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Hepatitis B Virus with Mutations in the Core Promoter for an e Antigen-Negative Phenotype in Carriers with Antibody to e Antigen

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Hepatitis B virus (HBV) DNA clones were propagated from 57 carriers with antibody to hepatitis B e antigen (HBeAg) and sequenced within nucleotides (nt) 1685 to 1926 including the core promoter (nt 1742 to 1849) and the pre-C region (nt 1814 to 1900). Mutations in the core promoter or those in the pre-C region, or both, were detected in 328 (97.9%) of 335 clones from them. Five carriers were infected with HBV mutants with mutations in the core promoter alone, while 20 carriers were infected only with those in the pre-C region to abort the translation of HBeAg precursor; the remaining 32 carriers were infected with HBV mutants with mutations in both the core promoter and pre-C region. Some carriers infected with HBV with mutations in the core promoter exclusively had high HBV DNA titers, comparable with those in carriers infected with wild-type HBV, thereby indicating that such mutations would not affect the transcription of the HBV pregenome extensively. Two point mutations in the core promoter, from A to T at nt 1762 and from G to A at nt 1764, were most prevalent. The other mutations included a point mutation at either of the two nucleotides and their deletion. All of these mutations involved the TTAAA sequence (nt 1758 to 1762) at 28 bp upstream of the initiation site for shorter pre-C mRNAs (nt 1790 ± 1). The ATAAATT sequence (nt 1789 to 1795) at 23 bp upstream of the initiation site for the pregenome RNA (nt 1818), however, remained intact in all 335 HBV DNA clones. HBV mutants with mutations in the core promoter, unaccompanied by pre-C mutations, prevailed and replaced wild-type HBV in two carriers as they seroconverted from HBeAg to the corresponding antibody. These results indicate that HBV mutants with an HBeAg$^-$ phenotype would be generated by mutations in the core promoter which might abort the transcription of pre-C mRNA but do not seriously affect that of pregenome RNA.

The precursor of hepatitis B e antigen (HBeAg) is coded for by the pre-C region and the C gene (13, 23, 28). It loses the amino-terminal 19 amino acids (2) and carboxyl-terminal 34 amino acids (32) for the secretion from hepatocytes and circulates in association with plasma proteins such as albumin and immunoglobulins (31). During persistent hepatitis B virus (HBV) infection, carriers seroconvert from HBeAg to the corresponding antibody (anti-HBe). The seroconversion has significance on both HBV and hosts (14). In general, HBV replicates more actively in carriers with HBeAg than those with anti-HBe. Carriers with HBeAg have a higher activity to transmit HBV than those with anti-HBe, typically in the perinatal transmission of carrier state from mother to baby (19).

HBV DNA clones from carriers who have seroconverted to anti-HBe have mutations in the pre-C region which abort the synthesis of HBeAg (3, 21). The seroconversion from HBeAg to anti-HBe, therefore, is believed to be induced by the immune pressure of hosts against wild-type HBV with an HBeAg$^+$ phenotype toward the predominance of mutants with an HBeAg$^-$ phenotype. Not all carriers with anti-HBe harbor pre-C mutants (16), however, indicating the abrogation of HBeAg synthesis by mutations not affecting the pre-C region.

HBeAg is translated from pre-C mRNAs of 3.5 kb (17, 22, 39). The other 3.5-kb mRNA (pregenome-C/P mRNA), which initiates 29 to 35 bp downstream of pre-C mRNA start points, is the viral pregenome (38), from which the core (C) and polymerase (P; DNA polymerase-reverse transcriptase) proteins are translated. The transcription of these mRNAs is controlled by the basic core promoter (BCP) and core upstream regulatory sequences (CURS) (41). Recently, a negative regulatory element that abolishes the function of CURS on BCP was described (12).

HBV mutants with an HBeAg$^-$ phenotype would be generated by mutations which interfere with the transcription of pre-C mRNA but do not affect that of pregenome-C/P mRNA. HBV DNA clones were propagated from HBV carriers with anti-HBe or HBeAg to determine whether they had mutations in the core promoter or pre-C region to interfere with the expression of HBeAg at either the transcriptional or translational level.

MATERIALS AND METHODS

Carriers of HBV. Sera were obtained from 40 asymptomatic carriers of HBV, including 14 positive for HBeAg and 26 positive for anti-HBe, as well as from 42 patients with chronic

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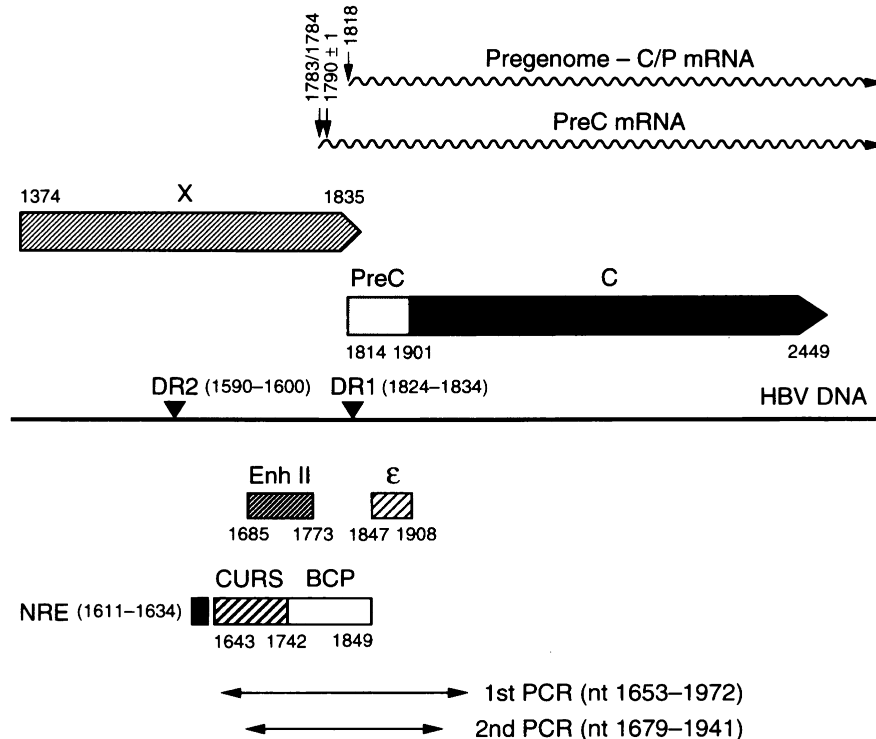

