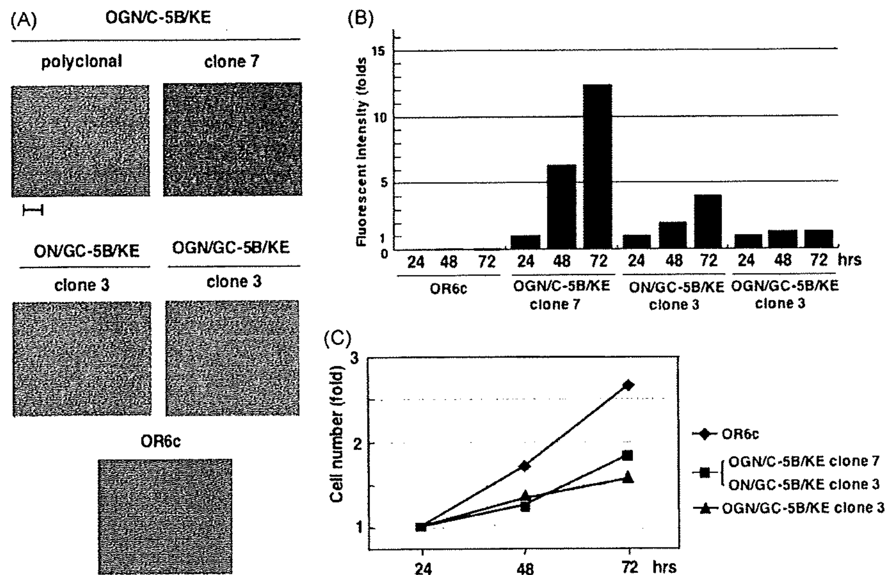


**Fig. 2.** Fluorescent intensities of G418-resistant cell lines. (A) Visualization of the fluorescence of G418-resistant cell lines under a fluorescence microscope. The panels show the fluorescence of expressed EGFP. Bar, 200 μm. (B) Time course of the fluorescent intensity of G418-resistant cell lines. The fluorescent intensity was measured at 24, 48, and 72 h after cell-seeding by a fluorometer as described in Section 2. For calculating the fluorescent intensity in each cell line, the intensity at 24 h after cell seeding was assigned a value of 1. OR6c cells were used as a negative control. (C) Growth curve of G418-resistant cell lines. The cells were plated onto 6-well plate ($1 \times 10^5$ cells per well), and the kinetics of cell proliferation during 72 h in culture were determined by Trypan blue treatment. OR6c cells were used as a control.

the fluorescence of EGFP in these selected cell lines was roughly equivalent to that in OGN/C-5B/KE polyclonal cells (Fig. 2A), we next examined the time course of the fluorescent intensities of these cells lines by using a fluorometer, and observed a remarkable, twelve-fold increase in the fluorescent intensity of OGN/C-5B/KE clone 7 cells at 72 h after the start of cell culture in comparison with the intensity at 24 h (Fig. 2B). The fluorescent intensity of ON/GC-5B/KE clone 3 cells was slightly increased at 72 h (approximately four-fold). In contrast to these cell lines, the fluorescent intensity of OGN/GC-5B/KE clone 3 cells did not change during the cell culture. Growth curve analysis of these G418-resistant cell lines revealed that these cell clones had a similar kinetics for cell proliferation, although the growth rate of these cell clones was significantly lower than that of OR6c cells (Fig. 2C). These results suggest that the efficiency of genome-length HCV RNA replication in OGN/C-5B/KE clone 7 cells is higher than that in the other clones. Therefore, we finally selected OGN/C-5B/KE clone 7 (herein designated OGF7) for further characterization.

First, to exclude the possibility that the HCV RNA sequence had become integrated into the genomic DNA, we assayed for the HCV 5′-UTR sequence in the genomic DNA isolated from OGF7 cells by PCR. As a positive control, we used a cloned cell line (Mori et al., 2008) in which the HCV 5′-UTR sequence was integrated into the genomic DNA. The HCV 5′-UTR sequence was not detected in the genomic DNA isolated from OGF7 cells, genome-length HCV RNA-replicating O cells (Ikeda et al., 2005), or OR6c cells (Fig. 3A), although an expected product (266 bp or 205 bp) was detected in the positive control (Fig. 3A, lane PC). These results suggest that the HCV RNA sequence (at least HCV 5′-UTR sequence) is not integrated into the genomic DNA in OGF7 cells. Consistent with these results, an approximately 12 kb RNA of the genome-length HCV RNA encoding EGFP in OGF7 cells was also detected by Northern blot analysis, and its accumulation level was almost the same as that in the O cells (Fig. 3B). In addition, we confirmed by Western blot analysis that OGF7 cells efficiently expressed not only HCV proteins but also the EGFP-Neo$^R$ fused protein, and the expression levels of HCV proteins in the OGF7 cells were also equivalent to those in the O cells (Fig. 3C). In summary, these results indicate that the OGF7 cell line harboring replicative genome-length HCV RNA encoding EGFP as a reporter was stably established.

### *3.2. OGF7 living cells are useful for direct monitoring of the level of HCV RNA*

First, we examined whether or not the expression level of EGFP in OGF7 cells was sufficient to allow direct visualization by confocal laser-scanning microscopy. As a consequence, we could detect the fluorescence in addition to the core protein expressed in OGF7 cells (Fig. 4). Furthermore, we confirmed that the detected fluorescence was derived from the EGFP expressed in OGF7 cells, because both the fluorescence and the core protein disappeared after IFN-α treatment (Fig. 4). These results suggest that the replication of genome-length HCV RNA encoding EGFP-Neo$^R$ fused protein occurs efficiently in OGF7 cells. We next examined whether or not the IFN sensitivity of the EGFP level was associated with that of the HCV RNA level in OGF7 cells. The levels of EGFP and HCV RNA were examined by the fluorometer and real-time LightCycler PCR, respectively. The results revealed that the level of reduction in the fluorescent intensity by IFN-α treatment was equivalent to the level of reduction in the HCV RNA level (Fig. 5A and B). In addition, we confirmed by Western blot analysis that the reduction pattern of the EGFP-Neo$^R$ fusion protein by IFN-α treatment was also similar to those of the core and NS3 proteins (Fig. 5C). These results indicate that the expression level of EGFP is sufficient for monitoring of the level of HCV RNA, and suggest that the direct measurement of the fluorescent intensity of the living OGF7 cells was an effective means of monitoring the level of HCV RNA replication.

### *3.3. The OGF7 system is useful as a quantitative assay system for various anti-HCV reagents*

To clarify whether or not the OGF7 system is useful as a quantitative antiviral assay system, we first compared the IFN-α sensitivity of the OGF7 fluorescent reporter system with that

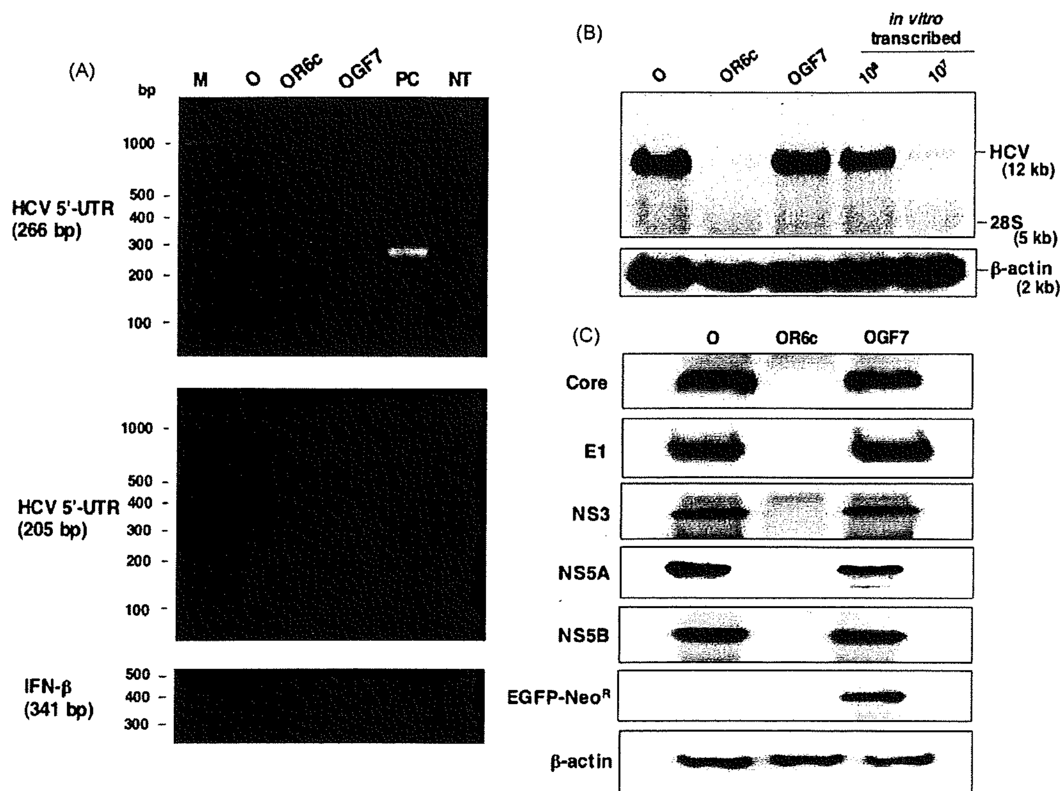


**Fig. 3.** Characterization of OGF7 cells replicating genome-length HCV RNA encoding EGFP as a reporter. Genome-length HCV RNA-replicating O cells (Ikeda et al., 2005) and OR6c cells (cured OR6 cells) were used for the comparison. (A) HCV genome-derived sequences were not integrated into the genomic DNA from OGF7 cells. Genomic DNA from the OGF7 cells was subjected to PCR for the detection of the HCV 5′-UTR and the IFN-β gene. Genomic DNAs from the O and OR6c cells were also used as negative controls. As a positive control, we used genomic DNA from a cell line (Mori et al., 2008) into which the HCV 5′-UTR sequence had been accidentally integrated (lane PC). PCR without genomic DNA was also performed (lane NT). PCR products (266 and 205 bp for HCV 5′-UTR, or 341 bp for the IFN-β gene) were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. The 100 bp DNA ladder was used as a size marker (lane M). (B) Northern blot analysis. Total RNAs (3 μg each) from the O, OR6c, and OGF7 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), respectively. In vitro-synthesized ORN/C-5B/KE (Ikeda et al., 2005) RNA ($10^7$ and $10^8$ genome equivalents spiked into normal cellular RNA) was used for the comparison of expression levels. (C) Western blot analysis. Production of core, E1, NS3, NS5A, and NS5B proteins in the O and OGF7 cells was analyzed by immunoblotting using anti-core, anti-E1, anti-NS3, anti-NS5A, and anti-NS5B antibodies, respectively. Production of EGFP-Neo$^R$ fusion protein and β-actin was also detected by anti-GFP and anti-β-actin antibodies, respectively.

