

Mutations in the Pre-core Region of Hepatitis B Virus DNA in Patients with Chronic Liver Diseases

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To investigate the prevalence of point mutation in the pre-core (pre-C) region of hepatitis B virus (HBV) DNA, we performed dot blot hybridization and sequencing of enzymatically amplified HBV DNA from the sera of 25 patients with HBeAg-positive and 32 patients with HBeAg-negative chronic liver diseases. The pre-C region of HBV DNA was successfully amplified by polymerase chain reaction (PCR) from 55 (96.5%) of 57 sera. According to the status of serum HBeAg, HBV DNA was amplified from all 25 sera of HBeAg-positive patients and 30 (93.8%) of 32 sera of HBeAg-negative patients. All amplified DNA from the sera of 25 patients with HBeAg-positive and that from 28 (93.3%) of 30 patients with HBeAg-negative chronic liver diseases hybridized with the wild type probe. In addition, that from 5 (20.0%) among 25 patients with HBeAg-positive and 16 (53.3%) among 30 patients with HBeAg-negative chronic liver diseases hybridized also with the mutant type probe. These results suggest that the prevalence of point mutation in the pre-C region of HBV DNA is relatively high in patients with HBeAg-negative chronic liver diseases and further study is mandatory to identify the significance of this mutation.

Key Words: Hepatitis B virus, pre-core, point mutation, HBeAg

HBeAg is generally considered as a marker of active ongoing viral replication during the natural course of hepatitis B virus (HBV) infection, while seroconversion to anti-HBe is a sign of remission and indicates the evolution of chronic HBV infection toward the carrier state (Realdi *et al.* 1980; Hoofnagle, 1983). Some patients, however, show persistence of serum HBV DNA, despite seroconversion to anti-HBe (Bonino *et al.* 1981; Krossgaad *et al.* 1986), and develop rapidly progressive chronic liver disease resistant to interferon therapy (Bonino *et al.* 1986; Brunetto *et al.* 1989; Kosaka *et al.* 1991; Omata *et al.* 1991). This

form of chronic hepatitis is frequently reported in the Mediterranean countries and in the Far East (Hadziyannis *et al.* 1983; Tong *et al.* 1990).

Recently, a translational stop codon at the end of the pre-core (pre-C) region was reported to occur in HBV isolated from HBeAg-negative, HBV DNA-positive patients (Carman *et al.* 1989; Brunetto *et al.* 1991). Since HBeAg is a peptide encoded by continuous translation from the pre-C through into the C region, this stop codon would prevent HBeAg production (Carman *et al.* 1989). The existence of these mutations in the pre-C region suggests that either HBeAg-negative chronic hepatitis is caused by a distinct HBV strain (Fagan *et al.* 1986; Okuda 1987) or, alternatively, that there exist in the HBV genome points of high-frequency mutations that, in some populations, are selected during the course of viral replication in order to escape the host immune defenses (Okamoto *et*

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al. 1990).

We performed dot blot hybridization and sequencing of enzymatically amplified HBV DNA to investigate the prevalence of point mutation of the pre-C region of HBV DNA in patients with HBeAg-positive and HBeAg-negative chronic liver diseases.

MATERIALS AND METHODS

Subjects

Fifty-seven patients with chronic HBsAg carrier were included in this study. Among them, 25 were HBeAg-positive and the remainder were HBeAg-negative. In all patients, liver biopsy was performed within 6 months of inclusion. Five were healthy HBsAg carriers, 21 were patients with chronic active hepatitis and the remaining 31 were patients with liver cirrhosis (Table 1).

HBV DNA (subtype adr) cloned into a plamid vector pBR322 (Kim *et al.* 1988) and genomic DNA of PLC/PRF/5 cell (CRL 8024, American Type Culture Collection, Rockville, MD, U.S.A.) to which HBV DNA is known to be integrated were used as positive controls. Serum from anti-HBs-positive healthy staff

member was used as negative control.

