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Emergence of and Takeover by Hepatitis B Virus (HBV) with Rearrangements in the Pre-S/S and Pre-C/C Genes during Chronic HBV Infection

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We have shown, by analyzing serial serum samples from a chronic hepatitis B virus (HBV) carrier, the emergence of HBV DNA molecules with nucleotide rearrangements in the pre-S/S and pre-C/C genes. Serum samples were obtained at four different times (1983, 1985, 1988, and 1989) from an HBsAg- and HBeAg-positive carrier with chronic hepatitis. The polymerase chain reaction was used to amplify the pre-S/S and pre-C/C genes. The amplified products were cloned, and 8 to 10 independent clones were sequenced. In 1983 and 1985 only one type of HBV DNA molecule was observed. Nucleotide divergence relative to the adw2 subtype was 4.7, 7.2, and 1.6%, for the pre-S1, pre-S2, and S regions, respectively, and 2.2 and 3.9% for the pre-C and C regions, respectively. In 1988 and 1989, HBV DNA forms with marked rearrangements of both the pre-S/S and pre-C/C regions were evidenced. In the pre-S/S region, they comprised two distinct HBV DNA molecules. The first showed nucleotide divergence of 20.4, 14.8, and 3.3% for the pre-S1, pre-S2, and S regions when compared with the adw2 sequence. In addition, nucleotide deletions in the pre-S1 region led to the appearance of a stop codon. The second was created by recombination between the original and mutated HBV DNA. In the pre-C/C region, the mutated viral DNA showed 11.7% divergence when compared with the adw2 sequence. A point mutation led to the creation of a stop codon in the pre-C region, together with an insertion of 36 nucleic acids in the core gene. Most of this DNA insertion was identical to that reported in an independent HBV isolate but showed no significant homology with known sequences. Semiquantitative estimation of the proportion of wild-type and mutated HBV DNA molecules showed a marked increase in the mutated forms during the period of follow-up. Sucrose gradient analysis indicated that the defective HBV DNA molecules were present in circulating virions. Western immunoblot analysis showed the appearance of modified translation products. Our findings thus indicate the emergence of and gradual takeover by mutated HBV DNA forms during the HBV chronic carrier state. The rearrangements we observed in the pre-S/S and pre-C/C genes might lead to changes in the immunogenicity of the viral particles and thus affect the clearance of the virus by the immune system.

Hepatitis B virus (HBV) is a small (3.2-kb) DNA virus whose replication cycle includes an intermediate RNA molecule (so-called pregenomic RNA) (8, 33). A reverse transcription step allows synthesis of the minus viral DNA strand in the core particle (8, 33). It has therefore been hypothesized that, owing to infidelity of the reverse transcriptase, HBV DNA undergoes a higher rate of mutation during replication than that observed for other viruses (21). The in vivo occurrence of genetic HBV variants has recently been reported (2–7, 10, 12, 13, 16, 19, 32). In particular, mutations in the pre-C/C open reading frame (ORF), coding for the viral c and e antigens (HBcAg and HBeAg, respectively), have been demonstrated in anti-HBe-positive HBV carriers with persistent HBV multiplication (2–6, 10, 19). One of these point mutations located in the pre-C region creates a stop codon and thus prevents synthesis of HBeAg. So far, the pre-C stop codon has been found only in anti-HBe-positive subjects. In addition, we recently identified in an HBV carrier a defective HBV with marked rearrangements on the pre-S gene coding for the viral envelope protein (8a).

The clinical implications of these mutations remain to be determined, although they might be associated with persistent viral infections and, possibly, severe chronic liver diseases (3, 4, 6, 19). In addition, there is controversy about whether the mutated forms are already present during the acute phase of HBV infection (5, 19).

To further elucidate these points, we analyzed HBV DNA sequences in serial serum samples obtained during a 6-year follow-up of an HBsAg-positive HBV carrier.

MATERIALS AND METHODS

Patient. Serial serum samples were obtained from an HBsAg-, HBeAg-, and HBV DNA-positive chronic carrier (referred to as B1). The HBeAg and HBV DNA titers remained high during the 6 years of follow-up. Liver biopsy showed moderately aggressive chronic hepatitis with no features of cirrhosis. Follow-up was initiated in 1983. In 1985, a 7-week course of adenine arabinoside was stopped as a result of side-effects. In 1987, the patient was treated with a combination of acyclovir and interferon for 3 months (27).

