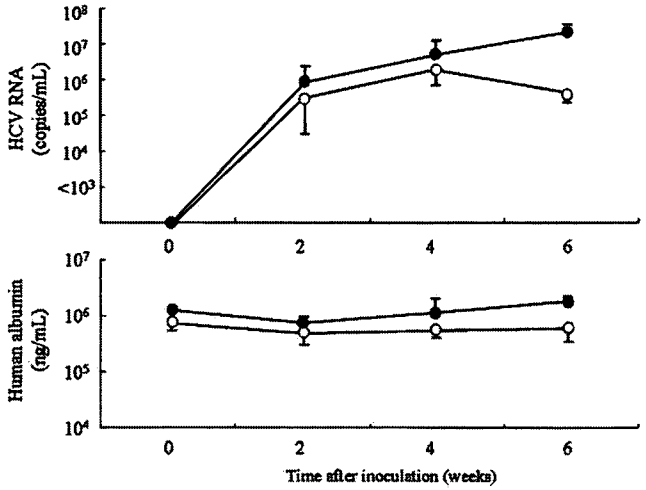

Fig. 2. Changes in HCV RNA and human albumin concentrations in serum of mice infected with clonal HCV. Each of three mice were inoculated intrahepatically with in vitro transcribed genotype 1a HCV RNA (closed circles) or intravenously with a culture medium collected from Huh7 cells transfected with JFH-1 genome intravenously (open circles). Data are mean ± S.D.

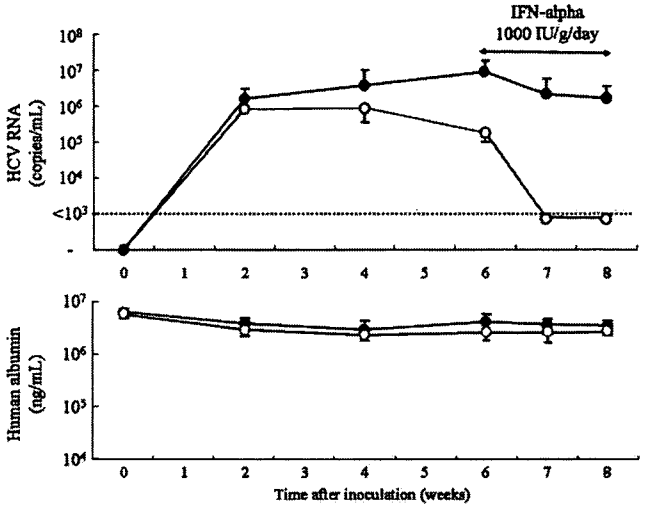

Fig. 3. Passage experiment and response to IFN-alpha therapy in mice infected with HCV genotypes 1a and 2a clones. Serum samples (10 μl) obtained from genotype 1a and 2a clonal HCV-infected mice sera (see Fig. 2) were inoculated intravenously into each of three naïve chimeric mice. Six weeks after infection, all six mice were injected intramuscularly with 1000 IU/g/day of IFN-alpha daily for 2 weeks. Closed circles: genotype 1a HCV-infected mice, open circles: genotype 2a HCV-infected mice. Data are mean ± S.D.

3.4. Variable susceptibility of HCV clones to IFN therapy

We treated each of the three mice infected with genotype 1a and 2a clones by passage experiments with 1000 IU/g of IFN-alpha daily for 2 weeks. Such treatment induced only a slight decrease in HCV in genotype 1a-infected mice; the viral load decreased only 0.6 and 0.7 log after 1 and 2 weeks of treatment, respectively (Fig. 3). In contrast, the same treatment reduced HCV genotype 2a RNA to undetectable levels after 1 and 2 weeks of IFN therapy. During IFN-treatment, serum HSA levels did not decrease in mice infected with genotype 1a or 2a HCV. Histopathological examination showed no morphological changes or apoptotic hepatocytes in replaced

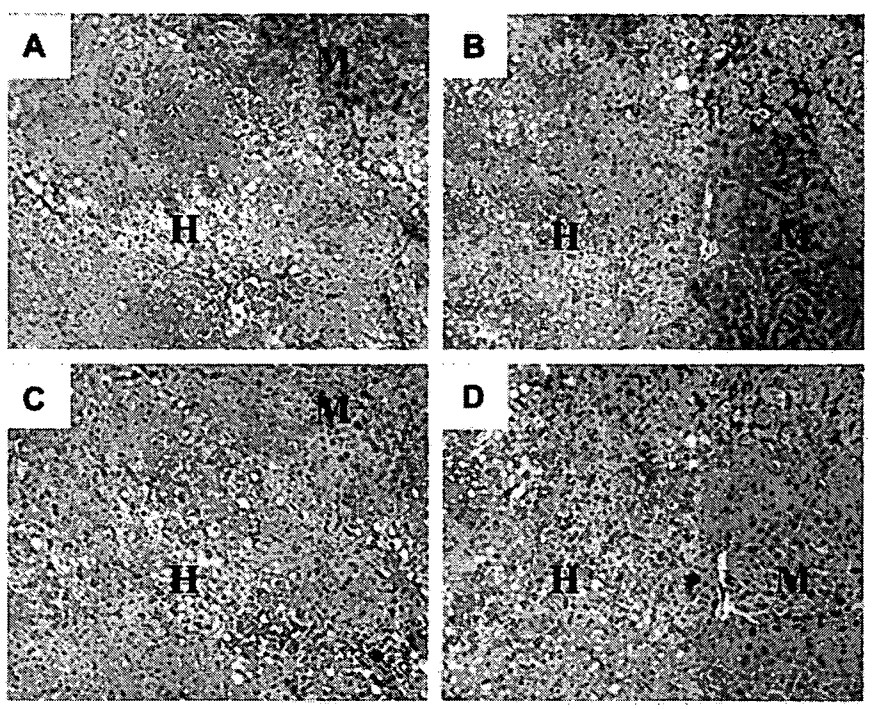

Fig. 4. Histochemical analysis of the tissues of infected chimeric mice. Liver samples obtained from mice infected with genotype 1a (A, C) and genotype 2a (B, D) stained with hematoxylin–eosin staining (A, B) or by immunohistochemical staining with anti-human serum albumin antibody (C, D). Regions are shown as human (H) and mouse (M) hepatocytes, respectively. (Original magnification, ×100.)

human hepatocytes in mice infected with each genotype after 2-week IFN-treatment (Fig. 4). These results suggest that the decrease in HCV is due to the direct anti-viral effect of IFN and not induced by liver cell damage. The difference in the virus titer and susceptibility to IFN are considered to be due to the characteristics of the genotypes.

4. Discussion

In this study, we established a reverse genetics system of HCV genotype 1a and 2a clones using human hepatocyte chimeric mice. The HCV genotype 2a clone, JFH-1, has remarkable features, i.e., infects cultured Huh7 cell line as well as establish infection in chimeric mouse [7]. It has been reported that HCV genotype 1a clone, H77-S, also infects Huh7 cell line and produces infectious virion [14]. In the present study, we intrahepatically inoculated genotype 1a infectious clone, CV-H77C. As reported in chimpanzee [13,15–17], we were able to establish genotype 1a infection using human hepatocyte chimeric mice. Using this technique, it is hoped that we can conduct further experiments in the future using genetically engineered HCV clones. Experiments using chimeric clone described by Lindenbach et al. [7] should also provide further information regarding the variable replication property of HCV genomes. Modifying genomes with nucleotide substitutions allowed examination of the functions of HCV peptides as we showed with HBV [12].

As reported recently by Kneteman et al. [10], the mouse model system is useful for evaluating the effect of anti-HCV drugs such as IFN, protease inhibitors and polymerase inhibitors. As we showed in this study, the response to IFN therapy varied according to HCV genotype. Further experiments are necessary to determine whether differences in response to IFN are due to the different replication ability (replication level of genotype 2a clone was slightly lower than that of genotype 1b, see Figs. 2 and 3) or differences in genotypes, as has been reported in clinical studies [18]. As we showed in this study (Fig. 4), there is no hepatocyte damage or inflammation in the liver of the infected chimeric mouse. Thus, this model is suitable for the study of mechanisms involved in HCV replication and IFN resistance.

The intrahepatic injection method used in this study simplified our experiments using genetically engineered virus. This is particularly important in studies of protease inhibitors and polymerase inhibitors because HCV will easily develop resistance against these small molecule agents.

Previous studies identified amino acid sequences that correlate with different susceptibilities of genotype 1b HCV against IFN therapy, namely, interferon sensitivity determining region [19] and the PKR-eIF2 phosphorylation homology domain [20,21]. To elucidate such issues, we are currently trying to establish genotype 1b infection system using the method described in this paper.

In summary, we showed in the present study the successful application of a genetically engineered HCV in human hepatocyte chimeric mice. Using this mouse model, we showed that genotypes 1a and 2a HCV clones exhibit different susceptibilities to IFN-alpha therapy. Our mouse model seems useful for the study of HCV virology and resistance of HCV against IFN and for the development of new anti-HCV therapy.

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Serum HBV RNA is a Predictor of Early Emergence of the YMDD Mutant in Patients Treated with Lamivudine

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Lamivudine (LAM) is a nucleoside analogue widely used for the treatment of chronic hepatitis B virus (HBV) infection. Emergence of resistant strains with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of reverse transcriptase is a serious problem in patients on LAM therapy. The amount of covalently closed circular DNA in the serum is reported to be higher in patients who develop YMDD mutants than in those without mutants. However, there is no useful serum marker that can predict early emergence of mutants during LAM therapy. Analysis of patients who were treated with entecavir (n = 7) and LAM (n = 36) showed some patients had high serum levels of HBV RNA. Median serum levels of HBV RNA were significantly higher in patients in whom the YMDD mutant had emerged within 1 year (n = 6, 1.688 log copies/ml) than in those in whom the YMDD mutant emerged more than 1 year after treatment (n = 12, 0.456 log copies/ml, $P = 0.0125$) or in whom the YMDD mutant never emerged (n = 18, 0.688 log copies/ml, $P = 0.039$). Our results suggest that HBV RNA is a valuable predictor of early occurrence of viral mutation during LAM therapy. (HEPATOLOGY 2007;45:1179-1186.)

