -treated replicon cells, we successfully obtained four clones (named 1, 3, 4, and 5) of HCV replicon cells that survived against IFN- α (200 IU/ml). These cloned cells were further treated with IFN- α or IFN- β (increased gradually to 2000 or 1000 IU/ml, respectively). This led to four replicon cell lines (α R series) possessing the IFN- α -resistant phenotype and four replicon cell lines (β R series) possessing the IFN- β -resistant phenotype. Furthermore, we obtained an additional replicon cell line (α Rmix) possessing the IFN- α -resistant phenotype by two rounds of prolonged treatment with IFN- α and RNA transfection as mentioned above. Characterization of these obtained HCV replicon cell lines revealed that the β R series were highly resistant to both IFN- α and IFN- β , although the α R series containing α Rmix were only partially resistant to both IFN- α and IFN- β . Genetic analysis of these HCV replicons found one common amino acid substitution in the NS4B and several additional amino acid substitutions in the NS5A of the β R series, suggesting that these genetic alterations are involved in the IFN resistance of these HCV replicons. These newly established HCV replicon cell lines possessing IFN-resistant phenotypes are the first useful tools for understanding the mechanisms by which HCV acquires IFN resistance in vivo.

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Persistent infection with the hepatitis C virus (HCV) is a major cause of chronic hepatitis (CH) [1,2], which progresses to liver cirrhosis (LC), and hepatocellular carcinoma (HCC) [3,4]. Since approximately 170 million individuals are estimated to be infected with HCV worldwide, this infection is a global health problem [5]. HCV belongs to the family *Flaviviridae*, whose gen-

ome consists of a positive-stranded 9.6 kilobase (kb) RNA and encodes a large polyprotein precursor of about 3000 amino acid residues [6,7]. This polyprotein is processed by a combination of the host and viral proteases into at least ten proteins: the core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. Six major HCV genotypes have been classified as HCV-1a, -1b, -2a, -2b, -3a, and -3b [8].

To prevent the progression to CH, LC, and HCC, it is essential to eliminate HCV immediately from the

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human body. Thus far, however, the only effective anti-HCV reagents used in current clinical therapy are interferon (IFN)- α and IFN- β . Moreover, IFN's effectiveness is limited to about 30% of the reported cases [9], although combined treatment of IFN and ribavirin has been found more effective (though still less than 50%) than treatment with IFN alone [10]. These clinical results suggest that HCV is rather resistant to the antiviral actions of IFN, and that HCV proteins directly or indirectly attenuate those actions [11].

Although many hypotheses have been proposed regarding the mechanisms of HCV's resistance to IFN [8,12], the lack of reproducible and efficient HCV proliferation in cell culture has been a serious obstacle to the clarification of such mechanisms [13].

In 1999, an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3–NS5B regions was first established using a human hepatoma cell line, Huh-7 [14]. Since then, several additional replicon systems, including ours (50-1 and 1B-2R1 replicons), have been established [15–20]. Recently, HCV replicons that autonomously replicate in human cervical carcinoma HeLa, human embryonic kidney 293, or mouse hepatoma cells have been introduced [21,22]. In these systems, replicated HCV RNAs and HCV proteins were detected by Northern and Western blot analyses, respectively. HCV replicon systems have become a powerful tool for basic studies of HCV, such as viral replication, virus–host interactions, and drug development [23]. Therefore, HCV replicon systems have been considered useful for clarifying the mechanisms underlying HCV's resistance to IFN.

However, unexpectedly, all HCV replicons established to date are found to be highly sensitive to IFN- α , IFN- β , and IFN- γ [19,24–27]. The mechanisms by which HCV replicons regulate the IFN-sensitive phenotype have not yet been clarified, although recent studies have proposed the involvement of proteasome subunits and ubiquitin-like proteins induced in replicon cells treated with IFN- α or IFN- γ [27,28]. The fact that HCV replicons are highly sensitive to IFNs seems to contradict the fact that more than 50% of patients with CH are resistant to current IFN therapy [10]. The elimination of this wide gap will contribute to the development of a method to eliminate HCV from the human body in vivo. Thus, we speculated that some stimuli might prompt IFN-sensitive HCV replicons to change into the IFN-resistant phenotype. According to this speculation, we attempted to develop IFN-resistant HCV replicons by a prolonged low-dose treatment of IFN against our established 50-1 replicon cells (termed 50-1 cells) [17].

Here, we report the successful establishment of HCV replicon cell lines possessing the IFN-resistant phenotype. We have also found several genetic alterations observed in only their HCV replicons.

Materials and methods

Cell cultures. Huh-7 and 50-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Our 50-1 cells were cultured in the presence of G418 (300 μ g/ml; Geneticin, Invitrogen). The 50-1 cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase (Neo) 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.

IFN treatment. For the initial treatment with IFN- α , 50-1 cells were plated onto six-well plates (1×10^5 cells/plate) and were cultured for one day immediately before IFN treatment. Human IFN- α (Sigma) was added to the cells at a final concentration of 1, 10, 100, or 1000 IU/ml, as described previously [19]. When the cells reached condition of confluence, they were passaged with several-fold dilutions. These cell cultures were continued for five months with the further addition of IFN- α at 5–6 day intervals. For further treatment with IFN- α , the replicon cells were plated onto 10 cm plates (1×10^6 cells/plate) and were cultured for one day immediately before IFN treatment. IFN- α was added to the cells at 4-day intervals, and the concentration of IFN- α was increased step by step to 400, 600, 800, 1000, and 2000 IU/ml. Human IFN- β (a gift from Toray Industries, Tokyo, Japan) was also added to the cells step by step at 4-day intervals, from concentrations of 400–600, 800, and 1000 IU/ml. The incubation was continued until apparent IFN-resistant colonies formed on the culture plates (in general, approximately one month). The analysis of the HCV replicon's sensitivity to IFN was performed as described previously [19]. Briefly, HCV replicon cells were plated in duplicate onto six-well plates (1×10^5 cells/plate) and were cultured for one day immediately before IFN treatment. IFN- α or IFN- β was added to the cells at a final concentration of 1, 10, 100, 500, 1000, or 2000 IU/ml, and incubation was continued. The cells were harvested 48 h after IFN treatment for the semi-quantitative analysis of HCV replicon RNA, or they were harvested five days after IFN treatment for the Western blot analysis of HCV proteins.

