Prevalence and Significance of Naturally Occurring Mutations in the Surface and Polymerase Genes of Hepatitis B Virus

Yuki Ogura,1 Masayuki Kurosaki,1 Yasuhiro Asahina,1 Nobuyuki Enomoto,1 Fumiaki Marumo,1 and Chifumi Sato1,2

The prevalence and clinical significance of naturally occurring mutations in the full-length surface and overlapping polymerase genes of hepatitis B virus (HBV) were analyzed in 42 patients with chronic hepatitis. Mutations were observed in 10 patients (24%) in the α determinant region, which is the neutralizing epitope within the major hydrophilic region of the surface gene. A high proportion of these mutations (17/18; 94%) occurred in the first loop, unlike mutations induced by immunization. The presence of serum antibody to hepatitis B surface antigen was significantly associated with these mutations. No other region of the surface gene contained any cluster of mutations. These results suggest that escape mutations commonly contribute to persistency in the natural course of HBV infection. In contrast, mutations affecting the major catalytic domains of the polymerase gene, which could alter susceptibility to antiviral nucleoside analogues, were not detected at all.

Several hundred million people throughout the world are chronically infected with hepatitis B virus (HBV), which eventually leads to life-threatening sequelae such as liver cirrhosis and hepatocellular carcinoma [1]. In general, the coordination of humoral and cellular immune responses to virus-encoded proteins is responsible for virus clearance. In the case of HBV infection, the humoral immune response to HBV surface protein contributes to the clearance of circulating HBV particles, whereas cellular immune responses are responsible for the elimination of infected hepatocytes [2]. Practically, the clearance of HBV occurs only in a small proportion of patients infected at birth, partly because of inadequate humoral and cellular immunity of the host [3]. Alternatively, the emergence of variant viruses that could escape from humoral and cellular immunity may be implicated in persistency, as reported in several other viruses [4, 5]. However, the contribution of immune escape variants in the natural course of HBV infection remains unclear.

The surface gene of HBV contains a dominant neutralizing epitope termed α determinant located within the major hydrophilic region of the surface gene. The production of an antibody to the α determinant after vaccination usually protects against HBV infection. Similarly, in patients with chronic HBV infection, clearance of HBV occurs in association with seroconversion from hepatitis B surface antigen (HBsAg) to the antibody to HBsAg (anti-HBs). Primary prevention of HBV infection, such as vaccination in infants born to HBsAg-positive mothers and immunoprophylaxis in orthotopic liver transplantation recipients, exerts strong selective pressure on HBV, which, in some cases, induces escape mutations within the α determinant region [6–12]. In these cases, HBV could replicate in the presence of anti-HBs. Similar escape mutations in the α determinant could also arise in the natural course of HBV infection, resulting in active viral replication and liver disease after seroconversion to anti-HBs in patients with chronic hepatitis B [13, 14]. The prevalence of these naturally occurring mutations in the α determinant, however, remains unknown because of the lack of comprehensive studies. The surface gene of HBV also contains putative HLA class I–restricted cytotoxic T lymphocyte (CTL) epitopes [2]. In general, viral mutations in CTL epitopes could evade cellular immunity and thus contribute to persistency [4, 5]. A recent study from Taiwan revealed a high frequency of mutations at amino acid positions 40 and 47 of the surface gene that coincides with the HLA-restricted CTL epitope in patients with chronic hepatitis B, and it was suggested that these mutations might contribute to chronic infection [15]. Because the number of patients analyzed in that study was relatively small, it is not clear whether these amino acid positions universally represent mutation-clustering regions or whether these mutations actually serve as immune escape mutations. Moreover, the frequency of mutations within the other regions of the HBV surface gene has not been studied comprehensively, and the occurrence and clinical significance of the mutations among patients with chronic hepatitis B remain to be established.

