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Ⅳ.研究成果の刊行物・別刷

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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

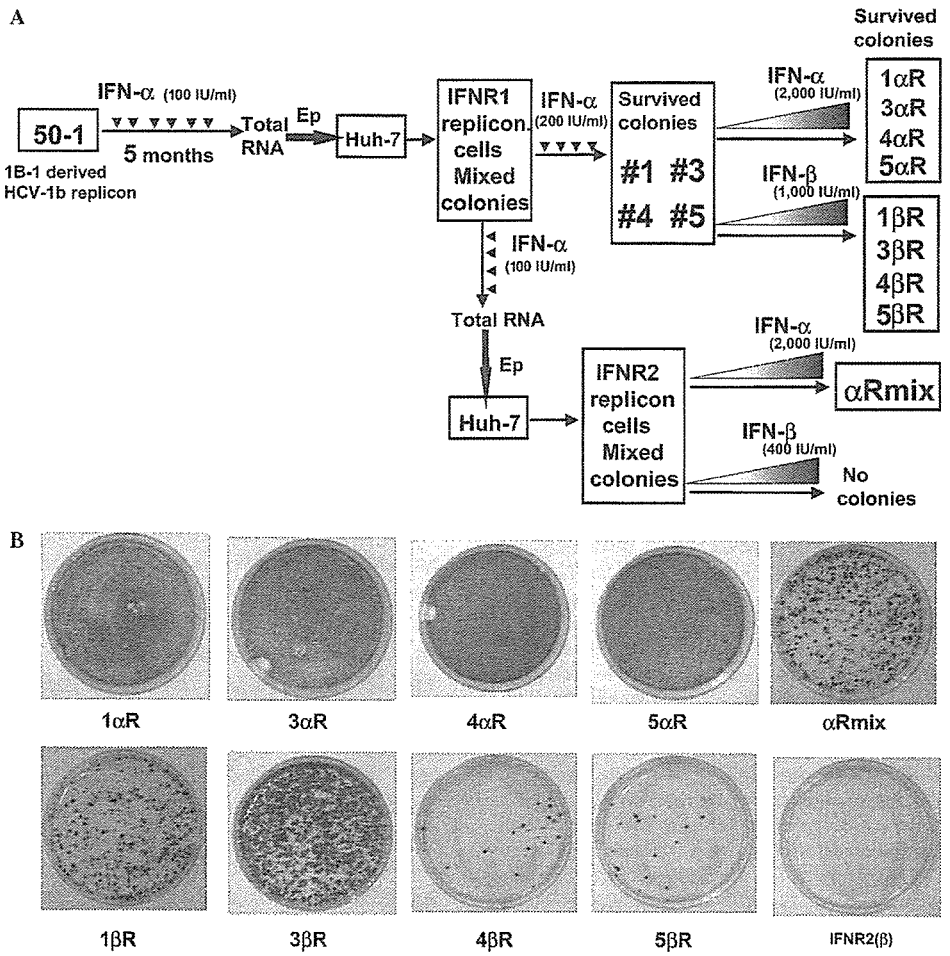

Fig. 1. Isolation of HCV replicon cell lines possessing IFN-resistant phenotype. (A) Outline of the isolation of HCV replicon cells possessing IFN-resistant phenotype. Ep indicates electroporation of total cellular RNA to Huh-7 cells. (B) HCV replicon cells possessing resistance against IFN-α (2000 IU/ml) 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

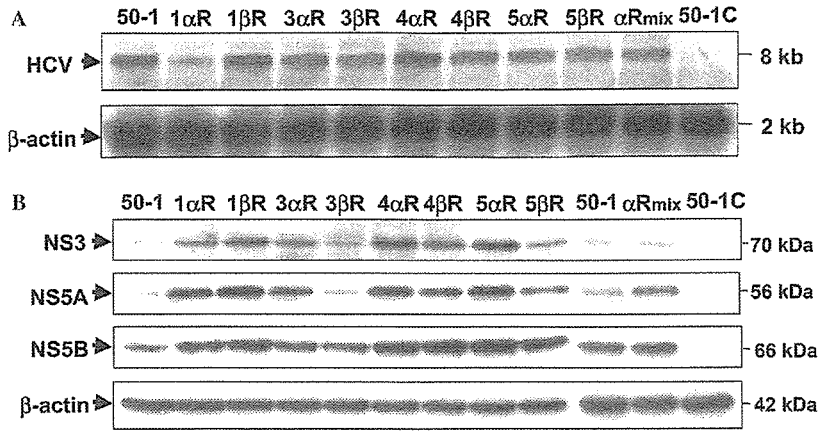

Fig. 2. Characterization of replicon cells possessing IFN-resistant phenotype. (A) Northern blot analysis. Total RNAs from 50-1 and nine replicon cell lines possessing IFN-resistant phenotype, as well as total RNA from 50-1C cells (cured cells), were analyzed by Northern blot analysis using a positive-stranded 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

