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Hepatitis B Viruses with Precore Region Defects Prevail in Persistently Infected Hosts along with Seroconversion to the Antibody against e Antigen

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The C gene of hepatitis B virus (HBV) codes for a nucleocapsid protein made of 183 amino acid residues and is preceded in phase by the precore (pre-C) region, encoding 29 residues. The pre-C-region product is required for the synthesis and secretion of hepatitis B e antigen (HBeAg), which is made of the C-terminal 10 amino acid residues of the pre-C-region product and the N-terminal 149 residues of the C-gene product. HBV mutants with pre-C-region defects prevailed in the circulation of three asymptomatic carriers as they seroconverted from HBeAg to the corresponding antibody (anti-HBe), and these mutants finally replaced nondefective HBV. HBV DNA clones were propagated from sera of an additional 15 carriers with anti-HBe and sequenced for the pre-C region. Essentially all HBV DNA clones (56 of 57 [98%]) revealed mutations that prohibited the translation of a functional pre-C-region product. A point mutation from G to A at nucleotide 83, converting Trp-28 (TGG) to a stop codon (TAG), was by far the commonest and was observed in HBV DNA clones from 16 (89%) of 18 carriers seropositive for anti-HBe. In addition, there were point mutations involving ATG codon to abort the translation initiation of the pre-C region, as well as deletion and insertion to induce frameshifts. Such mutations leading to pre-C-region defects were rarely observed in persistently infected individuals positive for HBeAg or in patients with type B acute hepatitis after they had seroconverted to anti-HBe. These results would indicate a selection of pre-C-defective mutants in persistently infected hosts, along with seroconversion to anti-HBe, by immune elimination of hepatocytes harboring nondefective HBV with the expression of HBeAg.

Genomic DNA of hepatitis B virus (HBV) has four open reading frames (42). They are the S gene, coding for an envelope protein (*env*); the C gene, coding for a core protein with group-specific antigen (*gag*); the P gene, coding for a putative DNA polymerase (*pol*); and the X gene, encoding a protein whose significance is not clear as yet.

The C gene codes for a core protein (p22), composed of 183 amino acid residues, and is preceded in phase by the precore (pre-C) region made of 29 codons that starts with an ATG initiation codon. The C-gene product assembles by itself to form core particles with the expression of hepatitis B core antigen (HBcAg) on the surface. Some core particles contain HBV DNA as well as DNA polymerase and make HBV particles when covered with envelope protein.

Recently, the pre-C region has attracted increasing attention because of its role in the synthesis and secretion of hepatitis B e antigen (HBeAg) (3, 15, 25, 28, 37, 40, 41, 43). Together with the C-gene product, the pre-C-region product forms the precursor of HBeAg. HBeAg precursor is directed to the endoplasmic reticulum membrane by a signal peptide encoded within the pre-C region and receives proteolytic modifications, for the eventual secretion of HBeAg protein (p15.5) from hepatocytes.

Individuals who are persistently infected with HBV have HBeAg in the circulation, but sooner or later they seroconvert to anti-HBe. The presence of HBeAg or anti-HBe in the circulation of HBV carriers is important in the clinical and epidemiological status of hosts, as well as on the biology of HBV (16). We determined the pre-C-region sequences of HBV DNA clones, propagated from sera of persistent carriers who had seroconverted to anti-HBe. Essentially all HBV DNA clones revealed various mutations that deprived the pre-C region of the capacity to direct the synthesis and secretion of HBeAg.

MATERIALS AND METHODS

Serological tests. HBsAg was determined by reverse passive hemagglutination with commercial assay kits (Mycell; Institute of Immunology Co. Ltd., Tokyo, Japan), and the titer was expressed as the highest twofold dilution of serum that gave a positive test. Subtypes of HBsAg were determined by solid-phase enzyme immunoassay (HBsAg SUBTYPE EIA; Institute of Immunology). HBeAg and anti-HBe were determined by solid-phase enzyme immunoassay (HBeAg/Ab EIA; Institute of Immunology).

Dot blot hybridization. HBV DNA was determined by dot blot hybridization (36) with a probe of cloned HBV DNA (pYRB259 [20]) labeled with [α-^{32}P]dCTP (Amersham Japan, Tokyo, Japan) by the multiprime labeling system (Amersham Japan).

