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CASE REPORT

Uncommon mutation pattern of a hepatitis B virus isolate from genotype F infecting a patient with AIDS

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Summary *Objective*. To study the genomic variations of a hepatitis B virus (HBV) isolate in a patient coinfected with human immunodeficiency virus type 1 (HIV-1) who developed severe hepatitis and died of AIDS.

Methods. Two blood samples were collected, the first one during the asymptomatic phase of HIV-1 infection, and the other, 3 years later, few months before the death of the patient. Both samples were HBsAg and anti-HBe positive. Pre-S/S and precore-core genome regions were PCR amplified and analyzed.

Results. The HBV isolate belonged to genotype F, cluster IV. A number of unique amino acid substitutions were found in the surface antigen gene and the overlapping polymerase coding region of HBV genomes derived from both samples. However, these substitutions reflected natural variations rather than mutations of clinical significance. The precore stop codon mutation A_{1896} was present in both genomes. Furthermore, the HBV genome derived from the second, but not first sample, showed two out-of-frame core interval deletions, one and 103 nucleotides in length, respectively.

Conclusions. This is the first report of an HBV isolate from genotype F with core internal deletions. Our results suggest an association between specific core mutations and the severe hepatitis developed by the patient.

Introduction

Hepatitis B virus (HBV), a member of the *Hepadnaviridae* family, is an etiologic agent of acute and chronic liver disease, including cirrhosis and hepatocellular carcinoma (HCC). HBV infection is commonly diagnosed by the presence of hepatitis B surface antigen (HBsAg) and antibodies to the hepatitis B core antigen (anti-HBc). HBV isolates have been classified into seven genotypes, A to G,[1-3] which show a distinctive geographic distribution in the world. Genotype F, indigenous to South and Central Americas, is the most divergent genotype. Due to their restricted geographic area of prevalence, HBV isolates from genotype F have not been extensively studied, and a limited number of mutants have been characterized until now.

Most of the mutations occurring during the natural course of chronic HBV infection are localized in the pre-S/S and precore/core genome

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regions. A number of rearrangements in the pre-S region, including deletions and start codon point mutations, have been identified in patients with fulminant or chronic hepatitis, as well as in patients with HCC.[4,5] Mutations in the S gene are of particular importance since they may affect the 'a' antigenic determinant of HBsAg (codons 124–147) and allow HBVs to escape neutralization by anti-HBs antibodies. Several types of vaccine escape mutations, as $Gly_{145} \rightarrow Arg$, $Asp_{144} \rightarrow Ala$, $Met_{133} \rightarrow Leu$, $Gln_{129} \rightarrow His$ and $Ile/Thr_{126} \rightarrow Ala$, have been detected in HBV isolates from different parts of the world.[6]

In the precore region, the most common mutation is a G → A substitution at nucleotide (nt) 1896, that prevents the production of hepatitis B 'e' antigen by introducing a premature stop codon into the precore open reading frame.[7] The occurrence of the A_{1896} mutation is restricted to the isolates showing a T, not a C, at position 1858, due to base pairing T_{1858}:A_{1896} in the pregenomic RNA.[8] This is the case for genotypes B, C, D, E, G, but not for genotype A. In genotype F, some isolates show a C at position 1858, while others show a T. On the other hand, naturally occurring variants containing core internal deletions (CID) have been found in HBV chronic carriers.[9] These CID variants can replicate preferentially at the expense of the wild-type helper virus.[10–12]

Characteristic mutations, such as deletions in the pre-S and C regions, premature termination codons in the pre-S1 and S region, and deletions/insertions in the X gene, have been found in HBVs infecting long-term immunosuppressed patients.[13–16] In patients coinfected with HBV and human immunodeficiency virus (HIV), it has been suggested that HIV may interfere with the natural history of HBV infection by enhancing HBV replication.[17]

In this study, we investigated the presence of mutations in pre-S/S and precore-core regions of an HBV isolate from genotype F present in two serum samples collected from an HBV–HIV coinfected patient. One sample was collected during the asymptomatic phase of HIV infection, and the other was obtained during the AIDS stage, few months before the patient died.