RNA transfection and selection of G418-resistant cells. RNA transfection into Huh-7 cells was performed by electroporation as described by Lohmann et al. [14]. Cells were selected in complete DMEM containing 300 μ g/ml G418 as described previously [19].

Northern blot analysis. Total RNAs from the cultured cells were prepared using the RNeasy extraction kit (Qiagen). Three micrograms of total RNA was used to detect the HCV replicon RNA and β -actin. Northern blotting and hybridization were performed as described previously [19,29]. 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 all performed as previously described [30]. The antibodies used to examine the expression levels of HCV proteins were those against NS3 (Novacastra Laboratories, UK), NS5A [30], and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science). Anti- β -actin antibody (AC-15, Sigma) was also used to detect β -actin as the internal control. To monitor the expression levels and phosphorylation status of the components involved in the IFN signal transduction pathway, HCV replicon cells were cultured for 30 min with or without IFN- α (500 IU/ml), and then cell lysates were used for immunoblotting analysis. Anti-JAK1, Tyk2, STAT1, STAT2, and STAT3 antibodies (BD Transduction Laboratories, Lexington, KY) were used to detect JAK1, Tyk2, STAT1, STAT2, and STAT3, respectively. Anti-*p*-JAK1(Tyr1022/1023) (Sigma), *p*-Tyk2(Tyr1054/1055), *p*-STAT1(Tyr701) (Cell Signaling Technology, Beverly, MA), *p*-STAT2(Tyr689) (Upstate Biotechnology, Lake Placid, NY), and *p*-STAT3(Tyr705) (Cell Signaling Technology) antibodies were used to

monitor the phosphorylation status of these proteins. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin–Elmer Life Sciences, Wellesley, MA).

Quantification of HCV replicon RNA. Total RNAs from the HCV replicon cells were prepared using the Isogen extraction kit (Nippon Gene, Toyama, Japan). Semi-quantitative analysis of HCV replicon RNA was performed by a previously described method [19,31]. Briefly, 0.5 µg of the RNA was used for reverse transcription (RT) with Superscript II (Invitrogen) using primer 319R. The synthesized cDNA was amplified by *Taq* DNA polymerase (Takara, Shiga, Japan) using primer set 319R and 196, resulting in a polymerase chain reaction (PCR) product of 266 bp containing the 5'-untranslated region (5'-UTR). In vitro synthesized positive-stranded HCV RNA containing the 5'-UTR (10^6 – 10^9 copies) was also subjected to RT-PCR as the standard in order to quantify the amount of replicon RNA. PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. The intensity of the band stained with ethidium bromide was quantified by a ChemiImager 4400 (Alpha Innotech, San Leandro, CA). The amount of HCV replicon RNA was estimated by comparing with the pattern of gradual amplification obtained by using in vitro synthesized HCV RNA containing the 5'-UTR, as shown previously [31]. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) was amplified by RT-PCR as described previously [32] and was used to standardize the level of HCV replicon RNA.

Dual luciferase assay. For the dual luciferase assay, we used the firefly luciferase reporter vector, pISRE(V2)-Luci [32], which contains five repeats of a 2'-5'-oligoadenylate synthetase (2'-5'-OAS)-type IFN-stimulated response element (ISRE). The assay was carried out as previously described [29]. After transfection of pISRE(V2)-Luci reporter plasmid and pRL-CMV (Promega) as an internal control reporter to the HCV replicon cells, the cells were cultured initially for 42 h and then again for an additional 6 h with or without IFN- α or IFN- β (500 IU/ml each). Triplicate transfection experiments were repeated in order to verify the reproducibility of the results. The relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). A manual Lumat LB 9501/16 luminometer (EG and G Berthold, Bad Wildbad, Germany) was used to detect luciferase activity.

Sequence analysis of HCV replicon RNA. Sequence analysis of HCV replicons was performed as previously described [19]. Briefly, to amplify HCV replicon RNA, RT-PCR using proofreading KOD-plus DNA polymerase (Toyobo, Japan) 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 2033 bp for the former part and 6107 bp for the latter part. The PCR products were subcloned into pBR322MC [17] as previously described [19] and plasmid inserts were sequenced in both the sense and antisense directions using Big Dye terminator cycle sequencing on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Cyclosporin A treatment. To prepare cured cells from which HCV replicons were eliminated, HCV replicon cells (1×10^6) were plated onto 10 cm plates and were cultured for one day immediately before cyclosporin A treatment. Cyclosporin A (Sigma) was added to the cells at a final concentration of 1 µg/ml, and incubation was continued in the absence of G418 for eight days as previously described [33].

Results

Isolation of HCV replicon cell lines possessing IFN-resistant phenotype

To clarify the molecular mechanisms of IFN resistance in patients with CH C and to develop a novel tool for antiviral therapy against persistent infection with HCV,