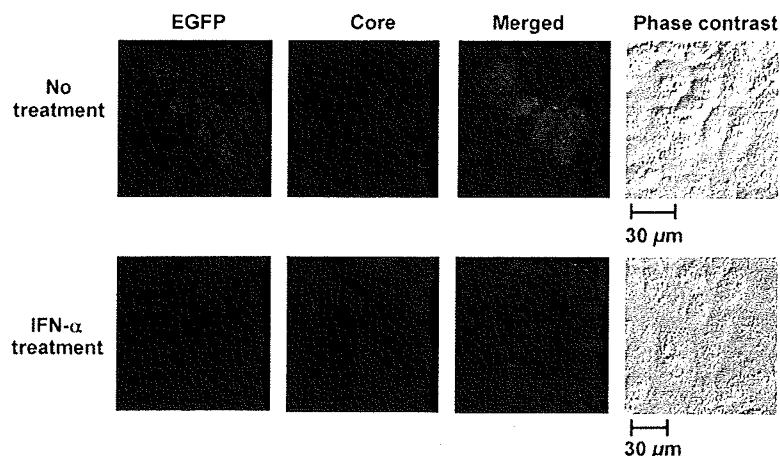


**Fig. 4.** The EGFP and core protein expressed in OGF7 cells disappeared following IFN-α treatment. OGF7 cells were examined by confocal laser-scanning microscopy. Cells were treated with IFN-α (500 IU/ml for 6 h). The cells were visualized with a fluorescence microscope, and then the cells were stained with anti-core antibody (CP11; Institute of Immunology, Tokyo, Japan) and Cy3-conjugated anti-mouse secondary antibody (Jackson Immuno Research, West Grove, PA) according to a method described previously (Naka et al., 2006). The merged panels show the two-color overlay images. Bar, 30 μm.

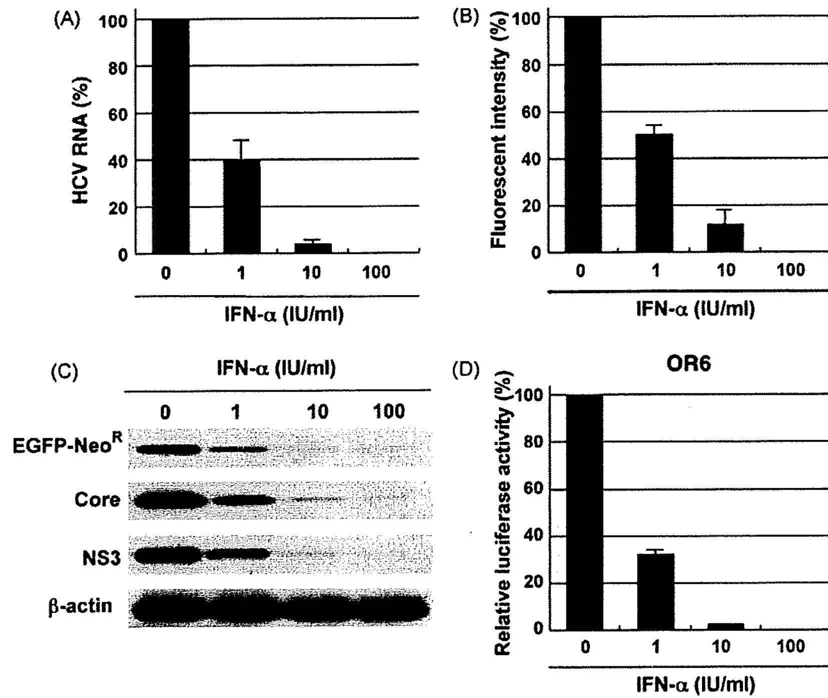


**Fig. 5.** Effect of IFN-α on genome-length HCV RNA replication in OGF7 and OR6 cells. OGF7 and OR6 cells were treated with IFN-α (0, 1, 10, and 100 IU/ml) for 72 h. After the measurements of the fluorescent intensity of OGF7 cells, the cells were subjected to quantitative RT-PCR analysis for HCV RNA and Western blot analysis. (A) Quantitative RT-PCR analysis of HCV RNA in OGF7 cells. Total RNA extracted from the cells was subjected to real-time LightCycler PCR analysis. The relative level of HCV RNA (%) calculated at each point, when the level of HCV RNA in untreated cells was assigned a value of 100%, is shown here. The experiments were performed in at least triplicate. (B) Fluorescent intensity of OGF7 cells. The fluorometer was used for the measurement of the fluorescent intensity of OGF7 cells. The relative level of the fluorescent intensity calculated, when the fluorescent intensity of untreated cells was taken as 100%, is shown here. The data indicate means from triplicate experiments. (C) Western blot analysis. The production of EGFP-Neo$^R$, core, and NS3 in OGF7 cells was analyzed by immunoblotting using anti-EGFP, anti-core, and anti-NS3 antibodies, respectively. β-Actin was used as a control for the amount of protein loaded per lane. (D) Renilla luciferase reporter assay using OR6 cells. The relative level of the luciferase activity calculated, when the luciferase activity of untreated cells was assigned a value of 100%, is shown here. The experiments were performed in at least triplicate.

of the OR6 luciferase reporter system. The results revealed that the profile of IFN-α sensitivity obtained by the OGF7 fluorescent system (Fig. 5B) was similar to that obtained using the OR6 luciferase system (Fig. 5D). Although the OGF7 system was slightly less sensitive than the OR6 system, the small difference may have been due to the different cell clones used. Because the results suggested that the OGF7 system is useful as a quantitative antiviral assay system, we proceeded to examine the activities of other anti-HCV reagents using the OGF7 system. The results revealed that the fluorescent intensity of OGF7 cells was decreased by the treatments of IFN-β, IFN-γ, CsA, and FLV in a dose-dependent manner (Fig. 6A), and that the level of the EGFP-Neo$^R$ fusion protein was also decreased by these anti-HCV reagents in a dose-dependent manner (Fig. 6B). These results suggest that the OGF7 system is useful as a quantitative anti-HCV assay system.

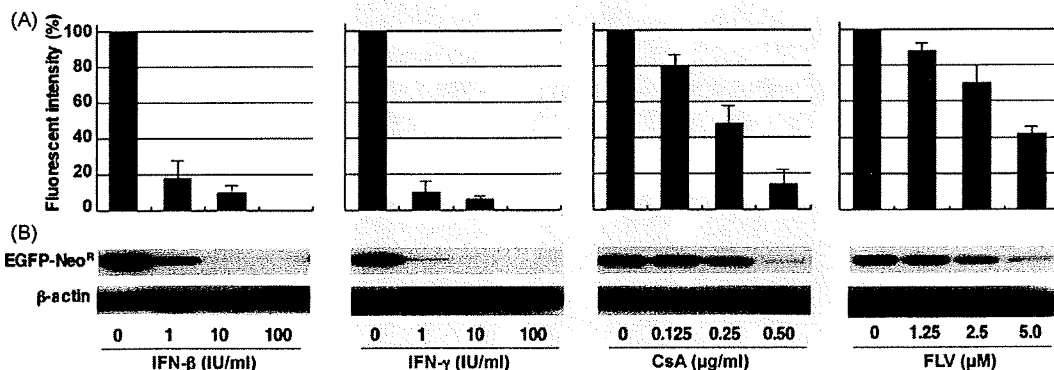


**Fig. 6.** Effects of IFN-β, IFN-γ, CsA, and FLV on genome-length HCV RNA replication in OGF7 cells. OGF7 cells were treated with IFN-β (0, 1, 10, and 100 IU/ml), IFN-γ (0, 1, 10, and 100 IU/ml), CsA (0, 0.125, 0.25, and 0.5 μg/ml) and FLV (0, 1.25, 2.5, and 5.0 μM). (A) Fluorescent intensity of OGF7 cells. After 72 h of treatment, the fluorescent intensity of OGF7 cells was measured by a fluorometer. The relative level of the fluorescent intensity calculated, when the fluorescent intensity of untreated cells was assigned a value of 100%, is shown here. The data indicate means from triplicate experiments. (B) Western blot analysis. The production level of EGFP-Neo$^R$ was analyzed by immunoblotting using anti-EGFP antibody. β-Actin was used as a control for the amount of protein loaded per lane.

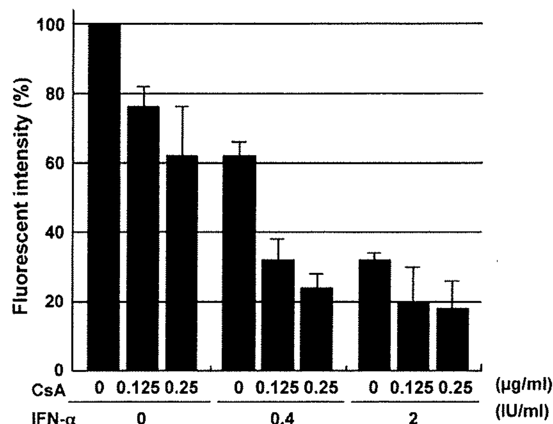


**Fig. 7.** Effect of IFN-α in combination with CsA on genome-length HCV RNA replication in OGF7 cells. OGF7 cells were co-treated with IFN-α (0, 0.4, and 2.0 IU/ml) and CsA (0, 0.125, and 0.25 μg/ml), and at 72 h after treatment, the fluorescent intensity of OGF7 cells was measured by a fluorometer. The relative level of the fluorescent intensity calculated, when the fluorescent intensity of untreated cells was taken as 100%, is shown here. The data indicate means from triplicate experiments.

### *3.4. The OGF7 assay system is also useful as a system for evaluating the efficacy of co-treatment with various anti-HCV reagents*

Since we demonstrated that the OGF7 system could be used effectively as either a quantitative anti-HCV assay system or OR6 assay system, we further examined whether or not the OGF7 system could be used to evaluate the efficacy of co-treatment with various anti-HCV reagents. The results showed that co-treatment with IFN-α and CsA was more effective than treatment with IFN-α alone (Fig. 7).

Together, the above results led us to conclude that the OGF7 living cell system is the most time-saving and low-cost anti-HCV assay system currently available.

## 4. Discussion

In the present study, we developed a new living cell-based reporter assay system (OGF7 assay system) for monitoring HCV RNA replication. We demonstrated that this OGF7 assay system was useful for the quantitative evaluation of anti-HCV reagents. Our study suggests that this new assay system is the most time-saving and inexpensive assay system for high-throughput screening of anti-HCV reagents.

To date, several cloned cell lines harboring HCV RNA (Con1 strain of genotype 1b) with EGFP have been reported (Liu et al., 2006; McCormick et al., 2006; Moradpour et al., 2004). However, regarding the Con1 strain, established cell lines are limited to the subgenomic replicon RNA, although several cloned cell lines harboring genome-length HCV RNA (JFH-1 strain of genotype 2a) with EGFP have been recently reported (Kim et al., 2007; Jones et al., 2007; Schaller et al., 2007). Since a quantitative reporter assay system for monitoring the level of HCV RNA replication has not been developed in these studies, we have tried to establish cell lines in which a genome-length HCV RNA encoding two or three copies of EGFP is efficiently replicating. However, from this study we have learned the limitation of RNA genome size. Although we tested seven different kinds of constructs for HCV RNA replication, most of the G418-resistant colonies were obtained from one copy type of EGFP (RNA genome size 11.8 kb) (Fig. 1). Although we obtained G418-resistant colonies from only OGN/GC-5B/KE construct containing two copies of EGFP (RNA genome size 12.5 kb), the fluorescent intensities of these colonies did not increase in a culture time-dependent manner, suggesting that the HCV RNA replication is not efficient in these cloned cells. These findings suggest that the genome size limitation in HCV RNA replication is approximately 12 kb. This suggestion is consistent with the previous finding (Ikeda et al., 2005) obtained in the process of development of the OR6 assay system. However, specific combination (Q1112R and K1609E) of adaptive NS3 mutations, which drastically enhanced the efficiency of genome-length HCV RNA replication (Abe et al., 2007), may overcome the genome size limitation (approximately 12 kb) in HCV RNA replication. When this genome size limitation is solved, a new cell line in which a genome-length HCV RNA encoding both EGFP-Neo$^R$ fused protein and another fluorescent reporter (e.g., EYFP)-NS5A fused protein replicate efficiently may be developed. Such a system would allow us to monitor the levels of HCV RNA and HCV proteins simultaneously.

