

of drug-resistant variants reduces considerably the effect of the drug [Honkoop et al., 1997b; Allen et al., 1998; Chayama et al., 1998; Niesters et al., 1998; Liaw et al., 1999; Ono-Nita et al., 1999a,b]. Lamivudine-resistant variants have unique amino acid substitutions in the Tyr-Met-Asp-Asp (YMDD) motif of the reverse transcriptase. One class of the variants, Tyr-Val-Asp-Asp (YVDD) variant, is usually associated with additional amino acid substitutions in the B domain of the reverse transcriptase, L528M. The other variant, Tyr-Ile-Asp-Asp (YIDD) variant, only occasionally exhibits this additional mutation. The YVDD variant associated with L528M shows the highest replication rate in the presence of lamivudine [Gutfreund et al., 2000; Ono-Nita et al., 2001].

YMDD variants emerge after a certain period of lamivudine therapy. This suggests that variants evolve during such therapy because the rate of nucleotide substitutions of the reverse transcriptase of HBV is quite high, though still controversial [Orito et al., 1989; Melegari et al., 1998]. However, some patients show relatively early emergence of drug-resistant strains, suggesting the existence of such strains prior to therapy. Viruses exist in the sera of infected patients as quasi-species. It is assumed that some of the quasispecies are inactive biologically because a proportion of these variants have stop codons that are thought to be fatal for the virus life cycle. Whether all the remaining variants without stop codons actually replicate in hosts is not known. To date, it has been difficult to analyse rare variants, for example, drug-resistant variants, before therapy, because they exist as rare quasispecies among the vast majority of wild-type virus. Previous studies that reported the presence of lamivudine-resistant variants in patients who received no lamivudine therapy [Kobayashi et al., 2001; Kirishima et al., 2002] did not determine the exact nature of such variants.

The aim of the present study was to detect these rare variants by using polymerase chain reaction (PCR) with peptide nucleic acid (PNA) clamping as reported previously [Kirishima et al., 2002]. PNA is a DNA analogue in which the ribose-phosphodiester backbone of DNA has been replaced by *N*-(2-aminoethyl) glycine linkages [Nielsen et al., 1991; Cherny et al., 1993; Egholm et al., 1993; Zhong et al., 1999]. The PNA anneals strongly to DNA like a complementary DNA, but with higher affinity. The annealing of the PNA to the target sequence prevents amplification of DNA in the PCR if the amplification conditions are appropriately optimised. We investigated nucleotide and amino acid sequences of resistant viral variants of HBV including YMDD variants that have amino acid substitutions in the YMDD motif of reverse transcriptase by using two-stage PCR with PNA clamping.

MATERIALS AND METHODS

Patients

Sixty-two Japanese adult patients with acute or chronic HBV infection before or without lamivudine

therapy were studied. These patients were negative for hepatitis B e antigen (HBeAg), but positive for anti-hepatitis B e antibody (anti-HBe). We preferentially selected these anti-HBe positive patients because recent reports [Kobayashi et al., 2001; Kirishima et al., 2002] showed that YMDD variants were often detected in anti-HBe positive patients. Nineteen of our patients were treated with lamivudine. All serum samples analysed in this study were collected prior to therapy with informed consent and stored at -80°C . All patients were negative for markers of hepatitis C virus (HCV) and HIV. The clinicopathological profiles of these patients are shown in Table I.

Blood Analysis

Hepatitis B surface antigen (HBsAg) was determined by enzyme immunoassay (Roche Diagnostics, Basel, Switzerland). HBeAg and anti-HBe were tested by radioimmunoassay (Abbott Diagnostics, Chicago, IL). HBV DNA was measured by branched DNA signal amplification technology (Chiron Corp., Emeryville, CA), and the results were expressed as 10^6 genomic equivalents (Meq) per millilitre. The lower limit of the assay was 0.7 MEq/ml. Antibody against HCV was tested by the third-generation enzyme immunoassay (Roche Diagnostics). HBV genotype was determined using the method of Naito et al. [2001].

Detection of YMDD Variants by Two-Stage PCR With PNA Clamping and Restriction Fragment Length Polymorphism (RFLP)

HBV DNA was extracted from 100 μl of serum using SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μl of H_2O . Nested PCR was performed using a first primer set (PLF1 and BR123) and a second PCR primer set (PLF2 and PLR2). Nucleotide sequences of primers and the sequence of PNA used in this study are listed in Table II. PNA-mediated PCR clamping was optimised by changing the PNA annealing

TABLE I. Clinicopathological Features of Anti-HBe Positive Patients

Variable	No lamivudine therapy (n = 62)
Age (years)	48.5 \pm 10.0
Sex (M/F)	48/14
Alanine aminotransferase (IU/L) ^a	55 (21–7,941)
HBV DNA (Meq/ml)	
ND	6
<0.7	34
≥ 0.7 to <100	17
≥ 100	5
Genotype (B:C:F:ND)	5:51:1:5
Clinicopathologic diagnosis	
Chronic hepatitis	26
Liver cirrhosis	8
Hepatocellular carcinoma	19
Acute hepatitis	1
Fulminant hepatitis	6
Normal	2

^aMedian (range).

TABLE II. Primers and Peptide Nucleic Acid (PNA) Used in the Present Study

Primer	Nucleotide sequence	Nucleotide
PLF1	Sense: 5'-GGTATGTTGCCCGTTTGTCC-3'	458-477
BR123	Antisense: 5'-TTCCAATTACATATCCCAT-3'	895-877
PLF2	Sense: 5'-CCTATGGGAGTGGGCCTCAG-3'	637-656
PLR2	Antisense: 5'-CCAATTACATATCCCATGAAGTTAAGGGA-3'	893-865
YNSspI	Sense: 5'-TTTCCCCCACTGTTTGGCTTTCAGTAATAT-3'	711-740
BR109	Antisense: 5'-AAGGGAGTAGCCCCAACGTT-3'	870-851
TMApaLI	Antisense: 5'-CAGACTTGGCCCCCAATACCACATCGTGCA-3'	769-740
PNA552	Antisense: H ₂ N-CACATCATCCATATAACT-CON ₂ H	750-733

Nucleotide sequence position numbers are those of Norder et al. [1994].

temperature from 68 to 75°C and the PNA concentration from 1.25 to 10 µM. Optimised PCR with PNA clamping was performed in a total volume of 25 µl, consisting of reaction buffer (100 mM Tris-HCl, [pH 8.3], 50 mM KCl, and 15 mM MgCl₂), 0.2 mM of each dNTP, 1 µl of the DNA solution, 12.5 pmol of each primer set, 150 pmol of PNA 552, and 1 U of Taq DNA polymerase (Gene Taq, Wako Pure Chemicals, Tokyo, Japan) with 0.2 µg of anti-Taq high (Toyobo Co., Osaka, Japan). The amplification conditions included initial denaturation at 95°C for 4 min, 25 cycles of amplification (denaturation at 95°C for 45 sec, PNA annealing at 73°C for 2 min, annealing and extension of primer at 63°C for 50 sec), followed by final extension at 63°C for 7 min. Detection of YVDD and YIDD variants was performed using primers TMApaLI and YNSspI, respectively, as described previously [Chayama et al., 1998].

The error rate of the Taq DNA polymerase was estimated to be 1.76×10^{-5} per site by amplifying about 100 copies of plasmid using the same conditions described above, followed by cloning and sequencing.

Cloning and Sequence Analysis of Rare Polymerase Variants

In this study, 1 µl of the above second PCR product amplified by two-stage PCR with PNA was amplified using primers PLF2 and BR109. Thirty-five cycles of PCR (94°C for 1 min; 58°C for 1 min; 72°C for 1.5 min) were performed after 4 min of initial denaturation at 94°C, followed by final extension for 7 min at 72°C. PCR-amplified DNA was purified after 2% agarose gel electrophoresis and cloned into pGEM-T Easy Vector (Promega Co., Madison, WI). Sequencing was performed using the ABI PRISMTM 310NT Genetic analyser (Applied Biosystems, Tokyo, Japan) with Big Dye terminator version 3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequences of independent clones from five of 62 anti-HBe patients were determined and analysed.

RESULTS

Detection of YMDD Variants in Serum Samples From 62 Anti-HBe Patients by Two-Stage PCR With PNA Clamping

By adding PNA in two-stage PCR and optimising the amplification conditions for suppression of wild-type virus, we were able to detect YVDD variants mixed in a

larger amount of wild-type sequences. We used clones with nucleotide sequences corresponding to YMDD wild-type and YVDD variant as described previously [Chayama et al., 1998]. The nucleotide sequences of these plasmids in these motifs are TAT ATG GAT GAT and TAT GTG GAT GAT, respectively. As shown in Figure 1, YMDD variants were detected when mixed with the same amount of wild-type in the absence of PNA. In contrast, by introducing PNA, only 10² copies of YMDD variants were detected when mixed with 10⁶ copies of wild-type. In fact, our method detected the YMDD variant mixed with the wild-type at a ratio of 1:10,000, and was 10,000-fold more sensitive than conventional RFLP without PNA (Fig. 1).

