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

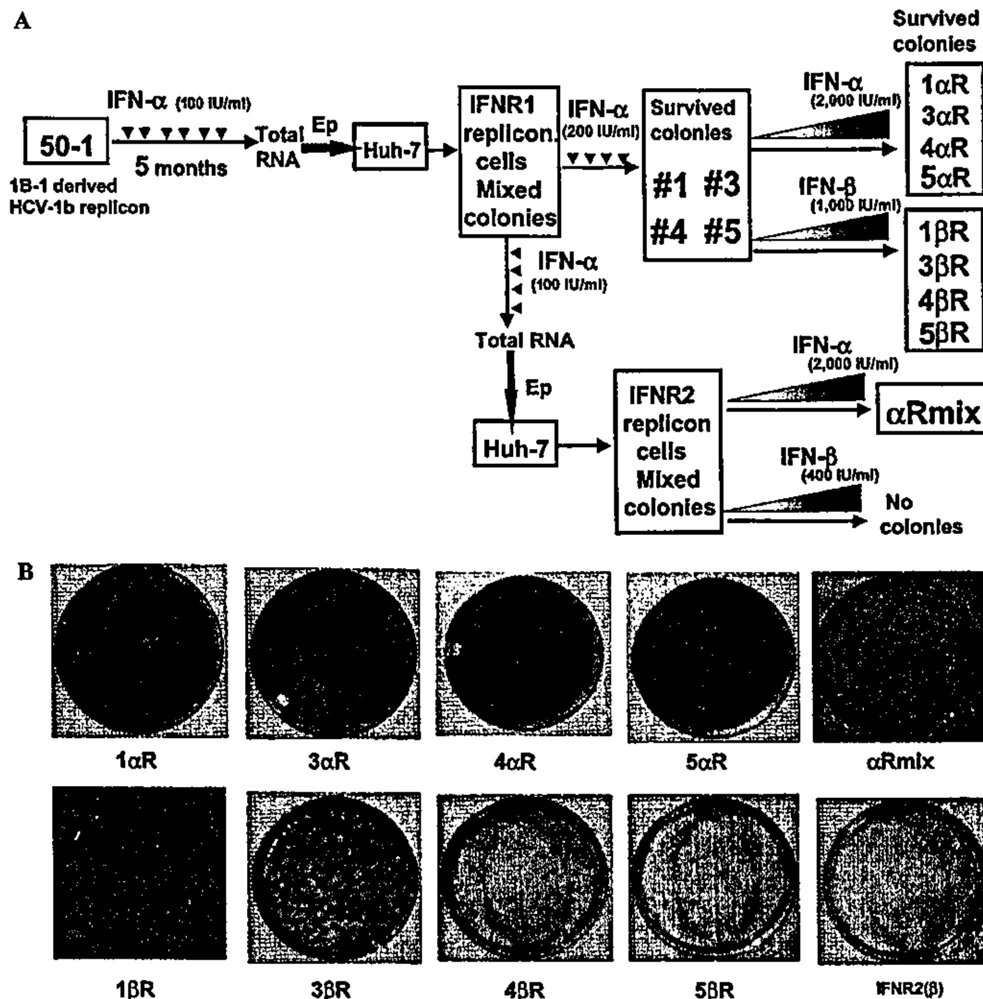

Fig. 1. Isolation of HCV replicon cell lines possessing IFN-resistant phenotype. (A) Outline of the isolation of HCV replicon cells possessing IFN-resistant phenotype. Ep indicates electroporation of total cellular RNA to Huh-7 cells. (B) HCV replicon cells possessing resistance against IFN-α (2000 IU/ml)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

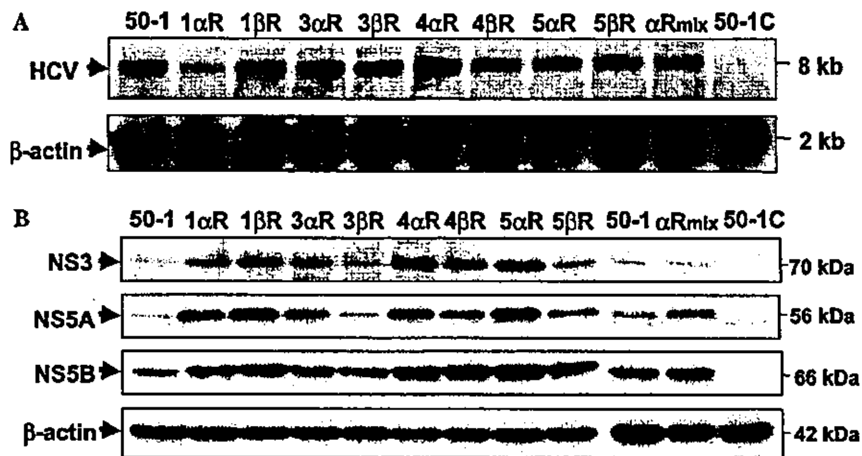

Fig. 2. Characterization of replicon cells possessing IFN-resistant phenotype. (A) Northern blot analysis. Total RNAs from 50-1 and nine replicon cell lines possessing IFN-resistant phenotype, as well as total RNA from 50-1C cells (cured cells), were analyzed by Northern blot analysis using a positive-stranded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

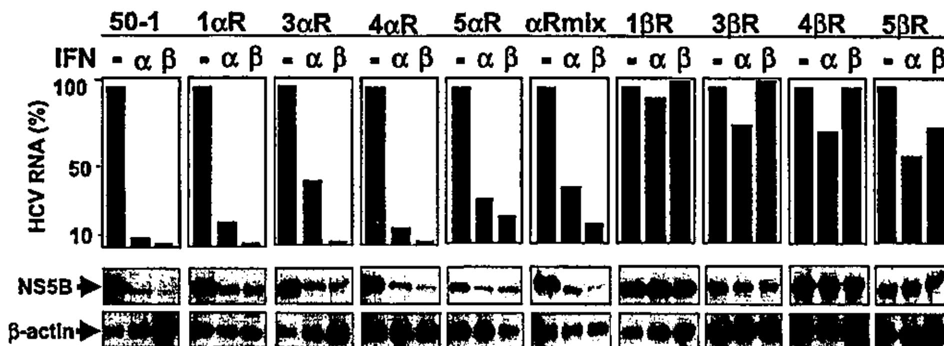

Fig. 3. IFN-resistant phenotypes of the established replicon cell lines. 50-1 and nine replicon cell lines possessing IFN-resistant phenotype were treated with IFN-α or IFN-β (500 IU/ml each) for two days for semi-quantitative RT-PCR analysis (upper panel) and for five days for Western blot analysis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

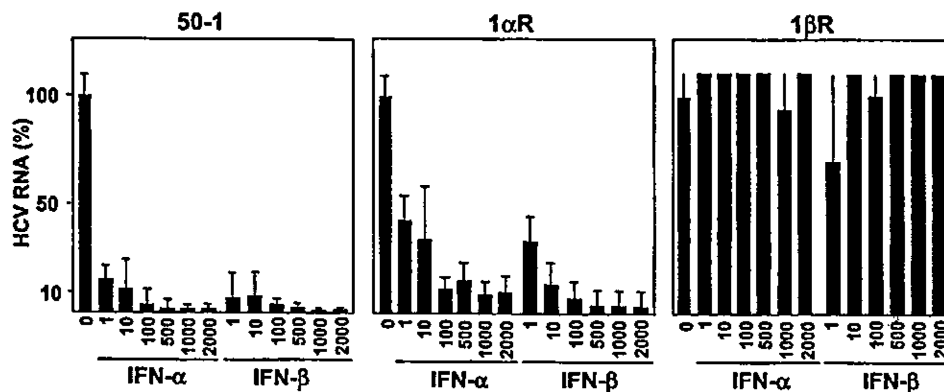

Fig. 4. IFN sensitivities of the replicons in 50-1, 1αR, and 1βR cells. Cells from each of these lines were treated with IFN-α or IFN-β (1, 10, 100, 500, 1000, and 2000 IU/ml each) for two days. Semi-quantitative RT-PCR was carried out to monitor the levels of replicon RNAs in the cells, as described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

