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 transfected with pCXpur retrovirus expression vector, in which the pyromycin 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 1a 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 until today (7, 8). The HCV genome encodes a large polyprotein precursor of approximately 3000 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: N1H2-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 lines (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 (29). 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 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 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 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 Biosystems 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 allowed to grow 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 plated 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. These cells were puromycin-resistant (pur+) colonies were stained with Coomassie Brilliant Blue (0.6 μg/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 pCMVF-G-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 core 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+ Colonies. The pur+ 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 MIE5' 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

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Direction</th>
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<tr>
<td>PU-NotI5'</td>
<td>aattaaCGCGCCCGGTTAACAATTGTGTAACCTAC</td>
<td>Forward</td>
</tr>
<tr>
<td>FCA3'</td>
<td>attaaGTGGCAGTTGTGTTGTTGTTGTTGTTGGT</td>
<td>Reverse</td>
</tr>
<tr>
<td>TA5'</td>
<td>GTTGGTCATGATTCTGTTGTTTCTAAAAAGGAAACC</td>
<td>Forward</td>
</tr>
<tr>
<td>PU-StuI3'</td>
<td>AGCGTCCCGGTGTTGCTTGCTGGGGCCAGTG</td>
<td>Forward</td>
</tr>
<tr>
<td>PU-5'</td>
<td>GAGGGGCTTACATCTGTTGCTTGCGGGCCCA</td>
<td>Reverse</td>
</tr>
<tr>
<td>MIE3'</td>
<td>ATTACCGGAGCAGGGCCCTGGCGTTGCTTAC</td>
<td>Reverse</td>
</tr>
<tr>
<td>hMLH1F</td>
<td>TTCTCAGGTATTAGGGACCCAGC</td>
<td>Reverse</td>
</tr>
<tr>
<td>hMLHR</td>
<td>CCTTGCCTAAAATCACCTGACTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>hMS2F</td>
<td>TACAGTGTTCAAGACACCTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>hMS2R</td>
<td>CTCTCTAACGGCTCAGGCTTCT</td>
<td>Forward</td>
</tr>
<tr>
<td>hMS60F</td>
<td>ACAAGGGGCTGGTGTAGCAAAAG</td>
<td>Reverse</td>
</tr>
<tr>
<td>hMS6R</td>
<td>AGTCCATTCATTCTCAGGCTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>hPMS2F</td>
<td>CAGGGTTCTTATTTACCCACCTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>hPMS2R</td>
<td>TTACCTTCAACATCAGGCAGTTGG</td>
<td>Reverse</td>
</tr>
<tr>
<td>hMS13R</td>
<td>GATGCGATTTTACCAAGGAGG</td>
<td>Forward</td>
</tr>
<tr>
<td>hMS15R</td>
<td>AGTCCATTCATTCTCAGGCTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>hPMS1R</td>
<td>GGGACACGGTCAGCCCTTCTCA</td>
<td>Reverse</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>GAGGAGACCACTTGTCTCAG</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

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 pCXpur/(CA)_{17}/out-of-frame retrovirus vector is presented schematically. The nucleotide sequences of the (CA) repeat unit of pCXbar/(CA)_{17}/out-of-frame and pCXbar/(CA)_{17}/in-frame are shown (bottom), and each codon is underlined. pur^{R}, puromycin-resistant.

RESULTS

Construction of the Retrovirus Vectors Containing the Micsatellite (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 (Sall 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}/out-of-frame were able to proliferate in the presence of puromycin (10 \mu g/ml; data not shown), indicating that the pur^{R} gene product from pCXpur/(CA)_{17}/out-of-frame is functional in SW480 cells (RER−), 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 able to survive in the presence of puromycin (10 \mu g/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 \beta-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 \mu g/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 \mu g/ml). In theory, although the cells transduced with pCXpur/(CA)_{17}/in-frame are able to proliferate in the presence of puromycin, the cells transduced 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, Zielinoldiny 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...
Therefore, HCT116, LoVo and LS174T were used as the RER+/H11001 cell lines, and HeLa and SW480 were used as the RER+/H11002 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-β-D-galactopyranoside, 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, purR 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 purR colonies in LoVo cell line despite RER+ phenotype. In all cases, the number of purR 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 purR 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 purR gene depends on the integration site of the retrovirus.

To further evaluate the reliability of our method, 7–10 independent purR 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. 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 purR colonies examined. In addition, (CA)19, (CA)10, and (CA)7 resulting in in-frame were obtained from four colonies in HCT116 cells. One interesting additional sequence, (CA)17A, 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-
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)7CC 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 purR colonies possessed the frameshift mutation (2-bp deletion) resulting in in-frame in the open reading frame of purR 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+/H11001 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 purR 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 purR colonies, although NS5A(1b-P) protein slightly decreased the number of purR colonies. Because the increase of purR 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(3a) retrovirus vectors

<table>
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<tr>
<th>PCR product</th>
<th>Colony no.</th>
</tr>
</thead>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>LS174T (resistant to puromycin 10 μg/ml)</td>
<td></td>
</tr>
<tr>
<td>(CA)17</td>
<td>out-of-frame</td>
</tr>
<tr>
<td>(CA)16</td>
<td>in-frame</td>
</tr>
<tr>
<td>(CA)13</td>
<td>in-frame</td>
</tr>
<tr>
<td>HCT116 (resistant to puromycin 10 μg/ml)</td>
<td></td>
</tr>
<tr>
<td>(CA)19</td>
<td>in-frame</td>
</tr>
<tr>
<td>(CA)17</td>
<td>in-frame</td>
</tr>
<tr>
<td>(CA)16</td>
<td>out-of-frame</td>
</tr>
<tr>
<td>(CA)15</td>
<td>out-of-frame</td>
</tr>
<tr>
<td>(CA)14</td>
<td>in-frame</td>
</tr>
<tr>
<td>(CA)13</td>
<td>CC in-frame</td>
</tr>
<tr>
<td>(CA)12</td>
<td>in-frame</td>
</tr>
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</table>

**Table 2. Sequence analysis of (CA) repeat region obtained from the purR colonies.**

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

![Fig. 3. Hepatitis C virus core protein promoted microsatellite instability in PH5CH8 cells.](image-url)
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 purR colonies obtained. In addition, we observed that the number of purK 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. 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 the addition of puromycin was 9 days. The puromycin-resistant colonies were counted by the method described in Fig. 2.](image)

![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.](image)
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 PH5CH 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 PH5CH 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 pure 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 pure colonies in PH5CH8 cells increased approximately 1.5-fold in the presence of FeSO₄ (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 and then suppresses their functions. Further 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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