we attempted to establish an IFN-resistant HCV replicon. In the first strategy to isolate an IFN-resistant HCV replicon (Fig. 1A), 50-1 cells were treated with several doses of IFN- α (final concentration 1, 10, 100, or 1000 IU/ml) as described in Materials and methods. This IFN treatment of the cells was continued for five months in the presence of G418. In the treatment using 1000 IU/ml of IFN- α , all cells were dead after the eighth IFN- α treatment. Contrary to this phenomenon, when the cells were treated with 1 or 10 IU/ml of IFN- α , most of the cells proliferated and the passage of cells was also easy. However, cells treated with 100 IU/ml of IFN- α survived in limited numbers and proliferated slowly as G418-resistant cells, suggesting that small portions of 50-1 replicon cell populations possess the IFN-resistant phenotype or become IFN-resistant during the IFN- α treatment. After five months of treatment with 100 IU/ml of IFN- α , the survived cells were transiently proliferated without IFN- α , and then the total RNA extracted from the cells was transfected into Huh-7 cells by electroporation. After selection with G418 for three weeks, a number of G418-resistant colonies were obtained and mixed (IFNR1 replicon cells). The IFNR1 replicon cells were then divided into two groups (Fig. 1A). The first group was treated with 200 and 400 IU/ml of IFN- α for one month. Although the cells treated with 400 IU/ml of IFN- α were completely dead, four colonies (termed 1, 3, 4, and 5) appeared as IFN- α (200 IU/ml)-resistant cells. The second group was treated with 100 IU/ml of IFN- α for one month, after which total RNA extracted from the IFN-treated cells was transfected again into Huh-7 cells by electroporation. As a consequence, a number of G418-resistant colonies were obtained and mixed (IFNR2 replicon cells). These obtained replicon cells (clones 1, 3, 4, and 5 and IFNR2) were treated again with IFN- α or IFN- β (gradually increased to 2000 or 1000 IU/ml, respectively). Regarding the four cloned cell lines treated with IFN- α , a number of colonies possessing the phenotype resistant to 2000 IU/ml of IFN- α were obtained and termed 1 α R, 3 α R, 4 α R, and 5 α R, respectively (Fig. 1B). The four lines of cloned cells treated with IFN- β also yielded many distinct colonies possessing the phenotype resistant to 1000 IU/ml of IFN- β ; these colonies were termed 1 β R, 3 β R, 4 β R, and 5 β R, respectively (Fig. 1B). Interestingly, there were fewer IFN- β -resistant colonies than IFN- α -resistant ones. Especially remarkable differences were observed by IFN treatment to the cloned cell lines, 4 and 5 (Fig. 1B), suggesting qualitative differences among these IFN-resistant colonies obtained from the four cloned cell lines. In addition, a number of colonies possessing the phenotype resistant to 2000 IU/ml of IFN- α were also obtained from IFNR2 replicon cells treated with IFN- α . These colonies were mixed and termed α Rmix (Fig. 1B). However, none of the IFNR2 replicon cells survived treatment with 400 IU/ml of IFN- β (Fig. 1B). In summary, we obtained four replicon cell lines (α R series) plus an α Rmix

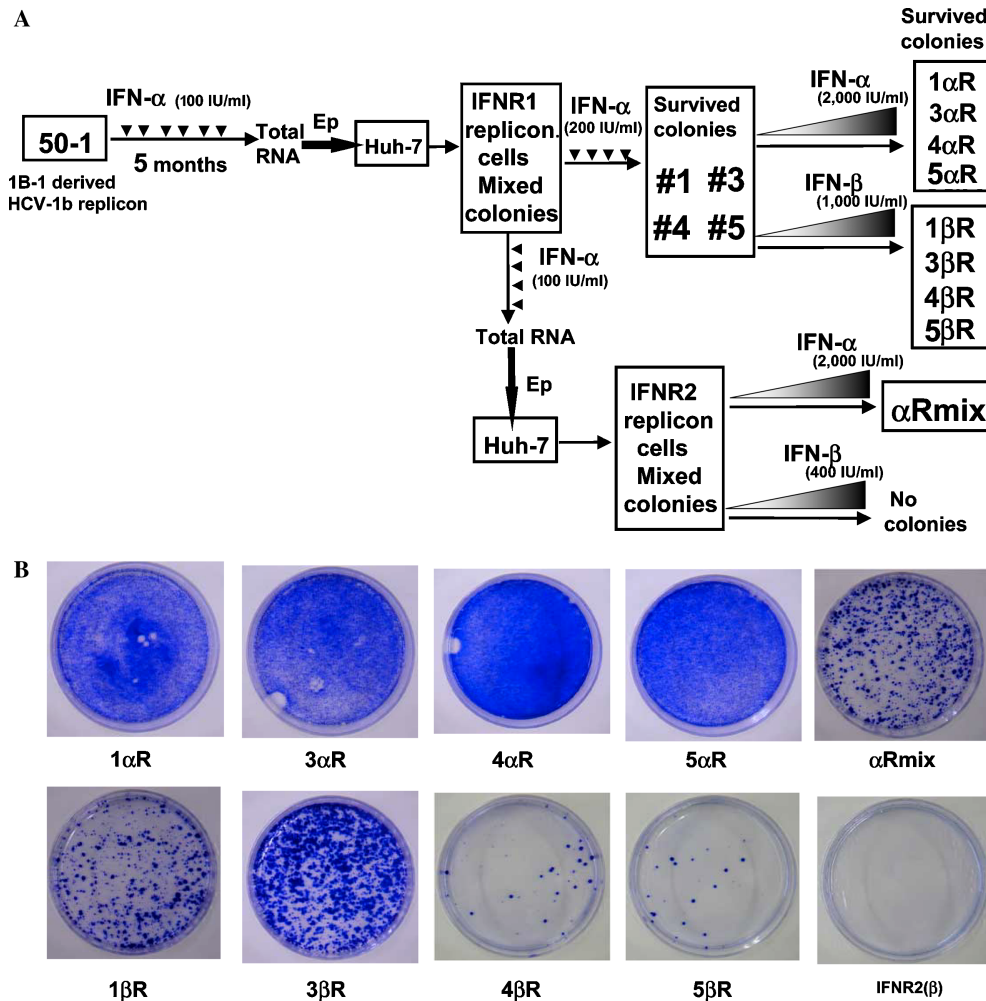


Fig. 1. Isolation of HCV replicon cell lines possessing IFN-resistant phenotype. (A) Outline of the isolation of HCV replicon cells possessing IFN-resistant phenotype. Ep indicates electroporation of total cellular RNA to Huh-7 cells. (B) HCV replicon cells possessing resistance against IFN- α (2000 IU/ml) and IFN- β (1000 IU/ml). A culture dish of each isolated cell line was stained with Coomassie brilliant blue as described previously [42]. IFNR2(β) indicates that no colonies have been obtained from IFNR2 replicon cells by the treatment with IFN- β (400 IU/ml).

cell line possessing the IFN- α -resistant phenotype, and four replicon cell lines (β R series) possessing the IFN- β -resistant phenotype.

