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Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal

(hepatitis B e antigen secretion/hepatitis B virus replication)

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ABSTRACT **We conducted a large-scale survey to determine the frequency and clinical significance of mutations in the pre-core region of hepatitis B virus (HBV). Sera from 263 patients with chronic HBV infection were analyzed by direct sequencing of PCR-amplified HBV DNA. Four major missense/nonsense mutations (M) were found: (M1) C → T at nucleotide position 1856, Pro → Ser at codon 15; (M2) G → A at position 1896, Trp → stop at codon 28; (M3) G → A at position 1898, Gly → Ser at codon 29; and (M4) G → A at position 1899, Gly → Asp at codon 29. The commonest conserved mutation was M0: T → C at position 1858, Pro → Pro at codon 15. We found that M1 and M2 were mutually exclusive, M3 was only found in association with M1, and M4 was predominantly found in association with M2. All patients with M1 but none of those with M2 had M0. The invariable coexistence of certain mutations in codon 15 and codons 28 and 29 and the mutual exclusion of other mutations in these two noncontiguous regions is related to the stem–loop structure of the pre-genome encapsidation sequence located in the pre-core/core region. M2 and M4 enhance the stability of the stem by providing two additional paired sites. M1 destroys an existing base pair. However, M1 only occurred in the presence of M0, which provides an extra paired site, and 50% of patients with M1 had M3, a compensatory mutation that restores base pairing at this site. Our data support the proposed secondary structure of the pre-genome encapsidation sequence. The primary function of the mutations in the pre-core region is to enhance stability of this secondary structure to ensure perpetuation of viral replication.**

Since the initial reports of mutations in the pre-core region of hepatitis B virus (HBV) in hepatitis B e antigen (HBeAg)-negative ($HBeAg^-$) patients with active liver disease (1, 2), numerous investigators from different parts of the world have reported the same mutation: G → A change at nucleotide position 1896, creating a stop codon at codon 28 (3–10). This mutation leads to premature termination of the pre-core/core protein, thus preventing the production of HBeAg. The reason for the frequent detection of this mutation is postulated to be due to immune selection. Several studies have shown that HBeAg may be a target antigen on HBV-infected hepatocytes (11, 12). Failure to produce a target antigen may be a means to evade immune clearance. Although mutations in other regions of the pre-core/core gene may also prevent production of HBeAg, practically all patients reported to date (except for one study) (13) had the same mutation: G → A change at nucleotide position 1896. The predilection for this site is not clear. Some patients have an additional mutation: G → A change at position 1899 (1, 3, 5, 7, 10, 13). The significance of this mutation is unknown, since it is downstream of the stop codon mutation.

In an earlier report on a small number of Chinese patients (13), most of whom were $HBeAg^-$, we found two unusual mutations—"M1," C → T change at position 1856 resulting in a Pro → Ser change at codon 15; and "M3," a G → A change at position 1898 resulting in a Gly → Ser change at codon 29—in addition to the stop codon mutation (G → A change at position 1896), which we called "M2," and the G → A change at position 1899, which we called "M4." M1 and M2 were mutually exclusive. M3 was only found in association with M1, and M4 was only found in association with M2. We hypothesized that M1 may prevent HBeAg secretion by blocking entry of the pre-core/core protein into the endoplasmic reticulum because of its close proximity to the signal peptide cleavage site at codon 19. We were unable to explain the mutual exclusion of M1 and M2 and the close association between M1 and M3 and between M2 and M4.

We have extended our study to include a larger number of patients to clarify: (*i*) the relation between M1 and HBeAg secretion, (*ii*) the role of M1 and M2 in the pathogenesis of liver disease, and (*iii*) the significance of M3 and M4. We also sought to confirm if M1 and M2 were truly mutually exclusive and to determine a biological basis for this phenomenon.

PATIENTS AND METHODS

Patients. Three hundred and eight Chinese patients from Hong Kong who had chronic HBV infection were studied: 201 were HBeAg-positive ($HBeAg^+$) and 107 were $HBeAg^-$. Patients with serum alanine aminotransferase (ALT) levels persistently below 1.5 times the upper limit of normal were considered to have inactive liver disease.

Methods. An aliquot of serum was collected at each clinic visit and stored at −20°C for serum HBV DNA assay. The earliest available sample of residual sera from each patient was analyzed. Follow-up samples over a 1- to 7-year period from 101 patients were also analyzed to determine the time of appearance of each mutation and the correlation with HBe seroconversion.