Patient and methods

Patient characteristics and serological markers

The patient under study was a 32-year-old homosexual man who was diagnosed HIV-1 positive in 1988. The patient remained asymptomatic until 1990 when he developed ganglionar tuberculosis and started azidothymidine treatment. In the following year, he presented Kaposi's sarcoma, and died of AIDS in 1992. The patient had a chronic active hepatitis which progressed to severe hepatitis in the year of his death.

The patient remained positive for HBsAg and anti-HBe antibody in all routine tests performed between 1988 and 1992. Regular liver function tests showed alanine aminotransferase levels 3–4 times the upper limit of the normal range, and alkaline phosphatase increases greater than five times the upper limit of normal. Two serial serum samples were available for HBV DNA analysis. The first one was collected in 1988 at the time of HIV diagnostics, and the other in 1991, few months before the death of the patient.

DNA extraction and PCR assays

HBV DNA was extracted from serum using phenol-chloroform after treatment with proteinase K, as previously described.[18] DNA samples were submitted to two different PCR assays, performed in a final volume of 50 μl under the following conditions: 94 °C, 30 s; 52 °C, 1 min; 72 °C, 2 min; 35 cycles, followed by a final elongation of 7 min at 72 °C. Precore-core region was amplified by semi-nested PCR using primers X4 (5′-AAGGTCTTACATAAGAGGAC-3′, sense, nt 1644–1663) and C2 (5′-CTAACATTGAGATTCCCGAGATTGAGA-3′, antisense, nt 2458–2432) in the first round, and primers PC1 (5′-GGCTGTAGGCATAAATTGGTCTG-3′, sense, nt 1781–1803) and C2 in the second round. Pre-S/S region was amplified with sense primer PS1 (5′-CCATATTCTTGGGAACAAGA-3′, nt 2826–2845) and a mix of antisense primers S2 (5′-GGGTTTAAATGTATACCCAAAGA-3′, nt 841–819) and S22 (5′-GTATTTAAATGGATACCCACAGA-3′, nt 841–819), able to amplify HBV from all genotypes. Amplification products (10 μl) were loaded on a 2% agarose gel, electrophoresed, stained with ethidium bromide, and visualized under UV light.

Restriction fragment length polymorphism analysis

Pre-S/S amplicons were digested with restriction endonucleases *Bam*HI, *Eco*RI and *Stu*I. The presence of restriction sites for these enzymes was determined after agarose gel electrophoresis and comparison of the band sizes with molecular weight markers. In addition, prediction of *Bam*HI, *Eco*RI, and *Stu*I restriction sites was performed for 172

HBV sequences available from GenBank, representative of genotypes A (42 sequences), B (22), C (41), D (27), E (7), F (23), and G (8). Among the 23 isolates from genotype F, three may be alternatively classified in a new genotype (H) whose existence has been recently proposed.[19]

The precore stop codon G → A_{1896} mutation was detected by using a PCR-restriction fragment length polymorphism (RFLP) method described previously.[20]

Molecular cloning and nucleotide sequencing

Pre-S/S and precore-core PCR products were cloned into pCRII plasmid vector using TA cloning kit (Invitrogen, San Diego, CA). For nucleotide sequencing recombinant plasmid DNAs were purified by ultracentrifugation in CsCl gradient. Nucleotide sequences were determined using the Cy5 Autoread Sequencing System (Amersham Biosciences, Little Chalfont, UK) with M13 universal and reverse primers, as well as internal, HBV specific, Cy5-labelled primers. Sequencing reactions were analyzed on an ALFexpress automated sequencer (Amersham Biosciences). Independent plus and minus strand sequencing were completed.

Phylogenetic analysis

Nucleotide sequences were aligned using PILEUP (Wisconsin Sequences Analysis Package GCG, Madison, WI). A phylogenetic tree was generated by neighbor-joining analysis of genetic distances, using the TREECON software package for Windows.[21] Sequence of woolly monkey HBV[22] was used as an outgroup for construction of the tree.

