Novel mutation in Hepatitis B virus preventing HBeAg production and resembling primate strains.

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Novel mutation in *Hepatitis B virus* preventing HBeAg production and resembling primate strains

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Chronic carriers of hepatitis B infection often harbour virus strains with mutations in the precore region. These mutations are temporally associated with the development of HBeAg loss and seroconversion to anti-HBe. The most common precore mutation is a stop codon at position 1896, but other mutations leading to abolished HBeAg secretion have been described. Here, a novel precore mutation introducing a lysine in the precore position 28, a sequence shared by non-human primates but not by other human isolates, is described. However, the insertion causes a frame-shift preventing the expression of HBeAg by introducing a stop codon 5 aa downstream of the mutation. Analysis of the predicted RNA secondary structure indicates that the insertion could occur without fatally affecting the stability of the stem–loop encapsidation signal.

A female asymptomatic chronic HBV carrier who seroconverted from HBeAg to anti-HBe after 4 years observation time was followed for an additional 12 years. She had suppressed from HBeAg to anti-HBe after 4 years observation time was followed for an additional 12 years. She had sup

**Hepatitis B virus** (HBV) belongs to the family *Hepadnaviridae*, whose members infect birds (genus *Aviparadnavirus*) and mammals (genus *Orthohepadnavirus*). Orthohepadnaviruses infecting mammals, other than humans, were first found in the serum of Eastern Woodchucks (*Woodchuck hepatitis B virus*, WHV) (Summers et al., 1978), and subsequently in California Beechey ground squirrels (*Ground squirrel hepatitis B virus*, GHSV). GHSV is slightly larger than HBV and is related more to WHV (82% nucleotide homology) than to HBV (55%) (Gerlich et al., 1980). The species of HBV comprises human isolates, but also strains from non-human primates such as chimpanzee (*Pan troglodytes*), gibbon (*Hylobates lar*), orangutan (*Pongo pygmaeus pygmaeus*) and gorilla (*Gorilla gorilla*) (Grethe et al., 2000; Norder et al., 1996; Warren et al., 1999; Vaudin et al., 1988). The *Woolly monkey hepatitis B virus* (WMHBV) represents the most divergent of the non-human primate strains and forms a separate species (Lanford et al., 1998).

Despite advances in HBV research leading to the development of efficient vaccines, it is estimated that approximately 400 million people worldwide are chronic carriers of the virus. The mortality within this group of diseases associated with the infection (primarily hepatocellular carcinoma, HCC) is significantly elevated, and those chronically infected also constitute a pool for the virus promoting its distribution to not yet infected individuals. The serological status of the chronic carrier provides valuable information about the actual status of the infection. Typically, HBsAg and anti-Hbc are present in serum, while HBeAg can be present or undetectable. The loss of circulating HBeAg and the development of antibodies specific for this antigen, anti-HBe, is temporally associated with a reduction in viral load, typically ranging from 3 to 4 orders of magnitude. This seroconversion has been shown to be associated with the selection of replication-competent precore HBV mutants that are unable to secrete HBeAg. The most frequent mutation is the guanine (G) to adenine (A) change at nt 1896 (Carman et al., 1989; Okamoto et al., 1990), but other mutations leading to abolished HBeAg secretion have also been described (Laras et al., 1998; Okamoto et al., 1994). In spite of its intrinsic reduction of viral load, this mutation has few positive consequences; late phase complications like the development of HCC are more frequent in anti-HBe-positive patients. Consequently, it is desirable to detect these mutations to facilitate possible future molecular screening methods at the nucleotide level. Here, we present a previously undescribed insertion near the precore/core junction.

DNA was extracted from serum that had been stored at -20°C, using the phenol/chloroform method with...
subsequent ethanol precipitation (Ljunggren & Kidd, 1991) or by using a DNeasy blood mini kit (Qiagen) according to the manufacturer’s protocol.

Estimation of differences in viral load was performed using a quantitative real-time PCR (Hennig et al., 2002). A RotorGene 2000 System (Corbett Research) was used. The method was standardized by using samples of known HBV DNA concentrations from a quality control panel (VQC Sanquin Diagnostics). The sensitivity was found to be 10–100 copies per ml by end-point titration. The viral load in samples acquired during the HBeAg-positive wild-type phase (1987) and the anti-HBe-positive mutated phase (1999), as measured by quantitative PCR, showed a reduction in calculated serum HBV DNA concentrations from $6 \times 10^7$ to $3 \times 10^4$ copies per ml.

The HBV core promoter and precore region were amplified as described previously (Ljunggren & Kidd, 1991). Strict measures were taken throughout the procedure to prevent contamination and there were no signs of DNA carryover at any stage of the investigation.