Sera of persistent HBV carriers and patients with type B acute hepatitis. Three asymptomatic carriers seroconverted from HBeAg to anti-HBe during an observation period of 1 to 5 years. Sera were harvested from them at least twice, once while they had HBeAg and once after they seroconverted to anti-HBe. Sera were obtained from six asympto-

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matic carriers and six patients with type B chronic liver disease, all of whom were seropositive for anti-HBe, and from two asymptomatic carriers as well as one patient with type B chronic active hepatitis who were positive for HBeAg. Six sera were from China, Indonesia, and Kenya (three from asymptomatic carriers positive for HBeAg and the other three from those positive for anti-HBe). In addition, sera were obtained from seven patients with type B acute hepatitis after they had seroconverted to anti-HBe.

Isolation of DNA from sera. Serum (100 μl) was mixed with 300 μl of Tris hydrochloride buffer (13.3 mM, pH 8.0) containing 6.7 mM EDTA, 0.67% (wt/vol) sodium dodecyl sulfate, and proteinase K (133 μg/ml) and incubated at 70°C for 3 h. DNA was extracted with phenol-chloroform, and precipitated with ethanol in the presence of carrier tRNA (10 μg/ml). The precipitate was dissolved in 20 μl of 10 mM Tris hydrochloride buffer (pH 8.0) supplemented with 1 mM EDTA (TE buffer).

For sera harvested from individuals with anti-HBe, HBV particles were concentrated before isolation of DNA. For this purpose, 1 ml of serum was diluted with an equal volume of TE buffer and centrifuged through a cushion of 20% (wt/vol) sucrose in a Beckman SW60 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 257,000 × *g* for 2 h. The resulting pellet was suspended in 100 μl of TE buffer and processed as described above.

Amplification of the pre-C region by the PCR. Oligonucleotide primers were synthesized in a 380B DNA synthesizer (Applied Biosystems Japan, Tokyo, Japan). They were a 20-mer (5′-CATAAGAGGACTCTTGGACT-3′; nucleotides [nt] 1653 to 1672 of plus-strand HBV DNA) and a 24-mer (5′-AAAGAATTCAGAAGGCAAAAAAGA-3′; nt 1949 to 1972 of minus-strand HBV DNA with the underlined T replacing G-1966 to create an *Eco*RI cleavage site) capable of multiplying nt 1653 to 1972 covering the pre-C region (nt 1814 to 1900). The polymerase chain reaction (PCR) was performed by a modification of the procedure originally described by Saiki et al. (29). Briefly, 10 μl of DNA sample was heated at 95°C for 7 min to denature proteases, spun in a microcentrifuge for 5 s, and allowed to cool at room temperature. Target sequences were amplified in a 100-μl reaction volume with the use of the Gene Amp DNA amplification reagent kit (Perkin-Elmer Cetus, Norwalk, Conn.) as recommended by the manufacturer. The amplification was carried on for 30 cycles in a programmable DNA thermal cycler (Perkin-Elmer Cetus). The reaction was allowed to proceed at 94°C for 1 min, at 55°C for 1.5 min, and at 72°C for 3 min in each cycle. In the last cycle the reaction at 72°C was continued for 10 min to ensure complete DNA extension.

Molecular cloning and DNA sequencing. HBV DNA amplified by the PCR was digested with *Eco*RI and *Hin*cII (Takara Biochemicals, Kyoto, Japan), and the resulting fragment of 283 base pairs (bp), representing nt 1682 to 1964 and containing the pre-C region (nt 1814 to 1900), was cloned into M13mp11 bacteriophage vector (Amersham Japan) that had been cleaved with *Eco*RI and *Hin*cII. The pre-C region was then sequenced by the dideoxy-chain termination method (30).

RESULTS

HBV mutants with pre-C-region defects prevailing in three asymptomatic carriers along with seroconversion to anti-HBe. Three asymptomatic carriers seroconverted from HBeAg to anti-HBe during follow-up. HBV DNA clones were propagated from their sera, after amplification by PCR, and sequenced for the pre-C region (Table 1). In the HBeAg phase few, if any, HBV DNA clones displayed defects in the pre-C region. After the carriers had seroconverted to anti-HBe, however, the great majority of HBV DNA clones from them (57 of 59 [97%]) exhibited defects in the pre-C region. There was a point mutation from G to A at nt 83, converting Trp-28 (TGG) to a stop codon (TAG). The other defects, such as a point mutation converting the ATG initiation codon of the pre-C region to ATA and a 2-bp insertion inducing a frameshift, were detected in a few HBV DNA clones propagated from two of the carriers while they had HBeAg in the circulation.