The genome of HBV encompasses 4 overlapping open-read-
ing frames, and the surface gene overlaps with the catalytic domains of the polymerase gene [16]. Thus, mutations in the surface gene may also affect the polymerase gene, which may alter viral replication. Conversely, mutations in the polymerase gene could have an impact on the surface gene. Recent studies have shown that some specific mutations in the catalytic domains B and C of the polymerase gene, especially in the YMDD motif of domain C, often appear in relation to the breakthrough during antiviral therapy with nucleoside analogues such as lamivudine or famciclovir [17–19]. Therefore, the susceptibility of surface gene mutants to the antiviral therapy may be altered because mutations in the surface gene may affect these critical regions of the polymerase gene.

The aims of the present study were to elucidate (1) the prevalence of naturally occurring mutations in the surface gene, especially immune escape mutations; (2) their clinical significance in the natural course of HBV infection; and (3) the effect of surface gene mutations on the overlapping polymerase gene.

Materials and Methods

Materials. Sera were obtained from 45 Japanese patients with chronic HBV infection who had been routinely followed up at our hospital. Presence of HBsAg, hepatitis B e antigen (HBeAg), and antibody to HBeAg (anti-HBe) was measured by commercially available EIA (Abbott Laboratories, Abbott Park, IL), and anti-HBs was measured by RIA (Abbott). Six of the patients were HBeAg-positive asymptomatic healthy carriers who had no history of liver disease and whose liver function had been persistently normal for ≥24 months. Thirty-nine were patients with chronic liver disease confirmed by radiologic and/or histologic evidence and had a history of elevated alanine aminotransferase (ALT) levels that had persisted for ≥6 months, and 39 of the 37 patients actually had elevated ALT levels at the time of serum sampling for the HBV sequence analysis. Sixteen patients were positive for HBeAg, 18 were positive for anti-HBe, and 5 were positive for both HBeAg and anti-HBe. All individuals were positive for HBsAg, and 11 patients were also positive for anti-HBs. No patients had a history of HB vaccination or therapy with hyperimmune globulin or nucleoside analogues. Sera were stored at −70°C until DNA was extracted. Antibodies to hepatitis C virus were measured by the second-generation EIA, and all the patients proved to be negative.

Primer synthesis. Nested primers for polymerase chain reaction (PCR) were synthesized by a DNA synthesizer (model 391; Applied Biosystems Japan, Tokyo) on the basis of the published HBV sequence pNDR260 [20]. Two sets of outer primers ([HBV 3, HBV 4] and [HBV 5, HBV 6]) were designed to amplify the surface region of HBV in 2 divided fragments. Three sets of inner primers ([HBV seq7, HBV seq8], [HBV seq9, HBV seq15], and [HBV seq11, HBV seq10]) were used in the second-round PCR to reamplify the product of the first-round PCR. The nucleotide sequence of T7 promoter primer (24 nt) or pUC/M13 reverse primer (24 nt) was attached to the 5’ end of the primers for second-round PCR. The T7 promoter primer and pUC/M13 reverse primer (Promega, Madison, WI) were used as sequencing primers. Sequences of the primers were as follows:

Outer primers for the first-round PCR: HBV 3, 5-GGGAAA-CTTACTGGGCTTATTCC-3’ (nt 2474–2498, sense); HBV 4, 5’-TAGAAATTAGAGAACTTCCACCA-3’ (nt 257–280, anti-sense); HBV 5, 5-TAGGACCCCTGCTGTTACGG-3’ (nt 180–203, sense); HBV 6, 5-AAGTTGGCGAGAATGTGAAG-CCT-3’ (nt 1085–1109, anti-sense). Inner primers for the second-round PCR: HBV seq7, 5-ATAATACGACTCATAAGGGC-AAAATCCAGATTGGGAC-3’ (T7 promoter primer sequence and nt 2960–2980, sense); HBV seq8, 5’-TCACACAGGAAAA-CAGCTATGAC-3’ (pUC/M13 reverse primer sequence and nt 237–257, anti-sense); HBV seq9, 5’-TAATACGACTCATAAGGGGCGGGTTTTTCTTGTTG-3’ (T7 promoter primer sequence and nt 202–211, sense); HBV seq15, 5’-TCACACAGGAAAACAGCTATGACGAAACGACTGAGCCAC-3’ (PUC/M13 reverse primer sequence and nt 646–675, anti-sense); HBV seq11, 5’-TAATACGACTCATAAGGGGACCATCAGACAGCTATGAC-3’ (T7 promoter primer sequence and nt 507–528, sense); HBV seq10, 5’-TCACACAGGAAAACAGCTATGAC-3’ (PUC/M13 reverse primer sequence and nt 1052–1073, anti-sense); T7 promoter primer, 5’-TAATACGACTCATAAGGGGCGGGTTTTTCTTGTTG-3’; and pUC/M13 reverse primer, 5’-TCACACAGGAAAACAGCTATGAC-3’.