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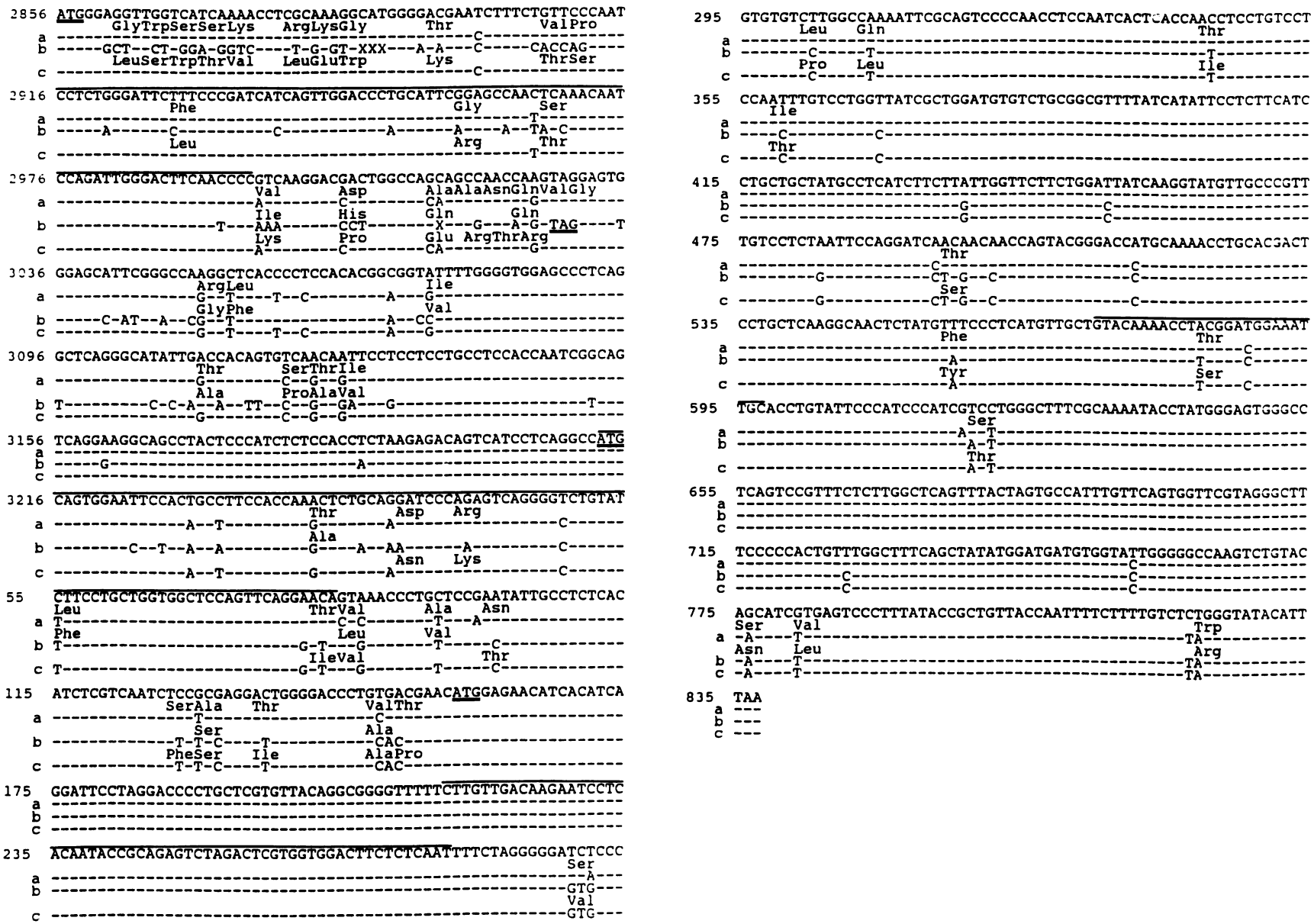