TABLE 1. Defect in the precore region sequence of HBV DNA clones from three asymptomatic HBV carriers prevailing along the seroconversion from HBeAg to anti-HBe

Carrier no. and date of cloning[a]	HBsAg titer[b]	HBeAg or Anti-HBe	Amt of HBV DNA (pg/50 μl)[c]	No. of clones with a pre-C defect[d]/total no. (%)
Carrier 1				
11 June 1983	2^{12}	HBeAg	1	1/23 (4%)[e]
5 Jan. 1985	2^{11}	HBeAg	0.3	2/18 (11%)
30 July 1988	2^{11}	Anti-HBe	<0.1	10/10 (100%)
Carrier 2				
23 Jan. 1988	2^{14}	HBeAg	1	4/34 (12%)[f]
2 Nov. 1988	2^{9}	Neither	<0.1	4/17 (24%)
21 Feb. 1989	2^{10}	Anti-HBe	<0.1	28/30 (93%)
Carrier 3				
19 May 1981	2^{10}	HBeAg	0.3	0/36 (0%)
27 Jan. 1984	2^{10}	Anti-HBe	<0.1	19/19 (100%)

[a] At the last date of cloning, carrier 1 was a 9-year-old female, carrier 2 was a 27-year-old male, and carrier 3 was a 47-year-old female.
[b] Determined by reverse passive hemagglutination. HBsAg samples from all three cases were of subtype adr.
[c] Determined by dot blot hybridization (36). HBV DNA was detected in all negative sera after amplification by PCR (29).
[d] G-to-A point mutation at nt 83 converting Trp-28 to a stop codon, unless otherwise specified.
[e] G-to-A point mutation at nt 3, converting the ATG initiation codon to ATA.
[f] One had a 2-bp insertion (TT) between nt 12 and 13.

Mutations in the pre-C-region sequence of HBV DNA clones from 12 HBV carriers seropositive for anti-HBe. HBV DNA clones were propagated from 12 HBV carriers with anti-HBe, including 6 without biochemical or histological evidence of hepatic disease and 6 with various type B chronic liver diseases diagnosed by histological criteria (Table 2). Of 47 HBV DNA clones from these carriers, 46 (98%) revealed mutations that affected the function of the pre-C-region product; only 1 of 7 HBV DNA clones from carrier 7 did not show any defects in the pre-C region. Of the 12 carriers tested, 11 possessed HBV genomes with a G-to-A point mutation at nt 83 in the pre-C region. The other mutations were detected in HBV DNA clones from three carriers. Two of them (carriers 6 and 12) had two different species of HBV DNA with distinct defects in the pre-C region, i.e., a G-to-A point mutation at nt 83 accompanied by either a deletion of G in any one of nt 83 to 86 for a frameshift or a T-to-C point mutation at nt 2 involving the ATG initiation codon. The remaining one (carrier 4) had HBV DNA with a T-to-C point mutation at nt 2.

Mutations in the pre-C-region sequence of HBV DNA clones from blood donors in China, Indonesia, and Kenya. All 15 HBV carriers with anti-HBe studied thus far were Japanese.

TABLE 2. Defect in the precore region sequence of HBV DNA clones propagated from 12 HBV carriers seropositive for anti-HBe with or without clinical disease

Carrier no.	Age (yr) and sex[a]	Diagnosis[b]	HBsAg titer[c]	Amt of HBV DNA (pg/50 μl)[d]	No. of clones with pre-C defect[e]	
					(+)	(−)
1	22 (M)	AC	2^7	<0.1	4	0
2	34 (M)	AC	2^7	<0.1	4	0
3	41 (M)	AC	2^4	<0.1	3	0
4	42 (F)	AC	2^{10}	<0.1	3[f]	0
5	44 (M)	AC	2^5	<0.1	3	0
6	46 (M)	AC	2^{13}	<0.1	3[g]	0
7	29 (M)	CAH	2^{10}	<0.1	7	1
8	37 (M)	CAH	2^{10}	<0.1	3	0
9	47 (M)	LC	2^{10}	1	4	0
10	66 (F)	LC	2^9	<0.1	4	0
11	76 (M)	LC	2^8	3	4	0
12	53 (F)	LC and HCC	2^{10}	<0.1	4[h]	0