We demonstrated that the established OGF7 cells were useful as a quantitative antiviral assay system (OGF7 assay system), because the anti-HCV activities of IFN-α, IFN-β, IFN-γ, CsA, and FLV were clearly shown in a dose-dependent manner just as in the evaluation using the OR6 assay system (Ikeda et al., 2005, 2006; Naka et al., 2005; Yano et al., 2007). Furthermore, since the OGF7 assay system allows us to measure, at different times, the same well containing OGF7 cells treated with the reagent, the OGF7 assay system can be considered superior to the OR6 assay system. Finally, since the OGF7 assay system is based on the simple measurement of the fluorescent intensity of living cells, this system has great advantages regarding time and cost for the antiviral assay of a number of reagents. Therefore, the OGF7 assay system is the most convenient method for high-throughput mass screening of a large compound library. Although we used 12-well plates for the assay in this study, we confirmed that we could monitor the level of HCV RNA replication on 24-well plates (data not shown). If the replication level of HCV RNA were to become higher than that in OGF7 cells due to additional adaptive mutation(s), such system might be capable of monitoring the replication level of HCV RNA in the living cells on 48- or 96-well plates. Such a system containing an OGF7 assay could be used to identify more effective and specific anti-HCV reagents in the future.

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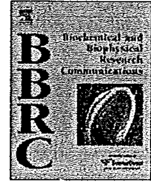

# New efficient replication system with hepatitis C virus genome derived from a patient with acute hepatitis C ☆

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ABSTRACT

We report for the first time a new RNA replication system with a hepatitis C virus (HCV) strain (AH1) derived from a patient with acute hepatitis C. Using an HCV replicon RNA library constructed with the AH1 strain (genotype 1b), we first established a cloned cell line, sAH1, harboring the HCV replicon. Cured cells obtained with interferon treatment of sAH1 cells were used for transfection with genome-length HCV RNA possessing four mutations found in sAH1 replicon. Consequently, one cloned cell line, AH1, supporting efficient replication of genome-length HCV RNA was obtained. By the comparison of AH1 cells with the O cells supporting genome-length HCV RNA (HCV-O strain) replication, we found different anti-HCV profiles of interferon-γ and cyclosporine A between AH1 and O cells. Reporter assay analysis suggests that the diverse effects of interferon-γ are due to the difference in HCV strains, but not the cellular environment.

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Hepatitis C virus (HCV) infection frequently causes chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV infection has now become a serious health problem because at least 170 million people worldwide are currently infected with HCV [1]. HCV is an enveloped virus with a positive single-stranded 9.6 kilobase (kb) RNA genome, which encodes a large polyprotein precursor of approximately 3000 amino acid (aa) residues [2,3]. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [3].

As a striking breakthrough in HCV research, in 1999, an HCV replicon system enabling robust HCV subgenomic RNA (Con-1 strain of genotype 1b) replication in specific human HuH-7 hepatoma cells has been developed [4]. After the first Con-1 replicon, several HCV replicon (genotypes 1a, 1b, and 2a) systems using HuH-7-derived cells have been developed. These replicon systems have become powerful tools for basic studies of HCV replication, HCV–host cell interactions, and screening of anti-HCV reagents, [5,6]. Furthermore, genome-length HCV RNA replication systems have been developed [7–9], since HCV replicons lacking HCV structural proteins are insufficient for further HCV research. We also established a genome-length HCV RNA-replicating cell line (HCV-O strain of genotype 1b; called O cell line) [10] using cured cells derived from sO cells [11], in which HCV replicon RNA (HCV-O strain) with an adaptive mutation (S2200R) is replicating. However, to date, established genome-length HCV RNA-replicating stable cell lines are limited to five HCV strains, H77 (1a), HCV-N (1b), Con-1 (1b), HCV-O (1b), and JFH1 (2a) [7–10,12], and there is no RNA replication system with an HCV strain derived from a patient with acute hepatitis C. Furthermore, there have been few reports comparing these HCV strains.

To clarify these problems, we have attempted to establish a new stable cell line, in which genome-length HCV RNA derived from a patient with acute hepatitis C is efficiently replicating. We report herein a new efficient RNA replication system with HCV derived from a patient with acute hepatitis C and provide a comparative analysis of RNA replication systems with AH1 and HCV-O strains regarding the sensitivities to anti-HCV reagents, including interferon (IFN)-α.

## Materials and methods

*Cell culture.* Cells supporting HCV replicon or genome-length HCV RNA, and cured cells, from which the HCV RNA had been eliminated by IFN treatment, were maintained as described previously [10].

*Reverse transcription (RT)-nested PCR.* RNA from a serum of patient AH1 [13] with acute hepatitis C was prepared using the ISOGEN-LS extraction kit (Nippon Gene Co., Japan). This RNA sample was used as a template for RT-nested PCR to amplify the HCV RNA. RT-nested PCR was performed separately in two parts; one part (3.5 kb) covered from HCV 5′UTR to NS3, and the other part (6 kb) covered from NS2 to NS5B. For the first part, the antisense primer AH3553R, 5′-CACACGCCGTTGATGC AGGTCG-3′ was used for RT. Primers 21 [11] and AH3519R, 5′-TGCGTGGCGG

☆ The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. AB429050.

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TGGAAACCACCTG-3′ were employed in the first round of PCR (35 cycles). An internal primer pair (21X [11] and AH3466RX: 5′-ATTATTCTAGAGGCCTGTGAGACTG GTGATGATGC-3′; containing a XbaI site (underlined)) was used for the second round of PCR (35 cycles). For the second part, the antisense primer 386R [11] was used for RT. Primers 542 and 9388R [11] were employed in the first round of PCR (35 cycles). An internal primer pair (3295X: 5′-ATTATTCTAGACTGACATGGA GACCAAGATCATCAC-3′; containing a XbaI site (underlined) and 9357RX: 5′-ATTATT CTAGACCCGTTCACCGGTTGGGGAGCAG-3′; containing a XbaI site (underlined)) was used for the second round of PCR (35 cycles). These fragments overlapped at the NS2 and NS3 regions and were used for sequence analysis for HCV RNA after cloning into the XbaI site of pBR322MC [11]. Superscript II (Invitrogen) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively.

*Plasmid construction.* PCR product (NS3 to NS5B of AH1 strain) with primers 542 and 9388R was further amplified with primers 3501S: 5′-ATTATACTAGTCTCACAGG CCGGGACAAGAACC-3′; containing a SpeI site (underlined) and 9162RB: 5′-ATTATC GTACGGCCCAGTTGAAGAGGTACTTGCC-3′; containing a BsiWI site (underlined). The amplified fragment was digested with SpeI and BsiWI, and ligated into the replicon cassette plasmid pNSS1RZ2RU [11], which was predigested with SpeI and BsiWI. Using this ligation reaction mixture, a replicon RNA library (AH1N/3-5B in Supplementary Fig. 1) was prepared by a previously described method [11]. To make the plasmid pAH1N/C-5B/PL, LS, $(VA)_2$ containing full-length HCV polyprotein of AH1 strain, pON/C-5B containing full-length HCV polyprotein of HCV-O strain [10] was utilized. First, to make a fragment for pAH1N/C-5B (Supplementary Fig. 1), overlapping PCR was used to fuse EMCV IRES to the core protein-coding sequence of the AH1 strain, as described previously [10]. The resulting DNA was digested with PmeI and ClaI, and then replaced with the PmeI–ClaI fragment of pON/C-5B (pON/C-5B/CoreAH was obtained). Second, the ClaI–AgeI fragment of pHCV-AH1 containing full-length HCV polyprotein of AH1 strain was replaced with the ClaI–AgeI fragment of pON/C-5B/CoreAH (pAH1N/C-5B was obtained). Finally, the SpeI–BsiWI fragment of pAH1N/3-5B clone 2 (see Fig. 1C) was replaced with the SpeI–BsiWI fragment of pAH1N/C-5B (pAH1N/C-5B/PL, LS, $(VA)_2$ was obtained).

*RNA synthesis.* Plasmid DNAs were linearized by XbaI and were used for RNA synthesis with T7 MEGAscript (Ambion) as previously described [11].

*RNA transfection and selection of G418-resistant cells.* The transfection of HCV replicon RNA or genome-length HCV RNA synthesized in vitro into HuH-7-derived cells was performed by electroporation, and the cells were selected in the presence of G418 (0.3 mg/ml; Promega) for 3 weeks as described previously [11].

*Quantification of HCV RNA.* The quantitative RT-PCR (RT-qPCR) analysis for HCV RNA was performed by LightCycler PCR as described previously [10]. Experiments were done in triplicate.

*Integration analysis.* Genomic DNA was extracted from the cultured cells using the DNeasy Blood & Tissue Kit (QIAGEN). The HCV 5′UTR and the IFN-β gene were detected according to a PCR method described previously [11].

*Western blot analysis.* The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as previously described [11]. The antibodies used in this study were those against Core, E2, NS3, NS4A, NS5A, and NS5B [10]. β-Actin antibody (AC-15, Sigma) was used as the control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA).

*Sequence analysis of HCV RNA.* To amplify replicon RNA and genome-length HCV RNA, RT-PCR was performed as described previously [10,11]. The PCR products were subcloned into the XbaI site of pBR322MC, and sequence analysis was performed as described previously [11].

*Northern blot analysis.* Total RNA was extracted from the cultured cells using the RNeasy Mini Kit (QIAGEN). Three micrograms of total RNA was used for the analysis. HCV-specific RNA and β-actin were detected according to a method described previously [10].

*Luciferase reporter assay.* For the dual-luciferase assay, firefly luciferase vectors, pGBP-1(-216)-Luc and p2′-5′-OAS(-159)-Luc [14], were used. The reporter assay was performed as previously described [14]. The experiments were performed in at least triplicate.

*Statistical analysis.* Differences between AH1 and O cell lines were tested using the Student's *t*-test. *P* values <.05 were considered statistically significant.