Amplification of HBV gene. DNA was extracted from sera by a sodium hydroxide method [21]. Briefly, 10 µL of serum was treated with 0.1 N sodium hydroxide in a volume of 20 µL at 37°C for 60 min and then was neutralized with hydrochloric acid. A 2-µL aliquot was amplified by hot-start PCR in a 50-µL mixture with 10 pmol of the outer primer sets for 35 cycles. Each cycle consisted of denaturation at 94°C for 15 s, annealing of primers at 55°C for 15 s, and extension at 72°C for 15 s. Then, second-round PCR was performed on 1 µL of the first PCR products in a 50-µL mixture with 10 pmol of the inner primer sets for 35 cycles under the same reaction conditions as the first-round PCR. The second-round PCR products were electrophoresed through agarose gel and visualized under ultraviolet light. Negative controls were included in each PCR round, and false-positive results were cautiously avoided by the strict application of contamination-prevention guidelines [22].

Direct sequencing of PCR products. Residual dNTPs and primers were removed from the PCR products by using a centrifugation filtration column (Suprec-02; Takara, Kyoto, Japan) and adjusted to a final volume of 50 µL with TRIS-EDTA buffer. Nucleotide sequences of the PCR products were directly determined by using Taq DyeDeoxy Terminator Cycle sequencing kits with an automated DNA sequencer (model 373A; Applied Biosystems Japan). In brief, 7 µL of purified PCR product was mixed with 3.2 pmol of the sequencing primers, 1 µL of each DyeDeoxy Terminator (A, G, C, T), 1 µL of a dNTP mixture (750 µM dITP, 150 µM dATP, 150 µM dTTP, and 150 µM dCTP), 4 U of AmpliTaq DNA polymerase, and 4 µL of the reaction buffer (400 mM Tris-HCl, 10 mM MgCl2, and 100 mM [NH4]2SO4 at pH 9.0) in a volume of 20 µL. Cycle sequencing reactions were performed for 25 cycles, each cycle consisting of 96°C for 30 s, 50°C for 25 s, and 60°C for 4 min. An excess of DyeDeoxy Terminators was removed from the completed reaction mixture by using Centri-Sep spin column (Princeton Separations, Adelphia, NJ). Samples were dried in a vacuum centrifuge (TOMY, Tokyo, Japan), dissolved in a loading buffer (5 µL of deionized formamide and 1 µL of 50 mM EDTA at pH 8.0), and
Figure 1. Amino acid mutations in the full-length surface gene of hepatitis B virus (HBV). Positions of mutations in deduced amino acid residues are indicated by vertical lines along the surface protein of HBV. The consensus residue of genotype C is shown in the first line. The consensus sequences of genotypes A, B, and D that are different from that of genotype C are indicated in parentheses. Dashes represent residues identical to these reference residues. Mutations that affect the $\alpha$ determinant region are indicated by a shaded box.