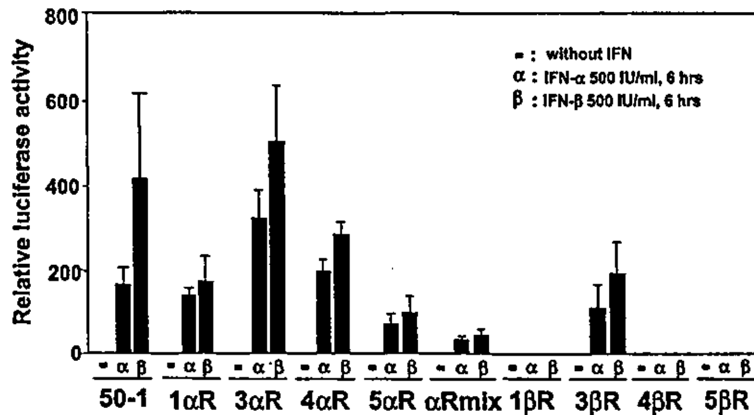

Fig. 5. IFN signal transduction in the established replicon cell lines. Regarding 50-1 and the nine replicon cell lines possessing IFN-resistant phenotype, dual luciferase reporter assay using pISRE(V2)-Luci [32] was performed as described previously [29]. The replicon cells were treated with IFN-α or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

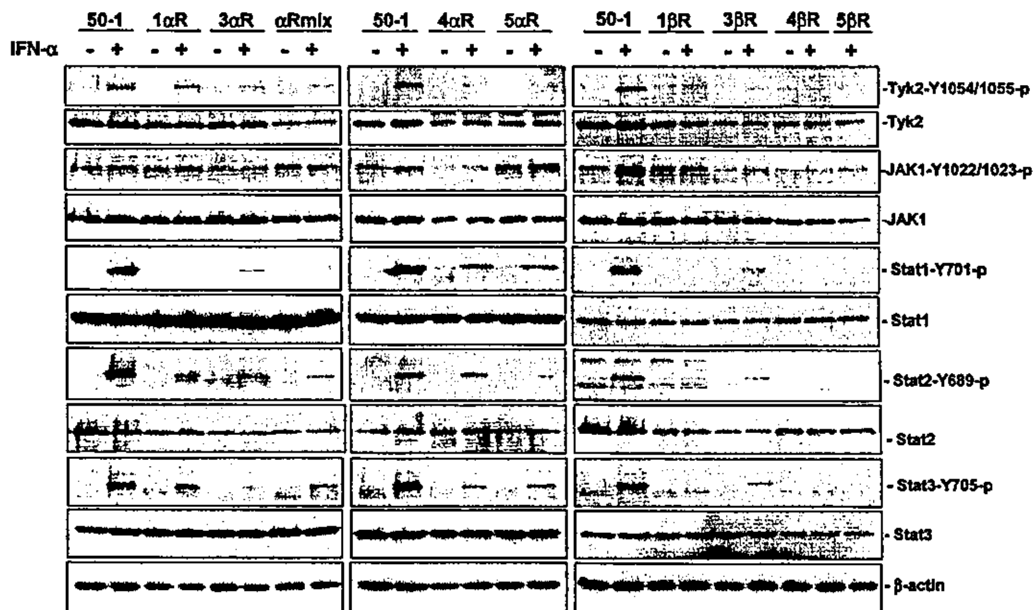

Fig. 6. Western blot analysis of the components involved in the IFN signal transduction pathway in the established replicon cell lines treated with IFN-α. The replicon cells were stimulated with or without IFN-α (500 IU/ml) for 30 min, and then Western blot analysis was performed as described under Materials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

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 *Sal*I 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 *Not*I-*Stu*I 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 *Sal*I 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 *Not*I-*Stu*I 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′	attatGTCGACTGTGTGTGTGTGTGTGTGTGTGTGTGTG GTGGTCATGGTATTATCGTGTTTTTCAAAGGAAAACC ACGTCCCGTGGTTCGGGGGGCCTAGAC	Reverse
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.

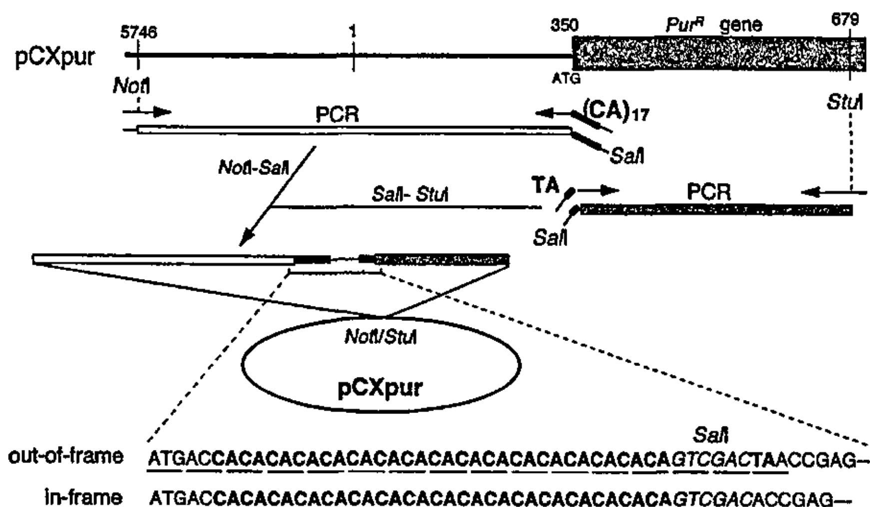

Fig. 1. Construction of pCXpur retrovirus vector containing the (CA) repeat sequence. The outline for the construction of the pCXbsr/$(CA)_{17}$/out-of-frame retrovirus vector is presented schematically. The nucleotide sequences of the (CA) repeat unit of pCXbsr/$(CA)_{17}$/out-of-frame and pCXbsr/$(CA)_{17}$/in-frame 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

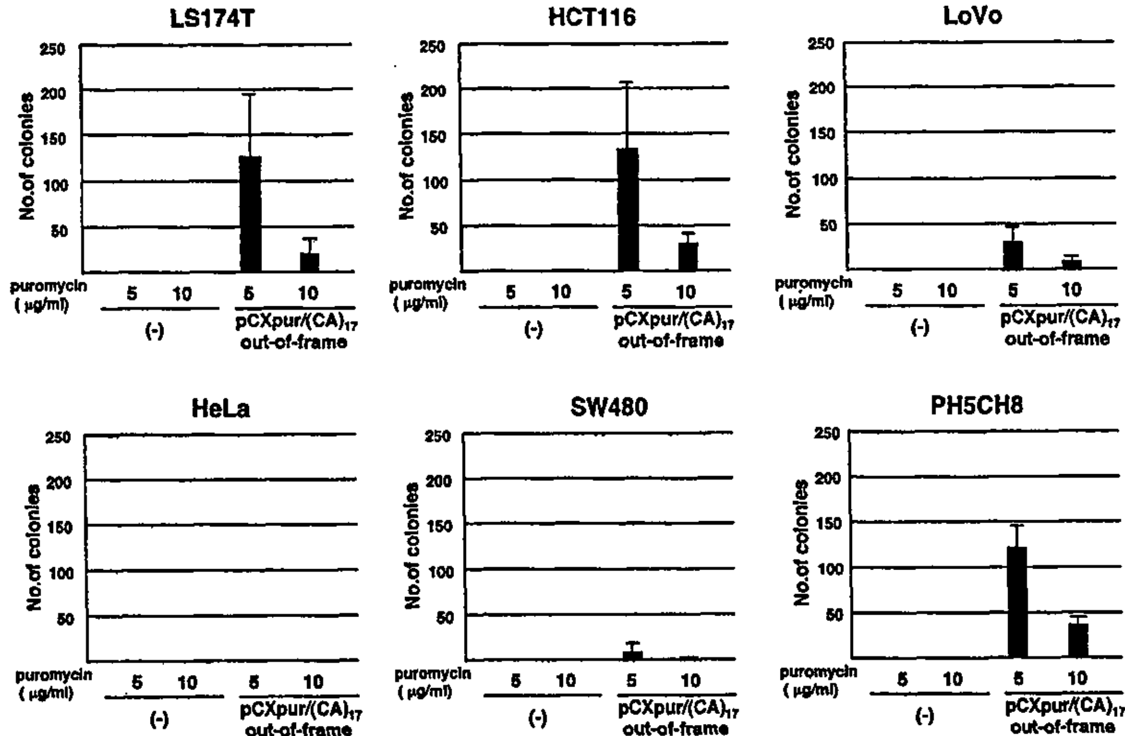

Fig. 2. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame in various cell lines. The puromycin-resistant colonies stained with Coomassie Brilliant Blue were automatically counted by a ChemiImager 4000. (-), mock infection.

(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-

Table 2 *Sequence analysis of (CA) repeat region obtained from the pur^R colonies*

The numbers in the table indicate the actual number of plasmid clones obtained and sequenced.

