# Mutations in Core Nucleotide Sequence of Hepatitis B Virus Correlate with Fulminant and Severe Hepatitis

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## Abstract

**Infection with hepatitis B virus leads to a wide spectrum of liver injury, including self-limited acute hepatitis, fulminant hepatitis, and chronic hepatitis with progression to cirrhosis or acute exacerbation to liver failure, as well as an asymptomatic chronic carrier state. Several studies have suggested that the hepatitis B core antigen could be an immunological target of cytotoxic T lymphocytes. To investigate the reason why the extreme immunological attack occurred in fulminant hepatitis and severe exacerbation patients, the entire precore and core region of hepatitis B virus DNA was sequenced in 24 subjects (5 fulminant, 10 severe fatal exacerbation, and 9 self-limited acute hepatitis patients). No significant change in the nucleotide sequence and deduced amino acid residue was noted in the nine self-limited acute hepatitis patients. In contrast, clustering changes in a small segment of 16 amino acids (codon 84–99 from the start of the core gene) in all seven adr subtype infected fulminant and severe exacerbation patients was found. A different segment with clustering substitutions (codon 48–60) was also found in seven of eight adw subtype infected fulminant and severe exacerbation patients. Of the 15 patients, 2 lacked precore stop mutation which was previously reported to be associated with fulminant hepatitis. These data suggest that these core regions with mutations may play an important role in the pathogenesis of hepatitis B viral disease, and such mutations are related to severe liver damage. (*J. Clin. Invest.* 1993. 91:1206–1213.) Key words: mutation • HBc gene • polymerase chain reaction • fulminant hepatitis • cytotoxic T lymphocyte**

## Introduction

Individuals infected with hepatitis B virus (HBV)[1] have several types of disease including self-limited acute hepatitis, fulminant hepatitis, and chronic active hepatitis going to cirrhosis or severe acute exacerbation with hepatic failure as well as asymptomatic healthy carrier. The hepatic injury caused by HBV is immune mediated (1). Although recent studies revealed that a precore defective mutant correlated with fulminant hepatitis and severe exacerbation of chronic hepatitis (2–4), the mechanism by which the precore defective mutant induces severe hepatocyte injury is not known yet (2–4). HBV DNA contains four open reading frames. One of them is the C gene, which encodes a core peptide (HBcAg) (5). The C gene encodes 183–185 amino acid residues of the nucleocapsid protein, and is preceded by the precore region starting with an initiation codon and encoding 29 amino acid residues (5). The hepatitis B core antigen (HBcAg) has been postulated to be an immunological target of cytotoxic T lymphocytes (CTL) (6–9).

Since studies on endogenously processed viral peptides demonstrated that a peptide as small as 8 amino acids could be recognized by CTL (10, 11), it should be supposed that if there is a "pressure" from CTL, one may be able to find substitutions in amino acid residues in a restricted segment of the core gene. In previous studies, it was found that clustering missense mutations in a certain region (codon 84–101) of core gene were related to the liver damage in patients with adr subtype chronic HBV infection (12, 13). Changes in such a region may help to predict the outcome of B viral liver disease. In this study, we analyzed the nucleotide sequence of the entire precore and core region of HBV DNA from patients who died of either fulminant hepatitis B or a severe exacerbation of chronic hepatitis B by the PCR and direct sequencing methods (14–16).

## Methods

*Patients.* Sera were taken from 15 patients with fatal type B hepatitis, including 5 patients with fulminant hepatitis and 10 patients with severe exacerbation of chronic hepatitis (Table I).

*Group A, adr subtype infected group (Table I).* Two fulminant hepatitis and 5 severe exacerbation patients were infected with adr subtype HBV (Table I). One of them appeared to be infected by his 30-y-old wife who was a type B chronic hepatitis patient and hepatitis B e antigen (HBeAg) positive. Serum samples obtained from the woman during her husband's hospitalization were also examined. At the same time, serum samples were taken from six self-limited acute hepatitis patients. One of them was infected by his HBeAg-positive wife who was a 25-yr-old asymptomatic healthy carrier. Serum sample was obtained from the infectious too.

*Group B, adw subtype infected group (Table I).* Three fulminant and five severe exacerbation patients were infected with adw subtype HBV (Table I). In one severe exacerbation patient (patient 20), sequential analysis was performed. At the same time, serum samples were taken from three self-limited acute hepatitis patients.

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1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, HBsAg, hepatitis surface antigen; HBV, hepatitis B virus.