DNA Extraction. Serum proteins were denatured by the addition of 2 μl of 1 M NaOH to 18 μl of serum (14). After a 1-hr incubation at 37°C, the samples were neutralized with 20 μl of 0.1 M HCl. The samples were then centrifuged for 15 min, and the supernatant was saved.

Polymerase Chain Reaction (PCR) Assay. PCR was performed on 10 μl of DNA extract in a 50-μl reaction mixture containing (final concentration) 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 2.5 units of *Taq* polymerase (Perkin–Elmer), and 10 pmol of each of the external primers. The reaction was carried out in 40

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Abbreviations: HBV, hepatitis B virus; wt, wild type; HBeAg, hepatitis B e antigen.

cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a 10-min extension step at 72°C at the end. Ten microliters of the first-round PCR products were used for the second-round PCR reaction under the same conditions except that 20 pmol of internal primers were used. Ten microliters of the second-round amplified products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide and visualized under UV light. The external primers were P1 (5'-GAGGAGTTGGGGGAGCACATT, positions 1734–1754) and P2 (5'-TCCAAGGGATACTAACATTGG, positions 2463–2443), while the internal primers were P3 (5'-TAGGAGGCTGTAGGCATAAATTGGT, positions 1774–1798) and P4 (5'-GGCGAGGGAGTTCTTCTTCTAGGGG, positions 2388–2364).

To prevent cross-contamination, all precautions recommended by Kwok and Higuchi (15) were observed. In addition, DNA extraction was performed within a hood; and first- and second-round PCR reactions were carried out on separate, designated benches. Negative controls (water and sera from subjects that were seronegative for HBV markers) were included in each assay. Positive bands were obtained in negative controls on two occasions only during this 2-year study. In both instances, contamination was traced to a buffer solution, which was discarded.

Sequencing. The amplified DNA was purified by Prep-A-Gene kit (Bio-Rad), and then used for direct sequencing with an internal primer P5 (5'-GGAAAGAAGTCAGAAGGCAA, positions 1974–1955). Dideoxynucleotide termination sequencing was performed with the Sequenase kit (United States Biochemical) according to the manufacturer's instructions except for minor modifications (16). Sequencing reactions were run on 6% polyacrylamide/urea gels, and autoradiography was performed with intensifying screens at 4°C overnight.

Statistical Analyses. These were performed by χ^2 or Fisher's exact test where appropriate.

RESULTS

Forty-five (15%) patients were repeatedly PCR-negative. All were HBeAg$^-$ and had persistently normal alanine aminotransferase levels. The sequencing results of the remaining 263 patients are presented in this paper. The pre-core HBV sequences of these patients were analyzed to determine the relation between M1 and HBeAg secretion, the role of pre-core HBV mutants in the pathogenesis of liver disease, and the biological basis for the mutual exclusion of M1 and M2.

Wild Type (wt). One hundred sixty-two (62%) patients, of whom 68% were HBeAg$^+$ and 42% were HBeAg$^-$, had no missense or nonsense mutations ($P = 0.0003$) (Fig. 1*a* and Table 1). However, 45% of these 162 patients had one or more conserved mutations that do not result in any change in amino acid sequence. Thirty-six (26%) of the HBeAg$^+$ patients with wt sequence lost HBeAg during follow-up (Table 2), and 69% of these patients had M2, but none developed M1 after loss of HBeAg. Only one patient with wt sequence in the initial sample developed M1 during follow-up. This patient's wife had M1 in all five samples studied. Eighty-seven (54%) patients with wt sequence, of whom 56% were HBeAg$^+$ and 42% were HBeAg$^-$, had active liver disease ($P = 0.2$) (Table 1).

M0: T → C Change at Nucleotide Position 1858 (Pro → Pro at Codon 15). The commonest change was a conserved mutation involving a T → C change at position 1858 (Fig. 1*b*). This was detected in 40% of the patients studied. The prevalence of M0 was the same in HBeAg$^+$ and in HBeAg$^-$ patients. M0 was present in all subsequent samples from patients in whom M0 was initially detected. M0 was detected in all of the patients with M1 but in none of the patients with M2 ($P < 0.0001$). HBeAg$^+$ patients with M0 were less likely to clear HBeAg during follow-up compared with those with T at position 1858 (16% vs. 32%; $P = 0.05$). None of the patients with M0 developed M2, while 93% of the patients with T at position 1858 had M2 after loss of HBeAg ($P < 0.0001$). Among the HBeAg$^-$ patients, none of those with M0 developed M2 during follow-up, while all patients with T at position 1858 had M2 in subsequent samples ($P < 0.0001$).