PCR product		Colony no. 1	2	3	4	5	6	7	8	9	10
LS174T (resistant to puromycin 10 μg/ml)											
$(CA)_{17}$	out-of-frame	1	1			1					1
$(CA)_{16}$	in-frame	3	3	4	4	3	4	3	4	4	3
$(CA)_{13}$	in-frame							1			
HCT116 (resistant to puromycin 10 μg/ml)											
$(CA)_{19}$	in-frame				3						
$(CA)_{17}A$	in-frame					3					
$(CA)_{16}$	in-frame	3	4	3	1	1	4	2			
$(CA)_{10}$	in-frame							2			
$(CA)_{7}$	in-frame	1		1							
PH5CH8 (resistant to puromycin 10 μg/ml)											
$(CA)_{17}$	out-of-frame							1	1	1	
$(CA)_{16}$	in-frame	4	4	3	2	2	4	3	2	1	2
$(CA)_{15}$	out-of-frame								1		
$(CA)_{14}$	out-of-frame									2	
$(CA)_{13}$	in-frame			1	2						
$(CA)_{9}$+CC	in-frame										2
$(CA)_{7}$	in-frame					2					

pared with the results from LS174T and HCT116 cells, PH5CH8-derived colonies showed a variety of mutation patterns. Although the $(CA)_{16}$ sequence was obtained from all colonies, $(CA)_{13}$ and $(CA)_7$ resulting in in-frame were obtained from two colonies and one colony, respectively, and $(CA)_9$CC resulting in in-frame was also obtained from one additional colony. In addition, $(CA)_{15}$ and $(CA)_{14}$ resulting in out-of-frame were obtained from a single colony, respectively, and the original $(CA)_{17}$ without mutation was also obtained from the three colonies. These results suggest that at least three copies of retrovirus were initially infected and integrated in a single target cell. In summary, sequence data on the (CA) repeat region indicated that the pur^R colonies possessed the frameshift mutation (2-bp deletion) resulting in in-frame in the open reading frame of pur^R gene. Taken together with these results, we concluded that our method can be used as an MSI assay at the cell-culture level.

HCV Core Protein Promoted MSI in PH5CH8 Cells. Because PH5CH8 cells did not show any tumorigenic potential when inoculated s.c. into thymic nude mice (23), we were surprised by the result that PH5CH8 cells showed the RER+ phenotype, as did the human colon cancer cell lines. Although the mechanism responsible for this finding is unclear, we speculate that HCV proteins may have further promoted MSI in PH5CH8 cells. Therefore, to evaluate this possibility, we initially prepared PH5CH8 cells stably expressing HCV protein [core(1b-P), E1(1b-P), E2(1b-P), or NS5A(1b-P)] as the recipient cells for the pCXpur/$(CA)_{17}$/out-of-frame retrovirus infection, by the pCXbsr/core(1b-P), pCXbsr/E1(1b-P), pCXbsr/E2(1b-P), or pCXbsr/NS5A(1b-P) retrovirus infection and following selection with blasticidin. As control recipient cells, we prepared PH5CH8 cells infected with retrovirus pCXbsr and selected with blasticidin. After retrovirus infection and following selection with blasticidin for 7 days, we monitored the growth curve of these blasticidin-resistant PH5CH8 cells, and we observed that the growth rates of these cells were almost the same (data not shown). We also confirmed by Western blot analysis the stable expression of core(1b-P), NS5A(1b-P), E1(1b-P), and E2(1b-P) proteins in PH5CH8 cells at day 10 and day 19 post-infection with retrovirus pCXbsr encoding HCV proteins (data not shown). Using these PH5CH8 cells, we performed an MSI assay, and found that the number of pur^R colonies obtained from the cells expressing the core(1b-P) protein was approximately 1.5-fold (selection with 5 μg/ml of puromycin) and approximately 2.5-fold (selection with 10 μg/ml of puromycin) higher than that from the control cells, as shown in Fig. 3. As compared with the core(1b-P) protein, the E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins did not increase the number of pur^R colonies, although NS5A(1b-P) protein slightly decreased the number of pur^R colonies. Because the increase of pur^R colonies in PH5CH8 cells expressing the core(1b-P) protein was reproducibly observed, it was suggested that core(1b-P) protein was able to further promote the MSI in PH5CH8 cells.

Promotion of MSI by the Core Protein Depends on HCV Genotype or Strain. Because the core protein is known to show some aa sequence heterogeneity among HCV genotypes (7, 8), we examined whether or not HCV core proteins other than the core(1b-P) protein are able to promote the MSI, using pCXbsr/core(1a), pCXbsr/core(2a), pCXbsr/core(2b), and pCXbsr/core(3a) retrovirus vectors

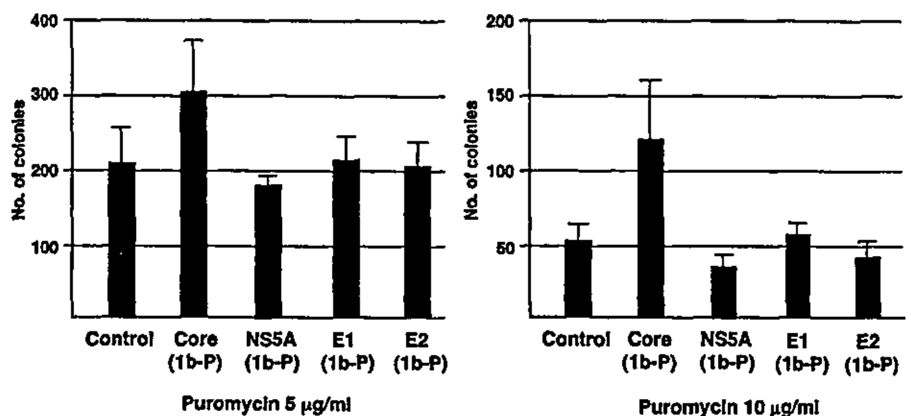

Fig. 3. Hepatitis C virus core protein promoted microsatellite instability in PH5CH8 cells. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame was carried out in PH5CH8 cells stably expressing core(1b-P), NS5A(1b-P), E1(1b-P), or E2(1b-P) protein. The culture period from retrovirus infection to addition of puromycin was 9 days. The puromycin-resistant colonies were counted by the method described in Fig. 2. *Control*, PH5CH8 cells infected with retrovirus pCXbsr.

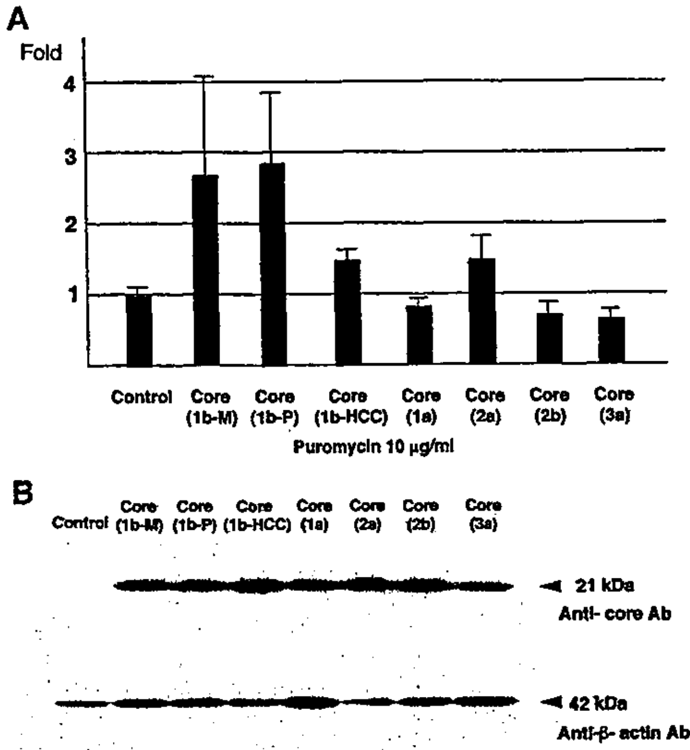

Fig. 4. *A*, promotion of microsatellite instability by the core protein depends on hepatitis C virus genotype. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame was carried out in PH5CH8 cells stably expressing the core protein derived from various hepatitis C virus genotypes. The culture period from retrovirus infection to addition of puromycin was 9 days. The puromycin-resistant colonies were counted by the method described in Fig. 2. *Control*, PH5CH8 cells infected with retrovirus pCXbsr. *B*, stable expression of the core protein in PH5CH8 cells. PH5CH8 cells were infected with retrovirus pCXbsr encoding the core protein belonging to various genotypes, and at 19 days postinfection, the lysate of cells was used for the detection of core protein and β-actin by Western blot analysis. *Control*, PH5CH8 cells infected with retrovirus pCXbsr.

encoding the core(1a), core(2a), core(2b), and core(3a) protein, respectively. In addition, pCXbsr/core(1b-HCC) was also used as a retrovirus vector encoding the core(1b-HCC) protein, which was derived from a cancerous HCC lesion. The pCXbsr/core(1b-M) retrovirus vector (32) encoding core(1b-M) protein, which possessed the consensus sequence of genotype 1b, was also used for the MSI assay. The core(1b-P), core(1b-HCC), core (1a), core(2a), core(2b), and core(3a) proteins differed by 1, 6, 3, 14, 22, and 17 aa from the core(1b-M) protein, respectively (30). Using these retrovirus vectors, including pCXbsr as a control vector, we initially prepared PH5CH8 cells stably expressing the core(1b-M), core(1b-P), core(1b-HCC), core(1a), core(2a), core(2b), and core(3a) proteins, respectively. We performed an MSI assay using these core protein-expressing cells. As shown in Fig. 4*A*, the results revealed that the number of pur^R colonies obtained from the cells expressing the core(1b-M), core(1b-P), core(1b-HCC), or core(2a) protein was 1.5- to 2.8-fold higher than that obtained in the control, whereas the number of pur^R colonies obtained from the cells expressing the core(1a), core(2b), or core(3a) protein was similar to that obtained in the control. Western blot analysis confirmed that these core proteins were stably and equally expressed in PH5CH8 cells at day 19 postinfection with the retrovirus pCXbsr expressing the core protein (Fig. 4*B*). These results suggest that the effectiveness of the core protein in promoting MSI, that is, in down-regulating MMR, is dependent on the HCV genotype or strain.