FIG. 1. Pre-S/S HBV gene sequences. The upper sequence represents the adw2 subtype nucleotide sequence (20). Nucleotides are numbered from the *Eco*RI site of the adw2 strain (20). (a) B1 wild-type molecule. (b) B1 mutated molecule. (c) B1 recombinant molecule. The dotted lines represent unchanged nucleotides, and X represents deleted nucleotides relative to the adw2 strain. Only the modified amino acids are indicated (under the nucleotide sequences). Pre-S1, pre-S2, and S start codons are underlined, as well as the TAG stop codon in the B1 pre-S1 mutated molecule. Major T- and B-cell epitopes are indicated (1, 9, 14, 30).

```
1816 ATGCAACTTTTTCACCTCTGCCTAATCATCTCTTGTACATGTCCCACTGTTCAAGCCTCC
                                          Thr         Val
   a ----------------------------------------------T-----------
                                                      Phe
   b ------------------------------------T-------T---------------
                                          Ser

1876 AAGCTGTGCCTTGGGTGGCTTTGGGGCATGGACATTGACCCTTATAAAGAATTTGGAGCT
   a --------------A-------------------------------------------
   b ---------------------TAG-----------------------------------

                  TAGAACAACTTTGCCATATGGCCTTTTTGGCTTAGA
            AspArgThrThrLeuProTyrGlyLeuPheGlyLeuAsp

1936 ACTGTGGAGTTACTCTCGTTTTTGCCTTCTGACTTCTTTCCTTCCGTCAGAGATCTCCTA
   a ---------------------------------------------C-G-----A---
   b -----------G----------------------T--C--G--T--TC-T-----T--C

1996 GACACCGCCTCAGCTCTGTATCGAGAAGCCTTAGAGTCTCCTGAGCATTGCTCACCTCAC
                                Ala         ProGlu
   a -----A---------T-------G-----------------------------------
   b --------T------T----C--G---T-----------CT----T-----T--G------
                                Ser         SerAsp

2056 CATACTGCACTCAGGCAAGCCATTCTCTGCTGGGGGGAATTGATGACTCTAGCTACCTGG
   a -----A-----------------------------------A------------------
   b -----A--------------A-----G--------T--G---------------------

2116 GTGGGTAATAATTTGGAAGATCCAGCATCTAGGGATCTTGTAGTAAATTATGTTAATACT
   a ------------------------------C--------A-----C---------------
   b ------------------------------C--A---T-G--G--C---------------

2176 AACGTGGGTTTAAAGATCAGGCAACTATTGTGGTTTCATATATCTTGCCTTACTTTTGGA
       Val
   a ---A----CC-------------------------------T------------------
       Met
   b --TA----------A------------------------C--T--C--T-----------G

2236 AGAGAGACTGTACTTGAATATTTGGTCTCTTTCGGAGTGTGGATTCGCACTCCTCCAGCC
   a -----A-----C-----G--------------T-------------------------
   b -----A--C--T-----G--------G-----T-----------------------T--T

2296 TATAGACCACCAAATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGA
   a ------------------------------------------------------------
   b ------------------------C----------------G------------------

2256 CGGGACCGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGCAGATCTCCA
                                                              Pro
   a --A-----------------------------------------------A-------A-
                                                              Gln
   b AXXXXXX--------------C---------------------------A-------A-

2416 TCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTCAATGTTAG
                            Gln   ArgGlu
   a ---------------------------------------------
   b ---------------------GC-----CA-CT--C---------
                            Ala   ProAla
```

FIG. 2. Pre-C/C HBV gene sequences. The upper sequence represents the adw2 subtype nucleotide sequence (20). Nucleotides are numbered from the *Eco*RI site of the adw2 strain (20). (a) B1 wild-type molecule. (b) B1 mutated molecule. The dotted line represents unchanged nucleotides, and X indicates deleted nucleotides relative to the adw2 strain. Only modified amino acids are indicated (under the nucleotide sequences). Pre-C and C start codons are underlined, as well as the TAG stop codon in the B1 pre-C mutated molecule.

Neither therapeutic regimen induced more than a slight and temporary decrease in HBeAg and HBV DNA titers. Serum samples from 1983 and 1985 (before treatment) and 1988 and 1989 (after treatment) were available for analysis.

Serological tests and serum HBV DNA detection. HBsAg and HBeAg were detected by a standard assay (Abbot Laboratories). Comparative dilution experiments were performed to estimate the titers of HBeAg in the serum samples. Serum HBV DNA was estimated semiquantitatively by the standard spot test procedure (29).

HBV DNA purification. HBV DNA was extracted from serum samples (100 μl) by using proteinase K (500 μg/ml) in a buffer consisting of 200 mM NaCl, 40 mM EDTA (pH 8), and 1.5% sodium dodecyl sulfate (SDS).