Primers and probes

Oligonucleotide primers, located upstream of the pre-C initiation codon and downstream of the C initiation codon, and probes as well as sequencing primer were synthesized (Fujiyama *et al.* 1983; Kobayashi and Koike, 1984; Tiollais *et al.* 1985; Okamoto *et al.* 1986) by DNA synthesizer (381 DNA synthesizer, Applied Biosystems, Foster City, CA, U.S.A.) (Fig. 1, 2).

Polymerase chain reaction (PCR)

Viral DNA was extracted twice with phenol-chloroform from 200 μ l of serum and precipitated with ethanol. Then, the pellet was dissolved in 100 μ l of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The reaction mixture for PCR contained 10 μ l of sample DNA, 200 μ M of each dNTPs, 2.5 units of *Taq* polymerase, 15 pmol of each primer, 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 2 mM of MgCl₂, and 0.01% (wt/vol) gelatin was adjusted as the final volume of 100 μ l by water and then 50 μ l of mineral oil

Table 1. Subjects

	Carrier	CAH	LC	Total
HBeAg(+)	2	9	14	25
HBeAg(-)	3	12	17	32
Total	5	21	31	57

Carrier=healthy HBsAg carrier, CAH=chronic active hepatitis B, LC=liver cirrhosis

P-1	5'-CGACCTTGAGGCATACTTCA-3'	(1692-1710)
P-2	5'-TCTTATCGAACGGACTCACG-3'	(2060-2079)
S-P	5'-TAGGCATAAATTGGTCTG-3'	(1692-1710)
W	5'-TGG CTT TGG GGC ATG GAC-3'	(1692-1710)
M	5'-TGG CTT TAG GGC ATG GAC-3'	(1692-1710)

Fig. 2. Sequences of primers and probes. P-1 and P-2 are primers for polymerase chain reaction, S-P is sequencing primer and W/M is wild or mutant type probe for hybridization.

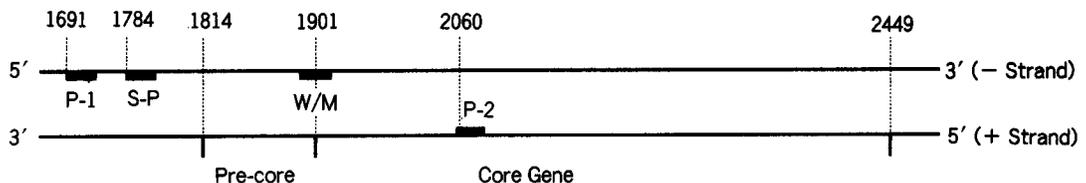


Fig. 1. Locations of primers and probes. P-1 and P-2 are primers for polymerase chain reaction, S-P is sequencing primer and W/M is probe for hybridization.

was overlaid to prevent evaporation. Thirty-two cycles of PCR were performed using a programmable DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.) using the following parameters: denaturation at 94°C for 1 minute, annealing of primers at 57°C for 1 minute and extension at 72°C for 1 minute except for 7 minutes of the final cycle (Sakai *et al.* 1985; Kaneko *et al.* 1991; Kim *et al.* 1992). PCR products were validated by ethidium bromide stain of agarose gel electrophoresis and purified by microglass bead kit (GeneClean II; Bio 101 Inc., La Jolla, CA, U.S.A.).

Dot blot hybridization

After denaturation, DNA was spotted to a nylon membrane and cross-linked by UV-crosslinker (UVC 1000, Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Then, the membrane was incubated with γ -³²P end-labelled oligonucleotide probes corresponding to sequences with or without mutation under stringent hybridization and washing condition (Scotto *et al.* 1983; Maniatis *et al.* 1988; Song *et al.* 1992). Autoradiography was performed overnight.

Sequencing

Asymmetric PCR using an amplified DNA as a template and primers at a ratio of 50:1 (P-1:P-2) was performed after purification and concentration by Centricon 30 (Amicon, Lexington, MA, U.S.A.) of pre-amplified DNA

using the following parameters: denaturation at 94°C for 0.5 minute, annealing of primers at 60°C for 0.5 minute and extension at 72°C for 0.5 minute except for 5 minutes of the final cycle. Then, the single stranded PCR product was directly sequenced by T7 sequencing™ kit (Pharmacia, Piscataway, NJ, U.S.A.).