The hepatitis B virus (HBV) is a member of the hepadnaviridae family. Worldwide, approximately 350 million people are estimated to be chronically infected with HBV.[1] Patients with chronic HBV infection develop chronic hepatitis, cirrhosis, and hepatocellular carcinoma, accounting for approximately 1 million deaths per year.[2] Recently, inhibitors of reverse transcriptase have been developed and widely used for patients with chronic HBV infection. Lamivudine (LAM), a cytosine nucleoside analogue, was first developed as an antiviral agent against HIV and later was used effectively against HBV because HBV also uses reverse transcriptase for replication.[3,4] Because LAM suppresses HBV replication, patients who are treated with LAM show a decreased level or disappearance of HBV DNA in serum and hepatitis B e antigen, normalization of serum alanine aminotransferase (ALT) level, and histological improvement.[5-12] However, discontinuation of therapy often leads to reactivation of HBV.[6,8,13,14] Therefore, long-term therapy is necessary for many patients with chronic HBV infection. During long-term LAM therapy, drug-resistant mutants with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) motif emerge, resulting in expression of HBV DNA increasing again and in worsening of hepatitis.[6,10,15-18] Moreover, some patients develop a severe flare-up of hepatitis that could lead to fatal hepatic failure. Therefore, prediction of the emergence of YMDD mutants is an important issue.

In our hunt for useful serum markers to detect the early emergence of YMDD mutants, we noticed some patients who showed a discrepancy in the expression of HBV DNA measured by the transcription-mediated amplifica-

Abbreviations: cccDNA, covalently closed circular DNA; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine; PCR, polymerase chain reaction; RT, reverse transcription; TMA-HPA, transcription-mediated amplification and hybridization protection assay; YMDD, tyrosine-methionine-aspartate-aspartate.

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Potential conflict of interest: Nothing to report.

Table 1. Clinical Characteristics of the 3 Groups

	Group A	Group B	Group C
Number	6	12	18
Age, median (range)	50 (37-67)	49 (31-66)	49 (27-68)
Sex (M:F)	3:3	9:3	13:5
Observation period (months)	34.5 (13-58)	38 (16-64)	34 (13-58)
Time before emergence of mutants (months)	8.5 (4-11)	19 (13-36)	
HBV DNA (LGE/ml)	7.8 ± 0.95	6.13 ± 0.84	6.64 ± 1.63
Hbe-antigen-positive	4 (66.7%)	6 (50%)	10 (55.6%)
Hbe-antibody-positive	1 (16.7%)	6 (50%)	9 (50%)
ALT (U/l)	136.1 ± 122.8	114.5 ± 104.1	129.8 ± 206.4

Group A: patients who showed early emergence of the mutants (within 1 year).
Group B: patients who developed resistance after 1 year of LAM therapy.
Group C: patients in whom mutants did not develop.

tion and hybridization protection assay (TMA-HPA) and that measured by the Amplicor HBV Monitor test. Because the former method detects both HBV DNA and HBV RNA, we thought that the difference in measurement by the 2 methods was a result of the presence of a large amount of HBV RNA.[19-21] We thus studied patients with chronic HBV infection who were being treated with LAM or entecavir (ETV) for the presence of HBV RNA. We also assumed that the presence of a large amount of HBV RNA would indicate that transcription and virus particle formation were still active in such patients. We thus assessed the value of this indicator in the prediction of the emergence of YMDD mutants during LAM therapy.

Patients and Methods

Patients. We studied 36 patients with chronic hepatitis B who were being treated with LAM from 2001 to 2006 at Hiroshima University Hospital, Kawakami Clinic, and Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital. We also analyzed 7 patients who were being treated with ETV from 2004 to 2006 at Hiroshima University Hospital. No patients showed clinical signs of cirrhosis or hepatocellular carcinoma. They were not treated with other antiviral agents, corticosteroids, or immunosuppressant drugs during LAM/ETV therapy. The LAM-treated patients were 25 men and 11 women whose median age was 52 years (range 27-68 years; Table 1). They were divided into 3 groups (groups A, B, and C) according to how long it took for YMDD mutants to appear. Group A (n = 6) was composed of patients who showed early emergence of the mutants (within 1 year); group B (n = 12) had patients who developed resistance after 1 year of LAM therapy; and group C (n = 18) was composed of patients who did not show resistance to LAM therapy. Each of the 36 patients received 100 mg of LAM daily for 4-58 months (median, 21.5 months). All patients continued LAM therapy throughout the course of the study. Patients in the ETV group were 6 men and 1 woman whose median age was 37 years (32-50 years). They received 0.01-0.5 mg of ETV daily for 21-28 months (median, 25 months), and all patients continued ETV therapy throughout the course of the study. Blood samples were obtained from patients of both groups just before commencement of antiviral therapy and every 4 weeks during therapy. Informed consent was obtained from each patient.

Quantification of HBV DNA. HBV DNA serum level was determined by using the TMA-HPA (Fujirebio Inc., Tokyo, Japan) and the Amplicor HBV monitor test (Roche Diagnostics, Tokyo, Japan). The measurement range of the former assay is $10^{3.7}$-$10^{8.7}$ genome equivalents (GE)/ml (3.7-8.7 LGE/ml),[22] whereas the range of the latter test was $10^{2.6}$-$10^{7.6}$ copies/ml (2.6-7.6 log copies/ml).[23] These quantitative assays of HBV DNA were performed at the Special Reference Laboratory (Tokyo, Japan).

Extraction of Nucleic Acid of HBV and Reverse Transcription. Nucleic acid was extracted from 100 μL of serum by the SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL of H_2O for DNA analysis or 8.8 μL of ribonuclease-free H_2O for RNA analysis. The latter solution was reverse-transcribed by using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan). In the next step, 25 pM of random primer was added to 8.8 μL of nucleic acid extract and heated at 65°C for 5 minutes. The samples were set on ice for 5 minutes. Then 4 μL of 5× reverse transcription (RT) buffer, 2 μL of 10 mM dNTPs, 2 μL of 0.1 M dithiothreitol (DTT), 8 units of ribonuclease inhibitor, and 100 units of M-MLV reverse transcriptase were added to each sample. The reaction mixture was incubated at 30°C for 10 minutes and 42°C for 60 minutes, followed by inactivation at 99°C for 5 minutes.

Quantitative Analysis of HBV DNA by Real-Time Polymerase Chain Reaction. One microliter of DNA solution or cDNA solution was amplified by real-time polymerase chain reaction (PCR) with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the instructions provided by the manufacturer. Amplification was performed in a 25-μL reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 200 nM of forward primer (5′-TTTGGGGCATGGACATTGAC-3′, nucleotides 1893-1912), 200 nM of reverse primer (5′-GGTGAACAATGGTCCGGAGAC-3′, nucleotides 2029-2049), and 1 μL of DNA or cDNA solution. After incubation for 2 minutes at 50°C, the sample was heated for 10 minutes at 95°C for denaturing, followed by a PCR cycling program consisting of 40 2-step cycles of 15 seconds at 95°C and 60 seconds at 60°C. The lower detection limit of this assay was 10^3 copies/ml.

Confirmation of Presence of HBV RNA in Serum by RNase Digestion. To confirm the presence of HBV RNA, nucleic acid extracted from the serum samples by SMITEST (Genome Science Laboratories, Tokyo) was digested with 1 μg/μL of RNase A (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 60 minutes, digested with proteinase K (New England Biolabs Inc., Ipswich, MA) at 37°C for 60 minutes, extracted with phenol/chloroform, precipitated with ethanol, and dissolved in water. Treated nucleic acid with or without RNase was analyzed by real-time PCR after reverse transcription with a random primer and reverse transcriptase, as already described.

Detection of YMDD Mutant. Mutations in the YMDD motif of reverse transcriptase of HBV were examined by PCR with peptide nucleic acid clamping, as described previously.[24]

Statistical Analysis. Differences between groups were examined for statistical significance using the Student t test, and correlations of parameters were examined by the Spearman's rank correlation. A difference with a P value less than 0.05 was considered statistically significant. All statistical analyses were performed with StatView version 5.0 (SAS Institute, Cary, NC).

Results

HBV DNA Levels Determined by TMA-HPA and Amplicor HBV Monitor Test During ETV Therapy. High expression of HBV RNA was initially observed by measuring HBV nucleic acid with the TMA-HPA and HBV DNA with the Amplicor HBV monitor test. As shown in Fig. 1, expression of HBV nucleic acid was higher than HBV DNA during the initial 6 months of ETV therapy. We assumed that the discrepancy in the measurements by these 2 methods was a result of the large amount of HBV RNA in the serum because the TMA-HPA measures both HBV DNA and HBV RNA, whereas the Amplicor HBV monitor test detects only HBV DNA. We measured the HBV nucleic acid levels in the 7 patients who received ETV therapy 3 and 6 months after the start of therapy. The HBV nucleic acid levels of all 7 patients determined by the TMA-HPA were 10-100 times higher than those determined by the Amplicor HBV Monitor test except for 2 patients who received a small amount (0.01 mg) of ETV (Fig. 2). The small dif-

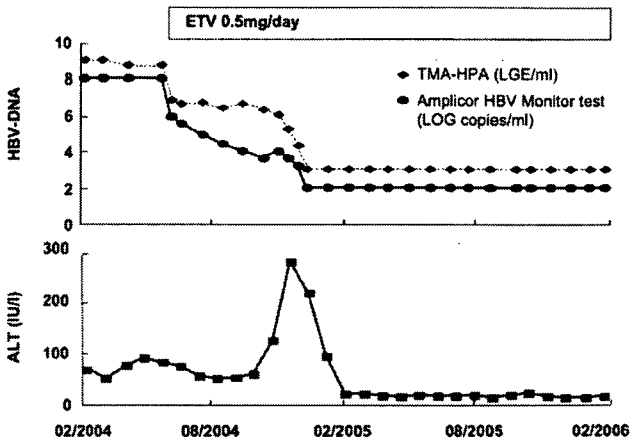

Fig. 1. Time courses of serum HBV DNA and ALT levels of patients treated with ETV. Expression of HBV nucleic acids determined by the TMA-HPA was higher than that determined by the Amplicor HBV Monitor test soon after beginning administration of ETV. The discrepancy was less marked when both measurements were low and when both were negative.

