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Relationship Between Mutation in PreS Region and Intracellular Retention of Envelope Protein in Patients with Chronic Hepatitis B

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It has been shown that accumulation of hepatitis B surface antigen (HBsAg) in hepatocytes is associated with liver cell damage. Release and retention of envelope protein is controlled by the major surface antigen promoter and the balance of large, middle and small envelope proteins. To investigate the mechanism of accumulation of envelope protein *in vivo*, we analyzed the relationship between the sequence of the preS region and retention of envelope protein histologically in patients with chronic hepatitis B. We studied 15 patients with histologically diagnosed chronic hepatitis B. Accumulation of envelope protein was studied immunohistochemically using polyclonal anti-HBs antibody. Sequence analysis was performed using direct sequencing methods following polymerase chain reaction (PCR) amplification. Strongly positive HBsAg staining was observed in 9 patients, and the remaining patients were negative for HBsAg. Sequence analysis revealed that the mutations were clustered in and around the Sp1 binding site within the major surface promoter region in the patients who exhibited accumulation of envelope protein. Mutations in and around the Sp1 binding site may affect activity of the major surface antigen promoter and the balance between the various envelope proteins, resulting in accumulation of envelope protein in hepatocytes.

Introduction

Hepatitis B virus (HBV) contains three distinct but related envelope surface proteins (L, M and S) produced by alternative translation of a single long open reading frame[1]. All of these proteins contain a 24-kDa C-terminal domain, which comprises the entirety of the S protein. The two larger proteins, M and L, also contain N-terminal chains of 55 and 174 amino acids, respectively. The expression of envelope proteins is regulated by several different promoters. Previous studies have shown that two of these promoters control transcripts that encode the three surface proteins. Transcripts 2.6 kb in length, derived from the upstream preS1 promoter, are translated into the full-length L protein. The 5′-heterogeneous transcripts 2.2 kb in length, derived from the downstream major surface antigen promoter, are translated into the M and S proteins, with translation initiated at internal in-frame ATG codons of the surface gene. The most numerous of these three envelope proteins is the S protein, which participates in virion formation and is secreted from host cells as a subviral envelope protein or

Table Summary of pertinent clinical information, serological profiles of hepatitis B virus (HBV) infection, and pathological findings of 15 patients with chronic hepatitis B

Case no.	Age/Sex	Serum HBsAg *	Serum HBeAg *	Serum anti-HBe **	Serum ALT	Serum DNA polymerase ***	Histology	Intra hepatic	
								HBsAg	HBcAg
1	37 M	173	+	+	33	85	CAH	+++	−
2	37 M	162	+	+	186	470	CAH	+++	n.d.
3	47 M	161	+	−	407	346	SN	+++	−
4	24 F	68	+	−	172	14,484	CAH	+++	diffuse
5	32 F	100	+	−	380	1,650	CAH	+++	focal
6	27 M	88	+	−	73	16,623	CPH	+++	diffuse
7	44 M	102	+	−	296	3,378	CAH	+++	diffuse
8	51 M	119	−	+	293	35,464	SN	+++	diffuse
9	50 M	156	−	+	79	447	CAH	+++	−
10	23 F	57	+	−	26	2,023	LH	−	focal
11	32 M	89	+	−	1,003	186	CAH	−	−
12	37 M	166	+	−	460	14	CAH	−	−
13	30 M	154	−	+	615	2,900	LH	−	−
14	44 F	142	−	+	361	430	CAH	−	−
15	48 F	71	−	+	73	15	LH	−	−

*: Values of HBsAg and HBeAg are shown as ratio of absorbance of sample to that of negative control. **: Values of anti-HBe are shown as inhibition percentage. ***: DNA polymerase and serum alanine aminotransferase are shown as count per minute and international unit /mL, respectively.
SN: submassive necrosis, CAH: chronic active hepatitis, CPH: chronic persistent hepatitis, LH: lobular hepatitis. n.d.: not done, −: not detected.

in viral particles[2]. This secretion process is inhibited by overexpression of L protein[3)~5)] ; the increase in the amount of L protein, relative to that of S protein, results in intracellular retention of envelope protein. The regulation of expression of the three types of envelope protein is not fully understood. However, Bulla et al[6] have reported that the major surface antigen promoter, which is located downstream of the preS1 promoter, can decrease the amount of preS1 transcripts. Therefore, it is possible that intracellular retention of envelope protein is caused by decreased activity of the major surface antigen promoter due to mutation in this region.

