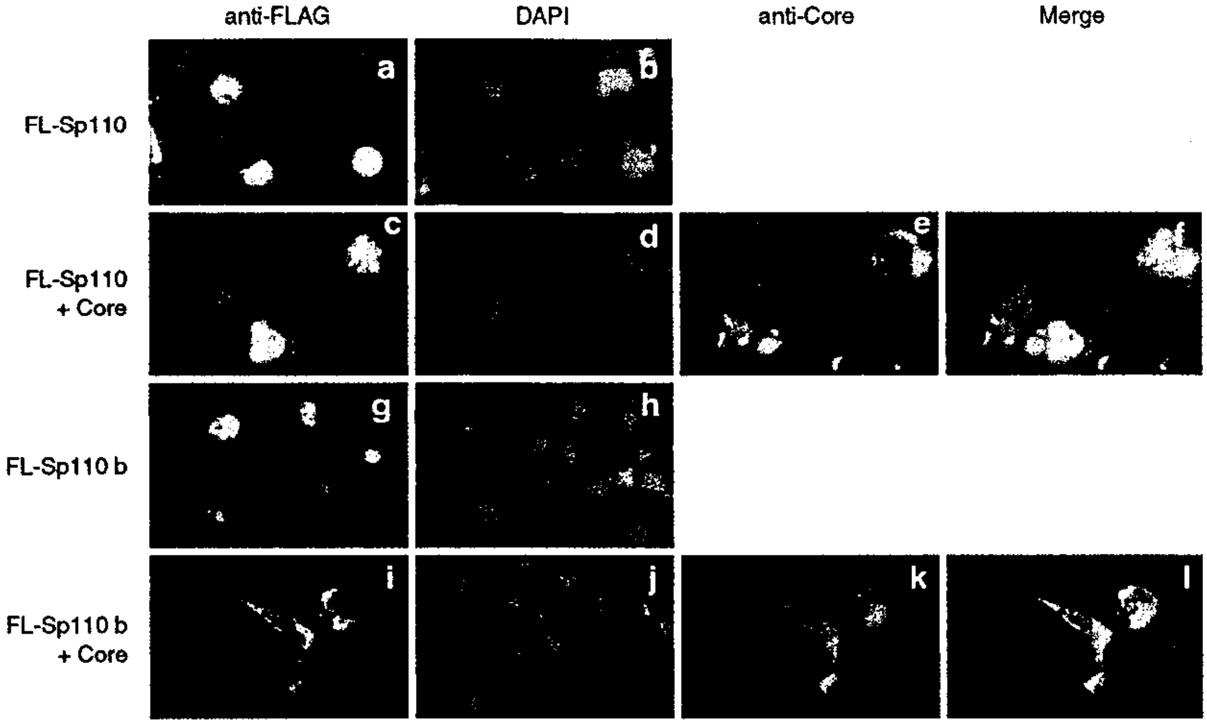

Fig. 2. Expression of the core changes the subcellular localization of Sp110b, but not Sp110 from the nucleus to the cytoplasm. Cells expressing FL-Sp110 (a–f) and FL-Sp110b (g–l) together with or without the core were examined to determine the subcellular localization by using antibodies against FLAG and core proteins. Nuclei were stained with DAPI. FL-Sp110 and FL-Sp110b, Flag-tagged Sp110 and Sp110b, respectively.

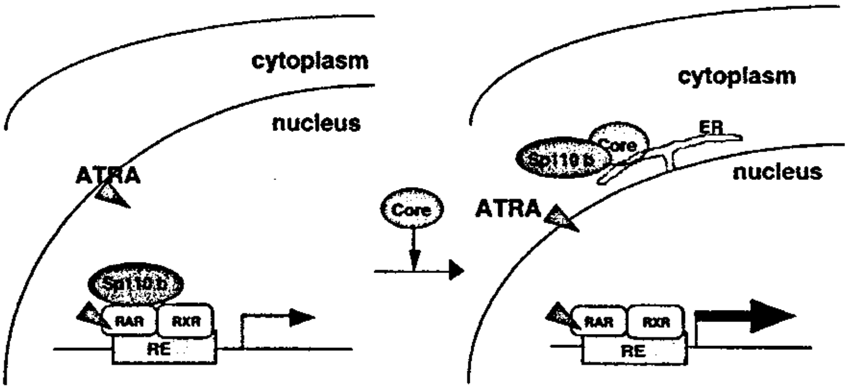

Fig. 3. Model of sequestration of the function of Sp110b by the core. The core protein associates with the transcriptional co-repressor, Sp110b, changing its subcellular localization from the nucleus to the cytoplasm. Thus, nuclear hormone-dependent transcription is enhanced by the expression of the core (shown by the thick arrow in the right). The mechanism underlying the transfer of Sp110b from nucleus to cytoplasm, however, remains to be clarified.

calization of Sp110b. From these results, the core likely plays an important role in regulating nuclear hormone receptor-mediated transcription through interactions with the transcriptional suppressor, Sp110b. In this model (Fig. 3), the core does not localize to the nucleus in order to regulate the transcription occurring in the nucleus. As transcriptional co-activators or co-repressors can often regulate the gene expression of multiple factors, the effects on various genes whose expression is thought to be modulated by the core may be explained by similar mechanisms. Furthermore, because Sp110b contains the motif, LXXLL, necessary for the interaction with additional nuclear hormone receptors such as RXR, Sp110b may also regulate RXR-dependent transcription. The various genes regulated by homo- and hetero-dimeric nuclear hormone receptors, such as RXR/RXR, RAR/RXR, and RXR/PPAR, may also be targeted by the core (Fig. 4). These nuclear hormone receptors regulate gene expression for several lipid metabolism-related proteins. Thus, it seems possible that this mechanism can explain the dysregulation of lipid metabolism often observed in patients with chronic hepatitis and in mice transgenic for the core. In addition, we confirmed upregulation of TGF-βII, downstream of RAR/RXR, by core expression. Since TGF-βII is responsible for gene expression of collagen, which may function in fibrosis,[40] we speculate that this mechanism contributes to the fibrosis that is frequently observed in patients with HCV infection.