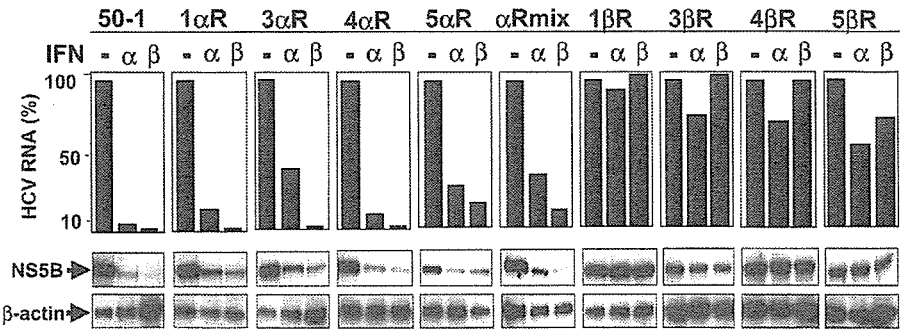

Fig. 3. IFN-resistant phenotypes of the established replicon cell lines. 50-1 and nine replicon cell lines possessing IFN-resistant phenotype were treated with IFN-α or IFN-β (500 IU/ml each) for two days for semi-quantitative RT-PCR analysis (upper panel) and for five days for Western blot analysis (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-

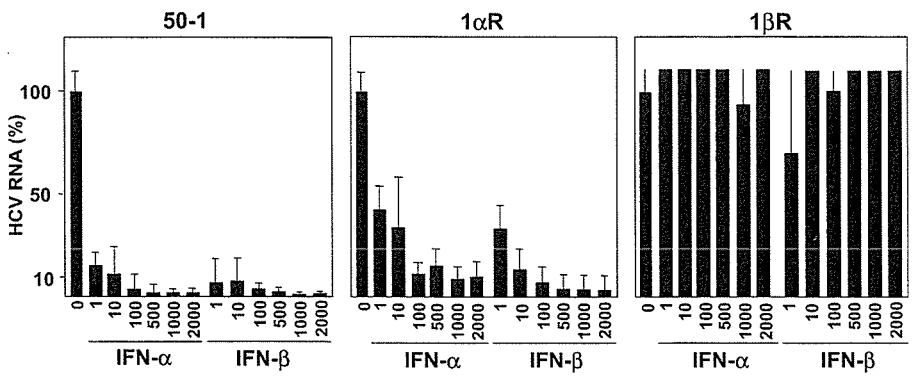

Fig. 4. IFN sensitivities of the replicons in 50-1, 1αR, and 1βR cells. Cells from each of these lines were treated with IFN-α or IFN-β (1, 10, 100, 500, 1000, and 2000 IU/ml each) for two days. Semi-quantitative RT-PCR was carried out to monitor the levels of replicon RNAs in the cells, as described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

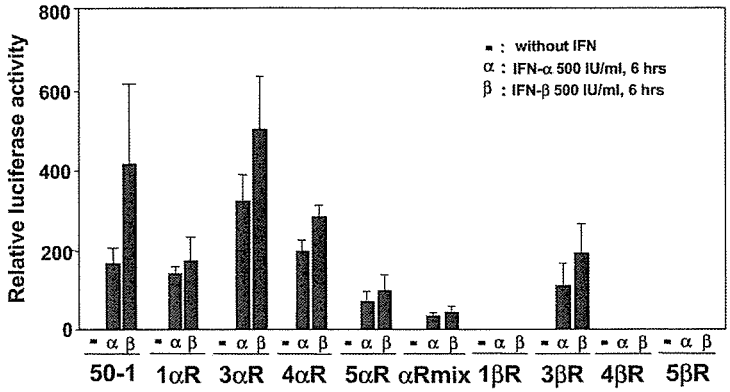

Fig. 5. IFN signal transduction in the established replicon cell lines. Regarding 50-1 and the nine replicon cell lines possessing IFN-resistant phenotype, dual luciferase reporter assay using pISRE(V2)-Luci [32] was performed as described previously [29]. The replicon cells were treated with IFN-α or 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 3 βR 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

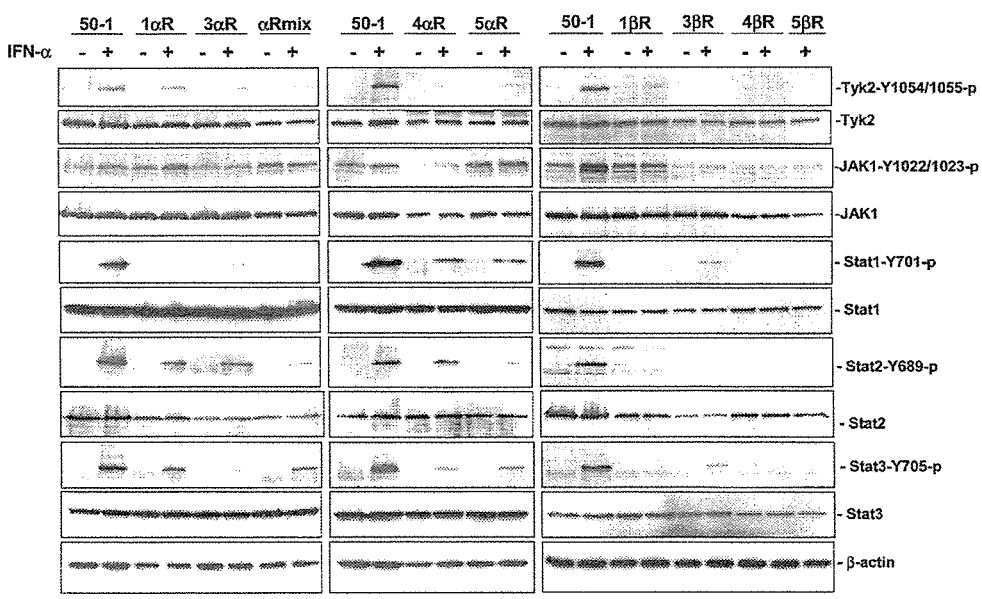

Fig. 6. Western blot analysis of the components involved in the IFN signal transduction pathway in the established replicon cell lines treated with IFN-α. The replicon cells were stimulated with or without IFN-α (500 IU/ml) for 30 min, and then Western blot analysis was performed as described under Materials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