Intracellular retention of envelope protein is known as the ground glass phenomenon. Ground glass cells contain large amounts of HBV surface protein that accumulates within dilated smooth vesicles [7]. This phenomenon is clinically important in terms of liver injury, because ground glass cells spontaneously die and are readily killed by interferon gamma[8]. Recently, an *in vitro* study has shown that retention of envelope protein is caused by mutations in major surface antigen promoter sequences in the preS region[9]. Also, mutations and a deletion have been detected in the major surface antigen promoter region in patients with HBV-induced fibrosing cholestatic hepatitis (FCH) who underwent liver transplantation[10].

In light of these findings, we decided to study the relationship between mutation in the preS region and intracellular retention of HBV envelope protein.

Patients and Methods

Patients

We studied 15 patients: 10 males and 5 females; age range, 23 to 51 years (mean, 32 ± 8years) (Table). They were diagnosed with HBV-induced chronic hepatitis based on histological findings,

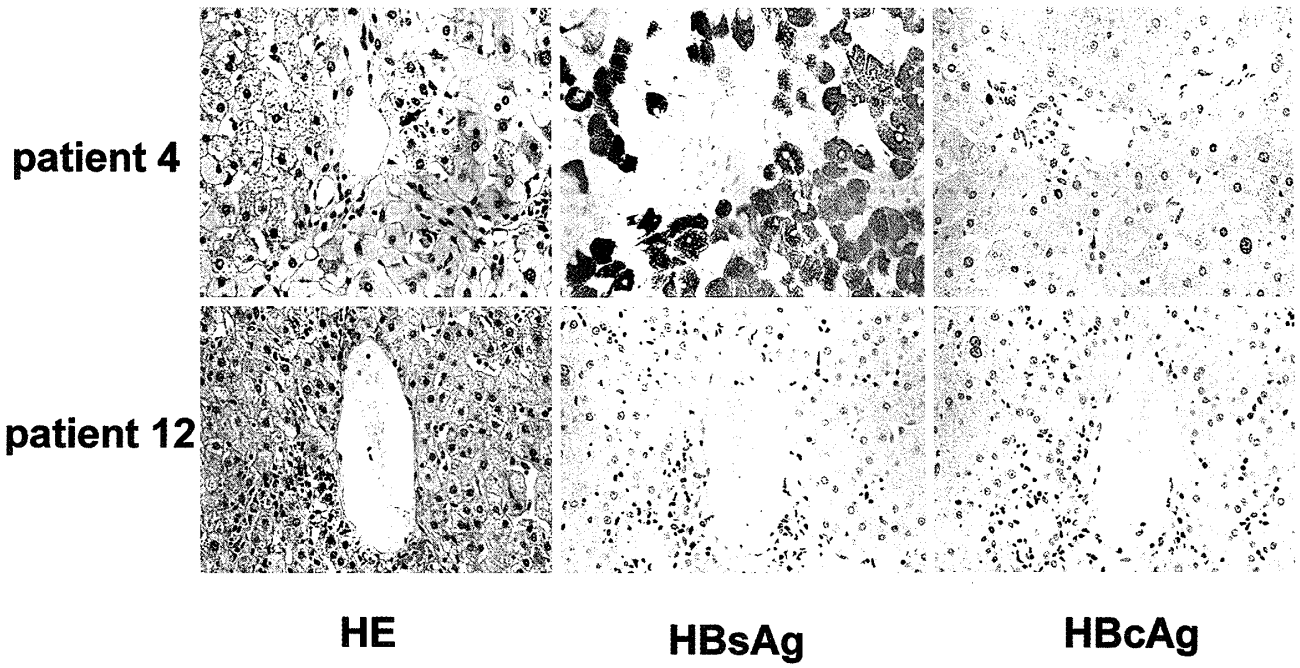

Fig. 1 Immunohistochemical staining for HBsAg (anti-HBs) and for HBcAg (anti-HBc) in liver tissue obtained from patient case no. 4 and no. 12. (original magnification, ×80)

clinical manifestations, blood biochemistry, and the continuous detection of HBsAg during at least a one-year follow-up. The clinical data shown in Table was obtained at the time of liver biopsy.