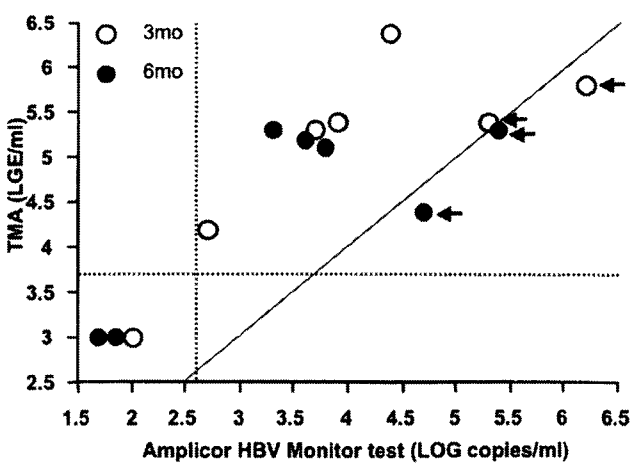

Fig. 2. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during ETV therapy. Serum samples obtained from the 2 patients who received low-dose ETV (0.01 mg) are indicated by arrows. The vertical and horizontal dotted lines indicate the lower detection limits of the Amplicor HBV Monitor test and the TMA-HPA, respectively.

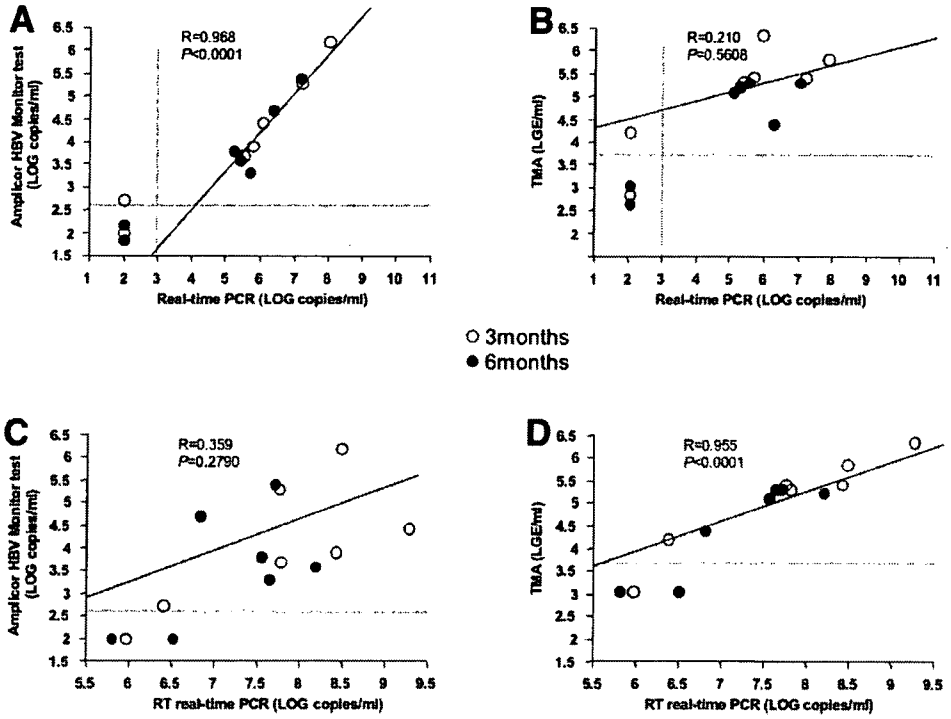

Fig. 3. Correlation between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of ETV therapy. (A) Correlation between HBV DNA level determined by Amplicor HBV Monitor test and that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA and of HBV DNA determined by real-time PCR. (C) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with HBV nucleic acid level determined by real-time RT-PCR. (D) Correlation of HBV nucleic acid level determined by the TMA-HPA with that determined by real-time RT-PCR. The vertical and horizontal dotted lines represent the lower detection limits of the Amplicor HBV Monitor test or TMA-HPA and in-house real-time PCR, respectively.

ference in nucleic acid level of these patients is probably a result of the small effect of the small amount of ETV.

Comparisons of HBV Nucleic Acid and DNA Values Determined by 4 Measurement Methods—TMA-HPA, Amplicor Monitor Test, In-House Real-Time PCR Assay, and Real-Time RT-PCR—in Patients Treated with ETV. We measured HBV DNA by in-house real-time PCR and HBV nucleic acid by real-time RT-PCR using serum samples obtained from the patients after 3 and 6 months of ETV therapy and compared these values with those obtained by the TMA-HPA and the Amplicor monitor test. HBV DNA determined by real-time PCR correlated well with that obtained by the Amplicor HBV Monitor test ($r = 0.968$, $P < 0.0001$; Fig. 3A), but not with HBV nucleic acid determined by the TMA-HPA ($r = 0.210$, $P = 0.5608$; Fig. 3B). Expression of HBV DNA determined by the in-house real-time PCR assay was $10^{1.5}$-10^{2} higher than that determined by the Amplicor HBV Monitor test. We confirmed the accuracy of our assay using limiting dilution and detection with nested PCR assay. When we diluted the standard samples used in our in-house assay to 1 copy/μL, we detected them by nested PCR using 1 μL of such samples. Three of the 10 (30%) samples tested positive by nested PCR. We thus conclude that our assay accurately measure the amount of HBV DNA in serum.

To examine if measurement by the TMA-HPA reflected the total amount of HBV RNA and HBV DNA in serum samples, we performed real-time RT-PCR using serum samples obtained from patients after 3 and 6 months of ETV therapy. In contrast to the values determined by real-time PCR without RT, the measurement of HBV nucleic acid determined by RT-PCR did not correlate well with that obtained by the Amplicor HBV Monitor test ($r = 0.359$, $P = 0.2790$; Fig. 3C), but did correlate well with that obtained with the TMA-HPA ($r = 0.955$, $P < 0.0001$; Fig. 3D). These results show that the TMA-HPA measures both HBV DNA and HBV RNA in serum. To further confirm the presence of HBV RNA, we digested 3 nucleic acid samples arbitrarily picked from serum samples obtained from patients treated by lamivudine for 3 months, by RNase A. As shown in Fig. 4, RNase treatment reduced the amount of HBV DNA detected by real-time RT-PCR to about 1% of that originally detected.

HBV DNA Levels Determined by TMA-HPA and Amplicor HBV Monitor Test during LAM Therapy. We then investigated the levels of HBV DNA in serum samples obtained from 36 patients after 3 and 6 months of LAM therapy. In some patients, HBV DNA was already negative after 3 and 6 months of therapy (Fig. 5). Similar to the results obtained from patients treated with ETV, comparisons of values obtained from patients who showed measurable HBV DNA levels revealed that HBV nucleic acid levels determined by the TMA-HPA tended to be higher than those determined by the Amplicor HBV Monitor test (Fig. 4).

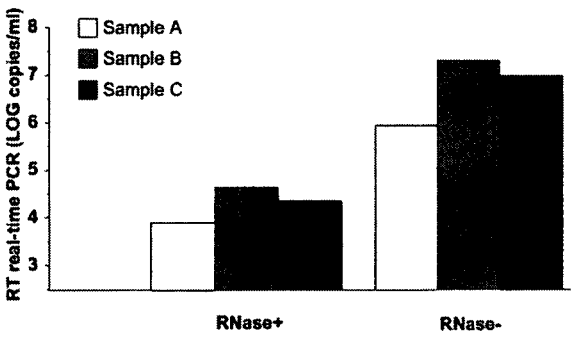

Fig. 4. Presence of HBV RNA confirmed by RNA treatment of 3 nucleic acid samples (samples A-C) obtained from patients after 3 months of LAM therapy. Extracted nucleic acid samples with or without RNase digestion were further digested by proteinase K and ethanol-precipitated after phenol/chloroform extraction. The amount of HBV DNA in each sample was then measured by real-time RT-PCR.

Comparisons of HBV Nucleic Acid Values and HBV DNA Determined by 4 Measurement Methods—TMA-HPA, Amplicor Monitor Test, In-House Real-Time PCR Assay, and Real-Time RT-PCR—in Patients Treated with LAM. We measured HBV nucleic acid and DNA levels by the same 4 methods and investigated the correlations between them after 3 and 6 months of LAM therapy (Fig. 6). HBV DNA levels determined by real-time PCR correlated better with those determined by the Amplicor HBV Monitor test (r = 0.653, P = 0.0083; Fig. 6A) than with those determined by the TMA-HPA (r = 0.456, P = 0.1173; Fig. 6B). Similarly, measurement of HBV nucleic acid by RT-PCR did not correlate well with that obtained by the Amplicor HBV Monitor test (Fig. 6C), but showed better correlation with that obtained by the TMA-HPA (r = 0.452, P = 0.0907, and r = 0.675, P = 0.0114, respectively; Fig. 6D). These results also showed that the TMA-HPA detects both HBV RNA and HBV DNA.

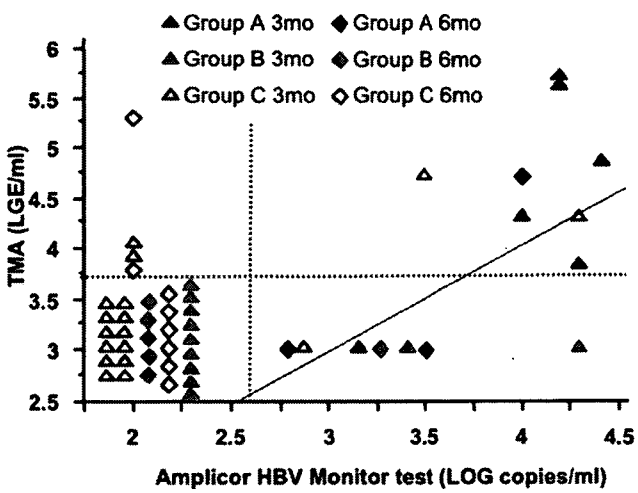

Fig. 5. Correlation of HBV nucleic acid levels determined by the TMA-HPA with HBV DNA levels determined by the Amplicor HBV Monitor test during LAM therapy. During ETV therapy the TMA-HPA showed higher expression of HBV DNA in patients regardless of the presence of the mutation than did the Amplicor HBV Monitor test. The vertical and horizontal dotted lines indicate the lower detection limits of the Amplicor HBV Monitor test and the TMA-HPA, respectively.