Results

Analysis of pre-S/S region

HBV DNAs were extracted from two serum samples collected from an HBV-HIV coinfected patient. Sample 88 was collected in 1988, during the asymptomatic phase of HIV infection, and sample 91 was obtained in 1991, during the AIDS stage, few months before the death of the patient. Pre-S/S genomic regions were amplified by PCR. After digestion of the amplicons with *Bam* HI, *Eco* RI and *Stu* I restriction endonucleases, it was observed that the restriction patterns of samples 88 and 91 were identical, but unique when compared with those deduced from 172 pre-S/S sequences available from GenBank and representative of all HBV genotypes. This was due to the presence of an uncommon *Stu* I site, absent in all 172 sequences. PCR products from samples 88 and 91 were then cloned, and clones S-88 and S-91, that displayed restriction patterns identical to that found for PCR products, were sequenced.

Phylogenetic analysis was performed with a limited number of sequences, representative of all HBV genotypes (Fig. 1). It was found that sequences of clones S-88 and S-91 differed slightly between them, and belonged to genotype F, in which they formed a separate group together with HBV isolate Fou (France, GenBank accession number X75658). Actually, this separate group corresponded to cluster IV of genotype F in which isolate Fou has been recently classified.[23] Sequences S-88 and S-91 were then aligned with all 30 HBV genotype F sequences available in GenBank. Two additional sequences (AF223962 and AF223965) from Argentinian isolates were found to belong to cluster IV (not shown). The uncommon *Stu* I site was localized at nt position 21 in sequences S-88 and S-91. Its presence was also depicted in sequence AF223965. Amino acid sequences were deduced for the whole surface antigen, as well as for residues 184–583 of the viral polymerase (due to gene overlapping). Table 1 shows the mutations occurring in isolate 88 and/or isolate 91, but not in any of the HBV sequences from isolates belonging to clusters I–III of genotype F. Three replacements common to the five isolates of cluster IV were observed: one in the S region (Leu_{110} → Ile), and two in the polymerase gene (Ile_{216} → Phe, Gln_{233} → His). Sequences S-88 and S-91 shared five unique amino acid residues, Gln_{15} and Ala_{51} in pre-S2 region, Ile_{23} and Ile_{126} in S region, and Ser_{346} in the polymerase. Nine mutations were exclusive for sequence S-88 and five were exclusive for S-91. No clinical significance has been previously associated with the mutations observed here, except for position 126 (within the 'a' determinant), where certain substitutions lead to the emergence of vaccine escape mutants. However, the Thr_{126} → Ile substitution, observed here, has been considered as a natural variation.[6,24]

Analysis of core region

The patient under study was anti-HBe positive in all routine tests performed between 1988 and 1992. The HBV genomes deriving from serum samples 88 and 91 were analyzed by a PCR-RFLP method designed to detect the presence of the precore G_{1896} → A stop mutation.[20] This mutation, that prevents the expression of the hepatitis B 'e' antigen, was identified in both genomes.