The patients were divided into two groups. The 'HBsAg-positive' group included patients whose hepatocytes showed strongly positive HBsAg staining, and the 'HBsAg-negative' group included patients whose hepatocytes were negative for HBsAg (Fig. 1).

Written informed consent was obtained from all patients or their next of kin to participate in all procedures associated with the study, which was approved by the Ethics Committee of Tokyo Women's Medical University.

The staining assays for HBsAg and HBcAg in hepatocytes were performed by incubating zinc formalin-fixed tissue sections with polyclonal rabbit anti-HBsAg and anti-HBcAg antisera (Dako, Carpinteria, CA, USA), respectively[11]. Serum was tested for HBsAg, HBeAg and anti-HBe antibody, using Ausria-II and Abbott-HBe kits (Abbott Laboratories, North Chicago, IL). Quantity of HBV was determined by measuring endogenous DNA polymerase activity[12]. Liver histology was performed, and serum ALT values were measured.

Amplification of HBV DNA by PCR and sequencing of PCR products

A 50-μL sample of serum was diluted with 50 μL of sterile distilled water and mixed with 100 μL of 25 mM sodium acetate containing 2.5 mM EDTA (pH 8.0), 1% SDS, 2 μg/mL of proteinase K and 10 μg/mL of yeast t-RNA as a carrier. Following digestion at 65 ℃ for 2 hours, viral nucleic acids were extracted (twice with phenol-chloroform and once with chloroform), ethanol-precipitated and suspended in water. The preS region and precore regions of the extracted HBV DNA, comprising an 808-bp sequence between nt 2762 and nt 293, were amplified by polymerase chain reaction (PCR). The primers were 5′-TGGAAGGCTGGCATTCTATA-3′ (sense; nu-

