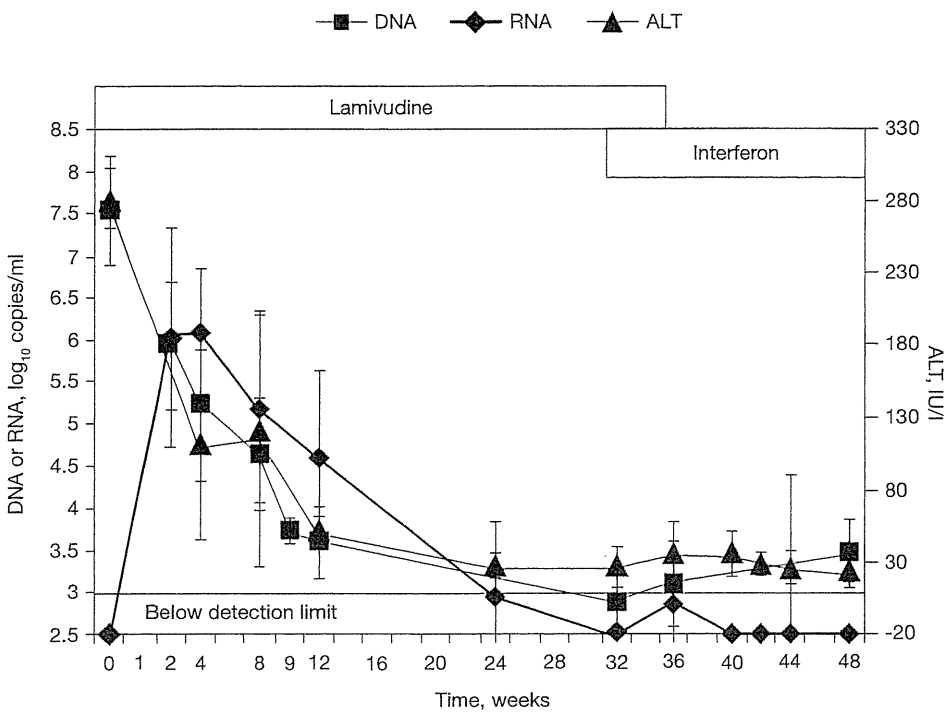
Figure 2. Sequential changes of serum HBV RNA, DNA and ALT levels in patients treated with lamivudine for 34–52 weeks with detectable serum HBV RNA (group II)

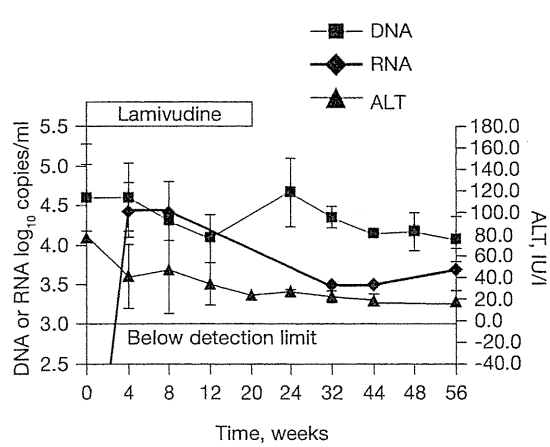


Patients were treated with lamivudine for 34–52 weeks and then shifted to conventional interferon therapy for 24–26 weeks; there was overlap of the two drugs for 4–20 weeks. Serum HBV RNA was undetectable at the end of treatment. ALT, alanine aminotransferase.

led to an improved sustained virological response compared with the use of interferon alone from the start [13]. Nevertheless, the underlying mechanisms of these convincing findings remain unclear and deserve further investigation. In this study, the inhibitory effect of interferon on serum HBV RNA in lamivudine-treated patients might explain why these patients have a higher sustained response rate than those treated with lamivudine monotherapy.

The weak point of this study is the small number of patients in groups II and III. Because this is a pilot study on the differential effects of various treatment regimens on serum HBV RNA, and because the treatment regimens in group II and group III patients are not the current standard of care, we did not intend to include additional patients treated with these regimens. In line with this study, however, our unpublished data on three patients also indicate that *de novo* combination therapy of pegylated interferon plus lamivudine therapy could inhibit serum HBV RNA levels. In these three patients, serum HBV RNA levels started to rise at 12–24 weeks of combination therapy and became undetectable at 48–72 weeks of therapy (YWH, JHK, *et al.*, unpublished data).

Figure 3. Sequential changes of serum HBV RNA, DNA and ALT levels in patients treated with short-term lamivudine for 20–24 weeks with detectable serum HBV RNA (group III)



Serum HBV RNA was detectable until the end of follow-up at 44–56 weeks. ALT, alanine aminotransferase.

The presence of serum HBV RNA in patients treated with nucleoside analogues could be explained by *in vitro* data obtained in HBV-transfected HepG2.2.15 cell lines. Doong *et al.* [7] demonstrated that HBV-specific RNAs in cell lysate were not reduced after lamivudine and other nucleoside analogue treatments. Our unpublished data also showed persistent detectable HBV RNA in supernatant from day 4 to 17 of nucleoside analogue treatment when all the cells died (YWH, JHK, *et al.*, unpublished data). Lamivudine and other nucleoside analogues do not affect the integrated HBV DNAs from which HBV RNAs are transcribed [7]. Further studies are needed to evaluate the effect of long-term treatment of nucleoside analogues on serum HBV RNA.

The inhibition of serum HBV RNA by interferon-α might be supported by previous studies on transgenic mice. Intrahepatic HBV replicative intermediates were cleared by a single injection of the interferon-α/β inducer polyinosinic-polycytidylic acid [14]. It was postulated that the mechanism of action of interferon involves the post-transcriptional steps of the HBV life cycle, as the intrahepatic HBV replicative intermediates were cleared while the steady-state content of HBV RNA was unaffected [14]. This same group of researchers further demonstrated that the inhibitory effect of interferon-α/β is at the level of the capsids containing pre-genomic RNA, acting either to accelerate their degradation or to prevent their assembly [15]. Interferon might directly inhibit HBV synthesis or could act through the cellular immune response against HBV-infected hepatocytes [16]. HBV inhibition in immortalized hepatocyte cell lines from HBV transgenic mice by interferon-β and interferon-γ confirms the non-cytolytic inhibition pathway [17]. This inhibition might act through the 2′,5′-oligoadenyl synthetase/RNAse L pathway [18]. Interferon can induce this multienzyme pathway that includes 2′,5′-oligoadenyl synthetase, endoribonuclease RNAse L and 2′,5′-oligoadenyl phosphodiesterase. Among these enzymes, RNAse L theoretically inhibits all viral replication that uses an RNA intermediate step [16]. Furthermore, activation of this ribonuclease has been proposed as the major driver by which interferon inhibits viral replication [18].

In the current study, we performed frequent detection of serum HBV DNA and RNA, in some cases as often as every 2 weeks, to determine the sequential change of serum HBV DNA and RNA during monotherapy or combination therapy. Our data showed that the peak serum HBV RNA level with entecavir treatment was significantly higher than that with lamivudine treatment (8.6 ±1.0 versus 5.6 ±1.0; $P<0.001$). There was also a trend towards higher detectability of serum HBV RNA in patients treated with entecavir in comparison with those treated with lamivudine (100% versus 71%; $P=0.48$). These findings suggest that the serum HBV RNA level might reflect the antiviral potency of nucleoside analogues [19]. Further studies are needed to clarify this interesting and important issue.

Although Rokuhara *et al.* [20] showed that HBV RNA was detectable before lamivudine therapy in serum samples of 24 patients, the detection rate was not specified. Their results of sucrose density gradient fractionation studies indicated that viral particles containing HBV DNA were dominant at the start of treatment, whereas those containing HBV RNA became more prevalent after 1 and 2 months of treatment. They also suggested that under untreated conditions, viral particles containing HBV RNA accounted for only about 1% of total HBV virions. However, these specific particles became the major component under lamivudine treatment [9]. They concluded, therefore, that HBV RNA particles seemed to exist in <1% of the HBV virions among patients without lamivudine treatment [21]. By contrast, the undetectable pre-treatment HBV RNA data in this study was consistent with our previous report, showing that serum HBV RNA levels increased soon after the administration of nucleoside analogues [10]. Furthermore, our data was supported by the *in vitro* data obtained in HBV-transfected HepG2.2.15 cell lines (YWH, JHK, *et al.*, unpublished data), suggesting that HBV RNA was undetectable in the supernatant before nucleoside analogue treatment but became detectable after administration of these agents. In addition, Rokuhara and colleagues [20] reported a more significant decline of the serum HBV DNA level than HBV RNA level during lamivudine therapy, which confirmed our findings.

Serum HBV RNA was persistently detectable even after discontinuation of short-term lamivudine therapy in group III patients. This finding suggested that, although new viral particles containing HBV RNA were no longer produced after discontinuation of lamivudine, the existing viral particles containing HBV RNA during lamivudine administration were not quickly degraded. The study by Rokuhara *et al.* [20] showed a more significant decline in serum HBV DNA than RNA during lamivudine therapy, also confirming our findings on the poor immediate inhibition of serum viral particles containing HBV RNA by nucleoside analogues. Nevertheless, further studies are needed to demonstrate how long the viral particles containing HBV RNA persist in serum.

