Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture

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Hepatitis C virus (HCV) genomic sequences are known to vary widely among HCV strains, but to date there have been few reports on the genetic variations and dynamics of HCV in an experimental system of HCV replication. In this study, a genetic analysis of HCV replicons obtained in long-term culture of two HCV replicon cells (50-1 and 1B-2R1), which were established from two HCV strains, 1B-1 and 1B-2, respectively, was performed. One person cultured 50-1 cells for 18 months, and two people independently cultured 50-1 cells for 12 months. 1B-2R1 cells were also cultured for 12 months. The whole nucleotide sequences of the three independent replicon RNA clones obtained at several time points were determined. It was observed that genetic mutations in both replicons accumulated in a time-dependent manner, and that the mutation rates of both replicons were approximately $3 \times 10^{-3}$ base substitutions/site/year. The genetic diversity of both replicons was also enlarged in a time-dependent manner. The colony formation assay by transfection of total RNAs isolated from both replicon cells at different time points into naive HuH-7 cells revealed that the genetic mutations accumulating with time in both replicons apparently improved colony formation efficiency. Taken together, these results suggest that the HCV replicon system is useful for the analysis of evolutionary dynamics and variations of HCV. Using this replicon cell culture system, it was demonstrated further that neither ribavirin nor its derivative mizoribine accelerated the mutation rate or the increase in the genetic diversity of HCV replicon.

INTRODUCTION

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (Choo et al., 1989; Kuo et al., 1989), which progresses to liver cirrhosis and hepatocellular carcinoma (Ohkoshi et al., 1990; Saito et al., 1990). HCV belongs to the family Flaviviridae, whose genome consists of a positive-stranded RNA molecule of 9-6 kb and encodes a large polyprotein precursor of about 3000 aa residues (Kato et al., 1990a; Tanaka et al., 1995). This polyprotein is processed by a combination of the host and viral proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, and non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Grakoui et al., 1993; Hijiikata et al., 1991, 1993; Mizushima et al., 1994). These HCV proteins not only function in virus replication but may also affect a variety of cellular functions, including gene expression, signal transduction and apoptosis (Bartenschlager & Lohmann, 2000; Kato, 2001).

The most characteristic feature of the HCV genome is its remarkable genetic diversity and variation. To date, more than 50 HCV genotypes have been identified worldwide (Bukh et al., 1995; Simmonds, 1995; Tokita et al., 1996). Each of these genotypes shows more than 20% difference at the nucleotide level and more than 15% difference at the amino acid level compared with any of the other genotypes, although the 5′ untranslated regions (5′ UTRs) and core protein-encoding regions are highly homologous among the 50 genotypes (homology of >90%). Comparisons of HCV genomes that belong to a single genotype have revealed 5–8% diversity in nucleotide sequences and 4–5% diversity in amino acid sequences (Kato et al., 1990b; Kato, 2001). An analysis of the genetic diversity among the HCV genomes in an individual revealed that the diversity in nucleotide sequences averaged 0-9%, and distributed throughout

Supplementary material is available in JGV Online.
the genome except in the 5′ UTR (Tanaka et al., 1992). This so-called 'quasispecies' nature of the HCV genome has generally been observed in a single patient with chronic hepatitis C (Kato et al., 1992; Martell et al., 1992). This remarkable genetic diversity of the HCV genome suggests that HCV frequently causes mutations of the viral genome.

To date, two groups have estimated the mutation rate of the HCV genome using specimens from a chimpanzee (interval of 8 years) and a patient (interval of 13 years) infected with HCV (Ogata et al., 1991; Okamoto et al., 1992). They estimated that the mutation rate of the HCV genome was 1.4–1.9 × 10⁻³ base substitutions/site/year; however, it is not clear whether this value indicates the actual mutation rate of the HCV genome, because complicated quasispecies are generally observed in patients or chimpanzees infected with HCV in vivo. On the other hand, Major et al. (1999) used chimpanzees that received intrahepatic inoculation with a full-length HCV RNA, and they estimated that the mutation rate of the HCV genome was 1.5 × 10⁻³ base substitutions/site/year. However, such experiments on HCV replication in humans are ethically problematic. Thus, there have been few reports on the genetic variations of HCV in an experimental system of HCV replication because of the lack of reproducible and efficient HCV proliferation in cell culture (Kato & Shimotohno, 2000).