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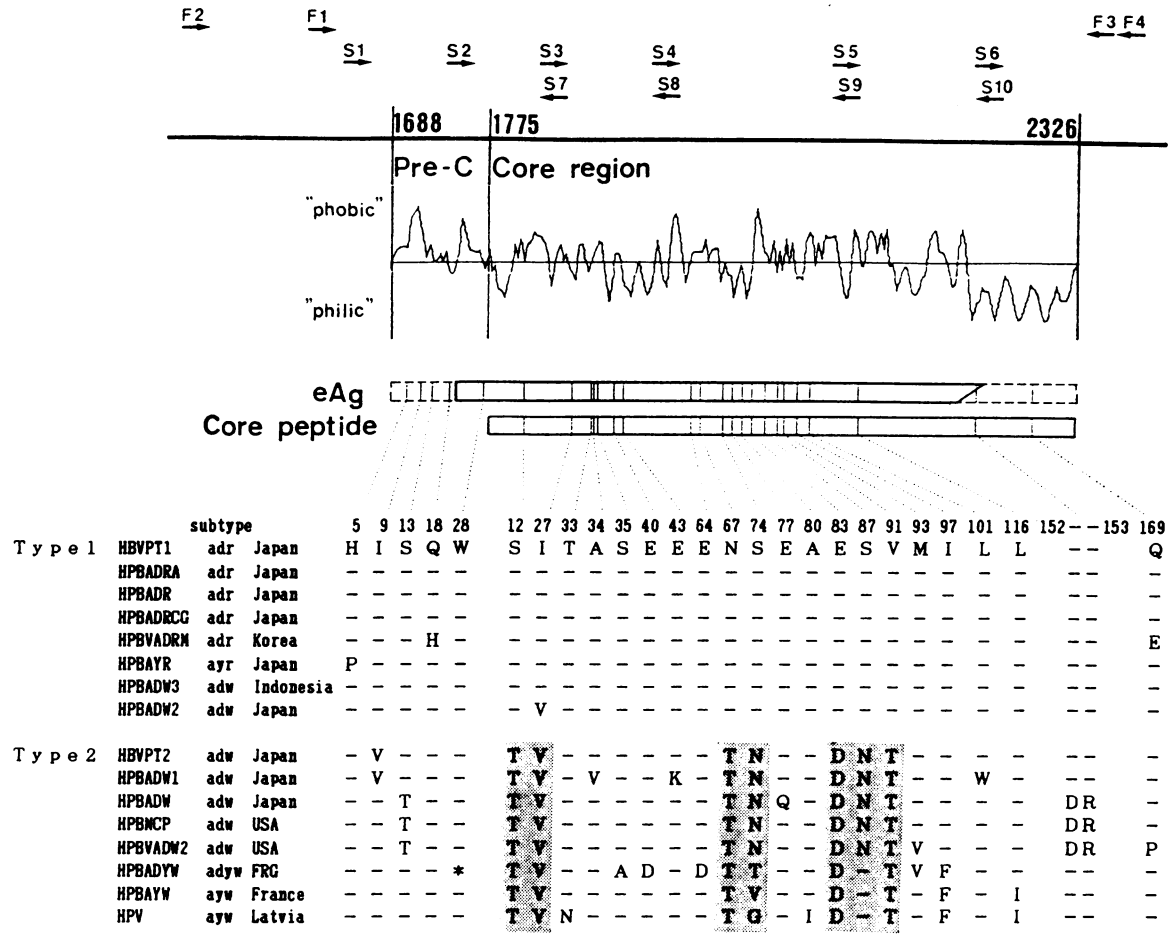
*Table I. Clinical and Laboratory Data of Patients with Fulminant Hepatitis, Severe Exacerbation, Self-limited Acute Hepatitis, and Donor*

| Age/Sex | HBeAg/Ab | IgM anti-HBc | Substitution in codons (84 99)* | Pre-C‡ mutation | Liver histology |
|---|---|---|---|---|---|
| Adr Subtype infected patients | | | | | |
| Fulminant patients | | | | | |
| 1. 35/M | +/− | + | + | − | Massive necrosis |
| 2. 50/M | −/− | + | + | + | Submassive necrosis |
| Severe exacerbation patients | | | | | |
| 3. 7/M | +/− | + | + | − | Submassive necrosis |
| 4. 53/M | −/+ | + | + | + | Submassive necrosis |
| 5. 68/M | −/+ | + | + | + | Submassive necrosis |
| 6. 42/F | −/+ | + | + | + | Submassive necrosis |
| 7. 60/M | −/+ | + | + | + | Submassive necrosis |
| Acute self-limited hepatitis patients | | | | | |
| 8. 31/M | +/− | + | − | − | Acute viral hepatitis |
| 9. 25/M | +/− | + | − | − | Acute viral hepatitis |
| 10. 38/M | +/− | + | − | − | Acute viral hepatitis |
| 11. 42/M | +/− | + | − | − | Acute viral hepatitis |
| 12. 38/— | −/+ | + | − | − | Acute viral hepatitis |
| 13. 31/M | −/+ | + | − | − | Acute viral hepatitis |
| Infectious sources, donors, of patient 1 and patient 8 | | | | | |
| 1′ 30/F | +/− | − | + | − | Chronic active hepatitis |
| 8′ 25/F | +/− | − | − | − | Nonspecific change |
| Adw Subtype infected patients | | | | | |
| Fulminant patients | | | | | |
| 14. 47/M | +/− | − | + | − | Submassive necrosis |
| 15. 50/F | −/− | + | + | + | Massive necrosis |
| 16. 59/F | −/− | + | + | + | Massive necrosis |
| Severe exacerbation patients | | | | | |
| 17. 68/F | −/+ | − | + | + | Submassive necrosis |
| 18. 64/M | −/+ | − | + | + | Submassive necrosis |
| 19. 67/F | −/+ | + | + | + | Cirrhosis and necrosis |
| 20. 64/F | −/+ | + | + | + | Submassive necrosis |
| 21. 50/M | −/+ | + | + | + | Cirrhosis and necrosis |
| Acute self-limited hepatitis patients | | | | | |
| 22. 56/M | +/− | + | − | − | Acute viral hepatitis |
| 23. 27/M | +/− | + | − | − | Acute viral hepatitis |
| 24. 22/F | +/− | + | − | − | Acute viral hepatitis |

* Number of codons from the start of C gene.
‡ A stop codon mutation at codon 28 from the start of precore gene.