HBV RNA in Serum after 3 Months of LAM Therapy Is Higher in Patients Who Showed Early Emergence of YMDD Mutants. In LAM-treated patients, it was assumed that a high serum level of HBV RNA was a marker of the active transcription form of covalently closed circular DNA (cccDNA) and packaging of HBV RNA in the liver. We assumed that YMDD mutants easily emerged under such condition. We compared HBV RNA values (HBV nucleic acid determined by real-time RT-PCR minus HBV DNA determined by real-time PCR) in patients who showed early emergence of mutants (within 12 months) with those who showed late emergence of mutants (more than 12 months) and those who did not show emergence of mutants (Table 1). As shown in Fig. 7, HBV RNA levels were significantly higher in patients who showed early emergence of mutants than the other 2 groups after 3 months of LAM therapy. There was no significant difference in the amount of HBV RNA between group A (patients who showed emergence of mutants within 12 months) and the other 2 groups at the beginning of LAM therapy (data not shown).

Discussion

In this study, we addressed the discrepant measurements of HBV nucleic acid by the TMA-HPA and the Amplicor Monitor test. The presence of HBV RNA in serum samples of patients with HBV infection has been previously reported.[19-21] Because the TMA-HPA uses RNA transcription and amplification of transcripts by T7 RNA polymerase,[22] we assumed that the discrepancy was a result of the presence of HBV RNA in the serum of LAM- and ETV-treated patients. The presence of HBV RNA in a patient treated with LAM was reported previously.[21] In that report, the authors mainly analyzed truncated HBV RNA, which they assumed was transcribed from the integrated genome.[20, 21] They showed a large difference between HBV DNA and truncated HBV RNA, which did not decrease during LAM therapy. We also detected HBV DNA and HBV nucleic acid by real-time PCR and real-time RT-PCR. The values determined by these 2 methods showed less than a 1 log difference (data not shown); we assume that the effect of truncated HBV RNA in serum was only minimal in our study. As we demonstrated in this study, HBV nucleic acid measured by real-time RT-PCR correlated with that determined by the TMA-HPA. This finding suggests that the

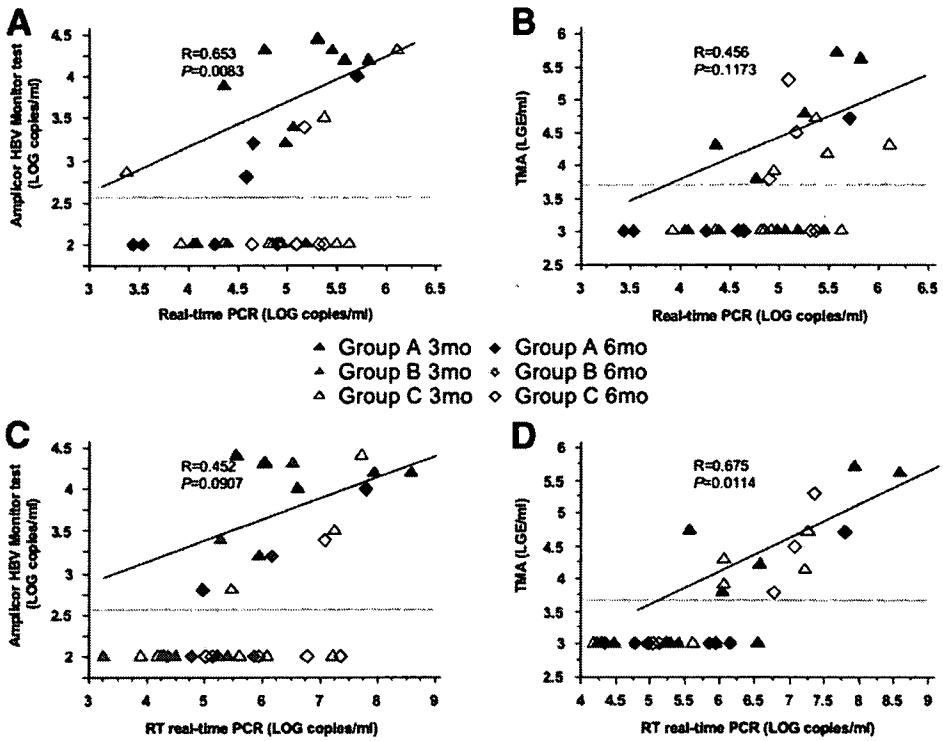

Fig. 6. Correlations between HBV nucleic acid and HBV DNA measurements after 3 and 6 months of LAM therapy. (A) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with that determined by in-house real-time PCR. (B) Correlation of HBV nucleic acid level determined by the TMA-HPA with HBV DNA by real-time PCR. (C) Correlation of HBV DNA level determined by the Amplicor HBV Monitor test with HBV nucleic acid level determined real-time RT-PCR. (D) Correlations of HBV nucleic acid level determined by the TMA-HPA with that determined by real-time RT-PCR. The vertical and horizontal dotted lines represent the lower detection limits of the Amplicor HBV Monitor test or TMA-HPA and in-house real-time PCR, respectively.

discrepancy in the values measured by the TMA-HPA and the Amplicor Monitor test is a result of the presence of HBV RNA in the serum.

We showed that a large amount of HBV RNA in the serum was produced during the early stage of ETV (Fig. 1) and LAM treatments (within 6 months). Because ETV and LAM work only on reverse transcription, it is difficult to conceive that the level of transcription from the cccDNA was altered by these drugs. Thus, the slow decrease in HBV RNA seems to reflect that a certain amount of cccDNA still existed in the liver and that the virus replication machinery was still actively operational. This is consistent with previous reports that showed that the amount of cccDNA in the liver tissues[25, 26] and in serum,[26] which correlated well with intrahepatic cccDNA,[27] reflected the effect of LAM and is a marker for cessation of therapy without viral level increasing again after stopping the therapy.

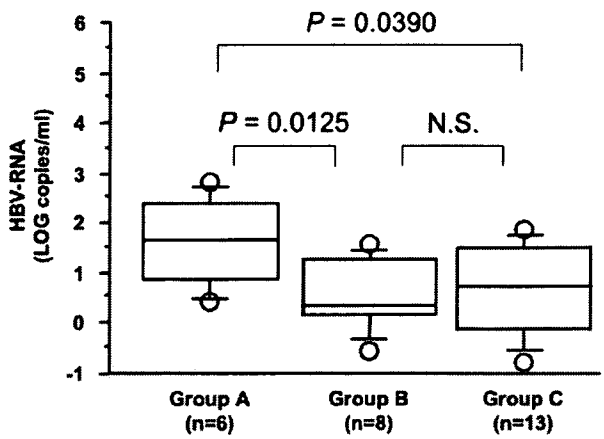

Fig. 7. Box plots of HBV RNA levels of patients in group A (patients who showed emergence of the mutants within 1 year, group B (those who developed resistance after 1 year of LAM therapy), and group C (patients who did not show resistance to LAM therapy). HBV RNA level represents the difference between HBV nucleic acid level determined by real-time RT-PCR minus HBV DNA level determined by in-house real-time PCR. Nine samples that tested negative for in-house real-time PCR were omitted from the analysis (4 samples of group B and 5 samples of group C).

Whether a large amount of HBV RNA originates from a large amount of cccDNA template in hepatocytes or from active transcription (or both) is actually unknown. However, it is assumed that the probability of developing mutants is high in patients who have large amounts of HBV RNA. We thus analyzed the amount of HBV RNA in patients treated with LAM and compared it in patients who showed early emergence of mutants and those who did not. As expected, the amount of HBV RNA in the serum was significantly higher in patients who showed early emergence of mutants than in those who showed late emergence and those who did not show emergence of mutants.

Using complex analysis, previous studies identified several factors predictive of emergence of YMDD mutants such as HBV genotype,[28] ALT level,[29, 30] HBV DNA level

before therapy,[28,30-32] degree of decline of HBV DNA level during therapy,[33,34] presence of hepatitis B e antigen,[17,29,31,32,35] presence of core promoter mutations,[36] deletion of pre-S region,[37] and HBV core–related antigen.[38] We also showed that a slow decrease in HBV nucleic acid measured by the TMA-HPA is a marker of early emergence of mutants. Our finding is important because this assay is routinely used in daily clinical practice. However, the results did not reach statistical significance, probably because of the small number of patients analyzed in our study and the low sensitivity of the assay (detection limit 3.7 log copies/ml). We assume that a sensitive measurement of HBV RNA is useful for predicting the emergence of mutants. Development of such an assay is needed for the proper treatment of patients using different nucleotide and nucleoside analogues. Mechanisms that control transcription of HBV from cccDNA deserve further investigation in order to develop more effective therapies for HBV infection.

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Dual effect of APOBEC3G on *Hepatitis B virus*

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G to A hypermutation of *Hepatitis B virus* (HBV) and retroviruses appears as a result of deamination activities of host APOBEC proteins and is thought to play a role in innate antiviral immunity. Alpha and gamma interferons (IFN-α and -γ) have been reported to upregulate the transcription of APOBEC3G, which is known to reduce the replication of HBV. We investigated the number of hypermutated genomes under various conditions by developing a quantitative measurement. The level of hypermutated HBV in a HepG2 cell line, which is semi-permissive for retrovirus, was 2.3 in 10^4 HBV genomes, but only 0.5 in 10^4 in permissive Huh7 cells. The level of APOBEC3G mRNA was about ten times greater in HepG2 cells than in Huh7 cells. Treatment of HepG2 cells with either IFN-α or -γ increased the transcription of APOBEC3G and hypermutation of HBV. These mRNAs and hypermutation of HBV genomes were induced more prominently by IFN-γ than by IFN-α. Both IFNs decreased the number of replicative intermediate of HBV. Overexpression of APOBEC3G reduced the number of replicative intermediate of HBV and increased hypermutated genomes 334 times, reaching 968 in 10^4 genomes. Deamination-inactive APOBEC3G did not induce hypermutation, but reduced the virus equally. Our results suggest that APOBEC3G, upregulated by IFNs, has a dual effect on HBV: induction of hypermutation and reduction of virus synthesis. The effect of hypermutation on infectivity should be investigated further.

INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus with partially double-stranded DNA as a genome (Ganem & Schneider, 2001; Seeger & Mason, 2000). The virus replicates through transcription of pregenome RNA and reverse transcription, like retroviruses (Skalka & Goff, 1993; Summers & Mason, 1982). Infection with HBV causes chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (Wright & Lau, 1993; Bruix & Llovet, 2003; Ganem & Prince, 2004).

Recent reports have shown that a cytidine deaminase, APOBEC3G, which is packaged in human immunodeficiency virus (HIV) virions in non-permissive cells, induces G to A hypermutation to a nascent reverse transcript of HIV and serves as part of the innate antiviral activity (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris *et al.*, 2003). Recent studies have demonstrated that a small number of HBV DNA in serum samples of patients with chronic HBV infection contains hypermutated genomes (Gunther *et al.*, 1997; Suspene *et al.*, 2005a; Noguchi *et al.*, 2005). We reported previously that there are small numbers of hypermutated genomes in serum samples of the majority of patients with chronic HBV infection and that G to A hypermutation could be induced in cultured liver cells derived from HepG2 cell lines (Noguchi *et al.*, 2005) using a peptide nucleic acid-mediated PCR clamping method. Suspene *et al.* (2005a) developed the more sensitive differential DNA denaturation (3D)-PCR method to detect hypermutated genomes and found that some APOBEC proteins induce G to A, and in some cases C to T, hypermutations in HBV DNA (Suspene *et al.*, 2005a). Why only a very small proportion of the HBV genome is hypermutated is unknown at present. Furthermore, the

mechanism that controls the level of APOBEC protein expression and degree of hypermutation has not been fully investigated. Recently, Tanaka *et al.* (2006) identified an interferon (IFN)-stimulated response element (ISRE) in the promoter region of APOBEC3G and showed that IFN-α upregulates transcription of APOBEC3G. Peng *et al.* (2006) also reported that IFN-α and -γ upregulate mRNA transcription of APOBEC proteins. However, these reports did not analyse whether increased numbers of APOBEC proteins actually increase hypermutation. More recently, Bonvin *et al.* (2006) demonstrated that IFN induces transcription of APOBEC proteins and increases hypermutation of HBV.

IFNs are cytokines that play a major role against many pathogens (Samuel, 2001; Colonna *et al.*, 2002; Grandvaux *et al.*, 2002). We also reported in a previous study that both IFN-α and -γ reduce virus replication in stably HBV-transfected cell lines without inducing a remarkable increase in G to A hypermutation (Noguchi *et al.*, 2005). However, the method used in previous experiments for detection of hypermutation was not as sensitive as the method of Suspene *et al.* (2005a, b) and not quantitative. To assess the level of hypermutation, a reliable measurement of hypermutated genome is needed. In the present study, we developed a new and sensitive method for the measurement of hypermutated genome levels. Using this method, we show here that both IFN-α and -γ increased the levels of hypermutated genomes in cultured cell lines. Furthermore, both IFNs increased the mRNA level of APOBEC3G. We also performed overexpression experiments to examine whether APOBEC3G and its inactive mutants increase the levels of hypermutation and reduce HBV replication.

METHODS

Plasmid constructs. The expression vector for haemagglutinin (HA)-tagged human APOBEC3G, pcDNA3/HA-A3G, was constructed as described previously (Kobayashi *et al.*, 2004). APOBEC3F cDNA was obtained by modifying APOBEC3F like (IMAGE clones from Open Biosystems) to have the same sequence as human APOBEC3F transcript variant 1 (GenBank NM_145298) and cloned into pcDNA3/HA (Invitrogen). APOBEC3G mutants were constructed using a QuikChange mutagenesis kit (Stratagene). The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, has been described previously (GenBank accession no. AB206816) (Tsuge *et al.*, 2005).

Cell culture and transfection. Huh7 and HepG2 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum at 37 °C in 5 % CO_2. Cells were seeded to semi-confluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 and Huh7 cell lines was performed using *Trans*IT-LT1 (Mirus) according to the instructions provided by the supplier. A plasmid encoding a secreted form of human placental alkaline phosphatase (SEAP) was co-transfected to adjust the transfection efficiency. The SEAP assay in the culture medium was performed using the Great EscAPe SEAP Reporter System 3 (BD Bioscience).

T23 cells are HepG2 cells stably transfected with the plasmid pTRE-HB-wt. They were cultured using a method described previously (Tsuge *et al.*, 2005). Cells were seeded to semi-confluence in six-well tissue culture plates and then treated with medium containing either IFN-α (Hayashibara Biochemical Laboratories) or IFN-γ (Shionogi & Co.). The cells were harvested 12–72 h after IFN treatment. Core-associated HBV DNA was extracted from the cells for HBV DNA quantification and quantitative analysis of G to A hypermutated genomes (Noguchi *et al.*, 2005).

Analysis of core-associated HBV DNA. The cells were harvested 4 days after transfection and lysed with 250 µl lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5 % (v/v) NP-40] followed by centrifugation for 2 min at 15 000 *g*. The core-associated HBV genome was immunoprecipitated from the supernatant by mouse anti-core monoclonal antibody anti-HBc determinant α (Institute of Immunology, Tokyo, Japan) and subjected to quantitative analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems). The primers used for amplification were #1, 5′-ACTTCAACCCCAACAMRRATCA-3′ (nt 2978–2999) [numbers are those of HBV subtype C reported by Norder *et al.* (1994)] and #2, 5′-AGAGYTTGKTGGAATGTKGTGGA-3′ (nt 24–1), where M is A/C, R is G/A, Y is T/C and K is G/T. The probe was a 6-carboxy-fluorescein (FAM)-labelled minor-groove binder (MGB) probe, 5′-(FAM)-TTAGAGGTGGAGAGATGG-(MGB)-3′ (nt 3184–3167). Real-time PCRs were set up in 25 µl TaqMan Universal Master Mix with 1 µl DNA solution, 0.9 µM each primer and 0.25 µM probe. The amplification conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 62 °C for 90 s).

Amplification and analysis of hypermutated HBV genomes by 3D-PCR. HBV DNA was extracted from 100 µl serum obtained from a chronic HBV carrier (genotype C) by SMITEST (MBL International) and was dissolved in 20 µl H_2O. Hypermutated genomes were detected by modified 3D-PCR using primers #1 and #2 and DNA solution from serum containing 8.0×10^7 or 2.3×10^5 copies of core-associated HBV DNA in 25 µl of 100 mM Tris/HCl pH 8.3, 50 mM KCl, 15 mM $MgCl_2$, 0.2 mM each dNTP, 10 pmol each primer and 1.25 U *Taq* DNA polymerase (Gene Taq, Nippon Gene Co.), together with 0.25 µg anti-*Taq* high (TOYOBO Co.). The amplification conditions included an initial denaturation step at 83–95 °C for 5 min, followed by 45 cycles of denaturation at 83–95 °C for 1 min, annealing at 50 °C for 30 s, extension at 72 °C for 30 s followed by 10 min of final extension. Amplicons were separated by electrophoresis on 2 % (w/v) agarose gel, cloned and sequenced in an ABI PRISM 3130 Genetic Analyzer with a BigDye Terminator version 3.1 cycle sequencing ready reaction kit (Applied Biosystems). The PCR products were also analysed on Hanse Analytik (HA)-yellow gel as described previously (Suspene *et al.*, 2005b; Tsuge *et al.*, 2005; Abu-Daya *et al.*, 1995).

Quantitative analysis of hypermutated genomes by real-time PCR. Hypermutated genomes were quantified by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems) and the above primers and probes. The amplification conditions included activation at 95 °C for 10 min followed by initial denaturation at 88 °C for 20 min and 45 cycles of amplification (denaturation at 88 °C for 15 s, annealing at 50 °C for 30 s and extension at 62 °C for 90 s). We chose 88 °C as this temperature is appropriate for detection of about 20 % hypermutated genomes. There are 200–300 such hypermutated genomes in 10^4 genomes present in HepG2 cells transiently transfected with APOBEC3G. The buffer comprised 10 mM Tris/HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 10 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 0.2 mM each dNTP, 0.9 µM each primer, 0.25 µM probe, 5×10^6 copies of HBV DNA