Nucleotide and deduced amino acid sequences encoding the full-length surface region of HBV were determined in 45 patients. Comparison with the published HBV sequences showed that 3 patients were infected with subtype adw/genotype B of HBV, and all the others were infected with subtype adr/genotype C. Patients infected with subtype adw/genotype B were excluded from further analysis. Sixteen (38%) of 42 patients had amino acid mutations in the surface gene, and 10 patients (24%) had amino acid mutations in the $\alpha$ determinant region (figure 1). Surface gene mutations occurred at 32 positions, but mutations to the consensus sequence of other genotypes were observed at only 2 positions (threonine $\rightarrow$ asparagine [genotype A] at position 131 and phenylalanine $\rightarrow$ tyrosine [genotype D] at 134), and thus the occurrence of other genotype-specific mutations seemed to be low. Among a total of 44 mutations, 18 were located within the $\alpha$ determinant region, 17 in the first loop (positions 124–137) and 1 in the second loop (positions 139–147). A cluster of mutations was observed solely within the $\alpha$ determinant region and not in the other regions of HBV. Cysteine residues that are believed to stabilize the conformational structure of the major hydrophilic region were reported to be frequently mutated in patients after therapeutic failure with hyperimmune globulin [28] or in those who were negative for serum HBsAg in spite of HBV viremia [29]. In the present study, however, these regions were also highly conserved. Cysteine residues that are believed to stabilize the conformational structure of the major hydrophilic region were
completely conserved. Positions 122 and 160, which determine the d/y and w/r subtypes of HBsAg, were also completely conserved. The number of mutations per amino acid residue was significantly high in the a determinant region compared with the other regions of the HBV surface genome (range, 0–1.20 \times 10^{-1} vs. 0–0.25 \times 10^{-1} mutations per site per patient; \(P = .02\) by Wilcoxon’s test; figure 2).

Among a total of 18 mutations at 7 amino acid positions in the a determinant, 16 were consistent with the previously characterized escape mutations, and 2 were novel mutations that had not been previously reported (phenylalanine \(\rightarrow\) lysine in position 134 and proline \(\rightarrow\) histidine in position 135). These 2 mutations were observed in a 47-year-old woman (patient 10). Although she was positive for anti-HBs with a relatively high titer (cutoff index of 10.0), she had active liver disease with elevated serum ALT levels (316 IU/mL) accompanied by active replication of HBV (serum HBV-DNA levels of 260 mEq/mL), suggesting that these mutations may be novel escape mutations.

Comparisons of various clinical features in terms of the presence of mutations in the a determinant region are presented in table 1. The presence of anti-HBe and anti-HBs was associated with mutations in the a determinant region (anti-HBe, \(P = .03\); anti-HBs, \(P = .01\); Fisher’s exact test for both). There was no significant association between presence of mutations in the a determinant and the other clinical features, including age, sex, serum ALT levels, and HBV-DNA polymerase levels. A multivariate analysis revealed that the presence of anti-HBs was the only independent factor associated with the a determinant mutations (table 2).

Because the surface gene overlaps with the major catalytic domains of the polymerase gene, the occurrence of mutations in the polymerase gene was also analyzed (figure 3). As a result, amino acid mutations were observed at 5 positions in >1 patient. Mutations at amino acid positions 403, 423, 471, 482, and 487 were observed in 2 (4.8%), 2 (4.8%), 4 (9.5%), 6 (14.3%), and 8 (19.0%) patients, respectively, and other mutations were present in only 1 patient. The mutation at position 423 that was observed in 2 patients affected the first amino acid position of domain A, but the other catalytic domains of the polymerase gene [30] were highly conserved. A cluster of mutations was observed between amino acids 482 and 487, a region upstream of domain B that overlaps with the a determinant region of the surface gene. The relationships between the mutations in the surface and the polymerase genes are shown in table 3. Amino acid mutations at positions 403 and 482 of the polymerase gene were accompanied by mutations at positions 47 and 126 of the overlapping surface gene, and 3 of 8 amino acid mutations at position 487 of the polymerase gene also altered

Table 1. Analysis of clinical factors in relation to the presence of mutations in the a determinant region in 42 patients with chronic hepatitis B virus (HBV) infection.