In 1999, an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions derived from the strain Con-1 was first established by using a human hepatoma cell line, HuH-7 (Lohmann et al., 1999). Since then, several additional replicon systems have been established (Ali et al., 2004; Blight et al., 2000, 2003; Ikeda et al., 2002; Kato et al., 2003a; Pietschmann et al., 2002; Zhu et al., 2003). In these systems, replicated HCV RNAs were detected by Northern blot analysis and the HCV proteins, which were produced, were detected by Western blot analysis. Therefore, HCV replicon systems are thought to be useful for the analysis of genetic variations and dynamics of HCV.

Recently, we also established two HCV replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively, using HuH-7 cells (Kato et al., 2003b; Kishine et al., 2002). The nucleotide sequences of the NS3-NS5B regions in the 50-1 replicon showed differences of 8.1% from those in the 1B-2R1 replicon (Kato et al., 2003b), although both HCV strains belonged to genotype 1b. In order to understand the genetic variations and dynamics of HCV, we performed genetic analysis of HCV replicons obtained in long-term culture of 50-1 and 1B-2R1 replicon cells (termed 50-1 and 1B-2R1 cells, respectively). Here, we show that the accumulation of genetic mutations and the acquisition of the genetic diversity among HCV replicons are time dependent. In addition, we evaluated the effect of ribavirin and mizoribine on the genetic variations and dynamics of HCV replicons.

### METHODS

**Cell cultures.** 50-1 and 1B-2R1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 300 μg G418 (Genetecinc; Invitrogen) ml⁻¹. The HCV replicon cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase (Neo³) was produced by the efficient replication of HCV replicon in the cells. Therefore, when an HCV replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418. 50-1 cells were also cultured in the presence of 5 or 25 μM ribavirin (Sigma) or 25 μM mizoribine (Sigma). In general, these replicon cells were passaged every 4 days.

**Northern blot analysis.** Total RNA from the cultured cells were prepared using an RNasea extraction kit (Qiagen). Total RNA (3 μg) was used to detect the HCV replicon RNA and β-actin mRNA. Northern blotting and hybridization were performed as described previously (Ikeda et al., 2002; Kato et al., 2003b). A digoxigenin-labelled, negative-sense RNA probe complementary to the NS5B region (positions 8935–9374 of the HCV genome) was used for the detection of the replicon RNA. A β-actin specific digoxigenin-labelled antisense RNA probe was used to check the amount of 18S RNA. The synthetic RNA transcribed from pNSS1RZ2RU (Kato et al., 2003b) (10⁶ and 10⁵ genome equivalents spiked into normal cellular RNA) was used to compare the level of replicon RNA. An RNA ladder (Invitrogen) was also used to mark the molecular length.

**Western blot analysis.** The preparation of cell lysates, SDS-PAGE and immunoblotting analysis with a PVDF membrane were performed as described previously (Hijikata et al., 1993; Naganuma et al., 2000). The antibodies used to examine the expression levels of HCV proteins were those against NS3 (Novocastra Laboratories) and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science, Japan). Anti-β-actin antibody (AC-15; Sigma) was also used to detect β-actin as an internal control. Immuno complexes on the membranes were detected by enhanced chemiluminescence assay (Renissance; Perkin-Elmer Life Sciences).

**RT-PCR.** To amplify HCV RNA RT-PCR was performed as described previously (Kato et al., 2003b). Briefly, the total RNA (2 μg) obtained from the replicon cells was used as a template for reverse transcriptase using SuperScript II (Invitrogen). PCR using proofreading KOD-plus DNA polymerase (Toyobo) 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 a 2033 bp fragment for the former part and a 6107 bp fragment for the latter part.

**cDNA cloning and sequencing.** The PCR products were subcloned into the XbaI site of pBR322MC (Kishine et al., 2002), which was derived from pBR322 and contained the multiple cloning site of pUC19, as described previously (Kato et al., 2003b). Plasmid inserts were sequenced in both the sense and antisense directions by using Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyser (Applied Biosystems).

**Molecular evolutionary analysis.** Nucleotide sequences of the clones obtained by RT-PCRs from 50-1 and 1B-2R1 cells were analysed by the neighbour-joining analysis using the program GENETY-MAC (Software Development).

**RNA transfection and selection of G418-resistant cells.** RNA transfection into HuH-7 cells was performed by electroporation as described previously (Lohmann et al., 1999). Briefly, total RNA (80 μg) isolated from the replicon cells was electroporated into 5 × 10⁶ HuH-7 cells, and then 1 × 10⁷ or 3 × 10⁷ cells were seeded into a 10 cm diameter dish. After 48 h, G418 was added to
0.3 mg ml⁻¹, and the medium was changed twice per week. After 3 weeks, the colonies obtained on the culture dish were stained with Coomassie brilliant blue as described previously (Naganuma et al., 2004).