Following sequential combination therapy of lamivudine and conventional interferon, the serum HBV DNA level declined but was still detectable in all patients until the end of treatment. By contrast, serum HBV RNA was inhibited and undetectable at the end of treatment. The persistent presence of serum HBV DNA was due to the discontinuation of nucleoside analogue treatment and, thus, the lack of

continuous inhibition. The shift to interferon led to the inhibition of serum HBV RNA, but the inhibitory effect of interferon on HBV DNA was not as efficient as that of the nucleoside analogue [8]. Although northern and Southern hybridization data of intracellular RNA and DNA were not available, our study and others have confirmed the possibility of detecting serum HBV RNA. We reported the discrepant measurement of HBV nucleic acid by the transcription-mediated amplification and hybridization protection assay (TMA-HPA) and the Amplicor HBV Monitor test [10]. Because TMA-HPA uses RNA transcription and amplification of transcripts by T7 RNA polymerase [22], we assumed that the discrepancy was a result of the persistence of serum HBV RNA in nucleoside-analogue-treated patients. Zhang *et al.* [9] reported the presence of serum HBV RNA in a patient treated with lamivudine. The study mainly analysed truncated HBV RNA, which was assumed to be transcribed from the integrated HBV genome; the authors showed a marked difference between truncated HBV RNA and HBV DNA. In this study, HBV DNA and HBV nucleic acid were assayed by real-time PCR and real-time reverse transcriptase PCR, and <1 $\log_{10}$ difference was shown; this observation suggests that the effect of truncated serum HBV RNA was minimal. In addition, Rokuhara *et al.* [20] investigated the incorporation of HBV RNA into virus particles using sucrose gradient analyses: HBV RNA made a single peak in one fraction, whereas both HBV DNA and HBV core-related antigen made single peaks at three different time points during lamivudine treatment.

In conclusion, interferon can inhibit serum HBV RNA induced by lamivudine therapy. The persistence of serum HBV RNA as a consequence of unaffected HBV RNA replicative intermediates might lead to indefinite nucleoside analogue therapy. By contrast, the inhibitory effect of interferon on HBV RNA replicative intermediates might potentiate the suppression of HBV replication.

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Disclosure statement

The authors declare no competing interests.

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G-to-A Hypermutation in Hepatitis B Virus (HBV) and Clinical Course of Patients with Chronic HBV Infection

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Background. The apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like family of cytidine deaminases induce G-to-A hypermutation in hepatitis B virus (HBV) genomes and play a role in innate antiviral immunity. The clinical relevance of this protein family is unknown.

Methods. We analyzed 33 instances in which 17 patients with chronic HBV infection experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level; we used a quantitative differential DNA denaturation polymerase chain reaction assay to quantify the hypermutated HBV genomes observed during 21 of these 33 increases in ALT level.

Results. Of the 9 increases in ALT level that involved a >5-fold increase (relative to basal levels) in the number of hypermutated genomes observed, 8 were associated with a >2-log reduction in plasma HBV DNA level. In contrast, a corresponding decrease in plasma HBV DNA level was observed for only 1 of the 12 increases in ALT level that did not involve an increase in the number of hypermutated genomes ($P < .001$). Hepatitis B e antigen clearance was often observed in patients who experienced an increase in the number of hypermutated genomes. Interferon treatment induced hypermutation in HBV genomes in an animal model. However, there was no apparent increase in the number of hypermutated genomes among the majority of patients who received interferon therapy, probably because the number of hypermutated genomes had already increased prior to the initiation of therapy.

Conclusion. Our results suggest that a marked increase in the number of hypermutated genomes represents a strong immunological host response against the virus and is predictive of hepatitis B e antigen clearance and plasma HBV DNA level reduction.

Despite the availability of safe and effective vaccines for >2 decades, hepatitis B virus (HBV) infection is still a global health problem. Worldwide, >2 billion people are infected with HBV, and chronic HBV infection affects ~400 million people [1, 2]. It is estimated that >500,000 people die annually because of cirrhosis and/or hepatocellular carcinoma due to HBV infection [3].

Recent reports have shown that cellular cytosine deaminase (apolipoprotein B messenger RNA [mRNA] editing enzyme, catalytic polypeptide–like 3G [APOBEC3G]), packaged in human immunodeficiency virus type 1 (HIV-1), induces G-to-A hypermutation to a nascent reverse transcript of HIV-1 and reduces the infectivity of HIV, thus contributing in part to innate antiviral activity [4–8]. HIV-1 overcomes this innate defense barrier in T cells with HIV virion infectivity factor, a protein that specifically targets APOBEC3G to proteasomal degradation [9–12]. HIV-1 can infect resting CD4 T cells in lymphoid tissues but not those circulating in peripheral blood [13–16]. Resting CD4 T cells in peripheral blood are protected from HIV infection through the action of the deaminase-active

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Table 1. Clinical profiles of 17 patients with chronic hepatitis B virus (HBV) infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level.

Patient	Sex	Age, years	ALT level, IU/L		Plasma HBV DNA level, log copies/mL	HBV serum marker status[a]		HBV subtype	Histologic result[b]	Receipt of IFN treatment
			Minimum	Maximum		HBeAg	HBeAb			
1	M	50	26	2000	8.1	+	−	C	F2, A2	Yes
2	M	31	22	230	8.2	+	−	C	F3, A2	Yes
3	F	23	14	313	8.7	+	−	C	F2, A2	Yes
4	M	22	16	846	6.9	+	−	C	F2, A1	Yes
5	F	42	10	100	7.8	+	−	C	L	No
6	F	33	21	748	8.8	+	−	C	F2, A3	Yes
7	M	23	22	339	8.4	+	−	C	L	Yes
8	F	54	22	108	6.7	−	+	C	F2, A2	No
9	M	44	17	512	9.5	+	−	C	F2, A3	No
10	M	27	39	115	8.8	+	−	C	F1, A1	Yes
11	M	36	16	452	3.8	+	−	C	F4, A3	Yes
12	M	20	21	1295	7.2	+	−	C	F2, A2	No
13	M	36	24	481	5.7	−	+	C	F2, A2	Yes
14	M	22	20	696	5.9	+	−	C	F1, A1	Yes
15	F	24	14	1544	7.7	+	−	C	F2, A2	Yes
16	M	35	10	1618	4.7	+	−	C	F2, A1	Yes
17	M	30	21	1655	6.7	+	−	C	L	Yes

NOTE. HBeAg, HBV e antigen; HBeAb, antibody against HBV e antigen; IFN, interferon; L, liver cirrhosis.

[a] Before increase in ALT level.

[b] Histologic evaluation of chronic hepatitis by use of the scoring system of Desmet et al. [29].

APOBEC3G [17]. Recent reports have shown that interferon (IFN)–α is a potent inducer of APOBEC3G [18–21]. It has also been reported that some of the HIV restriction exerted by APOBEC3G may be independent of its cytidine deaminase activity [17, 22–24].

We and others have reported the presence of small numbers of hypermutated genomes in serum samples obtained from HBV-infected patients [25–27]. Studies using HepG2 cell lines and primary human hepatocytes showed that such hypermutation is induced by the cytidine deaminase activity of the APOBEC family of proteins [27]. In our previous study, IFN induced little hypermutation in the HBV genome [27]. However, after extensive investigation supported by development of a quantitative analysis of hypermutation, we showed that both IFN-α and IFN-γ actually increase transcription of APOBEC3G mRNA in HepG2 cell lines and induce an increase in the number of hypermutated genomes [28]. We also showed that APOBEC3G induces hypermutation in HBV and reduces HBV replication levels in the absence of the deaminase activity. Thus, APOBEC3G has dual antiviral actions against HBV and is thought to be part of the host defense mechanisms, as has been shown for HIV infection. Although it is assumed that APOBEC3G is important in the host anti-HBV defense system, little is known about the clinical importance of this enzyme, because there are no methods available for the precise quantification of small amounts of hypermutated genomes.

Using a method that can measure small amounts of hypermutated genomes (differential DNA denaturation polymerase chain reaction [3D-PCR] combined with TaqMan PCR [28]), we analyzed fluctuations in the number of hypermutated genomes observed in patients with chronic HBV infection who experienced increased alanine aminotransferase (ALT) levels. The study group included patients who received IFN treatment and patients who did not.

METHODS

Patients. From 2002 through 2006 at Hiroshima University Hospital (Hiroshima, Japan), there were 17 consecutive patients with chronic hepatitis B who experienced >2 increases of >100 IU/L in ALT level and for whom stored serum samples were available. These 17 patients were enrolled in this study, among whom 33 such increases in ALT level were observed. Thirteen of 17 patients received IFN treatment, usually during an increase in ALT level. The clinical profiles of these 17 patients are shown in table 1. Written informed consent was obtained from all patients, and the study was approved by the Hiroshima University Ethics Committee.

HBV markers. Hepatitis B e antigen and antibody against e antigen were quantified by use of enzyme immunoassay kits (Abbott Diagnostics). HBV DNA was measured by use of real-time PCR performed with the 7300 Real-Time PCR System (Applied Biosystems), in accordance with the manufacturer's instructions. The primers used for amplification were 5′-TT-

TGGGCATGGACATTGAC-3' (nt 1893–1912; nucleotide numbers are those of HBV subtype C as reported by Norder et al. [30]) and 5'-GGTGAACAATGTTCCGGAGAC-3' (nt 2029–2049). For real-time PCR, we used 25 μL of SYBR Green PCR Master Mix (Applied Biosystems) with 1 μL of the DNA solution and 200 nmol/L of each primer. The amplification conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of amplification (denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min). The lower detection limit of this assay was 10^3 copies/mL.