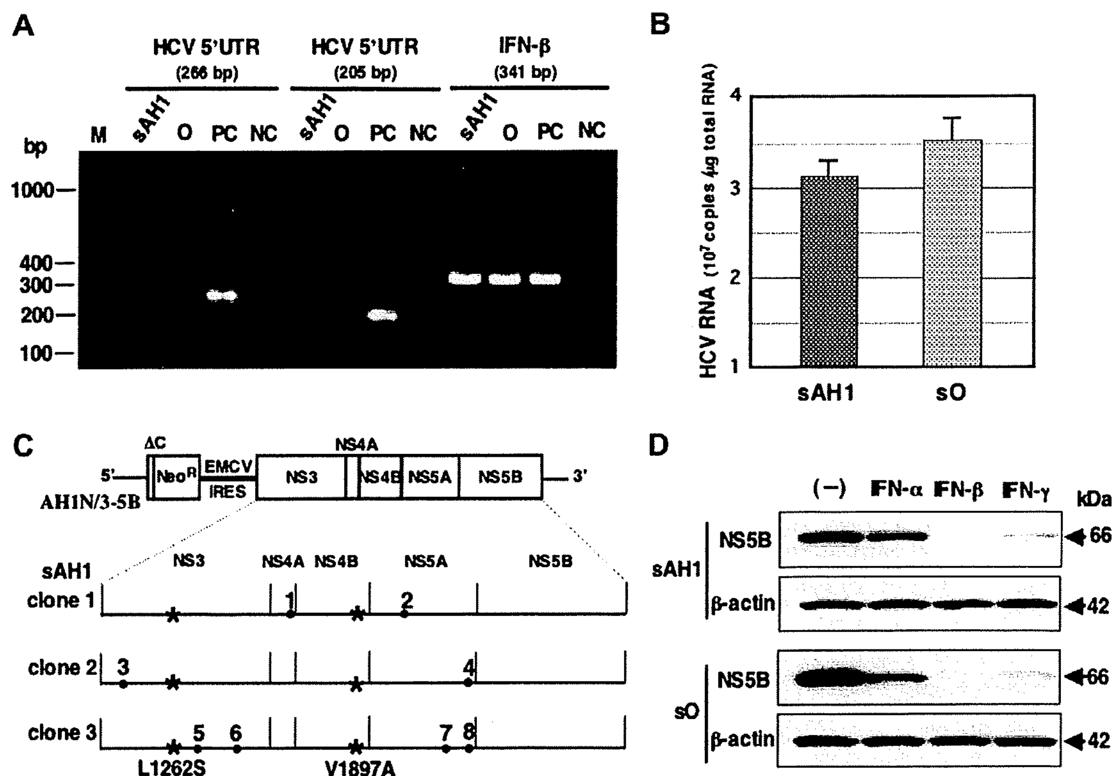


Fig. 1. Characterization of sAH1 cells harboring HCV replicon. (A) No integration of the HCV sequence in the genomic DNA. Genomic DNA from sAH1cells was subjected to PCR for the detection of the HCV 5′UTR and the IFN-β gene. O cells were used as a negative control. Lane PC, HCV sequence-integrated cells; lane NC, no genomic DNA; lane M, 100 bp DNA ladder. PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (B) Quantitative analysis of intracellular replicon RNA. The levels of replicon RNA were quantified by LightCycler PCR. sO cells harboring HCV-O replicon [11] were used for the comparison. (C) Amino acid substitutions detected in intracellular AH1 replicon RNA. NS3 to NS5B regions of three independent clones sequenced were presented. L1262S and V1897A conserved substitutions are indicated by asterisks. Clone-specific aa substitutions (indicated by the numbers with dots) are as follows: 1, K1691R; 2, M2105I; 3, P1115L; 4, V2360A; 5, K1368R; 6, A1533T; 7, I2285V; 8, D2377H. (D) IFN sensitivity of AH1 replicon. sAH1 cells were treated with IFN-α (Sigma), IFN-β (a gift from Toray Industries), and IFN-γ (Sigma) (20 IU/ml each) for 5 days. For the comparison, sO cells were treated as well as sAH1 cells. NS5B was detected by Western blot analysis.

## Results

### *Establishment of a G418-resistant cell line (sAH1) harboring HCV replicon RNA*

An HCV replicon RNA library prepared from the AH1 strain was first transfected into sOc cells (cured sO cells) [11], and the G418-resistant cells were selected as described previously [11]. Although several G418-resistant colonies were obtained, production of these colonies was due to integration of the HCV RNA sequence into the chromosomal DNA (PC in Fig. 1A). Therefore, we further cleaned up the replicon RNA library with additional DNase treatment, and it was then transfected into OR6c cells (cured OR6 cells) [10]. Consequently, a G418-resistant colony was obtained and successfully proliferated; this colony was referred to as sAH1. To exclude the possibility of integration of a replicon RNA sequence into the genomic DNA, we examined the presence of the HCV 5′UTR sequence in the genomic DNA isolated from sAH1 cells by a PCR method described previously [11]. Genome-length HCV RNA-replicating O cells were also examined as a negative control. The results revealed that the HCV RNA sequence was not integrated into the genomic DNA in either sAH1 cells or O cells (Fig. 1A).

Regarding the level of replicon RNA in sAH1 cells, RT-qPCR analysis revealed that the titer of replicon RNA was approximately $3 \times 10^7$ copies/μg total RNA, and its level was equivalent to that in sO cells (Fig. 1B), suggesting that the efficiency of RNA replication in sAH1 cells is similar to that in sO cells.

To exclude the possibility that sAH1 cells were derived from a small number of OR6 cells remaining after IFN treatment, and to determine whether replicon RNA in sAH1 cells possesses cell culture-adaptive mutations [5], which enhance the efficiency of RNA replication, we performed genetic analysis of the intracellular AH1 replicon. The sequences of three independent clones were determined and compared with each other to avoid PCR error. The obtained consensus nucleotide and aa sequences of NS3–NS5B regions of the AH1 replicon showed 7.3% and 3.7% differences, respectively, from those of the HCV-O replicon [11], indicating that sAH1 cells were not contaminated by the OR6 cells. In contrast, to find conserved mutations in the AH1 replicon, we determined the consensus nucleotide sequences of AH1 serum-derived HCV RNA by comparison of the nucleotide sequences of three independently isolated cDNA clones (Accession No. AB429050). The K1609E (NS3) and S2200R (NS5A) adaptive mutations found in O and OR6 cells were not detected in the AH1 replicon. However, instead of these mutations, L1262S (NS3) and V1897A (NS4B) conserved mutations were detected (Fig. 1C). Although V1897A has been detected as an adaptive mutation in Con-1 replicon [15], L1262S has until now remained undetected. In clone 2, the P1115L mutation (number 3 in Fig. 1C), which has been reported as an adaptive mutation [15,16], was detected.

To further characterize the sAH1 replicon, we compared the sensitivities of sAH1 and sO replicons against anti-HCV reagents (IFN-α, IFN-β, and IFN-γ) [5,6,11]. Western blot analysis of NS5B revealed that the IFN sensitivity of the sAH1 replicon was equivalent to that of the sO replicon (Fig. 1D).

### *Establishment of a genome-length HCV-AH1 RNA-replicating cell line, AH1*

To develop a genome-length HCV RNA replication system, we first constructed a pAH1N/C-5B/PL, LS, $(VA)_2$ by the replacement with sAH1 replicon clone 2 (Fig. 1C) into pAH1N/C-5B. AH1N/C-5B/PL, LS, $(VA)_2$ RNA was transfected into sAH1c cells, cured sAH1 cells. Following 3 weeks of culturing in the presence of

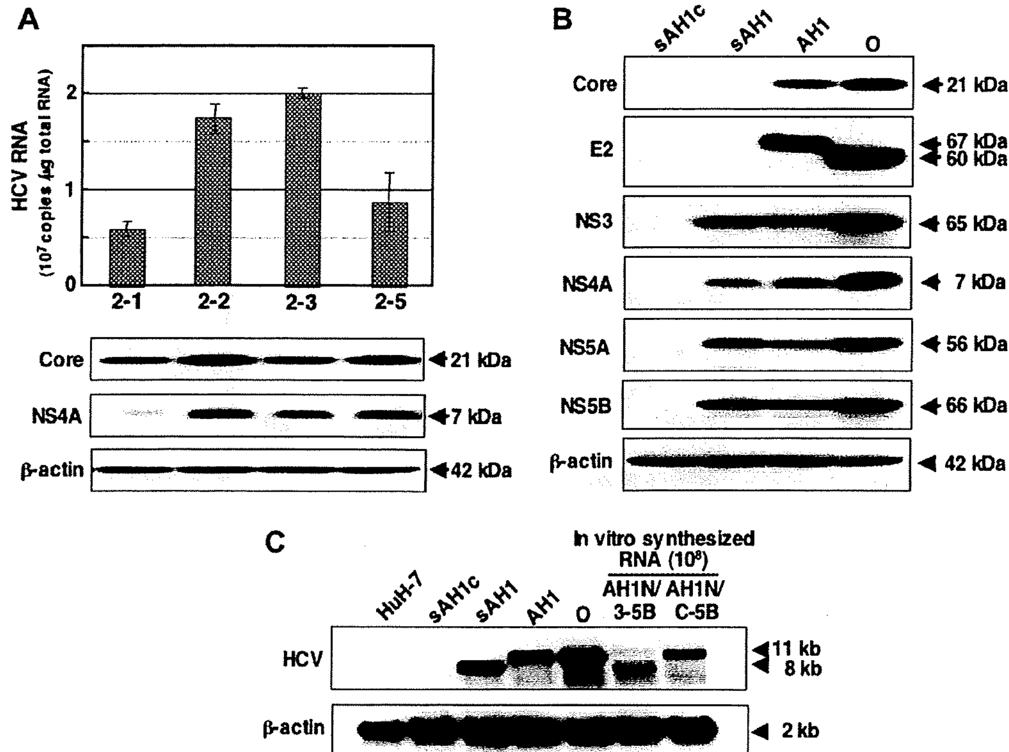


Fig. 2. Characterization of AH1 cells harboring genome-length HCV RNA. (A) Selection of G418-resistant cell lines. The levels of HCV RNA in G418-resistant cells were quantified by LightCycler PCR (upper panel). Core and NS4A were detected by Western blot analysis (lower panel). (B) Western blot analysis. AH1, O, sAH1, and sAH1c cells were used for the comparison. Core, E2, NS3, NS4A, NS5A, and NS5B were detected by Western blot analysis. (C) Northern blot analysis. AH1, O, sAH1, sAH1c, and HuH-7 cells were used for the comparison. In vitro-synthesized AH1N/3-5B and AH1N/C-5B RNAs were also used for the comparison.

G418, several colonies were obtained, and 4 colonies (2-1, 2-2, 2-3, and 2-5) then successfully proliferated. We selected colony 2-2 among them because it showed high levels of HCV RNA and proteins (core and NS4A) (Fig. 2A); this cell line was referred to as AH1. To compare the expression levels of HCV proteins in AH1 cells with those in O cells, Western blot analysis was further performed. Although the levels of HCV proteins in AH1 cells were slightly lower than those in O cells, the expression levels of NS proteins in AH1 cells were equivalent to those in sAH1 cells (Fig. 2B). In this analysis, we noticed that the size of the E2 protein in AH1 cells was 7 kDa larger than that in O cells (Fig. 2B). This difference may be due to the different numbers of *N*-glycosylation sites in E2 protein, since 11 and 9 *N*-glycosylation sites in E2 proteins are estimated in AH1 and HCV-O strains, respectively. Northern blot analysis also showed the presence of HCV-specific RNA with a length of approximately 11 kb in the extracts of total RNA prepared from AH1 cells, similar to that in the O cells (Fig. 2C). We confirmed the presence of replicon RNA (approximately 8 kb) in sAH1 cells (Fig. 2C). To check the additional adaptive mutations in the genome-length AH1 RNA, we performed sequence analysis of HCV RNA in AH1 cells. The results (Supplementary Fig. 2) revealed no additional mutations detected commonly among the three independent clones sequenced, suggesting that additional adaptive mutations are not required for genome-length HCV RNA replication. We therefore conclude that the AH1 cell line can be used as a genome-length HCV RNA replication system with acute hepatitis C-derived HCV strain.

*Diverse effects of anti-HCV reagents on HCV RNA replication in AH1 and O cells*

To compare the effects of anti-HCV reagents on RNA replication systems with different HCV strains, we examined the anti-HCV profiles of IFN-α, IFN-γ, and cyclosporine A (CsA) [17] using AH1 and O cells. Regarding IFN-α, the anti-HCV effect in AH1 cells was similar to that in O cells (Fig. 3A). Although RT-qPCR analysis showed a statistically significant difference in both cell systems when 1 IU/ml of IFN-α was used, such a difference was not observed in the Western blot analysis (Fig. 3A). In contrast, a significant different effect of IFN-γ was observed in both cell systems. RT-qPCR and Western blot analyses revealed that RNA replication of the AH1 strain was less sensitive than that of the HCV-O strain when 1 or 10 IU/ml of IFN-γ was used (Fig. 3B). Conversely, we observed that RNA replication of the AH1 strain was more sensitive to CsA than that of the HCV-O strain (Fig. 3C). These results suggest that anti-HCV profiles of IFN-γ and CsA are rather different between AH1 and O cell systems.