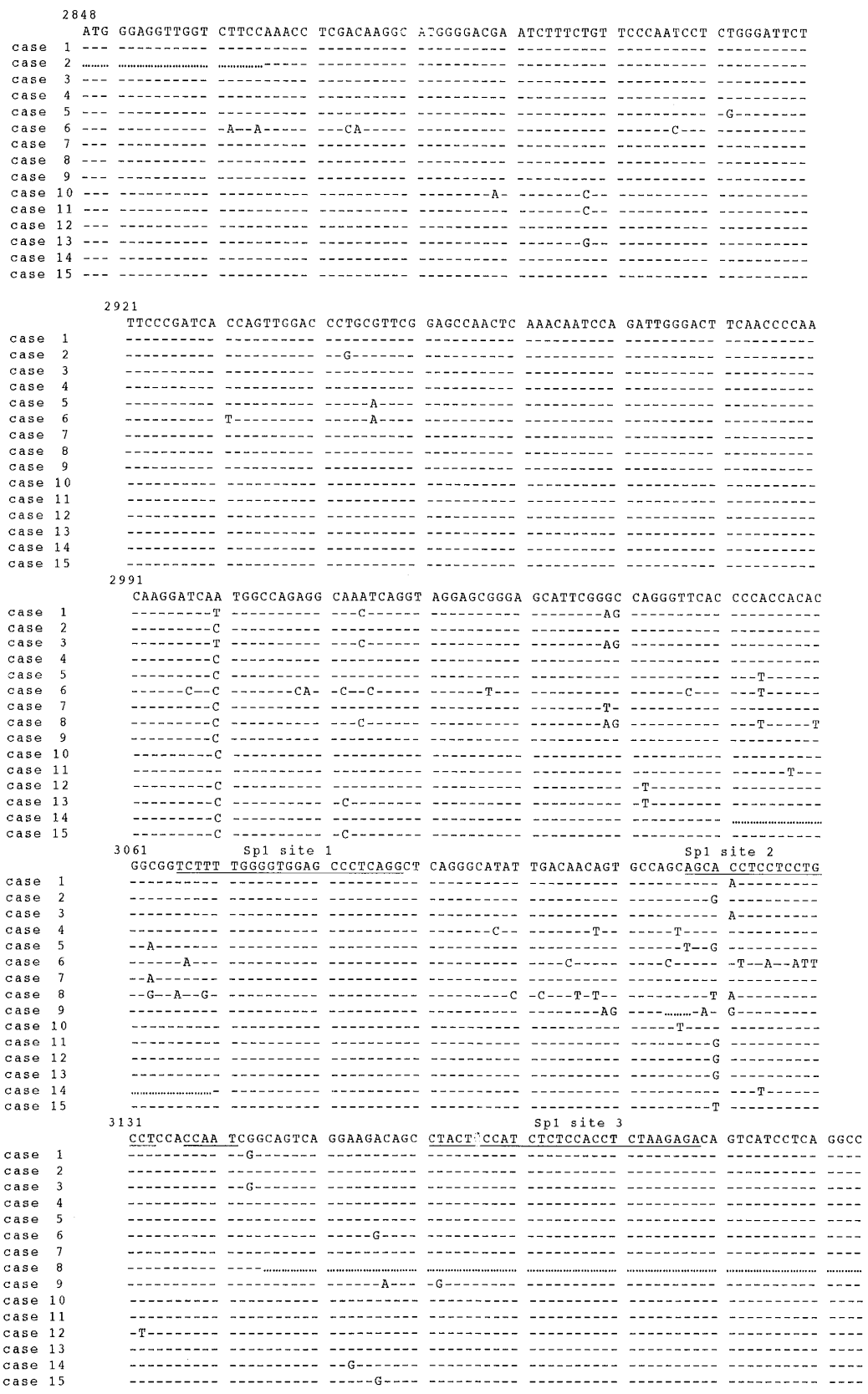

Fig. 2 Nucleotide sequences of the preS1 region from 15 patients, compared with reference sequences[13] indicated at the top.
Sp1 binding site and CCAAT box are underlined.