Extraction of HBV DNA and quantitative analysis of hypermutated genomes. HBV DNA was extracted from 100-μL serum samples by use of the SMITEST DNA Extraction Kit (Genome Science Laboratories) and dissolved in 20 μL of water. Hypermutated genomes were quantified by use of TaqMan 3D-PCR performed with the 7300 Real-Time PCR System (Applied Biosystems); we used a procedure described elsewhere [28], with slight modifications. In brief, the HBV DNA fragments were amplified by use of 3D-PCR in which the denaturation temperature was set lower than usual so that only G-to-A hypermutated genomes would be amplified. The amplification conditions were as follows: activation at 95°C for 10 min; followed by initial denaturation at 89°C for 20 min, to allow nonhypermutated genomes reanneal; and 45 cycles of amplification (denaturation at 89°C for 20 s, annealing at 50°C for 30 s, and extension at 62°C for 90 s). TaqMan PCR was performed using the following primers: 5'-ACTTCAACCCCAACAMRRATCA-3' (nt 2978–2999) and 5'-AGAGYTTGKTGGAATGTKGTGGA-3' (nt 24–1), where M is A or C, R is G or A, Y is T or C, and K is G or T. The probe was a 6-carboxyfluorescein (FAM)–labeled MGB probe, 5'-(FAM)-TTAGAGGTGGAGAGATGG-(MGB)-3' (nt 3184–3167). The detection limit of hypermutated genomes was 10^2 copies/mL, and nonhypermutated genomes were not amplified by 3D-PCR [28]. The reproducibility of the assay was quite high (as indicated by the small standard deviation relative to the results of the quantitative PCR control reaction), as reported in our previous study [28].

Cell culture and transfection. HepG2 cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum at 37°C in 5% CO_2. Cells were seeded to semiconfluence in 6-well tissue culture plates and transfected with the plasmid pTRE-HB-wt, which contained 1.4–genome length wild-type HBV genomes [31], by calcium phosphate precipitation. Seventy-two hours after transfection, the supernatant was collected for HBV DNA quantification by real-time PCR and for quantitative analysis of G-to-A hypermutated genomes [28]. The remaining supernatant was stored at −80°C for infection experiments using human hepatocyte–chimeric mice.

Quantitative analysis of G-to-A hypermutated genomes with human hepatocyte–chimeric mice. A human hepatocyte–chimeric mouse model was developed, as described previously [32], and used in infection and IFN-treatment experiments. The human hepatocytes progressively repopulated the murine host liver and were susceptible to HBV produced in cultured cell lines [31]. All animal protocols were in accordance with the guidelines of the local animal experimentation committee. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. Hepatocyte-chimeric mice were inoculated with 500 μL of the supernatant produced by transiently transfected cell lines. After confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-α, injected intramuscularly, for 14 days (the IFN-α was a gift from Hayashibara Biochemical Labs in Okayama, Japan). Human serum albumin in mouse serum was measured with the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories), used in accordance with the manufacturer's instructions.

Statistical analysis. Differences between clinical groups with respect to HBV DNA and e antigen levels were examined for statistical significance, using the Mann-Whitney *U* test. A *P* value $<.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with StatView (version 5.0; SAS Institute).

RESULTS

Clinical course of disease in patients with increased ALT levels and fluctuations in the number of hypermutated genomes. Figure 1*A*–1*D* shows clinical courses for 4 representative patients (patients 1–4 in Table 1) with chronic HBV infection who experienced increases in ALT level. We observed marked decreases in HBV DNA level in association with marked increases in hypermutated genomes (figure 1*A*–1*C*, black arrows). In contrast, there was no apparent reduction in HBV level in the absence of an increase in hypermutated genomes (1*A*–1*D*, white arrows). We also analyzed the effect of IFN therapy on the number of hypermutated genomes. In some patients, we observed an increase in the number of hypermutated genomes during IFN therapy (figure 1*B* and 1*C*) as well as a marked increase in the number of hypermutated genomes and a reduction of the virus accompanied by an increase in ALT level just after cessation of IFN therapy (1*A*–1*C*, black arrows). However, in some patients, such as patient 1 (figure 1*A*), we observed no apparent increase in the number of hypermutated genomes in response to IFN therapy. However, the number of hypermutated genomes observed in samples from this patient obtained just before the initiation of IFN therapy (996/10^6 genomes) was already higher than the baseline level (157/10^6 genomes). Samples from patient 4 (figure 1*D*) showed an increase in the number of hypermutated genomes during IFN therapy (1907/10^6 genomes), though this is less than the increase observed during natural exacerbation (12,404/10^6 genomes). In fact, there was no significant difference between IFN-treated patients and untreated patients with respect to the number of hypermutated genomes observed (data not shown). These results suggest that the host's antiviral immunity level was higher at baseline than it was after

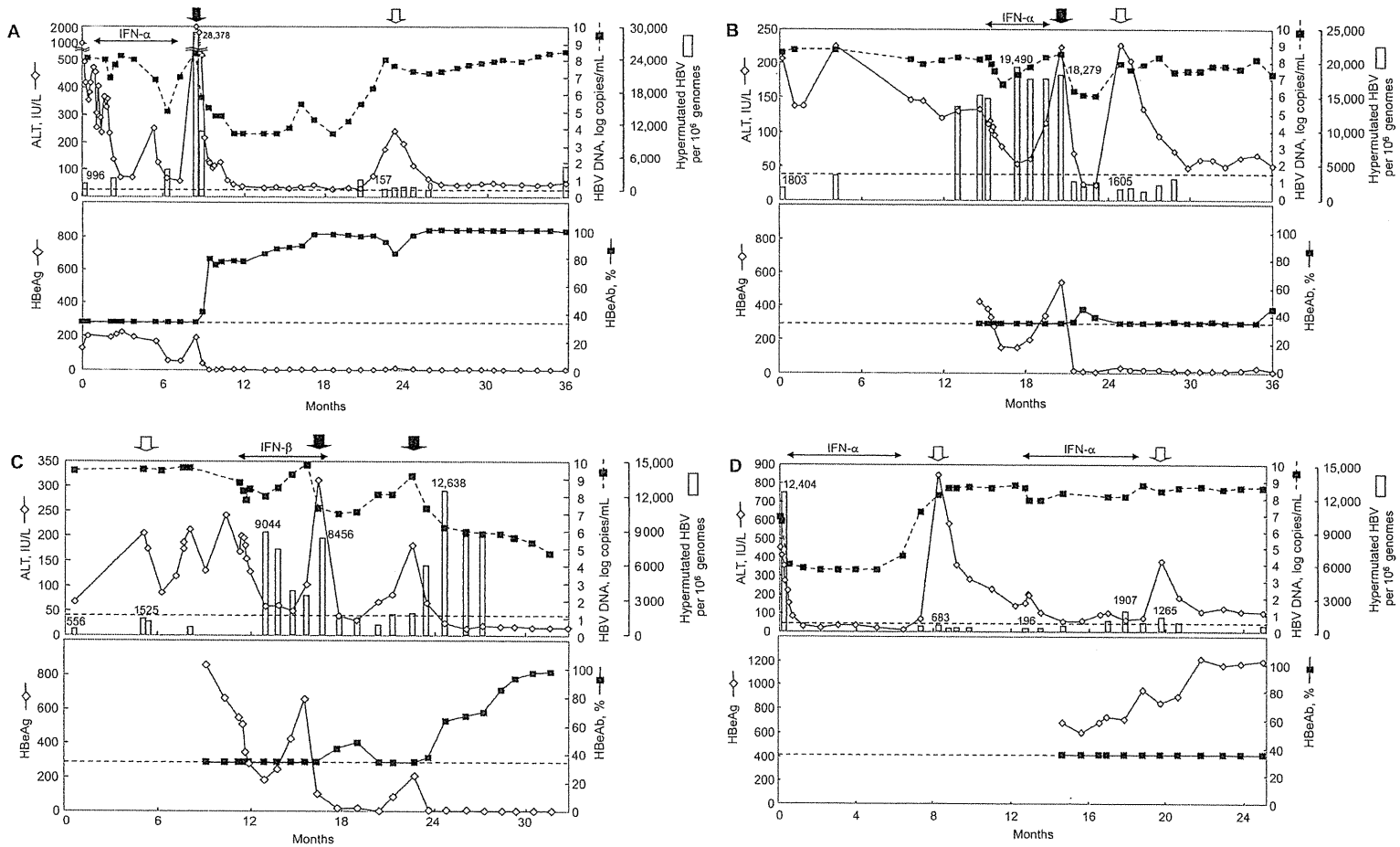

Figure 1. Clinical courses for 4 patients *(A–D)* with chronic hepatitis B virus (HBV) infection who experienced exacerbation of infection. *Black arrows,* exacerbation associated with an increase in the number of hypermutated genomes (>5 times basal levels); *white arrows,* exacerbation not associated with an increase in the number of hypermutated genomes; *horizontal dotted lines,* upper normal limit of alanine aminotransferase (ALT) (40 IU/mL; *upper panel, A–D*) and the detection limit for antibody against e antigen (HBeAb) (35%; *lower panel, A–D*). HBeAb, antibody against HBV e antigen; HBeAg, HBV e antigen; IFN, interferon.