[a] M, Male; F, female.
[b] AC, Asymptomatic carrier state; CAH, chronic active hepatitis; LC, liver cirrhosis; HCC, primary hepatocellular carcinoma.
[c] Determined by reverse passive hemagglutination. Carriers 1, 3, 4, 5, 7, and 11 had HBsAg of subtype adw, and the remaining carriers had HBsAg of subtype adr.
[d] Determined by dot blot hybridization (36). HBV DNA was detected in all negative sera after amplification by PCR (29).
[e] G-to-A point mutation at nt 83, converting Trp-28 to a stop codon, unless otherwise specified.
[f] T-to-C point mutation at nt 2, converting the ATG initiation codon to ACG.
[g] One had a deletion of G in any of nt 83 to 86, and the remaining two had a G-to-A point mutation at nt 83.
[h] Two had a G-to-A point mutation at nt 83, and the remaining two had a T-to-C point mutation at nt 2.

Pre-C-region defects were searched for in anti-HBe-positive carriers in the other countries. Serum from a Chinese donor contained HBsAg of subtype ayw with a hemagglutination titer of 2^{10}. All three HBV DNA clones propagated from it possessed a G-to-A point mutation at nt 83 of the pre-C region, resulting in a premature termination at codon 28. Similarly, serum from an Indonesian donor (HBsAg titer, 2^7; subtype adw) gave rise to four HBV DNA clones, all of which had a G-to-A point mutation at nt 83. Three HBV DNA clones propagated from serum from a Kenyan donor (HBsAg titer, 2^{11}; subtype adw) showed another defect in the pre-C region. They all had an insertion of T between nt 26 and 27, inducing a frameshift after codon 9.

Absence of pre-C-region defects in HBV DNA clones from sera of HBeAg-positive carriers in Japan, China, Indonesia, and Kenya. HBV DNA clones were propagated from sera of two asymptomatic carriers and one patient with type B chronic active hepatitis in Japan, all of whom were seropositive for HBeAg, and the pre-C-region sequences were determined. No defects were found in any of 88 HBV DNA clones (Table 3). Also tested were HBeAg-positive sera from a Chinese blood donor (HBsAg titer, 2^{14}; subtype adr), an Indonesian donor (2^{15}; adw), and a Kenyan donor (2^{13}; ayw). Of 90 HBV DNA clones propagated from the three sera, none displayed any defects in the pre-C-region sequence.

Absence of pre-C-region defects in HBV DNA clones from sera of patients with type B acute hepatitis after they seroconverted to anti-HBe. Seven patients seroconverted from HBeAg to anti-HBe after an episode of type B acute hepatitis. HBV DNA clones were propagated from sera harvested after the patients seroconverted to anti-HBe and were sequenced for the pre-C region. None of 71 HBV DNA clones (6 to 14 clones from each patient) displayed any defects in the pre-C-region sequence.

TABLE 3. Lack of precore region defects in HBV DNA clones propagated from three HBV carriers seropositive for HBeAg

Carrier no.	Age (yr) and sex[a]	Diagnosis[b]	HBsAg titer[c]	Amt of HBV DNA (pg/50 μl)[d]	No. of clones with a pre-C defect/total no. (%)
1	20 (M)	AC	2^{15}	100	0/30 (0%)
2	39 (F)	AC	2^{13}	1	0/38 (0%)
3	25 (F)	CAH	2^{12}	0.1	0/20 (0%)

[a] M, Male; F, female.
[b] AC, Asymptomatic carrier state; CAH, chronic active hepatitis.
[c] Determined by reverse passive hemagglutination. Carrier 1 had HBsAg of subtype adw, and carriers 2 and 3 had HBsAg of subtype adr.
[d] Determined by dot blot hybridization (36).

Various defects in the pre-C-region sequence of HBV DNA clones from sera of persistent carriers. Figure 1 and Table 4 summarize six different mutations observed in the pre-C region of HBV DNA clones from 18 carriers during or after seroconversion to anti-HBe. Two of them, (*c*) and (*f*), were found in minor populations of HBV DNA clones from sera of two HBeAg-positive asymptomatic carriers who seroconverted to anti-HBe within 5 and 3 years of infection, respectively. They were categorized into point mutation, deletion, and insertion. A point mutation from G to A at nt 83 (*a*), converting Trp-28 (TGG) to a stop codon (TAG), was by far the commonest and was detected in 16 carriers (89%). The other point mutations involved the second (*b*) or the third nucleotide (*c*) of the ATG initiation codon and aborted the translation of the pre-C/C gene product at the start.