Prospects

Recent studies have uncovered genetic and epigenetic changes in HCC related to HCV infection. The molecular mechanisms underlying hepatitis C-related hepatocarcinogenesis are still not clear. The quantity of HCV genomic DNA detected in cancerous HCC tissues is less than 0.1 copy per cell; thus, HCV genomic expression does not appear to function in the maintenance of the tumor state, unless it functions through a bystander effect. HCV infection, thus, may mainly contribute

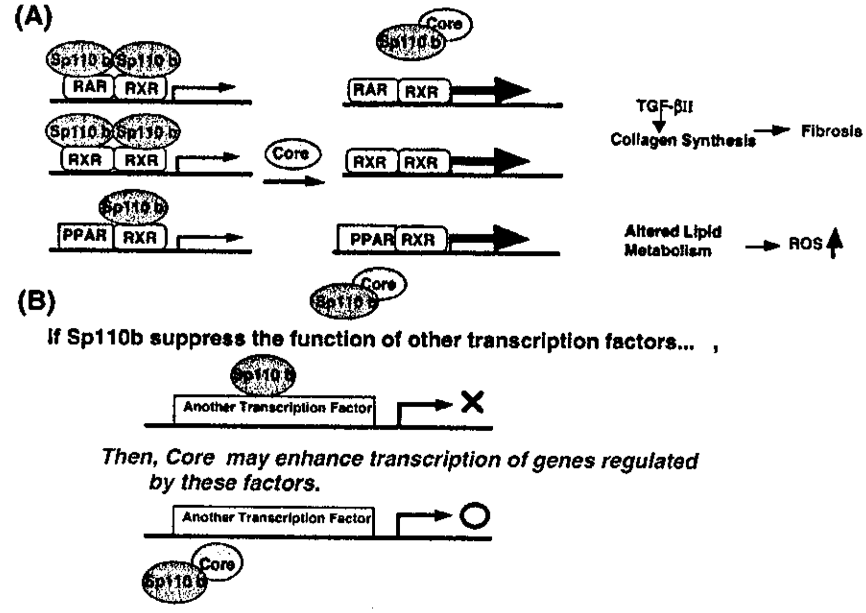

Fig. 4. The core may be involved in regulation of other transcriptional activities than that mediated by RAR/RXR heterodimer. (A) Possible mechanisms for the core to activate transcription regulated by nuclear hormone receptors, such as RAR/RXR, RXR/RXR, and PPAR/RXR. As Sp110b has a consensus sequence serving for interaction with liganded RARα and RXR, Sp110b is likely to regulate genes downstream of these nuclear hormone receptor signalings. Examples of the gene products regulated by these signals, related to HCV pathogenesis, are shown. (B) If Sp110b is a general transcriptional repressor, the core may regulate gene expression of additional genes, independently of those affected by the nuclear hormone receptor.

to pre-early stages of cancer development, beginning in the early stages of chronic hepatitis. Analysis of the genes altered in HCC has revealed several abnormalities, including changes in tumor suppressor genes, such as *p53*, *IGF2R*, *β-catenin*, *p16INK*, and *retinoblastoma*.[41] The alteration of these genes is detectable in specimens of HCC other than those derived from HCV-infection-related cancer; thus, a direct molecular link between HCV infection and the alteration of these genes remains obscure. Instead, HCV infection may enhance the genetic alterations occurring during the proliferation of hepatocytes. Various HCV infection-induced mechanisms, including inflammation and enhanced production of genotoxic agents such as ROS, may act as triggers inducing genomic instability.

Conclusion

Many reports have attempted to resolve the involvement of HCV proteins in the dysregulation of cellular functions. The role of the core in the regulation of cellular gene transcription, however, remains controversial. In particular, analyses conducted using an artificial C-terminally truncated form of the core, which localizes to the nucleus, have an unclear physiological significance, as the majority of the core produced in cells is normally localized to the ER through its hydrophobic C-terminal region. Due to these conflicting results using different forms of the core, there is still considerable debate over the role of the core in cellular functions. In addition, uncertainty in the evaluation of these results derives from uncertainty in the molecular mechanisms through which the core functions to modulate gene expression. Until now, there has not been any clear or probable mechanism for the modulation of gene expression. We therefore hope that the proposal introduced here regarding the mechanism through the core modulates RAR-dependent gene expression will stimulate further discussion and study of the roles of the HCV core.

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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 (αR series) possessing the IFN-α-resistant phenotype and four replicon cell lines (βR series) possessing the IFN-β-resistant phenotype. Furthermore, we obtained an additional replicon cell line (αRmix) 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 βR series were highly resistant to both IFN-α and IFN-β, although the αR series containing αRmix 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 βR 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.

Keywords: Hepatitis C virus; Huh-7; Replicon; Interferon resistance

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 genome 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 μg/ml; Geneticine, 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 μg/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 phRL-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 reproducibly 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 1αR, 3αR, 4αR, and 5αR, 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 1βR, 3βR, 4βR, and 5βR, 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 αRmix (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 (αR series) plus an αRmix