Expression Level of MMR-Related Genes in PH5CH8 Cells Expressing the Core Protein. To investigate the possibility that the core protein represses the expression of genes functioning in MMR, we examined the effect of the core protein on the expression level of MMR-related genes, including *hMLH1* and *hMSH2*, the frequent genetic mutations of which have been observed in the hereditary nonpolyposis colorectal cancer and a variety of sporadic cancers (25). As shown in Fig. 5, we were not able to find any significant differences in the expression level of *hMLH1*, *hMSH2*, *hMSH6*, *hPMS2*, *hMSH3*, and *hPMS1* genes between PH5CH8 cells expressing the core(1b-P) or NS5A(1b-P) protein, and PH5CH8 cells infected with retrovirus pCXbsr. This result suggests that the down-regulation of MMR by the core protein occurs by an as yet unknown mechanism other than the repression of MMR-related genes.

DISCUSSION

In this study, we first demonstrated that HCV core proteins were able to further repress the down-regulation of MMR activity in cultured human non-neoplastic hepatocytes, by a newly developed MSI assay system using a microsatellite sequence consisting of $(CA)_{17}$.

Regarding the MSI assay system developed in this study, we used retrovirus infection as a method for transduction of a microsatellite (CA) repeat sequence to the cells. However, it remains possible that the RER of pCXpur/$(CA)_{17}$/out-of-frame occurs in the packaging of Bosc23 cells and results in the production of the retrovirus possessing the (CA) repeat sequence altered in-frame. Although we cannot absolutely exclude this possibility, it is unlikely that such an event occurs in Bosc23 cells, because we observed a good correlation between the RER+ and RER− phenotypes of the examined cell lines with respect to the number of pur^R colonies obtained. In addition, we observed that the number of pur^R colonies increased in a culture-time-dependent manner. Therefore, the MSI assay developed in this study will be a useful method at the cell culture level.

The fact that non-neoplastic PH5CH8 cells showed remarkable RER+ phenotype was an unexpected result. Although the PH5CH8 cell line was cloned from PH5CH cells as an HCV-susceptible clone (24), we observed that not only the PH5CH8 cells but also the parental PH5CH cells showed the RER+ phenotype (data not shown). PH5CH cells were established from the non-neoplastic liver as a SV40 large T antigen-immortalized cell line and express hepatocyte characteristics (23). Therefore, the activity of p53 and pRb, two tumor suppressor

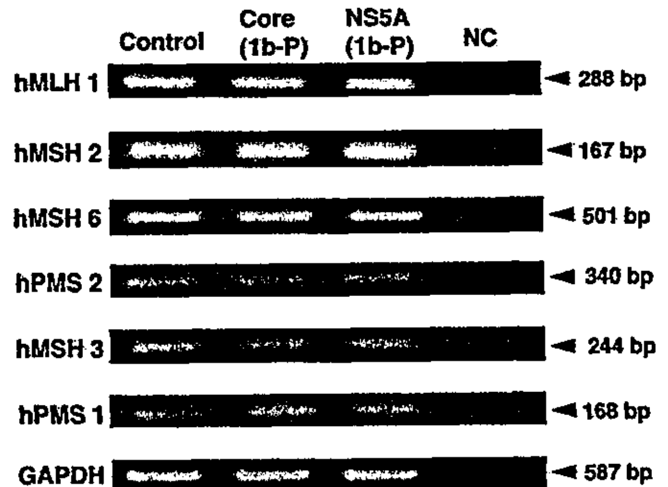

Fig. 5. Effect of the core protein on the expression level of mismatch-repair-related genes in PH5CH8 cells. PH5CH8 cells were infected with retrovirus pCXbsr/core(1b-P) or pCXbsr/NS5A(1b-P), and the cells were used for reverse transcription-PCR analysis of mismatch-repair-related genes. As a control, PH5CH8 cells infected with retrovirus pCXbsr were also used for the analysis. *Control*, PH5CH8 cells infected with retrovirus pCXbsr; *Core(1b-P)*, PH5CH8 cells stably expressing core(1b-P) protein; *NS5A(1b-P)*, PH5CH8 cells stably expressing NS5A(1b-P) protein; *NC*, no RNA.

proteins, in PH5CH cells should be partially repressed by the physical binding of the SV40 large T antigen (42). By complex with p53, the SV40 large T antigen blocks the apoptotic function of p53 and allows proliferation (43), and by binding pRb, the SV40 large T antigen induces the release of the E2F transcription factor, which activates the promoters of genes required for the S-phase transition (44). The functional repression of p53 or pRb may be involved in the repression of MMR activity, although no data suggesting such a relation has yet been reported. As an alternative possibility, the SV40 large T antigen may bind and repress some proteins that function in the MMR system, because it was reported recently that the SV40 large T antigen bound MRE11-NBS1-RAD50 complex, which was involved in homologous recombination, and, as a consequence, perturbed the double-strand break repair (45). Preliminary experiments using NKNT-3 cells (SV40-large T antigen immortalized non-neoplastic human hepatocytes) derived from primary normal human hepatocytes (46) and Saos-2 cells (derived from p53-deficient human osteogenic sarcoma; Ref. 47) revealed that NKNT-3 cells, like PH5CH8 cells, also showed the RER+ phenotype, but Saos-2 cells showed the RER− phenotype in our MSI assay. These results suggest that the activity of MMR is influenced by the SV40 large T antigen but not by p53; however, in addition to PH5CH8 cells, the analysis of cell lines derived from HCV-related HCC cases will be necessary to clarify the reason that PH5CH8 cells show the RER+ phenotype.

Although we found that the core protein promoted MSI in PH5CH8 cells, it is difficult to prove our findings in an HCV replication system because of the lack of a sufficiently reproducible and efficient HCV proliferation system (14). Alternatively, several HCV subgenomic replicons containing NS3-NS5B regions have been established using a human hepatoma cell line Huh-7 (48–50). These subgenomic replicon systems may be useful for the functional evaluation of the core protein. However, our preliminary results revealed that these subgenomic replicon cells showed the RER− phenotype and that no pur^R colonies were obtained from these subgenomic replicon cells stably expressing the core(1b-P) protein. These results suggest that these replicon cells have an intact MMR system that is not influenced by the core protein. To reproduce the promotion of MSI by the core protein in cells in which the HCV genome is replicated, we are currently establishing an HCV subgenomic replicon using PH5CH8 cells.

Our observation that the core proteins belonging to genotypes 1b and 2a, but not those belonging to genotypes 1a, 2b, and 3a, may promote MSI in human hepatocytes is interesting. Although it is not yet defined which region of the core protein is responsible for the promotion of MSI, comparison of aa sequences among these core proteins revealed that aa position 91 was a Cys residue in the core(1a), core(2b), and core(3a) proteins, whereas this position was a Leu residue in the core(1b-M), core(1b-P), and core(2a) proteins and a Met residue in the core(1b-HCC) protein. Only this aa position showed good correlation with the effect of the core proteins in the MSI assay. To clarify whether or not aa position 91 is important to promote MSI, further analysis using chimeric core proteins will be necessary. On the other hand, several studies have described an increased risk of HCC in patients infected with HCV genotype 1b (51, 52), although the contradictory result has also been reported (53). The fact that the core protein belonging to genotype 1b was most effective at promoting the MSI in hepatocyte cells may be related to the increased risk of HCC in patients infected with HCV genotype 1b. To examine this possibility, further MSI analysis using various core proteins derived from many HCV strains belonging to different genotypes will be needed. In addition, our preliminary experiment showed that the number of pur^R colonies in PH5CH8 cells increased approximately 1.5-fold in the presence of $FeSO_4$ (100 μM), suggesting that the Fe(II) compound promotes microsatellite mutations. Although the mechanism of this phenomenon has not yet been clarified, it has been reported that Nickel(II) also induces microsatellite mutations in human lung cancer cell lines (39). Future studies on the relationship between the core protein and these cation compounds will also be important to clarify their roles during the process of hepatocarcinogenesis.