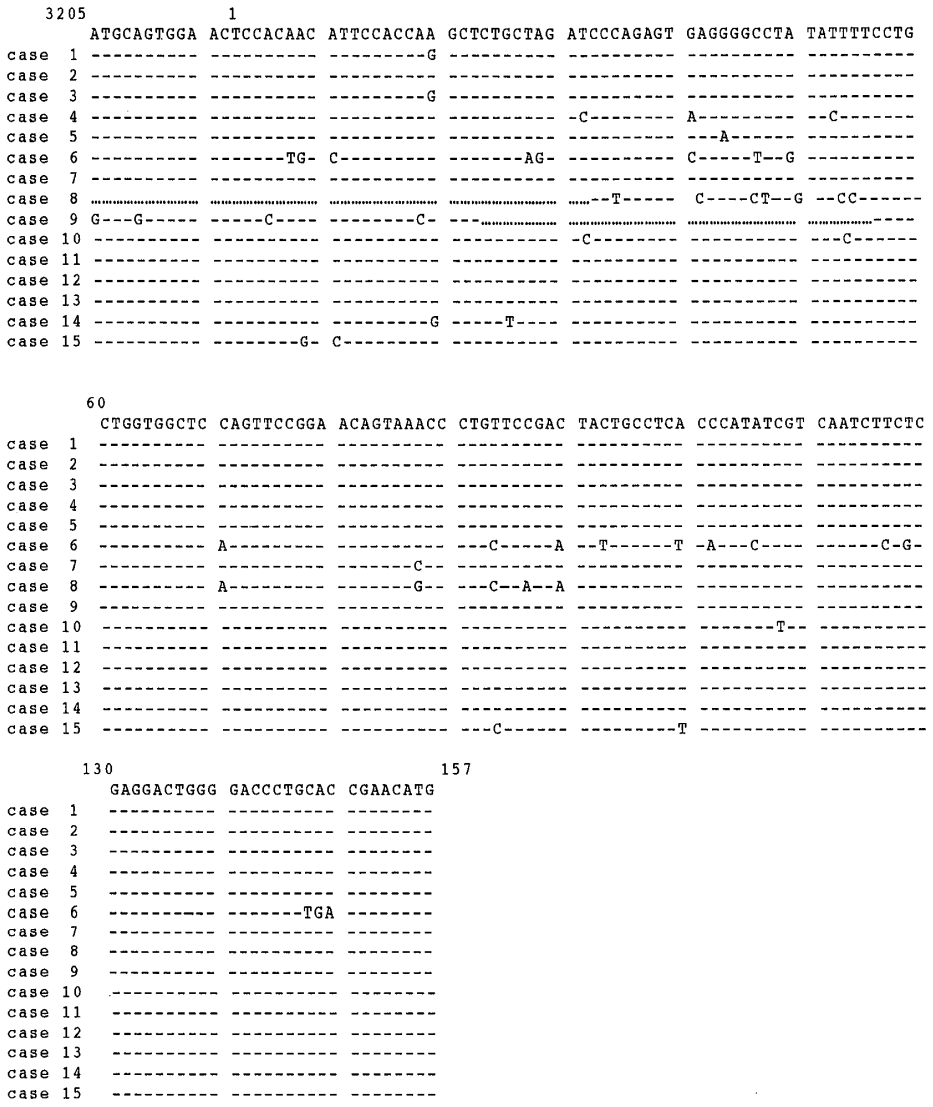

Fig. 3 Nucleotide sequences of the preS2 region from 15 patients, compared with reference sequences[13] indicated at the top.

cleotides 2762 to 2776) and 5′-CTTCTCTCAA-TTTTCTAGGGGAGCTCC-3′ (antisense; nucleotides 293 to 265). Five microliters of the extracted DNA was mixed with 45 μL of PCR reaction mixture containing 400 nM of the primers. Amplification was performed with 35 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 3 min, in an automatic DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The PCR products were directly sequenced by dideoxy-chain termination using an AutoRead DNA sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) and one of the PCR primers.

Statistical analysis

All results are expressed as means ± SD unless otherwise indicated. Clinical data of each group were compared using Student-t test. A p-value of <0.05 was considered significant in analyses.

Results

Clinical findings

Nine patients were classified as HBsAg-positive and 6 as HBsAg-negative. HBcAg was detected in 5 of the HBsAg-positive patients and in one of the HBsAg-negative patients. Liver histology showed that 2 patients had submassive necrosis, 9 had chronic active hepatitis, one had