Using this method, YVDD variants were detected in only one of 62 (1.6%) serum samples from anti-HBe patients. Further analyses of nine serial serum samples obtained from this patient revealed that another sample before the start of lamivudine therapy was positive for YVDD variants. Unexpectedly, the patient was later treated with lamivudine and showed a good response to the therapy during at least 23 months, and HBV DNA was not detected by two-stage PCR with PNA clamping after such treatment (data not shown).

Analyses of Nucleotide and Amino Acid Sequences of Clones Obtained From a Serum Sample of a Patient who Possessed YVDD Variant

We examined quasispecies in one of the two serum samples that tested positive for the YVDD variants by cloning and sequencing after amplification of HBV DNA by two-stage PCR with PNA clamping. Analyses of amino acid sequences of 43 clones showed the presence of multiple rare variants such as YRDD and YMDN that had not been previously reported (Fig. 2). Identified nucleotide substitutions showed preferential transitions, particularly transitions from G to A (Fig. 2A). Thirty-seven of 43 clones had nucleotide substitutions of methionine and asparagine residues but none had substitutions of tyrosine residues. Only one of 43 clones sequenced had the YVDD sequence. This variant did not have the L528M mutation that has been reported to be often associated with the YVDD variant (Fig. 2B). Thirteen of 43 clones had YIDD amino acid sequences. However, nucleotide sequences of these YIDD variants (TAT ATA GAT GAT) (Fig. 2A) differed from the reported lamivudine-resistant variants (TAT ATT

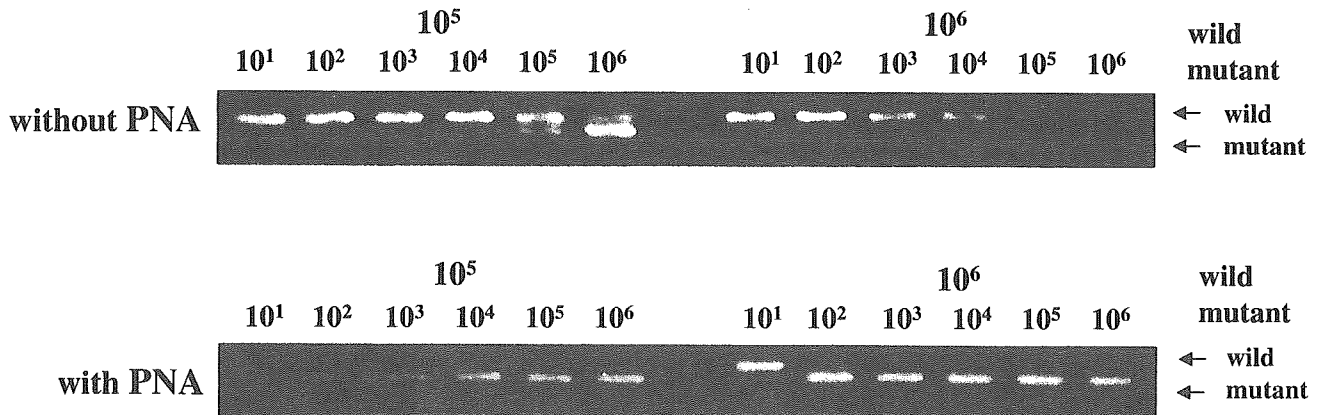


Fig. 1. Detection of the Tyr-Met-Asp-Asp (YMDD) variant by two-stage polymerase chain reaction (PCR) with peptide nucleic acid (PNA). 10^1 – 10^6 copies of Tyr-Val-Asp-Asp (YVDD) variant plasmid were mixed with 10^5 or 10^6 copies of wild-type plasmid. After amplification using primers PLF2 and TMApaL I, the amplified DNA was digested with restriction enzyme ApaI and separated in a 3% agarose gel. The restriction fragment length polymorphism (RFLP) method involving PCR with the PNA clamping could detect only 10^2 copies of YVDD variants in the presence of 10^6 copies of the wild-type hepatitis B virus (HBV). Remarkably, PNA completely blocked 10^6 copies of wild-type HBV in the presence of 10^1 – 10^6 copies of the mutant type (YVDD variants).

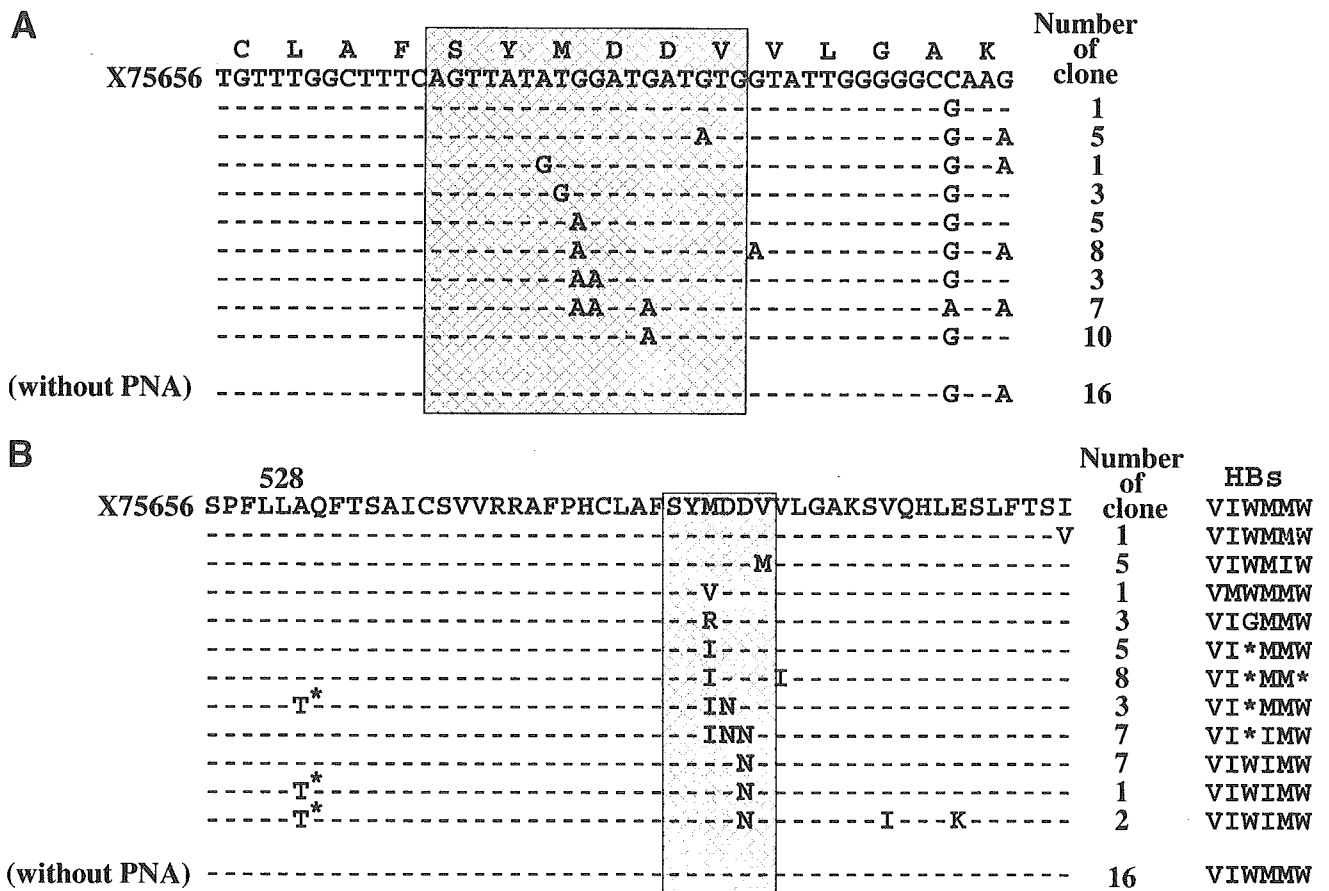


Fig. 2. Nucleotide (A) and amino acid (B) sequences of 43 clones obtained from a serum sample of a patient who possessed YVDD variant before the start of lamivudine therapy. Dark boxes indicate nucleotide and amino acid sequences where the PNA was assumed to anneal to the wild-type sequence of the YMDD motif. Nucleotide and amino acid sequences of 16 clones amplified without PNA are also shown. Created stop codons in the overlapping envelope (S) gene are labeled with asterisks.