*HBV markers.* Hepatitis B surface antigen (HBsAg), HBeAg, anti-HBe, and IgM class antibody to HBcAg (anti-HBc IgM) were detected with solid-phase radioimmunoassay (Abbott Laboratories, North Chicago, IL). Subtypes of HBsAg were assayed by enzyme immunoassay (special immunology Laboratories, Tokyo, Japan). Anti-hepatitis C virus antibody was measured by enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan). All the samples were negative for anti-hepatitis C virus antibody, and all the 15 fatal patients and 10 self-limited patients were positive for anti-HBc IgM.

*Amplification and sequencing of core and precore region of HBV DNA.* To amplify the precore (87 nucleotides or 29 amino acid residues) and the C gene (549–555 nucleotides or 183–185 amino acid residues), we prepared several sets of synthetic oligonucleotide primers according to the reported sequence of adr subtype by Kobayashi et al. (17) (Fig. 1): sense primers, F1 (nt 1618–1635, 5′-GGGAGGAGATTAGGTTAA-3′), F2 (nt 1518–1537, 5′-AAGCTCTTACATAAGAGGAC-3′); antisense primers, F3 (nt 2328–2347, 5′-ACCTTATGAGTCCAAGGGAT-3′), F4 (nt 2363–2382, 5′-GTACAGTAGAAGAATAAAGC-3′). By these primers, a segment of HBV DNA spanning 865 nucleotides from 1518 to 2382, which comprises the entire precore and core region, was amplified by PCR (14). These primers were synthesized by the phosphoramidite method. Amplification of HBV DNA was performed basically by the method described previously (15). Briefly, 100-μl reaction mixtures containing 10 μl of specimen DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 1 μM each of the two oligonucleotide primers, 200 μM dNTP, 200 μg/ml of gelatin, and 2 U of *Thermus aquaticus* DNA polymerase were overlaid with 100 μl of mineral oil. Samples were heated at 95°C for 1 min (denaturation), cooled to 50°C for 1 min (annealing), and heated to 70°C for 2 min (extension). These steps were repeated for 35




| | | subtype | | 5 | 9 | 13 | 18 | 28 | 12 | 27 | 33 | 34 | 35 | 40 | 43 | 64 | 67 | 74 | 77 | 80 | 83 | 87 | 91 | 93 | 97 | 101 | 116 | 152 | 153 | 169 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | H | I | S | Q | W | S | I | T | A | S | E | E | E | N | S | E | A | E | S | V | M | I | L | L | -- | Q |
| Type 1 | HBVPT1 | adr | Japan | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | HPBADRA | adr | Japan | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | -- | - |
| | HPBADR | adr | Japan | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | -- | - |
| | HPBADRCG | adr | Japan | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | -- | - |
| | HPBVADRM | adr | Korea | - | - | - | H | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | -- | E |
| | HPBAYR | ayr | Japan | P | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | -- | - |
| | HPBADW3 | adw | Indonesia | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | -- | - |
| | HPBADW2 | adw | Japan | - | - | - | - | - | - | V | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | -- | - |
| Type 2 | HBVPT2 | adw | Japan | - | V | - | - | - | T | V | - | - | - | - | - | - | T | N | - | - | D | N | T | - | - | - | - | -- | - |
| | HPBADW1 | adw | Japan | - | V | - | - | - | T | V | - | V | - | - | K | - | T | N | - | - | D | N | T | - | - | W | - | -- | - |
| | HPBADW | adw | Japan | - | - | T | - | - | T | V | - | - | - | - | - | - | T | N | Q | - | D | N | T | - | - | - | - | DR | - |
| | HPBNCP | adw | USA | - | - | T | - | - | T | V | - | - | - | - | - | - | T | N | - | - | D | N | T | - | - | - | - | DR | - |
| | HPBVADW2 | adw | USA | - | - | T | - | - | T | V | - | - | - | - | - | - | T | N | - | - | D | N | T | V | - | - | - | DR | P |
| | HPBADYW | adyw | FRG | - | - | - | - | * | T | V | - | - | A | D | - | D | T | T | - | - | D | - | T | V | F | - | - | -- | - |
| | HPBAYW | ayw | France | - | - | - | - | - | T | V | - | - | - | - | - | - | T | V | - | - | D | - | T | - | F | - | I | -- | - |
| | HPV | ayw | Latvia | - | - | - | - | - | T | V | N | - | - | - | - | - | T | G | - | I | D | - | T | - | F | - | I | -- | - |

*Figure 1.* Schematic presentation of precore and core peptide with hydorophobicity profile of HBV. Locations of amplification and sequencing primers used for PCR and direct sequencing are shown by arrows. Sense amplification primers, F1 and F2; antisense amplification primers, F3 and F4. Sense sequencing primers, S1 through S6; antisense sequencing primers, S7 through S10. Nucleotide sequences of these primers are described in the text. Deduced amino acid residues of precore and core peptide, different from prototype adr HBVPT1, are plotted against the corresponding position of peptides. Amino acid residues were numbered from start of precore or core gene. The dashed line indicates the same amino acid with prototype HBVPT1. *A*, alanine; *C*, cysteine; *D*, aspartic acid; *E*, glutamic acid; *F*, phenylalanine; *G*, glycine; *H*, histidine; *I*, isoleucine; *K*, lysine; *L*, leucine; *M*, methionine; *N*, asparagine; *P*, proline; *Q*, glutamine; *R*, arginine; *S*, serine; *T*, threonine; *V*, valine; *W*, tryptophan; *Y*, tyrosine; *termination codon. Quoted references are as follows: HPBADRA (17); HPBADR (18); HPBADRCG (19); HPBVADRM (20); HPBAYR (21); HPBADW3 (22); HPBADW2 (22); HPBADW1 (22); HPBADW (18); HPBNCP (23); HPBVADW2 (24); HPBADYW (25); HPBAYW (26); HPV (27).