Because we could find no effect of the core protein on the expression level of MMR-related gene, the mechanism by which the core protein promotes MSI in human hepatocytes is still unclear. However, it remains possible that the core protein directly interacts with these components involved in MMR and then suppresses their functions. An alternative possibility—that the core protein affects the functions of the other proteins involved in MMR, including DNA polymerase δ/ϵ, exonuclease 1, and endonuclease FEN1—remains to be examined. Future analyses to evaluate these possibilities may clarify the mechanism of the down-regulation of the MMR system by the core protein.

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Intramembrane Proteolysis and Endoplasmic Reticulum Retention of Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein is suggested to localize to the endoplasmic reticulum (ER) through a C-terminal hydrophobic region that acts as a membrane anchor for core protein and as a signal sequence for E1 protein. The signal sequence of core protein is further processed by signal peptide peptidase (SPP). We examined the regions of core protein responsible for ER retention and processing by SPP. Analysis of the intracellular localization of deletion mutants of HCV core protein revealed that not only the C-terminal signal-anchor sequence but also an upstream hydrophobic region from amino acid 128 to 151 is required for ER retention of core protein. Precise mutation analyses indicated that replacement of Leu^{139}, Val^{140}, and Leu^{144} of core protein by Ala inhibited processing by SPP, but cleavage at the core-E1 junction by signal peptidase was maintained. Additionally, the processed E1 protein was translocated into the ER and glycosylated with high-mannose oligosaccharides. Core protein derived from the mutants was translocated into the nucleus in spite of the presence of the unprocessed C-terminal signal-anchor sequence. Although the direct association of core protein with a wild-type SPP was not observed, expression of a loss-of-function SPP mutant inhibited cleavage of the signal sequence by SPP and coimmunoprecipitation with unprocessed core protein. These results indicate that Leu^{139}, Val^{140}, and Leu^{144} in core protein play crucial roles in the ER retention and SPP cleavage of HCV core protein.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (5, 19) and has been estimated to infect more than 170 million people throughout the world (15). Symptoms of persistent HCV infection extend from chronic hepatitis to cirrhosis and finally to hepatocellular carcinoma (18, 42). HCV belongs to the genus *Hepacivirus* in the family *Flaviviridae* and possesses a viral genome consisting of a single, positive-strand RNA with a nucleotide length of about 9.4 kb (6, 48). The genome encodes a large precursor polyprotein of approximately 3,000 amino acids (6, 17). The polyprotein is processed co- and posttranslationally into at least 10 viral proteins by host and viral proteases (2, 6, 10, 45). The structural proteins of HCV are located in the N-terminal one-fourth of the polyprotein and are cleaved by host membrane proteases (10, 44). Comparison with other flaviviruses suggests that HCV core protein forms the nucleocapsid, which is surrounded by the envelope containing glycoproteins E1 and E2 (6, 48). Functional analyses suggest that HCV core protein has regulatory roles in host cellular functions. In tissue culture systems, HCV core protein regulates signaling pathways and modulates apoptosis (4, 29, 40, 41, 46, 54, 55). Moreover, transgenic mice expressing HCV core protein developed liver steatosis and thereafter hepatocellular carcinoma (34, 36). Thus, it has been suggested that HCV core protein is a multifunctional molecule that acts as a structural protein but is also involved in the pathogenesis of hepatitis C. HCV core protein has two major forms, p23 and p21 (16, 25, 31, 43, 53). HCV core protein p23 represents a 191-amino-acid product in which the C-terminal hydrophobic region also acts as a signal sequence for E1. HCV polyprotein is cleaved between residues 191 and 192 by host signal peptidase to generate C-terminal and N-terminal polypeptides encompassing the core and E1 proteins, respectively. For the full maturation of HCV core protein, the C-terminal signal-anchor sequence was thought to be further processed by an unidentified microsomal protease (25, 30, 31, 43, 53), and the 21-kDa isoform of core protein is predominantly detected both in cultured cells by transfection with expression plasmid and in viral particles obtained from sera of patients with hepatitis C (53). These results suggest that p21 is the mature form of HCV core protein (53). Immunostaining revealed that most HCV core protein is distributed diffusely throughout the cell, probably in the endoplasmic reticulum (ER) (31, 53). However, a minor population was observed in the nucleus (53).

Recently, a presenilin-related aspartic protease, signal peptide peptidase (SPP), was identified (50). SPP is located in the ER membrane and promotes intramembrane proteolysis of signal peptides. The chemical compound $(Z\text{-}LL)_2$-keton inhibits processing of signal peptides by SPP, and it was shown to suppress intramembrane proteolysis of major histocompatibility complex class I molecules, preprolactin, HCV core protein, and others (21, 30, 51). Replacement of Asp^{265} with Ala in SPP resulted in a loss of catalytic function, although this mutant could bind to TBL_4K, a derivative of $(Z\text{-}LL)_2$-keton (50). HLA-A was processed into yeast microsomes following the addition of wild-type SPP but not mutant SPP, suggesting that SPP interacts with HLA-A (50). Processing of the signal sequence of HCV core protein by SPP was inhibited by the

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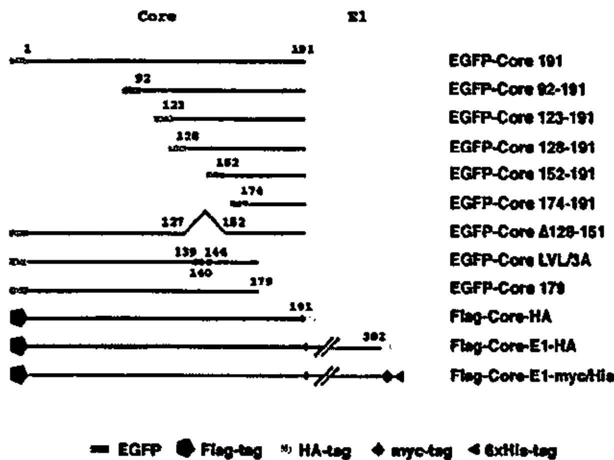

FIG. 1. Expression plasmids used in this study. The genes encoding HCV proteins and their mutants were cloned into pcDNA3.1FlagHA, pcDNA3.1/*myc*-His C, or pEGFP-C3 as described in Materials and Methods. Other plasmids are described in the text or in the other figure legends.

addition of $(Z\text{-}LL)_2$-keton, and Ser[183] and Cys[184] in the signal sequence of core protein were demonstrated to be important for flexibility and intramembrane proteolysis by SPP (23). Signal sequences generally have a tripartite structure, including a central hydrophobic H region and hydrophilic N- and C-terminal flanking regions (28). SPP recognizes the N- and C-terminal regions and cleaves in the middle of the H region (28). Mutational analyses suggested that the flexibility of signal peptides is generally required for substrate recognition of SPP (23). SPP contains the aspartic protease motifs YD and LGLGD, which are located in the predicted transmembrane region, and it is thought to cleave type II (N terminus in the cytosol and C terminus in the lumen)-oriented substrates (50). However, the effect of the cytoplasmic region of type II membrane substrates on intramembrane proteolysis by SPP is not known. In this study, we examined the regions of HCV core protein that are essential for ER retention and intramembrane cleavage by SPP.

MATERIALS AND METHODS

Plasmids. For expression of enhanced green fluorescence protein (EGFP)-fused HCV core proteins in culture cells, the core protein-coding region was amplified by PCR from cDNA encoding full-length HCV polyprotein type 1b (1). The PCR products were subcloned into SalI and BamHI sites 3' of the EGFP-coding region of pEGFP-C3 (Clontech, Palo Alto, Calif.). The cDNA fragments encoding amino acids 1 to 191, 1 to 179, 92 to 191, 123 to 191, 128 to 191, 152 to 191, and 174 to 191 of HCV core proteins were amplified by PCR and then introduced into pEGFP-C3; these constructs are designated EGFP-Core 191, EGFP-Core 179, EGFP-Core 123-191, EGFP-Core 128-191, EGFP-Core 152-191, and EGFP-Core 174-191, respectively. The genes encoding core proteins with the region between amino acids 128 and 151 deleted and replacement of Leu[139], Val[140], and Leu[144] with Ala were generated by the method of splicing by overlap extension (11, 14, 49) and introduced into pEGFP-C3; these constructs are designated EGFP-Core Δ128-151 and EGFP-Core LVL/3A, respectively (Fig. 1).