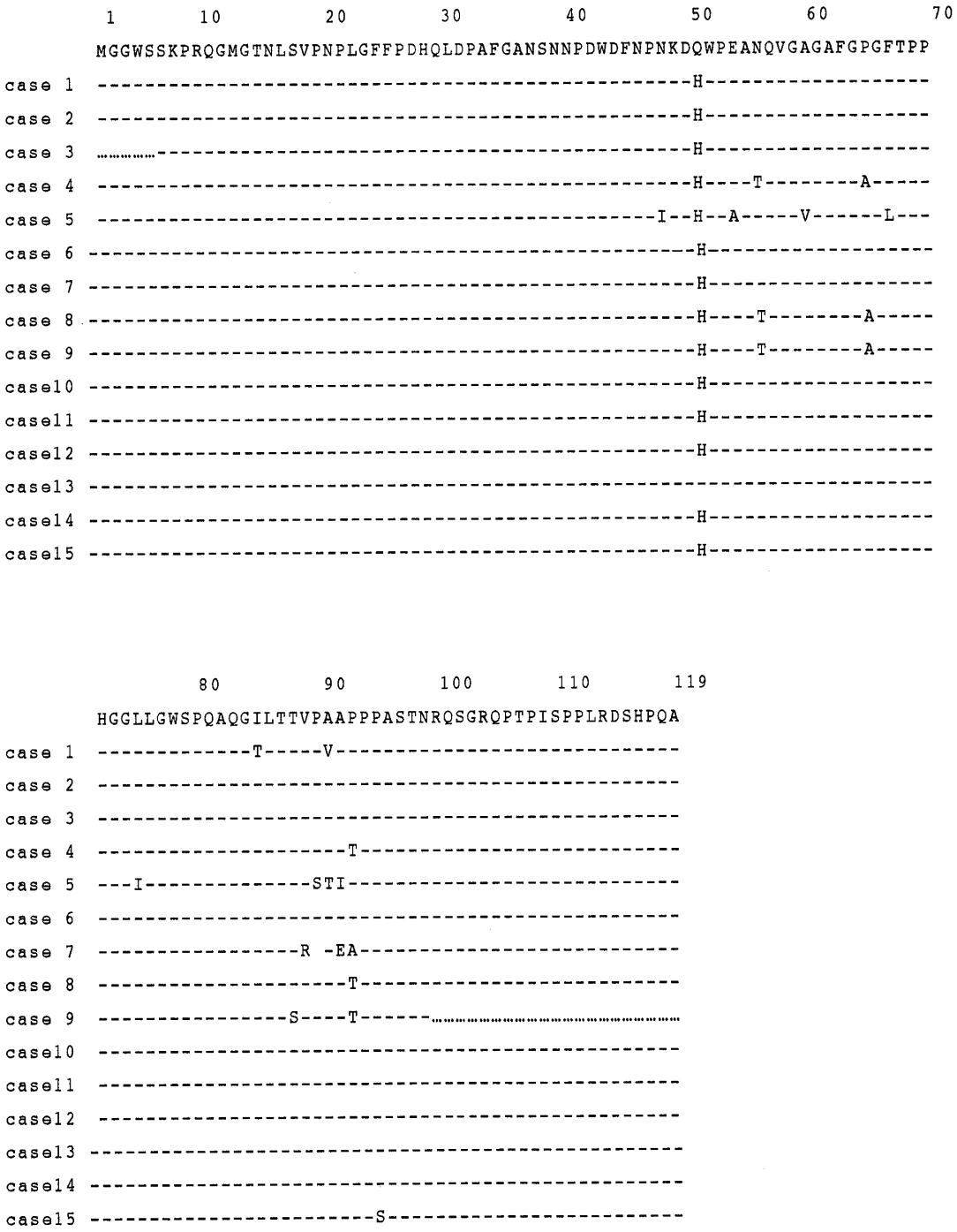

Fig. 4 Amino acid sequences of the preS1 region from 15 patients, compared with reference sequences[13] indicated at the top.

chronic persistent hepatitis, and 3 had lobular hepatitis. There were no significant differences between HBsAg-positive and HBsAg-negative patients in serum ALT (213 ± 137 vs. 423 ± 362 IU /L), serum HBsAg (125 ± 38 vs. 113 ± 46 S/N), or serum DNA polymerase (8105 ± 12079 vs. 928 ± 1229 cpm). HBeAg positivity was detected in 7 HBsAg-positive and 3 HBsAg-negative patients.

Mutational analysis of preS1 region

Results of sequencing analysis of the preS1 region are shown in Fig. 2. We compared the preS1 gene sequences between 15 patients with chronic

hepatitis B and a prototype HBV with adr[13]. In the region upstream of nt 3000, the number of mutations was small. Mutations in this region were found in both HBsAg-positive and HBsAg-negative patients. In the major surface antigen promoter, which is located in the coding region of the preS1 gene between nucleotides 3045 and 3180[14], mutations were frequently observed in HBsAg-positive patients (4.6 ± 4.0), with a lower frequency of mutations found in HBsAg-negative patients (1.7 ± 0.5). In the major surface antigen promoter region, three binding sites of nuclear factor Sp1 have been identified, and it is thought that these sites are necessary for transcription of the major surface antigen[15]. Of these three sites, mutations have been found to be concentrated within and around Sp1 site 2. All 5 HBsAg-negative patients had one mutation each within Sp1 site 2, and 4 of the HBsAg-positive patients had two or more mutations within Sp1 site 2. Four HBsAg-positive patients had one or more mutations upstream of Sp1 site 2, whereas none of the HBsAg-negative patients had mutations in the area surrounding Sp1 site 2. Case no. 9 had a 3-bp deletion in the 5′ end of Sp1 site 2, and case no. 8 had a 102-bp deletion spanning Sp1 site 3. No patients in either group had any mutations in the CCAAT box located between Sp1 sites 2 and 3.