GAT GAT) and were undetectable by RFLP because the amplified DNA fragment from this sequence using primers for the detection of the lamivudine-resistant YIDD variant (YNSspI) did not produce an SspI restriction site. These YIDD variants had stop codon(s) in the overlapping *HBs* gene, suggesting that all 13 YIDD variants might be biologically inactive. Eight of these 13 clones had an additional stop codon just adjacent to the YMDD motif. Similarly, Tyr-Ile-Asn-Asp (YIND) and Tyr-Ile-Asn-Asn (YINN) variants had stop codons in the *HBs* gene. Figure 2B shows the amino acid sequences surrounding the YMDD motif. There are a few amino acid substitutions in this area, and some of them had an additional stop codon (Fig. 2B).

We also determined the nucleotide sequences of DNA fragments amplified using similar amplification conditions without PNA. Although six of 43 clones obtained after amplification with PNA had the wild-type YMDD sequence, all 16 clones obtained without PNA did not have any nucleotide and amino acid substitutions in the YMDD motif (Fig. 2).

Analyses of Nucleotide and Amino Acid Sequences of Clones Obtained From Four of 62 Anti-HBe Patients With Chronic HBV Infection

We then examined the nucleotide and amino acid sequences of 41 clones obtained from four of 62 patients with chronic HBV infection, by cloning and sequencing after two-stage PCR with PNA. As shown in Figure 3, multiple YMDD variants with nucleotide and amino acid substitutions were identified in Patient 1. In contrast, none of ten clones obtained from Patient 2 had amino acid substitutions in the YMDD motif. There were many stop codons in the overlapping *S* gene in clones obtained from Patient 1, but none of 10 clones obtained from Patient 2 had any stop codon in the polymerase and *S* gene (Fig. 3). Further analyses of nucleotide and amino acid sequences of clones from remaining two patients (Patients 3 and 4) showed previously unknown variants such as Tyr-Met-Asn-Asn (YMNN). Many stop codons in the overlapping *S* gene were noted in clones obtained from Patient 3, but none of 11 clones obtained from Patient 4 had any stop codon (data not shown). Of 41 clones analysed, only ten clones had a wild-type sequence. Interestingly, none of the 41 clones had amino acid substitution of the tyrosine residue of the YMDD motif. Only two of these 41 clones had amino acid substitution of the serine residue, just one amino acid upstream of the YMDD motif, despite the fact that PNA was designed to anneal to the nucleotide sequence of this serine residue. Guanine to adenine transition was predominant in Patients 1, 3, and 4, but only a thymine to cytosine transition was seen in Patient 2 (Fig. 3).

Estimated Mis-Incorporation Rate of Taq DNA Polymerase Employed in This Study

We also studied the error rate of Taq DNA polymerase employed in this study by using cloned HBV plasmid

DNA under exactly the same conditions. We sequenced 6,665 bases from 31 clones (215 bases of each clone) and found only ten nucleotide substitutions. They included seven C to T substitutions (six were at the same position), two G to A and one T to C substitution at different positions. All of these substitutions were situated outside the PNA annealing site. The estimated mis-incorporation rate was only 1.76×10^{-5} substitutions per site.

DISCUSSION

Viruses, especially RNA viruses and retroviruses, exist in the hosts as quasispecies. HBV, despite being a DNA virus, replicate via an RNA intermediate [Summers and Mason, 1982; Miller et al., 1984; Will et al., 1987; Bavand and Laub, 1988] as retroviruses. HBV shows high-mutation frequency [Orito et al., 1989] partly because of the lack of proof-reading enzymes that assure fidelity of DNA replication [Holland et al., 1982]. The only method to analyse the quasispecies has been cloning of the genome of these viruses, with or without PCR amplification, and nucleotide sequencing. In this process, only major strains can be detected but no studies have focused on rare strains. For example, even analysis of the hypervariable region of the HCV revealed the existence of only a limited number of species [Koizumi et al., 1995; Pawlotsky et al., 1998]. The fact that amino acid sequences of the rare YMDD variants identified in this study had never been reported suggests that previous studies had observed only the relatively abundant strains. The importance of such rare strains during emergence of drug-resistant strains or vaccine escape variants remains to be elucidated [Carman et al., 1990; Fujii et al., 1992; Okamoto et al., 1992; Waters et al., 1992; Zuckerman, 2000]. Further studies using two-stage PCR with PNA clamping as described in this study should clarify such issues.

Mis-incorporation by Taq DNA polymerase must be taken into account in such analyses. We studied the error rate of Taq DNA polymerase and found that the mis-incorporation rate was only 1.76×10^{-5} substitutions per site. Furthermore, no substitutions were found in the PNA annealing site from clones amplified with PNA in the conditions described herein. These results indicate that almost all variants identified in this study actually exist in all five of the patients studied in the present study.

Drug-resistant variants usually emerge after a certain period of antiviral therapy [Honkoop et al., 1997; Allen et al., 1998; Chayama et al., 1998; Niesters et al., 1998; Liaw et al., 1999; Ono-Nita et al., 1999a,b]. It is debatable whether these strains actually existed prior to antiviral therapy were selected by antiviral drugs [Melegari et al., 1998] or whether resistant strains evolved from non-resistant virus by nucleotide substitution(s) during replication under pressure from the drug.

In this study, both the YVDD, which is reported to be resistant to lamivudine, and YIDD variants were identified in one patient before the start of lamivudine therapy. The detection rate of variants in this study is

Patient 1

A

	C	L	A	F	S	Y	M	D	D	V	V	L	G	A	K	Number of clone
X75656	TGTTTGGCTTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAG															
	--C-----				C-----											2
	-----				-----C-----											2
	-----				-----A-----											2
	-----				-----AA--A--AA-----										A	1
	-----				-----A-----										C-----	1
	--C-----				--A-----										A-----	1
	-----				-----A-----											1

B

	528	Number of clone	HBs
X75656	SPFLLAQFTSAICSVVRRAFPFCCLAF ^S YMDDVVLGAKSVQHLES ^L LFTSI		VIWMMW
	-----A-----V	2	AIWMMW
	-----A-----V	2	VIWMMW
	-----I-----H--A--V	1	VI*MMW
	-----I-----A--V	1	VI*MMW
	-----N-----IH--A--	1	VI*IM*
	-L-----N-----H--A--V	1	VI*MMW
	---T*-----N--N-----A--V	1	II*MMW
	-----N-----H--A--V	1	VI*MMW

Patient 2

A

	C	L	A	F	S	Y	M	D	D	V	V	L	G	A	K	Number of clone
X75656	TGTTTGGCTTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAG															
	-----T-----C-----															10

B

	528	Number of clone	HBs
X75656	SPFLLAQFTSAICSVVRRAFPFCCLAF ^S YMDDVVLGAKSVQHLES ^L LFTSI		VIWMMW
	-----AV	10	VTWMMW

Fig. 3. Nucleotide (A) and amino acid (B) sequences of clones obtained from two patients with anti-HBe (Patients 1 and 2). Created stop codons in the overlapping envelope (S) gene are labeled with asterisks.

quite different from those reported in previous studies. For example, Kirishima et al. [2002] used the same PNA method to detect the YMDD variant and found four of 18 subjects were positive for the variant. Since our method has similar or even better sensitivity than that of Kirishima et al., this difference might be due to different background of the patients'. The YVDD variant was detected in only one of 43 clones obtained from this patient (Fig. 2). Since RFLP showed that only part of the amplified DNA was digested with the enzyme ApaLI, the prevalence of YVDD variants in this patient was assumed to be very low. Furthermore, the variant detected was distinct from the double mutation variant

(M552V and L528M) that had been reported to have the strongest resistance to lamivudine [Gutfreund et al., 2000; Ono-Nita et al., 2001]. This might explain the excellent response to lamivudine in this patient. Alternatively, such variants might be defective viruses that lack the ability to replicate because of mutations in a different area that was not analysed in the present study. The data, however, show that care should be taken when interpreting results of detection of YMDD variants, especially when the sensitivity of the assay is very high. Whether such variants can evolve to drug-resistant variants needs to be studied in a larger number of patients, as the amount of HBV DNA fluctuates in

patients. Variants with the amino acid sequence YIDD identified in this patient had stop codon(s) in the overlapping *HBs* gene. Whether these variants, including the remaining newly identified sequences with stop codon(s) in the overlapping *HBs* gene, can replicate with trans-complementation by co-existing intact virus needs further studies. The enzymatic activity of reverse transcriptase/DNA polymerase in these variants, especially those without the *HBs* stop codon, should also be studied further.