A deletion of G in any one of nt 83 to 86 (*d*) changed the amino acid sequence after Ala-28; this frameshift gave rise to stop codon 71, bringing to a halt any translation thereafter. An insertion of a single base pair between nt 26 and 27 (*e*) changed the amino acid sequence after His-9 and resulted in stop codon 33. Another insertion of 2 bp between nt 12 and 13 (*f*) changed the amino acid sequence after Phe-5 and created stop codon 9. Two kinds of HBV DNA clones with different defects were simultaneously observed in 4 of the 18 carriers. One of the two defects was invariably a G-to-A point mutation at nt 83 (*a*), which was accompanied by another point mutation (*b*) or (*c*), a deletion (*d*), or an insertion (*f*).

DISCUSSION

Individuals infected with HBV raise antibodies against a variety of viral antigens. Among them, the antibody response to HBeAg is important for both the clinical status of the host and the biology of HBV. During a period when HBeAg is detected in the circulation of the host, HBV replicates actively to maintain viremia, which is responsible for a high infectivity of HBeAg-positive blood, especially in low-dose inoculations (1, 18). If the host has hepatitis, the disease tends to be active in the HBeAg phase (13, 27). After the host seroconverts to anti-HBe, in contrast, the replication of HBV usually becomes inactive; this is often accompanied by the resolution of hepatitis.

The evolving knowledge of the function of the pre-C-region product in eliciting serum HBeAg led us to search for any changes in the pre-C-region sequence of HBV DNA in the circulation of carriers after they had seroconverted to anti-HBe. The recently introduced technique for amplifying

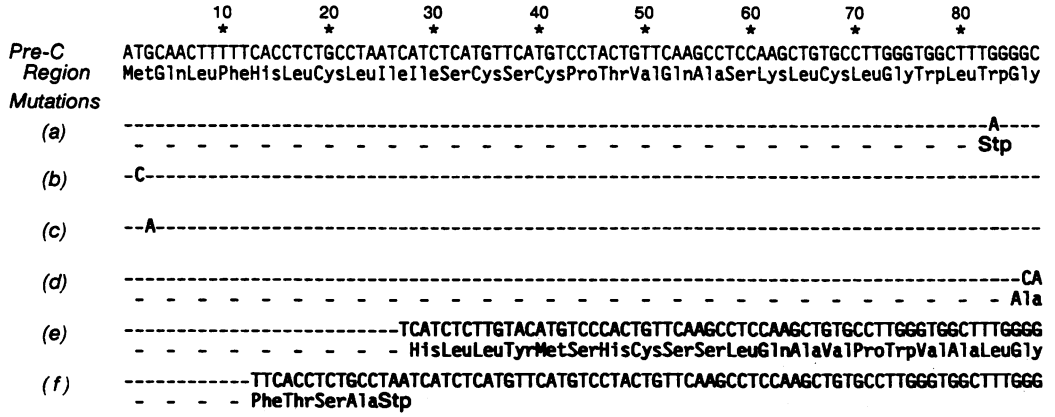

FIG. 1. Nucleotide sequence and deduced amino acid sequence of the pre-C region of HBV DNA. The sequence of nondefective HBV DNA clones is indicated at the top. The same sequence was observed in all 104 HBV DNA clones propagated from three asymptomatic carriers while they had HBeAg. The nucleotide sequence was also displayed by 178 HBV DNA clones from additional six HBeAg-positive carriers, as well as by reported HBV DNA clones from 14 HBeAg-positive carriers (7, 10, 19, 20, 22, 31–33), with minor substitutions not affecting the encoding of a functional pre-C-region product. Six different mutations are listed below. They are point mutations at nt 83 (*a*), nt 2 (*b*), and nt 3 (*c*); deletion of a G in any of nt 83 to 86 (*d*); and insertion of T after nt 26 (*e*) or TT after nt 12 (*f*). Origins of HBV DNA clones with mutations are as follows: (*a*) Table 1, carriers 1 to 3, Table 2, carriers 1 to 3 and 5 to 12, and two blood donors, one from China and the other from Indonesia; (*b*) Table 2, carriers 4 and 12; (*c*) Table 1, carrier 1; (*d*) Table 2, carrier 6; (*e*) a blood donor from Kenya; and (*f*) Table 1, carrier 2.