M1: C → T Change at Nucleotide Position 1856 (Pro → Ser Change at Codon 15). All of the patients with M1 had M0.

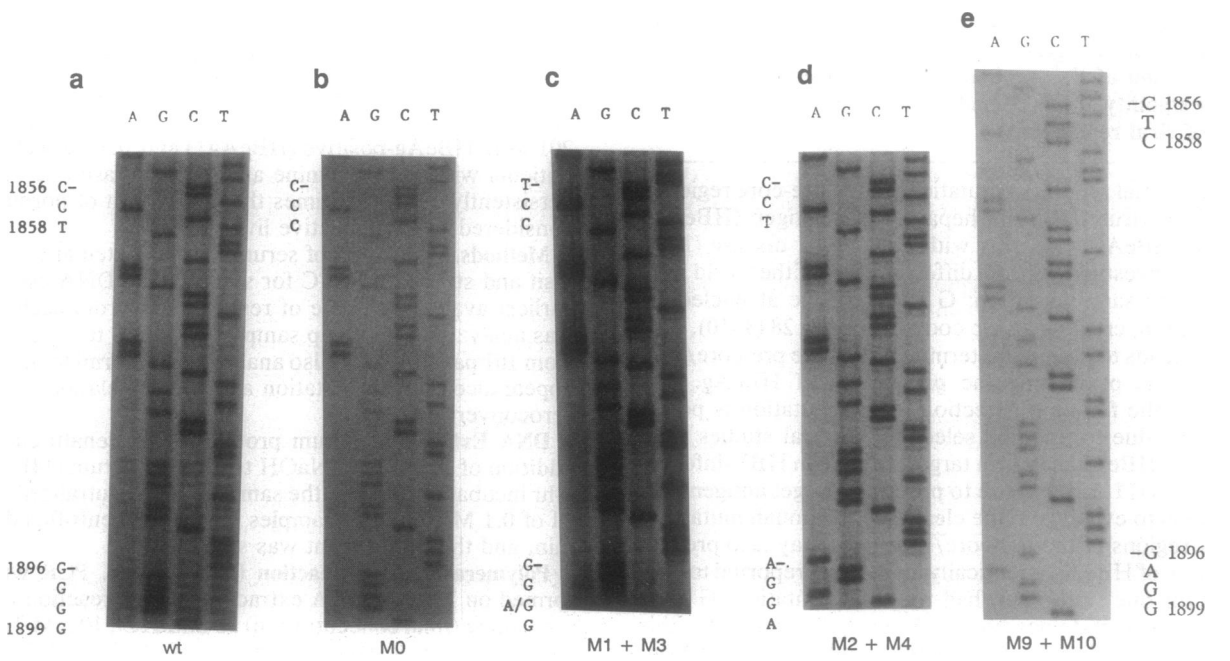

FIG. 1. Nucleotide sequence in the pre-core region of hepatitis B virus. (*a*) wt sequence. (*b*) M0: T → C at nucleotide position 1858 (Pro → Pro at codon 15). (*c*) M1 (and M0) + M3: C → T at position 1856, T → C at position 1858 (Pro → Ser at codon 15), and G → A at position 1898 (Gly → Ser at codon 29). (*d*) M2 + M4: G → A at position 1896 (Trp to stop at codon 28) and G → A at position 1899 (Gly → Asp at codon 29). (*e*) M9 (and M0) + M10: C → T at position 1857, T → C at position 1858 (Pro → Leu at codon 15), and G → A at position 1897 (Trp → stop at codon 28).