FIG. 1. Map of the X and pre-C/C genes and the HBV DNA fragment amplified for sequencing. Two kinds of 3.5-kb mRNAs and their initiation positions (17, 22, 38, 39) are indicated at the top. Liver-specific enhancer II (Enh II [36, 40, 42]), a pregenome encapsidation signal (ε [8]), BCP (41), CURS (41), and a negative regulatory element (NRE [12]) are indicated by boxes. HBV DNA fragments amplified by the first or second round of PCR are indicated at the bottom.

hepatitis B, including 11 positive for HBeAg and 31 positive for anti-HBe. A total of 766 HBV DNA clones (mean, 9.3 ± 8.3; range, 3 to 38) were propagated from the 82 sera and sequenced within the core promoter and pre-C region. Pre-C region sequences have been reported for HBV DNA clones from five asymptomatic carriers with HBeAg and three with anti-HBe and from three patients with HBeAg (1, 21, 33).

Sera from two carriers with HBeAg were tested for HBV DNA clones with mutations repeatedly while they seroconverted to anti-HBe. Also studied were a carrier mother with anti-HBe in serum and a baby born to her. Because she was positive for anti-HBe with little activity to transmit the persistent carrier state to offspring (19), the baby was not protected by hepatitis B immunoglobulin or vaccine. Unfortunately, hepatitis B surface antigen (HBsAg) developed in the baby 9 months after birth. HBV DNA clones propagated from sera of the mother taken 12 months after delivery and from the baby at the first appearance of HBsAg were sequenced within the core promoter and pre-C region.

Serological markers of HBV infection. HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay with commercial kits (HBeAg/Ab EIA; Institute of Immunology Co., Ltd., Tokyo, Japan). HBsAg and the corresponding antibody were determined by hemagglutination with commercial kits (MyCell; Institute of Immunology). Antibody to hepatitis B core antigen (anti-HBc) was determined by inhibition of hemagglutination (7). The results of hemagglutination assays were expressed by the highest twofold dilution (2^n) of sera which induced or inhibited hemagglutination.

Determination of HBV DNA. Nucleic acids were extracted from 0.1 ml of serum with phenol-chloroform in the presence of proteinase K-sodium dodecyl sulfate by a method described elsewhere (21). They were diluted 10-fold serially with Tris-HCl buffer (10 mM, pH 8.0) supplemented with 1 mM EDTA and 20 μg of glycogen (Boehringer, Mannheim, Germany) per ml. HBV DNA was amplified by PCR with nested primers deduced from the S gene (7), and the highest dilution (10^n) in which it was detectable was determined.

Amplification of a part of the X gene and pre-C region. On HBV DNA extracted from serum, a fragment of 263 bp corresponding to nucleotides (nt) 1679 to 1941 was amplified by PCR with nested primers which included enhancer II (nt 1685 to 1773 [36]), BCP (nt 1742 to 1849 [41]), and the 3′-terminal 64% of CURS (nt 1643 to 1742 [41]), as well as the pre-C region (nt 1814 to 1900) (Fig. 1); nucleotides are numbered from the unique *Eco*RI site in HBV DNA (21). Primers for the first PCR were 5′-CATAAGAGGACTCTTG GACT-3′ (sense, nt 1653 to 1672) and 5′-AAAGAATTCA GAAGGCAAAAAAGA-3′ (antisense, nt 1949 to 1972), and those for the second PCR were 5′-AATGTCAACGACC GACCTTG-3′ (sense, nt 1679 to 1698, with a *Hin*cII site [underlined]) and 5′-TCCACAGAAGCTCCGAATTC-3′ (antisense, nt 1922 to 1941, with an *Eco*RI site [underlined]).

Cloning and sequencing. HBV DNA amplified by PCR was digested with *Eco*RI and *Hin*cII (Takara Biochemicals, Kyoto, Japan). The products of 242 bp corresponding to nt 1685 to 1926 were ligated to phage vector M13 that had been cleaved with *Eco*RI and *Hin*cII, and clones carrying HBV DNA sequence were propagated. HBV DNA sequences were then determined by the dideoxy-chain termination method with a Sequenase DNA sequencing kit (7-deaza-dGTP edition version 2.0; United States Biochemical Corp., Cleveland, Ohio) or

TABLE 1. Mutations in the core promoter and pre-C region of HBV DNA clones from asymptomatic carriers with anti-HBe

Carrier no.	Age (yr) (sex[a])	HBV DNA titer	No. of clones tested	Clones with mutations in core promoter		No. (%) of clones with mutations in pre-C region at:	
				No. (%)	Pattern[b]	nt 1896	nt 1899
1	29 (F)	10^2	20	20 (100)	a	0	0
2	45 (F)	10^2	6[c]	5 (83)	k	0	0
3	26 (F)	10^2	5	5 (100)	a	5 (100)	0
4	27 (F)	10^3	5	5 (100)	a	5 (100)	0
5	28 (M)	10^2	5	5 (100)	a	5 (100)	0
6	29 (M)	10^3	6	6 (100)	a	6 (100)	6 (100)
7	29 (M)	10^1	5	5 (100)	a	5 (100)	0
8	30 (M)	10^2	5	5 (100)	a	5 (100)	0
9	33 (F)	10^2	5	5 (100)	a	5 (100)	0
10	34 (M)	10^1	10[d]	6 (60)	l	2 (20)	0
11	34 (F)	10^5	10	6 (60)	j	4 (40)	0
12	34 (F)	10^2	5	5 (100)	a	5 (100)	0
13	35 (F)	10^3	5	5 (100)	a	5 (100)	0
14	36 (F)	10^2	5	5 (100)	a	5 (100)	0
15	45 (M)	10^2	6	6 (100)	a	6 (100)	0
16	23 (M)	10^2	5	0		5 (100)	0
17	25 (F)	10^4	3	0		3 (100)	0
18	27 (M)	10^3	5	0		5 (100)	0
19	31 (M)	10^2	5	0		5 (100)	0
20	33 (M)	10^3	5	0		5 (100)	0
21	34 (F)	10^2	5	0		5 (100)	0
22	37 (F)	10^4	5	0		5 (100)	0
23	38 (M)	10^4	5	0		5 (100)	0
24	43 (M)	10^1	3	0		3 (100)	0
25	45 (M)	10^2	5	0		5 (100)	5 (100)
26	22 (M)	10^2	3[e]	0		0	0