DNA by PCR (29) was particularly helpful in dealing with minute quantities of HBV DNA available in anti-HBe-positive sera. By using this technique, Kaneko et al. (14) have succeeded in detecting HBV DNA in sera from eight of nine patients with type B chronic hepatitis who were seropositive for anti-HBe.

All 18 HBV carriers with anti-HBe in the present study, regardless of their clinical status, origin of birth, or subtypes of HBsAg, had circulating HBV mutants without the capacity to direct synthesis and secretion of HBeAg. Essentially all the HBV DNA clones propagated from their sera had pre-C-region defects (113 of 116 [97%]). This contrasted with few, if any, pre-C-defective mutants among HBV DNA clones from nine HBV carriers with HBeAg (7 of 289 [2%]). Furthermore, in all three asymptomatic carriers who were monitored closely, the replacement of nondefective HBV by pre-C-defective mutants was clearly demonstrated pari passu with the seroconversion to anti-HBe.

TABLE 4. Various mutations in the pre-C region of HBV DNA clones from 18 carriers undergoing seroconversion to anti-HBe

Mutation	Involved nucleotide(s)	Changes induced by the mutation	No. of carriers with defective HBV DNA (%)[a]
Point mutation			
(*a*)	G to A at nt 83	Termination at codon 28	16 (89%)
(*b*)	T to C at nt 2	Initiation failure	2 (11%)
(*c*)	G to A at nt 3	Initiation failure	1 (6%)
Deletion			
(*d*)	G in any of nt 83 to 86	Frameshift at codon 29[b]	1 (6%)
Insertion			
(*e*)	T between nt 26 and 27	Frameshift at codon 9[c]	1 (6%)
(*f*)	TT between nt 12 and 13	Frameshift at codon 5[d]	1 (6%)

[a] Four carriers possessed two species of HBV with different mutations in the pre-C region, and they were counted twice.
[b] Responsible for stop codon 71.
[c] Responsible for stop codon 33.
[d] Responsible for stop codon 9.

Association of pre-C-defective mutants with anti-HBe, however, was not observed in transient HBV infection. None of 71 HBV DNA clones propagated from sera of seven patients with type B acute hepatitis, after they had seroconverted from HBeAg to anti-HBe, exhibited any defects in the pre-C-region sequence. This indicates that mutations in the pre-C region and replacement of nondefective HBV by pre-C-defective mutants would take time, probably years.

Defects in the pre-C region are not observed in any of the reported HBV DNA clones from 14 HBeAg-positive plasma samples (7, 10, 19, 20, 22, 31–33). A point mutation at nt 83 from G to A, as well as an insertion of 6 bp after nt 14, was found in HBV DNA clones from a plasma sample whose HBeAg–anti-HBe status was not specified (26, 45). Another HBV DNA clone, propagated from a plasma sample from an infected chimpanzee seropositive for HBeAg, had a G-to-A point mutation at nt 83 (44); the animal might have been seroconverting to anti-HBe, along with spontaneous mutation in the pre-C region of HBV, and harbored nondefective HBV as well as pre-C-defective mutants at the time of cloning. We have found pre-C-region defects in minor populations of HBV DNA clones propagated from two HBeAg-positive asymptomatic carriers who later seroconverted to anti-HBe (Table 1).

HBV is prone to mutations, with a rate of nucleotide substitutions per site per year estimated at 1×10^{-5} to 3×10^{-5} (19). This reflects the replication strategy of HBV that involves the reverse transcription of an RNA intermediate (39). Such a process is highly susceptible to point mutations owing to the lack of proofreading enzymes for correcting errors in duplication (12, 38). In persistent carriers of HBV, for instance, an A-to-G point mutation at nucleotide 365 or 479 in the S gene occurs, which induces a change in subtypic determinants of HBsAg in alleles, from d to y or from w to r, respectively (21). It is reasonable to presume that muta-

tions would occur at any sequences of HBV DNA, with a frequency proportional to the length of time the host harbors HBV. A mutant HBV may survive so long as the mutation does not interfere with virus formation. For mutants to become dominant and finally replace nondefective predecessors, however, a selection would have to operate in favor of them.