One of the interesting findings of this study was that certain transition patterns were predominant in some patients. For example, 55 of 59 nucleotide substitutions in a patient who possessed YVDD variant before the start of lamivudine therapy were guanine to adenine transitions (Fig. 2A). In contrast, only a thymine to cytosine transition was identified in Patient 2 (Fig. 3A). A possible relationship between amino acid sequence/function of polymerase and patterns of nucleotide substitutions in these patients should be investigated. Amino acid substitutions in clones obtained from five patients were also distributed unevenly in the YMDD motif. None of 84 clones analysed from five patients had amino acid substitutions in the tyrosine molecule in this motif, and only two of 84 clones had amino acid substitution of the serine molecule just upstream of the YMDD motif. This uneven distribution of amino acid substitutions might be related to the function of the polymerase. Whether these rare polymerase variants are actually functional is important for designing new antiviral nucleotide analogs.

In conclusion, rare viral quasispecies were identified by using a sensitive method. The method might be further applied to study other nucleotide substitutions of other viruses. Analyses of rare variants might be useful for further understanding of the mechanism for emergence of drug-resistant HBV variants and for developing strategies for new antiviral drugs.

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G to A Hypermethylation of Hepatitis B Virus

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G to A hypermethylation of the human immunodeficiency virus type 1 (HIV-1) is induced by a deaminase APOBEC3G and is related to host antiviral defense. APOBEC3G has also been found to reduce the replication of HIV-1 by an unknown mechanism. This enzyme also reduces the production of hepatitis B virus, although the mechanism for this action has not been clearly elucidated. The hypermethylated hepatitis B virus (HBV) is rarely found in usual sequencing analyses. Using peptide nucleic acid mediated by polymerase chain reaction clamping, we detected the hypermethylated HBV DNA in 1 of 8 patients with acute HBV infection and 4 of 10 with chronic HBV infection. In the latter group, hypermethylated genomes were found only in eAb-positive patients. As much as 72.5% of G residues were mutated in the hypermethylated clones. G to A substitutions were predominant in almost all clones sequenced compared with other substitutions. G to A mutated viral genomes also were found in HepG2-derived cell lines that continuously produced HBV into the supernatant. Both alpha and gamma interferon reduced virus production in these cell lines, but they did not alter the frequency of the hypermethylation. Transcripts of APOBEC3G, as well as some other deaminases, were found in these cell lines. **In conclusion**, our results show that part of the minus strand DNA of HBV is hypermethylated both *in vitro* (HepG2 cell lines) and *in vivo*. The role and mechanism of hypermethylation in reducing HBV replication should be further investigated to understand the anti-HBV defense system. (HEPATOLOGY 2005;41:626-633.)

Hepatitis B virus (HBV) is a small enveloped DNA virus that replicates in hepatocytes in a noncytolytic manner. Chronic infection with the virus often leads to chronic hepatitis and liver cirrhosis. Hepatocellular carcinoma arises in chronic carriers at a higher frequency than noninfected individuals.¹⁻⁴

The replication cycle of the HBV includes pregenome RNA synthesis and reverse transcription, resulting in the production of the minus strand DNA, which serves as a template of the plus strand DNA.⁵ The life cycle of this virus resembles that of the human immunodeficiency virus 1 (HIV-1), which also replicates through reverse transcription.⁶

Recent reports showed that a cytosine deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G), which is packaged in HIV-1 virions, induces G to A hypermethylation to a nascent reverse transcript of HIV-1, which contributes in part to the innate antiviral activity.⁷⁻¹⁰ The antiviral activity of APOBEC3G is species specific^{11,12} and may represent the different actions of the protein.^{13,14} The virion infectivity factor encoded by lentivirus genomes associates with APOBEC3G to prevent the enzyme from being packaged into virions and triggers its proteasomal degradation.¹⁵⁻¹⁸ The negative strand DNA of the HBV might be a target of such antiviral deaminase activity. In fact, naturally occurring HBV genomes bearing the hallmarks of retroviral G to A hypermethylation have been reported in clones obtained from 2 HBV carriers.¹⁹ Both of these clones represented subgenomes arising from reverse transcrip-

Abbreviations: HBV, hepatitis B virus; HIV-1, human immunodeficiency virus type 1; APOBEC3G, apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; PCR, polymerase chain reaction; PNA, peptide nucleic acid.

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tion of packaged spliced mRNA. However, such hypermutated genomes have otherwise never been reported, nor deposited in DNA databases. Moreover, whether such hypermutated sequences are generated in liver cells or in leukocytes is unknown.

Inhibition of HBV replication by APOBEC3G was observed recently in a transient transfection system.²⁰ However, no induction of hypermutations to the HBV genome was observed. Instead, prevention of pre-genome RNA packaging was observed.

The aims of the current study were to determine the frequency of viral genomes with G to A substitutions in HBV carriers and patients with acute HBV infection, and to determine whether the hypermutated sequences are generated in hepatic cell lines. We identified such hypermutated viral genomes in 5 of 18 HBV carriers and patients with acute HBV infection and the expression of known deaminases that are potentially responsible for the hypermutation in cultured hepatoma cell lines.

Materials and Methods

Serum Samples. Serum samples from 18 adult Japanese patients with HBV infection were studied. At the time of the study, 8 of these patients had acute HBV infection and tested positive for immunoglobulin M anti-hepatitis B core antibody. The remaining 10 patients were chronic carriers. All serum samples were stored at -80°C until examined. All patients were negative for serum markers of both hepatitis C virus and HIV-1 infection, and none was on antiviral treatment.

Serological Markers of HBV Infection. Hepatitis B surface antigen (HBsAg) was detected by enzyme immunoassay (Roche Diagnostics, Basel, Switzerland), and hepatitis B early antigen (HBeAg) as well as anti-HBe were detected by radioimmunoassay (Abbott Diagnostics, Abbott Park, IL). HBV DNA was determined by transcription-mediated amplification and hybridization-protection assay (Chugai Diagnostics, Tokyo, Japan), and the results were expressed as log genome equivalents per milliliter. The lower detection limit of this assay is 3.7 log genome equivalents/mL (equivalent to 5,000 copies/mL). The antibody against hepatitis C virus was tested for by the third-generation enzyme immunoassay (Roche Diagnostics).

Analysis of HBV DNA in Cell Lines That Stably Produce HBV. Two cell lines known to produce wild-type HBV and one cell line known to produce lamivudine-resistant HBV (with mutations of L528M and M552V) were created by transfecting 1.4 genome length sequences of HBV to HepG2 cell lines. These cell lines produced HBV that showed a similar sedimentation in

sucrose density gradient centrifugation to HBV extracted from the serum of carriers (M. Tsuge et al., manuscript in preparation) and could infect human hepatocyte chimeric mice (manuscript in preparation). These cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C and 5% CO_2 . Cells were seeded to semiconfluence in 6-well tissue culture plates and then treated with media containing interferon alpha or gamma. After 3 days of interferon treatment, the cells were harvested and lysed with 250 μL lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 140 mmol/L NaCl, 0.5% [vol/vol] NP-40) followed by centrifugation for 2 minutes at 15,000g. Replicative intermediate of the HBV was immunoprecipitated and subjected to Southern blot analysis and quantitative analysis by light cycler. The effect of lamivudine was analyzed similarly, except that cells were harvested after 5 days of treatment.

Detection of Hypermutated Clones by Polymerase Chain Reaction With PNA Clamping, Cloning, and Sequencing. HBV DNA was extracted from 100 μL serum or culture supernatant by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μL H_2O . The first round of polymerase chain reaction (PCR) was performed with an outer primer set (PLF1 and BR112 [Table 1]) and a second-round PCR with an inner primer set (PLF2 and PLR2 [Table 1]). The peptic nucleic acid (PNA) oligonucleotide, initially designed to detect lamivudine-resistant variant genome,²¹ was an 18-mer (PNA 552 [Table 1]) that exactly matched the 18-nucleotide sequence of the original YMDD sequence of DNA polymerase/reverse transcriptase, which contained GG and TG sequences (AGT TAT ATG GAT GAT GTG). The PCR with PNA clamping was performed in a total volume of 25 μL , consisting of a reaction buffer (100 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl and 15 mmol/L MgCl_2), 0.2 mmol/L each of dNTPs, 1 μL of the DNA solution, 12.5 pmol each primer, 150 pmol PNA 552, and 1 unit of Taq DNA polymerase (Gene Taq, Wako Pure Chemicals, Tokyo, Japan) together with 0.2 μg anti-Taq high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denaturation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, annealing and extension of primer at 63°C for 50 seconds), followed by a final extension at 63°C for 7 minutes. Part of the X gene was amplified with an outer primer pair (HBV1 and HBV2) and an inner primer (PLF2 and HBV2) (Table 1) for the first- and second-round amplifications, respectively. The amplification for the first-round PCR included initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denatur-