<table>
<thead>
<tr>
<th>Clinical factor</th>
<th>Mutant (n = 10)</th>
<th>Wild (n = 32)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years, mean ± SD</td>
<td>4 ± 17</td>
<td>37 ± 17</td>
<td>.17</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>6/4</td>
<td>17/15</td>
<td>1.00</td>
</tr>
<tr>
<td>HBeAg</td>
<td>4 vs. 6 (40)</td>
<td>23 vs. 9 (72)</td>
<td>.13</td>
</tr>
<tr>
<td>Anti-HBe(^a)</td>
<td>8 vs. 2 (80)</td>
<td>12 vs. 20 (38)</td>
<td>.03</td>
</tr>
<tr>
<td>Anti-HBs(^a)</td>
<td>6 vs. 4 (60)</td>
<td>5 vs. 27 (16)</td>
<td>.01</td>
</tr>
<tr>
<td>ALT in IU/L, mean ± SD</td>
<td>119 ± 92</td>
<td>105 ± 174</td>
<td>.16</td>
</tr>
<tr>
<td>HBV-DNA polymerase</td>
<td>23 (0–9700)</td>
<td>160 (0–10,528)</td>
<td>.10</td>
</tr>
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</table>

\(^a\) Present versus absent (percent).
amino acid 131 of the surface gene. In contrast, amino acid mutations at positions 423 and 471 and 5 amino acid mutations at position 487 were silent for the surface gene. The presence of these mutations was not associated with any clinical features, including age, sex, serum ALT levels, HBV-DNA polymerase, and the presence of HBeAg and anti-HBe or anti-HBs (data not shown). There were no mutations known to be associated with a resistance to lamivudine or foscavir therapy, such as a methionine→valine or methionine→isoleucine mutation in the YMDD motif of domain C (position 552) or mutations in positions 514, 521, 525, and 528 of domain B [17–19].

**Table 2.** Multivariate analysis on clinical factors associated with mutations in the α determinant region.

<table>
<thead>
<tr>
<th>Clinical factor</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody to hepatitis B surface antigen</td>
<td>2.43</td>
<td>1.05–5.51</td>
<td>.04</td>
</tr>
<tr>
<td>Antibody to hepatitis B e antigen</td>
<td>2.17</td>
<td>0.88–5.36</td>
<td>.10</td>
</tr>
</tbody>
</table>

NOTE: Statistic program JMP, version 3.1 (SAS Institute), was used for multivariate analysis.

In the present study, mutations in the full-length surface gene were analyzed comprehensively in a natural population of 42 patients chronically infected with a single genotype of HBV. As a result, we observed a cluster of mutations only in the α determinant region, and we determined that the prevalence of these mutations among the studied population was as high as 24%. The calculated number of mutations per amino acid residue was significantly high in the α determinant region compared with the other regions of the HBV surface genome, suggesting the presence of a selective pressure for the mutations in the α determinant. Humoral immune pressures may have selected escape-variant viruses during the course of chronic infection, because the mutations in the α determinant were significantly associated with the presence of serum anti-HBs. This view is also supported by the fact that 16 of the 18 mutations observed in this study were consistent with the previously characterized escape mutations, which could replicate in spite of the presence of anti-HBs [6–14, 31–34]. Although the other 2 mutations were novel mutations that had not been reported, they occurred in a single patient who had active liver disease and replication of HBV in spite of the relatively high titer of anti-HBs, suggestive of escape mutations. Thus, the escape phenomenon from the neutralizing anti-HBs could be one of the mechanisms of persistency, even in patients who have not received vaccination or hyperimmune globulin therapy.

In 4 cases, mutations in the α determinant were seen without the presence of anti-HBs. The lack of serum anti-HBs in these patients does not necessarily indicate lack of humoral immune responses to the α determinant, because it has been reported that anti-HBs that is complexed with HBsAg could be detected in nearly all patients with chronic hepatitis B when analyzed by a highly sensitive immunoassay [35]. Therefore, our data suggest that these escape mutations could be induced by an immunological pressure exerted on the α determinant, even in patients who are found to be negative for serum anti-HBs by use of the usual assay. An alternative interpretation may be that these mutations occurred in another individual, and then the mutants were transmitted to these patients.