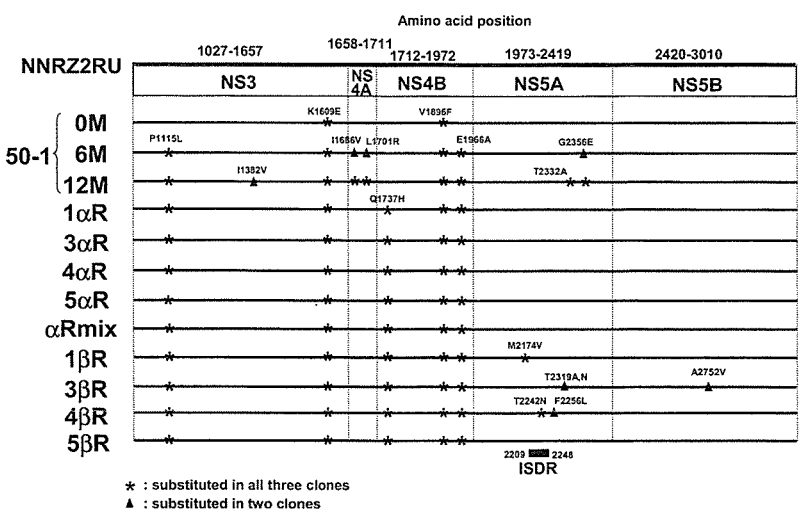

Fig. 7. Genetic analysis of the NS region of replicon RNAs in the established replicon cell lines possessing IFN-resistant phenotype. Compared with the amino acid sequences of NS region of the original replicon (NNRZ2RU), amino acid positions substituted in all thee clones and in two of three clones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

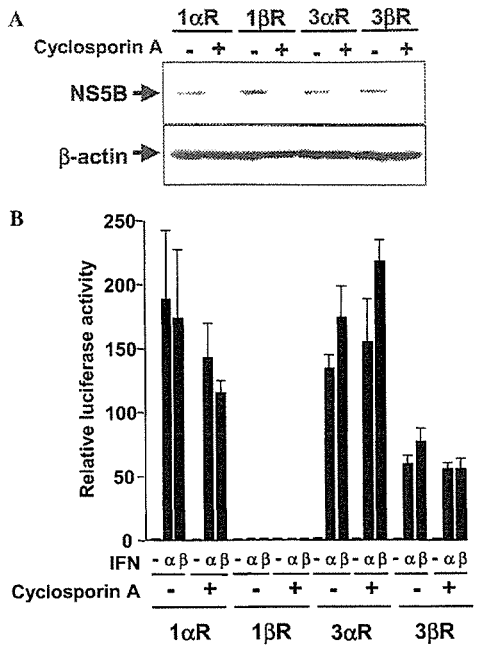

Fig. 8. Characterization of cured cells obtained from 1αR, 1βR, 3αR, and 3βR cells. (A) Western blot analysis. Regarding the four replicon cell lines possessing the IFN-resistant phenotype and their cured cell lines, NS5B was detected by immunoblot analysis using anti-NS5B antibodies. β-Actin was used 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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Promotion of Microsatellite Instability by Hepatitis C Virus Core Protein in Human Non-neoplastic Hepatocyte Cells

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ABSTRACT

Hepatitis C virus proteins exert an effect on a variety of cellular functions, including gene expression, signal transduction, and apoptosis, and because they possess oncogenic potentials, they have also been suggested to play an important role in hepatocarcinogenesis. Although the mechanisms of hepatocarcinogenesis remain poorly understood, we hypothesized that the disease may arise because of a disturbance of the DNA repair system by hepatitis C virus proteins. To test this hypothesis, we developed a reproducible microsatellite instability assay system for mismatch-repair using human-cultured cells transducted with pCXpur retrovirus expression vector, in which the puromycin resistance gene was rendered out-of-frame by insertion of a $(CA)_{17}$ dinucleotide repeat tract immediately following the ATG start codon. Using several human cancer cell lines known to be replication error positive or negative, we demonstrated that this assay system was useful for monitoring the propensity for mismatch-repair in the cells. This assay system was applicable to non-neoplastic human PH5CH8 hepatocytes, which could support hepatitis C virus replication. Using PH5CH8 cells, in which hepatitis C virus proteins were stably expressed by the retrovirus-mediated gene transfer, we found that the core protein promoted microsatellite instability in PH5CH8 cells. Interestingly, such promotion by the core protein only occurred in cells having the core protein belonging to genotype 1b or 2a and did not occur in cells having the core protein belonging to genotype 1a, 2b, or 3a. This is the first report to demonstrate that the core protein may disturb the DNA repair system.