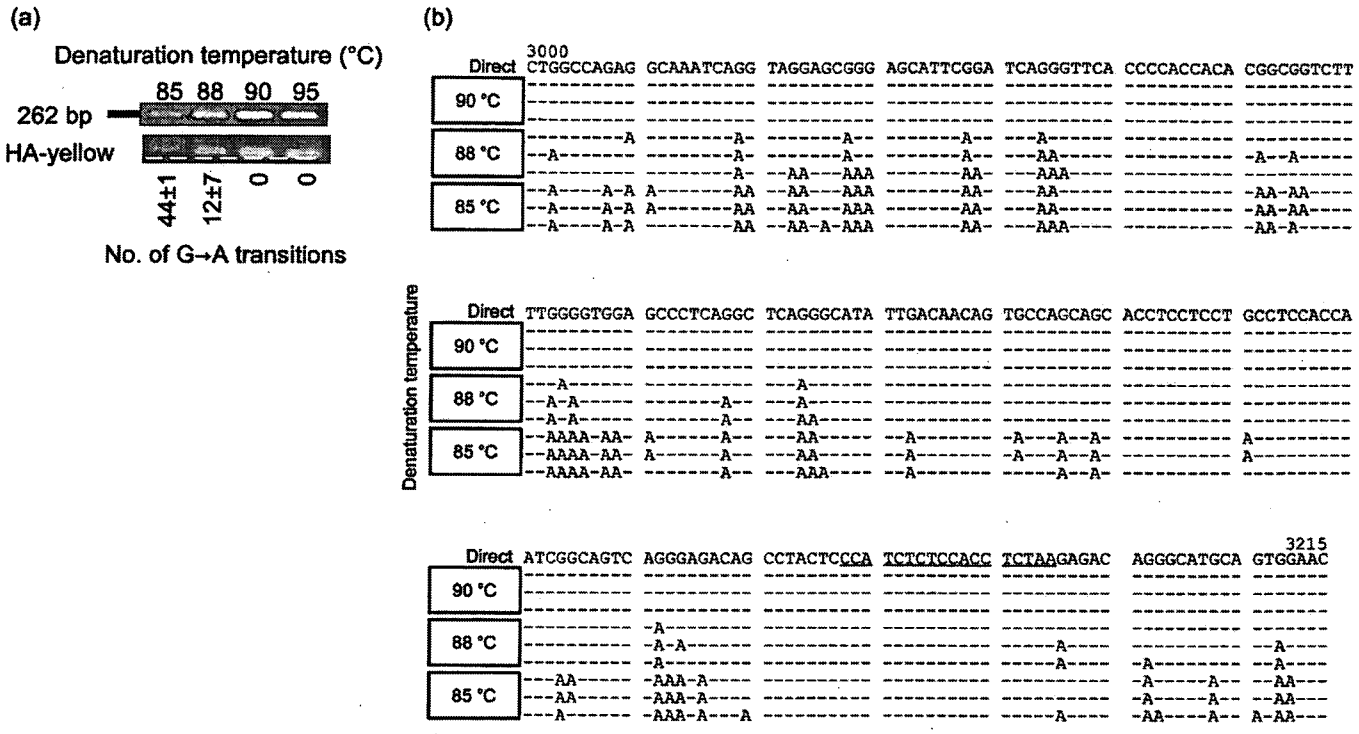

Fig. 1. Amplification of HBV DNA by 3D-PCR. (a) Detection of hypermutated genomes by HA-yellow agarose gel electrophoresis. The numbers of G to A transitions are expressed as means ± SD generated from the sequence analysis of five independent clones from PCR products. The white dotted line was added to help visualize the retardation of AT-rich DNA in HA-yellow agarose gel. (b) Nucleotide sequences of HBV amplified by 3D-PCR. The nucleotide sequences obtained by direct sequencing are used as a reference sequence. The nucleotide sequences where the probe hybridizes are underlined. Note that the number of G to A mutations correlates with denaturation temperature.

and 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 25 µl. A standard curve was constructed by the simultaneous amplification of serial dilutions of the 3D-PCR products.

Western blot analysis. Cell lysates were prepared as described above, resolved on 10 % (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman) via electro-blotting. The membranes were incubated with anti-haemagglutinin fusion epitope monoclonal antibody (Roche) or with anti-β-actin monoclonal antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit antibody or sheep anti-mouse immunoglobulin (Amersham Biosciences). Proteins were visualized via the ECL system (Amersham Biosciences).

Quantification of mRNA of APOBEC3G or APOBEC3F by reverse transcription and real-time PCR. Total RNA was extracted from HepG2 cell lines by using an RNeasy Mini kit (Qiagen). The RNA was reverse transcribed with random primers and Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, TOYOBO Co.) at 42 °C for 60 min according to the instructions provided by the manufacturer. Quantitative analysis of APOBEC3G and APOBEC3F cDNA was performed by real-time PCR using TaqMan Gene Expression assays (Applied Biosystems). To confirm that the APOBEC3G and -3F PCR primers specifically amplify the target genes, quantitative PCR on the expression plasmids encoding human APOBEC3G and -3F, used as templates, was performed. No cross amplification was observed, even when we used 10^7 copies of APOBEC3G plasmid in the amplification reaction of APOBEC3F and vice versa. A standard curve was constructed by the amplification of serial dilutions of the known number of plasmids containing human APOBEC3G and APOBEC3F. The target cDNA was normalized to the endogenous RNA level of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and FAM-labelled probe used to quantify GAPDH were purchased from Applied Biosystems.

Infectivity of luciferase reporter viruses produced from HepG2 and Huh7 cell lines. Luciferase reporter viruses with or without viral infectivity factor (Vif) were prepared by co-transfection of pNL43/ΔEnv-Luc (wild-type) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or expression vectors for A3G by Lipofectamine (Invitrogen) as described previously (Janini *et al.*, 2001; Shindo *et al.*, 2003). Productive infection was measured by luciferase activity. Values were presented as percentage of infectivity relative to the value of each virus without expression of APOBEC3G proteins.

RESULTS

Quantitative analysis of hypermutated genome by real-time PCR

Using serum samples from a patient with a high viral load, we amplified a large number of hypermutated genomes by 3D-PCR and detected them by HA-yellow agarose gel electrophoresis (Fig. 1a). Nucleotide sequence analysis

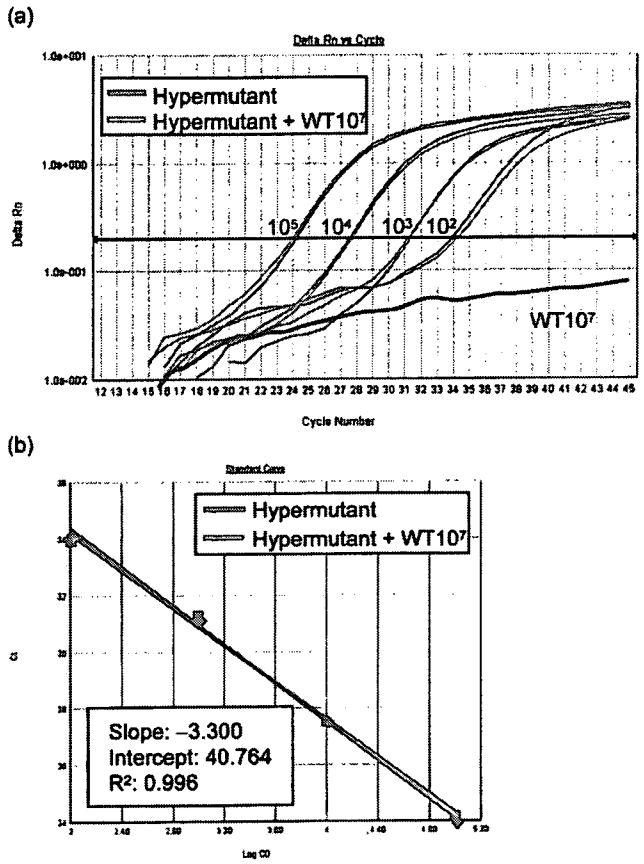

Fig. 2. Quantitative measurement of hypermutated HBV DNA using 3D-PCR combined with real-time PCR. The indicated numbers (10^2–10^5) of hypermutated genomes alone (orange lines) and a mixture of wild-type plus hypermutated genomes (green lines) were amplified by 3D-PCR. 3D-PCR did not result in amplification of wild-type sequence (purple line). Denaturation temperature was 88 °C.

showed detection of more heavily hypermutated genomes at lower denaturation temperatures (Fig. 1b). To develop quantitative measurement, we selected sequences with many G residues, designed primers that contained only a small number of G residues and used degenerate primers. A probe sequence was designed without a G residue. Using this primer and probe set, we could amplify only hypermutated genomes (Fig. 2). When hypermutated and non-mutated genomes were co-amplified, only hypermutated genomes were successfully amplified using the above primer and probe set (Fig. 2b). Non-hypermutated genomes (10^7 copies) were not amplified, although conventional PCR amplified both mutated and non-mutated genomes equally (data not shown). We also tried to detect only slightly (four of the 58 G residues) mutated genomes by 3D-PCR, but could not detect such genomes. It should thus be noted that the quantitative measurement we developed in this study detects only hypermutated genomes.

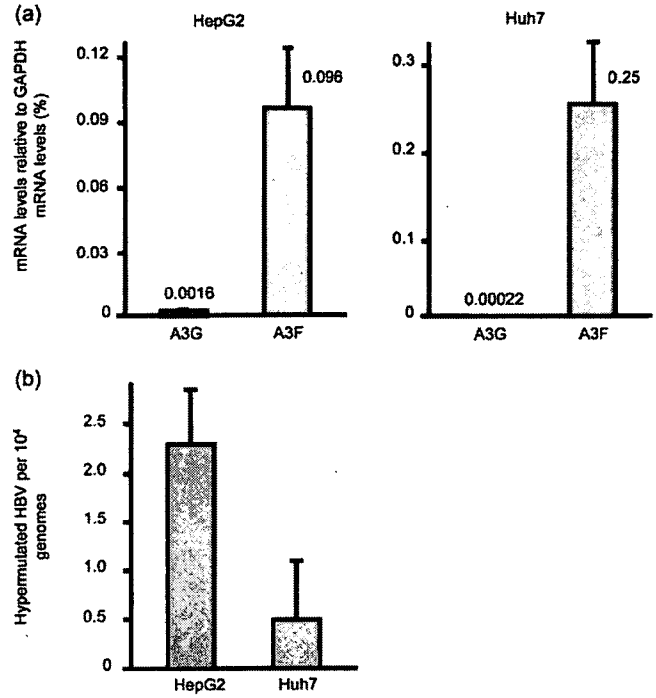

Fig. 3. Expression levels of APOBEC3G and -3F protein mRNAs in HepG2 and Huh7 cell lines. (a) mRNAs were extracted from cultured cell lines and the number of mRNA was quantified by real-time PCR with a probe for APOBEC3G and -3F. The expression levels were expressed as a percentage of GAPDH mRNA. (b) Number of hypermutated HBV genomes measured by real-time 3D-PCR in HepG2 and Huh7 cell lines transiently transfected with pTRE-HBV-wt. Results are means ± SD values of three independent experiments.

Detection of APOBEC3G mRNA and hypermutated genomes in semi-permissive and permissive cell lines

In retrovirus studies, it is known that some cell lines allow production of infectious retrovirus virions with Vif deficiency (permissive cells) while others do not. The difference between semi-permissive and permissive cell lines is the expression of APOBEC3G (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris *et al.*, 2003; Shirakawa *et al.*, 2006). Thus, we examined the expression of APOBEC3G in both HepG2 and Huh7 cell lines. The APOBEC3G mRNA level detected by real-time PCR was very low (approx. 0.002 % relative to GAPDH mRNA) and about ten times greater in HepG2 cells than in Huh7 cells (Fig. 3a).