[a] F, female; M, male.
[b] Patterns of mutations in the core promoter are shown in Fig. 2.
[c] One clone was without mutations in the core promoter of the pre-C region.
[d] Two clones were without mutations in the core promoter or pre-C region.
[e] All three clones possessed a T-to-C mutation at nt 1815 in codon 1 inducing initiation failure.

an AutoRead DNA sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Statistical analysis. Group means were compared by using Student's *t* test.

RESULTS

Mutations in HBV DNA clones from carriers with anti-HBe. Mutations in core promoter and pre-C region in HBV DNA clones from 26 asymptomatic carriers with anti-HBe are shown in Table 1. Mutations in the core promoter were detected in all or the majority of clones from carriers 1 to 15. The G-to-A mutation at nt 1896 in the pre-C region, converting codon 28 for tryptophan to a stop codon, was found in all clones from 21 carriers (carriers 3 to 9 and 12 to 25) and in some clones from two (carriers 10 and 11). One carrier (carrier 26) had a T-to-C point mutation at the initiation codon of the pre-C region to abort the translational initiation of HBeAg precursor. The second G-to-A mutation at nt 1899 in the pre-C region, converting codon 29 for glycine to that for aspartic acid, was detected in clones from only two carriers.

Clones from carriers 1 and 2 had mutations in the core promoter in the absence of those in the pre-C region, and clones from carriers 16 to 26 had mutations in the pre-C region only; clones from the remaining 13 carriers (carriers 3 to 15) had mutations in both core promoter and pre-C region. Thus, 149 (98%) of 152 HBV DNA clones from the 26 asymptomatic carriers with anti-HBe, except for 3 clones from two carriers (carriers 2 and 10), had mutations in either the core promoter or pre-C region or both.

Mutations in HBV DNA clones from 31 patients with chronic hepatitis B with anti-HBe are shown in Table 2. Mutations in the core promoter alone were detected in patients 1 to 3, and those in the pre-C region only were detected in nine (patients 23 to 31); clones from the remaining 19 patients (patients 4 to 22) had mutations in both the core promoter and pre-C region. The second G-to-A mutations at nt 1899 in the pre-C region was detected in clones from six patients. Mutations in either the core promoter or pre-C region or both were detected in 179 (98%) of the clones from the 31 anti-HBe-positive patients, except in 4 clones from two patients (patients 4 and 14).

The geometric mean titer of HBV DNA in patients with anti-HBe (5.0 ± 2.6 [Table 2]) was significantly higher ($P < 0.001$) than that in asymptomatic carriers with anti-HBe (2.4 ± 1.0 [Table 1]). Patients 3, 11, 18, and 21 (Table 2), who were infected with HBV mutants with mutations in the core promoter exclusively, had HBV DNA levels (10^8 or 10^9) comparable with those in HBeAg-positive carriers infected with wild-type HBV alone (10^6 to 10^9 [Table 3]).

Mutations in HBV DNA clones from carriers with HBeAg. Table 3 shows mutations in the core promoter and pre-C region from 276 HBV DNA clones from 14 asymptomatic carriers with HBeAg. Mutations in the core promoter were found only in 7 (30%) of 23 clones from a single carrier (carrier 1). No clones possessed mutations in the pre-C region to disturb the translation of HBeAg. The geometric mean titer of serum HBV DNA in asymptomatic carriers with HBeAg was higher than that in those with anti-HBe in Table 1 (7.9 ± 1.1 versus 2.4 ± 1.0, $P < 0.001$).

TABLE 2. Mutations in the core promoter and pre-C region of HBV DNA clones from hepatitis patients with anti-HBe

Patient no.	Age (yr) (sex[a])	HBV DNA titer	No. of clones tested	Clones with mutations in core promoter		No. (%) of clones with mutations in pre-C region at:	
				No. (%)	Pattern[b]	nt 1896	nt 1899
1	25 (M)	10^7	6	6 (100)	a	0	0
2	42 (M)	10^6	8	8 (100)	a	0	0
3	54 (M)	10^9	19	19 (100)	h	0	0
4	25 (M)	10^3	9[c]	5 (56)	a	2 (22)	0
5	27 (M)	10^2	3	3 (100)	i	3 (100)	1 (33)
6	29 (M)	10^7	3	1 (33)	e	3 (100)	0
7	33 (M)	10^8	10	1 (10)	m	10 (100)	0
8	37 (M)	10^1	3	3 (100)	l	3 (100)	0
9	39 (F)	10^7	9	7 (78)	p	9 (100)	0
10	41 (M)	10^4	3	3 (100)	d	3 (100)	3 (100)
11	42 (M)	10^8	3	3 (100)	a	3 (100)	3 (100)
12	45 (M)	10^7	3	3 (100)	a	3 (100)	0
13	45 (F)	10^4	3	3 (100)	b	2 (67)	1 (33)
14	47 (M)	10^2	8[d]	4 (50)	o	2 (25)	0
15	48 (M)	10^1	3	3 (100)	a	3 (100)	0
16	49 (M)	10^2	3	3 (100)	b	3 (100)	2 (67)
17	49 (M)	10^7	17	17 (100)	a	17 (100)	0
18	52 (M)	10^8	3	3 (100)	b	3 (100)	0
19	52 (F)	10^2	3	3 (100)	g	3 (100)	0
20	54 (F)	10^6	18	18 (100)	f	18 (100)	0
21	57 (M)	10^8	9	9 (100)	c	6 (60)	0
22	60 (M)	10^3	3	3 (100)	b	1 (33)	0
23	25 (M)	10^2	3	0		3 (100)	0
24	30 (M)	10^2	3	0		3 (100)	0
25	40 (M)	10^7	3	0		3 (100)	3 (100)
26	40 (F)	10^3	3	0		3 (100)	0
27	47 (F)	10^3	3	0		3 (100)	0
28	48 (M)	10^7	3	0		3 (100)	0
29	61 (F)	10^7	3	0		3 (100)	0
30	62 (M)	10^7	10	0		10 (100)	0
31	62 (F)	10^6	3	0		3 (100)	0