Since it has been known that the replication efficiency of an HCV replicon depends on cell proliferation [34], the possibility remains that only colonies with a growth-rate advantage were able to survive IFN treatment. To evaluate this possibility, we compared the growth rates of parental 50-1 and the nine replicon cell lines that possessed the IFN-resistant phenotype. However, no significant differences in cell growth rates were observed between 50-1 cells and the replicon cell lines (data not shown).

Characterization of HCV replicon cell lines possessing IFN-resistant phenotype

The levels of replicon RNAs and HCV proteins in the nine obtained replicon cell lines were examined by

Northern and Western blot analyses, respectively. Replicon RNAs approximately 8 kb long were detected in all specimens except those from the cured cells, from which the replicons had been eliminated from the replicon cells by the treatment with IFN- α (Fig. 2A). The number of copies of replicon RNAs in total RNAs (each 2 μ g) extracted from these replicon cells was estimated at approximately 10^8 (less than 10^8 in 1 α R cells) by comparing these replicon RNAs with replicon RNA synthesized *in vitro* from replicon cassette plasmid pNSS1RZ2RU [19] (data not shown). The NS3, NS5A, and NS5B proteins were also detected in all specimens except those from the cured cells (Fig. 2B). The expression levels of replicon RNAs and HCV proteins differed somewhat among these nine replicon cell lines, and no strong quantitative relationship between replicon RNA and HCV proteins was observed (Fig. 2). These results suggest that the stability of replicon RNA or HCV proteins produced from the replicon RNA, or

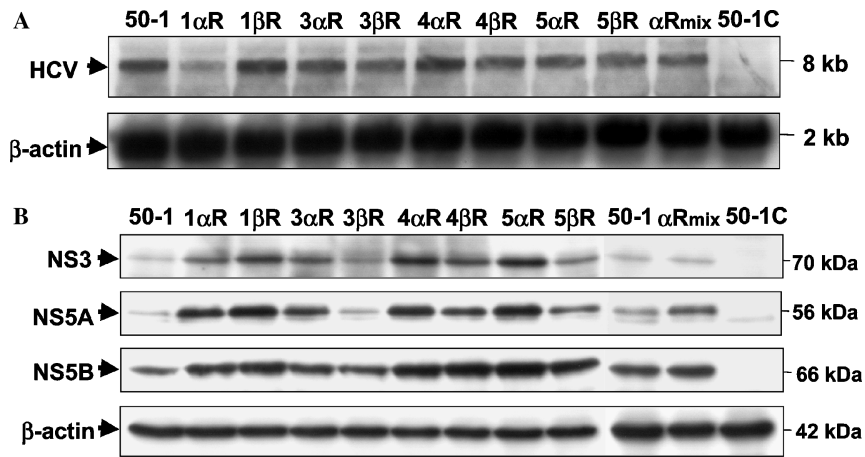


Fig. 2. Characterization of replicon cells possessing IFN-resistant phenotype. (A) Northern blot analysis. Total RNAs from 50-1 and nine replicon cell lines possessing IFN-resistant phenotype, as well as total RNA from 50-1C cells (cured cells), were analyzed by Northern blot analysis using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β -actin-specific RNA probe (lower panel). (B) Western blot analysis. Productions of NS3, NS5A, and NS5B in 50-1 and nine replicon cell lines possessing IFN-resistant phenotype were analyzed by immunoblotting using anti-NS3, anti-NS5A, and anti-NS5B antibodies, respectively. 50-1C cells were also analyzed as a negative control for NS3, NS5A, and NS5B. β -Actin was used as a control for the amount of protein loaded per lane.

the efficiency of translation, differs among these nine replicon cell lines. A similar phenomenon has been observed in other replicon cells [35]. In summary, we showed that the replication efficiencies of nine replicon cell lines possessing the IFN-resistant phenotype were highly maintained.

Two IFN-resistant phenotypes of the established HCV replicon cell lines

To assess the degree of IFN resistance among these newly established HCV replicons, we examined the levels of replicon RNA and NS5B protein in the cells (50-1 and each of the nine replicon cell lines established) treated with IFN- α or IFN- β (500 IU/ml each) by semi-quantitative RT-PCR analysis [19] and Western blot

analysis, respectively. Both analyses revealed that replicon RNA and NS5B were drastically decreased in 50-1 cells at two days (replicon RNA) and five days (NS5B) after treatment with IFN- α or IFN- β (Fig. 3). This indicated that 50-1 replicon was highly sensitive to IFNs as described previously [19]. However, five replicons (1 α R, 3 α R, 4 α R, 5 α R, and α Rmix) showed somewhat resistant phenotypes, especially against IFN- α . The levels of these replicon RNAs in the cells at two days after IFN- α treatment were maintained at about 15–40% of the levels in the untreated cells, whereas the level of 50-1 replicon RNA decreased to less than 10% that of the untreated cells (Fig. 3). This IFN resistance was confirmed by Western blot analysis (Fig. 3). These results indicate that the α R series (1 α R, 3 α R, 4 α R, and 5 α R) and α Rmix possessed partial IFN- α resistant phenotypes. Although