PCR. The polymerase chain reaction (PCR) was performed as previously described (26, 32). Each cycle included DNA denaturation for 1 min at 94°C, annealing of the primers at 55°C (1 min), and extension at 72°C (1 min). The last cycle was followed by a 10-min extension process to ensure complete DNA extension. The amplified products were analyzed by agarose gel electrophoresis followed by Southern blotting. Hybridization was performed by using specific oligonucleotide probes as previously described (32). HBV primers for the amplification of the pre-S/S and pre-C/C regions were designed by comparison of available hepadnavirus sequences and are given below (the localization refers to the genetic map of the ayw subtype). Pre-C/C: pre-C, 5′-GGGAGGAGGAGATTAGGTTAA-3′ (1763 to 1746); MD35, 5′-GGATAAAACCTGGCAGGCATAAT-3′ (2658 to 2636); MD27, 5′-ACTGTTCAAGCCTCCAAGCT-3′ (1961 to 1880); and MD31, 5′-AGTGCGAATCCACACTC-3′ (2286 to 2270). Pre-S/S: MD19, 5′-GGGTCACCATATTCT TGG-3′ (2816 to 2833); and MD13, 5′-TTAGGGTTTAAATG TATACCG-3′ (844 to 824). The following specific oligonucleotide probes were used: pre-C/C, 5′-CCTTGGGTGGCTT TGGGGCATGGACATCGACCCTTATAA (1884 to 1922); and pre-S/S, 5′-CCTGCTGGTGGCTCCAGTTCAGGAAC AGTAAACCCTG (652 to 692). HBV primers for the amplification of the mutated or wild-type B1 molecules were as follows: B1-1, 5′-TAGAACAACTTTGCCATATGGCCTT (nucleotides 1 to 25 of the 36-bp insert); B2-2, 5′-CCACTC GAGAGGGACCGTCCAAGAA (positions 2888 to 2864 on the B1 pre-S1 mutated sequence); B1-3, 5′-TTGGAACAG GGTTGAGTGTT (positions 100 to 81 on the B1 pre-S1 wild-type sequence); and B1-4, 5′-ATGGCTTTAGGGCAT GGACA (positions 1890 to 1909 on the B1 pre-C wild-type sequence).

Nucleotide sequence analysis. Amplified HBV DNA was cloned into bacteriophage M13mp18 or M13mp19 by using restriction sites (*Pst*I or *Hin*dIII) included 5′ of the HBV primer sequences. Eight to ten clones of each amplified product were sequenced by using the dideoxy-chain termination method. The reliability of the sequence results was ensured by analyzing clones from independent amplification experiments.

Sucrose gradient experiments and immunological assays. Serum samples (0.2 ml) from 1985 (before treatment) and 1989 (after treatment) were applied to isopycnic sucrose gradients. Each serum sample was loaded onto a 20 to 60% linear sucrose gradient in TN buffer (10 mM Tris-HCl, 140 mM NaCl [pH 7.2]) and centrifuged for 18 h at 200,000 × *g* and 4°C. Fractions were collected and probed for the presence of HBsAg, pre-S2Ag, and pre-S1Ag by means of polyclonal-monoclonal antibody (PAb-MAb) radioimmunoassays, as described in detail elsewhere (24). To characterize further the HBV-related proteins, the sucrose gradient fractions were denatured with 2% (wt/vol) SDS and 5% (vol/vol) 2-mercaptoethanol and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE; 12.5% polyacrylamide) followed by Western immunoblot analysis. Transfer blots were incubated with rabbit PAbs to serum-derived HBsAg or denatured liver-derived HBcAg (HBe/c) and with MAbs to the pre-S2-specific pHSA-binding site (22) or the pre-S1-specific hepatocyte receptor-binding site (23). Antibody binding to the transferred viral proteins was detected with the ^{125}I-labeled F(ab′)$_2$ fragment of anti-rabbit or anti-mouse immunoglobulin (Amersham France).

Electron microscopy. Fractions of the sucrose gradient were observed after negative staining under a 100 CX electron microscope (JEOL Ltd., Tokyo, Japan).

RESULTS

Nucleotide sequence analysis. Serum samples were obtained from the HBsAg-positive HBV carrier at four dif-