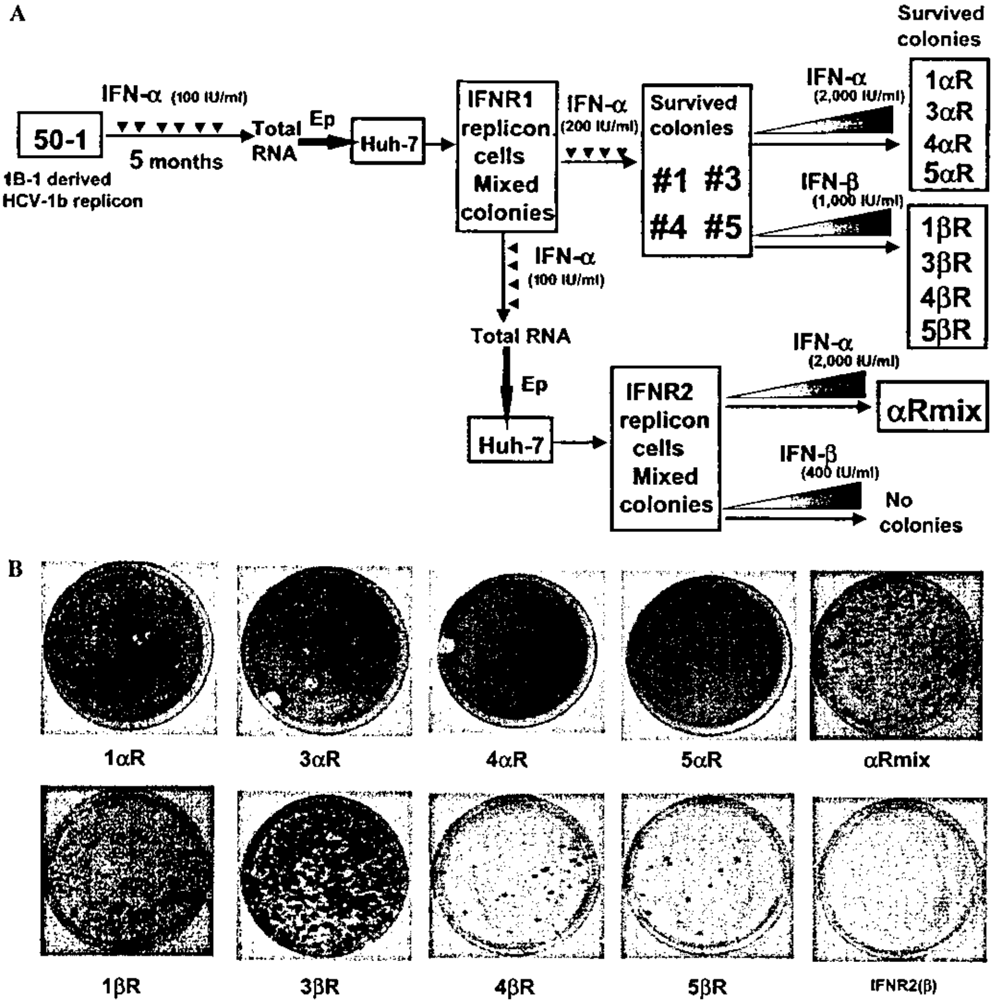

Fig. 1. Isolation of HCV replicon cell lines possessing IFN-resistant phenotype. (A) Outline of the isolation of HCV replicon cells possessing IFN-resistant phenotype. Ep indicates electroporation of total cellular RNA to Huh-7 cells. (B) HCV replicon cells possessing resistance against IFN-α (2000 IU/ml) 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 (βR 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 μg) extracted from these replicon cells was estimated at approximately 10^8 (less than 10^8 in 1αR 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

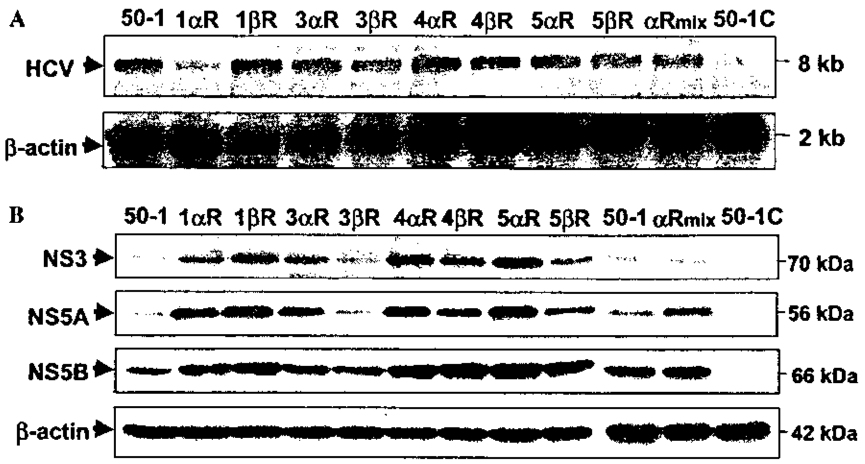

Fig. 2. Characterization of replicon cells possessing IFN-resistant phenotype. (A) Northern blot analysis. Total RNAs from 50-1 and nine replicon cell lines possessing IFN-resistant phenotype, as well as total RNA from 50-1C cells (cured cells), were analyzed by Northern blot analysis using a positive-stranded 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 (1αR, 3αR, 4αR, 5αR, and αRmix) 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 αR series (1αR, 3αR, 4αR, and 5αR) and αRmix possessed partial IFN-α resistant phenotypes. Although

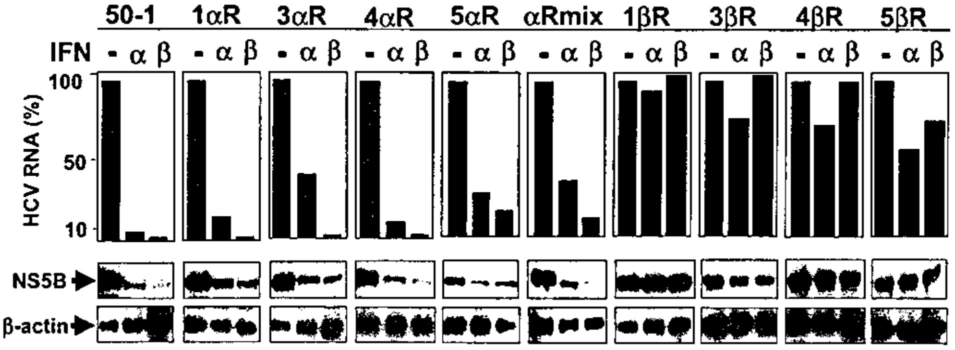

Fig. 3. IFN-resistant phenotypes of the established replicon cell lines. 50-1 and nine replicon cell lines possessing IFN-resistant phenotype were treated with IFN-α or IFN-β (500 IU/ml each) for two days for semi-quantitative RT-PCR analysis (upper panel) and for five days for Western blot analysis (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 αR series and αRmix, the βR series (1βR, 3βR, 4βR, and 5βR) showed almost complete resistance to both IFN-α and IFN-β (Fig. 3). Interestingly, the levels of replicon RNAs in 1βR, 3βR, and 4βR cells, though not in 5βR 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 βR series possesses phenotypes with severe resistance to both IFN-α and IFN-β.

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

To clarify whether or not the αR series obtained by treatment with IFN-α alone showed the IFN-β- resistant phenotype, we compared in detail the IFN sensitivities of 1αR with 50-1 and 1βR. The 50-1, 1αR, and 1βR 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 1βR 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 1βR 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 1αR against IFN-α or IFN-β treatment was in between that of 50-1 and that of 1βR (Fig. 4). This revealed that 1αR possesses a partially resistant phenotype against both IFN-α and IFN-β. This finding suggests that the other four replicon cell lines (3αR, 4αR, 5αR, and αRmix) 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 αR series as well as in the 50-1 cells. Meanwhile, these enhancements were remarkably lower in 5αR and αRmix cells than in 50-1 cells. These results suggest that both IFN-α and IFN-β are effectively transduced in the αR series cells (Fig. 5). However, the luciferase activities in the βR series cells, except for 3βR 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 1βR, 4βR, and 5βR cells but not in 3βR cells (Fig. 5). Although this reporter assay clarified the reason why 1βR, 4βR, and 5βR cells possessed the IFN-resistant phenotype, the reason for IFN resistance among the other replicons remained unclear. Since the luciferase activities in 5αR and αRmix 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-