RESULTS

Efficient replication of HCV replicons is maintained in long-term cell culture

In order to prepare the specimens for the genetic analysis of 50-1 and 1B-2R1 replicons, three people independently cultured 50-1 cells; one person cultured for 18 months (M) (K cell culture line; MK) and the two people cultured for 12 months (D and N cell culture lines; MD and MN), and one person cultured 1B-2R1 cells for 12 months. Using the specimens obtained at several time points (after 0, 4, 6, 12 and 18 months in culture), the levels of replicon RNAs and HCV proteins were examined by Northern and Western blot analyses, respectively. As shown in Fig. 1(a), 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 treatment with interferon-α. The number of copies of replicon RNAs in total RNA (each 3 μg) extracted from the replicon cells was estimated to be in the range of 10⁷ to 10⁸ by comparing these replicon RNAs with replicon RNA synthesized in vitro. The NS3 and NS5B were also detected in all specimens except those from the cured cells (Fig. 1b). The expression levels of replicon RNAs and HCV proteins differed somewhat among these specimens, and no strong quantitative relationship between replicon RNA and HCV proteins was observed (Fig. 1). These results suggest that the stability of replicon RNA or HCV proteins produced from the replicon RNA, or the efficiency of translation, changes during the periods of cell culture. In summary, we demonstrated that the replication efficiencies of the 50-1 and 1B-2R1 replicons remained high under the G418 selection pressure.

Sequence analysis of the 50-1 and 1B-2R1 replicon RNAs

To clarify the genetic variations and diversities of the replicons during the period of cell culture, we carried out sequence analysis of 50-1 and 1B-2R1 replicon RNAs obtained at several time points in the cultures of both replicon cells. 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 to NS5B regions) were amplified by RT-PCR, and three independent clones of each were sequenced after subcloning into pBR322MC, as described previously (Kato et al., 2003b).

Genetic variations of 50-1 and 1B-2R1 replicons during long-term cell culture

The determined nucleotide sequences of the 50-1 and 1B-2R1 replicon RNAs were compared with those of the
original 50-1 (Kishine et al., 2002; GenBank accession no. AB041927) and 1B-2R1 replicons (Kato et al., 2003b; AB109543), respectively. The results revealed that the numbers of base substitutions in the first 2-kb region and in the NS region (6·1 kb) of both replicon RNAs were time-dependently increased with linearity (Fig. 2). These substitutions were considered to be mutations that occurred during the intracellular replication of replicon RNA. Based on the results after 12 months in culture, the apparent mutation rates in 50-1 replicon RNA were calculated to be $3 \times 10^{-3}$ and $3 \times 10^{-3}$ base substitutions/site/year in the first 2 kb region and NS region, respectively, indicating that there was no difference in mutation rate between the two regions of 50-1 replicon RNA. Interestingly, almost the same mutation rates ($3 \times 10^{-3}$ base substitutions/site/year in the first 2 kb region; $3 \times 10^{-3}$ base substitutions/site/year in NS region) were obtained for the 1B-2R1 replicon RNA, suggesting that the replication efficiency of the 1B-2R1 replicon was almost equal to that of the 50-1 replicon.

Fig. 3(a) shows the schematic presentation of mutations detected in the first 2 kb region by comparison with the original sequences (NNRZ2RU) of 50-1 and 1B-2R1 replicon RNAs (Kato et al., 2003b; Kishine et al., 2002). The results revealed that there were no common mutations among the four cell culture lines (three for 50-1 and one for 1B-2R1) over at least 12 months of cell culture. However, genetic mutations in both replicons were time-dependently increased and accumulated, and several mutations became abundant during the subsequent cell culture (Fig. 3a).