Mutational analysis of preS2 region

In 2 patients, the initiation codon of the preS2 region had been destroyed: in patient case no. 8, by a 102-bp deletion; in case no. 9, by a change from ATG to GTG (Fig. 3). The numbers of mutations in the preS2 region for HBsAg-positive and HBsAg-negative patients were 3.8 ± 4.5 and 1.5 ± 1.8, respectively. However, this difference in the number of mutations in the preS2 region between the two groups was due entirely to the high numbers of mutations in case no. 6 and no. 8. When these 2 patients were excluded, the number of mutations in the preS2 region in the HBsAg-positive group was 1.6 ± 1.4, and there was no difference between the two groups. In contrast to the results of mutational analysis of the preS1 region, there was no clustering of mutations in any part of the preS2 region in HBsAg-positive or -negative patients. The initiation codon of the small S region was preserved in all patients, both HBsAg-positive and -negative.

Amino acid sequence of preS1 region

Amino acid sequences of the preS1 region are shown in Fig. 4. Two or more amino acid changes were observed in 6 HBsAg-positive patients, but only in one HBsAg-negative patient. In the HBsAg-positive group, 3 patients had the following changes: from asparagine to threonine at aa 56; from proline to asparagine at aa 65. Amino acid changes tended to be clustered between aa 87 and aa 92. In this area, amino acid changes were observed in 6 HBsAg-positive patients, but in none of the HBsAg-negative patients. The most common site for changes was aa 92: 3 patients, from proline to threonine; one patient, from proline to alanine.

Discussion

There have been reports of mutations in the major surface antigen promoter region in chronic hepatitis B patients with large amounts of HBsAg in hepatocytes. Previous studies have demonstrated an inverse relationship between the quantities of HBsAg in serum and liver tissue during the evolution of chronic HBV infection[16]. In other words, in the inactive phase of chronic hepatitis, the number of hepatocytes expressing HBsAg increases and the number of hepatocytes expressing HBcAg in the nucleus decreases. However, in the present patients, no relationship was found between serum ALT levels and accumulation of HBsAg in hepatocytes. Of the 9 HBsAg-positive patients, 3 had low levels of serum ALT (under 100 IU/mL), and 4 (case no. 3, 5, 7 and 8) had active hepatitis with serum ALT

over 200 IU/mL. Case no. 8 and no. 3 died from hepatic failure, and their liver biopsies showed submassive necrosis. Thus, in the present patients, accumulation of HBsAg in hepatocytes was not dependent upon the phase of chronic hepatitis.

Of the three S envelope proteins (large, middle and small), middle S protein is not necessary for viral particle formation. The ratio of these three envelope proteins is important for viral morphogenesis; the large S protein is trapped in the endoplasmic reticulum (ER), and is released in the presence of small S protein[17]. Therefore, a decrease in the amount of small S protein may result in the retention of envelope protein in hepatocytes. The expression of small S protein is regulated by the major surface antigen promoter region. It has been demonstrated that mutations in the transcription factor binding site in the major surface antigen promoter region reduce promoter activity[15]. Therefore, we studied the relationship between mutations in the major surface antigen promoter region and intracellular retention in patients with chronic hepatitis B.

In the present study, more mutations were observed in HBsAg-positive patients than HBsAg-negative patients, especially in Sp1 binding site 2 and its surrounding area. In case no. 9, a 3-bp deletion was observed in the 5′ end of Sp-1 site 2. Raney et al have reported that Sp1 site 2 exhibits higher promoter activity than the other two Sp1 sites[17]. Therefore, it is likely that mutations in Sp 1 site 2 reduce expression of the small S protein, although assessment of promoter activity is necessary to confirm this hypothesis. We observed several mutations in an area upstream of Sp1 site 2 in the HBsAg-positive patients. Although this area does not bind Sp1, it is involved in regulating expression of small S protein, as reported in a study by Raney et al[18]. They demonstrated that deletion of sequences in a region upstream of Sp1 site 2 (region C) resulted in an approximately 4-fold reduction in transcription promoted by the major surface antigen promoter. Thus, it appears that mutations in the region upstream of Sp1 site 2 affect promoter activity via reduced binding of Sp1 due to a change in the tertiary structure of the major surface antigen promoter.