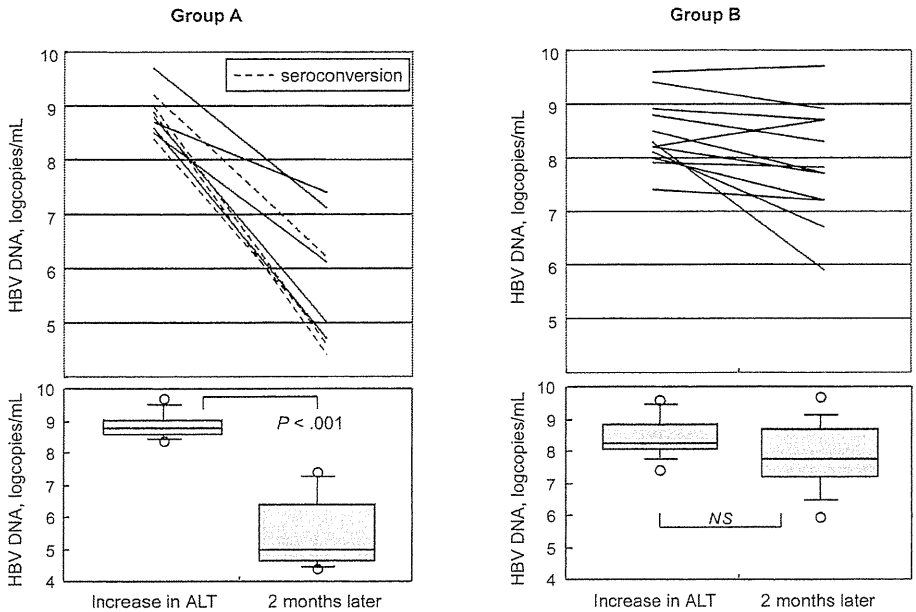

Figure 2. Relationship between increase in the number of hypermutated genomes and plasma levels of hepatitis B virus (HBV) DNA in 17 patients with chronic HBV infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level. Patients' exacerbations were divided into 2 groups, A and B, according to the extent of increase in the number of hypermutated genomes, relative to the basal number (group A included 9 exacerbations that involved a >5-fold increase in the number of hypermutated genomes; group B included 12 exacerbations that involved a ≤5-fold increase in the number of hypermutated genomes). *Upper panel* for groups A and B, individual HBV DNA levels at the time the ALT level increased and 2 months later; in the upper panel for group A, *dashed lines* indicate 4 exacerbations associated with seroconversion to positivity for antibody against e antigen. *Lower panel* for groups A and B, box-and-whisker plots for HBV DNA levels at same 2 time points. In the plots, the lines in the boxes indicate median values; the upper and lower lines of the boxes indicate the 25th and 75th percentiles, respectively; and the upper and lower whiskers represent the 90th and 10th percentiles, respectively.

IFN or that the feedback system for IFN signaling was already active before initiation of therapy.

We also compared the degree of reduction in the plasma HBV DNA level for exacerbations (i.e., increases in ALT level) associated with a marked increase in the number of hypermutated genomes (i.e., those in which the peak number was >5 times the number observed prior to exacerbation) and for exacerbations not associated with such an increase. As shown in figure 2, 8 of 9 exacerbations that were coupled with a marked increase in the number of hypermutated genomes (group A) were associated with a >2-log reduction in the HBV DNA level. In contrast, only 1 of the 12 exacerbations not associated with a marked increase in the number of hypermutated genomes (group B) was associated with a >2-log reduction in plasma HBV DNA level. The median serum HBV DNA level decreased from 8.8 to 5.0 log copies/mL among the patients in group A ($P < .001$) but did not decrease for patients in group B (figure 2).

In addition, we compared the reduction in e antigen level for these 2 groups. Levels were reduced in both groups, but the median reduction was more prominent for patients in group A than for those in group B (figure 3). All 4 exacerbations coupled with e antigen seroconversion (from positive to negative) were associated with marked increase in hypermutated genomes (figure 3).

Effect of IFN treatment on the rate of HBV hypermutation in chimeric mice. Next, we examined the effect of IFN treatment on G-to-A hypermutation in HBV genomes in human hepatocyte–chimeric mice. Two mice were intravenously injected with supernatant produced by HepG2 cells transiently transfected with a plasmid containing 1.4–genome length wild-type HBV genomes. Ten weeks later, after confirmation of high-level HBV viremia, the mice were treated with 7000 IU/g/day of IFN-α, injected intramuscularly, for 14 days. We observed an ~1.5-log reduction in plasma HBV DNA level accompanied by an increase in the number of hypermutated genomes in both mice (figure 4*A*). In a mouse inoculated with HBV but treated with phosphate-buffered saline, no increase of hypermutated genomes was observed (figure 4*B*). We also observed a 36-fold increase in the level of APOBEC3G mRNA, as determined by human oligonucleotide microarray (data not shown).

Infectivity of hypermutated genomes. To study the biological significance of hypermutated genomes, culture supernatant from HepG2 cells transfected with both HBV and APOBEC3G (5 μg each) was injected into a chimeric mouse. As shown in figure 5, the culture supernatant contained a large number of hypermutated genomes. In contrast, we could not detect hypermutated genomes in the chimeric mouse inoculated with this

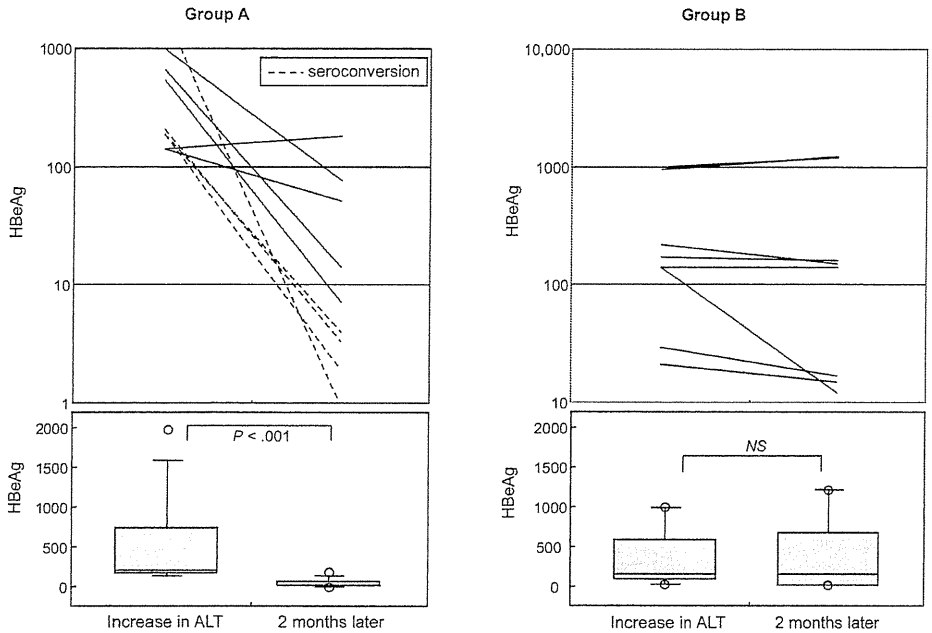

Figure 3. Relationship between increase in the number of hypermutated genomes and hepatitis B virus (HBV) e antigen (HBeAg) levels in 15 HBeAg-positive patients with chronic HBV infection who experienced >2 increases of >100 IU/L in alanine aminotransferase (ALT) level. Patients' exacerbations were divided into 2 groups, A and B, according to the extent of increase in the number of hypermutated genomes, relative to the basal number (group A included 9 exacerbations that involved a >5-fold increase in the number of hypermutated genomes; group B included 8 exacerbations that involved a ≤5-fold increase in the number of hypermutated genomes). *Upper panel* for groups A and B, individual e antigen levels at the time the ALT level increased and 2 months later; in the upper panel for group A, *dashed lines* indicate 4 exacerbations associated with seroconversion to positivity for antibody against e antigen. *Lower panel* for groups A and B, box-and-whisker plots for e antigen levels at these same 2 time points. In the plots, the lines in the boxes indicate median values; the upper and lower lines of the boxes indicate the 25th and 75th percentiles, respectively; and the upper and lower whiskers represent the 90th and 10th percentiles, respectively.

supernatant (figure 5*A* and 5*B*). These results suggest that the infectivity (or replication ability) of HBV with hypermutated genomes is very poor. It is possible that the inoculum contained less abundantly mutated genomes. To test this, we cloned and sequenced 72 clones of 217-bp DNA fragments amplified at a denaturation temperature of 95°C. Of 72 clones obtained from the inoculum, we found 1 clone with 8 G-to-A substitutions, 1 clone with 5 substitutions, 2 clones with 3 substitutions, and 1 clone with 1 substitution (figure 5*C*). In contrast, 1 of the 72 clones obtained from the mouse serum had 1 G-to-A substitution. If G-to-A substitutions were excluded, the only other nucleotide substitution observed in the 144 clones sequenced was a single C-to-T substitution.

DISCUSSION

In a previous study, we found that the majority of serum samples obtained from HBV-infected patients contained a small number of hypermutated genomes [27]. Recently, we developed a method (TaqMan 3D-PCR) to measure small numbers of hypermutated genomes [28]. Using this method, we reported dual antiviral effects for APOBEC3G, namely induction of hypermutation and reduction of viral replication. We also reported that IFN increased the transcription of APOBEC3G and enhanced the effect of the protein in vitro [28]. Other investigators also showed that IFN enhances the action of APOBEC proteins against HIV [18–21]. It is thus assumed that the antiviral effect of APOBEC proteins should be enhanced by IFN and other cytokines in vivo.

In the present study, we showed that an increase in ALT level accompanied by an increase in the number of hypermutated genomes was associated with reduction in the plasma HBV DNA level. In contrast, no decrease in HBV DNA level was observed if the increase in ALT level occurred in the absence of an increase in the number of hypermutated genomes. It is difficult to know which of the dual antiviral effects of APOBEC3G (or other APOBEC proteins) reduced the viral level. It is also impossible to estimate the importance of APOBEC proteins in this reduction. However, it is clear that the increase in the number of hypermutated genomes of HBV correlates with activation of the host antiviral defense against HBV.