The NS regions (6·1 kb) of the 50-1 and 1B-2R1 replicon RNAs were also analysed in addition to the first 2 kb region. The mutation sites that showed amino acid substitutions are schematically presented in Fig. 3(b). Regarding the 50-1 replicon, 2 aa substitutions (P1115L and E1966A) were newly detected after 6 months in culture in all three cell culture lines, in addition to 2 aa substitutions (K1609E and V1896F) already observed when the replicon was first established. These four substituted amino acids were stably maintained over at least 12 months of cell culture. However, such amino acid substitutions were not observed in the 1B-2R1 replicon even after 12 months of culture. After more than 12 months in culture, several culture line-specific amino acid substitutions (*1–5 for the K culture line; *6–8 for the D culture line; and *9–12 for the N culture line in Fig. 3b) were observed in the 50-1 replicon. Also in the 1B-2R1 replicon, 1 aa substitution (*13 in Fig. 3b) was detected after 12 months in culture; however, no common amino acid substitutions were observed between the 50-1 and 1B-2R1 replicons. The mean numbers of amino acid substitutions occurring after 6 and 12 months in culture were 4-2 and 8-9, respectively, for the 50-1 replicon, and 4-7 and 10-0, respectively, for the 1B-2R1 replicon. These values indicate a steady genetic evolution of 50-1 and 1B-2R1 replicons during the cell culture.

Fig. 2. Genetic variations of 50-1 and 1B-2R1 replicon RNAs. (a) First 2·0 kb region of replicon RNA. Filled squares indicate the mean numbers of base substitutions detected in nine (after 0, 6 and 12 months in culture) or three (after 4 and 18 months in culture) clones containing the first 2·0 kb region of 50-1 replicon RNA, by comparison with its original sequences (NNRZ2RU) (Kishine et al., 2002). Open squares indicate the mean numbers of base substitutions detected in three clones containing the NS region of 50-1 replicon RNA, by comparison with its original sequences (Kishine et al., 2002). (b) NS region (6·1 kb) of replicon RNA. Filled squares indicate the mean numbers of base substitutions detected in three clones containing the first 2·0 kb region of 1B-2R1 replicon RNA, by comparison with its original sequences (NNRZ2RU) (Kishine et al., 2002). Open squares indicate the mean numbers of base substitutions detected in three clones containing the NS region of 50-1 replicon RNA, by comparison with its original sequences (Kato et al., 2003b).
Fig. 3. Genetic variations of 50-1 and 1B-2R1 replicons in long-term cell culture. (a) Schematic presentation of mutations detected in first 2-0 kb regions of the replicon RNAs. Compared with the nucleotide sequences of the first 2-0 kb region of the original replicon RNA (NNRZ2RU), nucleotide positions mutated in all three clones, in two of three clones and in one of three clones are indicated by full-length, two-thirds and one-third vertical lines, respectively. Non-synonymous substitutions in the Neo<sup>R</sup> region are indicated by heavy vertical lines. (b) Schematic presentation of amino acid substitutions detected in the NS regions of the replicons. Compared with the amino acid sequences of NS region of the original 50-1 (Kishine et al., 2002) and 1B-2R1 replicons (Kato et al., 2003b), amino acid positions substituted in all three clones, in two of three clones and in one of three clones are indicated by full-length, two-thirds and one-third vertical lines, respectively. Four amino acid substitutions (P1115L, K1609E, V1896F and E1966A) are indicated by heavy vertical lines. Culture line-specific amino acid substitutions (indicated by the numbers with asterisks) are as follows: *1, I1686V; *2, L1701R; *3, T2332A; *4, G2336E; *5, A2372T; *6, A1243G; *7, I1797V; *8, S2053G; *9, L1701R; *10, T2051N; *11, R2279G; *12, L2476M; *13, I1097V.
Classification of mutations occurring in 50-1 and 1B-2R1 replicon RNAs during the long-term cell culture

To understand the mutation mode of the replicons in long-term cell culture, we examined the numbers of synonymous and non-synonymous mutations with transition or transversion. The results are summarized in Table 1. The ratio of synonymous to non-synonymous mutations in 50-1 replicon RNA was 0.81 to 1.50 (1.38 ± 0.14 after 6 months in culture and 1.03 ± 0.20 after 12 months in culture), and the ratio in 1B-2R1 replicon RNA was 0.63 after 6 months in culture and 0.59 after 12 months in culture. These values indicate that amino acid substitutions in the replicons occur frequently during the cell culture. The rate of mutations with transition in the 50-1 replicon was 1.82–4.06-fold (2.00 ± 0.18 after 6 months in culture and 2.85 ± 1.07 after 12 months in culture) greater than the rate of mutations with transversion. Similarly, the 1B-2R1 replicon showed a transition-to-transversion ratio of 2.69 (after 6 months in culture) or 2.86 (after 12 months in culture).

Regarding the mutation patterns over more than 12 months of culture, we observed that A→G and U→C mutations were the most and second-most common mutations, and these mutations were approximately two to three times more common than G→A and C→U mutations (Supplementary Table A, which is available as Supplementary material in JGV Online). The rarest mutation was G→U (Supplementary Table A).