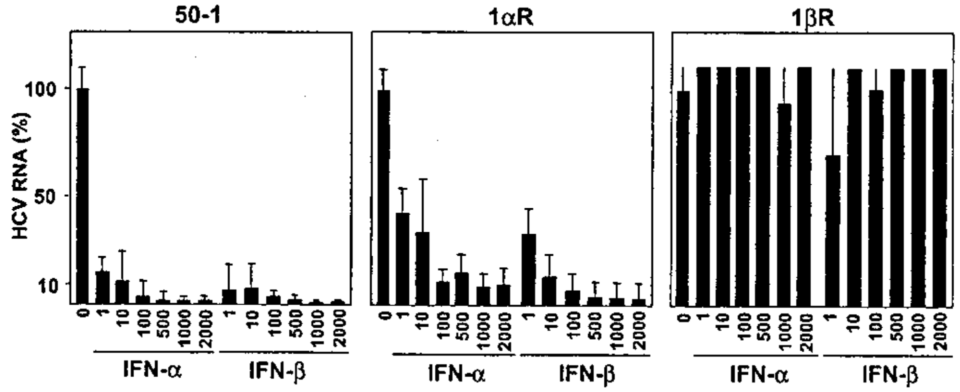

Fig. 4. IFN sensitivities of the replicons in 50-1, 1αR, and 1βR cells. Cells from each of these lines were treated with IFN-α or IFN-β (1, 10, 100, 500, 1000, and 2000 IU/ml each) for two days. Semi-quantitative RT-PCR was carried out to monitor the levels of replicon RNAs in the cells, as described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

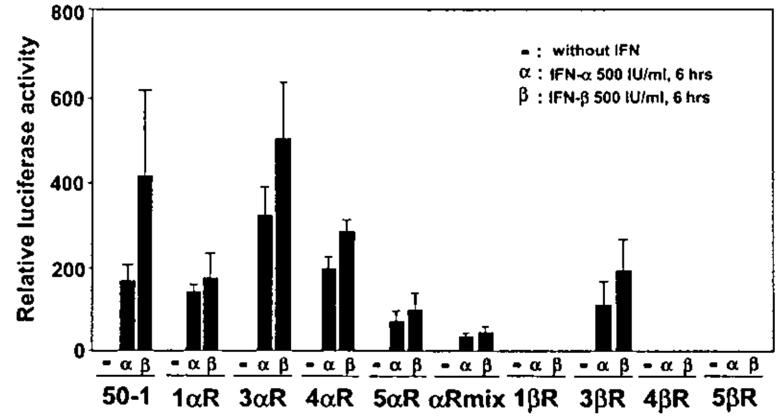

Fig. 5. IFN signal transduction in the established replicon cell lines. Regarding 50-1 and the nine replicon cell lines possessing IFN-resistant phenotype, dual luciferase reporter assay using pISRE(V2)-Luci [32] was performed as described previously [29]. The replicon cells were treated with IFN-α or 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 αR series and 3βR cells after IFN-α treatment than in 50-1 cells, and that phosphorylation of these proteins was barely observed in 1βR, 4βR, and 5βR cells in spite of the IFN-α treatment (Fig. 6). The results for the αR series cells are consistent with their partially IFN-resistant phenotype (Figs. 3 and 4), although the IFN-resistant phenotype of 3βR 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 αR series plus the αRmix cell line) and a completely IFN-resistant phenotype (four cell lines of the βR 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

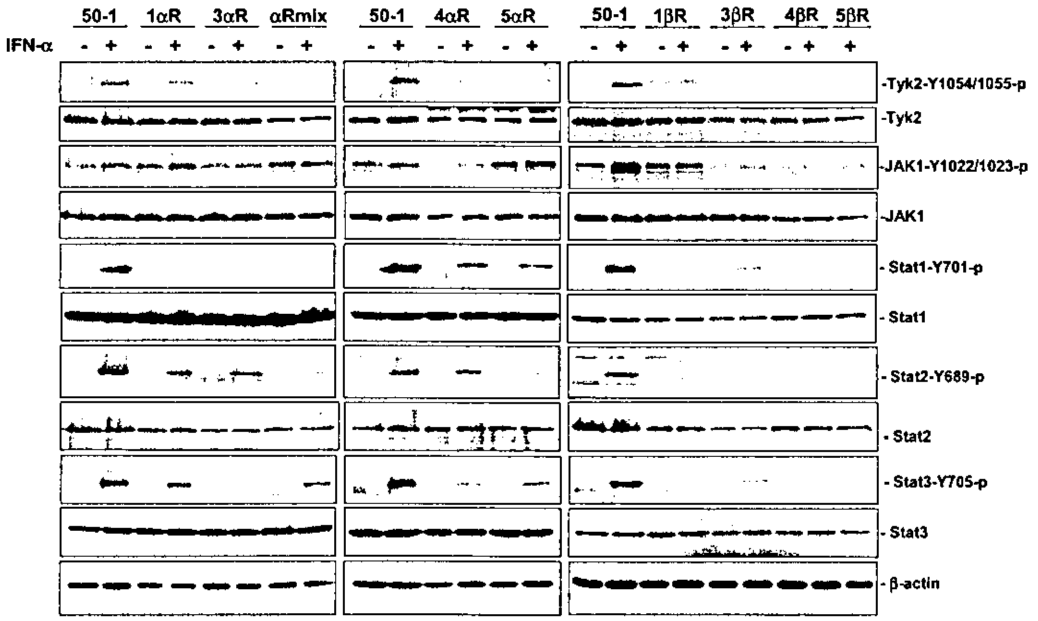

Fig. 6. Western blot analysis of the components involved in the IFN signal transduction pathway in the established replicon cell lines treated with IFN-α. The replicon cells were stimulated with or without IFN-α (500 IU/ml) for 30 min, and then Western blot analysis was performed as described under Materials 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 αR-series-specific or βR-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 1βR; T2319A and N for 3βR; and T2242N and F2256L for 4βR) and NS5B (A2752V for 3βR) in the βR series only, although no other common amino acid substitutions were found in the βR 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 αR series, αRmix, βR 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 αR series, αRmix, or βR 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]. 1αR and 3αR cells possessing a partially IFN-resistant phenotype, and 1βR and 3βR 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 1αR, 1βR, 3αR, or 3βR 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 1αR, 3αR, and 3βR cells, IFN response was not restored in the cured cells obtained from 1βR cells. This result suggests that some host factor(s) rather than replicon RNA(s) contributed to the

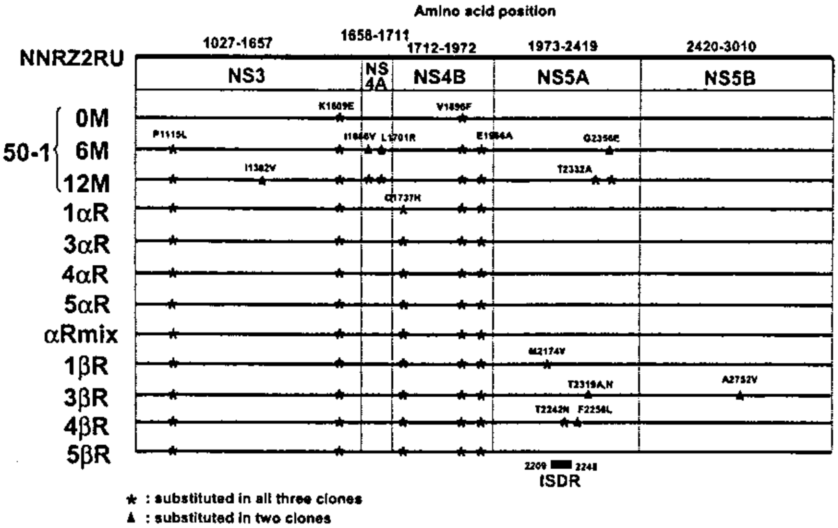

Fig. 7. Genetic analysis of the NS region of replicon RNAs in the established replicon cell lines possessing IFN-resistant phenotype. Compared with the amino acid sequences of NS region of the original replicon (NNRZ2RU), amino acid positions substituted in all thee clones and in two of three clones 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.