Fragments encoding Flag and hemagglutinin (HA) tags were inserted at both ends of the multicloning site of pcDNA3.1 (pcDNA3.1FlagHA). PCR products encoding either HCV core protein alone, core protein followed by E1 (Core-E1), or their mutants were cloned into pcDNA3.1FlagHA, resulting in plasmids encoding recombinant proteins sharing Flag and HA tags at the N and C termini, respectively (Fig. 1). In Flag-Core-HA and its derived mutants, Ala[191] was replaced by Arg to avoid processing by signal peptidase for determination of cleavage by SPP, as previously shown for the processing of the E1-E2 junction (7). In addition, the region encoding Flag-Core-E1 or its mutants was cleaved from pcDNA3.1FlagHA constructs and then introduced between the SacI and XhoI sites of pcDNA3.1/*myc*-His C (Invitrogen Corp., Carlsbad, Calif.). The resulting plasmids encode HCV proteins sharing Flag and myc/His epitopes at the N and C termini, respectively (Fig. 1). Genes encoding core protein with a single amino acid (Leu[139], Val[140], or Leu[144]), double amino acids (Leu[139] and Val[140], Leu[139] and Leu[144], or Val[140] and Leu[144]), or triple amino acids (Leu[139], Val[140], and Leu[144]) replaced with Ala were generated by splicing by overlap extension and introduced into pcDNA3.1FlagHA and pcDNA3.1/*myc*-His C (Fig. 1; see Fig. 4).

The genes encoding the ER-targeting and ER retrieval sequences of calreticulin fused with DsRed at the N and C termini, respectively (8, 37, 39), were inserted between the EcoRV and XbaI sites of pcDNA3.1 (pcDNA ER-DsRed) to visualize the ER in culture cells. This recombinant protein is designated ER-DsRed in this study.

Cloning of SPP. The cDNA encoding SPP was amplified from human liver mRNA (Clontech) by reverse transcription-PCR and cloned into T-vector prepared from pBluescript II SK(−) (27). The gene encoding SPP with an attached HA tag and ER retrieval signal, KEKK, at the C terminus (SPP-HAER) was cloned into pcDNA3.1 to eliminate the possibility that the HA tag suppresses the endogenous ER retrieval signal of SPP. SPP-HAER was colocalized with ER-DsRed on the ER membrane and glycosylated upon transfection into cells (data not shown).

Subcellular localization of wild-type and mutant HCV core proteins. HeLa cells were maintained in the Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HeLa cells were seeded on an eight-well chamber slide at 2×10^4 cells per well 24 h before transfection. The cells were transfected with the various plasmids by lipofection with Lipofectamine 2000 (Invitrogen). To determine protein subcellular localizations, transfected cells were fixed with phosphate-buffered saline (PBS) containing 3% paraformaldehyde at 18 h posttransfection and then observed with a confocal laser-scanning microscope (Bio-Rad, Tokyo, Japan). To confirm subcellular localization of the core proteins, transfected cells were fractionated with a subcellular proteome extraction kit (Calbiochem, Darmstadt, Germany). Stepwise extraction resulted in four distinct fractions, which contain mainly cytosolic, membrane-organelle, nuclear, and cytoskelton proteins, respectively. Each fraction was precipitated with trichloroacetic acid and analyzed by immunoblotting, and the densities of the bands were measured with Multi Gauge version 2.2 (Fujifilm, Tokyo, Japan).

Immunoblotting. After transfection, 293T cells were harvested, washed twice with PBS, and lysed in 20 mM Tris-HCl (pH 7.4) containing 135 mM NaCl, 1% Triton X-100, and 10% glycerol (lysis buffer) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and 1 mM Na_2VO_3. The lysate was centrifuged at 6,500 × *g* for 5 min at 4°C. The resulting supernatants were subjected to sodium dodecyl sulfate (SDS)–13.5% polyacrylamide gel electrophoresis. The separated proteins were electroblotted onto a Hybond-P polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, N.J.). These membranes were blocked with PBS containing 5% skim milk and 0.05% Tween 20 (Sigma, St. Louis, Mo.) and incubated with mouse monoclonal anti-Flag M2 (Sigma), anti-HA 16B12 (HA.11; BabCO, Richmond, Calif.), or monoclonal mouse anti-His_6-AD1.1.10 (Genzyme/Techne, Tokyo, Japan) immunoglobulin G (IgG) at room temperature for 30 min and then with horseradish peroxidase-conjugated anti-mouse IgG antibody at room temperature for 30 min. Immunoreactive bands were visualized by using the enhanced chemiluminescence Super Signal West Femto substrate (Pierce, Rockford, Ill.).

Immunoprecipitation. Immunoprecipitation analysis was carried out as described previously (32). Plasmids were transfected into 293T cells by lipofection. Transfected cells were harvested at 18 h posttransfection and lysed in lysis buffer with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid (CHAPSO) (Dojindo, Kumamoto, Japan). Cell lysates were incubated with monoclonal anti-HA, anti-Glu-Glu (anti-EE) (BabCO), or anti-Flag antibody at 4°C for 1.5 h and then with protein G-Sepharose CL-4B (Amersham Bioscience) at 4°C for 1.5 h. After centrifugation at 6,500 × *g* for 3 min at 4°C, the pellets were washed five times with lysis buffer. Immunoprecipitates were subjected to immunoblotting.

Deglycosylation. Plasmids encoding core and E1 proteins were transfected into 293T cells by lipofection, and cell lysates were immunoprecipitated with anti-HA antibody at 18 h posttransfection. Immunoprecipitates were eluted from protein G-Sepharose in 0.5% SDS and 1% 2-mercaptoethanol and digested with endo-β-*N*-acetylglucosaminidase H (Endo H) or peptide-*N*-glycosidase F

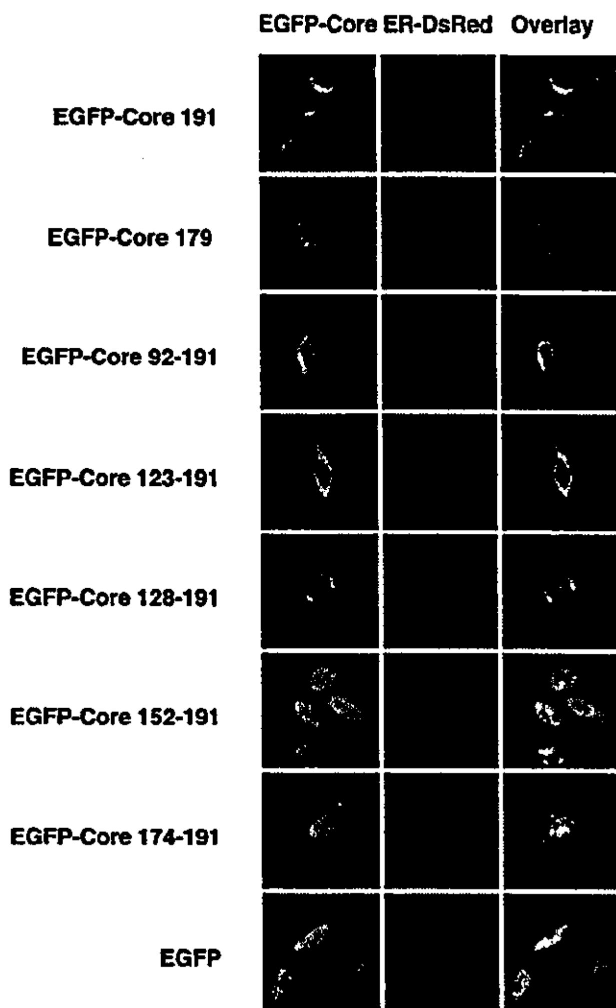

FIG. 2. Intracellular localization of EGFP-Core mutants. EGFP-Core and its deletion mutants were coexpressed with ER-DsRed in HeLa cells, and the localization of core proteins was examined by confocal microscopy.

(PNGase F) according to the protocol of the manufacturer (Roche, Mannheim, Germany). The resulting mixtures were subjected to immunoblotting.

RESULTS

Region required for ER retention of HCV core protein. To determine the regions within HCV core protein that are responsible for ER retention, EGFP-fused, N-terminally truncated HCV core protein (Fig. 1) was coexpressed with the ER marker ER-DsRed. EGFP-Core 191 colocalized with ER-DsRed to the ER (Fig. 2), whereas EGFP-Core 179 was localized primarily to the nucleus as reported previously (3, 33, 43, 47), suggesting that the C-terminal signal sequence is essential for anchoring HCV core protein to the ER membrane. However, EGFP-Core 174-191 exhibited diffuse staining similar to that of EGFP, suggesting that the signal sequence alone is not sufficient for ER localization. EGFP-Core 92-191, EGFP-Core 123-191, and EGFP-Core 128-191 were colocalized with ER-DsRed in the ER, but EGFP-Core 152-191 stained similarly to EGFP-Core 174-191 and EGFP. These data suggest that not only the C-terminal signal sequence but also the region from amino acids 128 to 151 is required for ER retention of HCV core protein.