Genetic diversity of the 50-1 and 1B-2R1 replicons arising during long-term cell culture

To clarify whether or not the replicons acquire a quasispecies nature during long-term cell culture, we estimated the genetic diversities of the 50-1 and 1B-2R1 replicon populations. First, based on the sequence data of all clones obtained in this study, we constructed phylogenetic trees for the first 2 kb region and the NS region. The results revealed that the genetic diversity of 50-1 replicon populations was expanded in a time-dependent manner (Fig. 4). Similar phylogenetic trees were obtained for the 1B-2R1 replicon populations as well (data not shown). Next, as another index of genetic diversity, we calculated the mean number of nucleotide differences among three independent clones at each time point. The schematic presentation of such analysis on the NS regions of 50-1 and 1B-2R1 replicon RNAs was shown in Supplementary Fig. A, which is available as Supplementary material in JGV Online. The results also showed a time-dependent expansion of genetic diversity. After 12 months in culture, 0.32 % (mean of three cell culture lines) and 0.55 % diversities in nucleotide sequences were observed in the NS region of 50-1 and 1B-2R1 replicon RNAs. A similar time-dependent expansion of genetic diversity was also observed in the first 2 kb regions of both replicon RNAs (data not shown). These results indicate that the quasispecies nature of replicon RNA was easily acquired during the replication of the replicons.

Table 1. Base substitutions occurring in 50-1 and 1B-2R1 replicon RNAs during long-term cell culture

The counting of base substitutions was performed by comparison with the consensus sequence obtained from the 0M series of 50-1 or 1B-2R1 replicon.

<table>
<thead>
<tr>
<th>Replicon series</th>
<th>No. base substitutions</th>
<th>Synonymous/ non-synonymous transversion</th>
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<tbody>
<tr>
<td></td>
<td>Transition</td>
<td>Transversion</td>
</tr>
<tr>
<td></td>
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<td>Non-synonymous</td>
</tr>
<tr>
<td></td>
<td>NeoR NS</td>
<td>NeoR NS</td>
</tr>
<tr>
<td></td>
<td>Non-coding region</td>
<td></td>
</tr>
<tr>
<td>50-1</td>
<td>4MK</td>
<td>1 13 0 8 4 0 5 0 6 2 1.36 2.00</td>
</tr>
<tr>
<td></td>
<td>6MK</td>
<td>0 20 2 10 8 3 8 1 9 1 1.41 1.82</td>
</tr>
<tr>
<td></td>
<td>12MK</td>
<td>3 29 6 19 13 4 9 4 9 2 1.18 2.50</td>
</tr>
<tr>
<td></td>
<td>18MK</td>
<td>5 43 8 26 16 3 10 4 14 5 1.17 2.72</td>
</tr>
<tr>
<td></td>
<td>6MD</td>
<td>3 20 3 9 2 0 5 4 7 1 1.22 2.18</td>
</tr>
<tr>
<td></td>
<td>12MD</td>
<td>5 29 2 26 3 2 5 1 8 0 1.11 4.06</td>
</tr>
<tr>
<td></td>
<td>6MN</td>
<td>2 19 2 8 3 2 4 0 8 3 1.50 2.00</td>
</tr>
<tr>
<td></td>
<td>12MN</td>
<td>3 25 2 21 9 1 6 5 15 3 0.81 2.00</td>
</tr>
<tr>
<td>1B-2R1</td>
<td>6M</td>
<td>1 14 5 14 1 1 3 5 3 0.63 2.69</td>
</tr>
<tr>
<td></td>
<td>12M</td>
<td>2 22 4 29 6 1 2 3 10 6 0.59 2.86</td>
</tr>
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</table>
Enhancement of HCV replication is associated with the expansion of the replicons’ genetic diversity

To assess whether or not the mutations accumulating in the replicons increase the replication efficiencies of the replicons, the efficiency of colony formation (ECF) of the replicon was examined at each time point of the culture. An ECF assay was performed by transfection of total RNAs isolated from 50-1 and 1B-2R1 replicon cells at different time points into naïve HuH-7 cells. After 3 weeks of G418 selection, only a few colonies were obtained when RNAs from 50-1 replicon cells cultured less than 4 month were used (Fig. 5). However, ECF was apparently increased when RNAs from cells cultured 6 months, in particular the D and N cell culture lines, were used, and much higher numbers of colonies were obtained when RNAs from cells cultured 12 months were used (Fig. 5). Interestingly, ECFs of RNAs from D and N cell lines cultured more than 6 months were higher than those in the K cell culture line. These results indicated that ECF of the replicon was increased with the cultured periods of the replicon cells and suggested that ECF enhancement is associated with the expansion of the 50-1 replicon’s genetic diversity.