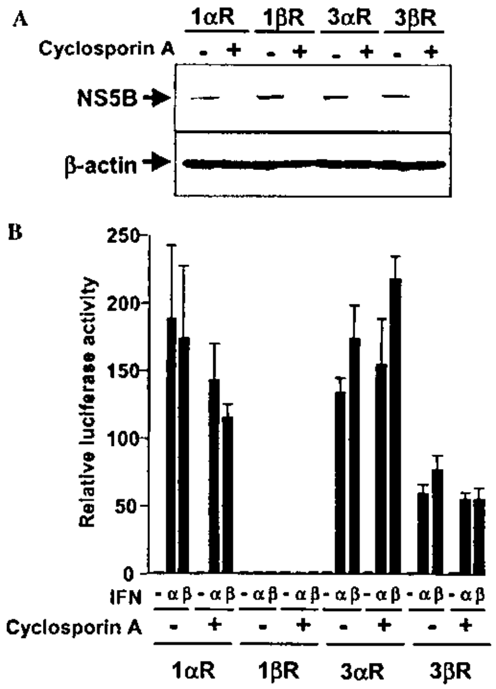

Fig. 8. Characterization of cured cells obtained from 1αR, 1βR, 3αR, and 3βR cells. (A) Western blot analysis. Regarding the four replicon cell lines possessing the IFN-resistant phenotype and their cured cell lines, NS5B was detected by immunoblot analysis using anti-NS5B antibodies. β-Actin was used 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 NA5A 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 1βR cells. It is considered that one reason why we have obtained HCV replicon cells that are deficient in IFN signaling (such as the 1βR 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.

Acknowledgments

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cDNA microarray analysis to compare HCV subgenomic replicon cells with their cured cells

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Abstract

The hepatitis C virus (HCV) replicon system carrying autonomously replicating HCV subgenomic RNA in human hepatocyte cells is a potent tool for basic studies of HCV, such as viral replication and drug development. Recently, we developed two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively. Since the expression of HCV proteins is thought to affect the host cells' gene expression profiles, we attempted to identify target genes of HCV proteins using microarray analysis (9970 genes) by comparing 50-1 and 1B-2R1 replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with interferon-α. The results showed that HCV replicons could have a variety of expression profiles in human hepatocytes. The results also showed that 2 and 6 genes were commonly up-regulated (more than 2.0-fold) and down-regulated (less than 0.50-fold), respectively, in both 50-1 and 1B-2R1 replicon cells compared with their cured cells. The differential expression profiles of genes selected by the microarray analysis were confirmed with standard RT-PCR and real-time LightCycler PCR. It was noteworthy that the commonly down-regulated genes contained large multifunctional proteases 2 and 7, which are known as catalytic subunits of immunoproteasome, and serine proteinase inhibitor clade C. Our microarray analysis demonstrated that HCV subgenomic replicons can change the gene expression profiles of host cells, and it allowed us to compile the first list of genes that the replicons transcriptionally regulate.

Keywords: Hepatitis C virus; HCV subgenomic replicon; Cured cells; cDNA microarray; Gene expression profile

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (Choo et al., 1989; Kuo et al., 1989), which progresses to liver cirrhosis and hepatocellular carcinoma (Ohkoshi et al., 1990; Saito et al., 1990). HCV belongs to the family Flaviviridae, whose genome consists of a positive-stranded RNA molecule of about 9.6 kb and encodes a large polyprotein precursor of about 3000 amino acids (Kato et al., 1990; Tanaka et al., 1995). This precursor protein is cleaved by the host and viral proteases to generate at least ten proteins: the core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. Although many hypotheses have been proposed over the past decade regarding the functions of the viral proteins (Bartenschlager and Lohmann, 2000; Kato, 2001), the lack of reproducible and efficient HCV proliferation in cell cultures (Kato and Shimotohno, 2000) has been a serious obstacle in understanding those proteins' actual functions.

However, in 1999, an HCV replicon system carring autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions was first established using a human hepatoma cell line, Huh-7 (Lohmann et al., 1999). Since then, several additional replicons have also been established (Blight et al., 2000, 2003; Ikeda et al., 2002; Kato et al., 2003b). In these systems, replicated HCV RNAs were detected by Northern blot analysis, and the HCV proteins pro-

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duced were detected by Western blot analysis. Therefore, the system of HCV replicons has become a powerful tool for basic studies in HCV, such as viral replication and drug development (Bartenschlager, 2002).

Recently, we also established two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively, using Huh-7 cells (Kato et al., 2003a; Kishine et al., 2002). We demonstrated that the 50-1 and 1B-2R1 subgenomic replicons (Kato et al., 2003a) were sensitive to interferon (IFN)-α, IFN-β and IFN-γ as are the other replicons (Frese et al., 2001, 2002). The nucleotide sequences of the NS3-NS5B regions in the 50-1 subgenomic replicon showed differences of 8.1% from those in the1B-2R1 subgenomic replicon (Kato et al., 2003a), although both the 1B-1 and 1B-2 strains belong to genotype 1b. Although the efficient replication of an HCV subgenomic replicon expressing HCV proteins is considered to affect the gene expression profiles of host cells (Bartenschlager and Lohmann, 2000; Kato, 2001), few reports have demonstrated inclusive searches for HCV's target genes (Zhu et al., 2003). Therefore, we thought a comprehensive search for HCV subgenomic replicon-regulated cellular genes would be important in understanding the molecular interplay exerted by HCV in vivo.

In the present study, to obtain the candidates of HCV's target genes, we performed cDNA microarray analysis by comparing two types of HCV subgenomic replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with IFN-α. Here we report on the differential gene expression profiles in the replicon cells, and we first provide a list of genes that the replicons transcriptionally regulate.

2. Materials and methods

2.1. Cell cultures

50-1 and 1B-2R1 cells possessing 50-1 and 1B-2R1 subgenomic replicons, respectively, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and G418 (300 mg/ml; Geneticine, Invitrogen). The 50-1 and 1B-2R1 cells were known to possess the G418-resistant phenotype, because neomycin phophotransferase (NEOR) was produced by the efficient replication of HCV subgenomic replicon in the cells. When an HCV subgenomic replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418. Therefore, the cured cells obtained from 50-1 and 1B-2R1 cells were maintained in the absence of G418.