cycles. After the final step of amplification, each sample (10 $\mu$l) was applied to an 8% acrylamide gel.

For direct sequencing of a portion of HBV DNA, the PCR products were centrifuged in microconcentrators (Centricon 30; Amicon Corp., Danvers, MA). To sequence the amplified segment bi-directionally, we prepared several sequencing primers: sense primers, S1 (nt 1644–1662, 5′-GTACTAGGAGGCTGTAGGC-3′), S2 (nt 1739–1758, 5′-CAAGCCTCCAAGCTGTGCCT-3′), S3 (nt 1828–1847, 5′ - TTTGCCTTCTGACTTCTTTC - 3′), S4 (nt 1934 – 1953 , 5′-GCACTCAGGCAAGCTATTCT-3′), S5 (nt 2103–2121, 5′-TTGGAAGAGAAACTGTTCT-3′), S6 (nt 2228–2247, 5′-CGAGGCAGGTCCCCTAGAAG-3′); antisense primers, S7 (nt 1828–1847, 5′-GAAAGAAGTCAGAAGGCAAA-3′), S8 (nt 1934–1953, 5′-AGAATAGCTTGCCTGAGTGC-3′), S9 (nt 2103–2121, 5′-AGAACAGTTTCTCTTCCAA-3′), S10 (nt 2228–2247, 5′-CTTCTAGGGGACCTGCCTCG-3′) (Fig. 1). One sequencing primer was radiolabeled with [$^{32}$P]ATP and T4 polynucleotide kinase. From 1 to 10 pmol of microconcentrator purified PCR product and 5 pmol of $^{32}$P-labeled sequencing primer were combined in 12 $\mu$l of 50 mM KCl, 50 mM Tris (pH 8.0), 5 mM $MgCl_2$ and 10 mM dithiothreitol. The direct sequencing of the PCR products was performed as previously described (16).

## Results

### *Comparison with reported amino acid residues of core region*

14 previously reported and two of our own prototype (HBVPT1, HBVPT2) amino acid residues of the precore and core region of HBV are aligned in Fig. 1 according to their subtypes (17–27). HBVPT1 is the sequence of HBV from acute hepatitis patient 8 who was infected with adr subtype HBV, and HBVPT2 is the sequence of HBV from acute hepatitis patient 22 who was infected with adw subtype HBV. We plotted the codons which showed amino acid residues different from the prototype adr sequence HBVPT1 against the corresponding positions of the precore and core peptide (Fig. 1). These sequences were divided into two groups. The first group

(from HVBPT1 to HPBADW2) had almost the same core amino acid sequence and was defined as type 1 core sequence. Four reported sequences of adr subtype showed well-conserved amino acid residues of core peptides (Fig. 1). The second group (from HVBPT2 to HPV) was different from type 1 core sequence at almost the same positions (core codons No. 12[T], 27[V], 67[T], 74[N or T or V or G], 83[D], 87[N], 91[T]) and was defined as type 2 core sequence. The core amino acid sequences of all our patients infected with adw subtype (group B) were type 2 core sequence. All the HBV core sequences in these patients (group B) were different from adr prototype HBVPT1 at precore codon #9 (V) and core codon No. 12(T), 27(V), 67(T), 74(N), 83(D), 87(N), 91(T).

*Nucleotide sequence and deduced amino acid residue of core gene*

*Group A: adr subtype infected patients (Table I).* The entire nucleotide sequence of the core gene of HBV was explored in all 13 patients and 2 infectious source, "donors," of sexual partners. Different nucleotides from HBVPT1 were found at 94 locations (Table II). The distributions of substitutions in different disease groups were as follows: 14 substitutions in two fulminant hepatitis patients with a mean of 7.0 substitutions, 44 substitutions in five severe exacerbation patients with a mean of 8.8, and 36 substitutions in six self-limited acute hepatitis with a mean of 6.0. Among those 94 substitutions, 65 were "silent" (without changes in the deduced amino acid residue) and 29 were "missense" (with alteration in the deduced amino acid residue). All the 36 nucleotide changes observed in self-limited acute hepatitis patients were silent and the amino acid residues were identical to HBVPT1 (Fig. 2, group *A*). In contrast, all the 29 missense changes were only found in fulminant and severe acute exacerbation patients (Table II).