Region essential for processing of the signal sequence of HCV core protein by SPP and signal peptidase. Based on hydrophobicity and a cluster of basic amino acids, HCV core protein was proposed to possess three regions (domains 1 to 3) (Fig. 3A, upper panel) by Hope and McLauchlan (12). To assess the involvement of the region encompassing amino acids 128 to 151 in proteolysis of the signal sequence of HCV core protein by signal peptidase and SPP, three hydrophobic amino acids, Leu^{139}, Val^{140}, and Leu^{144}, in the most hydrophobic peak in domain 2 were replaced with Ala to reduce hydrophobicity, and Ala^{191} was replaced with Arg to eliminate processing by signal peptidase (Fig. 3A, lower panel). When a wild-type Flag-Core-HA construct was expressed in 293T cells, a single band of 23 kDa was detected by blotting with anti-Flag, but not with anti-HA, suggesting that the HA-fused signal sequence was properly processed by SPP and that Flag-core protein of 23 kDa was generated (Fig. 3B, lanes 2 and 11). In cells expressing the substitution mutants, 25- and 23-kDa bands were detected by the anti-Flag antibody (Fig. 3B, upper panel, lanes 3 to 9) and 25-kDa bands were detected by the anti-HA antibody (Fig. 3B, lower panel, lanes 3 to 9), indicating that the 25- and 23-kDa bands correspond to core proteins that are unprocessed and processed by SPP, respectively. Cleavability of the signal sequence of mutant core proteins by SPP was suppressed in accordance with the number of substitutions, and almost no processing of the signal sequence was observed in cells expressing Flag-Core LVL/3A-HA, which has three amino acid substitutions (Fig. 3B, lane 9). These results indicate that Leu^{139}, Val^{140}, and Leu^{144} play crucial roles in the processing of the signal-anchor of HCV core protein by SPP. Furthermore, deletion of the hydrophobic region including amino acids 128 to 151 from HCV core protein completely eliminated processing by SPP, and this species was seen only as a single band of 23.5 kDa which was detected by both the anti-Flag and anti-HA antibodies (Fig. 3B, lane 10). Taken together with the observation that Ala^{180}, Ser^{183}, and Cys^{184} in the signal sequence of HCV core protein of the type 1a Glasgow strain were demonstrated to be essential for SPP proteolysis (13, 23), these results indicate that the hydrophobic region from amino acid 139 to 144 in domain 2 of HCV core protein also participates in the processing of the signal sequence by SPP.

To examine the role of the region from amino acid 139 to 144 in the cleavage of the HCV core protein signal sequence by signal peptidase and SPP in more detail, substitutions of Leu^{139}, Val^{140}, and/or Leu^{144} with Ala were introduced into the Flag-Core-E1-HA polyprotein (Fig. 1). Flag-Core-E1-HA protein was cleaved to the expected molecular mass of 23 kDa of Flag-Core protein by signal peptidase and SPP (Fig. 3C, lanes 2 and 11), whereas slightly larger bands corresponding to a core protein unprocessed by SPP were detected in cells expressing polyproteins possessing mutations within amino acids 139 to 144 (Fig. 3C, lanes 3 to 9). A lack of processing by SPP was detected mainly in core proteins containing double amino acid changes of Leu^{139}, Val^{140}, and/or Leu^{144} to Ala

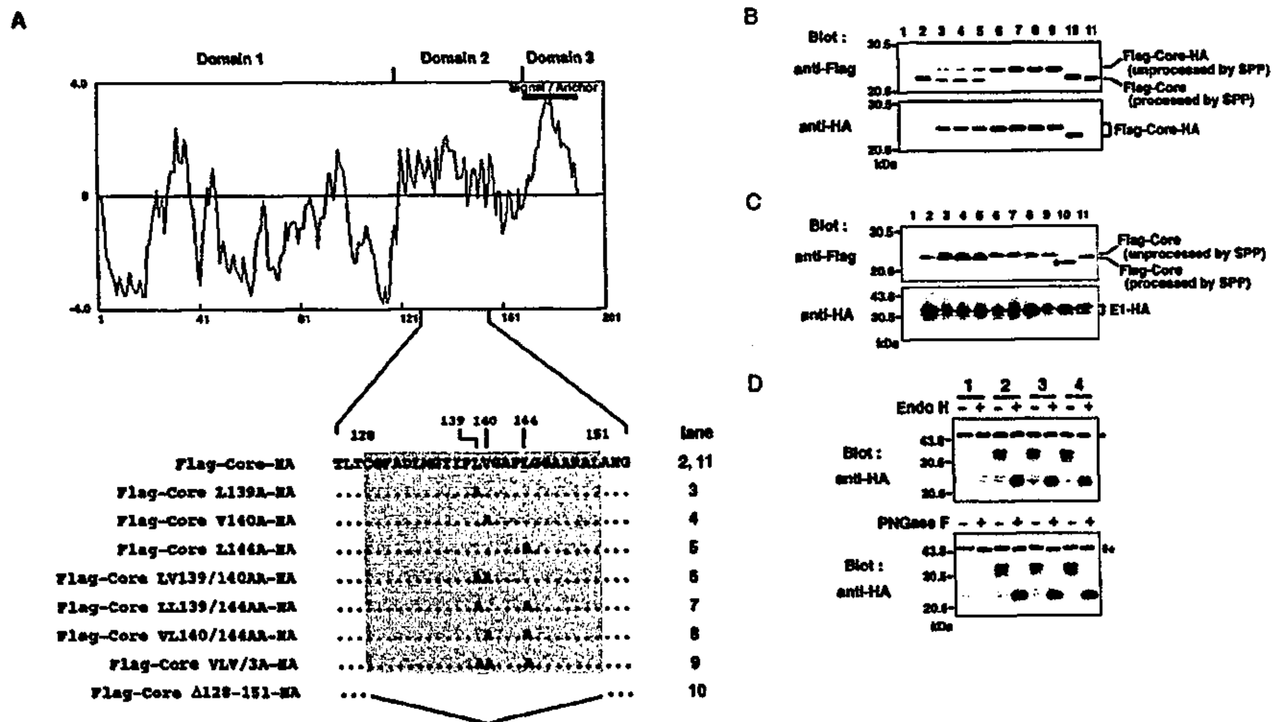

FIG. 3. Identification of the region responsible for processing of the signal sequence of HCV core protein by SPP and signal peptidase. (A) The hydrophobicity profile of HCV core protein was predicted by the method of Kyte and Doolittle (20). Hope and McLauchlan separated the HCV core protein into three regions, domains 1 to 3 (12). Two hydrophobic regions are predicted in the regions from amino acid 128 to 151 and from amino acid 164 to 186 in the C terminus of the HCV core protein. Mutations and deletions in the region from amino acid 128 to 151 of Flag-Core-HA and Flag-Core-E1-HA constructs are indicated. Dots indicate unchanged amino acids. (B) Expression of Flag-Core-HA polyproteins with changes of Ala[191] to Arg in 293T cells. Flag-Core-HA (lanes 2 and 11), Flag-Core L139A-HA (lane 3), Flag-Core V140A-HA (lane 4), Flag-Core L144A-HA (lane 5), Flag-Core LV139/140AA-HA (lane 6), Flag-Core LL139/144AA-HA (lane 7), Flag-Core VL140/144AA-HA (lane 8), Flag-Core LVL/3A-HA (lane 9), and Flag-Core Δ128-151-HA (lane 10) were analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. Cells transfected with an empty plasmid were used as a negative control (lane 1). (C) Expression of Flag-Core-E1-HA mutants in 293T cells. Flag-Core-E1-HA (lanes 2 and 11), Flag-Core L139A-E1-HA (lane 3), Flag-Core V140A-E1-HA (lane 4), Flag-Core L144A-E1-HA (lane 5), Flag-Core LV139/140AA-E1-HA (lane 6), Flag-Core LL139/144AA-E1-HA (lane 7), Flag-Core VL140/144AA-E1-HA (lane 8), Flag-Core LVL/3A-E1-HA (lane 9), and Flag-Core Δ128-151-E1-HA (lane 10) were analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. The asterisk indicates unprocessed Flag-Core Δ128-151. Cells transfected with an empty plasmid were used as a negative control (lane 1). (D) The deglycosylation procedure is described in Materials and Methods. After transfection, cell lysates were immunoprecipitated with anti-HA antibody and immunoprecipitates were digested with Endo H (upper panel) or PNGase F (lower panel). Following digestion, proteins were separated by SDS-polyacrylamide gel electrophoresis, and material from cells transfected with vector (lane 1), Flag-Core-E1-HA (lane 2), Flag-Core LVL/3A-E1-HA (lane 3), and Flag-Core Δ128-151-E1-HA (lane 4) was detected by blotting with anti-HA. Nontreated and Endo H- or PNGase F-treated samples are indicated by − and +, respectively. Asterisks indicate mouse IgG heavy chains.