We also demonstrated that exacerbations of HBV infection associated with a marked increase in the number of hypermutated genomes were associated not only with a decrease in the plasma HBV DNA level but also with clearance of e antigen.

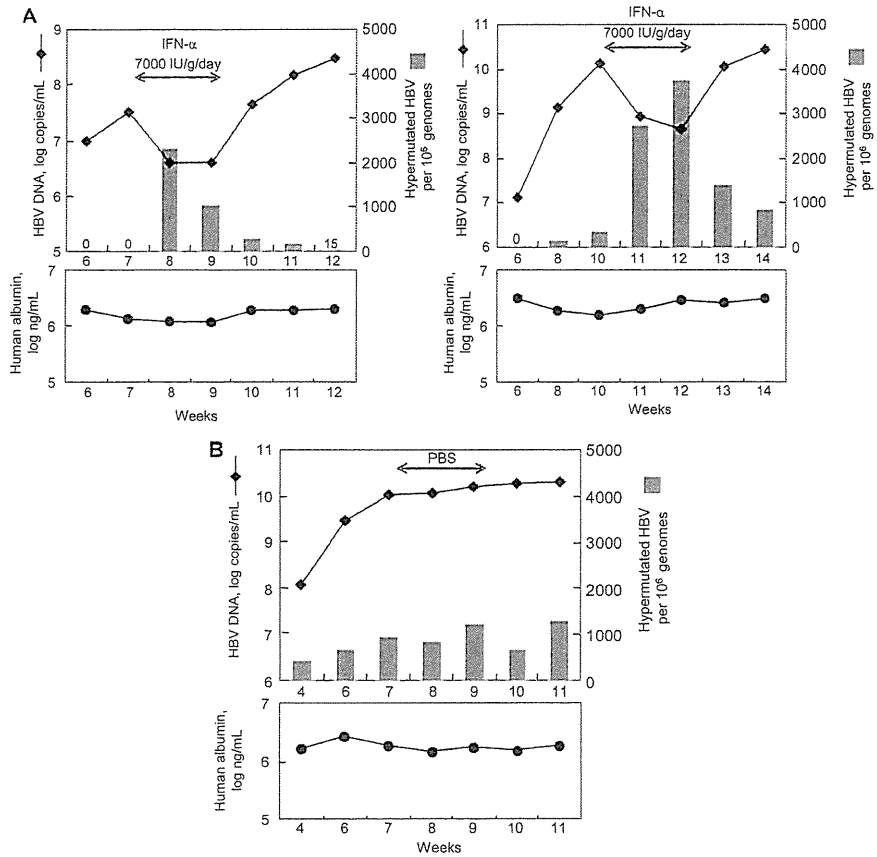

Figure 4. Effect of interferon (IFN)–α therapy on hepatitis B virus (HBV) hypermutation in HBV-infected, human hepatocyte–chimeric mice. Two chimeric mice *(A)* were inoculated with recombinant wild-type HBV produced by transfected HepG2 cells; 10 weeks later, after confirmation of high-level HBV viremia, they were treated with IFN-α at 7000 IU/g/day for 14 days, by intramuscular injection. *Upper panels* in both parts of *A*, serum HBV DNA levels and the number of hypermutated genomes; *lower panels* in both parts of *A*, human serum albumin concentrations. Note that the albumin levels are stable during IFN-α therapy. A control mouse *(B)* was inoculated with recombinant wild-type HBV produced by transfected HepG2 cells and treated with phosphate-buffered saline (PBS). Upper and lower panels of *B* show the same information as in *A*.

Furthermore, all exacerbations followed by seroconversion to positivity for antibody against e antigen were associated with a marked increase in the number of hypermutated genomes. Clearance of e antigen often results from a G-to-A nucleotide substitution at the first position of a 5'-GGGG stretch in the precore coding sequence (the G1896A mutation). Because this substitution (changing TGGGG to TAGGG) is in agreement with the dinucleotide pattern preferentially edited by APOBEC3G, one might assume that G-to-A substitution in this region could be caused by this enzyme and is related to the clearance of e antigen. However, we observed that hypermutation was induced in only some genomes, whereas the majority of genomes were unaffected. Thus, it seems unlikely that APOBEC proteins play a role in seroconversion to positivity for antibody against e antigen, although it is still possible that the 5'-GGGG stretch in the precore region is the preferred editing site for the enzyme. Importantly, such substitution of the 5'-GGGG stretch should result in the occurrence of multiple stop codons (TAG, TGA, and TAA) in HBV genomes, as we observed and reported in our previous study [28], which makes the replication of mutated genomes impossible.

In the present study, we did not observe any increase in the number of hypermutated genomes during IFN therapy in some patients. This finding is discrepant from the results of previous in vitro experiments that showed increased numbers of hypermutated genomes after the application of IFN [28]. Interestingly, our experimental results also showed the induction of APOBEC3G gene expression, an increase in the number of hypermutated genomes, and a reduction of plasma HBV DNA level in 2 human hepatocyte–chimeric mice treated with IFN (figure 4). What is the reason for the lack of increase in hypermutation in some IFN-treated patients? We usually administer IFN to patients who have high ALT levels. The patients in this study had abnormal ALT levels prior to treatment with IFN—that is, their livers were inflamed, and the levels of many cytokines produced by the immune cells in the liver were already high. We presume that the effect of these elevated cytokine levels masked the effect of the IFN we administered. It could also be argued that the effect

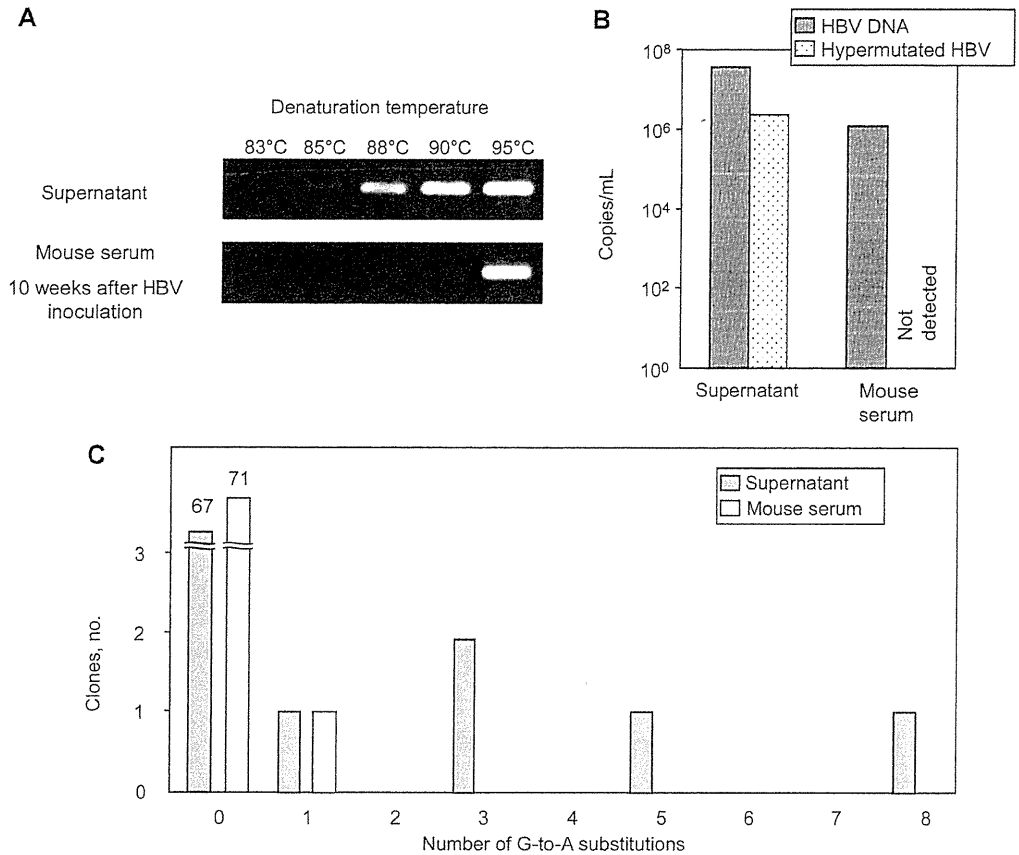

Figure 5. Results for a human hepatocyte–chimeric mouse inoculated with hepatitis B virus (HBV) produced by HepG2 cells transfected with an equal amount (5 μg each) of HBV and apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like 3G plasmids. The inoculum contained ~6.25% hypermutated genomes. A serum sample was obtained 10 weeks after the inoculation. *A,* HBV DNA was amplified by polymerase chain reaction (PCR) that used different denaturation temperatures and run on 2% agarose gel. *B,* Quantitative measurement of HBV DNA and hypermutated DNA in the inoculum and mouse serum. *C,* Number of G-to-A substitutions found in each of 72 clones obtained from products of PCR of culture supernatant or mouse serum.

observed in mice represents the absence of the immune response in mice, whereas the lack of a clear response to IFN in the study patients was the result of the complex immune response in human beings. Alternatively, the concentrations of IFN in treated patients might be lower than those used for the cell culture or the chimeric mice. Although we did not perform this analysis in the present study, it would be interesting to determine the expression levels of APOBEC proteins and IFN-stimulated genes in the liver of IFN-treated patients.