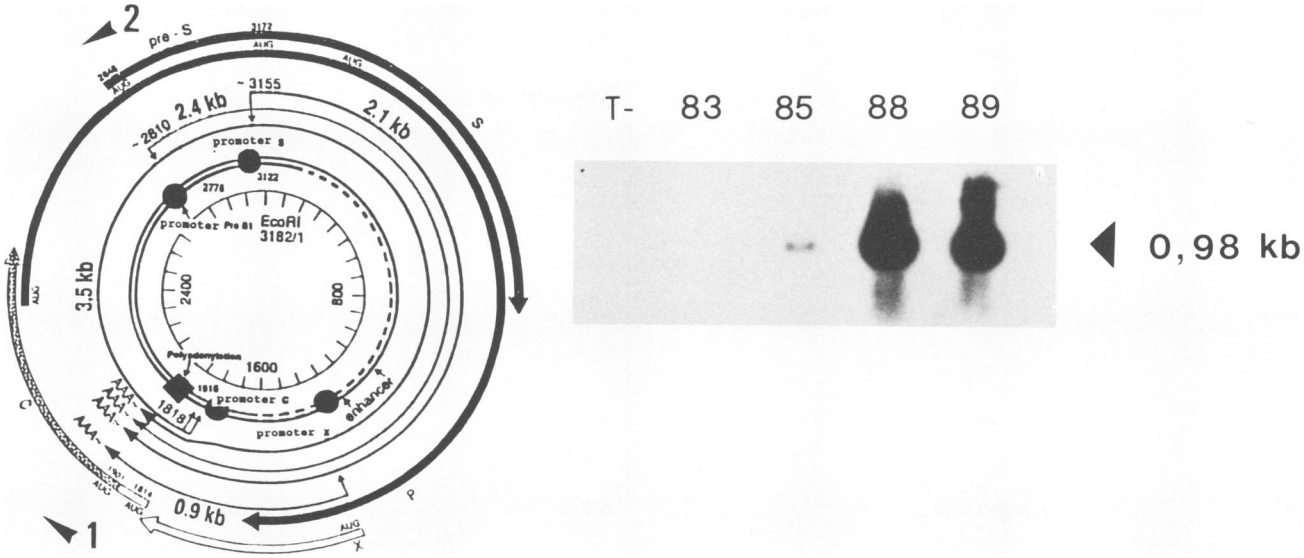

FIG. 3. Detection of mutant molecules by PCR. HBV DNA extracted from B1 serum samples obtained in 1983, 1985, 1988, and 1989 was amplified by using B1-1 and B1-2 primers designed from the sequence data of the mutated molecule (see Materials and Methods). B1-1 and B1-2 primers are localized on the genetic HBV map (left). Amplified products were transferred onto nylon membranes and hybridized with a pre-C/C-specific oligonucleotide probe (right).

ferent times (1983, 1985, 1988, and 1989). HBsAg and HBe titers did not change significantly during this period. Pre-S/S and pre-C/C HBV DNA sequences were amplified by PCR and sequenced. The sequence data are reported in Figs. 1 and 2. In 1983 and 1985, only one type of HBV DNA molecule was found. When the DNA sequences were compared with various HBV DNA sequences available, the greatest homology was obtained with the adw2 subtype (20) (Fig. 1 and 2). Nucleotide divergence from the adw2 subtype was 4.7, 7.2, and 1.6% for the pre-S1, pre-S2, and S regions, respectively (Fig. 1, row a) and 2.2 and 3.9% for the pre-C and C regions, respectively (Fig. 2, row a). These molecules will be referred to as the wild-type molecules. By contrast, in 1988, in addition to the wild-type molecules, HBV DNA molecules with marked rearrangements in the pre-S/S and pre-C/C genes were observed. Analysis of the pre-S/S region showed that the serum contained three distinct molecules, one of which was identical to that identified in 1983 and 1985. The second showed a nucleotide divergence of 20.4, 14.8, and 3.9% in the pre-S1, pre-S2, and S regions, respectively, from the adw2 sequence (Fig. 1, row b). In the pre-S1 region, mutations were particularly numerous between nucleotides 2861 and 2886. In addition, deletion of four nucleotides at positions 2888, 2889, 2890, and 3016 led to the appearance of a stop codon at positions 3028 to 3030. The third HBV DNA molecule showed a sequence identical to that of the wild-type molecule for the pre-S1 and the 5′ part of the pre-S2 regions and a sequence identical to that of the mutated molecule for the 3′ part of the pre-S2 region and the S gene (Fig. 1, row c). Analysis of the pre-C/C sequence showed the presence of two viral DNA molecules (Fig. 2). One was identical to the one detected in 1983 and 1985 (i.e., wild type) (Fig. 2, row a), whereas the second showed a nucleotide divergence of 11.7% from the adw2 sequence (Fig. 2, row b). In the pre-C region, a stop codon (TAG) was evidenced at positions 1897 to 1899. An in-frame 36-bp insertion located 6 bp after the initiation codon and a 6-bp deletion (2257 to 2262) were observed in the C ORF. Further analysis of the 36-bp sequence showed no significant homology to hepadnavirus sequences or any available nucleic acid sequences (in the EMBL and GenBank data bases). A 36-bp oligonucleotide probe synthesized from this sequence did not hybridize to normal human cellular DNA (data not shown). Interestingly, 60% of the mutations found in the wild-type molecules were also present in the mutated HBV DNA molecules. In the serum sample obtained in 1989, the pre-S/S region showed only the mutated DNA molecules. By contrast, the pre-C/C region contained both the wild-type and mutated molecules.

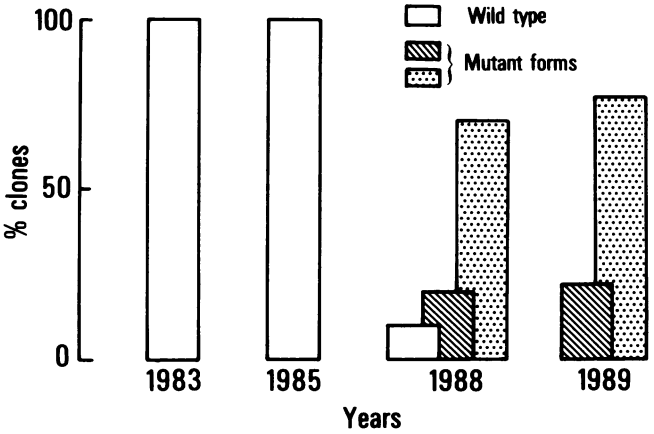

FIG. 4. Semiquantitative analysis of wild-type and mutated HBV DNA molecules. The number of clones with the different pre-S/S sequences was calculated at the different periods of follow-up. For each amplified product, 8 to 10 independent clones were sequenced. Symbols: ▭, B1 wild-type molecule; ▩, B1 mutant molecule; ▧, B1 recombinant molecule.