In hepatocytes infected with pre-C-defective HBV mutants, the synthesis and secretion of HBeAg would be prohibited. In hepatocytes infected with nondefective HBV, in contrast, HBeAg would be synthesized and secreted. As the host develops immunologic responses to HBeAg, hepatocytes secreting HBeAg would be subject to immune elimination, whereas hepatocytes incapable of secreting HBeAg would escape it. Such an immune selection may explain how pre-C-defective HBV replaced nondefective HBV along with seroconversion to anti-HBe in persistent carriers.

A variety of mutations were observed in HBV DNA clones from carriers, along with seroconversion to anti-HBe. Point mutation, deletion, and insertion all occurred. Among them, a point mutation from G to A at nt 83, converting Trp-28 (TGG) to a stop codon (TAG), was by far the commonest and was found in 16 (approximately 90%) of 18 carriers studied. The other two point mutations affected nt 2 and 3 and they both involved the ATG codon, resulting in interference with the translation initiation of the pre-C region. Deletion of a G in any of nt 83 to 86, as well as insertion of 1 and 2 bp after nt 26 and nt 12, respectively, induced a frameshift to inhibit coding for a functional pre-C-region product. It is likely that additional mutations in the pre-C region, different from those described here, will emerge as sequence data accumulate on HBV DNA clones.

Of possible note is a G-to-A point mutation at nt 83, which was observed in 104 (90%) of 116 clones propagated from 18 carriers with anti-HBe. There are 10 candidate positions in the pre-C region (nt 4, 21, 36, 42, 52, 69, 77, 78, 83, and 84) for which a single nucleotide replacement can create an in-phase stop codon. The fact is that only a point mutation at nt 83 was observed. This may reflect some regulations that prohibit random mutations, even though a substantial part of the pre-C region is singly encoded (62 bp [71%] spanning nt 1839 to 1900). The pre-C region is involved in a terminal redundant region of pregenomic RNA (6, 17, 46), which might prevent haphazard mutations. Clonal selection after random mutations provides another plausible explanation, however.

After the introduction in the early 1980s of molecular hybridization methods to detect HBV DNA in serum, some patients were noticed who were HBsAg and anti-HBe positive and also HBV DNA positive. Specific attention to this finding and its potential importance was first paid by Hadziyannis et al. (11), who found that 50% of patients in one HBsAg- and anti-HBe-positive carrier group in Greece were serologically HBV DNA positive and that most HBV DNA-positive patients had chronic liver disease independent of the HBeAg–anti-HBe status. The present study provides a molecular explanation for these findings and also indicates that spontaneous mutation of viral genomes and host selection of mutated virus leads to continued replication of virus in these HBsAg- and anti-HBe-positive carriers.

Whether or not HBV mutants, defective in the pre-C region, are capable of infecting hepatocytes is of virological interest. Persistent HBV infection with pre-C-defective mutants, after the host seroconverts to anti-HBe, would support an infectious activity of such mutants. Furthermore, duck hepadnavirus mutants defective in the pre-C region can infect susceptible ducks, thereby indicating that virus formation, replication, and infection are independent of the expression of the pre-C region (5, 34). Of possible relevance are deletion mutants of Moloney murine leukemia virus, incapable of coding for a pre-C-like region with an initiator codon upstream of the *gag* gene, which can replicate and infect susceptible mice (9, 35).

It is now necessary to find whether there are any differences in the pathogenic potential between nondefective HBV and pre-C-defective mutants. A clue might be hidden in a group of patients with type B chronic active hepatitis who have anti-HBe together with high-titer HBV DNA in the serum (2, 11). It is reasonable to presume that judging from anti-HBe in the serum, patients with this disease would circulate HBV with pre-C-region defects. Since hepatocytes of these patients are abundant in core protein (p22), with the expression of HBcAg and HBeAg determinants (40), humoral and cellular immune responses against HBcAg and/or HBeAg would attack them for causing continued hepatic injuries. In patients with HBeAg-positive hepatitis, such an assault might be diverted by circulating HBeAg that would act as a decoy. Indeed, HBV coexisting with anti-HBe may be associated with high virulence. Fulminant hepatitis B, the most drastic sequel of HBV infection, often leading to death, frequently occurs in recipients of blood from anti-HBe-positive carriers (8, 23, 24).

ADDENDUM

In work published after submission of this report, Carman et al. (4) described a G-to-A point mutation at nt 83 in the pre-C region of HBV DNA in sera from patients with anti-HBe- and HBV DNA-positive hepatitis.

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