2.2. IFN treatment

To prepare the cured cells, 50-1 and 1B-2R1 cells (each 1×10^6) were plated onto 10-cm plates and were cultured for 1 day immediately before IFN treatment. Human IFN-α (Sigma) was added to the cells at a final concentration of 3000 IU/ml as described previously (Kato et al., 2003a). The incubation in the absence of G418 was continued for 3 weeks with the addition of IFN-α (3000 IU/ml) at 4-day intervals. The cured cells obtained from 50-1 and 1B-2R1 cells were named 50-1C and 1B-2R1C cells, respectively.

2.3. 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 (Ikeda et al., 2002; Kato et al., 2003a). As a molecular length marker, replicon RNA synthesized in vitro from replicon cassette plasmid pNSS1RZ2RU (Kato et al., 2003a) was also utilized.

2.4. 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 (Hijikata et al., 1993). The antibodies used in this study were those against NS3 (Novacastra Laboratories, UK), NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science), and β-actin (Sigma). Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences).

2.5. cDNA microarray analysis

The 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells (each 1×10^6 cells) were plated onto 10 cm plates, and each plate was cultured for 5 days in the absence of G418. The confluent cells were harvested and total RNAs were prepared using the RNeasy extraction kit (Qiagen). Using the obtained total RNAs, cDNA microarray analysis (CodeLink^{TM}, Uniset human I containing 9970 spots of 30-mer oligonucleotides; Amersham Biosciences) was performed by Kurabo Industries Ltd. (Osaka, Japan) with the authorization of Amersham Biosciences.

2.6. Analysis of mRNA expression by RT-PCR

The total RNAs (each 2 μg) that were the same as those subjected to cDNA microarray analysis were reverse-transcribed with Superscript II using an oligo dT primer (Invitrogen). One-tenth of the synthesized cDNA was subjected to PCR. The PCR primers are listed in Table 1. After 10 min at 98 °C, PCR was performed with Taq DNA polymerase (TaKaRa, Japan). Each cycle consisted of annealing at 60 °C (64 °C for LMP2 and LMP7 only) for 45 s, primer extension at 72 °C for 1 min, and denaturation at 94 °C for 20 s. The cycle numbers and the size of PCR products were also

Table 1
The primers used for RT-PCR analysis of mRNA expression

Genes	Orientation	Nucleotide sequence	Product (bp)	Cycles
Large multifunctional protease2 (LMP2)	Forward	ATGGAACCCTGGGAGGAATGCTG	145	30
	Reverse	GCAATAGCGTCTGTGGTGAAGCG		
Large multifunctional protease 7 (LMP7)	Forward	CTGGGATAAGAAGGGTCCTGGAC	293	27
	Reverse	TACTGGTGCAGCAGGTCACTGGAC		
Serine proteinase inhibitor (serpin) clade C	Forward	TGGATGAATTGGAGGAGATGATGC	249	25
	Reverse	CAATCACAACAGCGGTACTTGCAG		
S100-type calcium binding protein A14	Forward	CAGAGGATGCTCAGGAATTCAGTG	256	27
	Reverse	CTCTTGGCCGCTTCTCCAATGAG		
Latent transforming growth factor β binding protein 1 (LTBP1)	Forward	GCCTTGGTTGACTTCAGTGAACAG	325	27
	Reverse	CAGAAGGCACGTAGCCTGGCAG		
Weakly similar to zinc finger protein 91	Forward	CCAGAACCACATCCAAACCATCC	299	33
	Reverse	CCATCCCTTCGAAGCTGTGCTC		
Transgelin	Forward	GATTCTGAGCAAGCTGGTGAACAG	254	25
	Reverse	AGTGCCCATCATTCTTGGTCACTG		
Annexin A1	Forward	GATGCCAGGGCCTTGTATGAAGC	264	25
	Reverse	AACACCTTTCATGGCTTGATGAAGC		
Solute carrier family 7	Forward	AGTCCTTCGCTGGAAGAAGCCTG	314	27
	Reverse	CCATGTCCTCATTAGCCTCCTCTG		
Protein phosphatase 1 regulatory subunit 1A	Forward	CCACGGCAACGGAAGAAGATGAC	302	27
	Reverse	GCTCCCTTGGAATCCAGTGGTGG		
Phosphatidylserine-specific phospholipase A1α	Forward	GAGAAACAAGGACACCAACATCGAG	288	28
	Reverse	GTCACACTTGCTTGTAAGTTCACTG		
Oncostatin M receptor	Forward	CAGAAAAGAGTCACTCTGGCCCTG	292	27
	Reverse	GGTGCCTCTACTGGGTTTGTTGG		
Similar to interferon-induced protein 35	Forward	CCGTATGTGAATGGGGAGATCCAG	222	27
	Reverse	GCCTGACTCAGAGGTGAAGACTG		
Caspase 1	Forward	AGAAACACTCTGAGCAAGTCCCAG	278	30
	Reverse	AACATTATCTGGTGTGGAAGAGCAG		
Neutrophil cytosolic factor 2	Forward	GACATGGTGTCTAAGAAACTGGAG	277	27
	Reverse	CTCATAACTGAAGAGTGCCTCCAC		
Putative secred protein ZSIG13	Forward	CTGGTTATGACAATGACCGACCAG	272	25
	Reverse	GCAGATCTGGGCATATTTGAGAGG		
GAPDH	Forward	GACTCATGACCACAGTCCATGC	334	22
	Reverse	GAGGAGACCACCTGGTGCTCAG		

listed in Table 1. The PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels. RT-PCR was performed in duplicate experiments. The mRNA levels of target genes were monitored by a ChemiImager 4400 (Alpha Innotech), which measured the intensities of bands stained with ethidium bromide as described previously (Kato et al., 2003a). As an internal control, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was amplified by RT-PCR, and the products were used to normalize the mRNA levels of the target genes.

2.7. LightCycler PCR

One-twentieth of the cDNA synthesized above was subjected to real-time LightCycler PCR as described previously (Nozaki and Kato, 2002; Nozaki et al., 2003). The primers listed in Table 1 were also used for LightCycler PCR. Temperature cycling conditions for each primer set consisted of 10 min at 95 °C followed by 35 cycles for 1 s at 94 °C, 5 s at 60 °C (64 °C for LMP2 and LMP7 only), and 6–14 s (25 bp per second) at 72 °C. All reactions were performed in a LightCycler$^{\mathrm{TM}}$ Quick System 330 (Roche) using FastStart DNA Master SYBR Green I mix (Roche) according to the manufacturer's instructions. The experiments were performed in at least triplicate. The relative mRNA expression ratios of the target genes were calculated based on crossing-point analysis using a second derivative maximum method (LightCycler analysis software version 3.5). To correct for differences in RNA quality and quantity between the samples, data were normalized using the ratio of the target cDNA concentration to that of GAPDH. This ratio was assessed by a different reaction in the same experimental round.