(Fig. 3C, lanes 6 to 8), and only an unprocessed band was detected in a triple amino acid substitution mutant (Fig. 3C, lane 9) and a deletion mutant lacking amino acids 128 to 151 (Fig. 3C, lane 10). In contrast to the processing of core protein, E1 protein processed from the mutant polyproteins exhibited the same molecular mass of 32 to 35 kDa and the same deglycosylation patterns following digestion with Endo H or PNGase F (Fig. 3D). These results indicate that the internal hydrophobic region from amino acid 139 to 144 of HCV core protein is essential for processing by SPP but not for cleavage of the core-E1 junction by signal peptidase and the subsequent translocation of E1 protein into the ER. It was suggested that signal peptides must be liberated from the precursor protein by cleavage with signal peptidase in order for them to become substrates for SPP (23). Our data indicate that processing by SPP is not a prerequisite for cleavage of the core-E1 junction by signal peptidase.

Amino acid sequence essential for SPP cleavage of the signal sequences of HCV core proteins of genotypes 1a and 1b. Martoglio and colleagues reported that HCV core protein is processed by SPP after cleavage by host signal peptidase and that Ala[180], Ser[183], and Cys[184] residues in the signal sequence of HCV core protein of type 1a Glasgow strain are essential for SPP proteolysis, as they maintain the structure of the breaking α-helix (23, 30). To determine the amino acids essential for SPP cleavage of the signal sequence of type 1b HCV core protein, Flag-Core-E1-HA and its substitution mutants were expressed in 293T cells (Fig. 4). Mutation of one, two, or three amino acids, except for Flag-Core IF176/177AL-E1-HA (Fig. 4B, lane 9), did not affect the processing of the core protein signal sequence. Flag-Core IF176/177AL-E1-HA exhibited the same molecular size as Flag-Core LVL/3A-E1-HA (Fig. 4B, lane 2), suggesting that Ile[176] and Phe[177] in the signal sequence of core protein are essential for cleavage by SPP in our system.

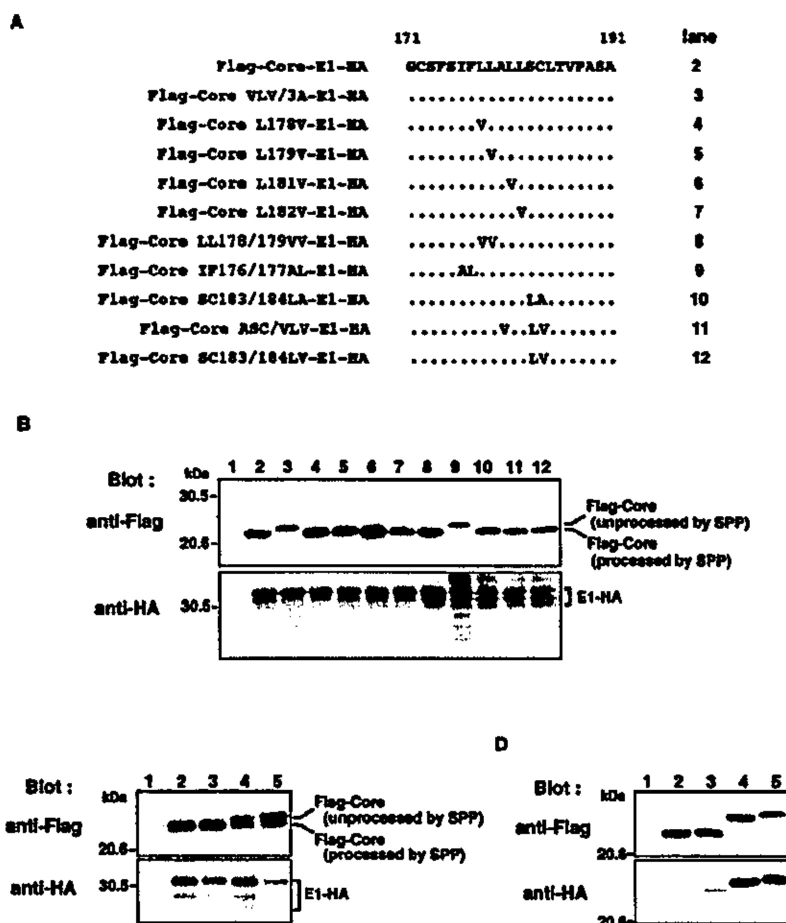

FIG. 4. Amino acid residues essential for SPP cleavage of the HCV core protein signal sequence of genotype 1a and 1b strains. (A) Mutations in the amino acid residues in the signal sequence of Flag-Core-E1-HA are indicated. Dots indicate unchanged amino acids. (B) Flag-Core-E1-HA (lane 2), Flag-Core LVL/3A-E1-HA (lane 3), Flag-Core L178V-E1-HA (lane 4), Flag-Core L179V-E1-HA (lane 5), Flag-Core L181V-E1-HA (lane 6), Flag-Core L182V-E1-HA (lane 7), Flag-Core LL178/179VV-E1-HA (lane 8), Flag-Core IF176/177AL-E1-HA (lane 9), Flag-Core SC183/184LA-E1-HA (lane 10), Flag-Core ASC/VLV-E1-HA (lane 11), or Flag-Core SC183/184LV-E1-HA (lane 12) was expressed in 293T cells. (C) The gene encoding core and E1 polyprotein of the genotype 1a H77c strain of HCV was introduced into pcDNA3.1FlagHA. Flag-H77c Core-E1-HA (lane 2), Flag-H77c Core ASC/VLV-E1-HA (lane 3), Flag-H77c Core LVL/3A-E1-HA (lane 4), or Flag-H77c Core IF176/177AL-E1-HA (lane 5) was expressed in BHK cells. Cell lysates were analyzed by immunoblotting with anti-Flag (upper panel) and anti-HA (lower panel) antibodies. (D) Expression of Flag-Core 191-HA mutants in 293T cells. The gene encoding core protein with a change of Ala^{191} to Arg was introduced into pcDNA3.1FlagHA. Flag-Core-HA (lane 2), Flag-Core ASC/VLV-HA (lane 3), Flag-Core LVL/3A-HA (lane 4), and Flag-Core IF176/177AL-HA (lane 5) were analyzed by immunoblotting with anti-Flag (upper panel) and anti-HA (lower panel) antibodies. Cells transfected with an empty plasmid were used as a negative control (lanes 1 in panels B, C, and D).

However, the triple amino acid substitution (Ala^{180}, Ser^{183}, and Cys^{184}) in the type 1b J1strain (Flag-Core ASC/LVL-E1-HA) (Fig. 4B, lane 11), which is the same as the spmt mutant of the type 1a Glasgow strain (23, 30), did not affect the processing of the signal sequence of HCV core protein by SPP. All derived E1 proteins exhibited a molecular mass of 32 to 35 kDa irrespective of the presence of mutations, and deglycosylation by digestion with endoglycosidases generated uniform 22-kDa bands of E1 proteins (data not shown). These results indicate that Ile^{176} and Phe^{177}, but not Ala^{180}, Ser^{183}, and Cys^{184}, in the signal sequence of type 1b HCV core protein are essential for processing by SPP and confirm that processing of signal sequence by SPP is not required for cleavage by signal peptidase and translocation of E1 protein into the ER. To determine whether the difference in cleavage of signal sequence depends on the genotype of HCV, Ala^{180}, Ser^{183}, and Cys^{184} in the HCV core protein of the genotype 1a H77c strain were replaced with Val, Leu, and Val, respectively. The spmt construct of the type 1a H77c strain did not affect the processing of core and E1 proteins in BHK cells (Fig. 4C, lane 3) and 293T cells (data not shown). In contrast, replacement of Leu^{139}, Val^{140}, and Leu^{144} by Ala and of Ile^{176} and Phe^{177} by Ala and Leu suppressed the processing of the core protein signal sequence of the type 1a H77c strain in BHK cells (Fig. 4C, lanes 4 and 5). These results indicate that three hydrophobic amino acids Leu^{139}, Val^{140}, and Leu^{144} in the hydrophobic peak in domain 2 and the two amino acids Ile^{176} and Phe^{177} in the transmembrane domain play important roles in the in-