RESULTS

The pre-C region of HBV DNA was successfully amplified by PCR from 55 (96.5%) of 57 sera (Fig. 3). According to the status of serum HBeAg, HBV DNA was amplified from all 25 sera of HBeAg-positive patients and 30 (93.8%) of 32 sera of HBeAg-negative patients. According to the status of liver diseases, HBV DNA was amplified from all 5 sera of healthy HBsAg carrier as well as 21 sera of patients with chronic active hepatitis, and 29 (93.5%) of 31 sera of patients with liver cirrhosis (Table 2).

All of the amplified DNA from the sera of 25 patients with HBeAg-positive and that from 28 (93.3%) of 30 patients with HBeAg-negative chronic liver diseases hybridized with the wild type probe. In addition, that from 5 (20.0%) among 25 patients with HBeAg-positive and 16 (53.3%) among 30 patients with HBeAg-negative chronic liver diseases hybridized also with the mutant type probe (Fig. 4). This point mutation was confirmed by direct sequencing of the amplified DNA

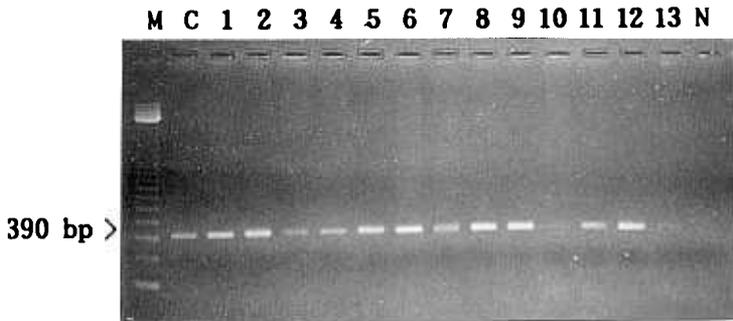


Fig. 3. Agarose gel electrophoresis of PCR products. Lane C and N are positive and negative control respectively. Lane 1 to 12 are PCR product from sera of patients with chronic liver disease B showing amplified 390 base pairs long DNA bands.

fragment (Fig. 5). Among HBeAg-positive chronic liver diseases, the mutant type virus was not detected from any serum of 2 healthy HBsAg carriers, but detected from 1 (11.1%) of 9 sera of patients with chronic active hepatitis and 4 (28.6%) of 14 sera of patients with liver cirrhosis. Among HBeAg-negative liver disease, the mutant type virus was detected from 2 (40.0%) of 5 sera of healthy HBsAg carriers, 7 (58.3%) of 12 sera of patients with chronic active hepatitis and

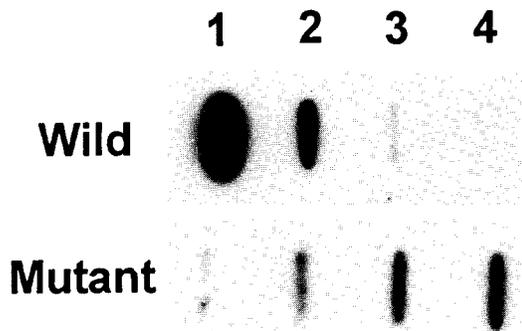


Fig. 4. Oligonucleotide hybridization of PCR products. Homogenous wild type and mutant type HBV are seen in serum 1 and serum 4 respectively. Sera 2 and 3 show mixed population of wild and mutant type viruses.