In the pre-S/S region, amino acid divergence from the adw2 amino acid sequence was 4.7% (pre-S1, 8.4%; pre-S2, 10.9%; S, 1.3%) for the wild type, 11.2% (truncated pre-S1,

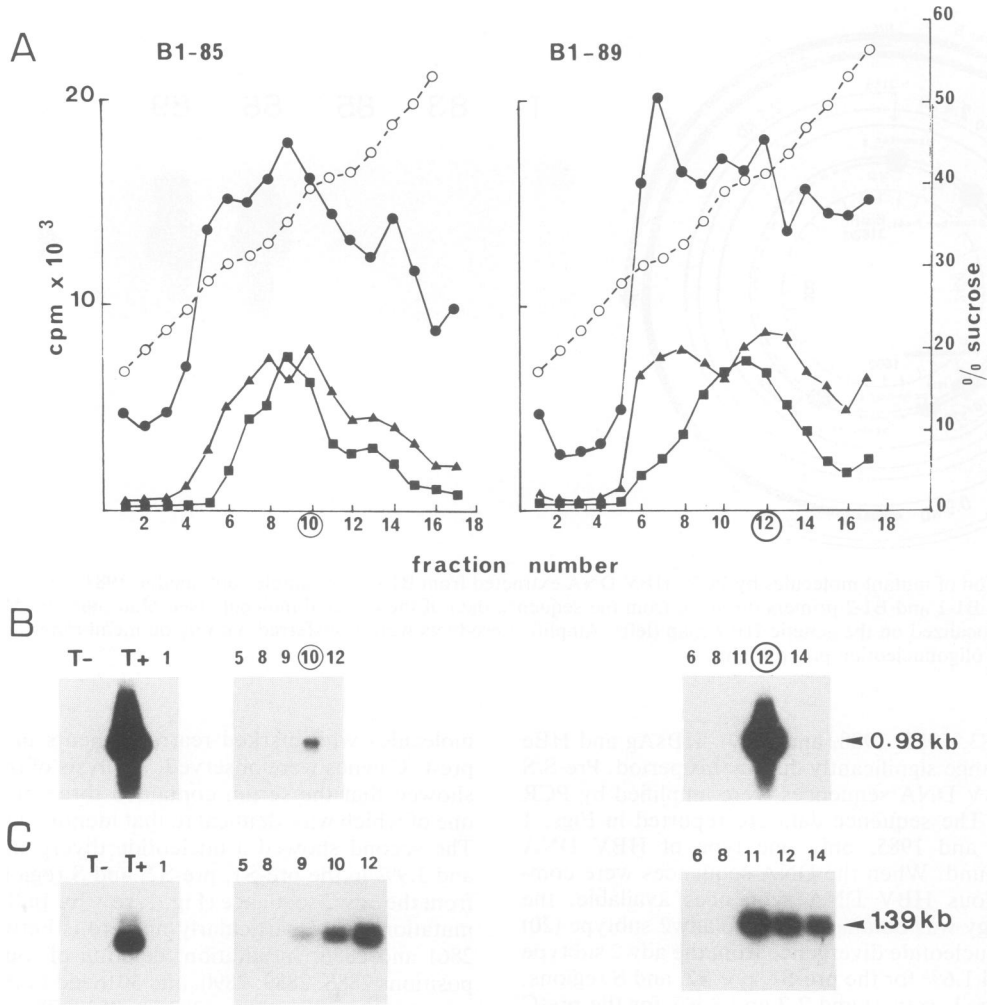

FIG. 5. Sucrose gradient analysis. (A) Serum samples (0.2 ml) from 1985 (B1-85) and 1989 (B1-89) were subjected to isopycnic sucrose gradient centrifugation (see Materials and Methods). Fractions were probed with the HBsAg-specific MAb F39.20 (●), the pre-S2-specific MAb F124 (▲) and the pre-S1-specific MAb F35.25 (■) by means of PAb-MAb radioimmunoassays with polyclonal anti-HBs in the solid phase. The sucrose concentration (○) was assayed by refraction. Fractions 5, 8, 9, 10, and 12 from B1-85 and fractions 6, 8, 11, 12, and 14 from B1-89 were further subjected to PCR analysis, with B1-1/B1-2 (B) or B1-3/B1-4 (C) primers. The negative controls correspond to PCR without DNA (Lanes T−) and normal serum (lanes 1); the positive controls contain B1-89 or B1-85 serum (lanes T+).