Table 1. Oligonucleotides and PNAs Used in the Current Study

Primer	Sequence
HBV amplification	
PLF1	5'-GGT ATG TTG CCC GTT TGT CC-3'
BR112	5'-TTC CGT CGA CAT ATC CCA T-3'
PLF2	5'-CCT ATG GGA GTG GGC CTC AG-3'
PLR2	5'-CCA ATT ACA TAT CCC ATG AAG TTA AGG GA-3'
HBV1	5'-CCG GAA AGC TTG AGC TCT TCT TTT TCA CCT CTG CCT AAT CA-3'
HBV2	5'-CCG GAA AGC TTG AGC TCT TCA AAA AGT TGC ATG GTG CTG G-3'
BR109	5'-AAG GGA GTA GCC CCA ACG TT-3'
PNA	
PNA552	H2N-CAC ATC ATC CAT ATA ACT-CON2H
PNA552V	H2N-CAC ATC ATC CAC ATA ACT-CON2H
Amplification of mRNAs of deaminases	
APO1a	5'-CAG AGC ACC ATG ACT TCT-3'
APO1d	5'-ATT GTG GCC AGT GAG CTT CA-3'
APO2a	5'-AGA AGG AAG AGG CTG CTG TG-3'
APO2b	5'-AGA ACG GCT GCC TGC CAA CT-3'
APO2c	5'-GAA GGC TGG CAG GAT GGT GT-3'
APO2d	5'-CAG GTG ACA TTG TAC CGC AG-3'
APO3Aa	5'-TCT TAA CAC CAC GCC TTG AG-3'
APO3Ad	5'-GAA GAT GCG CAG TCT CAC GT-3'
APO3Ba	5'-AGA GCG GGA CAG GGA CAA GC-3'
APO3Bb	5'-GCG TAT CTA AGA GGC TGA AC-3'
APO3Bd	5'-CGA AGG ACC AAA GGG TCA TT-3'
APO3Be	5'-ACA AGT AGG TCT GGC GCC GT-3'
APO3Ca	5'-AGG ACG CTG TAA GCA GGA AG-3'
APO3Cb	5'-CCG ATG AAG GCA ATG TAT GG-3'
APO3Cc	5'-GTC GTC GCA GAA CCA AGA GA-3'
APO3Cd	5'-GAT GTG TAC CAG GTG ACC TG-3'
APO3Da	5'-CTG GGA CAA GCG TAT CTA AG-3'
APO3Dd	5'-AGT CTG AGA TGA AGA GGT GG-3'
APO3Fa	5'-CTT GGG TCC TGC CGC ACA GA-3'
APO3Fd	5'-TCA TCC TTG GCC GGC TAG TC-3'
APO3Ga	5'-GAC TAG CCG GCC AAG GAT GA-3'
APO3Gb	5'-CAC AGT GGA GCG AAT GTA TC-3'
APO3Gc	5'-GTT CCG AAT ACA CCT GGC CT-3'
APO3Gd	5'-ACT CCT GGT CAC GAT GCA GC-3'

ation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, primer annealing at 60°C for 1 minute, and extension of primer at 63°C for 4 minutes), followed by the final extension at 63°C for 7 minutes. The second-round amplification was performed under the same conditions without a primer extension for 3 minutes. The estimated error rate of the Taq DNA polymerase was 1.76×10^{-5} per site in amplifying approximately 10^2 copies of plasmid under the same conditions as described previously and cloning and sequencing.²¹ Products (1 μ L each) of the second-round of PNA PCR were subjected to PCR with primers PLF2 and BR109 for 35 cycles (94°C, 1 minute; 58°C, 1 minute; 72°C, 1.5 minutes) after initial denaturation at 94°C for 4 minutes and followed by the final extension at 72°C for 7 minutes. Amplicons were purified by electrophoresis on 2% (wt/vol) agarose gel and cloned into pGEM-T Easy Vector (Promega, Madison, WI) with the standard method, and then transformed

into *Escherichia coli* JM 109 (Takara Shuzo Co., Otsu, Japan). Sequencing was performed in the ABI PLISM TM 310NT Genetic analyzer (Applied Biosystems, Tokyo, Japan) with Big Dye terminator version 3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems). Ten independent clones from each serum sample of patients or supernatant of cell cultures were sequenced for analysis and compared for nucleotide sequences obtained by direct sequencing of PCR products. Hypermutation was defined as clones with a statistically significant number of G to A substitutions.

Sequence Analysis. Nucleotide sequences were aligned and parameters of hypermutation were evaluated with Hypermut Program Package²² (<http://www.hiv.lanl.gov/HYPERMUT/hypermut.html>). We used nucleotide sequences obtained by direct sequencing as reference sequences and tentatively labeled clones with a statistically significant ($P < .05$ by Fisher's exact test) number of G to A substitutions as "hypermutated."

Detection of mRNA of Known Deaminases by Reverse Transcription and PCR. Total RNA was extracted from HepG2 cell lines by using cell-to-cDNAII kit (Ambion, Austin, TX). The extracted RNA was reverse transcribed with random primer and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO, Osaka, Japan) at 42°C for 60 minutes according to the instructions provided by the manufacturer. Synthesized cDNAs were used to detect mRNAs of known deaminases using primers listed in Table 1. Each of these primers was carefully designed to amplify only the target member of the APOBEC families. Amplification of specific deaminases was confirmed by amplifying each deaminase cDNA by using cDNAs obtained from organs reported to be positive for the expression of each deaminase. The amplicons were analyzed in 2% agarose gel, and the nucleotide sequences were confirmed by direct sequencing.

Results

Frequent Detection of G to A Substituted HBV Genomes by PCR With PNA Clamping in Patients With Acute or Chronic Hepatitis B Virus Infection. Using PCR with PNA clamping, clones with multiple G to A substitutions were found (Table 2). In contrast, only small numbers of other substitutions were identified in these clones. A hypermutated genome of HBV was found in 1 of 8 patients with acute HBV infection and 4 of 10 patients with chronic HBV infection (Table 2). We cloned and sequenced more than 20 clones without PNA and found no hypermutated clones. Among patients with chronic HBV infection, hypermutated clones were identified only in eAb-positive patients (Table 2). Figure 1

Table 2. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping and Clinical Features of Patients With Acute and Chronic Hepatitis B Virus Infections

Patient	No. of Substitutions*		No. of Clones†	Pre-core‡	CPS	eAg	eAb	HBV DNA	ALT
	G to A	Other							
A-1	27	3	8 (1)	G	A/G	42	0	5.1	2,517
A-2	13	4	8	G	A/G	7.8	88	6.1	3,778
A-3	12	2	5	A/G	A/G	190	0	<3.7	1,417
A-4	11	0	4	G	A/G	58.3	0	4.5	2,550
A-5	11	3	9	G	A/G	170	0	8.3	175
A-6	7	7	9	A/G	Mixed	260	0	7.8	28
A-7	1	2	4	G	Mixed	0.1	99.4	4.1	2,295
A-8	1	1	3	A	T/A	0.7	91	7.1	6,183
C-1	152	2	10 (10)	A	T/A	0.3	100	5.5	394
C-2	44	12	9 (4)	A/G	T/A	18.2	73.4	6.2	340
C-3	30	4	10 (1)	A/G	T/A	0.3	97	7.3	53
C-4	23	1	3	G	A/G	140	0	5.9	2,770
C-5	22	1	8 (1)	A	T/A	0.4	95	6.5	105
C-6	19	9	9	A/G	Mixed	200	0	8.2	113
C-7	18	5	7	G	T/A	170	0	6.6	31
C-8	17	1	7	G	A/G	200	0	7.7	92
C-9	12	4	7	G	T/A	180	0	>8.8	56
C-10	6	4	7	A	A/G	2.5	95	8.3	267

*Total number of nucleotide substitutions in 10 clones compared with sequences obtained by direct sequencing.

†Number of different clones of 10 clones sequenced. Figures in parentheses represent the number of clones with hypermutation (those with a statistically significant number of G to A substitutions).

‡Nucleotide sequence of codon 28 of pre-core protein (nucleotide 1896).

§Nucleotide sequence of basic core promoter (nucleotides 1762 and 1764). Mixed represents mixture of A/G and T/A.

illustrates hypermutations found in an eAb-positive patient with chronic HBV infection (C-1 in Table 2). As much as 72.5% (29 of 40) of G residues were mutated in such hypermutated clones. Hypermutation was found in both the envelope/polymerase region (Fig. 1A) and x region (Fig. 1B) of HBV genome obtained from this patient. Preference of G to A mutation was similar with those reported in HIV-1; that is, G residues in GA sequences were the most frequently hypermutated (Fig. 2).

In contrast, the G residues in CxG context were less frequently substituted (Fig. 2). Numerous G to A nucleotide substitutions were identified in clones lacking a statistically significant number of G to A hypermutations (Table 2). The number of such substitutions was apparently greater than "other substitutions" (Table 2). There was no relationship between the degree of hypermutation and serum alanine aminotransferase concentration or HBV DNA level (Table 2).