Table 1. Prevalence of pre-core HBV sequences in HBeAg$^+$ and HBeAg$^-$ patients

Patient phenotype	No. (%) of patients with pre-core HBV sequences				
	wt	M1	M2	Misc.	Total
HBeAg$^+$	136 (68)*	43 (21)†	20 (10)‡	2 (1)	201
Active disease	76 (63)§	24 (20)¶	19 (16)‖	1 (1)	120
Inactive disease	60 (74)	19 (24)	1 (1)	1 (1)	81
HBeAg$^-$	26 (42)*	8 (13)†	24 (39)‡	4 (6)	62
Active disease	11 (35.5)§	6 (19)¶	11 (35.5)‖	3 (10)	31
Inactive disease	15 (48)	2 (7)	13 (42)	1 (3)	31
Total	162 (62)	51 (19)	44 (17)	6 (2)	263

*136/201 vs. 26/62; $P = 0.0003$.
†43/201 vs. 8/62; $P = 0.14$.
‡20/201 vs. 24/62; $P < 0.0001$.
§76/136 vs. 11/26; $P = 0.2$.
¶24/43 vs. 6/8; $P = 0.45$.
‖19/20 vs. 11/24; $P = 0.0008$.

Thus, the sequence at codon 15 was changed from CCT to TCC (Fig. 1*c*). M1 was detected in 51 (19%) of the total patients; of these 51, 43 were HBeAg$^+$ (21% of HBeAg$^+$ patients) and 8 were HBeAg$^-$ (13% of HBeAg$^-$ patients) ($P = 0.14$) (Table 1). Twenty-one (41%) of the 51 patients had concomitant M3. Twelve (28%) HBeAg$^+$ patients with M1 lost HBeAg during follow-up (Table 2). All 12 continued to have M1 after loss of HBeAg, and none developed M2. Fifty-nine percent (30 of 51) of the patients with M1—56% (24 of 43) of the HBeAg$^+$ M1 patients and 75% (6 of 8) of the HBeAg$^-$ M1 patients—had active liver disease ($P = 0.45$) (Table 1). One patient had a mixture of C and T at position 1856.

M2: G → A Change at Nucleotide Position 1896 (Trp → Stop at Codon 28). M2 (Fig. 1*d*) is the pre-core stop codon mutation that has been described by many investigators. Interestingly, none of the patients with M2 had M0 or M1. Thus, the nucleotide sequence at codon 15 was invariably CCT in these patients. M2 was detected in 44 (17%) patients; of these 20 were HBeAg$^+$ (10% of HBeAg$^+$ patients) and 24 were HBeAg$^-$ (39% of HBeAg$^-$ patients) ($P < 0.0001$) (Table 1). Fifteen (75%) HBeAg$^+$ patients with M2 lost HBeAg, and 9 (45%) did so within the first year of follow-up (Table 2). Thirty (68%) patients with M2 (95% of the HBeAg$^+$ M2 patients and 46% of the HBeAg$^-$ M2 patients) had active liver disease ($P = 0.0008$) (Table 1).

M3: G → A Change at Nucleotide Position 1898 (Gly → Ser Change at Codon 29). M3 (Fig. 1*c*) was invariably associated with the presence of M1 and the absence of M2. Thus, all patients with M3 had TCC at codon 15 and TGG at codon 28. M3 was detected in 21 (8%) patients. M3 tended to emerge later than M1, being more frequently present in HBeAg$^-$ than in HBeAg$^+$ patients with M1 (63% vs. 36%, $P = 0.24$). In addition, serial studies showed that 14% of patients who had M1 only in the initial sample developed M3 in subsequent samples. The concomitant presence of M1 and M3 was associated with more active liver disease: 86% of patients with M1 + M3, and 39% of patients with M1 only had active liver disease ($P = 0.001$).

Table 2. Correlation between initial pre-core HBV sequence and subsequent loss of HBeAg

Pre-core sequence	Total no. of patients	No. (%) of patients losing HBeAg	
		During follow-up	Within year 1
wt	136	36 (26)*	16 (12)†
M1	43	12 (28)*	6 (14)†
M2	20	15 (75)*	9 (45)†
Misc.	2	1 (50)	0 (0)
Total	201	64 (32)	31 (15)

*$P = 0.0001$.
†$P < 0.0001$.

M4: G → A Change at Nucleotide Position 1899 (Gly → Asp at Codon 29). M4 (Fig. 1*d*) was found in 10 (4%) patients as the only mutation or in association with M1 or M2. Thus, both nucleotides 1856 and 1858 could be T or C. The association with M2 was stronger than that with M1 or the wt sequence ($P < 0.0001$). Seven (70%) patients had concomitant M2, and all were HBeAg$^-$. In these patients, M4 appeared to emerge later than M2, being detected in 29% of HBeAg$^-$ patients but in none of the HBeAg$^+$ patients ($P = 0.01$). In addition, serial studies showed that 11% of patients who had M2 only in their initial samples developed M4 in subsequent samples. The concomitant presence of M4 and M2 was not associated with more active liver disease: 43% of patients with M2 and M4 and 64% of patients with M2 only had active liver disease ($P = 0.65$). Two (20%) patients with M4 had concomitant M1; one was persistently HBeAg$^-$ with inactive liver disease, and the other had active liver disease and reverted from being HBeAg$^-$ to HBeAg$^+$ during follow-up. One patient had isolated M4; he was HBeAg$^-$ negative and had inactive liver disease.