34.4%; pre-S2, 23.6%; S, 5.3%) for the nonrecombinant mutant, and 8.2% (pre-S1, 8.4%; pre-S2, 20%; S, 5.3%) for the recombinant mutant (Fig. 1). In the pre-C/C region, the amino acid divergence was 1.4 and 3.3% for the wild-type and mutant molecules, respectively (Fig. 2).

To determine whether the pre-S/S and pre-C/C mutated regions found in the 1988 and 1989 serum samples were located on the same HBV molecules, we performed PCR with a new set of primers. The first primer (B1-1) was designed from the 36-bp DNA sequence insert. The second (B1-2) was designed from the pre-S1 mutated sequence. HBV DNA extracted from serum obtained in 1983 failed to amplify with these primers (Fig. 3). In contrast, amplified HBV DNA sequences were detected in 1985, 1988, and 1989 serum samples; the amount of amplified products was significantly larger in 1988 and 1989 than in 1985. These results are consistent with the sequence data and indicate that the mutated sequences of the pre-S/S and pre-C/C regions were located on the same molecule.

For each amplified product, 8 to 10 independent clones were sequenced. From the sequence data of the pre-S/S region, a semiquantitative estimation of the respective amounts of the wild-type and mutated HBV DNA molecules was obtained (Fig. 4). In 1983 and 1985 only the wild-type molecules could be detected. In 1988 the mutant HBV DNA forms were identified in 9 of 10 clones; there were 7 nonrecombinant and 2 recombinant mutants, but only 1 clone corresponded to the wild type. In 1989, the wild type was not detected and all the clones corresponded to mutated HBV DNA forms. For the serum sample obtained in 1985, by contrast to the cloning and sequences data, the presence of mutated molecules was observed when a set of primers specific for mutated DNA was used (Fig. 3). This indicates that a small amount of mutated virus was present before the patient first underwent antiviral therapy. Overall, these results indicate the emergence of HBV DNA mutants and their gradual takeover during the period of follow-up.

Viral particles and HBV protein analysis. To determine whether the mutated HBV DNA molecules were present in circulating viral particles (VPs), serum samples obtained in

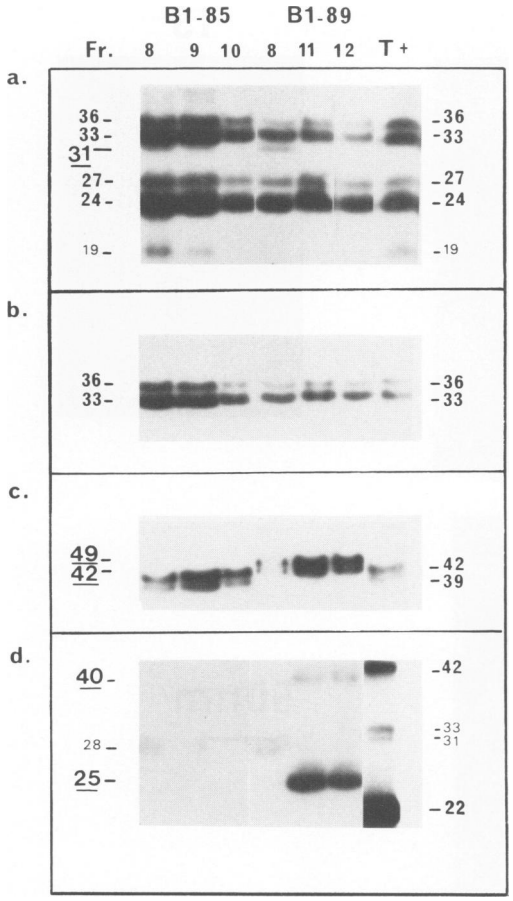

FIG. 6. HBV protein analysis. Immunoblotting of the surface- and core-specific proteins from fractions 8, 9, and 10 (B1-85) and 8, 11, and 12 (B1-89) of the sucrose gradient (Fig. 5A). The positive controls (lanes T+) used were complete HBV particles (ad subtype) purified from the serum of an HBeAg-positive chronic carrier positive for pre-S2 and pre-S1 antigens (a, b, and c) or HBcAg particles purified from human liver (d). Test samples from B1-85 and B1-89 and positive controls were run on the same gel and transferred on the same blot. (a and d) Transfer blots were incubated with rabbit PAbs to HBsAg or denatured HBcAg, respectively; (b and c) transfer blots were incubated with MAbs to pre-S2- or pre-S1-specific epitopes, respectively. Antibody binding to the transferred proteins was detected with ^{125}I-labeled anti-rabbit or anti-mouse immunoglobulin F(ab')$_2$. The numbers on the left and right indicate the molecular masses of the polypeptides in kilodaltons.