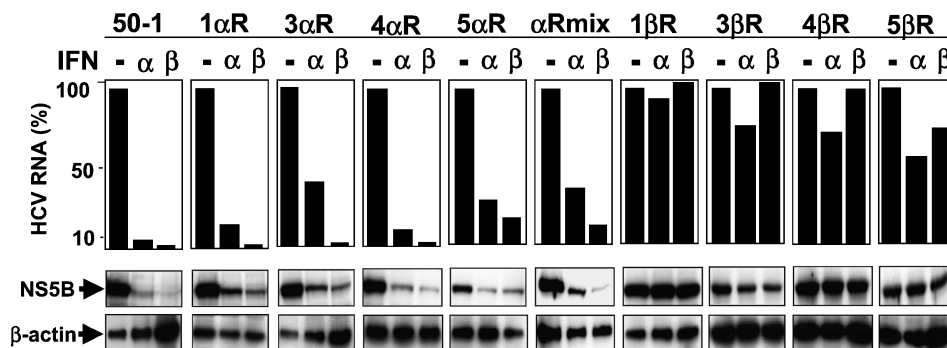


Fig. 3. IFN-resistant phenotypes of the established replicon cell lines. 50-1 and nine replicon cell lines possessing IFN-resistant phenotype were treated with IFN- α or IFN- β (500 IU/ml each) for two days for semi-quantitative RT-PCR analysis (upper panel) and for five days for Western blot analysis (middle panel for NS5B and lower panel for β -actin). Semi-quantitative RT-PCR was carried out to monitor the levels of replicon RNAs in the cells, as described in Materials and methods. The data, obtained from duplicate assays, were averaged for the presentation (upper panel). NS5B was detected by immunoblot analysis using anti-NS5B antibodies (middle panel). β -Actin was used as a control for the amount of protein loaded per lane (lower panel).

the IFN- β resistance of these replicons was also suggested, the differences between these replicons and the 50-1 replicon were not so clear (Fig. 3). In contrast to the α R series and α Rmix, the β R series (1 β R, 3 β R, 4 β R, and 5 β R) showed almost complete resistance to both IFN- α and IFN- β (Fig. 3). Interestingly, the levels of replicon RNAs in 1 β R, 3 β R, and 4 β R cells, though not in 5 β R cells, were barely reduced, in spite of the treatment with IFN- β , although the levels in these cells were somewhat reduced by the treatment with IFN- α (Fig. 3). This IFN resistance was confirmed by Western blot analysis (Fig. 3). These results indicate that the β R series possesses phenotypes with severe resistance to both IFN- α and IFN- β .

1 α R possesses a partially resistant phenotype against both IFN- α and IFN- β

To clarify whether or not the α R series obtained by treatment with IFN- α alone showed the IFN- β -resistant phenotype, we compared in detail the IFN sensitivities of 1 α R with 50-1 and 1 β R. The 50-1, 1 α R, and 1 β R cells were treated for two days with IFN- α and IFN- β (1, 10, 100, 500, 1000, and 2000 IU/ml each), and then the levels of replicon RNAs in the treated cells were examined by semi-quantitative RT-PCR analysis [19]. The IFN-sensitive phenotype of 50-1 and the IFN-resistant phenotype of 1 β R were clearly reconfirmed, because the level of replicon RNA in 50-1 cells treated with only 1 IU/ml of IFN- α or IFN- β was decreased to less than 15% that of the untreated cells, and the level of replicon RNA in 1 β R cells treated with 2000 IU/ml of IFN- α or IFN- β was the same as that of the untreated cells (Fig. 4). However, the responsiveness of 1 α R against IFN- α or IFN- β treatment was in between that of 50-1 and that of 1 β R (Fig. 4). This revealed that 1 α R possesses a partially resistant phenotype against both IFN- α and IFN- β . This finding suggests that the other four replicon cell lines (3 α R, 4 α R, 5 α R, and α Rmix) also possess the

partially resistant phenotype against both IFN- α and IFN- β .

Repression of IFN signal transduction pathway in established HCV replicon cell lines

To examine whether or not the IFN signal is transduced in the HCV replicon cells possessing the IFN-resistant phenotype, we carried out a luciferase reporter assay using synthetic promoters possessing five repeats of a 2'-5'-OAS-type ISRE [32]. The results revealed that the luciferase activities were remarkably enhanced by the treatment with IFN- α or IFN- β in the cells of the α R series as well as in the 50-1 cells. Meanwhile, these enhancements were remarkably lower in 5 α R and α Rmix cells than in 50-1 cells. These results suggest that both IFN- α and IFN- β are effectively transduced in the α R series cells (Fig. 5). However, the luciferase activities in the β R series cells, except for 3 β R cells, were barely enhanced in spite of the treatment with IFN- α and IFN- β , suggesting that the IFN signaling pathway is completely repressed in 1 β R, 4 β R, and 5 β R cells but not in 3 β R cells (Fig. 5). Although this reporter assay clarified the reason why 1 β R, 4 β R, and 5 β R cells possessed the IFN-resistant phenotype, the reason for IFN resistance among the other replicons remained unclear. Since the luciferase activities in 5 α R and α Rmix cells were lower than that in 50-1 cells, we next evaluated the possibility that the IFN signaling pathway in the replicon cells possessing the IFN resistance phenotype becomes weaker than that in 50-1 cells by exposure to IFN- α . To accomplish this, we examined the phosphorylation status of the components (JAK1, Tyk2, STAT1, and STAT2) of the JAK-STAT signaling transduction pathway in these replicon cells after treatment with IFN- α . Since it has been reported that STAT3 is also activated by IFN- α treatment [36] phosphorylation status of STAT3 in these replicon cells after treatment with IFN- α was also examined. The results revealed signifi-