The location of 29 amino acids substitutions in the core region were shown in Figs. 2 and 3, group *A*. Of these 29 amino acid changes, 12 (41.4%) clustered in a small segment of 16 amino acids (codon 84–99 from the start of the core gene, 8.7% of the core peptide (Figs. 2 and 3, group *A*). At least one amino acid substitution in this segment was found in the two fulminant and five severe exacerbation patients (Table I, Figs. 2 and 3, group *A*). At other regions of the core peptide, 5 amino acid substitutions were found in a 15 amino acid segment from 20 to 35 and 6 amino acid substitutions were found in a 26 amino acid segment from 130 to 156 (Figs. 2 and 3, group *A*). Sporadic amino acid substitutions were recognized in codons 5, 13, 60, 74, and 105 (Figs. 2 and 3, group *A*). Most of the substitutions (L to A at 84, S to G or N at 87, Y to H at 88, I to L at 97, Q to H at 99) (Fig. 3, group *A*) were not identified in any of the

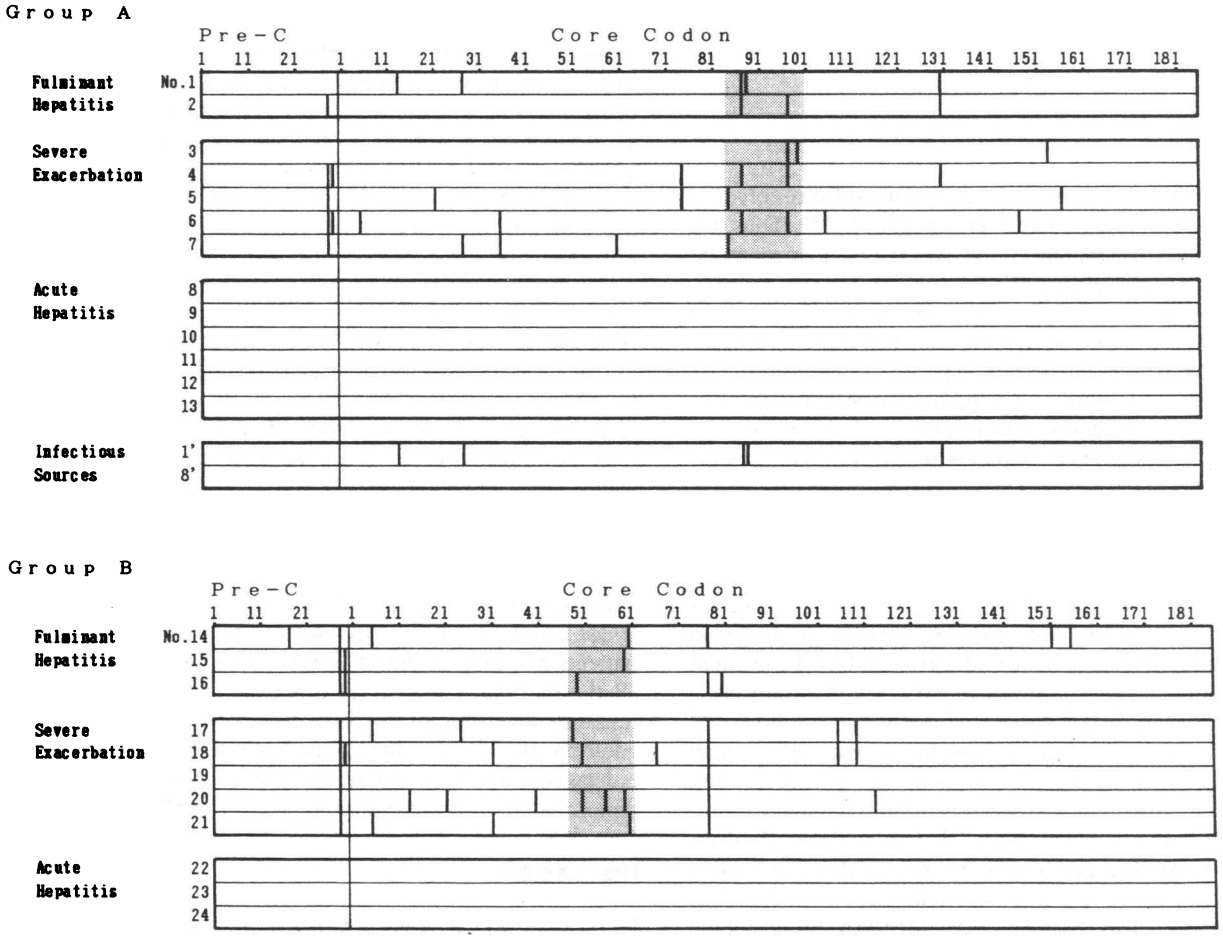



*Figure 2.* Deduced amino acid residues of precore and core peptide from Group *A*, 13 adr subtype infected patients and two infectious sources (donors) and Group *B*, 11 adw subtype infected patients. Only the deduced amino acid residues different from the prototypes HBVPT1 and HBVPT2 are indicated by vertical solid lines. The mutation clustering regions (codons 48–60 and codons 84–99) are shadowed.