The present study showed that the number of hypermutated genomes increased during some increases in ALT level, probably as a result of IFN-activated APOBEC proteins and other cytokines in patients with chronic hepatitis B. However, the number of hypermutated genomes was very small, only 28,378 in 10^6 HBV genomes at most (figure 1*A*). Because it was possible that the less abundantly hypermutated genomes were not detected (i.e., that genomes with only 1 or 2 G-to-A substitutions were not amplified by 3D-PCR), cloning and sequencing were performed to detect such genomes. However, the number of genomes containing G-to-A substitutions was still low (5 [6.9%] of 72 clones), even in the culture medium of HepG2 cells cotransfected with APOBEC3G and HBV (figure 5*C*). This means that the number of genomes with only a small number of G-to-A substitution was not high, suggesting that only selected DNA molecules were heavily mutated while the remaining DNA was not. Does this mean that the effect of APOBEC proteins in antiviral defense is trivial in patients with chronic HBV infection? It is a possible that the heavily deaminated genomes are an easy target for uracil DNA glycocylase. Although the dual antiviral effects of APOBEC proteins are currently known to reduce the amount of HBV, the importance and magnitude of APOBEC proteins with respect to in vivo virus reduction should be investigated further.

Treatment of patients with chronic HBV infection has improved with the advent of new nucleoside and nucleotide analogues. However, reactivation of HBV and flare-ups of hepatitis are often seen in patients who stop such therapy. Furthermore, hepatitis B surface antigen clearance is rare in patients treated

with these antiviral drugs. On the other hand, most patients with chronic HBV infection achieve sufficient viral suppression and disease quiescence through immunological suppression of the virus. As we showed in this study, the immunological suppression of HBV is much stronger than that achieved with IFN therapy, but it is often transient. It is thus necessary to clarify the mechanism of transient immune response and to develop treatment that produces persistent suppression of HBV. Quantitative measurement of hypermutated genomes should be useful in monitoring the immune response in this context.

Acknowledgments

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Original Article

Effects of structural variations of *APOBEC3A* and *APOBEC3B* genes in chronic hepatitis B virus infection

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Aim: Human APOBEC3 deaminases induce G to A hypermutation in nascent DNA strand of hepatitis B virus (HBV) genomes and seem to operate as part of the innate antiviral immune system. We analyzed the importance of APOBEC3A (A3A) and APOBEC3B (A3B) proteins, which are potent inhibitors of adeno-associated-virus and long terminal repeat (LTR)-retrotransposons, in chronic HBV infection.

Methods: We focused on the common deletion polymorphism that spans from the 3′ part of *A3A* gene to the 3′ portion of *A3B* gene. An association study was carried out in 724 HBV carriers and 469 healthy control subjects. We also analyzed hypermutated genomes detected in deletion and insertion (non-deletion) homozygous patients to determine the effect of *APOBEC3* gene deletion. Further, we performed functional analysis of *A3A* gene by transient transfection experiments.

Results: The association study showed no significant association between deletion polymorphism and chronic HBV carrier state. Context analysis also showed a negligible effect for the deletion. Rather, mild liver fibrosis was associated with *APOBEC* gene deletion homozygosity, suggesting that *A3B* deletion is not responsible for chronic HBV infection. Functional analysis of A3A showed that overexpression of *A3A* induced hypermutation in HBV genome, although the levels of hypermutants were less than those introduced by *A3G*. However, overexpression of *A3A* did not decrease replicative intermediates of HBV.

Conclusion: These results suggest that A3A and A3B play little role in HBV elimination through anti-viral defense mechanisms. The significance of hypermutation induced by A3A should be investigated further.

Key words: APOBEC3A, APOBEC3B, APOBEC3G, deaminase, hypermutation, structural variation

INTRODUCTION

APOBEC3 CYTIDINE DEAMINASE family consists of at least seven tandem arrayed genes *APOBEC3A (A3A), A3B, A3C, A3DE, A3F, A3G,* and *A3H* on chromosome 22.[1,2] The anti-viral effect of A3G was initially identified in 2002 when it was found to inhibit the replication of human immunodeficiency virus (HIV).[3] Similarly, A3F, A3B and A3DE have been reported to inhibit HIV replication.[4–8]

APOBEC3 proteins also act on many other viruses such as simian immunodeficiency virus,[9] adeno-associated virus[10] and retrotransposons.[11–13] With regard to hepatitis B virus (HBV), A3G was also reported to inhibit HBV replication and induction of hypermutation, although the significance of the latter on viral inactivation is still controversial.[14–23] Among the APOBEC3 family members, A3B, A3C, A3G and A3F have been

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extensively analyzed in these reports for induction of hypermutation and inhibition of replication of HBV. In contrast, the function of A3A on HBV has not been evaluated despite its potent inhibitory effects on adeno-associated virus and retrotransposons.[9–13] Recently, Henry *et al.*[24] reported that, among the APOBEC3 family, A3A is the most efficient editor in induction of hypermutation in the HBV genome. This finding is not consistent with the previous reports. However, the relationship between genomic DNA editing by A3A and its effect on HBV replication have not been elucidated. This background prompted us to examine the effects of A3A on HBV replication and induction of hypermutation.

A recent study[25] identified a common deletion polymorphism of *APOBEC* gene spanning from the 3′ end of *A3A* gene to the 3′ portion of *A3B* gene (the segment extending from exon 5 of *A3A* to exon 8 of *A3B* was removed by the deletion, positions 37, 683, 131–37, 712, 716 on chromosome 22). The deletion results in complete elimination of the *A3B* coding region and the resultant fusion gene has a protein sequence identical to A3A, but has 3′ untranslated region of A3B. This polymorphism might modulate the expression levels of A3A peptide because the transcription levels and stability of this fusion mRNA could be altered by replacement of the 3′ untranslated region sequences. Analyzing the association between this deletion polymorphism and chronic HBV infection should clarify the effect of *A3B* on the establishment of chronic HBV carrier state.

The aims of the present study were to determine the association between *APOBEC3* gene deletion polymorphism and chronic HBV infection and the effect of *A3A*, which might be up- or down-regulated by the deletion polymorphism, on HBV replication and induction of hypermutation, by *in-vitro* overexpression experiments.

PATIENTS AND METHODS

Study subjects

BLOOD SAMPLES WERE obtained from 724 patients with chronic HBV infection at the hospitals of the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html) and Toranomon hospital. We also collected 469 control samples from healthy individuals who agreed to join the BioBank Japan Project at the Institute of Medical Science, the University of Tokyo. The study protocols were approved by the ethics committees of the University of Tokyo and the Center for Genomic Medicine, Riken. All participants were ethnically Japanese and provided written informed consent. Histological activity and fibrosis was assessed in liver biopsy specimens by the Metavir score.[26]

HBV markers

We measured DNA polymerase by the method of Robinson *et al.*[27] The quantity of HBV DNA was assessed by the following tests. Quantiplex HBV DNA probe assay (Chiron Corporation, Emeryville, CA), PCR (Amplicor Cobas TaqMan HBV Auto; Roche Molecular Diagnostic, Basel), transcription mediated amplification (TMA) assay (Fujirevio Diagnostic, Tokyo). The level of HBV in serum was assessed as high or low according to the following criteria (< 200 or ≥ 200 for DNA polymerase, < 200 or ≥ 200 for probe assay, < 6.0 or ≥ 6.0 for PCR assay, < 6.0 or ≥ 6.0 for TMA assay).

HBV-e antigen (HBeAg) and HBV-e antibody (HBeAb) were measured by commercially available chemiluminescent enzyme immunoassay kit (Abbott Laboratories, Chicago, IL). The cut off levels were 1.0 (cut off index) for HBeAg and 70% for HBeAb.

Genotyping

First, we genotyped genomic samples of 94 individuals by the PCR assay using the Deletion and Insertion specific primer sets reported by Kidd *et al.*[25] Since we observed some non-specific amplification, which was confirmed by sequencing analysis, we used the invader probes,[28] which specifically recognize *A3A* and *A3B*. These probes were designed and synthesized by Third Wave Technologies (Madison, WI). Deletion and two-insertion (non-deletion) PCR assays were performed separately as described previously,[25] then pooled (Deletion : Insertion1 : Insertion2 = 3:1:1), and subjected to Invader assay.

Cell culture and transfection

Human liver cancer cell line, HepG2, was purchased from RIKEN Cell Bank (Tsukuba). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. Cells were seeded to semi-confluence in six-well tissue culture plates. Transient transfection experiments were performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier.

Plasmid construction

The expression vector for hemagglutinin (HA)-tagged human A3G was kindly provided by Dr. Takaori (Kyoto University).[29] We constructed A3A cDNA expression

plasmid by cloning DNA fragment, which was amplified by PCR from cDNA obtained from lymphocytes of a deletion homozygous patient, into pcDNA3.1/nV5-DEST (Invitrogen, Carlsbad, CA). Construction of the wild-type HBV 1.4 genome length plasmid, pTRE-HB-wt was described previously (Tsuge *et al.*;[30] GenBank accession no. AB206816).

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]. The lysate was then centrifuged for 2 min at 15 000 × *g*. The core particles were immunoprecipitated from the supernatant by mouse anti-core monoclonal antibody (anti-HBc determinant α, Institute of Immunology, Tokyo). Genomic DNA was separated from the core particles by SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed using the above HBV DNA by RT-PCR using the RT-PCR system (Applied Biosystems, Foster City, CA). The primers and the probe used were described previously.[31] The real-time PCR was performed in a 25-μl reaction volume containing 2×TaqMan Gene Expression Master Mix, 0.9 μM of each primer, 0.25 μM probe and 1 μl DNA solution. The thermal profile was 50°C for 2 min, 95°C 10 min, followed by 40 cycles of amplification (denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 62°C for 90 sec).