INTRODUCTION

Hepatitis C virus (HCV), discovered in 1989, is the major causative agent of parenteral non-A, non-B hepatitis worldwide (1). Following the development of a method of diagnosing HCV infection (2), it became apparent that HCV infection frequently causes chronic hepatitis, and the persistent infection with HCV is implicated in liver cirrhosis and hepatocellular carcinoma (HCC; 1–4). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae* (5, 6). The HCV genome shows remarkable genetic heterogeneity and at least six major HCV genotypes, further grouped into >50 subtypes, have been identified to date (7, 8). The HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues, and this precursor protein is cleaved by the host and viral proteinases to generate at least 10 proteins in the following order: NH_2-core-envelope 1 (E1); E2; p7; nonstructural protein 2 (NS2); NS3; NS4A; NS4B; NS5A; NS5B; and COOH (9–11). These HCV proteins not only play a role in viral replication but also affect a variety of cellular functions, including gene expression, signal transduction, and apoptosis (12, 13).

HCV replication and the viral protein expression have been observed in HCCs, but the molecular mechanism of HCV-associated hepatocarcinogenesis remains poorly understood. One major reason for this is the lack of reproducible and efficient HCV proliferation in cell culture (14). In HCV-related hepatocarcinogenesis, it has been speculated that repeated hepatocytic regeneration processes also occur in HCV-infected individuals to offset the damage caused by HCV multiplication and maintain sufficient liver function. Such a process of damage and regeneration probably enhances the likelihood of genetic alteration (15). In addition, it has also been reported that no significant differences were found in the number and type of chromosomal imbalances between hepatitis B virus- and HCV-infected HCCs (16). This finding is consistent with models suggesting that hepatitis B virus and HCV cause cancer through nonspecific inflammatory and regenerative processes (17). On the other hand, it has been demonstrated that HCV proteins significantly influence a variety of oncogenic processes. For example, the HCV core protein may cooperate with H-ras in the process of transforming the cells into malignant phenotypes (18), and the constitutive expression of core protein in transgenic mice has been shown to induce HCC (19). Furthermore, it has been reported that the HCV NS3, NS4B, and NS5A proteins also have oncogenic potential (20–22). Therefore, it is likely that HCV proteins contribute to the initiation or development of HCC.

We reported previously that PH5CH8 cells cloned from PH5CH cell line (23) could support HCV replication (24), although the level of HCV proliferation was fairly low. PH5CH cell line was established by immortalization with SV40 large T antigen using non-neoplastic liver tissue from a patient with HCV-related HCC (23). PH5CH8 cells are considered to be useful in examining the role of HCV proteins during the process of hepatocarcinogenesis. In addition, PH5CH8 cells possess wild type of p53 and Rb protein and show nonmalignant phenotype (23), although SV40 large T antigen would partially repress the function of p53. Then, we speculated that the DNA repair system of host cells may be one of the target sites of HCV proteins, because the constant operation of this system is crucial to the process of inflammation and regeneration of hepatic lesions in patients with chronic hepatitis C. Although DNA damages caused by such damaging agents as X-rays, UV light, and alkylating agents are repaired by base excision, nucleotide excision, recombinational repair, and so forth, the mismatch-repair (MMR) system is used to repair A-G or T-C mismatches, insertion, and deletion caused by the replication errors (RER) during the regenerative process (25). In addition, studies on genetic instability using clinical specimens from patients with HCC have revealed that microsatellite instability (MSI) was found in approximately 20% of the patients examined (26, 27), whereas no MSI was found in the histologically normal liver (26). In this study, we focused on the MMR system to examine the effects of HCV proteins. For this purpose, we developed a novel MSI assay system in human cultured cells using the retrovirus expression vector containing the (CA) repeat sequence. Our results indicate that the core protein may promote MSI in PH5CH8 cells.

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MATERIALS AND METHODS

Cell Lines. The human colon cancer cell lines LS174T, LoVo, and SW480 were provided by the Cell Resource Center of the Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University). LS174T, LoVo, and SW480 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), F12 supplemented with 20% FBS, and RPMI 1640 supplemented with 10% FBS, respectively. The additional human colon cancer cell line HCT116 was a kind gift of K. Shimizu (Okayama University) and was maintained in DMEM supplemented with 10% FBS. Cells of the human cervical carcinoma cell line HeLa were maintained in DMEM supplemented with 10% FBS. PH5CH8 hepatocytes were maintained as described previously (23). Bosc 23 cells were also maintained in DMEM supplemented with 10% FBS.

Construction of Expression Vectors. pCX retroviral vector pCXpur (28), a kind gift of T. Akagi (Osaka Bioscience Institute), which contains the resistance gene for puromycin, was used for the construction of pCXpur containing 17 CA repeats [pCXpur/$(CA)_{17}$/out-of-frame and pCXpur/$(CA)_{17}$/in-frame]. pCXpur/$(CA)_{17}$/out-of-frame was generated by inserting a fragment that was ligated with *SalI* sites of the PCR products of two primer sets (primers PU-NotI5′ and FCA3′; primers TA5′ and PU-StuI3′; Table 1) using pCXpur as a template, into the *NotI-StuI* sites of pCXpur. A schematic of the construction of the pCXpur/$(CA)_{17}$/out-of-frame retrovirus vector is shown in Fig. 1. pCXpur/$(CA)_{17}$/in-frame was also generated by inserting a fragment that was ligated with *SalI* sites of the PCR products of two primer sets (primers PU-NotI5′ and FCA3′; primers PU-5′ and PU-StuI3′; Table 1) using pCXpur as a template, into the *NotI-StuI* sites of pCXpur. The nucleotide sequences of the obtained pCXpur/$(CA)_{17}$/out-of-frame and pCXpur/$(CA)_{17}$/in-frame retrovirus vector were confirmed by Big Dye termination cycle sequencing using an Applied Biosystem 310 automated sequencer (Applied Biosystems, Norwalk, CT).