In contrast to the case with 50-1 replicon cells, a number of colonies were obtained even when RNA from the initial culture of 1B-2R1 replicon cells was used (Fig. 5). However, ECF was apparently increased when RNAs from cells cultured 6 months, in particular the D and N cell culture lines, were used, and much higher numbers of colonies were obtained when RNAs from cells cultured 12 months were used (Fig. 5). Interestingly, ECFs of RNAs from D and N cell lines cultured more than 6 months were higher than those in the K cell culture line. These results indicated that ECF of the replicon was increased with the cultured periods of the replicon cells and suggested that ECF enhancement is associated with the expansion of the 50-1 replicon’s genetic diversity.

Effect of ribavirin and mizoribine on the genetic evolution and dynamics of the 50-1 replicon

Combined treatment of interferon plus ribavirin for patients with chronic hepatitis C has been shown to be more effective than treatment with interferon alone (McHutchison et al., 1998), although it has been shown that ribavirin alone does not cause a decrease of HCV level in patients with chronic hepatitis C. Recently, several groups have reported that ribavirin might cause ‘error catastrophe’ of HCV genome (Contreras et al., 2002; Tanabe et al., 2004; Zhou et al., 2003), however, controversial results have also been reported (Schinkel et al., 2003). Therefore, to clarify whether or not ribavirin affects the genetic alterations of HCV, we cultured parent 50-1 cells (corresponding to 0M in Fig. 1) for 6 months in the presence of ribavirin (5 or 25 μM) or its derivative molecule, mizoribine (25 μM). As a control, the parent 50-1 cells were also cultured for 6 months in the absence of ribavirin or mizoribine. After 6 months in culture, the levels of replicon RNAs and HCV proteins were examined by Northern and Western blot analyses, respectively. As shown in Fig. 6(a), the level of replicon RNA in the cells treated with ribavirin or mizoribine was almost the same as that in the cells without ribavirin or mizoribine treatment. The NS3 and NS5B were also expressed at similar levels in the cells irrespective of ribavirin or mizoribine treatment (Fig. 6b). These results indicate that even 6 months of treatment with ribavirin or mizoribine did not prevent the replication of replicon RNA under the G418 selection pressure. Using the 50-1 cells cultured for 6 months with or without ribavirin or mizoribine, we performed sequence analysis of replicon also contributes to the enhancement of ECF, as was the case with the 50-1 replicon.
RNAs as described above. As shown in Table 2, the results revealed that the numbers of mutations in the first 2·0 kb and NS regions of the replicon RNAs sequenced were not significantly different among the specimens, although the number in the NS region derived from the cells treated with 25 μM of ribavirin was a little lower than those of the other specimens. These results suggest that the treatment of replicon cells with either ribavirin or mizoribine does not increase the mutation rate of replicon RNA. The ratio of synonymous and non-synonymous mutations, and the ratio of transition and transversion mutations were also not altered by ribavirin or mizoribine treatment (data not shown). In addition, we did not observe any ribavirin- or mizoribine-specific common amino acid substitutions in either the first 2 kb or NS regions of the replicon RNA, although P1115L and E1966A were detected after 6 months in culture in all cell culture lines. The above-described analysis of genetic diversity among the replicon RNAs did not reveal any significant differences in the mutation rate or in the ratio of synonymous and non-synonymous mutations.

**Fig. 5.** ECF of the RNAs isolated from 50-1 and 1B-2R1 replicon cells at different time points in the culture. Total RNAs obtained from the replicon cells were transfected into HuH-7 cells as described in Methods. The panels show the cell colonies that were recovered after 3 weeks of G418 selection.