The number of hypermutated genomes in HepG2 cells transiently transfected with pTRE-HB-wt was about five times that in Huh7 cells (Fig. 3b). Vif-deficient HIV-1 virions produced from HepG2 cell exhibited very low infectivity compared with wild-type (Fig. 4a). In contrast, the infectivity of HIV-1 virions produced by Huh7 was

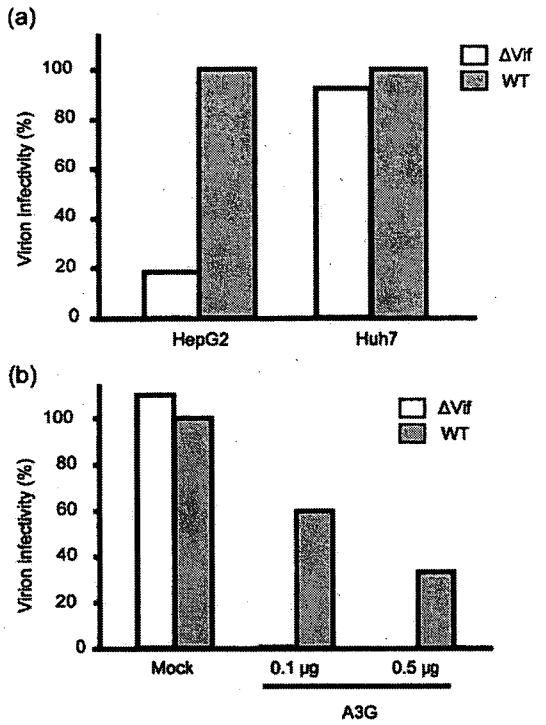

Fig. 4. Infectivity of HIV-1 virions produced from HepG2 and Huh7 cell lines. (a) Wild-type and mutant viruses lacking Vif protein produced from the two cell lines were examined for infectivity as described in Methods. The relative infectivity of the wild-type is shown. (b) Effect of APOBEC3G (A3G) expression on infectivity. HIV-1 virions produced by Huh7 cells co-transfected with the indicated number of APOBEC3G expression plasmid were used for measurement of infectivity.

similar to that of the wild-type virus (Fig. 4a). Transient expression experiments showed that the expression of APOBEC3G in Huh7 cell lines reduced infectivity of wild-type HIV-1 produced in these cell lines in a dose-dependent manner (Fig. 4b). Infectivity of Vif-deficient HIV-1 was reduced to almost undetectable levels (Fig. 4b). Thus, APOBEC3G effectively suppressed the production of infectious HIV in these cell lines.

Both IFN-α and -γ induce APOBEC3G mRNA expression and hypermutation of HBV genomes and reduce replication of HBV

We treated HepG2 cell lines stably transfected with 1.4 genome length construct HBV (Tsuge *et al.*, 2005) with either IFN-α or -γ to examine their influence on the expression of APOBEC3G mRNA and G to A hypermutation of HBV genomes. Chronological studies showed that the core-associated HBV DNA in the stably HBV-producing cell line gradually decreased until 36 h after IFN-α treatment (Fig. 5a). Expression levels of APOBEC3G mRNA, but not those of APOBEC3F, increased in this cell line at 12 h after the IFN treatment (Fig. 5a). Hypermutated genomes in this cell line increased with time until 36 h after IFN-α

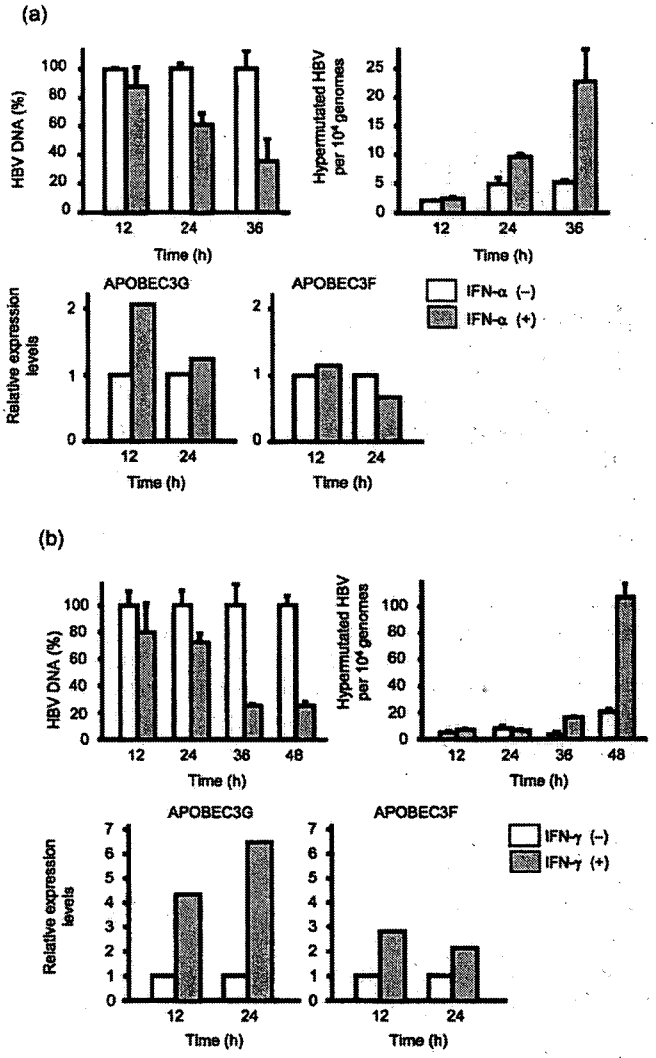

Fig. 5. Effects of IFN-α and -γ on HBV-producing cells. (a) The IFN-α-treated and -untreated HBV-producing T23 cell line was harvested at the indicated time after IFN treatment and examined for the number of core-associated HBV DNA, the number of hypermutated genome and mRNAs of APOBEC3G and APOBEC3F. (b) IFN-γ-treated and -untreated HBV-producing T23 cell line was examined as described in (a). Results are means ± SD values of three independent experiments.

treatment. Similarly, the core-associated HBV DNA decreased gradually to about 20 % of the levels in untreated cells after IFN-γ treatment (Fig. 5b). The increase in APOBEC3G mRNA expression was more prominent after IFN-γ than after IFN-α treatment. The level of APOBEC3F mRNA was also about double that of untreated cells. G to A hypermutation of HBV genomes increased markedly with time after IFN-γ treatment (Fig. 5b).

We further examined the effect of IFN on reduction of HBV replication and induction of hypermutation by comparing the effects of different doses of IFN-α and -γ. Both IFN-α

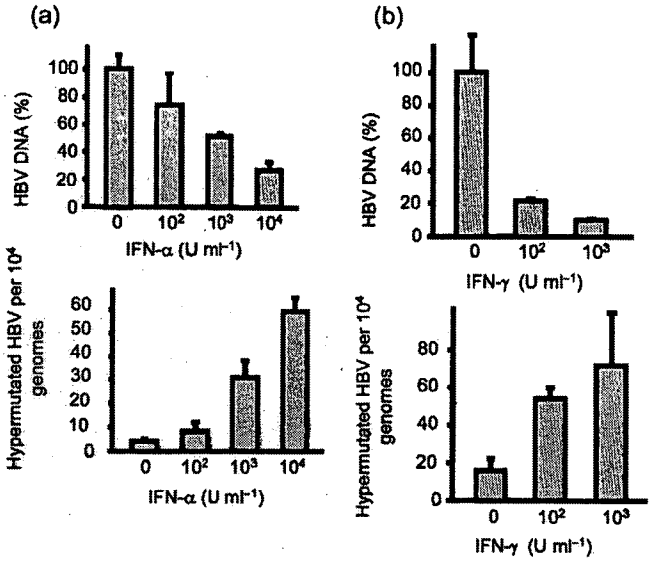

Fig. 6. Dose-dependent reduction of HBV replication and hypermutation of genomic sequences. HBV-producing cell line T23 was harvested after (a) IFN-α and (b) IFN-γ treatment for 72 h. The number of core-associated HBV DNA and the number of hypermutated genomes were measured. Results are means ± SD values of three independent experiments.

and -γ treatment decreased core-associated HBV DNA in a dose-dependent manner (Fig. 6). Hypermutation of HBV genomes also increased with higher doses of IFN (Fig. 6).

Expression of APOBEC3G increases hypermutation of the HBV genome

To confirm that the increase in hypermutation of the HBV genome is induced by the effect of APOBEC3G, we performed expression experiments of APOBEC3G and its deaminase function-deficient mutants into HepG2 cell lines and measured the number of hypermutated HBV genomes. Transient expression experiments showed that the number of HBV DNA was decreased by co-transfection of APOBEC3G in HepG2 cells (Fig. 7a). 3D-PCR and detection with HA-yellow agarose gel electrophoresis showed the presence of heavily hypermutated genomes (Fig. 7b). No amplification was observed at the 81 °C denaturation temperature (data not shown). Quantitative analysis showed an about 334-fold increase in hypermutated genomes compared with mock-transfected control cells (Fig. 7c). However, the proportion of hypermutated genomes was 9.68 % (968 in 10^4 genomes).

To confirm the effect of APOBEC3G on HBV hypermutation, we transfected wild-type and inactive mutants of APOBEC3G (Fig. 8a, b) into Huh7 cells. Wild-type APOBEC3G effectively induced hypermutation of HBV genomes and reduced the replication of HBV. In contrast, insufficient deaminase activity in the E67Q mutant induced less hypermutation of HBV genomes than in the wild-type. No increase in hypermutation was observed in cell lines transfected with deamination-defective E259Q and E67Q/E259Q mutants, although the number of HBV replication was reduced in these cells (Fig. 8a). We observed similar reduction in HBV replication by transient transfection of APOBEC3F. Induction of hypermutation by APOBEC3F was less efficient than by wild-type and the E67Q mutant of

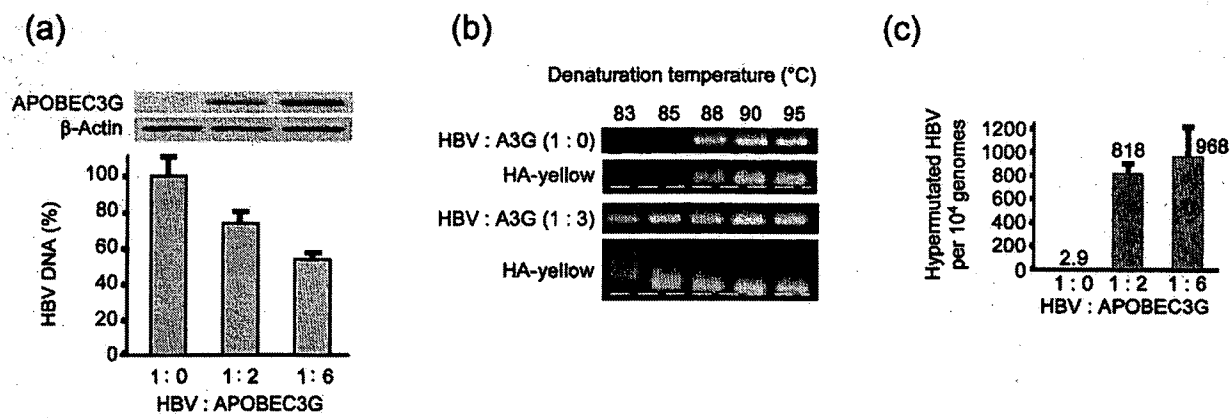

Fig. 7. Effects of APOBEC3G expression on HBV hypermutation. A plasmid containing 1.4 genome length HBV DNA was co-transfected with pcDNA3/HA-A3G into HepG2 cells. At 72 h after transfection, the cells were harvested. (a) Quantification of core-associated HBV DNA and Western blot analysis of cytoplasmic extracts with anti-HA or anti-β-actin antibody. (b) Detection of hypermutated genomes by HA-yellow agarose gel electrophoresis. Hypermutated genomes in the presence or absence of APOBEC3G-HA were amplified by 3D-PCR. The white dotted line was added to help visualize the retardation of AT-rich DNA in HA-yellow agarose gel. (c) Quantification analysis of hypermutated genomes by real-time 3D-PCR. Results are means ± SD values of three independent experiments.