Retrovirus Infection of the Recipient Cell Lines. Transient transfections were performed with 2 μg of DNA and 6 μl of FuGENE6 (Boehringer Mannheim, Mannheim, Germany) in 6-well plates according to the manufacturer's instructions. Retrovirus infection was performed by a method described previously (29, 30). At 3 h postinfection, the cells were cultured with fresh medium until reaching confluence (approximately 3 days), and then the cells were used for the selection with puromycin (5 μg/ml) or blasticidin (20 μg/ml).

MSI Assay Using Human Cultured Cells. Various human-cultured cells (2×10^5 cells) were plated on a 6-well plate for 24 h before retrovirus infection and were infected with the retrovirus pCXpur/$(CA)_{17}$/out-of-frame or pCXpur/$(CA)_{17}$/in-frame. Mock infection was also performed as a control. At 3 days postinfection, cells were passaged to a 10-cm dish and further cultured for 2 days, and then aliquots of 1×10^5 cells were placed in 10-cm dishes for selection by puromycin (5 or 10 μg/ml) and grown for 14 days with a medium change every 3–4 days. In some experiments, the culture period from retrovirus infection to addition of puromycin was changed to 9–21 days from 5 days. The obtained puromycin-resistant (pur^R) colonies were stained with Coomassie Brilliant Blue (0.6g/liter in 50% methanol-10% acetic acid) and were automatically counted using a ChemiImager 4000 (α Innotech Corp.). Triplicate infection experiments were repeated at least three times to verify the reproducibility of the results. To examine the efficiency of retrovirus infection, the retrovirus pCLMFG-LacZ (31) was used, and at 2 days postinfection, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Preparation of PH5CH8 Cells Stably Expressing HCV Proteins. PH5CH8 cells were infected with retrovirus pCXbsr encoding various HCV proteins, as described above. Because the HCV core protein constructs, pCXbsr/core(1b-M) (32), pCXbsr/core(1b-P) (32), pCXbsr/core(1b-HCC) (33), pCXbsr/core(1a) (30), pCXbsr/core(2a) (30), pCXbsr/core(2b) (30), and pCXbsr/core(3a) (30), which are able to express core(1b-M), core(1b-P), core(1b-HCC), core(1a), core(2a), core(2b), and core(3a) proteins, respectively (the numbers and letters in parentheses indicate the HCV genotype), were used to obtain the PH5CH8 cells stably expressing the core protein. Because the other HCV protein constructs, pCXbsr/E1(1b-P), pCXbsr/E2(1b-P), and pCXbsr/NS5A(1b-P), which are able to express E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins, respectively, were also used to obtain the PH5CH8 cells stably expressing E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins, respectively. pCXbsr was used as a control. At 2 days postinfection, the PH5CH8 cells were changed with fresh medium containing blasticidin (20 μg/ml), and the culture was continued for 7 days to select the cells expressing HCV proteins.

MSI Assay Using PH5CH8 Cells Stably Expressing HCV Proteins. The PH5CH8 cells (2×10^5 cells) stably expressing HCV protein [core, E1, E2, or NS5A] were used for the MSI assay as described above. The PH5CH8 cells, which became resistant to blasticidin after infection of the retrovirus pCXbsr, were used as a control. These cells were further infected with the retrovirus pCXpur/$(CA)_{17}$/out-of-frame, and then an MSI assay was performed according to the method described above, with the exception that double-selections were performed using both blasticidin (20 μg/ml) and puromycin (5 or 10 μg/ml).

Sequence Analysis of the (CA) Repeat-Surrounding Region Recovered from pur^R Colonies. The pur^R colonies (7–10 independent colonies) obtained in the MSI assay were randomly selected and grown to confluence in 6-well plates, and their genomic DNAs were prepared using an ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan) as indicated by the manufacturer. These DNAs (each 1 μg) were used for the amplification of the (CA) repeat-surrounding region by PCR using a primer set (primers MIB5′ and MIE3′; Table 1). Thirty-five PCR cycles were performed, and each cycle consisted of annealing at 63°C for 45 s, primer extension at 68°C for 1 min, and denatur-