**Table 2.** Base substitutions occurred in 50-1 replicon RNA during 6 months culture in the presence of ribavirin or mizoribine

<table>
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<th>Series</th>
<th>First 2-kb region</th>
<th>NS region</th>
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<tr>
<td>6M (Fig. 2)</td>
<td>5·4 ± 1·9*</td>
<td>16·4 ± 1·8</td>
</tr>
<tr>
<td>6M</td>
<td>5·7 ± 2·5</td>
<td>16·0 ± 0·0</td>
</tr>
<tr>
<td>6MR5</td>
<td>5·7 ± 1·5</td>
<td>16·3 ± 1·5</td>
</tr>
<tr>
<td>6MR25</td>
<td>5·7 ± 1·5</td>
<td>10·7 ± 1·2</td>
</tr>
<tr>
<td>6MM25</td>
<td>3·7 ± 0·6</td>
<td>18·7 ± 4·0</td>
</tr>
</tbody>
</table>

*Numbers of base substitutions ± SD.
not reveal any significant differences between the specimens derived from the replicon cells with and those without ribavirin or mizoribine treatment (data not shown). Taken together, these results suggest that neither ribavirin nor mizoribine accelerated the mutation rate of HCV replicons or the development of their quasispecies nature.

**DISCUSSION**

In this study, we analysed the genetic evolution and dynamics of HCV replicons, and time-dependent genetic mutations of HCV replicons were observed. Time-dependent expansions of their genetic diversities were also revealed. Our results should provide useful fundamental information for understanding the remarkable genetic diversity and variation among the HCV genomes observed in patients with chronic hepatitis C.

Although RT-PCR techniques were used to amplify the replicon RNAs in this study, it is unlikely that the detected mutations were due to errors related to the use of the KOD-plus DNA polymerase in the PCR reaction, because we previously showed that KOD-plus DNA polymerase possessed a high proofreading activity (Alam et al., 2002; Naganuma et al., 2004). Furthermore, in the present study, we sequenced several clones (containing a 2–0 or 6–1 kb fragment) obtained by PCR using KOD-plus DNA polymerase and a single sequenced clone as a template, but no mutations were detected in these sequenced clones, indicating that KOD-plus DNA polymerase possesses extremely high fidelity. However, we are not able to completely exclude the possibility that some substitutions resulted from the erroneous use of KOD-plus DNA polymerase during the PCR. Even if such errors occurred, the error frequency is estimated to be less than one nucleotide per sequenced clone. This is explained as follows. Fig. 2 shows that the numbers of substitutions time-dependently increased with linearity in both HCV replicons. Interestingly, when these linear lines are extrapolated to zero base substitutions, the crossing points show approximately −2−3 months in the time axis. These range of months is in accord with the time of initial electroporation of HCV replicon RNA to HuH-7 cells. Therefore, PCR-induced mutations are considered to be very rare and such mutations would have very little effect on the results shown in Fig. 2. In addition, to avoid a sampling effect, we sequenced three independent clones derived from each time point.

We showed that the mutation rates for the 50-1 and 1B-2R1 replicon RNAs were almost the same – about $3 \times 10^{-3}$ base substitutions/site/year. However, the actual mutation frequency of the replicon RNAs would be higher than this value, because the mutations that occurred in positions that were critical for the replication of replicon RNA should not have been passed on to the progeny. Our observed mutation rates of the replicon RNAs were approximately two times higher than those previously obtained in chimpanzees and clinical patients with chronic hepatitis C (Major et al., 1999; Ogata et al., 1991; Okamoto et al., 1992). Since the selective pressure of the immune system also functions in vivo (Kato et al., 1993), the mutation rate in cell culture obtained in this study may be reasonable value as a potential mutation rate of HCV. However, direct comparison of these mutation rates would be difficult, because both the experimental model and analytical method were different in this study compared with the previous studies. It would be interesting to examine whether this mutation rate ($3 \times 10^{-3}$ base substitutions/site/year) would be maintained during longer-term culture of the replicon cells. If so, approximately 3% of nucleotide sequences of the replicon RNAs might be mutated after 10 years in cell culture. Alternatively, the mutations might become saturated during further long-term culture of the replicon cells. To clarify this point, further long-term culture of replicon cells is in progress.

Although the mutations detected in this study were dispersed throughout the entire length of the replicon RNAs (Fig. 3), the mutation frequencies in the 5' UTR and NS5B region were lower than those in other regions, and the NS5A region showed the highest mutation frequency. These observations are consistent with the genetic diversities of HCVs in patients with chronic hepatitis C reported to date (Kato, 2001). In addition, the positions in which amino acid substitutions were observed during the cell culture did not appear to be critical for replication of the HCV genome.

Time-dependent expansions of genetic diversities of HCV replicons were also found in this study. However, this finding seems to be different from the previous findings that HCV populations in the cells infected in vitro gradually altered with time and converged to the limited populations (Kato et al., 1998; Kato, 2001). This gap may have been due to the differences in the HCV sources used: a patient's inoculum containing a quasispecies of HCV was used for the in vitro infection experiment, and a single HCV species was used for the replicon system. Alternatively, the gap may have been due to the overwhelming difference between the replication level of the HCV genome in the cells infected in vitro and that in the replicon cells.