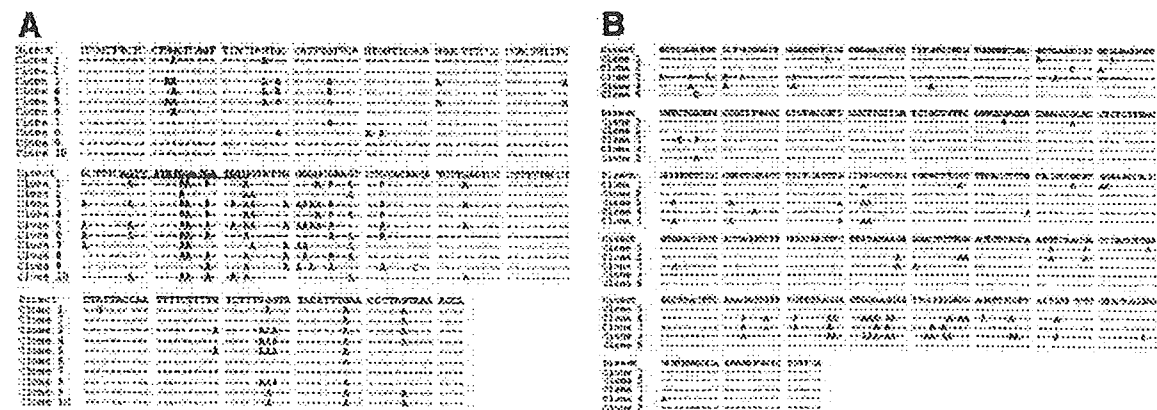


Fig. 1. G to A hypermutations detected in sequences of HBV DNA in sera extracted from an HBe antibody-positive HBV carrier (Patient C-1, Table 2) by PCR with PNA clamping. (A) DNA sequence alignment in the HBs antigen/polymerase region of the HBV. The nucleotide sequences that were obtained by direct sequencing were used as a reference sequence (top line). The target sequence of PNA annealing is underlined. (B) DNA sequence alignment in the x region of the HBV.

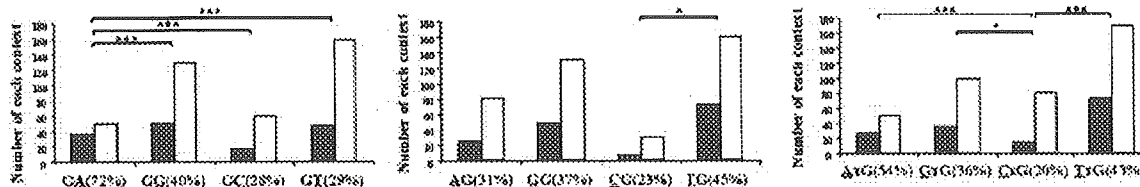


Fig. 2. Preferred nucleotide contexts of G to A hypermutation in 10 clones from patient C-1 (Table 2). The preferred nucleotide letter one letter after (left), one letter before (middle), and two places before (right, x = any) the target G residue. **Open bars:** number of occurrences of each context in the sequence analyzed. **Gray bars:** number of G residues mutated to A. The percentage in parentheses represents the rate of mutated G residues. * $P < .05$, *** $< .001$ (Fisher's exact test or chi-square test).

G to A Hypermutation in HBV-Producing Cell Lines. We established HepG2 cell lines that continuously produced HBV into the medium and examined the frequency of hypermutation. Hypermutated clones were identified in one of these cell lines (Table 3 and Fig. 3). The preference of G to A mutation was similar to that found in serum samples obtained from patients (data not shown). Various levels of HBsAg, HBeAg, and HBV DNA were released into the medium from these cells (Table 3). No relationship was found between the frequency of the hypermutated genome and intracellular intermediates of HBV DNA and HBsAg and HBeAg levels (Table 3). Figure 4 shows replicative intermediates of the HBV produced in these cell lines detected by Southern blot analysis (Fig. 4). No noticeable difference was observed between a cell line with hypermutated genomes and those without hypermutated genomes (lanes 1 and 2 in Fig. 4).

G to A Hypermutation During Antiviral Treatment. We treated the cell lines with alpha and gamma interferon and lamivudine. Both interferons reduced HBV DNA production from these cells in a dose-dependent manner (Fig. 5). The frequency of G to A hypermutation did not increase in those treated cell lines (Fig. 6), suggesting that G to A hypermutation is not responsible

for antiviral defense through these interferons. Treatment of a cell line with lamivudine resulted in marked reductions in the production of HBV in the supernatant as well as intracellular viral intermediates (Fig. 7) and completely abolished identification of G to A substitution (Fig. 6). A similar reduction of detection of hypermutated clones was observed in serum samples obtained from patients who were treated with lamivudine (data not shown).

Expression of Deaminases in HepG2 Cell Lines. We examined the expression of known deaminases to see whether any such enzymes are active in HepG2 cells. As shown in Fig. 8, mRNA expression of 5 of 8 of these deaminases was detected, although the expression level of some deaminases was very low. mRNA of Apobec3G, a key enzyme for the hypermutation of HIV-1, was expressed in HepG2 cells, but the cDNA of this enzyme was only found by nested PCR. The expression level of the mRNA was similar in HBV-producing cells with various levels of hypermutations of HBV as well as parent HepG2 cells (detected by only nested PCR).

Discussion

In this study, we detected the mutated HBV genome in some patients by using PCR with PNA clamping. PNA is a DNA analog in which the ribose-phosphodiester backbone of DNA has been replaced by *N*-(2-aminoethyl) glycine linkages.²³ The PNA anneals strongly to DNA like a complementary DNA, but with higher affinity.²³ The annealing of the PNA to the target sequence thus prevents amplification of the target DNA in the PCR. In our previous study,²¹ we attempted to block the amplification of lamivudine-sensitive wild-type YMDD motif strain and detected a very small amount (1/10,000) of YMDD motif mutant. Because the target sequence of this system contained many Gs with GA and GG (AGT TAT ATG GAT GAT GTG), we assumed that we could detect very rare hypermutated genomes.

Because we did not detect any hypermutated sequence without PNA, we assumed that the rate of the hypermutated genome is very low. This low frequency of hyper-

Table 3. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping in Three Cell Lines That Produce the Hepatitis B Virus

Cell Line	No. of Substitutions*		No. of Clones†	eAg	HBs Ag	HBV DNA
	G to A	Other				
Cell line 1	102	0	10 (7)	17	4.7	5.2
Cell line 2	19	0	7	10	4.9	4.6
Cell line 3	21	1	6	14	2.8	4.6

*Total number of nucleotide substitutions in ten clones compared with sequences of the transfected clone.

†Number of different clones of 10 clones sequenced. The figure in parentheses represents the number of clones with hypermutation (those with a statistically significant number of G to A substitutions). Codon 28 of the pre-core gene of the transfected clone was wild (Trp), and nucleotides 1762/1764 were T/A.

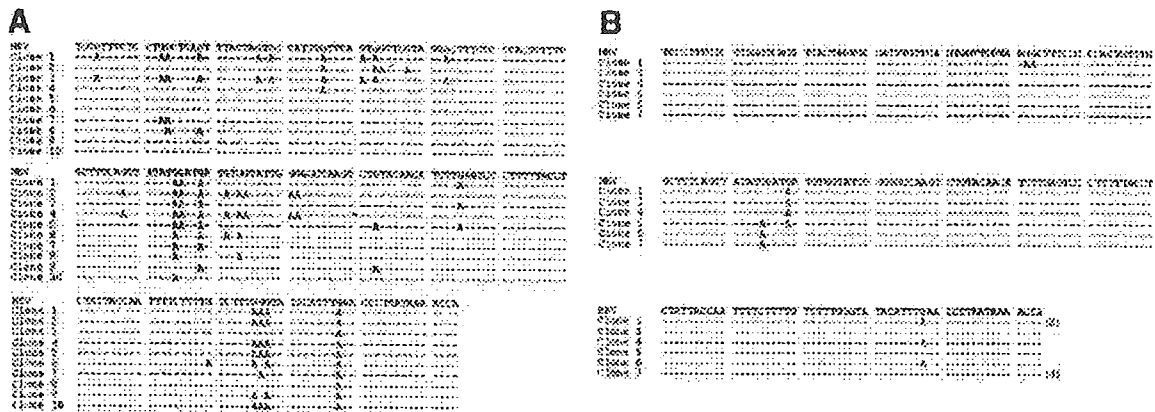


Fig. 3. G to A hypermutations detected in sequences of the HBV DNA (produced by HBV DNA-transfected cell lines to the supernatants). The nucleotide sequences of the transfected clone were used as a reference sequence (top line). DNA sequence alignments in the HBs antigen/polymerase region of cell line 1 (A) and cell line 2 (B) of the HBV. Numbers in parentheses are numbers of clones.

mutated genomes accounts for the lack of reports of such sequences with only one exception until recently,¹⁹ in which the presence of two clones of hypermutated sequences in spliced genomes was reported. One may assume that the rare hypermutated genome might be produced in peripheral blood mononuclear cells because the HBV genome was previously found in such cells.²⁴⁻²⁸ However, we showed that these genomes are found in HBV-transfected cell lines. Our results clearly demonstrate that hypermutation actually occurs in hepatocytes. The reason(s) for such a low frequency of hypermutation

is not clear. The low expression level of deaminases in hepatocytes might account for the low frequency. In fact, we observed a very low expression level of APOBEC3G (transcripts was only detected by nested PCR [Fig. 8]) in HepG2 cell lines.