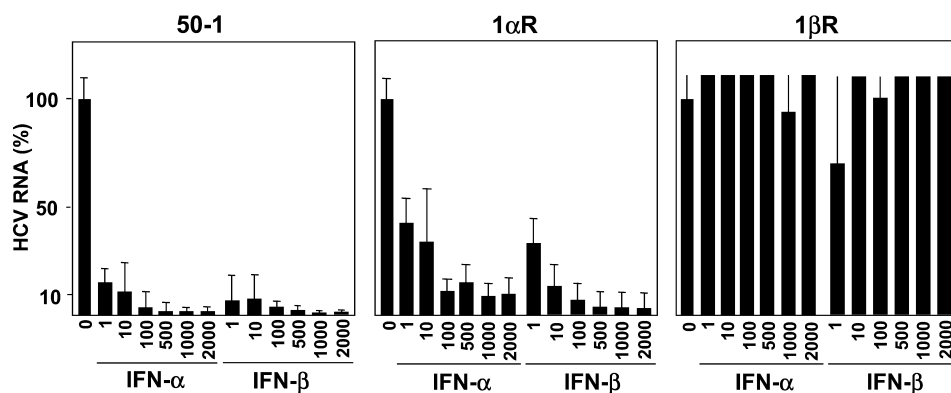


Fig. 4. IFN sensitivities of the replicons in 50-1, 1 α R, and 1 β R cells. Cells from each of these lines were treated with IFN- α or IFN- β (1, 10, 100, 500, 1000, and 2000 IU/ml each) for two days. Semi-quantitative RT-PCR was carried out to monitor the levels of replicon RNAs in the cells, as described under Materials and methods. The data, obtained in at least triplicate assays, were averaged for the presentation.

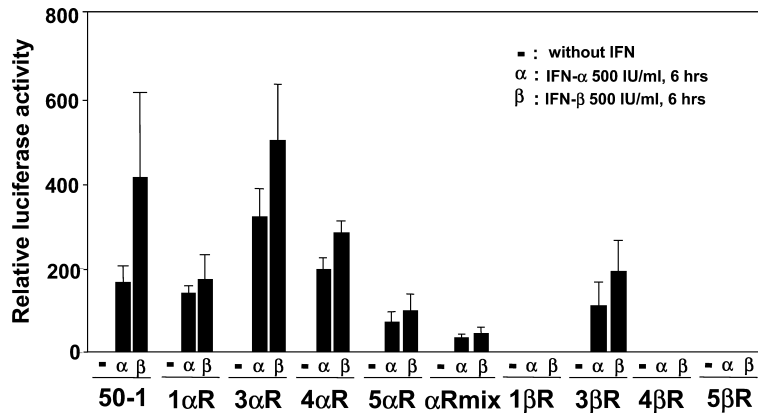


Fig. 5. IFN signal transduction in the established replicon cell lines. Regarding 50-1 and the nine replicon cell lines possessing IFN-resistant phenotype, dual luciferase reporter assay using pISRE(V2)-Luci [32] was performed as described previously [29]. The replicon cells were treated with IFN- α or IFN- β (500 IU/ml each) for 6 h.

cantly lower levels of phosphorylation of JAK1, Tyk2, STAT1, and STAT2 in the cells of the α R series and 3 β R cells after IFN- α treatment than in 50-1 cells, and that phosphorylation of these proteins was barely observed in 1 β R, 4 β R, and 5 β R cells in spite of the IFN- α treatment (Fig. 6). The results for the α R series cells are consistent with their partially IFN-resistant phenotype (Figs. 3 and 4), although the IFN-resistant phenotype of 3 β R is not simply explained. We concluded that the nine HCV replicon cell lines established in this study could be divided into two phenotypes: a partially IFN-resistant phenotype (four cell lines of the α R series plus the α Rmix cell line) and a completely IFN-resistant phenotype (four cell lines of the β R series).

Genetic analysis of the newly established HCV replicons and their comparison with 50-1 replicon

In order to examine whether or not genetic mutations on replicon RNA confer the mutated replicons with the IFN-resistant phenotype, we carried out a genetic analysis of all HCV replicons established in this study. Two separate RNA fragments (one was 2.0 kb in length, containing the 5'-UTR to the amino-terminal of the NS3 region; the other was 6.1 kb in length, containing the NS3–NS5B regions) were amplified by RT-PCR, and three independent clones of each were sequenced after subcloning into pBR322MC, as described previously [19]. The determined nucleotide

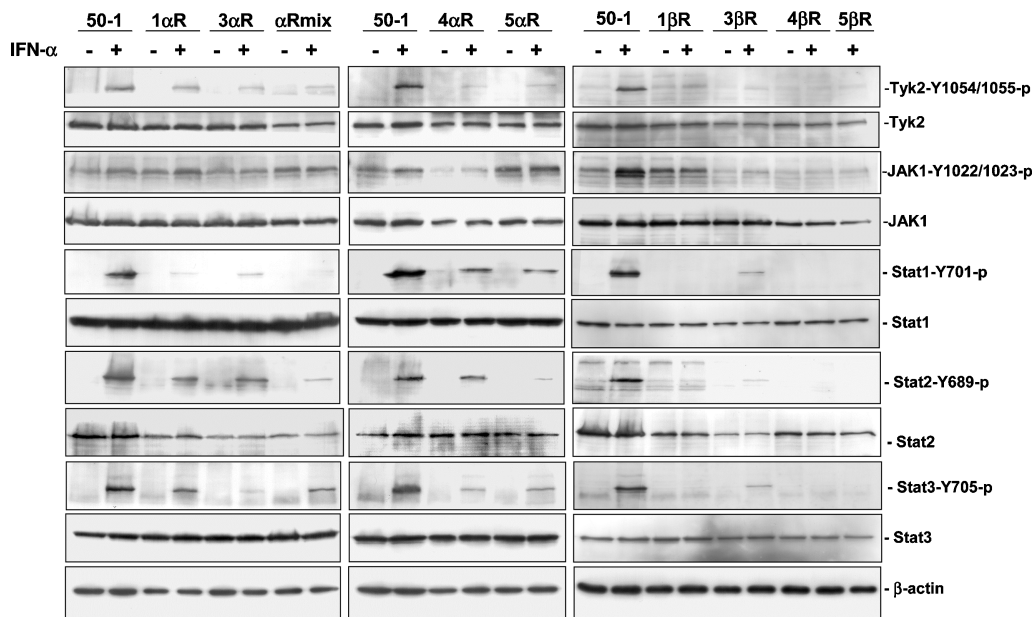


Fig. 6. Western blot analysis of the components involved in the IFN signal transduction pathway in the established replicon cell lines treated with IFN- α . The replicon cells were stimulated with or without IFN- α (500 IU/ml) for 30 min, and then Western blot analysis was performed as described under Materials and methods.

sequences were compared with those of the original 50-1 replicon and with 50-1 replicons after 6 and 12 months in cell culture.