To date, a number of amino acid substitutions belonging to adaptive mutations that enhance the frequency with which the replicon is established in vitro have been found in established HCV replicons (Bartenschlager, 2002; Blight et al., 2000, 2003; Ikeda et al., 2002; Krieger et al., 2001; Lanford et al., 2003; Lohmann et al., 2001, 2003; Pflugheber et al., 2002). Although none of the amino acid substitutions detected in the long-term cultures of the 50-1 and 1B-2R1 replicons were the same as those reported as adaptive mutations, ECF analysis of the replicons using naïve HuH-7 cells suggested that adaptive mutations accumulated in the replicon populations in a time-dependent manner. In particular, drastic enhancement of ECF was observed in the 50-1 replicon after 6 months of culture. However, this result suggests that the four common amino acid substitutions (P1115L, K1609E, V1896F and E1966A) do not contribute much to the drastic enhancement of ECF,
because the ECFs of 4MK and 6MK samples possessing these substitutions did not increase much. Therefore, we estimate that some uncommon amino acid substitutions accumulated as so-called adaptive mutations. The candidates for such adaptive mutations are culture-line-specific amino acid substitutions (Fig. 3b, *1–12), and many amino acid substitutions sporadically appeared in the replicons in the long-term cell cultures. To identify which amino acid substitution is the main contributor to the drastic enhancement of ECF, further transfection experiments using replicon RNAs possessing mutations will be needed. Based on the results of this study, S2200R substitution in the 1B-2R1 replicon is considered an adaptive mutation. This description is supported by the previous result that we were unable to obtain any G418-resistant colonies when the original 1B-2 replicon RNA library, used in the isolation of the 1B-2R1 replicon, was transfected into naïve HuH-7 cells (Kato et al., 2003b). Since the ECF of 1B-2R1 replicon RNA from 12 months of culture was further enhanced, it may be that the I1097V substitution, detected commonly at 12 months of culture, functions as an additional adaptive mutation.

Interestingly, once a new mutation was observed in all three clones at a particular time point, the clones which went back to the original sequences were never obtained in the subsequent cell culture, except for one clone (a mutation in the HCV IRES region) derived from 1B-2R1 replicon cells after 12 months in culture (Fig. 3a). This finding suggests that the genetic evolution of HCV replicons is irreversibly progressing.

Although the mechanism of action of ribavirin for patients with chronic hepatitis C is ambiguous, an ‘error catastrophe’ theory of ribavirin has been proposed by several groups (Contreras et al., 2002; Tanabe et al., 2004; Zhou et al., 2003). However, our results obtained in this study were not able to support this ‘error catastrophe’ theory, because ribavirin had no effect on the genetic variation and diversity of the 50-1 replicon. The concentration (5 and 25 μM) of ribavirin used in this study was considered to be reasonable, because the growth rate of 50-1 cells decreased at a ribavirin concentration of more than 50 μM, and approximately 10 μM of ribavirin is the maximum plasma concentration in current clinical usage (Tanabe et al., 2004). Higher concentration (more than 50 μM) of ribavirin used in previous studies may be required for causation of the error catastrophe. Recently, a single amino acid substitution (F2834Y) was identified as a ribavirin-resistant NS5B mutation in genotype 1a (Young et al., 2003); however, it is difficult to evaluate that finding in this study, because most of the HCV strains belonging to genotype 1b, including 1B-1 (50-1) and 1B-2 (1B-2R1), already possess a Tyr residue at position 2834. No amino acid substitution at position 2834 in NS5B was observed in the replicon cells treated with ribavirin. This study provided the fact that the genetic diversity of HCV replicons was enlarged in a time-dependent manner during long-term cell culture. Since all the HCV replicons established to date have been shown to be highly sensitive to interferon-α, -β and -γ (Kato et al., 2003b), and most of the HCV replicons established to date are able to replicate in only HuH-7 cells, the extensive genetic polymorphism of HCV replicon populations obtained by long-term cell culture may change the sensitivity against interferon or the ability of replication in the cells except for HuH-7. In the future, it will be necessary to clarify these points. Thus, HCV replicon populations obtained by long-term cell culture may be useful not only for analysis of the genetic variations and dynamics of HCV but also for analysis of the variable properties of HCV.

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