Analysis of hypermutated HBV genomes by 3D-RT-PCR

Hypermutated genomes were detected and quantified by modified 3DRT-PCR using the primers, probe and reagents described previously.[31] The thermal profile was 50°C for 2 min, 95°C for 10 min followed by initial denaturation at 85°C for 20 min and 45 cycles of amplification (denaturation at 85°C for 15 sec, annealing at 50°C for 30 sec and extension at 62°C for 90 sec).

Detection of A3A-A3B fusion mRNA by RT-PCR

We extracted total RNA from lymphocytes of each allele patients using RNeasy Mini Kit (Qiagen, Hilden) and reverse-transcribed using ReverTra Ace (TOYOBO, Osaka) with random primer in accordance with the instructions supplied by the manufacturer. We then amplified cDNAs by 35 cycles of PCR using primers specific for exon 1 of *A3A* and 3′-untranslated region of *A3B* in a 25μl reaction volume containing 1 × KOD-Plus buffer [0.3 μM each primers, 0.2 mM $MgSO_4$, 1 μl DNA solution and 1 unit of KOD-Plus (TOYOBO Co.)]. The thermal profile was initial denaturation at 98°C for 2 min, followed by 35 cycles of amplification (denaturation at 98°C for 15 sec, annealing at 58°C for 15 sec and extension at 68°C for 60 sec). Nucleotide sequences of the amplified fusion cDNA sequences were confirmed by direct sequencing.

Western blot analysis

Cell lysates prepared as described above were separated by sodium dodecyl sulfate polyacrylamide electrophoresis on a 12% poly acrylamide gel and transferred to polyvinylidene fluoride (Pall Corporation, Pensacola, FL). The membranes were incubated with anti-V5 (Invitrogen), anti-hemagglutinin fusion epitope monoclonal anti-body (Roche) or with anti-β-actin monoclonal antibody (Sigma-Aldrich, St Louis, MO) followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse antibody (GE Healthcare UK, Buckinghamshire). We detected signals using the ECL system (GE Healthcare).

Nucleotide sequencing analysis of hypermutated HBV genomes by 3D-PCR, cloning and nucleotide sequencing

We analyzed hypermutated HBV DNA genomes obtained from serum samples of each genotype patient by 3D PCR (denaturation at 85°C) and cloning and sequencing. The amplified DNA fragments were cloned into pGEM T Easy vector (Promega Corporation, Madison, WI) by TA cloning. Nucleotide sequences were determined using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The nucleotide sequences were compared with those obtained by direct sequencing of amplified PCR products by normal PCR protocol.

Statistical analysis

The allele frequencies was calculated and fit to Hardy-Weinberg equilibrium was tested by the chi-square test between cases and controls using Excel software (Microsoft, Redmond, WA).[32] We also compared differences in allele frequency and genotype distribution of the deletion between cases and controls with χ^2-test. Continuous data were compared by analysis of variance (ANOVA). Differences in categorical data were analyzed by the χ^2-test. Differences in core-associated HBV and hypermutated HBV genomes per 1×10^4 copies of HBV genomes, were analyzed by Student's *t*-test.

Table 1 Characteristics of subjects

	Patients	Control	*P*-value
Number of patients	724	469	–
Sex			NS
Male	499	373	
Female	224	95	
Age (years)	53.1 (20.6–86.4)	55 (18–93)	NS
ALT	66 (5–3634)	–	–
Fibrosis stage		–	–
F0	13		
F1	80		
F2	149		
F3	114		
F4	46		
Activity		–	–
A0	1		
A1	50		
A2	125		
A3	47		
Platelet ($\times 10^4/mm^3$)	16.5 (2.2–29.8)	–	–
HBV DNA		–	–
High	137		
Middle	108		
Low	156		
HBeAg/HBeAb		–	–
+/−	207		
−/+	184		
Hepatocellular carcinoma	65	–	–

Data are number of patients or median (range) values. Differences in age between case and control were compared by Mann–Whitney *U*-test. The sex ratio was analyzed by the χ^2-test. ALT, alanine aminotransferase; HBVeAb, hepatitis B virus e antibody; HBVeAg, hepatitis B virus e antigen; NS, not significant.

RESULTS

Association between chronic HBV carriers, clinical parameters and the APOBEC3 gene deletion

TABLE 1 SUMMARIZES the clinicopathological features of the patients and control subjects. If A3B contributes to the prevention of chronic HBV infection, there should be an association between chronic HBV carrier state and *APOBEC* gene deletion polymorphism. However, we did not find any association between the two (Table 2). Furthermore, all clinical parameters, with the exception of the extent of liver fibrosis associated with chronic HBV, did not associate with the polymorphism (Tables 3,4). Advanced histopathological stages were associated with insertion homozygosity. These findings also suggest that A3B does not play any important role in anti-viral immunity in the development of chronic HBV infection.

Table 2 Case-control analysis of *APOBEC3B* deletion

	Frequency (%)		Additive mode		
	Ins	Del	*P*-value	OR	95% CI
HBV ($n = 724$)	0.709	0.291	0.599	0.964	0.624–1.489
Control ($n = 469$)	0.719	0.281			

P-values were calculated from case-control analysis by χ^2-test. OR, odds ratio; CI, confidence interval; Del, deletion homozygote; Ins, insertion homozygote.

Table 3 Correlation between deletion and clinical parameters

	Genotype			*P*-value
	I/I	I/D	D/D	
Genotype frequency	0.50	0.42	0.08	NS
Age (years)	54.0 ± 12.8	52.0 ± 12.6	50.4 ± 13.3	NS
ALT	169.0 ± 320.6	149.5 ± 322.9	196.8 ± 309.3	NS
Platelets ($\times 10^4/mm^3$)	16.8 ± 5.2	16.6 ± 6.1	17.0 ± 5.8	NS

Data are number of patients or mean ± SD. Age, ALT and platelet count were compared by ANOVA. ALT, alanine aminotransferase; D/D, deletion homozygote; H, heterozygote; I/I, insertion homozygote; NS, not significant.

Context analysis of hypermutated genomes obtained from deletion homozygous and insertion homozygous patients

The amount of hypermutated genomes was not analyzed in this study because it is known to fluctuate during the clinical course.[33] Instead, we searched for the target context of G to A mutation in hypermutated HBV genomes using serum obtained from patients with deletion homozygotes and with insertion homozygotes. As shown Figure 1, multiple G to A hypermutations were observed in deletion homozygote and insertion homozygote patients. The results of context analysis showed no significant difference between the contexts obtained from deletion homozygotes and those form non-deletion homozygotes (Fig. 2). In fact, the preferred contexts were similar in all three deletion homozygous patients and one insertion homo patient (DD1-3 and II1 in Fig. 2) but slightly different from those of the remaining two (II2 and II3). These results suggest that the effect of deletion is not strong in these preferred context patterns.

Detection of A3A-A3B fusion mRNA

We then analyzed whether the resultant *A3A* and A3B fusion was actually transcribed. We designed primers specific for exon 1 of *A3A* and the 3′-untranslated region

Table 4 Association of clinical parameters and *APOBEC* gene polymorphism (categorical data)

	Genotype frequency				Additive mode	I/I vs I/D, D/D	D/D vs I/I, I/D
	I/I	I/D	D/D				
Sex (Male/Female)				*P* value	0.76	0.85	0.30
Male ($n = 328$)	154 (0.47)	143 (0.44)	31 (0.09)	OR	0.75	1.03	0.72
Female ($n = 166$)	78 (0.47)	74 (0.45)	14 (0.08)	95% CI	0.40–1.41	0.75–1.41	0.40–1.33
Fibrosis stage (F0-F1/F2-F4)				*P* value	0.0054	0.0019	0.48
F0-F1 ($n = 62$)	22 (0.35)	34 (0.55)	6 (0.10)	OR	0.51	0.47	0.74
F2-F4 ($n = 187$)	95 (0.51)	77 (0.41)	15 (0.08)	95% CI	0.21–1.24	0.30–0.76	0.31–1.73
Activity (A0-A1/A2-A3)				*P* value	0.31	0.46	0.30
A0-A1 ($n = 51$)	22 (0.43)	23 (0.45)	6 (0.12)	OR	0.56	0.80	0.60
A2-A3 ($n = 168$)	81 (0.48)	75 (0.45)	12 (0.07)	95% CI	0.20–1.56	0.45–1.44	0.22–1.60
HBV DNA (High/Low)				*P* value	0.12	0.12	0.47
High ($n = 194$)	82 (0.42)	94 (0.48)	18 (0.09)	OR	0.66	0.73	0.77
Low ($n = 206$)	103 (0.50)	88 (0.43)	15 (0.07)	95% CI	0.32–1.40	0.49–1.09	0.38–1.57
HBeAg/HBeAb ((+/−)/(−/+))				*P* value	0.52	0.34	0.84
+/− ($n = 207$)	89 (0.43)	99 (0.48)	19 (0.09)	OR	0.96	0.82	1.07
−/+ ($n = 184$)	88 (0.48)	78 (0.42)	18 (0.10)	95% CI	0.47–1.95	0.55–1.23	0.54–2.11
HCC				*P* value	0.85	0.89	0.64
(−) ($n = 648$)	323 (0.50)	266 (0.41)	59 (0.09)	OR	0.73	1.04	0.69
(+) ($n = 65$)	34 (0.52)	31 (0.47)	0 (0.00)	95% CI	0.25–2.13	0.62–1.73	0.24–1.98

ALT, alanine aminotransferase CI, confidence interval; D/D deletion homozygote; H, heterozygote; HBVeAg, hepatitis B virus e antibody; HBVeAg, hepatitis B virus e antigen; HCC, hepatocellular carcinoma; I/I, insertion homozygote; OR, odds ratio.