Regarding the first 2.0 kb fragment of the replicon RNA, none of the common mutations were found among any of the replicons obtained from the cells possessing the IFN-resistant phenotype (data not shown). No α R-series-specific or β R-series-specific mutations were found either, although several sporadic mutations or deletions were observed in the nonfunctional region upstream of the encephalomyocarditis virus internal ribosome entry site or in the Neo^R region.

Contrary to the first 2 kb fragment of replicon RNA, in the NS region (6.1 kb) we found that at position 5552 (the nucleotide number in the HCV genotype 1b genome), uridine was commonly exchanged for adenosine among all replicons obtained from the cells possessing the IFN-resistant phenotype. This mutation results in the substitution of histidine (H) for glutamine (Q) at amino acid position 1737 in the NS4B (Q1737H in Fig. 7). Furthermore, several amino acid substitutions were found in NS5A (M2174V for 1 β R; T2319A and N for 3 β R; and T2242N and F2256L for 4 β R) and NS5B (A2752V for 3 β R) in the β R series only, although no other common amino acid substitutions were found in the β R series. The amino acid substitutions we found did not appear in long-term culture (to at least 12 months) of 50-1 cells. These results suggest that the amino acid substitutions found may contribute to the acquisition of the IFN-resistant phenotype. In addition, although four amino acid substitutions (P1115L, K1609E, V1896F, and E1966A) were observed in the α R series, α Rmix, β R series, and 50-1 replicon after six months in cell culture, it is interesting to note that

two additional amino acid substitutions (I1686V and L1701R) found in the 50-1 replicon after six months in cell culture were barely detected in the α R series, α Rmix, or β R series (Fig. 7).

Characterization of cured cells obtained from HCV replicon cells possessing the IFN-resistant phenotype

To further examine whether or not IFN resistance depends on the presence of IFN-selected HCV replicon RNAs, we prepared cured cells from established HCV replicon cells by treatment with cyclosporin A, which was recently found to be a potent inhibitor of HCV replication [33]. 1 α R and 3 α R cells possessing a partially IFN-resistant phenotype, and 1 β R and 3 β R cells possessing a completely IFN-resistant phenotype, were treated with cyclosporin A as described under Materials and methods. As shown in Fig. 8A, we demonstrated by Western blot analysis that NS5B proteins were no longer detected in 1 α R, 1 β R, 3 α R, or 3 β R cells after eight days of cyclosporin A treatment. We further confirmed by RT-PCR [19] for the detection of 5'-UTR that replicon RNAs were excluded from the cells (data not shown). Using these cured cells and their parental cells, we examined whether or not the cured cells' IFN responses were altered after elimination of the replicon RNAs. The results of the luciferase reporter assay shown in Fig. 5 revealed that IFN responses were not remarkably changed in the cured cells (Fig. 8B). Although both IFN- α and IFN- β were still transduced in 1 α R, 3 α R, and 3 β R cells, IFN response was not restored in the cured cells obtained from 1 β R cells. This result suggests that some host factor(s) rather than replicon RNA(s) contributed to the

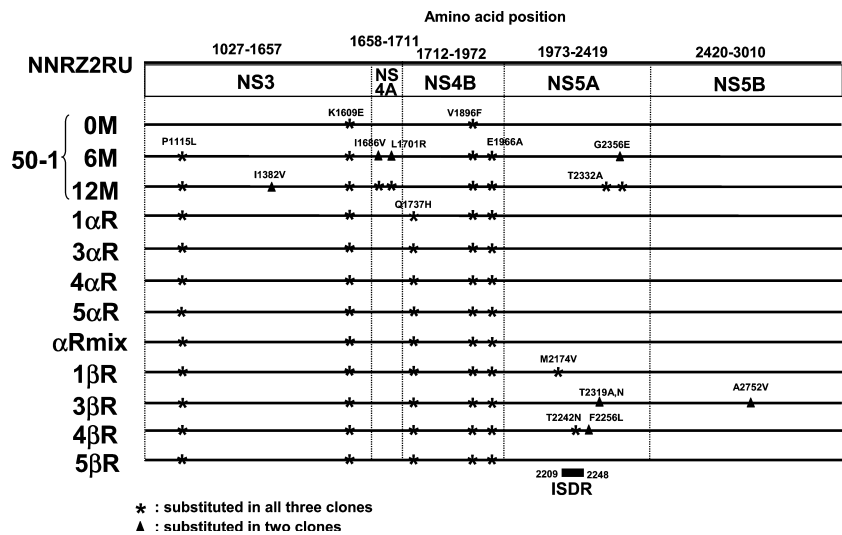


Fig. 7. Genetic analysis of the NS region of replicon RNAs in the established replicon cell lines possessing IFN-resistant phenotype. Compared with the amino acid sequences of NS region of the original replicon (NNRZ2RU), amino acid positions substituted in all three clones and in two of three clones are indicated by asterisk and triangle, respectively. The results of genetic analysis of parental 50-1 replicon (0 M), and 50-1 replicons (6 and 12 M) after 6 and 12 months in culture are presented for comparison.