[a] M, male; F, female.
[b] Patterns of mutations in the core promoter are shown in Fig. 2.
[c] Two (22%) of the clones were without mutations in the core promoter or pre-C region.
[d] Two (25%) of the clones were without mutations in the core promoter or pre-C region.

Mutations in 155 clones from HBeAg-positive patients with chronic hepatitis are shown in Table 4. Mutations in the core promoter were detected in all clones from patients 2, 8, and 10 and in some clones from the remaining eight patients. The G-to-A point mutations at nt 1896 was found in some clones from four patients.

Mutations in the core promoter and induced amino acid changes. Patterns of point mutations in the core promoter are illustrated in Fig. 2, and induced amino acid changes are shown in Fig. 3. Most frequently seen were two point mutations, A to T at nt 1762 and G to A at nt 1764, converting amino acids 130 and 131 in the X protein from lysine to methionine and from valine to isoleucine, respectively (patterns a to h). Other point mutations involved either nt 1762 or nt 1764 (patterns i and j). We observed one to four additional point mutations making different patterns, some of which induced amino acid substitutions. The other mutations were 8-, 20-, or 21-bp deletions involving nt 1762 and 1764 or nt 1764 alone, as well as a 3-bp insertion of TTG between nt 1766 and 1767. They created premature termination, deletion of seven amino acids, or insertion of one amino acid.

Three AT-rich regions were identified in the core promoter (Fig. 2). The first AT-rich region (ATTA) spanned nt 1752 to 1755, the second (TTAAA) spanned nt 1758 to 1762, and the third (ATAAATT) spanned nt 1789 to 1795. They were 23 to 28 bp upstream of the initiation sites for longer pre-C mRNA (nt 1783 and 1784), shorter pre-C mRNA (nt 1789 to 1791), and the pregenome-C/P mRNA (nt 1818), respectively. Most mutations in the core promoter, except patterns j, k, and p, involved the second AT-rich region. HBV DNA clones from some carriers had a point mutation (patterns e to i) or a deletion (pattern o) in the first AT-rich region. The third AT-rich region remained intact in all 766 clones from the 82 carriers with either anti-HBe or HBeAg.

Mutations in the core promoter unaccompanied by pre-C mutations prevailing as hosts seroconverted from HBeAg to Anti-HBe. Sera from an asymptomatic carrier and a patient with chronic hepatitis were monitored for mutations in the core promoter and pre-C region as they seroconverted from HBeAg to anti-HBe (Table 5). Pre-C mutations were detected in none of 117 clones from them. As these subjects seroconverted to anti-HBe, HBV mutants with mutation in the core promoter of pattern a or h gradually prevailed and finally replaced wild-type HBV. After seroconversion to anti-HBe, the HBV DNA titer decreased sharply in the asymptomatic carrier but stayed at high levels in the patient with chronic hepatitis.

Mutations in the core promoter of HBV DNA clones from a carrier mother with Anti-HBe and the baby born to her. Table 6 shows markers of HBV infection in sera from an anti-HBe-positive mother and her baby, as well as mutations in the core promoter and pre-C region of HBV DNA clones propagated

TABLE 3. Mutations in the core promoter and pre-C region of HBV DNA clones from asymptomatic carriers with HBeAg

Carrier no.	Age (yr) (sex[a])	HBV DNA titer	No. of clones tested	Clones with mutations in core promoter		No. (%) of clones with mutations in pre-C region at:	
				No. (%)	Pattern[b]	nt 1896	nt 1899
1	31 (M)	10^9	23	2 (9)	n	0	0
				5 (22)	o		
2	4 (F)	10^9	23	0		0	0
3	18 (M)	10^9	6	0		0	0
4	18 (F)	10^9	6	0		0	0
5	19 (M)	10^8	23	0		0	0
6	20 (M)	10^9	30	0		0	0
7	20 (M)	10^9	10	0		0	0
8	23 (M)	10^8	25	0		0	0
9	26 (M)	10^7	34	0		0	0
10	31 (M)	10^7	6	0		0	0
11	39 (F)	10^6	38	0		0	0
12	43 (M)	10^8	10	0		0	0
13	44 (F)	10^7	36	0		0	0
14	47 (M)	10^8	6	0		0	0

[a] M, male; F, female.
[b] Patterns of mutations in the core promoter are shown in Fig. 2.

from them. Four of the ten clones from the mother had the pre-C mutation at nt 1896. The remaining six possessed a mutation in the core promoter of pattern j. The identical mutation in the core promoter, unaccompanied by pre-C mutations, was detected in all 10 clones from the baby, who had extremely high titered HBV DNA without detectable HBeAg or anti-HBe in serum.