*Table II. Numbers of Nucleotide Substitutions and Amino Acid Changes in Precore Region and Core Gene*

| | Nucleotide substitution | Silent substitution | Missense mutation |
|---|---|---|---|
| Adr Subtype infected patients | | | |
| Core gene | | | |
| 2 fulminant | 14 | 6 | 8 |
| 5 exacerbation | 44 | 23 | 21 |
| 6 acute hepatitis | 36 | 36 | 0 |
| Total | 94 | 65 | 29 |
| Precore region | | | |
| 2 fulminant | 1 | 0 | 1 |
| 5 exacerbation | 8 | 2 | 6 |
| 6 acute hepatitis | 0 | 0 | 0 |
| Total | 9 | 2 | 7 |
| Adw Subtype infected patients | | | |
| Core gene | | | |
| 3 fulminant | 24 | 15 | 9 |
| 5 exacerbation | 47 | 24 | 23 |
| 3 acute hepatitis | 7 | 7 | 0 |
| Total | 78 | 46 | 32 |
| Precore region | | | |
| 3 fulminant | 6 | 0 | 6 |
| 5 exacerbation | 6 | 0 | 6 |
| 3 acute hepatitis | 0 | 0 | 0 |
| Total | 12 | 0 | 12 |

14 previously reported sequences except one substitution at 87 (S to N change) (Figs. 1 and 3, group *A*) (17–27). Thus, the majority of missense mutations in type 1 core sequences of this study were de novo substitutions.

The nucleotide sequences of core gene in the two infectious sources, "donors," were completely matched those of infected patients. Donor 1′ was a chronic hepatitis patient and the infected partner had fulminant hepatitis (patient 1, donor 1′, Table I). This pair had core substitutions at 13, 27, 87, 88, 130 (Figs. 2 and 3, group *A*). The other is an asymptomatic healthy carrier and the infected patient was with self-limited acute hepatitis (patient 8, donor 8′, Table I). This pair had no core substitution (Fig. 2, group *A*). Both donors (1′, 8′) were HBeAg positive.

*Group B: adw subtype infected patients (Table I).* The entire nucleotide sequence of the core gene of HBV was studied in all 11 patients. Different nucleotides from HBVPT2 (patient 22) were found at 78 locations (Table II). The distributions of substitutions in different disease groups were as follows: 24 substitutions in three fulminant hepatitis patients with a mean of 8.0 substitutions, 49 substitutions in five severe exacerbation patients with a mean of 9.8, and 7 substitutions in three self-limited acute hepatitis with a mean of 2.3. Among those 80 substitutions, 46 were silent and 34 were missense. All seven nucleotide changes observed in self-limited acute hepatitis were silent and the amino acid residues were identical to prototype HBVPT2 (Fig. 2, group *B*). In contrast, all the 34 missense changes were only found in fulminant and severe acute exacerbation patients (Table II).

The location of 34 amino acids substitutions in the core region were shown in Figs. 2 and 3, group *B*. Of these 34 amino acid changes, 9 (26.5%) clustered in a small segment of 13 amino acids (codons 48–60 from the start of the core gene, 7.1% of the core peptide) (Figs. 2 and 3, group *B*). At least one amino acid substitution in this segment was found in all the three fulminant and four of five severe exacerbation patients (Table I, Figs. 2 and 3, group *B*). Seven out of eight fatal patients had amino acid substitution at codon 77. And 5 amino acid substitutions were found in a 9 amino acid segment from 105 to 113 (Figs. 2 and 3, group *B*). Sporadic amino acid substitutions were recognized in codons 5, 13, 21, 24, 31, 40, 66, 80, 150, and 155 (Figs. 2 and 3, group *B*). All of the substitutions were not identified in any of the 14 reported sequences except one substitution at 77 (E to Q change) (patients 16 and 18, 19) (Figs. 1 and 3, group *B*) (17–27). Thus, the majority of missense mutations in type 2 core sequences of this study were also de novo substitutions.

The clinical course of a patient with severe exacerbation of chronic hepatitis B is shown in Fig. 4. Patient 20 was a 64-yr-old woman who had been known to be positive for HBsAg and anti-HBe since 1980. In 1982, her serum levels of alanine aminotransferase increased, but she recovered from the episode (Fig. 4). Thereafter, she was asymptomatic. Jaundice was found in November 1983, so she was admitted to Chiba University Hospital. Hepatic failure developed gradually, and she died in February 1984. A postmortem examination revealed submassive necrosis of the liver. HBV-DNA sequences in precore and core region were studied in serum samples on five different occasions (Fig. 4). The sequences of three samples before severe exacerbation were completely identical. Interestingly two additional amino acid substitutions were found at core codons 21 (S to V change) and 55 (L to I change) after the severe exacerbation (Fig. 4). These two substitutions were de novo too.

*Precore mutation and core substitutions*

A defective virus with a stop codon mutation at the 3′ end of the precore region incapable of encoding HBeAg has been found in various liver diseases (2–4, 28–30). In the present study, presence of a precore stop codon at the 28th codon from the beginning of precore region was analyzed in relation to the changes of core region (Figs. 2 and 3). In fulminant hepatitis patients, one had no defective precore mutation and the others were with defective precore mutation (Figs. 2 and 3). In severe exacerbation patients, one had no defective precore mutation and the other nine patients had defective precore mutations (Figs. 2 and 3). None of the nine acute self-limited hepatitis patients had precore defective mutations (Fig. 2). Therefore, all the fulminant and severe exacerbation patients had core mutations, but not all the patients had precore mutations. In addition, all the self-limited acute hepatitis patients were without precore and core mutations. The two infectious sources, donor, had no precore mutation (Figs. 2 and 3).