M5: A → T (5a) or A → C (5b) Change at Nucleotide Position 1846 (Ser → Ser at Codon 11). M5 was detected in 17% (45) of the patients—intermittently in 5% and consistently in 12%. All except 2 patients had M5a. Of the patients with M5a (43), 45% had no missense or nonsense mutation, 11% had concomitant M1, 44% had M2. Moreover, of the patients with M5a, 48% were initially HBeAg$^+$; of these, 77% lost HBeAg during follow-up. Of the 2 patients with M5b, 1 also had M4.

M6–M12. Five (2%) patients had M6 [C → T change at position 1877 (Leu → Leu at codon 22)]. All came from the same family. All had M0 but no missense or nonsense mutation. All were HBeAg$^+$ and remained so during follow-up. One patient had M7 [C → T change at position 1873 (Ser → Ser at codon 20)], and one had M8 [G → A change at position 1887 (Gly → Glu at codon 25)]. Both patients also had M0 but no missense or nonsense mutation. One (0.4%) patient had coexistent M9 [C → T change at position 1857 (Pro → Leu at codon 15)] and M10 [G → A change at position 1897 (Trp → stop at codon 28)] (Fig. 1*e*). This patient also had M0. He was HBeAg$^-$ and had active cirrhosis. One patient had M11 [A → T change at position 1814 (Met → Leu at codon 1), leading to loss of the start codon. This patient was HBeAg$^-$ and had active cirrhosis. One patient had M12 (insertion of A-A between positions 1817 and 1820, leading to a frameshift mutation creating a stop codon at codon 9). This patient was HBeAg$^-$ and had chronic active hepatitis.

DISCUSSION

In this extended study, we found that, as in our earlier report (13), the most frequent missense or nonsense mutations in the pre-core region were M1, M2, M3, and M4. We also confirmed that M1 and M2 are mutually exclusive and that M3 was found only in association with M1. Although M4 was mainly found in association with M2, it was also found in association with M1 as well as wt sequence.

Our current data showed that M1 can be detected in similar proportions of HBeAg$^+$ and HBeAg$^-$ patients and is unlikely to affect HBeAg secretion. M1 alone had no effect on the activity of liver disease, but the combination of M1 and M3 was frequently associated with active liver disease. The presence of M2 in HBeAg$^+$ patients was associated with active liver disease in 95% of these patients and impending HBeAg clearance in 45%. In contrast, only 46% of HBeAg$^-$ patients with M2 had active liver disease. Thus, M2 on its own is not the cause of active liver disease, but the immune process by which wt sequence is eliminated and M2 is selected may be the responsible factor. M4 did not seem to

confer increased risk of active liver disease in patients with M2.

The most common conserved mutation was M0. A few published sequences have also reported a C at position 1858 (17–19), so M0 may be a common variant rather than a true mutation. In view of the conserved nature of this change, M0 was ignored in our earlier report. We were astonished that M0 was present in 100% of patients with M1 even though codon 15 can be changed from encoding proline to encoding serine with C → T change at position 1856 only (TCT also codes for serine). We were even more surprised by the complete absence of M0 in patients with M2.

The invariable coexistence of certain mutations in codon 15 and codons 28 and 29 and the mutual exclusion of other mutations in these two noncontiguous regions is intriguing. Revelation came from the paper by Junker-Niepmann *et al.* (20) on the localization of a short cis-acting sequence for HBV pre-genome encapsidation in the pre-core/core region. The authors found that this encapsidation sequence contains a number of inverted repeat sequences with the potential of forming secondary structures such as a stem–loop. Codon 15 and codons 28 and 29 are in fact located opposite each other on the stem (Fig. 2). Ogata *et al.* (21) showed that M2 and M4 serve to stablize the stem. Tong *et al.* (22) demonstrated that M2 and M4 had no negative effect on pre-genome encapsidation and viral replication; in contrast, some mutations involving other parts of the pre-core region may result in defective replication, thus accounting for the absence of such mutants in nature. Wang and Seeger (23) recently reported that this pre-genome encapsidation signal also serves as a template for the initiation of reverse transcription from pre-genomic RNA to minus-strand DNA.