Recently, Turelli et al.^{20,29} suggested that overexpression of APOBEC3G inhibits the replication of HBV by preventing encapsidation of the virus. However, they did not observe an increase in G to A hypermutation. In contrast, Rosler et al.³⁰ reported that G to A substitutions significantly increased in HepG2 cells when co-transfected with APOBEC3G cDNA. They found only 50 G to A substitutions by cloning 223 clones,³⁰ suggesting that the frequency of G to A substitutions is rare despite overexpression of APOBEC3G. Our preliminary data suggest that overexpression of APOBEC3G does not produce a

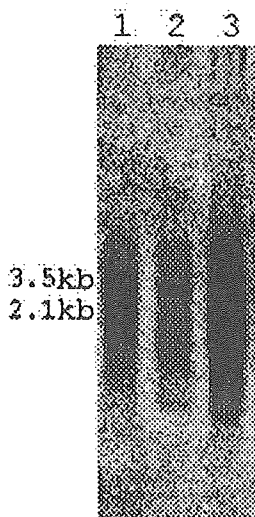


Fig. 4. Southern blot analysis of the HBV DNA extracted from cell lines that stably produce HBV into the supernatant. Two YMDD wild-type virus sequences (lanes 1 and 2) and one YVDD mutant virus sequence (lane 3) were transfected into the HepG2 cell line.

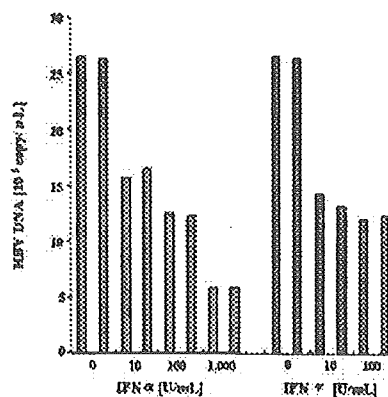


Fig. 5. Effects of interferon alpha and gamma on production of HBV DNA by cell line 1. Experiments were performed in duplicate with increasing amounts of each interferon.

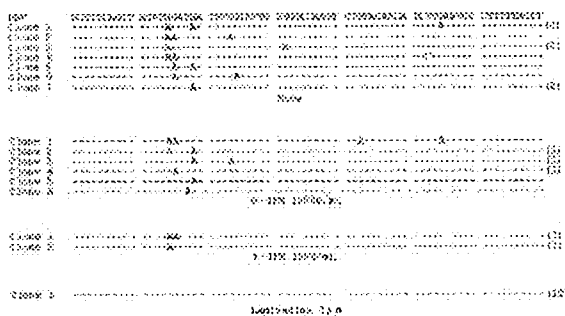


Fig. 6. Nucleotide sequence substitutions around YMDD motif of reverse transcriptase detected by PCR with PNA clamping after treating a HepG2 cell line (cell line 2 in Table 3). The nucleotide sequence of the transfected clone was used as a reference sequence (top line). Cells were treated with interferons and lamivudine as shown in Figs. 5 and 7, respectively.

noticeable increase in HepG2 cells by our detection method (C. Noguchi and K. Chayama, unpublished data). However, the method employed to detect hypermutation is not quantitative. Moreover, no antibody to detect APOBEC3G is available. Measurement of activity of this enzyme might be necessary to address this issue.

Because the patterns of hypermutations found in patients as well as cell lines are in agreement with strong dinucleotide preferences of a retroviral genome³¹⁻³⁵ edited by APOBEC3G,⁷⁻⁹ we assume that hypermutations might also be induced by a similar enzyme. As pointed out by Turelli et al.,²⁰⁻²⁹ another deaminase including APOBEC3F might be responsible for the generation of hypermutation. We actually detected the expression of deaminases in HepG2 cell lines. The expression levels of these deaminases are very low because they were detected by only two-stage PCR with one exception (only APOBEC3F was detected by a single-stage PCR).

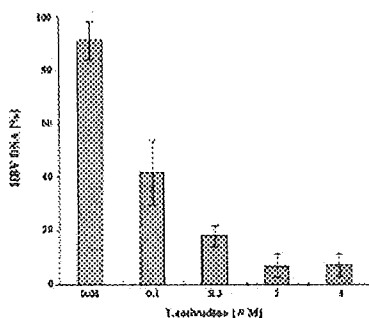


Fig. 7. Effects of lamivudine on production of HBV DNA by cell line 1. After 5 days of lamivudine treatment, the HBV DNA in core particles was immunoprecipitated and quantitated by real-time PCR. Data are mean \pm SD of 4 independent experiments.

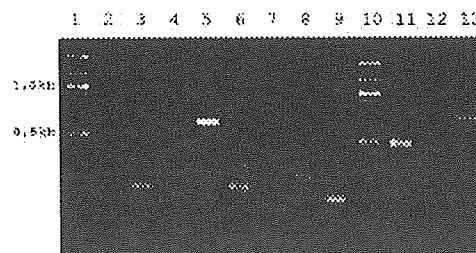


Fig. 8. Agarose gel electrophoresis of mRNAs of known deaminases amplified by reverse transcription-polymerase chain reaction. Lane 1: molecular weight size marker; lane 2: APOBEC1; lane 3: APOBEC2; lane 4: APOBEC3A; lane 5: APOBEC3B; lane 6: APOBEC3C; lane 7: APOBEC3D; lane 8: APOBEC3F; lane 9: APOBEC3G; lane 10: molecular weight size marker. Only mRNA of APOBEC3F was detected by one-stage PCR. To confirm the predictability of the assay, 3 negative mRNAs in Hep3G (APOBEC1, 3A and 3D) were amplified by using mRNAs from tissues known to express it. Lanes 11 and 12: APOBEC1 and APOBEC3A from the ileum; lane 13: APOBEC3D from the duodenum. All detected cDNAs were cloned, and nucleotide sequences were confirmed.

However, other possibilities should not be ignored. For example, some viral proteins might prevent such editing activity of deaminase by associating with this enzyme, as virion infectivity factor does in HIV-1-infected cells. Possibly the edited HBV genomes are degraded in liver cells rapidly by removal of the U residues by uracil DNA glycosylase followed by cellular nucleases.³⁶

We found hypermutated genomes only in patients positive for eAb. The G to A nucleotide substitution of codon 28 of pre-core protein, which induces premature stop of this protein and basal core promoter mutations (A1762T/G1764A), might be related to the clearance of eAg.²⁸ Further studies should be conducted to investigate the relationship between G to A substitutions in these regions by deaminase(s), production of eAg, and replication efficacy of the virus.

A recent study showed that the amount of HBV DNA reduction occurs noncytologically through the action of cytokines, especially interferon alpha/beta and gamma.^{37,38} We thus examined whether interferon can alter the occurrence of hypermutation. However, the results showed no increase in the number of hypermutation in HepG2-derived cell lines treated by interferon alpha and gamma (Fig. 6). Thus, the antiviral action of the mechanism responsible for G to A substitution in liver cells is likely to be independent of the action of interferon.

In conclusion, numerous innate intracellular defense systems exist, and the precise pathways of such systems are not fully understood. The role of editing of the HBV genome in such defense systems should be further investigated to understand the natural antiviral mechanisms and to develop an antiviral strategy against HBV.