## Discussion

It is not known why there are differences in the severity of liver disease among HBV-infected individuals (31). Previous studies focused primarily on host factors. For example, an attempt was made to define a relation between B viral liver disease and

Group A

| | | Pre-C | | Core Codon | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 28 | 29 | 5 | 13 | 21 | 27 | 35 | 60 | 74 | 84 | 87 | 88 | 97 | 99 | 105 | 130 | 147 | 153 | 156 |
| | | W | G | P | V | S | I | S | L | S | L | S | Y | I | Q | I | P | T | G | P |
| Fulminant Hepatitis | No. 1 | – | – | – | A | – | V | – | – | – | – | N | H | – | – | – | L | – | – | – |
| | 2 | * | – | – | – | – | – | – | – | – | – | G | – | L | – | – | T | – | – | – |
| Severe Exacerbation | 3 | – | – | – | – | – | – | – | – | – | – | – | – | L | H | – | – | – | C | – |
| | 4 | * | D | – | – | – | – | – | – | G | – | G | – | L | – | – | T | – | – | – |
| | 5 | * | – | – | – | V | – | – | – | G | A | – | – | – | – | – | – | – | – | T |
| | 6 | * | D | Q | – | – | – | A | – | – | – | G | – | L | – | T | – | A | – | – |
| | 7 | * | – | – | – | – | V | T | V | – | A | – | – | – | – | – | – | – | – | – |
| Donor | 1' | – | – | – | A | – | V | – | – | – | – | N | H | – | – | – | L | – | – | – |

Group B

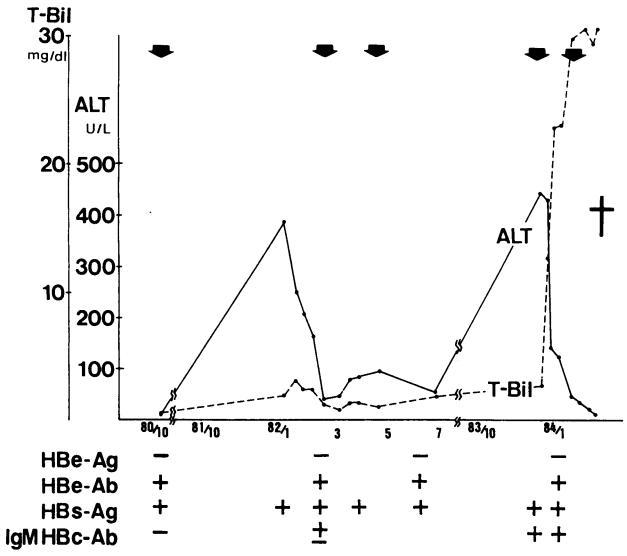
| | | Pre-C | | | Core Codon | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 17 | 28 | 29 | 5 | 13 | 21 | 24 | 31 | 40 | 48 | 49 | 50 | 55 | 59 | 60 | 66 | 77 | 80 | 105 | 109 | 113 | 151 | 155 |
| | | V | W | G | P | V | S | F | L | E | C | S | P | L | I | L | M | E | A | I | T | E | R | S |
| Fulminant Hepatitis | No. 14 | F | * | – | T | – | – | – | – | – | – | – | – | – | – | V | – | D | – | – | – | – | Q | T |
| | 15 | – | * | D | – | – | – | – | – | – | – | – | – | – | V | – | – | – | – | – | – | – | – | – |
| | 16 | – | * | D | – | – | – | – | – | – | – | T | – | – | – | – | – | Q | G | – | – | – | – | – |
| Severe Exacerbation | 17 | – | * | – | H | – | – | Y | – | – | V | – | – | – | – | – | – | D | – | V | M | – | – | – |
| | 18 | – | * | D | – | – | – | – | I | – | – | – | A | – | – | – | T | Q | – | V | M | – | – | – |
| | 19 | – | * | – | – | – | – | – | – | – | – | – | – | – | – | – | – | Q | – | – | – | – | – | – |
| | 20 | – | * | – | – | A | V | – | – | D | – | – | S | I | V | – | – | D | – | – | – | D | – | – |
| | 21 | – | * | – | T | – | – | – | I | – | – | – | – | – | – | V | – | D | – | – | – | – | – | – |

*Figure 3.* All the changes of amino acid residues differ from the prototypes (HBVPT1, HBVPT2) are shown. Codon numbers and prototype amino acid sequence are given in the top two rows. Dashes indicate identity to the prototype. Core codons are numbered from the beginning of the core region. Amino acid residues are expressed by universal genetic codes (see the legend for Fig. 1). The underlined amino acid residues indicate the coexistence of prototype sequence. (Group *A*) adr subtype infected patients and infectious source (donor). (Group *B*) adw subtype infected patients.

the HLA system (32). Recently, several reports indicated that precore defective mutant hepatitis B virus was related to fulminant hepatitis and severe exacerbation (2–4). However, the mechanism of severe hepatic injury caused by precore defective mutant virus was still not understood. Furthermore, it had been suggested that the core peptide (HBcAg) was an immunological target of CTL in HBV infection (6–9). So we analyzed the precore and C gene of HBV in fatal patients.