*Different anti-HCV profile of IFN-γ is not correlated with the cellular potentials of the IFN-γ signaling pathway*

To clarify whether the different effects of IFN-γ observed between AH1 and O cells are dependent on the cellular potentials of the IFN-γ signaling pathway, we performed a dual-luciferase reporter assay using an IFN-γ-inducible intrinsic GBP-1 gene promoter. As a control, IFN-α-inducible intrinsic 2′-5′-OAS gene promoter was also used for the analysis of the IFN-α signaling pathway. The results revealed that a good response of both AH1 and O cells to IFN-α and IFN-γ stimulation, with their potentials for both signaling pathways being almost the same (Fig. 4). These results suggest that the diverse anti-HCV effects of IFN-γ are dependent on the HCV strain, but not on the cellular potentials of the IFN-γ signaling pathway.

## Discussion

In the present study, we established for the first time an HCV RNA replication system with AH1 strain derived from a patient

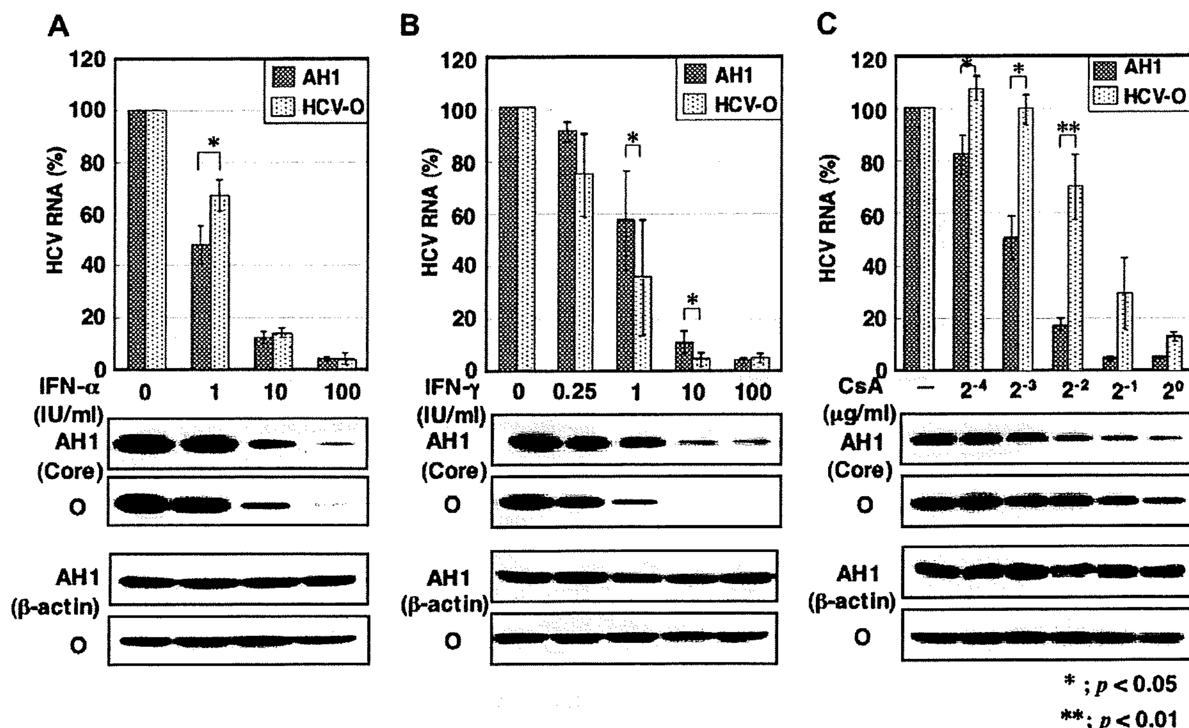


**Fig. 3.** The diverse effects of anti-HCV reagents on AH1 and HCV-O RNA replications. AH1 and O cells were treated with anti-HCV reagents for 72 h, and then extracted total RNAs and cell lysates were subjected to RT-qPCR for HCV 5′ UTR (each upper panel) and Western blot analysis for the core protein (each lower panel), respectively. (A) Effect of IFN-α. (B) Effect of IFN-γ. (C) Effect of CsA (Sigma).

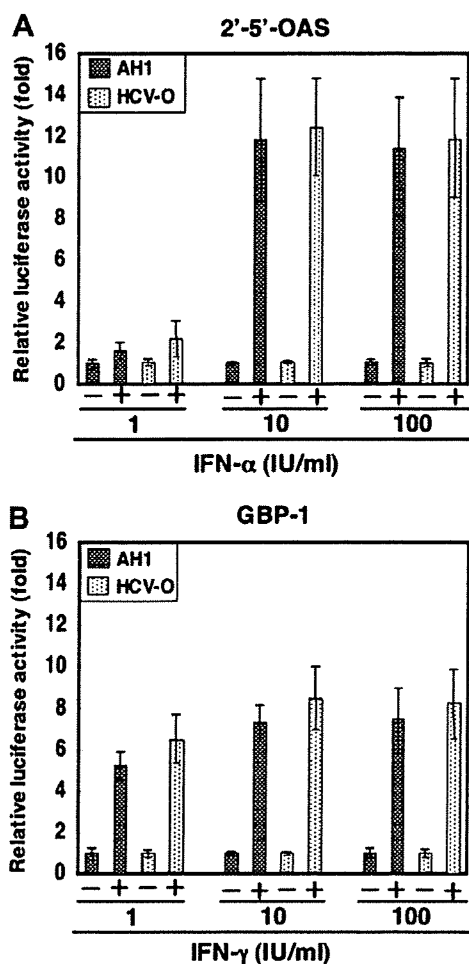


**Fig. 4.** Dual-luciferase reporter assay of IFN-α or IFN-γ-inducible gene promoter. AH1 and O cells were treated for 6 h with IFN-α or IFN-γ before the reporter assay. (A) 2′-5′-OAS gene promoter. (B) GBP-1 gene promoter.

with acute hepatitis C, and found diverse anti-HCV effects of IFN-γ and CsA between AH1 and HCV-O strains.

The levels of HCV replicon RNA and genome-length HCV RNA in sAH1 and AH1 cells were assigned to $3 \times 10^7$ and $2 \times 10^7$ copies/μg total RNA, respectively. These values are similar to those obtained from previously established HCV RNA replication systems [5]. Since known adaptive mutations (P1115L and V1897A) and additional conserved mutations (L1262S) were detected in the developed sAH1 replicon, these mutations may contribute to enhanced levels of RNA replication. The expression levels of genome-length HCV RNA and proteins observed in the present study suggest that genome-length HCV RNA replication efficiently occurs in AH1 cells, and that this RNA replication system is useful for comparison with already developed genome-length HCV RNA replication systems with HCV-N [7], Con-1 [8,9], or HCV-O [10] strains.

In the comparative analysis of genome-length HCV RNA replication systems with AH1 and HCV-O strains, we found that IFN-γ and CsA showed different anti-HCV profiles between AH1 and HCV-O strains. Regarding IFN-γ, RNA replication of the AH1 strain ($EC_{50}$ = 1.9 IU/ml) was less sensitive than that of the HCV-O strain ($EC_{50}$ = 0.3 IU/ml). Windisch et al. [18] have previously reported that RNA replication in an HCV replicon system using HuH-6 hepatoma cells is highly resistant ($EC_{50}$ was more than 100 IU/ml) to IFN-γ, and that its resistant phenotype is not due to a general defect in the IFN-γ signaling pathway. In that study, they speculated that some mutations within a critical effector gene in HuH-6 cells might account for the inability of the cells to reduce the number of replicon RNAs in response to IFN-γ. Although such a possibility is not completely excluded, the diverse effects of IFN-γ observed in the present study were likely due to the difference in viral strains because RNA replication of the AH1 strain is still sensitive to IFN-γ. To clarify this point, development of an additional HCV RNA replication system such as an OR6 assay system with more quantitative reporter genes [10] is needed.

Regarding CsA, RNA replication of the AH1 strain ($EC_{50}$ = 0.13 μg/ml) showed more sensitivity than that of the HCV-O strain ($EC_{50}$ = 0.35 μg/ml). Ishii et al. [17] have previously reported that RNA replication of the JFH1 strain (genotype 2a) is less sensitive to CsA than genotype 1b strains, including the HCV-O strain. In that study, they concluded that the difference in sensitivity of JFH1 and genotype 1b strains to CsA could be attributed to characteristic differences in the HCV strains, not to the parent cell strain. In addition, sensitivity to CsA was almost the same among genotype 1b strains in that study. Therefore, we estimate that the AH1 strain is more sensitive to CsA than these genotype 1b strains examined to date. Further analysis will be necessary to clarify the mechanism underlying differences in sensitivity to CsA among genotype 1b strains.

In conclusion, an HCV RNA replication system with the AH1 strain would be useful for comparison with other strain-derived systems in various HCV studies, including analysis of the effects of anti-HCV reagents.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.005.

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MAJOR ARTICLE

# Supportive Role Played by Precore and PreS2 Genomic Changes in the Establishment of Lamivudine-Resistant Hepatitis B Virus

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***Background.*** Hepatitis B virus (HBV) establishes lamivudine resistance via the resistance-causative rtM204V/I mutation and the replication-compensatory rtL180M mutation. However, both lamivudine-resistant viruses with and those without rtL180M can exist in clinical settings. To elucidate the differences between viruses with and those without rtL180M, we conducted full-length sequencing analysis of HBV derived from patients with type B chronic hepatitis showing lamivudine resistance.

***Methods.*** The full-length HBV DNA sequences derived from 44 patients showing lamivudine resistance were determined by polymerase chain reaction direct sequencing. Viral replicative competence was examined by in vitro transfection analysis using various HBV-expressing plasmids.

***Results.*** Throughout the HBV genome, a precore-defective A1896 mutation and a short deletion in the preS2 gene were detected more frequently in viruses without rtL180M than in those with it (64% vs. 17% [$P < .005$] and 50% vs. 10% [$P < .01$], respectively). In vitro transfection analysis revealed that the level of reduction in intracellular viral replication caused by the introduction of lamivudine resistance–associated mutations was lower in precore-defective and preS2-deleted viruses than in wild-type virus.

***Conclusions.*** Both the precore-defective mutation and the preS2 deletion may play a supportive role in the replication of lamivudine-resistant HBV, which may be a reason for there being no need for the compensatory rtL180M mutation in lamivudine-resistant HBV possessing the precore and preS2 genomic changes.

Therapeutic concepts for hepatitis B virus (HBV) infection have been strikingly modified by the introduction of nucleos(t)ide analogues. Nucleos(t)ide analogues, such as lamivudine, adefovir dipivoxil, entecavir, tenofovir disoproxil fumarate, emtricitabine, telbivudine, and clevudine, have been shown to lead to suppression of viral replication and improvement of liver diseases in chronic HBV infection [1–10]. However, the effectiveness of nucleos(t)ide analogues is debilitated by the emergence of drug-resistant mutant virus. Treatment with lamivudine has been shown to lead to a higher rate of emergence of drug-resistant virus than with other newly developed nucleos(t)ide analogues, such as adefovir dipivoxil and entecavir [11–14]. The incidence of lamivudine resistance has been reported to be 24% at 1 year and 70% at 4 years of therapy [11].