DISCUSSION

HBV mutants which have various mutations in the X gene, including deletions or insertions of nucleotides and point mutations, have been reported (5, 6, 9, 18, 26, 27). It is not clear, however, whether such mutations have any influence on either HBV or hosts. Essential elements controlling viral replication, such as BCP (41), CURS (41), and a negative regulatory element have been identified in the X gene (12). The liver-specific enhancer II overlaps with BCP and CURS (36, 40, 42). Should mutations in the X gene involve any of these essential elements, they would modify the replication of HBV and expression of viral proteins. Mutations disturbing the expression of the HBeAg precursor at the transcriptional level may result in HBV mutants with an HBeAg$^-$ phenotype, which would be selected by immune responses of the host against HBeAg.

The results obtained in this study support such a hypothesis. Mutations in the core promoter were detected frequently in asymptomatic HBV carriers with anti-HBe (15 of 26, or 58%) but rarely in those with HBeAg (1 of 14, or 7%). They were detected in essentially all clones from the majority (22 of 31, or 71%) of anti-HBe-positive patients with chronic hepatitis.

Mutations in the core promoter were also detected in at least some clones from 11 HBeAg-positive patients with chronic hepatitis (Table 4). Mutants with such mutations, however, co-occurred with wild-type HBV in eight of them. The three patients with mutants with invariable mutations in the core promoter might have been seroconverting to anti-HBe; some HBV DNA clones from two of them had mutations in the pre-C region. HBeAg in their sera might be attributable to coinfecting wild-type HBV that escaped detection, which

TABLE 4. Mutations in the core promoter and pre-C region of HBV DNA clones from hepatitis patients with HBeAg

Patient no.	Age (yr) (sex[a])	HBV DNA titer	No. of clones tested	Clones with mutations in core promoter		No. (%) of clones with mutations in pre-C region at:	
				No. (%)	Pattern[b]	nt 1896	nt 1899
1	27 (M)	10^7	20	9 (45)	a	0	0
2	31 (M)	10^8	10	10 (100)	a	0	0
3	36 (M)	10^6	20	16 (80)	b	0	0
4	41 (M)	10^7	19	18 (95)	a	0	0
5	44 (M)	10^8	10	6 (60)	a	0	0
6	54 (F)	10^7	10	9 (90)	a	0	0
7	64 (M)	10^8	17	10 (59)	a	0	0
8	32 (M)	10^6	10	10 (100)	a	5 (50)	0
9	33 (M)	10^8	19	12 (63)	b	6 (32)	0
10	39 (M)	10^8	8	8 (100)	a	4 (50)	0
11	55 (M)	10^7	12	7 (58)	a	1 (8)	0
				2 (17)	k		

[a] M, male; F, female.
[b] Patterns of mutations in the core promoter are shown in Fig. 2.

```
           1741                1762 1764                                1800          PreC-Start   Cases
           |                    | |                                    |                       ↓     (N)
Wild Type: TGGGGGAGGAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCATAAATTGGTCTGTTCACCAGCACCATG
Mutants a: ---------------------T-A----------------------------------------------------   28
        b: -----------------C---T-A----------------------------------------------------    6
        c: ---------------------T-A-T--------------------------------------------------    1
        d: ---------------------T-A--------T-------------------------------------------    1
        e: ------------G--------T-A-T--------------------------------------------------    1
        f: ------------C--------T-A-------C--------------------------------------------    1
        g: ------------G--------T-A--------T-------------------------------------------    1
        h: ------------C---A----T-A----------G-----------------------------------------    1
        i: ------------G--------T---T-A----T-------------------------------------------    1
        j: -----------------------A-T-A------------------------------------------------    1
        k: ----------------------////////----------------------------------------------    2
        l: ---------------------T////////--T-------------------------------------------    2
        m: ----------------A////////---------------------------------------------------    1
        n: ----------------/////////////////////---------------------------------------    1
        o: ------------////////////////////--------------------------------------------    2
        p: ----------------------------------------------------------------------------    1
                                     Δ
```

FIG. 2. Various mutations in the core promoter of HBV DNA clones from 49 carriers. The sequence of a wild-type HBV of genotype B (20) is indicated at the top; three AT-rich regions are indicated by bars, and initiation sites for pre-C mRNAs are indicated by arrows. Dashes represent nucleotides identical to those of the wild-type HBV, and slashes represent deletions of nucleotides. The triangle indicates a 3-bp insertion (TTG) found in HBV DNA clones from an asymptomatic carrier with anti-HBe. Numbers of carriers with mutations of the specified types are indicated on the right. Two subjects (carrier 1 in Table 3 and patient 11 in Table 4) had two distinct mutants with different patterns of mutations.

would have to be searched for by PCR with specific primers. Some HBV DNA clones from two asymptomatic carriers (carriers 2 and 10 in Table 1) and two patients with chronic hepatitis B (patients 4 and 14 in Table 2), all of whom were positive for anti-HBe, had no mutations in the pre-C region or the core promoter. HBeAg would have occurred in their circulation but escaped the detection because it complexed with antibodies.

Mutations affecting the core promoter in 32 (86%) of 37 carriers with anti-HBe were found in association with pre-C mutations, which can abort the synthesis of the HBeAg precursor (3, 21). The remaining five anti-HBe-positive carriers, however, were infected with HBV mutants with mutations in the core promoter unaccompanied by mutations in the pre-C region. This finding indicates that mutations in the core

```
           124   130 131                 154
           |     ||                      |
Wild Type: GEEIRLKVFVLGGCRHKLVCSPAPCNFFTSA
Mutants a: ------MI-----------------------
        b: ------MI-----------------------
        c: ------MI-----------------------
        d: ------MI-----------------------
        e: ---S--MI-----------------------
        f: ---T--MI-----------------------
        g: ---S--MI-----------------------
        h: ---T--MI-----------------------
        i: ---S--M-Y----------------------
        j: -------IY----------------------
        k: ------NTRR-
        l: ------IIRR-
        m: ------CTRR-
        n: -----///////-------------------
        o: ---T-RL
        p: -------------------------------
                   Δ
```

FIG. 3. Amino acid substitutions in the carboxyl-terminal part of the X protein in HBV DNA clones from 49 carriers with mutations in the core promoter. The triangle indicates an insertion of leucine between amino acids 131 and 132.

promoter, by themselves, would be able to induce an HBeAg⁻ phenotype. It is possible that mutations in the core promoter would disturb the transcription of pre-C mRNA for an HBeAg⁻ phenotype, so that the hepatocytes harboring mutants may escape the immune elimination by anti-HBe responses of hosts.