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3. 非B型, 非C型肝炎

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key words GBV-C/HGV, HCV, TTV, SENV, occult HBV

動 向

肝炎ウイルスとしてA, B, C, D, E型までの5種類が知られている。しかし、劇症肝炎の50%, 急性肝炎の10~20%, 慢性肝疾患の5-10%はB型肝炎ウイルス(HBV)もC型肝炎ウイルス(HCV)もnegativeであることから、いわゆる非A-E型肝炎ウイルスが存在するのではないかと考えられてきた。近年、G型肝炎ウイルス(GBV-C/HGV), TTウイルス(TTV), などが相次いで同定され、原因不明の非A-E型肝炎ウイルスの候補として報告されているが、これらのウイルスにおける肝炎の病原性についての報告は少なく、否定的である。次に同定されたSENVウイルス(SENV)も非A-E型輸血後肝炎の原因ウイルスではないかと議論されたが、確証となるものではない。本稿では、これらのウイルスの遺伝子構造、感染実態、病態、臨床的意義を中心に解説する。そして、近年、HBsAg陰性、HBcAb陽性のドナーから肝移植をした場合に、レシピエントがB型肝炎を発症するという報告からさらに重要視されるようになったoccult HBVの臨床的意義についても解説を加えた。

A. GBV-C/HGV

1995年-1996年にかけて、米国の2つの異なっ

た研究グループから非A-E型肝炎患者血清から分子クローニング法により新型RNAウイルスが同定され、それぞれGBウイルスC(GBV-C), G型肝炎ウイルス(HGV)と命名された¹⁻⁴⁾。両者の全塩基配列と、ポリプロテインのアミノ酸配列を比較すると、ともに高い相同性を有していることから、両者が同一のウイルスであることが判明した。通常、GBV-C/HGVあるいは、単にHGVと呼称される。GBV-C/HGVは、HCVと同じフラビウイルス科(Flaviviridae)に属し、約9.4kbからなる1本鎖プラス鎖RNAウイルスである。現在、HGVには3種類の遺伝子型(G1, G2, G3)が存在し、分布からG1型はアフリカ型、G2型は欧米型、G3型はアジア型ともいわれている。HGV感染者の約50%に輸血歴があり、経静脈的薬物常用者のHGV-RNA陽性率がきわめて高いことから、感染経路は血液を介すると考えられる⁴⁾。

非A-C型慢性肝疾患について行った我々の検討では、急性肝炎27例中0例(0%), 慢性肝疾患78例中11例(14.1%)にHGV RNAを検出した⁵⁾。また、わが国では献血者の1-2%がHGV-RNA陽性であることが判明した。しかし、HGV感染の結果、肝炎を発症することは少なく、肝炎となっても症状は軽微で、不顕性感染が多いと考えられる。その後、肝炎発症との関連は薄いという報告

が相次ぎ, さらにHGV-RNAの局在も肝細胞ではないことも明らかにされ, 現在は肝炎ウイルスの認証を得ていない。

B. TTV

TTVは, 1997年にNishizawa⁶⁾らにより最初に同定され, 発見の発端となった患者のイニシャル(TT)から, 「TTウイルス」と命名された。TTVはエンベロープをもたない全長約3.9kbからなる1本鎖マイナス鎖DNAウイルスで, サークウイルス科(Circoviridae)に属する。感染経路は血液, 体液, 糞便を介しており, 主に血液感染のB型, C型肝炎と経口感染のA型, E型肝炎の両方の感染経路をもち合わせている。その後Okamoto⁷⁾らにより, TTVの全遺伝子構造が解明され, きわめて遺伝子変異が多く, 多数のサブタイプが存在することが明らかにされ⁸⁻¹⁰⁾, 現在では少なくとも16のゲノタイプが存在している。病原性がみられるのは, 日本人の大人で約10%が感染している遺伝子型1型(1a型と1b型)とされている。

非A-C型慢性肝疾患に関して, 我々のORF1のprimerを用いた方法(第1, 2世代)による検討では, 急性肝炎27例中3例(11%), 慢性肝疾患78例中9例(11.5%)にTTV DNAを検出したが⁵⁾, 健常人でも25例中3例(12%)にTTV DNA陽性の症例が存在したことから¹¹⁾, TTVの病態への影響の可能性は低いと考えられる。さらに, TTVの宿主はヒトのみならず多くの動物種への感染が確認されているが, チンパンジーの実験では感染は成立するものの肝炎は発症していない¹²⁾。また, TTVは糞便中に高濃度に検出されることから, 胆管上皮や胆嚢上皮などで増殖している可能性があり, 胆管病変を引き起こしている可能性も考えられる。したがって, 現在, TTVは, 肝炎発症の原因ウイルスとしては否定

的な報告が多く, 確証はされていない。

C. SENV

SENVウイルス(SENV)は, 最近, HIV感染者の経静脈的薬物常用者の血清から同定された約3.8kbの1本鎖環状DNAウイルスである¹³⁾。系統樹により遺伝子型は8型の存在が報告されている¹⁴⁾。構造的には, TTVに類似しているが, TTVのプロトタイプとの比較では, 塩基配列では55%, アミノ酸配列では37%程度の相同性しか示さない。感染経路は血液を介する。

SENV-AとSENV-Eは非A-E型肝炎患者と献血者においては, ともに低い検出率で差がほとんど認められなかった。非A-E型輸血後肝炎とSENV-DとSENV-H感染の関与の検討では¹⁵⁾, 輸血後例のSENV感染の頻度は286例中86例(30%)で, 非輸血コントロール例の97例中3例(3%)と有意に輸血後例で多く認められた。また, 436例の献血者におけるSENV陽性率は1.8%であった。また, 非A-E型輸血後肝炎患者のSENV陽性率は, 輸血後肝炎を起こさなかった症例と比較し有意に高かったことから, SENVが輸血後肝炎の原因ウイルスである可能性を示唆した。

しかし, SENVの検出率が, 肝疾患の有無とあまり関係ないとする報告や¹⁶⁾, SENVが献血者と非A-E型肝炎患者において同じ程度に存在するという報告¹⁷⁾がなされていることから, 非A-E型肝炎疾患に関して, SENV感染が原因ウイルスであることを十分に示すデータはいまだない。

D. その他

近年, HBsAg陰性, HBcAb陽性のドナーから肝移植をした場合に, レシピエントがB型肝炎を発症することから, たとえ, ドナーのHBsAgが陰性であってもドナーの肝組織中にはHBV

DNAが存在することが明らかにされた¹⁸⁾。そして、近年になって、Torbenonらは、HBsAgは陰性であるが、血清あるいは肝組織におけるHBV DNAが陽性である状態を、occult HBVと定義した¹⁹⁾。occult HBV感染に関しては、移植後のような、高度の免疫抑制状態など、特殊な状況下では実際に問題になりうるが、通常の臨床において、どのような意義を有するかに関しては、研究者間で考えも異なっている。occult HBV感染患者のHBV DNA量は定性的なPCRで捉えられる程度の低ウイルス量であるため、occult HBV感染の診断には、高感度のPCRアッセイが必要であり、コンタミネーション（偽陽性）を除外することも重要である。

B型急性肝炎から回復した後に、occult HBV感染となり、長年にわたり続くことがある²⁰⁾。

また、occult HBV感染は、B型慢性肝疾患患者において、血清中のHBsAgが自然経過で消失する場合、あるいは、抗ウイルス療法によって消失した後などにみられる場合がある。さらに、非B非C型肝癌患者およびHCV感染患者においても高頻度にoccult HBV感染が報告されている^{21,22)}。我々のXおよびS遺伝子領域のprimerを用いた検討では、非B非C型肝癌において、HBcAb陽性群では21例中5例（24%）、HBcAb陰性群では21例中2例（9.5%）にHBV DNAを検出した（表1）。また、HBsAg消失例では、9例中4例（44%）がHBV DNA陽性で、陽性例のうち2例ではHBsAg消失後に発癌した（表2）。

このような、極少量のウイルスの臨床的意義についてはよく議論をされるところであるが、occult HBVが臓器移植、輸血、血液透析などを

表1 非B非C型肝癌症例におけるHBV DNA陽性例

症例	年齢	性別	anti-HBs	anti-HBc (×1)	組織	HBV DNA	
						X gene	S gene
1	66	M	-	-	LC	+	-
2	54	F	+	-	LC	+	+
3	75	M	-	100%	CH	+	+
4	79	M	-	95%	LC	+	+
5	55	F	-	90%	LC	+	+
6	82	F	+	83%	CH	+	-
7	82	F	+	61%	LC	+	+

表2 HBsAg消失例におけるHBV DNAの検出

症例	年齢	性別	* genotype	anti-HBc (×200)	組織	HBV DNA	
						X gene	S gene
1	47	M	C	23%	CH	+	+
2	56	F	C	83%	CH	-	-
3	58	M	ND	80%	CH	-	-
4	62	M	B	61%	CH	-	-
5	44	F	C	40%	CH	-	-
6	48	M	C	16%	CH	+	-
7	57	M	F	72%	CH	-	-
8**	71	F	ND	89%	CH	+	-
9**	55	M	ND	48%	LC	+	-

* HBs抗原消失前の血清

** HBs抗原消失後に発癌した症例

通してHBV感染の危険をもっていること, 原因不明の肝疾患の原因となりうること, 肝癌の原因となりうること²³⁾などから, 臨床的に重要な問題であるとする考えもある. 非B非C型肝炎患者におけるoccult HBVの関与についても, 今後, 高感度で, 特異的で, 定量的な, HBV DNAの検出を行い, HBV DNAの複製レベルでの検討と病態との関連について前向きに調べていくことが解明の手がかりになると思われる.

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