A recent study on HB hyperimmune globulin therapy failure in liver transplant recipients revealed that therapeutic failure was associated with mutations in the α determinant region in some, but not all, patients. In other patients without a determinant mutation, therapeutic failure was associated with mutations in the major hydrophilic region of the surface gene outside the α determinant (positions 99–169) [28]. Because the exact border of the neutralizing domain is unknown, the authors suggested that these mutations may have contributed to the therapeutic failure. In some patients being studied here, HBV without mutations in the α determinant region replicated actively in the presence of anti-HBs. The major hydrophilic regions other than the α determinant were also highly conserved, suggesting that escape mutations were not involved in these patients. Thus, anti-HBs in these patients may not be directed to the neutralizing epitope. They were possibly directed against the epitopes within the surface gene, such as d/y or w/r subtype determinants [31].

Two of the characteristics of the α determinant mutations observed in the present study were that they occurred preferentially at the first loop (94%) and that position 126 of the first loop was affected most frequently (33%). In contrast, originally described vaccine-induced escape mutations involved the glycine→arginine mutation at position 145, which is located in the second loop of the α determinant [6]. In subsequent studies, a high proportion of escape mutations induced by vaccination or hyperimmune globulin therapy were also located in the second loop, position 145 being the hottest position [7, 10–12]. These observations in immunization recipients are consistent with the report that most anti-HBs in sera from vaccinees are targeted to an epitope between amino acids 139 and 147 [36]. In the present study, a glycine→arginine mutation at position 145 was observed in only 1 patient. These differences in the location of mutations may represent the distinctive feature of naturally occurring mutations and may suggest the differences in the target epitopes between naturally acquired anti-HBs and that obtained by immunization. Alternatively, because the vast majority of studies on vaccine- or hyperimmune globulin-induced escape mutations have dealt with the subtype adw or ayw of HBV, in which position 126 is divergent from adw (threonine in subtypes adw and ayw [genotypes A, B, and D] and isoleucine in subtype ad [genotype C]), differences in the genotypes may be another reason for the preferred mutations in the first loop, especially at position 126 in the present study dealing with the subtype adr/genotype C.

In theory, naturally occurring mutations within the CTL ep-
Figure 3. Amino acid mutations in the overlapping polymerase gene of hepatitis B virus (HBV). Positions of alterations in deduced amino acid residues are indicated by vertical lines along the polymerase protein of HBV. The consensus residue of genotype C is shown in the first line. Consensus sequences of genotypes A, B, and D that are different from that of genotype C are indicated in parentheses. Dashes represent residues identical to these reference residues. The major catalytic domains A–E and a region overlapping the a determinant of the surface gene are shown as the closed box.

Amino acid could lead to epitope inactivation and T cell receptor antagonism [37]. In fact, several studies have shown that patients with chronic active hepatitis often carry missense mutations or deletions in the middle of the core region of HBV, whereas mutations are not observed among patients with inactive disease, suggesting that these mutations in the core region may be CTL escape mutations [38–41]. A previous study from Taiwan revealed a high frequency of mutations at positions 40 and 47 of the surface gene, which coincide with mouse H-2 Ld–restricted and HLA A2–restricted CTL epitopes, respectively, and the authors suggested that these mutations could alter CTL recognition and predispose these patients to chronic infection. In the present study, however, mutations at amino acid residues 40 and 47 were observed in only 1 (2%) and 2 (5%) patients, respectively, and the other putative CTL epitopes were also conserved. Therefore, we could not confirm similar frequent mutations in the CTL epitopes of the surface gene. Because mutations at position 40 were preferentially seen in the HBV adw subtype (genotype B) in the study from Taiwan, they may be specific to genotype B, and thus we did not observe them in our study. However, the reason for the lack of frequent mutations at position 47 is not clear.