Advanced molecular biological technology such as polymerase chain termination method permitted the analysis on very little amounts of viral nucleotide sequences from specimens of patients with various hepatitis B liver diseases. In previous studies, precore and core gene had been investigated in patients with chronic HBV infection (12, 13). Despite active virus replication, no significant hepatocellular injury has been observed for 3–8 yr in 10 asymptomatic healthy carriers and the deduced amino acid residue sequences of the core peptide were identical to the prototype sequences in all 10 patients (13). In contrast, a mutation clustering region of 18 amino acid residues (codons 84–101 of core region) was found in 15 chronic active liver disease patients infected with adr subtype HBV (13). In this study, it was shown that all the fatal hepatitis cases infected with adr subtype HBV had amino acid substitutions in this mutation clustering region (84–99 of core gene) and that all except one patient infected with adw subtype HBV had amino acid substitutions in another mutation clustering region (48–60 of core gene). On the contrary, all the precore and core sequences from self-limited acute hepatitis patients were identical to prototypes of each subtype (HBVPT1 and HBVPT2). The results indicated that HBV with the missense mutations in mutation clustering regions of core gene might be related to the severity of liver damage in fulminant hepatitis and severe exacerbation of chronic hepatitis and that mutation clustering regions were different between core sequences type 1 and 2 (Fig. 2). Therefore, it could be supposed that codons 84–99 in type 1 core sequences might be with strongly immunological epitopes and that the type 2 core sequences might have clustering substitutions at different region (core codons 48–60) because frequent amino acid substitutions already existed in codons 84–99 of type 2 core sequences.

The fact that wild-type hepatitis B virus could be transmitted from mother to children in generations implied that wild-type HBV without core mutation might cause a lesser immune response than HBV with core mutation in humans. This phenomenon also emphasized that HBV with core mutation might have the higher possibility of being strongly attacked by




| | | 9/27/80 | 2/22/82 | 4/14/82 | 12/17/83 | 1/7/84 |
|---|---|---|---|---|---|---|
| Prototype 2 Pre-C Codon 28 | W | * | * | * | * | * |
| Core Codon 13 | V | A | A | A | A | A |
| 21 | S | – | – | – | V | V |
| 50 | P | S | S | S | S | S |
| 55 | L | – | – | – | I | I |
| 59 | I | V | V | V | V | V |
| 77 | E | D | D | D | D | D |
| 113 | E | D | D | D | D | D |

*Figure 4.* Clinical course of a 64-yr-old patient with a severe exacerbation of chronic hepatitis B (patient 20). Serum total bilirubin (*dashed line*) and alanine aminotransferase (*solid line*) levels and the result of assays for HBe antigen, anti-HBe, and anti-HBc IgM are shown. The patient had been known to be positive for HBsAg and ani-HBe since 1980. Jaundice and hepatic failure developed in November 1983, and the assay for anti-HBc IgM became positive. HBV DNA sequences in the precore and core regions were studied in serum samples taken on five occasions at the arrows (27 October 1980; 22 February 1982; 14 April 1982; 17 December 1983; 7 January 1984). Amino acid substitutions from prototype 2 are shown in these five samples.

CTL. By the sequential analysis of a patient with a severe exacerbation of chronic hepatitis B (Patient 20) (Fig. 4), the core sequences of early three samples before hepatic failure were completely matched (Fig. 4). However, two additional amino acid substitutions were found at core codons 21 (S to V change) and 55 (L to I change) after severe exacerbation (Fig. 4). These data suggested that the target of her severe exacerbation should be core protein with the former sequence and this HBV was almost wiped out, and that the HBV with the latter core sequence might be an escaped mutant and remained after immune clearance.

Studies on influenza and vascular stomatitis viruses displayed that the targets of cytotoxic T lymphocytes are endogenously processed viral peptides derived from nucleocapsid protein which are as small as 8–10 amino acid residues (10, 11). Thus, it is tempting to speculate that the mutation clustering region found only in patients with hepatic injury might be immunological targets of CTL.

The precore protein is highly hydrophobic, and acts as a "leader sequence" to engage the precore and core peptide into the membrane of the endoplasmic reticulum (33, 34). It has been shown that the secretory HBeAg peptide is derived from the cleavage of the precore and core peptide at its $NH_2$ and COOH terminus after translocation into the endoplasmic reticulum (33, 34). Secretory HBeAg consists of a part of the 3′ end of the precore region (10 amino acid residues) and the majority of the core region (lacking only 34–36 amino acid residues at its COOH terminus) (35) (Fig. 1). Thus, secretory HBeAg contains a mutation clustering regions. Because all the fatal patients had core substitutions but not all the patients were with precore mutations and because the precore mutation was found only in patients who already had core mutations or after the appearance of a mutation in the core region (13), it might be assumed that the virus mutates to induce a stop codon at the 3′ end of the hydrophobic leader sequence to cease continuous secretion of the immunological target (modified core peptide), and to avoid the attack from CTL. Of particular interest was that two HBeAg positive infectious donors apparently induced different outcome in the recipients, namely one fatal (patient 1) and the other completely resolved (patient 8). Both donors lacked the precore mutation, but the one who induced fatal outcome in the recipient showed core gene mutations. These data indicate that only the presence of core mutations (without the precore stop codon) is sufficient to produce the most severe liver injury. This notion is compatible with the previous findings that fulminant hepatitis is not necessarily associated with precore mutation (4, 36, 37).

The natural course of B viral liver diseases is quite variable (31), and a good prognostic marker is lacking. In this study, absence of substitutions in the mutation clustering regions was related to an uneventful course, and by contrast the presence of such changes was associated with severe B viral liver disease. Thus, the most important clinical implication of our findings, perhaps, is that the presence of an amino acid substitution in the mutation clustering regions might be used as an indicator of worsening of the disease, and the most dangerous infectious source may be chronic hepatitis carriers with the core mutations.

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