HBV DNA clones with mutations in the core promoter unaccompanied by pre-C mutations gradually prevailed in two carriers as they seroconverted from HBeAg to anti-HBe and finally replaced wild-type HBV. Such a gradual shift, from wild-type HBV to pre-C mutants, is observed in carriers who seroconvert to anti-HBe (21). Mutations in the core promoter and those in the pre-C region co-occurred frequently, and they would collaborate toward the seroconversion to anti-HBe. The second G-to-A mutation at nt 1899 in the pre-C region, which is reported in HBV DNA clones with an HBeAg⁻ phenotype (3), was rather rare in HBV DNA clones with the G-to-A mutation at nt 1896 from the carriers studied.

Typical patterns emerged for mutations in the core promoter. Most frequent were an A-to-T mutation at nt 1762 and a G-to-A mutation at nt 1764, which were detected in HBV DNA clones from 40 (82%) of 49 carriers with core promoter mutants. Without point mutations in these hot spots, clones from some carriers possessed an 8-, 20-, or 21-bp deletion which involved both nt 1762 and nt 1764. All of these mutations affected the second of the three AT-rich regions in the core promoter, which is positioned 27 to 29 bp upstream of the transcriptional initiation sites (nt 1790 ± 1) for shorter pre-C mRNAs (39). AT-rich regions are detected about 25 bp upstream of the mRNA start points in cellular and viral promoters (4), and they are thought to bind with RNA polymerase for initiating transcription. Should the observed mutations in the core promoter interfere with the transcription of pre-C mRNA, they would disturb the synthesis of HBeAg. Recently, Laskus et al. (10) described HBV mutants with an 8-bp deletion involving the core promoter, as we observed in patterns k and l in Fig. 2, and proposed its negative effect on the transcription of pre-C mRNAs for an HBeAg⁻ phenotype.

TABLE 5. Mutations in the core promoter in HBV DNA clones from an asymptomatic carrier and a patient with chronic hepatitis who seroconverted from HBeAg to anti-HBe

Date of sampling	HBeAg or anti-HBe	HBV DNA titer	No. of clones tested	Clones with mutations in core promoter		No. (%) of clones with mutations in pre-C region at:	
				No. (%)	Pattern[a]	nt 1896	nt 1899
Asymptomatic carrier[b]							
5 Apr. 1983	HBeAg	10^9	18	0		0	0
21 July 1985	HBeAg	10^6	13	4 (31)	a	0	0
18 May 1993	Anti-HBe	10^2	20	20 (100)	a	0	0
Hepatitis patient[c]							
10 Feb. 1985	HBeAg	10^9	13	3 (21)	h	0	0
21 June 1986	HBeAg	10^9	16	14 (88)	h	0	0
22 Apr. 1989	Anti-HBe	10^8	18	17 (94)	h	0	0
2 Mar. 1990	Anti-HBe	10^9	19	19 (100)	h	0	0

[a] Patterns of mutations in the core promoter are shown in Fig. 2.
[b] A female asymptomatic carrier who was 29 years old at the date of the last sampling (corresponds to carrier 1 in Table 1).
[c] A male patient with chronic hepatitis who was 54 years old at the date of the last sampling (corresponds to patient 3 in Table 2).

This view is supported by the low in vitro expression of HBeAg by HBV mutants with an 8-bp deletion in the X gene (25).

Point mutations in the TATA sequence motif in eucaryotic promoters drastically decrease specific in vitro transcription (15, 37). Should this negative influence be extended to AT-rich regions in viral promoters, the change of the last A for T in the second AT-rich region (TTAAA) of most clones with mutations in the core promoter would be expected to decrease the transcription of shorter pre-C mRNAs starting at nt 1790 ± 1. The validity of this hypothesis could be evaluated by transfecting hepatoma cell lines with HBV mutants with various mutations involving the second AT-rich region in the core promoter for the expression of HBeAg.

The third AT-rich region (ATAAATT), which is preserved very well in reported HBV isolates, is proposed for the transcription of pregenome-C/P mRNA 23 bp downstream of it (39). It is remarkable that the third AT-rich region remained intact in all 766 HBV DNA clones from the 82 carriers examined. This observation reflects the indispensability of the ATAAATT sequence for transcription of the HBV pregenome and suggests distinct controls of pregenome-C/P mRNA and pre-C mRNAs for the evolution of HBV mutants with an $HBeAg^-$ phenotype. An anti-HBe-positive carrier mother transmitted HBV to her baby perinatally. Both mutants with mutations in the core promoter and pre-C mutants were detected in the mother. Mutants with mutations in the core promoter were found exclusively in the baby, who had high-titered HBV DNA without detectable HBeAg or anti-HBe in serum. These results indicate that HBV mutants with mutations in the core promoter are replication competent and that they would not be able to direct the synthesis of HBeAg in hosts. The possibility of HBeAg complexed with anti-HBe, preventing the detection of either antigen or antibody, needs to be excluded, however.

Some arguments against our hypothesis have to be addressed. HBV DNA clones with the core promoter mutations were detected predominantly or exclusively in some HBeAg-positive carriers. This finding indicates that mutations in the core promoter would reduce but probably not eliminate the expression of HBeAg. More importantly, the core promoter mutations could have reduced the level of all mRNAs derived from the core promoter rather than selectively inhibited pre-C mRNA transcription, which in effect reduces the production of HBeAg for anti-HBe seroconversion. High serum HBV DNA titers were observed in some carriers with anti-HBe who were infected with the core promoter mutants exclusively, as well as in a carrier who had seroconverted from HBeAg to anti-HBe along with gradual shift from wild-type HBV to core promoter mutants in his circulation, however. They suggest that mutations in the core promoter might not seriously affect transcription of the HBV pregenome. In vitro transfection studies are required to examine the effect of various core promoter mutations on the level of expression of various transcripts derived from the core promoter before our hypothesis is validated.