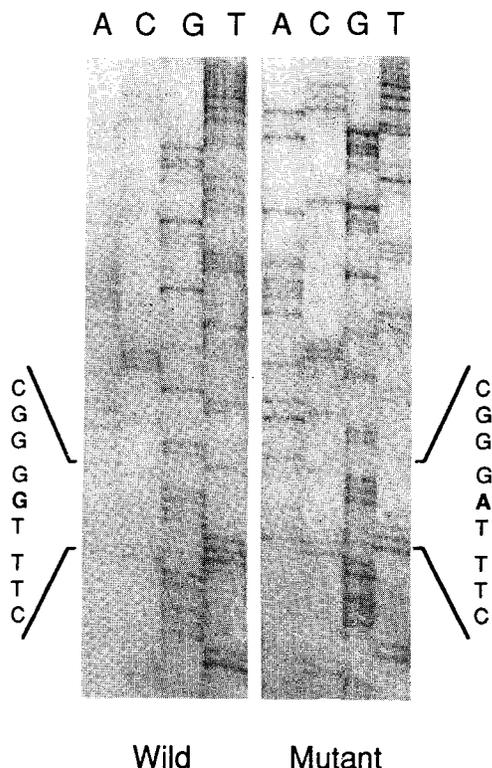


Fig. 5. Direct sequencing of asymmetric PCR products around distal pre-core region of HBV DNA. Tracks of A,C,G, and T are marked. Bolded characters indicate point-mutated nucleotides.

Table 2. HBV DNA amplification rate

	Carrier	CAH	LC	Total
HBeAg(+)	2/2(100.0)	9/ 9(100.0)	14/14(100.0)	25/25(100.0)
HBeAg(-)	3/3(100.0)	12/12(100.0)	15/17(88.2)	30/32(93.8)
Total	5/5(100.0)	21/21(100.0)	29/31(93.5)	55/57(96.5)

() : %

Table 3. Incidence of G to A point mutation in 83 th nucleotide of pre-core region of HBV DNA

	Carrier	CAH	LC	Total
HBeAg(+)	0/2(0.0)	1/9(11.1)	4/14(100.0)	5/25(20.0)
HBeAg(-)	3/3(66.7)	7/12(58.3)	7/17(46.7)	16/30(53.3)
Total	2/5(40.0)	8/21(38.1)	11/29(37.9)	21/55(38.2)

() : %

7 (46.7%) of 15 sera of patients with liver cirrhosis (Table 3).

DISCUSSION

The pre-C/C open reading frame (ORF) of HBV DNA has two initiation codon separated by 87 nucleotides named the pre-C region and encodes two proteins (Ganem and Varma, 1987). The C gene, which consists of 549 nucleotide starts from the second ATG of pre-C/C ORF, codes the nucleocapsid protein of HBV, named HBcAg. While, the entire pre-C/C ORF of HBV, starting from the first ATG located upstream of the pre-C region through into the C region, encodes the precursor protein of HBeAg (Uy *et al.* 1986). This protein is secreted into the blood stream as HBeAg after cleavage of the amino terminus and proteolysis of carboxyterminal sequences (Schlicht *et al.* 1987; Jean-Jean *et al.* 1989). The pre-C region is not necessary for production of viral particles (Chang *et al.* 1987; Schlich *et al.* 1987), yet is highly conserved in all of the hepadna viruses (Ono *et al.* 1983; Miller *et al.* 1989) and encodes signal sequence, which directs the precursor protein of HBeAg to the endoplasmic reticulum (Ou *et al.* 1986; Chang *et al.* 1987; Garcia *et al.* 1988).

HBV is a partially double stranded DNA virus and utilizes reverse transcription of pregenome RNA in replication like retroviruses (Girones and Miller, 1989). Thus, the mutation rate is known to be relatively high particularly in the pre-C region (Fiordalisi *et al.* 1990; Santantonio *et al.* 1991). Recently, a translational stop codon at the end of the pre-C region due to a point mutation from guanosine (G) to adenosine (A) at nucleotide 1896 (TGG to TAG), was reported to occur in HBV isolated from HBeAg-negative, HBV DNA-positive patients (Carman *et al.* 1989; Okamoto *et al.* 1990; Tong *et al.* 1990; Bonino *et al.* 1991; Brunetto *et al.* 1991; Liang *et al.* 1991; Naoumov *et al.* 1992). Since HBeAg is a peptide encoded by continuous translation from the pre-C through into the C region, this stop codon would prevent HBeAg production (Carman *et al.* 1989). In addition to a mutation from G to A at nucleotide 1896 in

the pre-C region, mutation in the pre-C initiation codon as well as frameshift mutations prevent expression of HBeAg (Santantonio *et al.* 1991). A second nucleotide substitution at position 1989 was also found in isolates from several patients (Carman *et al.* 1989; Fiordalisi *et al.* 1990), but the functional implications of this mutation are still undefined.