Precore–core regions of HBV isolates from

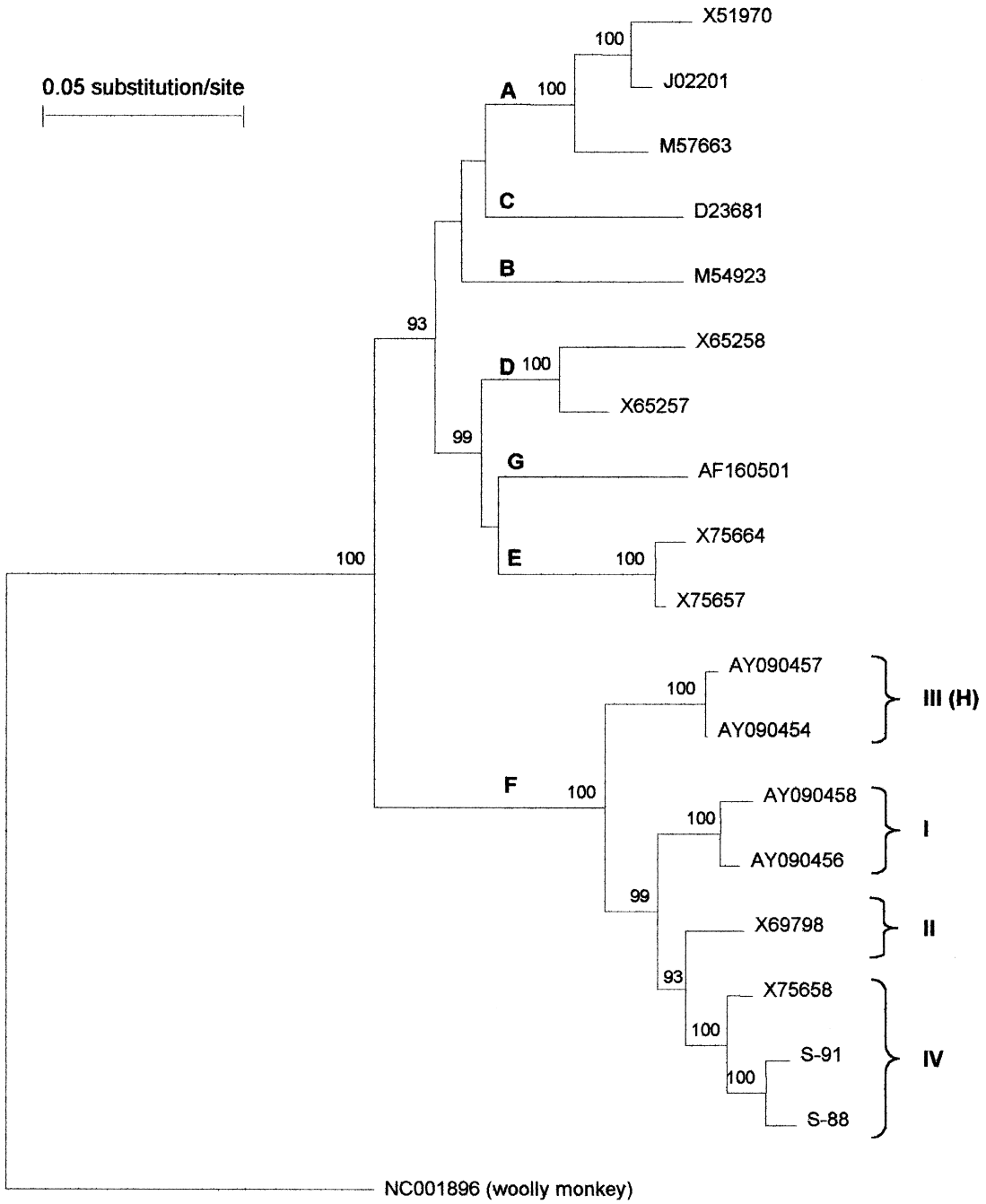

Figure 1 Phylogenetic tree of HBV isolates constructed with the neighbor-joining method, and based on the nucleotide sequences of the entire pre-S/S region (nt positions 2854 to 833). Isolates S-88 and S-91 are from this work. The other isolates are representative of all genotypes (A to G) and are designated by their GenBank accession numbers. The four clusters (I–IV) of genotype F are indicated. Isolates of cluster III have been recently classified into a new genotype named H.

samples 88 and 91 were PCR amplified, and amplicons were analyzed in agarose gel electrophoresis. In amplicon 91, the DNA band of expected size (678 nt) was faint, and an additional, major band of smaller size, indicating the presence of a CID variant, was observed. In amplicon 88, only the band of expected size was seen. Precore–core PCR products from sample 91 were cloned and three recombinant plasmids were sequenced. The sequences of the clones were closely related to that of isolate AF223965 (cluster IV), but presented the T_{1858}:A_{1896} pattern. Interestingly, all three clones presented two frameshift deletions in the core region, one of them of only one nucleotide

Table 1 Unique amino acid replacements in S-88 and S-91 sequences (genotype F, cluster IV) when compared to all HBV isolates from genotype F, clusters I–III.