Table 1 *Oligonucleotides used in this study*

Oligonucleotide	Sequence	Direction
PU-NotI5′	aattaaGCGGCCGCGGTAACAATTGTTAACTAAC	Forward
FCA3′	attatGTCGACTGTGTGTGTGTGTGTGTGTGTGTGTG	Reverse
	GTGGTCATGGTATTATCGTGTTTTTCAAAGGAAAACC	
	ACGTCCCGTGGTTCGGGGGGCCTAGAC	
TA5′	attatGTCGACTAACCGAGTACAAGCCCACGGTG	Forward
PU-StuI3′	attatAGGCCTTCCATCTGTTGCTGCGCGGCCAG	Reverse
PU-5′	attatGTCGACACCGAGTACAAGCCCCACGGTG	Forward
MIB5′	attatGGATCCGCACATGCTTTACATGTGTTTAGTCG	Forward
MIE3′	attatGAATTCAGGCGCACCGTGGGCTTGTACTC	Reverse
hMLH1F	TTCTCAGGTTATCGGAGCCAGCAC	Forward
hMLH1R	CTTCGTCCCAATTCACCTCAGTGG	Reverse
hMSH2F	TACATGTCACAGCACTCACCACTG	Forward
hMSH2R	CTCCTCAAGTTCCAGGGCTTTCTG	Reverse
hMSH6F	ACAAGGGGCTGGGTTAGCAAAAG	Forward
hMSH6R	AGTCACCATTCTCTTCCGCTTTCG	Reverse
hPMS2F	CAGGTTTCATTTCACAATGCACGC	Forward
hPMS2R	TTACCTTCAACATCCAGCAGTGGC	Reverse
hMSH3F	GATGGCATTTTCACAAGGATGGG	Forward
hMSH3R	CTGGCGGATAATGGGTGACAAAC	Reverse
hPMS1F	GGCAACAGTTCGACTCCTTTCAAG	Forward
hPMS1R	TTGATACCCTCCCCGTTATCTCGC	Reverse
GAPDHF	GATGCTGGCGCTGAGTACGTCG	Forward
GAPDHR	GAGGAGACCACCTGGTGCTCAG	Reverse

The recognition sites of restriction enzymes are underlined.

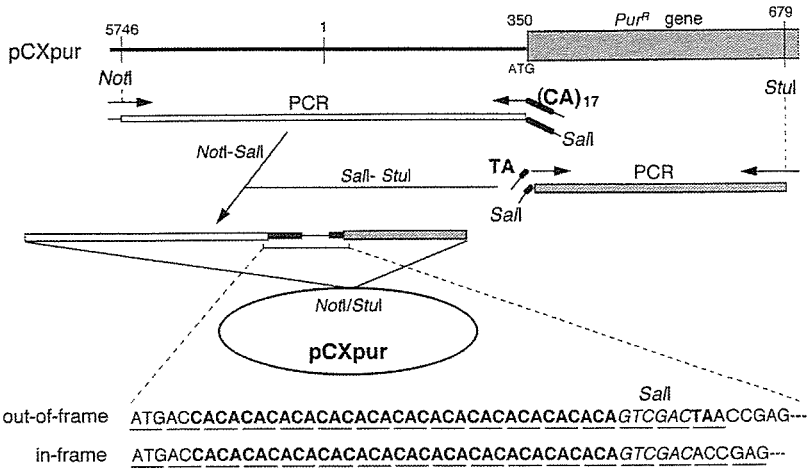

Fig. 1. Construction of pCXpur retrovirus vector containing the (CA) repeat sequence. The outline for the construction of the pCXbsr/$(CA)_{17}$/out-of-frame retrovirus vector is presented schematically. The nucleotide sequences of the (CA) repeat unit of pCXbsr/$(CA)_{17}$/out-of-frame and pCXbsr/$(CA)_{17}$/in-frame are shown (*bottom*), and each codon is *underlined*. pur^R, puromycin-resistant.

ation at 97°C for 45 s using proofreading KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). PCR products (186 bp) containing the (CA) repeat sequence were cloned into the *Bam*HI and *Eco*RI sites of pCRII-TOPO (Invitrogen, Carlsbad, CA). Plasmid inserts were sequenced in both the sense and antisense.

Western Blot Analysis. The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as described previously (10). Anti-core monoclonal antibody (2ZCP9; Institute of Immunology Co., Tokyo, Japan), anti-E1 monoclonal antibody (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-E2 monoclonal antibody (34), and anti-NS5A antibody (a generous gift from A. Takamizawa, Osaka University) were used for the detection of core, E1, E2, and NS5A proteins, respectively. Anti-β-actin antibody (AC-15; Sigma) was also used for the detection of β-actin as an internal control. Immunocomplexes on the filters were detected by enhanced chemiluminescence assay (Renaissance; NEN, Boston, MA).

Reverse Transcription (RT)-PCR. Total cellular RNA was extracted using an ISOGEN extraction kit for the RT-PCR analysis. RT-PCR was performed by a method described previously (30). The sequences of hMLH1 (accession number U07418), hMSH2 (accession number U03911), hMSH6 (accession number U54777), hPMS1 (accession number U13695), hPMS2 (accession number U14658), hMSH3 (accession number U61981), and glyceraldehyde-3-phosphate dehydrogenase (accession number NM 002046) were used to design the primers listed in Table 1. Twenty-five cycles of PCR (20 cycles for glyceraldehyde-3-phosphate dehydrogenase only) were performed, and the amplified DNA was detected by staining with ethidium bromide after separation by 3% agarose gel electrophoresis.