The product of the X gene transactivates viral and host genes (29, 30, 35). Observed mutations in the core promoter may or may not affect the transactivating function of the X protein. High HBV DNA levels in some anti-HBe-positive patients infected with mutants with mutations in the core promoter indicate that such mutations would have little, if any, influence on transactivation of the core promoter.

The G-to-A point mutation at nt 1896 in the pre-C region is proposed to be dependent on HBV genotypes, because it

TABLE 6. Mutations in core promoter of HBV DNA clones from a carrier mother with anti-HBe and her baby

Subject	Age (yr) (sex[a])	HBeAg/ anti-HBe	HBsAg titer	Anti-HBc titer	HBV DNA titer	No. of tested clones	No. (%) of clones with mutations in:	
							Core promoter[b]	Pre-C region[c]
Mother[d]	34 (F)	−/+	2^9	$\geq 2^{12}$	10^5	10	6 (60)	4 (40)
Baby	0.8 (M)	−/−	$\geq 2^{12}$	$\geq 2^{12}$	10^7	10	10 (100)	0

[a] F, female; M, male.
[b] Mutations were of pattern j in Fig. 2.
[c] G-to-A point mutation at nt 1896 to abort the synthesis and secretion of HBeAg; it was detected in all four clones without mutations in the core promoter.
[d] Corresponds to carrier 11 in Table 1.

rarely occurs in HBV DNA clones from anti-HBe-positive carriers in France, where genotype A HBV is prevalent (11). Genotype A HBV has C as nt 1858 in place of T in the other genotypes (20). In genotype A HBV, therefore, conversion of A to G at nt 1896, which makes a pair with nt 1858, disturbs the formation of the stem-loop structure (24) required for the pregenome encapsidation signal (8). In contrast, the G-to-A mutation at nt 1896 in HBV of the other genotypes strengthens the conformation of the stem-loop structure by generating an additional matched pair (34). These lines of evidence invite speculation that genotype A HBV mutants with an HBeAg$^-$ phenotype, should they occur, may possess mutations in the core promoter repressing the transcription of pre-C mRNAs.