Genotype F isolates (cluster)	Pre-S1 residue	Pre-S2 residues				S residues				Polymerase residues												
	27	15	24	26	33	51	23	101	110	114	121	126	161	210	216	233	313	333	334	336	346	374	458	469	477	517
S-88 (IV)	E	Q	V	A	T	A	I	Q	I	T	G	I	C	T	F	H	L	A	D	S	S	G	G	H	R	M
S-91 (IV)	D	Q	A	G	N	A	I	P	I	P	C	I	Y	P	F	H	L	A	E	P	S	R	R	S	M	I
AF223962 (IV)	D	P	A	G	N	G	T	Q	I	T	C	T	Y	T	F	H	P	T	E	S	N	G	R	H	M	I
AF223965 (IV)	E	P	A	G	N	G	T	Q	I	T	C	T	Y	P	F	H	L	A	E	S	N	G	R	H	M	I
X75658 (IV)	D	P	A	G	N	G	T	Q	I	T	C	T	Y	P	F	H	S	A	E	S	N	G	R	H	M	I
All others[a] (I–III)	D	P	A	G	N	G	T	Q[b]	L	T	C	T	Y	P	I	Q	S[c]	T	E	S	N	G	R	H[d]	M[e]	I

[a] Thirty isolates.
[b] R in one isolate.
[c] T in two isolates.
[d] K in three isolates and Y in two others.
[e] L in four isolates.

(position 1941), and the other of 103 nt (positions 2132–2234) (Fig. 2). As both deletions were localized upstream of the polymerase initiation codon, the open reading frame for polymerase was preserved.

Discussion

Deletions removing the pre-S2 start codon, as well as substitutions introducing premature termination codons in the pre-S/S region, have been frequently found in HBV isolates infecting immunosuppressed patients.[13–15] On the other hand, deletions in the pre-S region have been recently associated with the development of liver cirrhosis and end-stage liver disease.[16] In the present study, no such mutation was found in the HBV isolate infecting the HBV-HIV coinfected patient under study. Several unique amino acid substitutions were found in pre-S/S region, that seem, however, to be natural variations rather than mutations of clinical significance.

Seroconversion to anti-HBe usually correlates with a decreased level of HBV replication. However, the presence of anti-HBe antibodies can also be associated with the precore G → A stop mutation at nt 1896.[7] In this case, the patients present high levels of viremia, and serum HBV DNA is detectable by PCR. Several studies have associated the A_{1896} stop mutation with an exacerbation of clinical symptoms of liver disease caused by HBV.[25,26] Few reports, however, have mentioned the presence of the precore A_{1896} stop mutation in genotype F strains. We performed a screening of all 30 genotype F complete nucleotide sequences available in GenBank (21 had C_{1858} and nine had T_{1858}). None of them showed the A_{1896} stop mutation. However, when considering other genotype F isolates, deriving exclusively from anti-HBe positive patients, and whose nucleotide sequences have been only partially determined, a notable proportion (about one-half) show the mutation,[26–28] In this study, the mutation was found in an HBV-HIV coinfected patient who developed a progressive liver disease. The mutation was detected during both the asymptomatic phase of HIV infection (1988) and the AIDS stage (1991).

According to several longitudinal studies, CID variants have been often demonstrated in HBeAg positive patients,[9,13,29] but rarely in those who were negative.[30] CID variants can accumulate in long-term immunosuppressed patients, and their persistence has been associated with progressive liver disease.[13] Here, HBV genome derived from sample 91 showed two out-of-frame deletions, one and 103 nt in length,