RESULTS

Construction of the Retrovirus Vectors Containing the Microsatellite (CA) Repeat Sequence. The retrovirus expression vector pCXpur (28) contains a pur^R gene to select for transduced cells. Initially, we made a pCXpur/$(CA)_{17}$/in-frame, in which 42 nucleotides [AC +17 CA repeats + GTCGAC (*Sal*I site)] were inserted immediately following the ATG initiation codon of the pur^R gene, and examined the influence of this insert on the pur^R activity. We confirmed that the human colon cancer SW480 cells (35), which were known to possess RER− (MMR proficient) phenotype, infected with the retrovirus pCXpur/$(CA)_{17}$/in-frame were able to proliferate in the presence of puromycin (10 μg/ml; data not shown), indicating that the pur^R gene product from pCXpur/$(CA)_{17}$/in-frame is functional in the cells. We next constructed pCXpur/$(CA)_{17}$/out-of-frame, in which the pur^R gene was rendered out-of-frame by the insertion of 44 nucleotides [AC +17 CA repeats + GTCGAC (*Sal*I site) + TA (to make a TAA stop codon)] immediately following the ATG initiation codon, as shown in Fig. 1. By this modification, the pur^R gene product should not be produced from pCXpur/$(CA)_{17}$/out-of-frame. Using the SW480 cells (RER−), we confirmed that cells infected with the retrovirus pCXpur/$(CA)_{17}$/out-of-frame were also unable to survive in the presence of puromycin (10 μg/ml; data not shown), indicating that the pur^R gene product is not produced from pCXpur/$(CA)_{17}$/out-of-frame, as we expected (Fig. 1). With regard to the plasmid vector for MSI assay at the cell-culture level, to date, several similar vector systems using the neomycin resistance gene, hygromycin B phosphotransferase gene, or β-galactosidase gene have been reported (36–39), but there has been no system using the pur^R gene. Puromycin has an advantage for the fast (within a few days) and keen-edged selection of the cells. In the present study, none of the cells lines examined were able to survive in the presence of 1 μg/ml of puromycin.

Establishment of the MSI Assay System. In this assay, after the transduction of pCXpur/$(CA)_{17}$/out-of-frame [pCXpur/$(CA)_{17}$/in-frame as a positive control], the recipient cells were cultured for 5 days, and then the cells were selected with puromycin (5 or 10 μg/ml). In theory, although the cells transducted with pCXpur/$(CA)_{17}$/in-frame are able to proliferate in the presence of puromycin, the cells transducted with pCXpur/$(CA)_{17}$/out-of-frame should not be able to survive in the presence of puromycin, as we confirmed in RER− cells. However, if some frameshift mutations do occur in the vicinity of the $(CA)_{17}$ sequence during the 5 days of culture before addition of puromycin, such cells would become pur^R cells and grow up even in the presence of puromycin. As a consequence, we therefore considered the colonies to be pur^R colonies at about 2 weeks after addition of puromycin. Because the microsatellite insert puts the pur^R gene in the −1 reading frame, detectable dinucleotide frameshift mutations include the deletions of 2, 8, 14, 20, 26, or 32 bp and insertions of 4, 10, or 16 bp, and so forth. As the method of gene transduction, we used retrovirus infection because of its highly efficient gene transfer into cells. Recently, Zienolddiny *et al.*(38) also used a retrovirus infection system for MSI assay.

We initially verified our method using several human cell lines. It has been reported that HCT116 and LoVo cells exhibited marked dinucleotide repeat instability, because HCT116 cells possessed a nonsense mutation in exon 9 in *hMLH1* gene, and LoVo cells were *hMSH2*-deficient (deletion of exons 4–8; 40). LS174T cells have been also reported to possess RER+ (MMR deficient) phenotype by the analysis of 32 microsatellite loci (41). On the other hand, HeLa and SW480 cells are known to possess RER− phenotype because of accurately replication of repetitive DNA and correction of mismatches