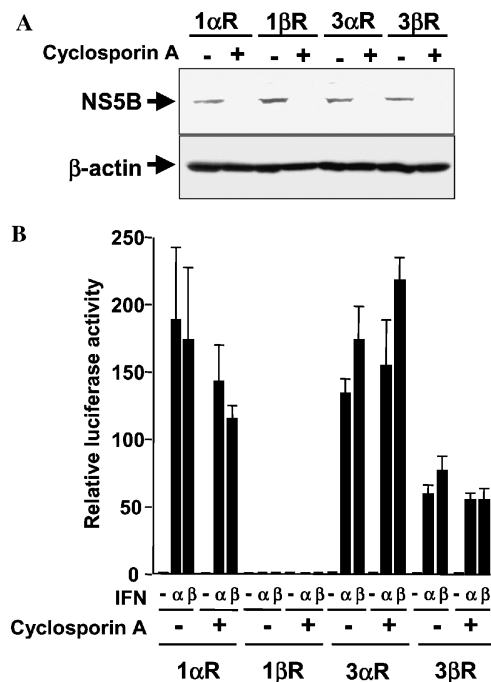


Fig. 8. Characterization of cured cells obtained from 1αR, 1βR, 3αR, and 3βR cells. (A) Western blot analysis. Regarding the four replicon cell lines possessing the IFN-resistant phenotype and their cured cell lines, NS5B was detected by immunoblot analysis using anti-NS5B antibodies. β-Actin was used as a control for the amount of protein loaded per lane. (B) Analysis for IFN signal transduction. Regarding the four replicon cell lines possessing the IFN-resistant phenotype and their cured cell lines, a dual luciferase reporter assay using pISRE(V2)-Luci [32] was performed as shown in Fig. 5.

IFN-resistant phenotype in at least the 1βR cells, although further experiments are needed to obtain conclusive results.

Discussion

In this study, we first established nine replicon cell lines possessing the IFN-resistant phenotype from 50-1 cells possessing an IFN-sensitive phenotype. Interestingly, we were able to divide these nine replicon cell lines into two types according to their IFN resistance. The first type included four cell lines of the αR series plus the αRmix cell line; treated with IFN-α alone, these lines showed a partially resistant phenotype against both IFN-α and IFN-β. The second type included four cell lines of the βR series treated with IFN-α and IFN-β; these lines showed a severely resistant phenotype against both IFN-α and IFN-β. Therefore, these findings suggest that these two IFN-resistant phenotypes were caused by different mechanisms. To clarify these mechanisms, it will be important to determine which viral and cellular factors contribute to the acquisition of IFN resistance of the replicons.

To identify such viral factors, genetic analysis found that all of these newly established replicons had one

common amino acid substitution (Q1737H) in the NS4B, and several amino acid substitutions were found in the NS5A and NS5B of the βR series. Since these amino acid substitutions did not appear during the long-term culture (at least 12 months) of 50-1 cells, the genetic alterations observed in the replicons established in this study are considered to have appeared during the prolonged IFN treatment, and may induce the replicons' IFN resistance. NS4B possesses four fixed and one flexible transmembrane (TM) structures, located on the endoplasmic reticulum [37]. Since it has been proposed that the amino-terminal region of the first TM may play an important role not only for the topology of NS4B but also for the efficiency of HCV replication [37], the Q1737H substitution in this region may affect the function of NS4B and contribute to the acquisition of IFN resistance. However, even if this hypothesis were correct, additional factors would be necessary to acquire severe IFN resistance, because the IFN resistance of the αR series possessing the Q1737H mutation was weaker than that of the βR series possessing that mutation. Such factors that might be involved in the acquisition of severe IFN resistance are the additional cell-line-specific amino acid substitutions observed in NS5A and NS5B of the βR series. Although such cell-line-specific amino acid substitutions were not found in 5βR (Fig. 7), three clones of 5βR each possessed S1269Y, K1270R, and R1135K substitutions in NS3, which were not observed in any of the three clones of 5αR. Such amino acid substitutions may contribute to the acquisition of IFN resistance. To clarify these points, further analysis, such as the characterization of HCV replicon cells re-established by the transfection of these HCV replicon RNAs to Huh-7 cells, will be necessary.

To date, the IFN sensitivity-determining region (ISDR; amino acids 2209–2248 in the HCV-1b genotype), in which substitutions correlate well with IFN sensitivity in patients with CH, has been known as a good prediction factor for current IFN therapy [38,39]. Contrary to this phenomenon, all HCV replicons established thus far show high sensitivity to IFNs via unknown mechanisms. Nevertheless, most HCV replicons, including the 50-1 replicon, possess the IFN-resistant type of ISDR sequence, according to Enomoto's criteria. Interestingly, ISDR sequences of all HCV replicons except 4βR were barely altered, suggesting that unknown factors other than ISDR can regulate the IFN sensitivity in an HCV replicon system. Since it has been thought that NS5A blocks a signal of IFNs by interacting with PKR, a double-strand RNA-dependent protein kinase [40,41], amino acid substitutions in the NS5A protein found in the βR series may exert the function of PKR.

Although several genetic mutations were observed in the HCV replicons established in this study, the possibility is also considered that some cellular factors,

either alone or in combination with viral factors, contributed to the acquisition of IFN resistance. In an experiment to explore this possibility, we examined the IFN responses of cured cells from which replicon RNAs were eliminated by cyclosporin A. The obtained data suggested that some cellular factor(s) determined the IFN-resistant phenotype of at least the 1 β R cells. It is considered that one reason why we have obtained HCV replicon cells that are deficient in IFN signaling (such as the 1 β R cells) is their spontaneous appearance and their selection during prolonged IFN treatment. However, we are not able to exclude the possibility that persistent HCV replication induces some irreversible genetic mutations, which result in deficient IFN signaling, because it was recently reported that HCV replication induces a mutator phenotype that involves enhanced mutations of many somatic genes [43]. Therefore, it is important to evaluate these possibilities in future studies. Although the mechanism underlying the acquisition of IFN resistance is still ambiguous in the present study, our newly established HCV replicon cell lines possessing the IFN-resistant phenotype will be a very useful tool to further our understanding of molecular mechanisms for IFN resistance by HCV. Moreover, these replicon cells may be useful in the evaluation of combination therapies, such as IFN plus ribavirin.

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