Genetic heterogeneity of HBV significantly influences the course and outcome of chronic hepatitis B. Brunetto *et al.* (1991) hypothesized that wild type HBV secreting HBeAg induces immunologic tolerance and causes chronic infection. Once chronic infection is established, the mutant type HBV, unable to secrete HBeAg, prevail and displace the wild type virus by positive selection. Clinical studies have also shown that anti-HBe-positive chronic hepatitis B has specific features that are different from those of hepatitis in HBeAg-positive chronic carriers (Bonino *et al.* 1981; Krossgaad *et al.* 1986), and such patients were shown to be infected with pre-C mutant viruses unable to produce HBeAg (Fagan *et al.* 1986; Okuda 1987; Carman *et al.* 1991). Antigenicity of HBeAg is found not only in hepatitis B e protein but also on the viral core protein (HBcAg). Both HBcAg and HBeAg are believed to be major targets for the host immune response (Ou *et al.* 1986; Bruss and Gerlich, 1988). The recent demonstration of selective elimination of wild type virus and the selective survival of the pre-C mutant type virus indirectly suggests that HBeAg can also represent a critical target for virus elimination (Okamoto *et al.* 1990; Naoumov *et al.* 1992).

The more severe and rapidly progressing liver disease in patients infected with mutant type HBV have been reported, but it is not the rule (Raimondo *et al.* 1990). The mechanisms, why the mutant type HBV induces more severe liver disease, is not clarified but could be explained as follows. If HBe epitopes are presented on the hepatocyte surface, the absence of circulating HBeAg may allow more aggressive cell-mediated immune lysis of infected hepatocytes, because circulating HBeAg are known to down-regulate cell-mediated immunity and thus induce immunologic tolerance (Milich *et al.* 1990). A truncated pre-C peptide, terminating at the stop codon produced by the mutation, may be directly

cytopathic to hepatocytes or may be transported to the cell membrane in association with major histocompatibility complex glycoprotein, where it could become a new target for cytotoxic T cells (Carman *et al.* 1989). The mutant type HBV synthesizes more HBeAg rather than HBeAg, thus could be more cytopathic (Roossink *et al.* 1983).

The results in our study showed that most if not all HBeAg-negative patients with chronic liver disease have HBV DNA in their serum. In addition, most of them infected with the mutant type virus were coinfecting with the wild type virus also, especially in the HBeAg-negative group. The reasons of HBeAg-negativity in these patients could be explained in that HBeAg in the serum is complexed with anti-HBe and therefore is undetectable (Castillo *et al.* 1990) or that HBeAg expression is too low for detection (Naoumov *et al.* 1992). However, sometimes, HBeAg may be detected in cases of the mutant type virus infection. Explanations for this finding are that intracellular core particles may be released as a result of hepatocellular necrosis, accounting for the cross immunoreactivity and the mutant type virus may be coinfecting with the wild type virus (Liang *et al.* 1991). The mutant type virus was much more frequently detected from sera of HBeAg-negative patients than from that of HBeAg-positive patients (53.3% vs. 20.0%) in our study. In HBeAg-negative patients, the detection rate of the mutant type virus was not different between disease status, however, in HBeAg-positive patients, the detection rate of the mutant type virus became higher as the liver disease progressed. These results suggest that the prevalence of point mutation in the pre-C region of HBV DNA is relatively high in patients with chronic liver diseases B particularly in HBeAg-negative patients and further study is mandatory to identify the significance of this mutation.

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