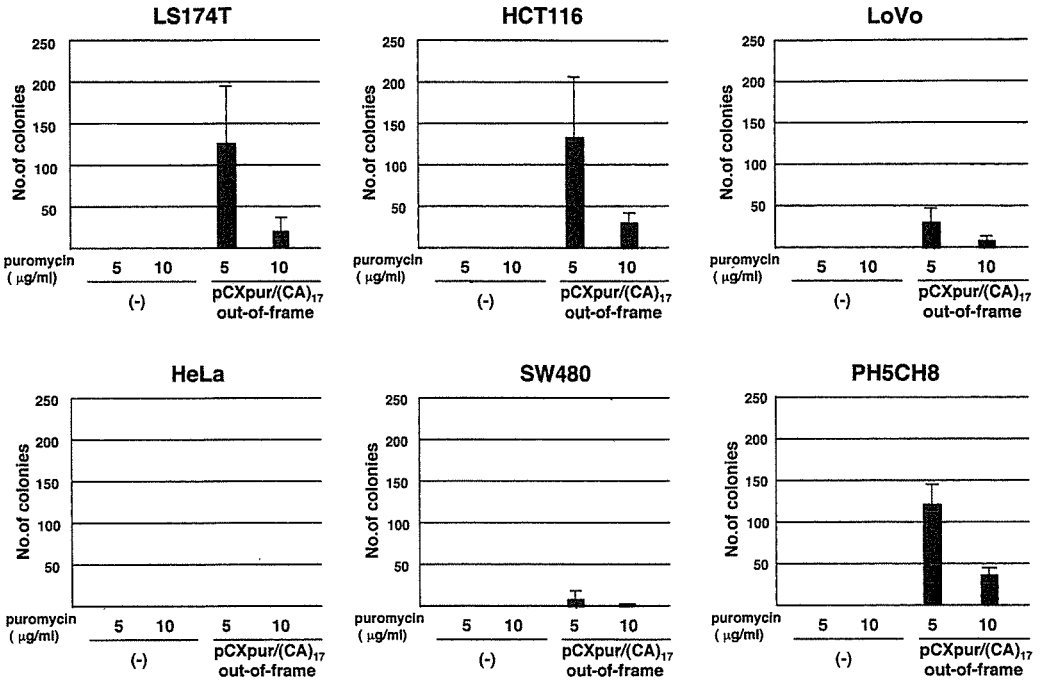

Fig. 2. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame in various cell lines, The puromycin-resistant colonies stained with Coomassie Brilliant Blue were automatically counted by a ChemiImager 4000. *(-)*, mock infection.

(35). Therefore, HCT116, LoVo and LS174T were used as the RER+ cell lines, and HeLa and SW480 were used as the RER− cell lines. PH5CH8 cells were also used for the analysis using our method, although the state of MMR system has not yet been determined by the analysis of microsatellite loci.

All cell lines examined at 2 days postinfection with the retrovirus pCLMFG-LacZ were efficiently stained with 5-bromo-4-chloro-3-indolyl-b-D-galactropyranoside, although the level of staining and the percentage of stained cells in the LoVo and HeLa cell lines were somewhat lower than in the other cell lines (data not shown). At 2 weeks after the selection with puromycin, pur^R colonies were counted after staining with Coomassie Brilliant Blue. As shown in Fig. 2, a substantial number of colonies were obtained in the RER+ cell lines (LS174T, HCT116, and LoVo), whereas no or only a few colonies were obtained in RER− cell lines (HeLa and SW480). Because the growth rate of LoVo cells was rather lower than those of LS174T and HCT116 cells, it might cause the low number of pur^R colonies in LoVo cell line despite RER+ phenotype. In all cases, the number of pur^R colonies obtained in the presence of puromycin (10 μg/ml) was lower than that obtained in the presence of puromycin (5 μg/ml), suggesting that the colonies expressing pur^R gene at low level were not able to survive in the presence of puromycin (10 μg/ml). This phenomenon may be explained by the reason that the expression level of pur^R gene depends on the integration site of the retrovirus. All cell lines infected with the retrovirus pCXpur/$(CA)_{17}$/in-frame became fully confluent up to 2 weeks after the selection with puromycin (data not shown), and no colonies were obtained from any of the mock-infected cell lines (Fig. 2). These results revealed that the number of pur^R colonies obtained indicated a good correlation with the RER phenotypes. Interestingly, however, nonmalignant PH5CH8 cells showed the RER+ phenotype, because the number of pur^R colonies obtained in PH5CH8 cells was similar to that obtained in LS174T and HCT 116 cells showing the RER+ phenotype. In addition, the modification of the culture period (from 5 days to 14 or 21 days) before addition of puromycin in the MSI assay using LS174T cells revealed that the number of pur^R colonies increased in a time-dependent manner at both of two different concentrations (5 and 10 μg/ml) of puromycin (data not shown).

Sequence Analysis of the Integrated (CA) Repeat Unit in the pur^R Colonies. To further evaluate the reliability of our method, 7–10 independent pur^R colonies derived from LS174T, HCT116, and PH5CH8 cells were isolated and expanded. Using the pCXpur/$(CA)_{17}$/out-of-frame vector DNA, we initially confirmed that KOD-plus DNA polymerase was superior to nonproofreading *Taq*DNA polymerases, as described previously (33), because 3 of 10 clones obtained by *Taq*DNA polymerases showed deletions of 1–3 nucleotides, whereas all 10 clones obtained by KOD-plus DNA polymerase showed the exact $(CA)_{17}$ sequence. Therefore, using the genomic DNA from each colony, a fragment of 186 bp containing the CA repeat unit was amplified by proofreading KOD-plus DNA polymerase and was cloned into pCRII-TOPO for sequencing analysis. In most cases, four-independent clones were obtained from each pur^R colony and sequenced. Table 2 provides a summary of all of the sequenced clones. As can be seen, at least one clone, which became in-frame by the deletion of 2 bp (CA) from $(CA)_{17}$, was obtained from all pur^R colonies examined. In addition to $(CA)_{16}$, $(CA)_{13}$ resulting in in-frame was obtained from one colony in LS174T cells, and $(CA)_{19}$, $(CA)_{10}$, and $(CA)_7$ resulting in in-frame were obtained from four colonies in HCT116 cells. One interesting additional sequence, $(CA)_{17}A$, which resulted in in-frame was also obtained from one colony in HCT116 cells. Although all of the clones obtained from HCT116-derived colonies showed the expected pattern of frameshift mutation resulting in in-frame, a single clone possessing the original $(CA)_{17}$ without mutation was also obtained from 4 LS174T-derived colonies. Because each of the remaining three clones from these four colonies possessed $(CA)_{16}$ resulting in in-frame, it is suggested that more than two copies including the retrovirus possessing $(CA)_{17}$ sequence were infected and integrated in a single target cell. Com-