The stem–loop structure consists of 14 nucleotides on each limb of the stem; 3 of the nucleotides are unpaired: 1858 with 1896, 1855 with 1899, and 1850 with 1904. Interestingly, in all 3 unpaired sites, the nucleotide on the ascending limb is a T, while that on the descending limb is a G. Codon 15 and codons 28 and 29 are involved in 2 of the 3 unpaired sites. M2 and M4 change nucleotides 1896 and 1899 in the descending limb from G to A, thereby increasing the stability of this stem. However, a G → A change at position 1904 has not been reported, nor is it observed in any of the 263 patients that we studied. This may be related to the fact that nucleotide 1850, which is situated opposite to nucleotide 1904 on the stem, lies outside the functionally important region (nucleotides 1852–1930) (20) of the pre-genome encapsidation sequence.

Surprisingly, mutations that involve T → C change in two of the three unpaired sites of the ascending limb, which also would serve to stabilize the stem, have not been reported. We found that 40% of our patients had a T → C change at the third unpaired site (position 1858: M0). M0 provides base pairing with the G at position 1896, thus obviating the need for a G → A change in the descending limb. This explains why none of our patients with M0 develop M2.

M1 involves a C → T change at position 1856, thus destroying the C-G pairing between nucleotides 1856 and 1898. In our study, M1 was only found in the presence of M0. It is possible that the additional base pairing provided by M0 may permit other mutations in the vicinity without jeopardizing the function of the encapsidation sequence and the replication of the virus. Nevertheless, approximately half the patients with M1 also had M3, a G → A change at position 1898, which restores base pairing at this site. Theoretically, either M1 or M3 could appear first. However, our data suggest that M3 tended to follow M1. While M1 was present on its own in 59% of patients, M3 was always found in association with M1. The scarcity of reports of M1 may be related to the following facts: M0 is an uncommon variant in