Lamivudine resistance is known to be caused by a point mutation within the reverse transcriptase (rt) domain of the HBV polymerase gene, either rtM204V or rtM204I [15–17]. In addition, an rtL180M mutation has been shown to be frequently found together with the rtM204V/I mutation associated with lamivudine resistance [15–17]. Previous studies using in vitro transfection with the HBV-expressing plasmid have demonstrated that the rtM204V/I mutation principally confers lamivudine resistance but results in a decrease in viral replicative activity [18, 19]. It has also been shown that

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the rtL180M mutation has no relevance to lamivudine resistance in itself but restores the reduced replicative activity caused by the lamivudine resistance–associated rtM204V/I mutation [20]. In light of these findings, the rtL180M mutation has been recognized as being compensatory for the support of replication in lamivudine-resistant HBV. Among patients with type B chronic hepatitis (CH-B) showing lamivudine resistance, almost all rtM204V mutations have been detected in conjunction with the rtL180M mutation, and rtM204I mutations have been found either in isolation or together with the rtL180M mutation [15–17]. Thus, the compensatory rtL180M mutation is not always necessary for generating replicative-competent lamivudine-resistant HBV in the clinical setting. Virus without the rtL180M mutation is speculated to possess specific features in the genome that support viral replicative activity, compared with virus with the mutation. However, differences between lamivudine-resistant viruses with and those without the rtL180M mutation have not been elucidated.

To clarify this, we determined the nucleotide sequences of full-length HBV DNA in 44 patients with CH-B who showed lamivudine resistance, by means of the direct sequencing method. Differences in the whole HBV genome were comprehensively investigated in relation to the presence or absence of the rtL180M mutation.

## METHODS

***Patients.*** The subjects were 44 consecutive patients with CH-B (37 males and 7 females) who received lamivudine therapy and became refractory to it at Osaka University Hospital and National Hospital Organization Osaka National Hospital. At the beginning of therapy, all patients tested positive for hepatitis B surface antigen (HBsAg) and were positive for HBV DNA by a branched DNA assay (Quantiplex HBV DNA; Chiron) or a polymerase chain reaction (PCR)–based assay (Amplicor HB Monitor; Roche Diagnostics). All patients were negative for antibodies to hepatitis C virus and HIV; none showed evidence of alcoholic liver disorder, autoimmune hepatitis, or drug-induced liver injury. Eight patients (18%) had previously received interferon (IFN) therapy.

All patients were treated with 100 mg of lamivudine daily, and liver function tests and monitoring of HBV markers were conducted during follow-up. In 16 patients (36%), natural IFN-$\alpha$ therapy (Sumiferon; Sumitomo Pharmaceuticals) was administered in combination with lamivudine for the initial 24 weeks (total dose, 432 million units). For all 44 patients, the lamivudine-resistant rtM204V/I mutation was detected by a PCR enzyme-linked minisequence assay (Sumitomo Metal Industries) [21] after an initial reduction and subsequent increase in HBV DNA during therapy. All serum samples for sequencing analysis of full-length HBV DNA were collected after the emergence of the lamivudine-resistant mutant virus and were stored at $-80$°C until use. The serum sampling points ranged from 0.8 to 5.5 years (median, 2.7 years) after the commencement of lamivudine therapy. In addition, pairwise serum samples obtained before therapy were used to determine the nucleotide sequences in portions of HBV DNA as baseline controls for 23 patients (52%).

Patient characteristics at the point of analysis were as follows. Age ranged from 25 to 74 years (median, 51 years). Hepatitis B e antigen (HBeAg) was found in 31 patients (70%), and antibody to HBeAg developed in all 13 HBeAg-negative patients (30%). Serum HBV DNA levels ranged from 3.5 to >7.6 $\log_{10}$ copies/mL (median, 7.2 $\log_{10}$ copies/mL). Serum alanine aminotransferase (ALT) levels ranged from 11 to 393 IU/L (median, 66 IU/L). Chronic hepatitis was diagnosed in 34 patients (77%), cirrhosis in 6 (14%), and hepatocellular carcinoma in 4 (9%), on the basis of liver biopsy and/or abdominal imaging procedures. Informed consent was obtained from all patients.

***Genomic analysis of full-length HBV nucleotide sequences.*** From the serum sample, full-length HBV DNA was amplified by PCR and directly sequenced as described elsewhere [22]. The full-length HBV DNA sequences derived from the 44 patients with lamivudine-resistant CH-B (GenBank accession numbers AB367392–AB367435) were aligned together with the 12 representative HBV strains of various genotypes by means of CLUSTALW software. Phylogenetic tree analysis was then conducted [23, 24].

***Plasmid and transfection.*** The HBV-expressing plasmid pHBC was derived from the genotype C2 HBV strain adr4 (GenBank accession number X01587) [25]. pHBC was constructed by inserting the 1.2-fold HBV genome into pBluescriptIISK$^+$. pHBC-PC and pHBC-$\Delta$PS2, which were generated by site-directed mutagenesis, possessed the precore-defective A1986 mutation and the short deletion of 45 bp (nt 11–55) within the preS2 gene. Further site-directed mutagenesis was done to introduce rtM204V and rtL180M, rtM204I and rtL180M, or rtM204I alone into pHBC, pHBC-PC, and pHBC-$\Delta$PS2. pCMV-SEAP was a secreted alkaline phosphatase–expressing plasmid.

For transfection, $3 \times 10^5$ Huh7 cells were seeded on a 35-mm-diameter culture dish and transfected with 1 $\mu$g of various HBV-expressing plasmids and 0.06 $\mu$g of pCMV-SEAP, using FuGENE6 reagent (Roche Diagnostics). On day 5, the culture supernatant and cell lysate were collected. Transfection efficiency was evaluated by measuring the secreted alkaline phosphatase activity.

***Detection of viral progeny DNA and antigen in HBV-expressing cells.*** For detection of intracellular HBV DNA, cells were lysed in buffer containing 50 mmol/L Tris-Cl (pH 7.5), 1 mmol/L EDTA, and 1% Nonidet-P40. After a 15-min incubation on ice, nuclei were removed by brief centrifugation. Then, the sample was incubated at 37°C for 30 min in the presence of 0.1 mg/mL DNaseI and 10 mmol/L $MgCl_2$. After the reaction was stopped by adding EDTA, the sample was subjected to over-

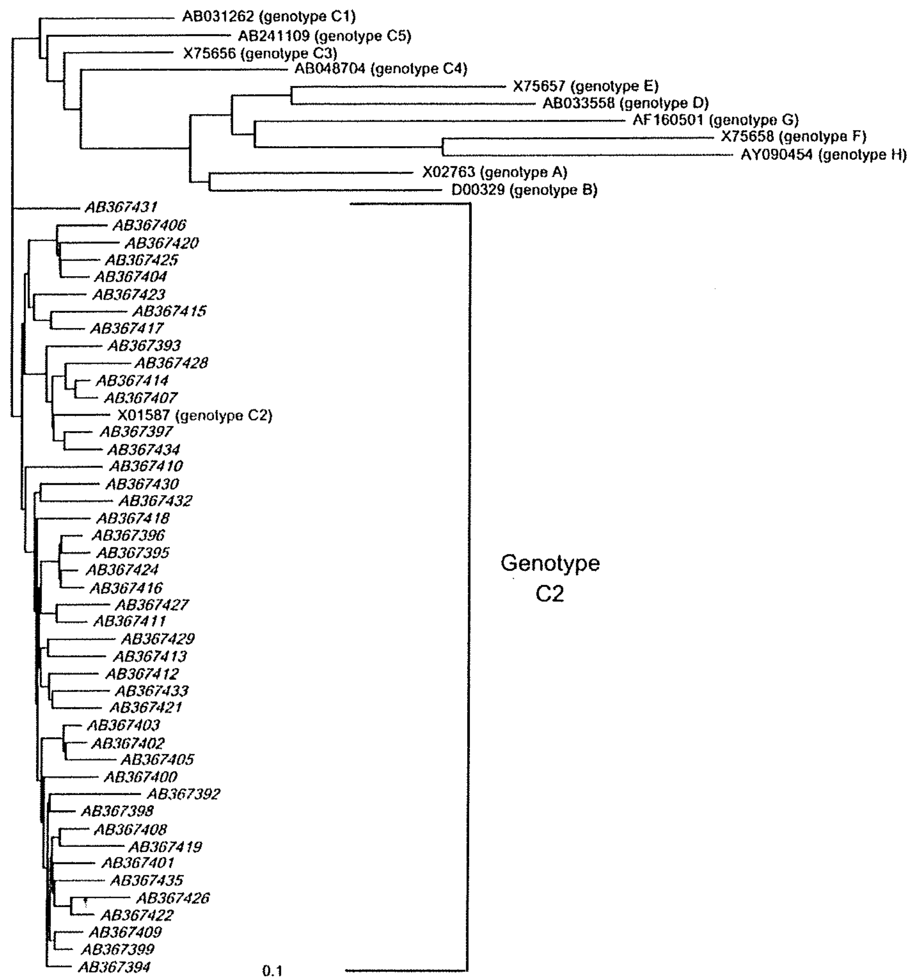


**Figure 1.** Phylogenetic tree analysis including 44 lamivudine-resistant hepatitis B virus (HBV) strains obtained in the present study and 12 representative HBV strains of various genotypes. All HBV strains are represented as GenBank accession nos., and the 44 lamivudine-resistant HBV strains are indicated by italics.

night incubation at 37°C in buffer containing 1% sodium dodecyl sulfate and 0.5 mg/mL proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The DNA sample was subjected to Southern blot analysis to detect HBV DNA, using a nonradioactive detection system (Alkphos Direct; GE Healthcare Life Sciences). Finally, the signals were analyzed quantitatively using image analyzing software (ImageJ; version 1.38). To detect extracellular HBV DNA, the transfection was scaled up to the 60-mm-diameter culture dish. After clarification by centrifugation at 8300 *g* for 30 min, the culture medium (3 mL) was centrifuged through a 20% sucrose cushion at 192,000 *g* for 4 h, using the Beckmann SW55Ti rotor. Then, DNA was extracted from the pellet and subjected to Southern blot analysis as described above. HBsAg and HBeAg in the culture medium were measured by chemiluminescent immunoassay.

***Statistical analysis.*** Statistical analysis was performed by the $\chi^2$ test, Fisher's exact test, and the Mann-Whitney *U* test. The results for the in vitro transfection study were examined by 1-way analysis of variance, and pairwise comparison was done by Fisher's protected least significant difference test. $P < .05$ was considered to indicate statistical significance.