Table 2
Genes whose expression levels were commonly altered in 1B-2R1 and 50-1 cells compared with their cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
Up-regulation (more than 2-fold)			
Phosphatidylserine-specific phospholipase A1α[a]	2.2	2.9	NM_015900
Oncostatin M receptor[a]	2.1	2.2	NM_003999
Down-regulation (less than 0.50-fold)			
LMP2[a]	0.14	0.30	NM_002800
LMP7[a]	0.21	0.44	NM_004159
Similar to interferon-induced protein 35[a]	0.31	0.32	BC001356
Weakly similar to zinc finger protein 91[a]	0.36	0.42	AK027354
Protein phosphatase 1, regulatory subunit 1A[a]	0.40	0.32	NM_006741
Serpin clade C[a]	0.49	0.31	NM_000488

[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

3. Results

3.1. Preparation of the cured cells from 50-1 and 1B-2R1 cells

To obtain cured cells for the microarray analysis, 50-1 and 1B-2R1 cells were cultured with prolonged IFN-α treatment as described Section 2. After 3 weeks of this treatment, we demonstrated by Northern blot analysis that the replicon RNAs were not detected in the IFN-α-treated (50-1C and 1B-2R1C) cells, although approximately 10^8 copies of replicon RNA were detected in the total RNA (3 mg) extracted from 50-1 and 1B-2R1 cells (Fig. 1A). We further confirmed by RT-nested PCR (Mizutani et al., 1996) for the detection of the 5′-untranslated region that the replicon RNAs were absolutely excluded from the cells (data not shown). Western blot analysis also showed that the NS3 and NS5B proteins were no longer detected in 50-1C and 1B-2R1C cells, but were detected in 50-1 and 1B-2R1 cells, as shown in Fig. 1B.

3.2. cDNA microarray analysis

To examine the effects of HCV replicons on gene expression in host cells, cDNA microarray analyses (CodeLink™, Amersham Biosciences; 9970 human genes) were performed by comparing 1B-2R1 with 1B-2R1C cells and 50-1 with 50-1C cells. The majority of genes examined showed only small differences, with ratios ranging between 2.0 and 0.50 (data not shown). There were 55 and 101 up-regulated genes (those

Table 3
Genes whose expression levels were up-regulated (more than 3-fold) in either 1B-2R1 or 50-1 cells compared with the cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
AU62G04.X1	**8.5**	1.4	AI929792
Homeobox 1(HESX1)	**4.2**	0.50	NM_003865
Microsomal NAD+ dependent retinol dehydrogenase 4	**3.4**	0.92	NM_003708
Advillin	**3.3**	0.61	NM_006576
SSFV proviral integration oncogene Spi1	**3.1**	1.0	NM_003120
Napsin 2 precursor	**3.1**	0.94	AF098485
Transgelin[a]	0.85	**8.5**	NM_003186
Uncharacterized bone marrow protein BM040	0.81	**5.8**	AF217516
Annexin A1[a]	1.0	**4.2**	NM_000700
Putative secreted protein ZSIG13[a]	1.7	**3.9**	AF193611
Protease serine 23	1.2	**3.8**	NM_007173
Colon cancer antigen NY-CO-45	1.3	**3.7**	AF039442
HSPC157 protein	1.1	**3.5**	NM_014179
Uronyl-2-sulfotransferase	1.0	**3.5**	NM_005715
Cadherin, EGF lag seven-pass G-type receptor 2	0.68	**3.5**	NM_001408
Hypothetical protein (LOC51321)	1.1	**3.4**	NM_016627
Kidney-specific membrane protein (NX-17)	1.0	**3.3**	NM_020665
Neutrophil cytosolic factor 2[a]	1.8	**3.2**	NM_000433
Amphiregulin	1.4	**3.1**	NM_001657
Fibrillin 1	0.83	**3.1**	NM_000138
LTBP1[a]	1.6	**3.0**	NM_000627

The numbers of more than 3-fold were indicated by bold letters.

[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

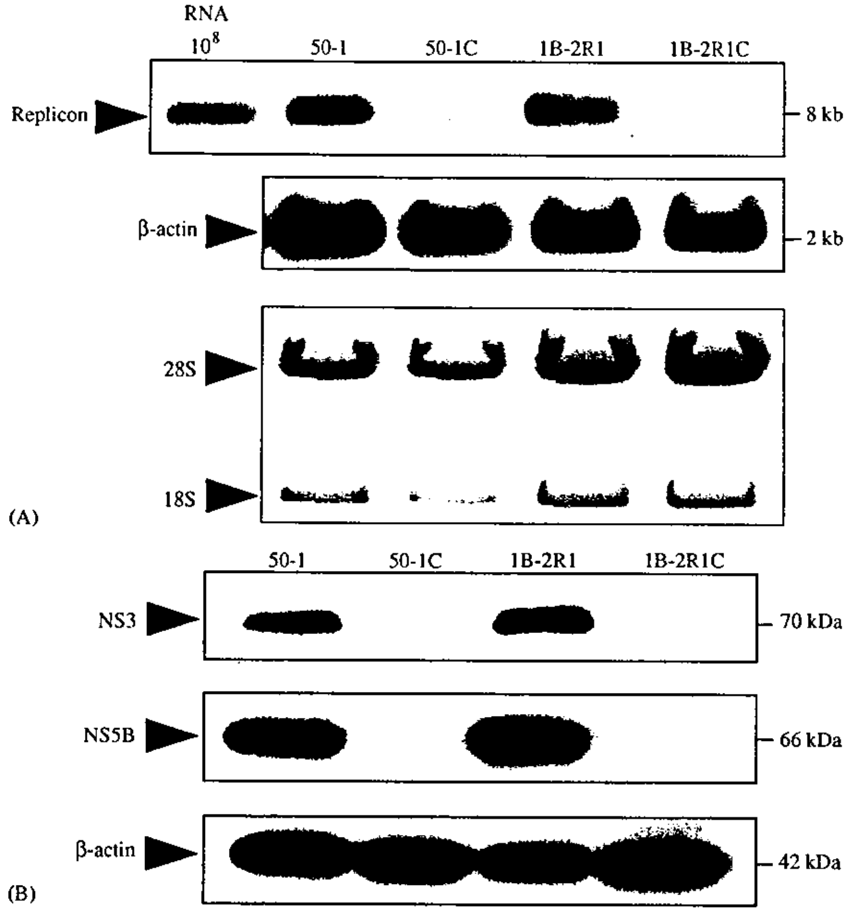

Fig. 1. Characterization of the replicon cells and their cured cells. (A) Northern blot analysis. Total RNAs from 50-1 and 1B-2R1 cells, as well as total RNAs from the cured cells, were analyzed by Northern blotting using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific RNA probe (middle panel). RNA samples were equalized for 28S and 18S ribosomal RNAs stained with ethidium bromide (lower panel). A synthetic RNA transcribed from pNSS1RZ2RU (10^8 genome equivalents spiked into normal cellular RNA) was used as a positive control. (B) Western blot analysis. Productions of NS3 and NS5B in 50-1 and 1B-2R1 cells were analyzed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. 50-1C and 1B-2R1C cells were also analyzed to confirm the lack of NS3 and NS5B proteins. β-actin was used as a control for the amount of protein loaded per lane.