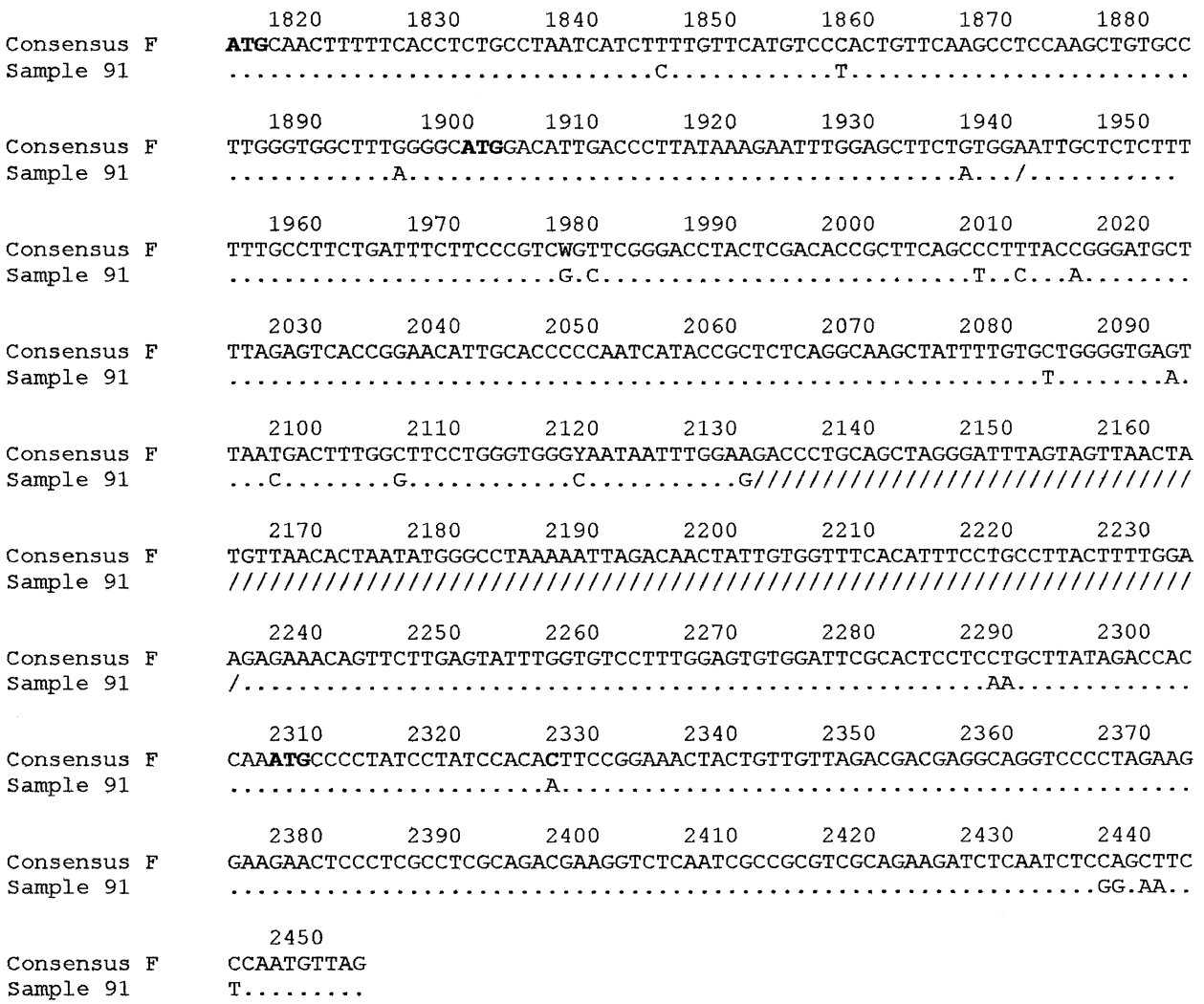

Figure 2 Alignment of nucleotide sequences of the precore–core region. Consensus F is the consensus sequence of thirty HBV isolates from genotype F. A dot represents an identical nucleotide in comparison with the top sequence, and a slash indicates a deletion. All variations indicated on the bottom line were observed in all three clones derived from sample 91, with the exception of positions C_{2097} and A_{2327}, which were present in only two clones. The initiation codons for precore (nt 1814), core (nt 1900), and polymerase (nt 2307) open reading frames are indicated in bold.

respectively. Both in-frame and out-of-frame CID variants behave like defective interfering particles which, although defective for autonomous replication, are able to replicate preferentially at the expense of the wild-type helper virus.[10–12] These findings have been demonstrated with HBV isolates from genotypes other than F. No such interference, however, has been observed with out-of-frame CID variants of another member of the *Hepadnaviridae* family, namely woodchuck hepatitis virus.[12] Our observations that core region PCR products of shorter length became the predominant population suggests that out-of-frame CID variants from genotype F presents defective interfering properties.

In most studies, precore stop $G_{1896} \rightarrow A$ mutation and core gene deletions have not been found together.[9,31] Here we demonstrated that both types of mutations may coexist on the same HBV molecule. In conclusion, the data presented here suggest an association between specific mutations (precore stop codon $G \rightarrow A_{1896}$ mutation and core gene deletions) in the genome of an HBV isolate from genotype F and the exacerbation of liver disease in an AIDS patient.

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