## RESULTS

***Patient clinical characteristics and lamivudine resistance–associated mutations.*** All 44 HBV strains obtained from the patients with lamivudine-resistant CH-B comprised 3161–3230 nt in length and belonged to genotype C2, the most prevalent type in Japan (figure 1). As for lamivudine resistance–associated mutations in these strains, the rtM204V mutation was observed in 16 strains (36%), whereas the remaining 28 strains (64%) had the rtM204I mutation. The compensatory rtL180M mutation was found in 30 strains (68%). All 16 strains with rtM204V and 14 (50%) of the 28 strains with rtM204I possessed the rtL180M

**Table 1. Clinical features of patients with lamivudine-resistant type B chronic hepatitis, according to the mutational status of rt180 and rt204.**

| | rt180 status | | | rt204 status | | |
|---|---|---|---|---|---|---|
| Clinical feature | rtL180M positive ($n$ = 30) | rtL180M negative ($n$ = 14) | $P$ | rtM204V ($n$ = 16) | rtM204I ($n$ = 28) | $P$ |
| Age, years | 48 (25–74) | 55 (27–71) | NS | 48 (25–74) | 51 (27–71) | NS |
| Sex, M/F, no. | 25/5 | 12/2 | NS | 13/3 | 24/4 | NS |
| Liver disease, chronic hepatitis/cirrhosis/ hepatocellular carcinoma, no. | 26/3/1 | 8/4/2 | NS | 13/2/1 | 21/5/2 | NS |
| ALT level, IU/L | 66 (11–331) | 67 (25–393) | NS | 85 (17–261) | 54 (11–393) | NS |
| HBeAg, positive/negative, no. | 23/7 | 8/6 | NS | 13/3 | 18/10 | NS |
| HBV DNA level, $\log_{10}$ copies/mL | 7.5 (3.5 to >7.6) | 7.1 (3.6 to >7.6) | NS | 7.5 (3.8 to >7.6) | 7.1 (3.5 to >7.6) | NS |
| Previous IFN therapy, no. (%) | 6 (20) | 2 (14) | NS | 3 (19) | 5 (18) | NS |
| Combination therapy with IFN, no. (%) | 10 (33) | 6 (43) | NS | 4 (25) | 12 (43) | NS |
| Duration of lamivudine administration until point of analysis, years | 2.9 (1.5–5.5) | 2.2 (0.8–4.8) | NS | 2.9 (1.5–5.5) | 2.2 (0.8–4.8) | NS |

**NOTE.** Data are median (range) values, unless otherwise indicated. HBeAg, hepatitis B e antigen; IFN, interferon; NS, not significant.

mutation, in agreement with previous reports with respect to the emergence pattern of the rtM204V/I and rtL180M mutations [15–17].

Various patient clinical characteristics were first correlated with the presence or absence of the rtL180M mutation or with the alternative of the rtM204V or rtM204I mutation in our 44 patients with CH-B (table 1). No differences were observed between patients with and those without the rtL180M mutation with respect to age, sex ratio, disease severity, ALT level, HBeAg positivity, serum HBV DNA level, frequency of previous IFN therapy, frequency of combination therapy with IFN, and total duration of lamivudine administration until the point of analysis. Also, there were no significant differences concerning these 9 characteristics between patients with virus having the rtM204V mutation and those with virus having the rtM204I mutation.

***Genomic changes throughout the HBV genome associated with lamivudine resistance–associated mutations.*** Next, the genomic changes, which were significantly correlated with the occurrence of rtL180M or the preference for rtM204V or rtM204I, were investigated for the 44 HBV strains derived from the patients. As shown in table 2, 8 mutations and 1 deletion were identified as viral genomic changes significantly associated with the presence or absence of rtL180M. Among them, the A1896 mutation, which forms the in-frame stop codon in the

**Table 2. Differences in the viral genome between lamivudine-resistant hepatitis B virus (HBV) strains with and those without the rtL180M mutation.**

| | | | rtL180M, no. (%) | | |
|---|---|---|---|---|---|
| Viral genomic changes | Consensus nucleotide[a] | Amino acid substitution | Positive ($n$ = 30) | Negative ($n$ = 14) | $P$ |
| Mutation | | | | | |
| A373 | C | Pol-L428M (rtL82M) | 0 (0) | 3 (21) | <.05 |
| T619 | C | None | 0 (0) | 3 (21) | <.05 |
| G739 | T | Pol-M550V (rtM204V), surface-I95R | 16 (53) | 0 (0) | <.001 |
| T/C/A741 | G | Pol-M550I (rtM204I), surface-W96L/S/stop | 14 (47) | 14 (100) | <.001 |
| A1896 | G | Precore-W28stop | 5 (17) | 9 (64) | <.005 |
| T2102 | C | None | 0 (0) | 3 (21) | <.05 |
| A/G2660 | C | Pol-N118K | 0 (0) | 3 (21) | <.05 |
| A2860 | T | PreS1-S5T, pol-V184D[b] | 0 (0) | 4 (29) | <.01 |
| Deletion | | | | | |
| 6–54-bp deletion within nt 1–55 | . . . | Truncation of 2–18 amino acids in preS2[c] | 3 (10) | 7 (50) | <.01 |

[a] Consensus nucleotides are derived from the genotype C2 HBV strain adr4 (GenBank accession no. X01587) [25].
[b] One patient had the pol-V184Q amino acid substitution due to a mutation in the adjacent nucleotide position.
[c] Detailed patterns of the preS2 deletion are shown in figure 2.

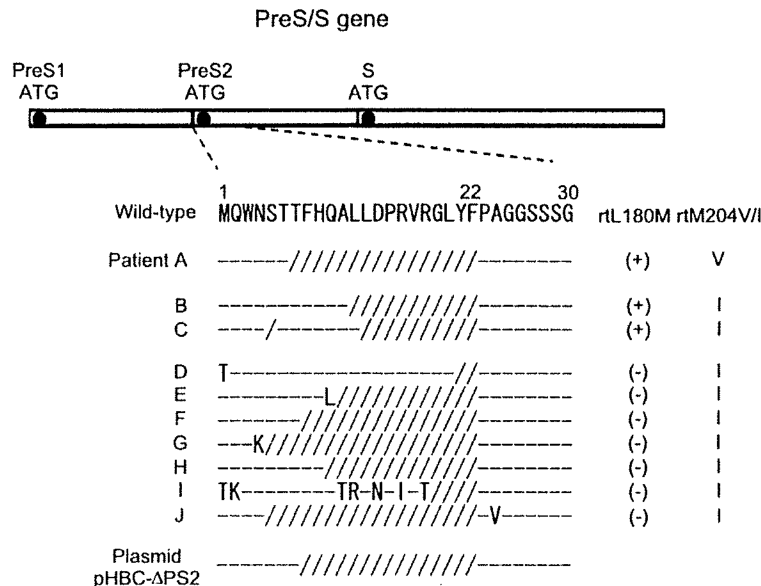


**Figure 2.** Patterns of the short deletion in the preS2 gene observed in lamivudine-resistant hepatitis B virus (HBV) strains. Ten of the 44 patients (patients A–J) had virus with the deletion in the preS2 gene of various patterns. The top sequence represents the amino acid sequence of the genotype C2 HBV DNA strain adr4 [25] as a representative strain. As for sequences derived from the patients, residues identical to the top sequence are indicated by dashes, whereas deletions of amino acid residues are shown by slashes. All deletions were found within the codon positions 5–22 of the preS2 gene. The bottom sequence represents the deletion pattern of the plasmid (pHBC-ΔPS2) used for in vitro transfection analysis (see figure 3), which expresses HBV DNA with the short deletion in the preS2 gene.

precore gene and results in the disability of HBeAg synthesis [26, 27], was found more frequently in viral strains without rt180M than in those with it (64% vs. 17%; $P < .005$). Viral strains lacking rtL180M possessed the short deletion in the preS2 gene more frequently than those with rtL180M (50% vs. 10%; $P < .01$). The lengths of the deletion ranged from 12 to 54 bp, and all deletions were located within codon positions 5 to 22 of the preS2 gene (figure 2). Significant differences were also seen in the occurrences of 5 additional mutations—A373, T619, T2102, A/G2660, and A2860—between strains with and those without rtL180M. The detection rate of these 5 mutations was generally low among the lamivudine-resistant HBV strains obtained in this study. The G739 and T/C/A741 mutations are the causes of the rtM204V and rtM204I amino acid changes, and the occurrences of these mutations differed between viral strains with and those without rtL180M ($P < .001$), as described above.

Throughout the HBV genome, 5 mutations were significantly associated with the preference for the rtM204V or rtM204I mutation in the 44 lamivudine-resistant HBV strains (table 3). Of them, 3 mutations—C565, A853, and C1568—were found more frequently in strains with rtM204V than in those with rtM204I, but the frequencies of these mutations were considerably low in our lamivudine-resistant HBV strains. The occurrence of the A667 mutation, which accounts for rtL180M, was

**Table 3. Differences in the viral genome between lamivudine-resistant hepatitis B virus (HBV) strains with the rtM204V and rtM204I mutations.**

| Mutation | Consensus nucleotide[a] | Amino acid substitution | No. (%) rtM204V ($n$ = 16) | No. (%) rtM204I ($n$ = 28) | $P$ |
|---|---|---|---|---|---|
| C565 | T | None | 4 (25) | 0 (0) | <.05 |
| T/C646 | A | Pol-V519L (rtV173L) | 5 (31) | 0 (0) | <.005 |
| A667 | T | Pol-L528M (rtL180M) | 16 (100) | 14 (50) | <.001 |
| A853 | C | None | 3 (19) | 0 (0) | <.05 |
| C1568 | T | Pol-L826P | 3 (19) | 0 (0) | <.05 |

[a] Consensus nucleotides are derived from the genotype C2 HBV strain adr4 (GenBank accession no. X01587) [25].

**Table 4. Changing pattern of the precore defective A1896 mutation and short deletion in the preS2 gene from the pretreatment baseline to development of lamivudine resistance in relation to the presence or absence of the rtL180M mutation.**

| | Pattern of mutation | | rtL180M, no. | |
|---|---|---|---|---|
| Type of mutation | Before therapy | After therapy[a] | Positive ($n$ = 15) | Negative ($n$ = 8) |
| Precore-defective A1896 mutation | − | − | 8 | 2 |
| | + | + | 4 | 4 |
| | − | + | 1 | 2 |
| | + | − | 2 | 0 |
| Short deletion in the preS2 gene | − | − | 12 | 5 |
| | + | + | 0 | 1 |
| | − | + | 2 | 2 |
| | + | − | 1 | 0 |

[a] After development of lamivudine-resistant mutant virus.

higher in viral strains with rtM204V than in those with rtM204I ($P < .001$), as shown above. The T/C646 mutation, which causes the rtV173L change, was detected in 5 strains (31%) with rtM204V, compared with none of those with rtM204I ($P < .005$). It has been reported that the rtV173L mutation was detected together with the rtM204V and rtL180M mutations and was considered to be associated with lamivudine resistance [17, 28]. Our finding concerning the rtV173L mutation agreed with those of previous reports.

According to these observations, the relevance of the precore-defective A1896 mutation and the preS2 deletion to the absence of rtL180M was the most distinctive feature of the lamivudine-resistant HBV strains on screening of the whole genome. We therefore directed our attention to these precore and preS2 genomic changes and further investigated their role in the establishment of lamivudine-resistant virus.

***Serial changes in the precore mutation and the preS2 deletion in lamivudine-resistant virus before and after lamivudine therapy.*** Serial changes in the precore-defective A1896 mutation, the short deletion in the *preS2* gene, and the drug resistance–associated rtM204V/I, rtL180M, and rtV173L mutations were investigated in the 23 (52%) of 44 patients with CH-B whose serum samples obtained before lamivudine therapy were available (table 4). Of the 11 patients with virus having the precore-defective mutation after the development of lamivudine resistance, 8 had virus that already possessed the mutation before therapy. Thus, the precore-defective mutation was generally a preexisting genomic change in most patients showing lamivudine resistance. On the other hand, of the 5 patients with virus that had the deletion in the preS2 gene after the development of drug resistance, 4 had virus that did not possess the deletion before therapy. The frequent detection of the preS2 deletion in lamivudine-resistant virus compared with virus before therapy indicates that this deletion may be coselected with drug resistance–associated mutations during the establishment of lamivudine-resistant mutant virus. As for the lamivudine-resistant rtM204V/I, rtL180M, and rtV173L mutations, they were not detected in any of the 23 viruses before lamivudine therapy, as expected.