In the analysis of the overlapping polymerase gene, mutations at only 5 amino acid positions were observed in >1 patient. About half of these polymerase mutations simultaneously had mutations in the overlapping a determinant region of the surface gene, suggesting that they were probably affected by the primary mutations in the a determinant. The rest of the polymerase mutations, namely, at positions 423, 471, and 487, were not accompanied by the surface gene mutation and may have been primary mutations selected by undetermined selective pressures on the polymerase gene. Position 423 was located in
the first amino acid position of the catalytic domain A, but this position seemed variable, according to the comparative study of the known RNA-dependent DNA polymerases [30]. The other mutations were outside of the 5 conserved catalytic domains of the polymerase gene and thus did not appear to affect the critical regions of the polymerase gene. In fact, the presence of these mutations was not associated with various clinical features, including serum HBV-DNA polymerase levels, HBV-DNA levels, ALT levels, and the presence of HBsAg, anti-HBe, or anti-HBs, suggesting that these mutations may not affect replication competency of the virus. The characterization of the functional significance of these mutations would require further in vitro studies. Mutations at several amino acid positions in the polymerase gene are reported to exert resistance to lamivudine or famiciclovir therapy: phenylalanine→leucine in position 514, valine→leucine in 521, proline→leucine in 525, and leucine→methionine in 528 of the catalytic domain B [17, 18] and methionine→valine or methionine→isoleucine in position 552 in domain C, affecting the YMDD motif [17]. None of these mutations were observed in the present study. Therefore, naturally occurring mutations in the polymerase gene that could alter susceptibility to antiviral therapy seemed to be absent in those who have not received antivirus nucleoside analogues.

In conclusion, we have shown that the prevalence of naturally occurring mutations in the a determinant was unexpectedly high in patients with chronic hepatitis B. A high proportion of these natural mutations, unlike escape mutations induced by vaccine or immunoglobulin therapy, occurred in the first loop. These mutations were significantly associated with the presence of anti-HBs, suggesting that they may cause escape from the neutralizing antibody, thus contributing to persistent infection in some patients. In contrast, escape mutations in the putative CTL epitopes seemed rare, and natural mutations affecting the major catalytic domains of the polymerase gene that could alter susceptibility to therapy with antivirus nucleoside analogues were not observed at all.

### Table 3. Relationship between the mutations in the a determinant region and in the overlapping polymerase gene.

<table>
<thead>
<tr>
<th>Surface gene</th>
<th>Polymerase gene</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>T47</td>
<td>H403</td>
<td>2</td>
</tr>
<tr>
<td>Silent</td>
<td>S423</td>
<td>2</td>
</tr>
<tr>
<td>Silent</td>
<td>N471</td>
<td>4</td>
</tr>
<tr>
<td>I126</td>
<td>D482</td>
<td>5</td>
</tr>
<tr>
<td>I126</td>
<td>D482</td>
<td>1</td>
</tr>
<tr>
<td>G130</td>
<td>R468</td>
<td>1</td>
</tr>
<tr>
<td>T131</td>
<td>N487</td>
<td>3</td>
</tr>
<tr>
<td>Silent</td>
<td>N487</td>
<td>5</td>
</tr>
<tr>
<td>M133</td>
<td>Silent</td>
<td>3</td>
</tr>
<tr>
<td>M133</td>
<td>Y489</td>
<td>1</td>
</tr>
<tr>
<td>F134</td>
<td>Silent</td>
<td>1</td>
</tr>
<tr>
<td>F134</td>
<td>V490</td>
<td>1</td>
</tr>
<tr>
<td>P135</td>
<td>S491</td>
<td>1</td>
</tr>
<tr>
<td>G145</td>
<td>R501</td>
<td>1</td>
</tr>
</tbody>
</table>

* Polymerase mutations not accompanied by surface gene mutations.  
  † Novel mutations in the a determinant that have not been reported.

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### References