The CCAAT sequence is located between Sp1 site 2 and Sp1 site 3. It has been shown that CCAAT binds NF-Y and is important for the regulation of the S-gene controlling the ratio of preS mRNA to S mRNA[19]. The CCAAT sequence within the major surface antigen promoter negatively regulates the amount of preS1 transcripts via a posttranscriptional mechanism[20]. Previously, it has been reported that point mutation within CCAAT sequences causes intracellular retention of HBsAg in hepatocytes[10][21]. Surprisingly, in the present study, no mutations were found in the CCAAT sequence in any of the patients.

We found that changes in amino acid sequence were common at aa 56 and aa 65 of the preS1 region. Also, amino acid changes tended to cluster in the region between aa 87 and aa 92. However, in the present patients, it is not likely that HBV would escape immune surveillance and accumulate within hepatocytes as a result of these mutations, because the T cell epitope is located at the amino terminus of the preS1 region, upstream from the area in which mutations were clustered[22]. Therefore, the clinical significance of the mutations found in these patients is still unclear.

In 2 of the present patients, HBV middle S protein was not produced, because of the destruction of the initiation codon of the preS2 region. The function of preS2 protein is unclear. Lack of preS2 does not prevent virion assembly or secretion[3]. It has been reported that preS2 protein binds human serum albumin and is involved in viral entry into hepatocytes[23]. However, in another study,

lack of preS2 protein did not prevent infection of hepatocytes[24]. In the present study, case no. 8 and no. 9 lacked preS2 protein but had active hepatitis, despite testing negative for HBeAg. Also, no clustering of mutations was observed in the preS2 region, and there were no significant differences in numbers of mutation between HBsAg-positive and-negative patients. These findings suggest that changes in the preS2 region do not contribute to intracellular retention of HBV envelope protein.

Finally, it has been reported that FCH is caused by a mutant HBV with mutations in the major surface antigen promoter region[10]. Histopathological examination of our patients showed that none of them had liver histology that resembled FCH. However, the 3 patients who died of hepatic failure showed retention of HBsAg. Two of these patients had deletion mutations, and all 3 had more mutations in the surface antigen promoter region than the other patients. Thus, studies of the retention of HBsAg and sequence analysis of the surface antigen promoter region may provide useful information about the prognosis of the course of chronic hepatitis B.

Conclusion

The present study revealed intracellular retention of HBsAg may be caused by mutation in the preS region of HBV. Since retention was associated with severity of liver diseases in some cases, sequential study of the preS region may be useful for the prediction of prognosis of chronic hepatitis B.

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Pre S 領域の変異による肝細胞内の HBs 抗原蓄積のメカニズム

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これまでに，HBs 抗原の肝細胞への蓄積は肝障害の重症度と関連していることが，トランスジェニックマウスを用いた実験モデルで証明されている．また envelope 蛋白の分泌の制御の一部は，large S，middle S，small S などによって担われていることが知られている．従って，preS 領域の塩基変異による envelope 蛋白の産生の変化は，envelope 蛋白の分泌と細胞内の蓄積に影響を与えることが予想される．我々は，B 型慢性肝炎患者における肝細胞内の HBs 抗原の蓄積，preS 領域の塩基配列の関連について検討した．慢性 B 型肝炎患者 15 例を対象とし血清より DNA を抽出後，PreS 領域を PCR 増幅した．肝細胞中の HBs 抗原強陽性は 9 例で変異は SP1 結合部周辺に強くみられ，それらの症例は，肝細胞内にエンベロープ蛋白の蓄積を多く認めた．SP1 結合部周囲の変異は，HBs 抗原プロモーターと他のエンベロープ蛋白との活性化に影響を与える可能性が示唆された．