***Effect of the precore mutation and the preS2 deletion on the replicative competence of lamivudine-resistant HBV in vitro.*** We further conducted in vitro transfection analysis to explore the influence of the precore-defective mutation and the preS2 deletion on the replicative competence of lamivudine-resistant HBV. Three plasmids that expressed wild-type virus, precore-defective virus, and virus with the preS2 deletion were prepared. Next, plasmids with rtM204V plus L180M, rtM204I plus L180M, and rtM204I alone were synthesized in each of the 3 HBV-expressing backbone constructs. The level of intracellular HBV DNA was examined in cells transfected with these HBV-expressing plasmids. As shown in figure 3*A* and 3*B*, the introduction of lamivudine resistance–associated mutations into the virus with the wild-type backbone led to a decrease in viral replication (lanes 1–4). In addition, the replicative competence of the drug-resistant virus lacking rtL180M tended to be lower than that of the virus having rtL180M, although the difference was not statistically significant. As for the precore-defective virus, its replicative activity at baseline was higher than that of the wild-type virus (lanes 1 and 5). The decline in HBV replication due to the insertion of drug resistance–associated mutations was also observed for the virus with the precore-defective backbone. However, unlike for the virus with the wild-type backbone, the replicative activity of the precore-defective virus with lamivudine-resistant mutations was maintained at a considerable level (lanes 5–8). As for the virus with the preS2-deleted backbone, a reduction in viral replication due to the introduction of lamivudine resistance–associated mutations was also seen, but the degree of the reduction was not as great as that in the wild-type virus (lanes 9–12). Thus, both the precore-defective mutation and the preS2 deletion possessed activity supporting the viral replicative competence of lamivudine-resistant HBV, although the activity with the preS2 deletion was not as strong as that with the precore-defective mutation. The

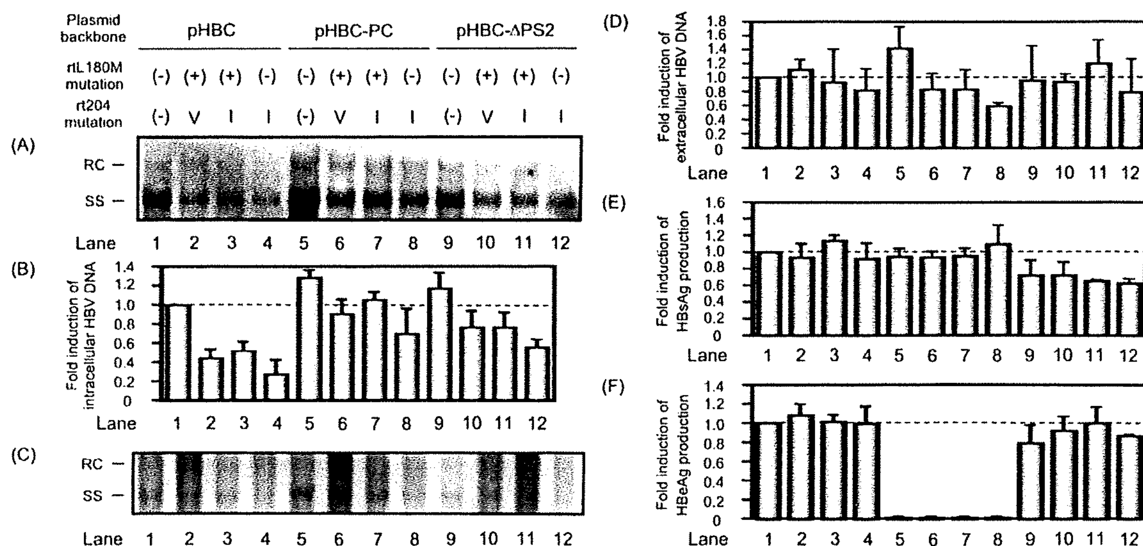

**Figure 3.** Levels of intracellular and extracellular progeny viral DNA and viral antigen production in cultured cells transfected with wild-type, precore-defective, or preS2-deleted hepatitis B virus (HBV)–expressing plasmids with or without lamivudine resistance-associated mutations. *A,* Representative result of Southern blot analysis to detect the intracellular progeny HBV DNA in cells transfected with various HBV-expressing plasmids. *B,* Quantitative analysis of the level of intracellular progeny HBV DNA. The progeny HBV DNA level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean ± SD values. Statistically significant differences ($P < .05$) are as follows: lane 1 vs. 2–4, 1 vs. 5, 2 vs. 6 and 10, 3 vs. 7 and 11, 4 vs. 8 and 12, 5 vs. 6 and 8, and 9 vs. 10–12. *C,* Representative result of Southern blot analysis to detect extracellular progeny HBV DNA in cells transfected with various HBV-expressing plasmids. *D,* Quantitative analysis of the level of extracellular progeny HBV DNA. The progeny HBV DNA level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 4 times, and results are shown as mean ± SD values. A statistically significant difference was not observed by 1-way analysis of variance. *E,* Levels of hepatitis B surface antigen (HBsAg) in culture medium of cells transfected with various HBV-expressing plasmids. The HBsAg level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean ± SD values. Statistically significant differences ($P < .05$) are as follows: lanes 1 and 5 vs. 9, 3 and 7 vs. 11, and 4 and 8 vs. 12. *F,* Levels of hepatitis B e antigen (HBeAg) in culture medium of cells transfected with various HBV-expressing plasmids. The HBeAg level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean ± SD values. Statistically significant differences ($P < .05$) are as follows: lanes 1 and 9 vs. 5, 2 and 10 vs. 6, 3 and 11 vs. 7, and 4 and 12 vs. 8. RC, relaxed circular HBV DNA; SS, single-stranded HBV DNA.

tendency appeared to be more evident in the drug-resistant virus without the rtL180M mutation. This may be a reason for the compensatory rtL180M mutation not being necessary during the establishment of lamivudine resistance in the HBV strain having the precore and preS2 genomic changes.

When the level of extracellular HBV DNA was examined in cells transfected with various HBV-expressing plasmids (figure 3*C* and 3*D*), no significant differences were observed among wild-type, precore-defective, and preS2-deleted viruses with respect to the reduction of viral secretion caused by the introduction of the lamivudine resistance–associated mutation. The discrepant results between the intracellular and extracellular viral DNA levels likely occurred because the extracellular viral DNA assay was less sensitive to minute changes in viral replication than the intracellular viral DNA assay.

As for the levels of production of HBsAg and HBeAg, the virus with the preS2-deleted backbone produced less HBsAg than did the viruses with the wild-type and precore-defective backbones (figure 3*E*). The wild-type and preS2-deleted viruses secreted HBeAg, whereas the precore-defective virus did not (figure 3*F*). The lamivudine resistance-associated mutations did not affect the production levels of HBV antigens.

## DISCUSSION

HBV establishes lamivudine resistance via the resistance-causative rtM204V/I mutation and the replication-compensatory rtL180M mutation [15–20]. The present study aimed to investigate the genomewide peculiarity of lamivudine-resistant HBV. In particular, we elucidated the differences between viruses with and those without the compensatory rtL180M mutation. For this purpose, we conducted full-length sequencing analysis of lamivudine-resistant viruses derived from patients with CH-B by means of the PCR direct sequencing method. In some patients, the results were also confirmed by the PCR-subcloning method (data not shown). As a result, the precore-defective

A1896 mutation and the short deletion in the preS2 gene were identified as genomic changes significantly associated with the occurrence of the rtL180M mutation. These 2 viral genomic changes were found to be highly relevant to the observation that the rtL180M mutation was not needed for the establishment of the lamivudine-resistant mutant virus. This suggests that the precore-defective mutation and the preS2 deletion may function as surrogates for the compensatory rtL180M mutation and assist replication of lamivudine-resistant HBV. In the serial analysis of the mutations examined before and after lamivudine therapy, the preS2 deletion tended to be coselected with the drug resistance–associated mutation after therapy, although this tendency was not seen in the case of the precore-defective mutation. This also indicates that the preS2 deletion may have some advantage for establishment of lamivudine-resistant HBV.

We further conducted in vitro transfection analysis to verify the possible supportive role played by the precore and preS2 genomic changes in replication of lamivudine-resistant virus. The intracellular viral DNA was measured as a marker of viral replicative competence. In the wild-type virus, lamivudine resistance–associated mutations reduced viral replicative competence, and the rtL180M mutation compensated for viral replication to a certain degree. This agreed with previous findings of some other investigators [18–20]. On the other hand, the reduction in the viral replication level caused by the lamivudine-resistant mutations was lower in the precore-defective and preS2-deleted viruses than in the wild-type virus. Even the lamivudine-resistant virus without the rtL180M mutation maintained a substantial level of replicative activity in the viruses with precore and preS2 genomic changes. Thus, our results contribute evidence for a supportive role of both precore and preS2 genomic changes in the replicative competence of lamivudine-resistant HBV. This tendency was not evident in the case of the extracellular viral DNA assay, which may have been due to this assay's lower ability to detect slight changes in viral replicative activity.

As for the functional role played by the precore-defective A1896 mutation in the replication competence of lamivudine-resistant HBV, enhanced replicative activity of virus with lamivudine resistance caused by introduction of the precore-defective mutation has been reported for the recombinant HBV–expressing baculovirus system using the genotype D HBV strain [29]. Another previous in vitro transfection analysis using the genotype A HBV strain revealed that experimental insertion of the precore-defective mutation together with the T1858 mutation compensated for the replication competence of the virus possessing lamivudine-resistant mutations [30]. Our experimental result using the genotype C2 HBV strain is consistent with these previous findings. In addition, we showed in the present study that the preS2 deletion may also play a supportive role in the replication yield of lamivudine-resistant HBV, although the enhancement of viral replication caused by the preS2 deletion was not as strong as that caused by the precore-defective mutation.

It remains unclear why the precore-defective mutation leads to an increase in the viral replication of drug-resistant HBV. Previous in vitro transfection analyses have shown that the precore-defective mutation had no influence on viral replicative competence [29–31]. However, in our transfection analysis using the genotype C2 HBV strain, the replicative competence of the precore-defective virus tended to be higher than that of the wild-type virus, even when viruses without the lamivudine resistance–associated mutations were compared. It has recently been shown that the precore-defective mutation caused an elevation in viral replication in the particular HBV strain of genotype B1 [32]. According to this, the precore-defective mutation may in some way enhance HBV replication irrespective of the lamivudine resistance.

As for the involvement of the preS2 deletion in the replicative advantage of lamivudine-resistant HBV, the deletion results in truncation of the polymerase protein as well as the surface protein. Such truncation of the polymerase protein may increase the enzymatic activity and replication capacity of drug-resistant virus. As another possibility, the surface protein with the preS2 deletion may link to incomplete envelopment and subsequent intracellular accumulation of immature viral particles, resulting in an elevated intracellular HBV DNA level. However, this is improbable, because viral envelopment and secretion may be achieved efficiently in preS2-deleted virus as well as wild-type and precore-defective viruses, as was shown in the extracellular viral DNA assay.

In summary, our findings indicate that a precore-defective A1896 mutation and a short deletion in the preS2 gene may support viral replicative activity and substitute for the compensatory rtL180M mutation. Both the precore-defective mutation and the preS2 deletion have been shown to be frequently found during chronic HBV infection [26, 27, 33]. It is noteworthy that such naturally occurring frequent genomic changes in HBV significantly affect the establishment of drug-resistant viral strains. The lamivudine-resistant rtM204V/I mutation has also been reported to be completely or partially involved in resistance to other nucleos(t)ide analogues (emtricitabine, telbivudine, entecavir, and clevudine) [8, 9, 14, 34]. Our findings reveal novel aspects about the establishment of drug-resistant virus possessing the rtM204V/I and rtL180M mutations during the antiviral treatment of patients with CH-B.

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