HBV DNA isolated from the patient in 1999 had been sequenced as described previously (Kretz et al., 1989; Ljunggren & Kidd, 1991). Newly amplified aliquots from the patient’s serum from 1999 and 1987 (the HBeAg-positive wild-type phase) were subsequently sequenced using the ABI Prism BigDye Terminator version 3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems) using primers KL6 and KL28 (Ljunggren & Kidd, 1991). Each strand was analysed with an ABI 3100 Genetic Analyser (Applied Biosystems) by the Biomolecular Resource Facility at Lund University. BioEdit software (Tom Hall, Department of Microbiology, North Carolina State University, NC) was used for the analysis of the results. HBV DNA isolated from the patient in 1999 contained an insertion clearly visible in the original sequence gel. The presence of the insertion of thymine (T) at 1895 was confirmed by direct sequencing in both the forward and reverse sequences of the sample isolated after seroconversion, the absence of T of the same isolate from the HBeAg-positive wild-type phase (data not shown) was also confirmed by direct sequencing. These observations were further confirmed by reamplifying and sequencing extracted DNA from the patient sample. The sequence has been given the GenBank accession no. DQ223127.

Multiple alignments revealed a similarity at the nucleotide level with isolates from non-human primates: namely a T in position 1896 (Fig. 1). However, translation of the sequences constituting the precore region revealed a translational stop codon 5 aa downstream of the insertion (Fig. 2), a result of the insertion and the resulting frame-shift.

Figure 1 shows a multiple alignment of the precore region from human isolates including the described mutant strain (1895T Insertion; GenBank accession no. DQ223127) along with strains infecting non-human primates. A thymine (T, arrow) in the mutant strain is shared by non-human primate strains, but not by other human isolates.

Fig. 1. Multiple alignments of the precore region from human isolates including the described mutant strain (1895T Insertion; GenBank accession no. DQ223127) along with strains infecting non-human primates. A thymine (T, arrow) in the mutant strain is shared by non-human primate strains, but not by other human isolates.

MFOLD prediction (Zuker, 2003) of RNA secondary structures suggested that stem–loop structures of the HBV encapsidation signal were largely unaltered in the mutated variant as compared with the wild-type structure. The same analysis performed on an HBV strain infecting chimpanzee and other non-human primate strains revealed an RNA secondary structure different from that of HBV strains infecting humans (Fig. 3).

The disappearance of HBeAg from the blood of hepatitis B-infected patients is temporally associated with the emergence of anti-HBe antibodies and a concomitant reduction in viral load. In a large proportion of these patients the precore nucleotide mutation G1896A is present. This position lies within the functionally important encapsidation signal necessary for the packaging of the HBV pregenome into core particles (Junker-Niepmann et al., 1990). Upon examination of calculated secondary structures it is apparent that nt 1896 is located opposite to nt 1858 in the stem–loop.

Fig. 2. Translated sequences reveal a lysine (L, arrow) in the precore position 28 of the mutant strain (1895T Insertion). This sequence is shared by non-human primates, but not by other human isolates. However, a translational stop codon (asterisk) 5 aa downstream in the mutated strain explains the inability of this isolate to secrete HBeAg.
The nucleotide in the latter position is a C in genotype A strains and some genotype F strains, but a T in the other genotypes. As a consequence, the G1896A nucleotide mutation stabilizes the structure of the encapsidation signal in genotypes B–E and is thus favoured in these genotypes. Conversely, if the mutation occurs in a genotype A strain, this has negative consequences for the stability of the stem–loop structure – a notion reflected in the relatively low frequency of anti-HBe-positive HBV patients infected by genotype A strains (Li et al., 1993). However, A and F strains have been recorded to be able to harbour the G1896A mutation if this is temporally preceded by a C1858T mutation, thus conserving the structural stability of the encapsidation signal (Bla¨ ckberg & Kidd-Ljunggren, 2000; Li et al., 1993). The necessity of these sequential alterations of the genome in order to maintain the stability of secondary structures in genotypes A and F evokes further questions when the same region in non-human primates is studied. These strains have a T in position 1896 and a T in position 1858 (Fig. 1). The resulting RNA secondary structure as calculated through the MFOLD algorithm (Fig. 3c) is identical to that of a hypothetical genotype A strain with a G1896A mutation, but without the C1858T mutation alleged to be a necessity for its stability.

Insertions leading to HBeAg negativity have previously been described both in cases where the function of the stem–loop encapsidation signal is retained because of compensatory mutations or because the mutation occurs in the distal part of the gene (Li et al., 1993; Okamoto et al., 1990), but also within the functionally important encapsidation signal (Santantonio et al., 1991; Tong et al., 1993), resulting in an impaired packaging of pregenomic RNA. However, the relatively low frequency of insertions in the precore region is obvious in works like the NMR examination of the apical stem–loop by Flodell et al. (2002). In their report, 1217 human HBV isolates representing all seven HBV genotypes (A–G) were studied (205 strains from their own laboratory and 1012 strains from the literature) and no insertions were detected. We describe a novel precore mutation resulting in a frame-shift preventing the expression of HBeAg by introducing a stop codon 5’ aa downstream of the mutation. Examination of calculated RNA secondary structure indicates that the insertion could occur without fatally affecting the stability of the encapsidation signal stem–loop; however, a single nucleotide bulge is observed. Whether this structural change is the cause of the observed reduction of viral load requires further investigations and a thorough analysis of possible steric disturbances.

Fig. 3. RNA secondary structures as predicted by the MFOLD algorithm. Genotype A (GenBank accession no. V00866) (a), HBeAg-negative frame-shift mutant (b) and HBV isolated from chimpanzee (GenBank accession no. AF242585) (c). Secondary structures for HBV strains isolated from other non-human primates discussed are identical to the chimpanzee strain.
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References