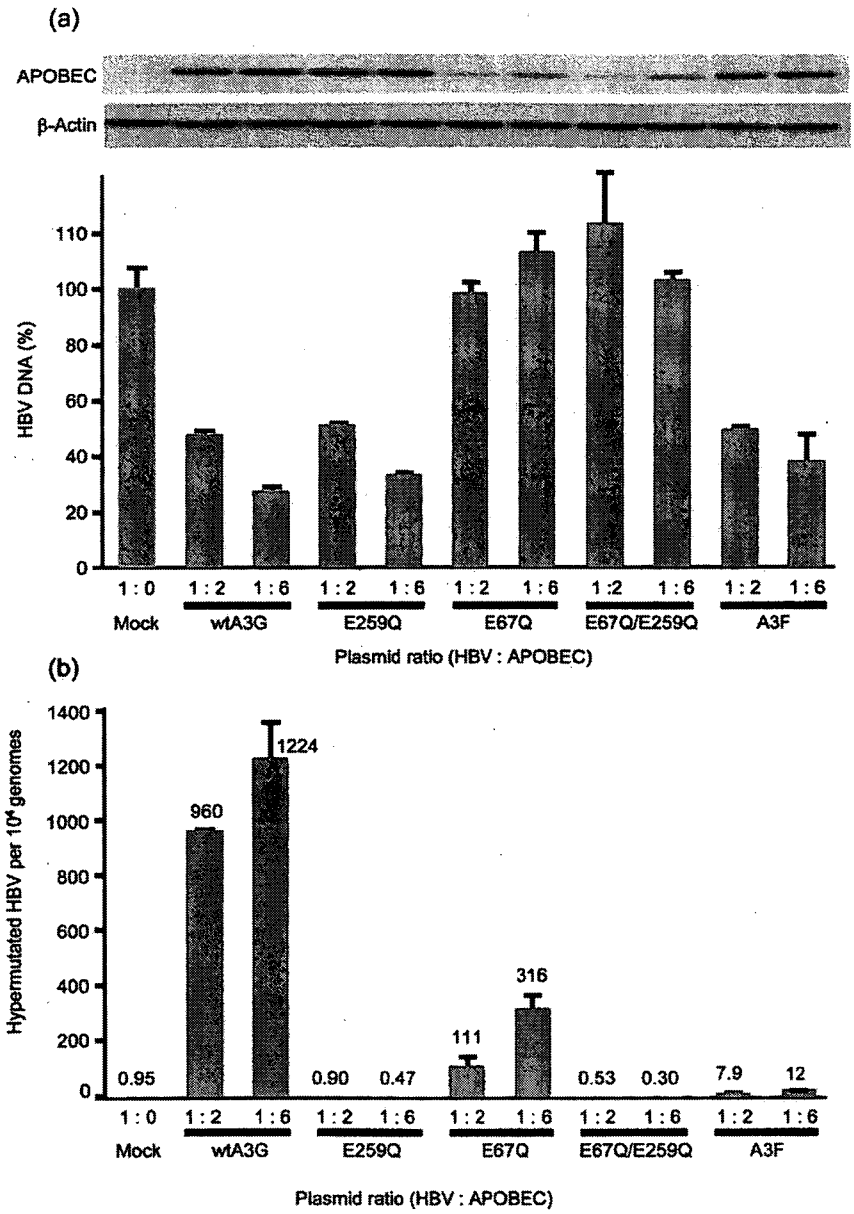

Fig. 8. Effect of APOBEC proteins on HBV hypermutation. A plasmid containing 1.4 genome length HBV DNA was co-transfected with wild-type, enzymically impaired APOBEC3G mutants (E67Q, E259Q, E67Q/E259Q) and APOBEC3F into Huh7 cells (plasmid ratio HBV : APOBEC = 1 : 2 or 1 : 6). The cells were harvested at 96 h after transfection. (a) Quantification of core-associated HBV DNA and Western blot analysis of cytoplasmic extracts with anti-HA or anti-β-actin antibody. (b) Quantification of hypermutated genomes by real-time 3D-PCR. Results are means ± SD values of three independent experiments.

APOBEC3G. These results suggest that hypermutation of HBV contributes very little to reduce the number of replicative intermediate.

DISCUSSION

Induction of G to A hypermutation in HIV has been reported as part of host innate immunity against virus infection (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris *et al.*, 2003; Sheehy *et al.*, 2002). We and others have reported the presence of hypermutated genomes of HBV in serum samples of chronically infected patients and in HepG2 cell lines (Gunther *et al.*, 1997; Suspene *et al.*, 2005a; Noguchi *et al.*, 2005; Rosler *et al.*, 2004). Hypermutation of HBV was induced in hepatocytes (Noguchi *et al.*, 2005), and expression of APOBEC proteins in liver cell-derived cell lines increased hypermutation (Suspene *et al.*, 2005b; Rosler *et al.*, 2004). However, the estimated number of hypermutated genomes in chronically infected patients is very low (Noguchi *et al.*, 2005; Suspene *et al.*, 2005b). The reason for the partial hypermutation of HBV remains an enigma. It might be due to the low expression levels of APOBEC proteins in liver cells (Jarmuz *et al.*, 2002). Alternatively, rapid packaging of pregenome RNA into capsid might prevent access of APOBEC3G to the first strand DNA. Furthermore, rapid degradation of edited HBV genomes by uracil DNA glycosylase in liver cells might also explain the low number of hypermutated genomes.

The mechanism that controls the activities of APOBEC proteins to cause hypermutation has not been analysed until

recently. Tanaka *et al.* (2006) reported that IFN-α increases the expression levels of APOBEC3G mRNA. They reported the presence of ISRE elements in the promoter region of APOBEC3G and that the promoter was activated by IFN-α. However, they did not examine the occurrence of G to A hypermutation in their experiments. Moreover, Peng *et al.* (2006) showed that IFN-α and -γ cooperatively induce APOBEC3G expression and that the inhibition of HIV production by a small number of IFN is cancelled by a small interfering RNA (siRNA) against APOBEC3G. More recently, Bonvin *et al.* (2006) demonstrated that IFN-α induces transcription of APOBEC proteins. They showed that IFN treatment increased APOBEC3B, -3C, -3F and -3G mRNAs, particularly when they used primary cultured hepatocytes. They also reported that they were able to detect hypermutated genomes after transfection of APOBEC3 plasmids, but did not measure the direct effect of IFN on G to A hypermutation.

These studies did not analyse quantitatively the increase in hypermutation of viral genomes. The studies that analysed the expression of APOBEC protein and reduction of HBV DNA also did not analyse quantitatively the number of hypermutated genome (Suspene *et al.*, 2005a; Noguchi *et al.*, 2005; Turelli *et al.*, 2004a, b; Rosler *et al.*, 2005). In the present study, we developed a method that accurately measures the level of hypermutation using real-time PCR. It is often difficult to design a primer set and a probe to detect G to A hypermutation because they are located in a region with many G residues, but the primer and probe sequences should not contain any. It is thus possible that we did not see any C to T substitution because we did not design a primer–probe set to detect this substitution. We also tried to select such a primer–probe set applicable for all genotypes of HBV, but were able to select only one suitable for genotype C.

Using this method, we demonstrated that both IFN-α and -γ increased G to A hypermutation of the HBV genome. Although the expression levels of APOBEC3G increased after IFN treatment, we did not observe an apparent shift of preferred dinucleotide sequence of APOBEC proteins from 3F to 3G. This is probably because the increase in APOBEC3G is only slight (Fig. 5).

The exact mechanism by which IFNs activate the transcription of APOBEC3G is unknown. Furthermore, what kind of sensor(s) detects HBV infection and how the signal is communicated for the production of IFNs and subsequent induction of effector molecules have not been analysed yet. Although the importance of the IFN system in eliminating HBV and its possible mechanism have been reported (Wieland *et al.*, 2004a, b, 2005), further studies are needed to fully describe the mechanism of action of IFNs including the activation of APOBEC3G.

We also demonstrated that the number of hypermutated genomes increased with the expression of APOBEC3G and APOBEC3F (Fig. 8), but not in deaminase-inactive mutants, as demonstrated previously in HIV studies (Shindo *et al.*, 2003; Newman *et al.*, 2005). However, these mutants also reduced the replication of HBV almost to the wild-type level. This suggests that the contribution of hypermutation of HBV to the reduction of virus replication is only minimal and supports the previous report that showed that APOBEC3G reduced the replication of HBV through inhibition of packaging of the pregenome (Turelli *et al.*, 2004a). However, the effect of hypermutation on infectivity of the virus should be investigated further. The effects of APOBEC proteins, including other family members, especially under physiological conditions, should also be examined further. Whether any HBV protein inhibits deamination of the genomic DNA awaits further investigation. Furthermore, the mechanism that enables HBV to cause chronic infection, especially escape from innate antiviral immunity, should also be clarified in order to control chronic HBV infection and reduce HBV-related morbidity.

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