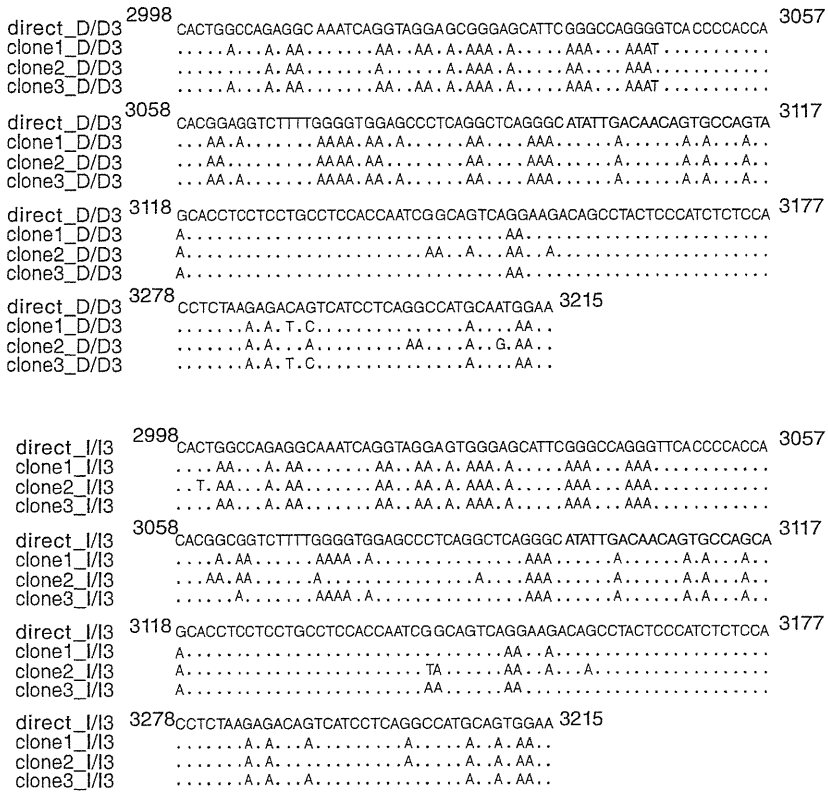

Figure 1 Nucleotide sequences of hypermutated genomes detected from deletion homozygous and insertion homozygous patients. Nucleotide sequences of 3D-PCR amplified hebatitis B virus (HBV) DNA clones are compared with those obtained by usual PCR and direct sequencing. Upper panel, nucleotide sequences obtained from a deletion homozygous patient. Lower panel, nucleotide sequences obtained from a homozygous patient. Nucleotide numbers are those from GenBank accession no. AB206816.

of *A3B*, and performed RT-PCR using cDNAs obtained from patients of each genotype. We obtained amplified DNA fragments of expected size only from deletion homozygotes and heterozygotes (Fig. 3). These results confirmed the transcription of the fusion mRNA with the coding region of *A3A* and the 3′ untranslated region of *A3B*.

Inhibition of HBV replication and induction of hypermutation by A3A

We then analyzed the antiviral effect and induction of hypermutation by A3A. Although the expression of both A3A and A3G was confirmed by western Blot analysis (Fig. 4A), transient expression of A3A did not reduce the amount of the core-associated HBV DNA in HepG2 cells (Fig. 4B). However, A3A transfection increased the hypermutated genomes of HBV in a dose-dependent manner albeit the level of induction was much lower than that observed when transfected with A3G. These results suggest that A3A has negligible anti-viral effect although it induces hypermutation of HBV genomes.

DISCUSSION

THE MAIN FINDINGS of the present study were: (i) no association between *APOBEC3* deletion and chronic HBV infection (Table 2). (ii) Mild liver fibrosis and low alanine amino transferase (ALT) levels were associated with APOBEC gene deletion homozygous genotype. (iii) The absence of *A3B* is not responsible for chronic HBV carrier status, although A3B is known as a potent inhibitor of adeno-associated virus and retrotransposons.[12] This suggests different antiviral activities for APOBEC proteins against viruses and that A3B plays little role in inhibition of HBV. (iv) The preferred context analysis showed no differences between insertion homozygotes and deletion homozygotes. Only one of the six patients examined showed different context pattern (Fig. 2). These results suggest that A3B protein has only small effect on the formation of hypermutated genomes in the serum of chronic carriers. The protein has been reported to induce hypermutation on the negative and positive strands of HBV.[18] However, our results showed that the effect of *A3B* is almost negligible in

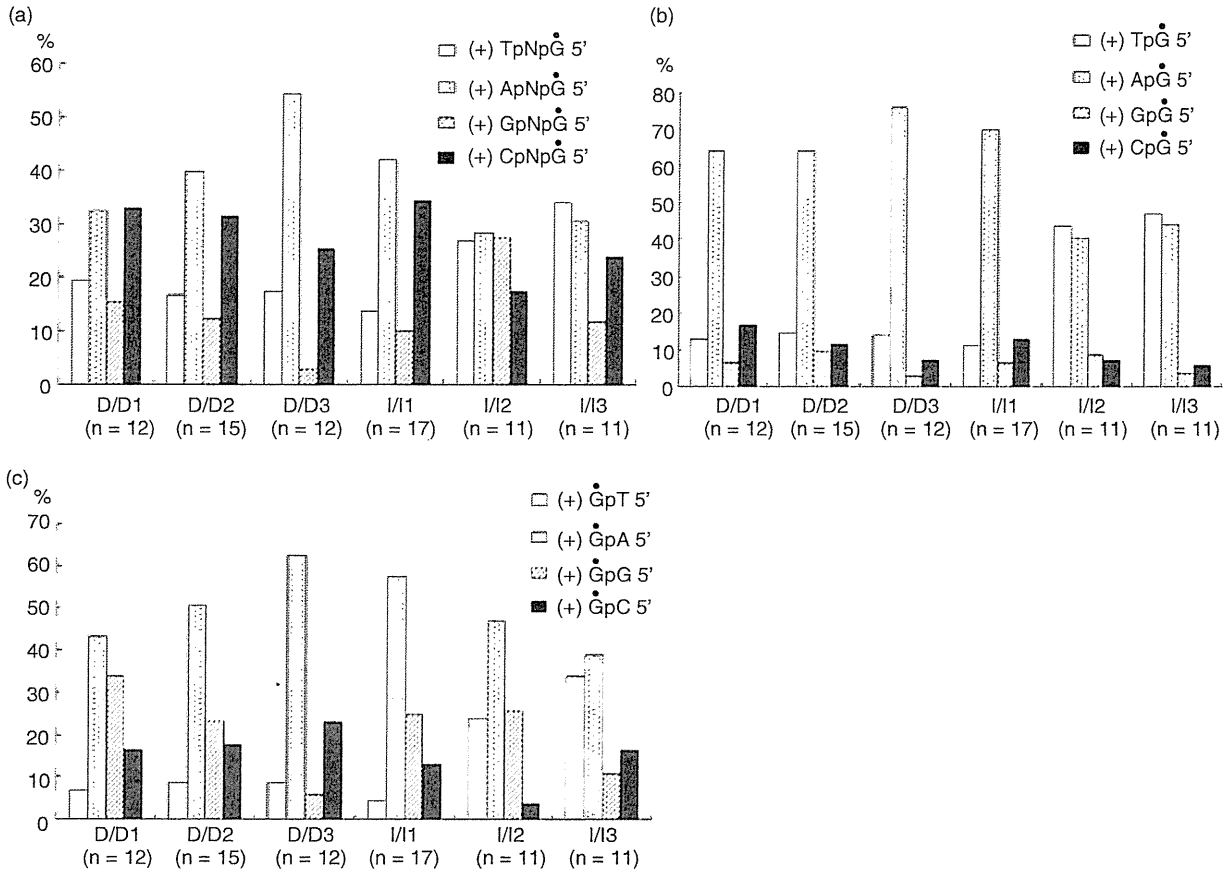

Figure 2 Context analysis of hypermutated genomes from deletion homozygous and insertion homozygous patients. Context of G to A hypermutation in hypermutated genome clones obtained from three deletion homozygous patients (D/D1, D/D2 and D/D3) and three insertion homozygous patients (I/I1, I/I2 and I/I3) were analyzed. Numbers after each patient represent the number of clones analyzed in each patient. (a) Two letters up-stream, (b) one letter upstream and (c) one letter downstream of mutated G residue were analyzed.

chronic HBV carriers compared to that of A3G. It is assumed that the other APOBEC3 family proteins mainly induce hypermutation of HBV genomes in HBV carriers to compensate for the function of deleted A3B. It is also assumed that the expression pattern of the remaining six APOBEC3 proteins is different from patient to patient.

As discussed above, our results suggest that A3B protein has almost no effect on prevention of chronic HBV infection and induction of hypermutation. It is thus assumed that A3B is not part of the innate anti-viral immune system against HBV. This is consistent with the finding that deletion is commonly seen in normal populations[25] irrespective of HBV carrier rates. Other association studies are required to clarify the role of A3B protein on other pathogens. The functional relevance of other APOBEC3 proteins on HBV infection as anti-viral immunity should be clarified further.

We also found that A3A protein induced hypermutation on the negative strand of HBV. However, the level of induction of hypermutation was much less than that of A3G (Fig. 4). Recent reports showed quite different effects for *A3A* on induction of hypermutation on HBV genomes. Henry *et al.*[24] reported that A3A is the most efficient editor of seven APOBEC3 proteins. In contrast, Zang *et al.*[23] did not detect induction of hypermutation on HBV. Although these different results might come from different cell lines and conditions used in each experiment, our results clearly showed that *A3A* induced hypermutation on the negative strand of HBV genome.