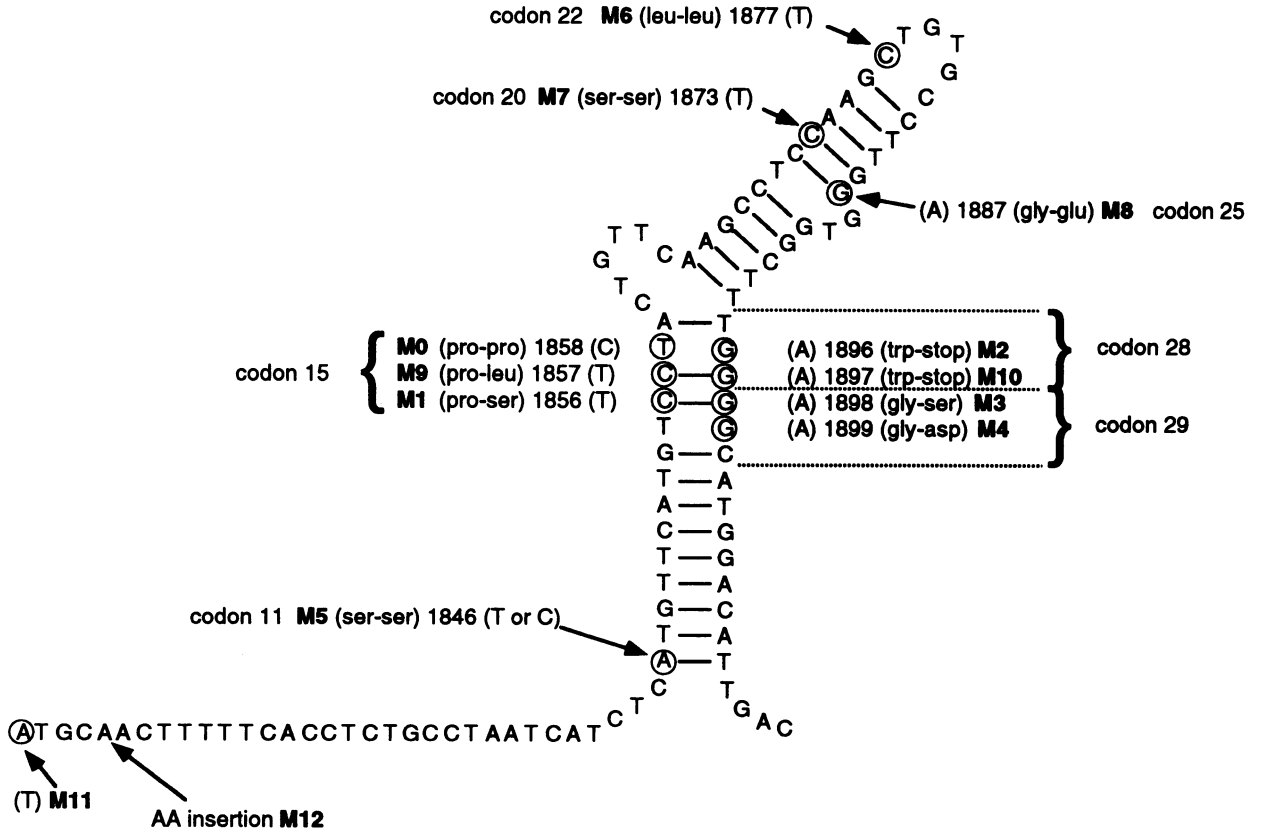

FIG. 2. Location and nature of the mutations detected in relation to the nucleotide sequence and proposed secondary structure of the HBV pre-genome encapsidation sequence (20). Substituted nucleotides are shown in parentheses. The most frequently detected mutations are clustered around a 4-nt segment of the stem.

other ethnic groups, other studies had a smaller number of patients, and some studies used biased techniques (hybridization with oligonucleotide probes corresponding to wt or the M2 sequence). Among 19 published complete HBV sequences, only 3 (16%) had M0 (17–19, 21). Of 13 studies on pre-core HBV mutations, only 3 (23%) reported the presence of M0 in a small proportion of the clones sequenced (1, 3, 4, 7–9, 24–31).

The single patient with coexistent M9 and M10 further illustrates the importance of base pairing in this stem–loop structure. These two mutations involve the replacement of the C·G pair between nucleotides 1857 and 1897 by a T·A pair. This patient also had M0, emphasizing that mutations that destabilize the 3-nt segment of the stem from position 1856 to 1858 and from 1896 to 1898 can only occur if there are already two paired sites.

M4 provides base pairing between nucleotides 1855 and 1899, thus enhancing the stability of the stem–loop structure. It is located beyond the 3-nt segment that encompass M0, M1, M2, and M3. Thus, it is not surprising that M4 can be associated with any of these mutations. However, it is unclear why M4 was more frequently associated with M2, nor is it known why M4 emerged later than M2.

The only other mutations involving the stem are M5a and 5b which change nucleotide 1846 from A to T or C, thus destroying base pairing at this site. However, compensatory mutations at nucleotide 1908 that would restore stability to this site were not observed. This may be related to the position of this site: at the mouth of the stem–loop structure, so the need for base pairing here is less critical; and the fact that this site is outside the functionally important region of the pre-genome encapsidation sequence. Furthermore, in some patients, M5a was only intermittently detected.

Some of the mutations identified in this study have been reported in other published full-length HBV sequences. Interestingly, M0 and M1 were also not detected in the five sequences with M2 (31, 32–34). However, neither M1 nor M3 was found in the three sequences with M0 (17–19). M9 was not detected in the two sequences with M10 (31), but both sequences had M2, again indicating that at least two of three sites in the 3-nt segment (1856–1858 and 1896–1898) need to be paired.

Our results support the proposed secondary structure of the pre-genome encapsidation sequence (20) and the importance of maintaining this secondary structure. Our data also suggest that the 3-nt segment (1856–1858 and 1896–1898) and perhaps the adjacent nucleotide (1855 or 1899) are crucial for the function of this encapsidation sequence, possibly because of its close proximity to the start codon of the core gene. The frequent occurrence of M2 and M4 is easy to understand, since they serve to stabilize the stem structure. However, the frequent occurrence of M1, which destabilizes the stem structure, is intriguing. Although half of the patients with M1 had M3, a compensatory mutation that restores stability of the stem, we do not rule out the possibility that M1 and M3, both being missense mutations, have specific functional roles including modification of the secondary structure and hence biological effects of the pre-genome encapsidation sequence. The absence of mutations in the bulge (nucleotides 1860–1865) of this stem–loop structure may be related to the recent finding that mutations in this region can interfere with synthesis of minus-strand DNA (23).

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