with ratios of more than 2.0) in 1B-2R1 and 50-1 cells, respectively. Between the two types of replicon cells, only two genes were commonly up-regulated. There were 56 and 74 down-regulated genes (those with ratios of less than 0.50) in 1B-2R1 and 50-1 cells, respectively, of which 6 genes were commonly down-regulated in both types of replicon cells. Table 2 summarizes the genes that the replicons commonly affected. Among these genes, it is noteworthy that large multifunctional proteases 2 (LMP2) and LMP7, which have been known as catalytic subunits in immunoproteasome (Akiyama et al., 1994; Tanaka and Kasahara, 1998), and serine proteinase inhibitor (serpin) clade C (Gettins, 2002) were down-regulated in both types of replicon cells (discussed below). However, no common genes were directly linked to the transformation of the cells. Since the standard of selection seemed to be rather strict, we further selected the genes whose expression levels were up-regulated or down-regulated with ratios of more than 3.0 or less than 0.33, respectively, in either 1B-2R1 or 50-1 cells. By this method, we selected 6 and 15 genes as up-regulated genes in 1B-2R1 and 50-1 cells, respectively (as shown in Table 3); and 6 and 9 genes as down-regulated genes in 1B-2R1 and 50-1 cells, respectively (as shown in Table 4). These selections allowed us to find several additional genes, including latent transforming growth factor β binding protein 1 (LTBP1) and caspase 1, that were commonly regulated in both types of replicons.

3.3. RT-PCR confirmation of the alteration of gene expression by HCV replicons

To confirm the results of our microarray selection, we examined the levels of several mRNAs by RT-PCR in duplicate. As shown by the stars in Tables 2-4, 16 genes (7 up-regulated and 9 down-regulated) were subjected to RT-PCR analysis. As shown in Fig. 2, RT-PCR confirmed that the expressions of most of these genes changed. This result suggests that the relative mRNA expression ratio obtained by the microarray analysis reflects the differential expression profiles of the replicon and its cured cells. Of the 16 genes, 9 (4 up-regulated and 5 down-regulated) were fur-

Table 4
Genes whose expression levels were down-regulated (less than 0.33-fold) in either 1B-2R1 or 50-1 cells compared with the cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
Hephaestin	**0.14**	1.7	NM_014799
Solute carrier family 7[a]	**0.15**	0.62	NM_003982
Caspase 1[a]	**0.18**	0.65	NM_033292
Protease inhibitor 3	**0.19**	1.1	NM_002638
Collagen type II α1	**0.31**	1.6	NM_033150
C-terminal binding protein 2	**0.31**	0.71	NM_022802
ATPase α polypeptide (ATP 12A)	0.57	**0.26**	NM_001676
Hypothetical protein FLJ20043	0.79	**0.27**	NM_017637
CM2-HT0948-070900-368-D08 cDNA	1.0	**0.28**	BF089733
S100-type calcium binding protein A14[a]	0.62	**0.30**	NM_020672
Hypothetical protein MGC2827	0.65	**0.31**	NM_023940
EGFL6	2.4	**0.32**	NM_015507
ISL1 transcription factor	0.94	**0.32**	NM_002202
Pre-α globulin inhibitor	1.2	**0.32**	NM_002217
Regulator of G-protein signalling 16	0.65	**0.33**	NM_002928

The numbers of less than 0.33-fold were indicated by bold letters.

[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

ther subjected to real-time LightCycler PCR analysis in order to obtain the actual ratios of mRNA expression. As shown in Table 5, the resultant relative mRNA expression ratios actually correlated with those obtained by our microarray analysis. Regarding the selected genes in this study, we confirmed by RT-PCR the reproducibility of the relative mRNA ratios using different lots of RNA specimens derived from 1B-2R1 and 1B-2R1C cells (data not shown). Taken together, our results suggest that these altered mRNA expressions are caused by the multiplication of HCV subgenomic replicons.

4. Discussion

This study yielded evidence of alterations in gene expression by HCV subgenomic replicons in human hepatocytes, as observed through microarray analysis (9970 genes), and first provided a list of genes including LMP2, LMP7, and serpin clade C that the replicons transcriptionally regulate.

To date, only one report of cDNA microarray analysis (832 cytokine-related genes) has been conducted by comparing HCV subgenomic replicon cells with parental Huh-7 cells (Zhu et al., 2003). That analysis obtained 14 up-regulated genes (those with ratios of more than 2.0) in the replicon cells. However, the parental Huh-7 cells may not be appropriate for use as control cells in such microarray analyses, because the HCV subgenomic replicon cells used are derived from a single cloned cell. Therefore, it is very important to avoid the clone-based differences for microarray analysis. From this principal reason, we used two types of cured cells derived from 50-1 and 1B-2R1 cells as the control cells for our microarray analysis. The cured cells are considered to have the same background as the replicon cells. The possibility remains that the genes selected in this study were obtained by the effect of IFN-α that was used to

Table 5
LightCycler RT-PCR analysis of genes whose expression levels were altered by HCV replicons

Genes	Relative mRNA expression ratio (mean ± S.D.)	
	1B-2R1/1B-2R1C	50-1/50-1C
Up-regulation		
Phosphatidylserine-specific phospholipase A1α	2.03 ± 0.09	3.09 ± 0.74
Oncostatin M receptor	2.58 ± 0.20	2.46 ± 0.49
Transgelin	0.83 ± 0.11	13.72 ± 0.56
Annexin A1	1.19 ± 0.17	4.23 ± 0.72
Down-regulation		
LMP2	0.06 ± 0.00	0.40 ± 0.12
LMP7	0.09 ± 0.02	0.33 ± 0.08
Serpin clade C	0.39 ± 0.11	0.37 ± 0.11
Solute carrier family 7	0.13 ± 0.08	0.77 ± 0.18
S100-type calcium binding protein A14	0.37 ± 0.21	0.32 ± 0.17