REFERENCES

1. **Akahane, Y., T. Yamanaka, H. Suzuki, Y. Sugai, F. Tsuda, S. Yotsumoto, S. Omi, H. Okamoto, Y. Miyakawa, and M. Mayumi.** 1990. Chronic active hepatitis with hepatitis B virus DNA and antibody against e antigen in the serum. Disturbed synthesis and secretion of e antigen from hepatocytes due to a point mutation in the precore region. Gastroenterology **99:**1113–1119.
2. **Bruss, V., and W. H. Gerlich.** 1988. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. Virology **163:**268–275.
3. **Carman, W. F., M. R. Jacyna, S. Hadziyannis, P. Karayiannis, M. J. McGarvey, A. Markis, and H. C. Thomas.** 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet **ii:**588–591.
4. **Corden, J., B. Wasylyk, A. Buchwalder, C. P. Sassone, C. Kedinger, and P. Chambon.** 1980. Promoter sequences of eukaryotic protein-coding genes. Science **209:**1406–1414.
5. **Feitelson, M., L.-X. Duan, N. Horiike, and M. Clayton.** 1991. Hepatitis B X open reading frame deletion mutants isolated from atypical hepatitis B infections. J. Hepatol. **13:**S58–S60.
6. **Horikita, M., S. Itoh, K. Yamamoto, T. Shibayama, F. Tsuda, and H. Okamoto.** 1994. Differences in the entire nucleotide sequence between hepatitis B virus genomes from carriers positive for antibody to hepatitis B e antigen with and without active disease. J. Med. Virol. **44:**96–103.
7. **Iizuka, H., K. Ohmura, A. Ishijima, K. Satoh, T. Tanaka, F. Tsuda, H. Okamoto, Y. Miyakawa, and M. Mayumi.** 1992. Correlation between anti-HBc titers and HBV DNA in blood units without detectable HBsAg. Vox Sang. **63:**107–111.
8. **Junker-Niepmann, M. R. Bartenschlager, and H. Schaller.** 1990. A short cis-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J. **9:**3389–3396.
9. **Kim, S. H., S. P. Hong, S. K. Kim, W. S. Lee, and H. M. Rho.** 1992. Replication of a mutant hepatitis B virus with a fused X-C reading frame in hepatoma cells. J. Gen. Virol. **73:**2421–2424.
10. **Laskus, T., J. Rakela, M. J. Tong, M. J. Nowicki, J. W. Mosley, and D. H. Persing.** 1994. Naturally occurring hepatitis B virus mutants with deletions in the core promoter region. J. Hepatol. **20:**837–841.
11. **Li, J. S., S. P. Tong, Y. M. Wen, L. Vitvitski, Q. Zhang, and C. Trepo.** 1993. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. J. Virol. **67:**5402–5410.
12. **Lo, W. Y., and L. P. Ting.** 1994. Repression of enhancer II activity by a negative regulatory element in the hepatitis B virus genome. J. Virol. **68:**1758–1764.
13. **McLachlan, A., D. R. Milich, A. K. Raney, M. G. Riggs, J. L. Hughes, J. Sorge, and F. V. Chisari.** 1987. Expression of hepatitis B virus surface and core antigens: influences of pre-S and precore sequences. J. Virol. **61:**683–692.
14. **Miyakawa, Y., and M. Mayumi.** 1985. Hepatitis B e antigen and antibody (HBeAg/anti-HBe), p. 47–76. *In* R. J. Gerety (ed.), Hepatitis B. Academic Press, Inc., Orlando, Fla.
15. **Myers, R. M., K. Tilly, and T. Maniatis.** 1986. Fine structure genetic analysis of a beta-globin promoter. Science **232:**613–618.
16. **Naoumov, N. V., R. Schneider, T. Grotzinger, M. C. Jung, S. Miska, G. R. Pape, and H. Will.** 1992. Precore mutant hepatitis B virus infection and liver disease. Gastroenterology **102:**538–543.
17. **Nassal, M., M. Junker-Niepmann, and H. Schaller.** 1990. Translational inactivation of RNA function: discrimination against a subset of genomic transcripts during HBV nucleocapsid assembly. Cell **63:**1357–1363.
18. **Ogata, N., R. H. Miller, K. G. Ishak, and R. H. Purcell.** 1993. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology **194:**263–276.
19. **Okada, K., I. Kamiyama, M. Inomata, M. Imai, Y. Miyakawa, and M. Mayumi.** 1976. e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N. Engl. J. Med. **14:**746–749.
20. **Okamoto, H., F. Tsuda, H. Sakugawa, R. I. Sastrosoewignjo, M. Imai, Y. Miyakawa, and M. Mayumi.** 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J. Gen. Virol. **69:**2575–2583.
21. **Okamoto, H., S. Yotsumoto, Y. Akahane, T. Yamanaka, Y. Miyazaki, Y. Sugai, F. Tsuda, T. Tanaka, Y. Miyakawa, and M. Mayumi.** 1990. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J. Virol. **64:**1298–1303.
22. **Ou, J. H., H. Bao, C. Shih, and S. M. Tahara.** 1990. Preferred translation of human hepatitis B virus polymerase from core protein- but not from precore protein-specific transcript. J. Virol. **64:**4578–4581.
23. **Ou, J. H., O. Laub, and W. J. Rutter.** 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc. Natl. Acad. Sci. USA **83:**1578–1582.
24. **Pollack, J. R., and D. Ganem.** 1993. An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. J. Virol. **67:**3254–3263.
25. **Preisler-Adams, S., H. Schlayer, T. Peters, B. Hiller, W. Gerok, and J. Rasenack.** 1994. In vitro HBV gene expression of immunologically negative HBV variants. Gastroenterology **106:**A965. (Abstract.)
26. **Preisler-Adams, S., H. J. Schlayer, T. Peters, F. Hettler, W. Gerok, and J. Rasenack.** 1993. Sequence analysis of hepatitis B virus DNA in immunologically negative infection. Arch. Virol. **133:**385–396.
27. **Repp, R., C. Keller, A. Borkhardt, A. Csecke, S. Schaefer, W. H. Gerlich, and F. Lampert.** 1992. Detection of a hepatitis B virus variant with a truncated X gene and enhancer II. Arch. Virol. **125:**299–304.
28. **Roossinck, M. J., S. Jameel, S. H. Loukin, and A. Siddiqui.** 1986. Expression of hepatitis B viral core region in mammalian cells. Mol. Cell. Biol. **6:**1393–400.
29. **Seto, E., P. J. Mitchell, and T. S. Yen.** 1990. Transactivation by the hepatitis B virus X protein depends on AP-2 and other transcription factors. Nature (London) **344:**72–74.
30. **Spandau, D. F., and C. H. Lee.** 1988. *trans*-activation of viral enhancers by the hepatitis B virus X protein. J. Virol. **62:**427–434.
31. **Takahashi, K., M. Imai, Y. Miyakawa, S. Iwakiri, and M. Mayumi.** 1978. Duality of hepatitis B e antigen in serum of persons infected with hepatitis B virus: evidence for the nonidentity of e antigen with immunoglobulins. Proc. Natl. Acad. Sci. USA **75:**1952–1956.
32. **Takahashi, K., A. Machida, G. Funatsu, M. Nomura, S. Usuda, S. Aoyagi, K. Tachibana, H. Miyamoto, M. Imai, T. Nakamura, Y. Miyakawa, and M. Mayumi.** 1983. Immunochemical structure of hepatitis B e antigen in the serum. J. Immunol. **130:**2903–2907.
33. **Takeda, K., Y. Akahane, H. Suzuki, H. Okamoto, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1990. Defects in the precore region of the HBV genome in patients with chronic hepatitis B after sustained seroconversion from HBeAg to anti-HBe induced spontaneously or with interferon therapy. Hepatology **12:**1284–1289.
34. **Tong, S. P., J. S. Li, L. Vitvitski, A. Kay, and C. Trepo.** 1993. Evidence for a base-paired region of hepatitis B virus pregenome encapsidation signal which influences the patterns of precore mutations abolishing HBe protein expression. J. Virol. **67:**5651–5655.
35. **Twu, J. S., and R. H. Schloemer.** 1987. Transcriptional *trans*-

activating function of hepatitis B virus. J. Virol. **61**:3448–3453.

36. **Wang, Y., P. Chen, S. Wu, A. L. Sun, H. Wang, Y. A. Zhu, and Z. P. Li.** 1990. A new enhancer element, ENII, identified in the X gene of hepatitis B virus. J. Virol. **64**:3977–3981.
37. **Wasylyk, B., and P. Chambon.** 1981. A T to A base substitution and small deletions in the conalbumin TATA box drastically decrease specific in vitro transcription. Nucleic Acids Res. **9**:1813–1824.
38. **Will, H., W. Reiser, T. Weimer, E. Pfaff, M. Buscher, R. Sprengel, R. Cattaneo, and H. Schaller.** 1987. Replication strategy of human hepatitis B virus. J. Virol. **61**:904–911.
39. **Yaginuma, K., Y. Shirakata, M. Kobayashi, and K. Koike.** 1987. Hepatitis B virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA. Proc. Natl. Acad. Sci. USA **84**:2678–2682.
40. **Yee, J. K.** 1989. A liver-specific enhancer in the core promoter region of human hepatitis B virus. Science **246**:658–661.
41. **Yuh, C. H., Y. L. Chang, and L.-P. Ting.** 1992. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. J. Virol. **66**:4073–4084.
42. **Yuh, C. H., and L. P. Ting.** 1990. The genome of hepatitis B virus contains a second enhancer: cooperation of two elements within this enhancer is required for its function. J. Virol. **64**:4281–4287.