1985 and 1989 were subjected to ultracentrifugation in a sucrose gradient and the fractions positive for HBV surface antigens (HBsAg, pre-S2Ag, and pre-S1Ag), as revealed by PAb-MAb radioimmunoassays, were tested by means of PCR. B1-1 and B1-2 primers were used, allowing the amplification of only a mutated HBV DNA (see above). In the serum sample from 1985 (B1-85; Fig. 5), only one population of VPs positive for HBsAg, pre-S2Ag, and pre-S1Ag was found in fractions 8 to 10 (32 to 40% sucrose) (Fig. 5A). An HBV DNA mutant was faintly detected only in fraction 10 (Fig. 5B). Molecules with nonmutated S and C genes were observed in fractions 9, 10, and 12 by PCR with primers specific for wild-type HBV DNA (B1-3/B1-4) (Fig. 5C).

In the serum sample from 1989 (B1-89; Fig. 5), two distinct HBsAg-positive populations were identified: one positive for pre-S2Ag was recovered from fractions 6 to 8 (30 to 32% sucrose), and another positive for both pre-S2Ag and pre-S1Ag was found in fractions 10 to 12 (40 to 42% sucrose) (Fig. 5A). PCR analysis demonstrated the presence in fraction 12 of this mutated HBV DNA, which was absent from the other fractions tested (Fig. 5B) (nonmutated molecules were found in fractions 11, 12, and 14 [Fig. 5C]).

Fractions 8, 9, and 10 from B1-85 and fractions 8, 11, and 12 from B1-89 were further analyzed for the size of HBV proteins by means of the Western immunoblotting technique (Fig. 6). Size-modified HBV surface- and core-specific proteins were identified in the fractions from B1-89. In fraction 8 (32% sucrose), anti-HBs showed an additional 31-kb HBsAg-specific protein (Fig. 6a). Anti-HBx revealed an increased amount of the 28-kDa HBx-specific protein (Fig. 6b). In fraction 12 (42% sucrose), in which the HBV DNA mutant was found (Fig. 5B), the pre-S1 proteins were increased in size (42 and 49 kDa) (Fig. 6c). In addition, a 25-kDa protein was found to react strongly with rabbit PAbs to denatured HBcAg (anti-HBe/c) (Fig. 6d).

Electron-microscopic examination of fractions 10 to 12 (40 to 42% sucrose) from B1-89 revealed the presence of empty (55%) and full (45%) modified VPs with an average diameter of 30 nm (Fig. 7). Small numbers of complete 42-nm HBV particles, corresponding to the wild type, were observed in fraction 13 (44% sucrose).

DISCUSSION

Our findings indicate that during the course of chronic HBV infection, there is an emergence of and takeover by HBV DNA molecules with marked rearrangements of both pre-C/C and pre-S/S coding regions. The presence of a heterogeneous population of HBV DNA molecules in the same patient is consistent with previous findings (10, 12, 13, 17, 18).

The HBV mutant forms that we have identified in this patient have several interesting genetic features. First, the point mutation identified in the pre-C ORF which induces a stop codon is identical to that previously found in several anti-HBe- and HBV DNA-positive chronic HBV carriers (6, 19). It has been suggested that chronic carriers bearing this mutant are more likely to develop severe chronic hepatitis (3, 4). This is not, however, a general feature, since the pre-C stop codon has also been found in asymptomatic Japanese HBV carriers at the time of HBe/anti-HBe seroconversion (19). Finally, it has been hypothesized that these viral molecules might be present during the acute phase of HBV infection in patients with fulminant hepatitis (5).

A second interesting feature of our results is the detection of a DNA insertion of 36 bp in the N-terminal part of the C ORF. The first 26 bp of this sequence is homologous with the last 26 bp of an insert found in the pre-C ORF of an independent HBV isolate (2). The origin of this insert is not clear. We detected no homology with HBV or with cellular or other viral DNA sequences available in data banks. The finding of the same DNA insert in the pre-C/C genes of two independent isolates suggests the occurrence of a specific recombination event. We are currently investigating a possible origin of this insert by HBV integration into the host genome or by recombination with other pathogens.

The defective HBV strain present in our patient also shows significant rearrangements in the pre-S coding region. A stop codon is created in the pre-S1 gene, together with point mutations and deletions in the pre-S1, pre-S2 and S ORFs. Using PCR, we were able to show that the pre-C/C

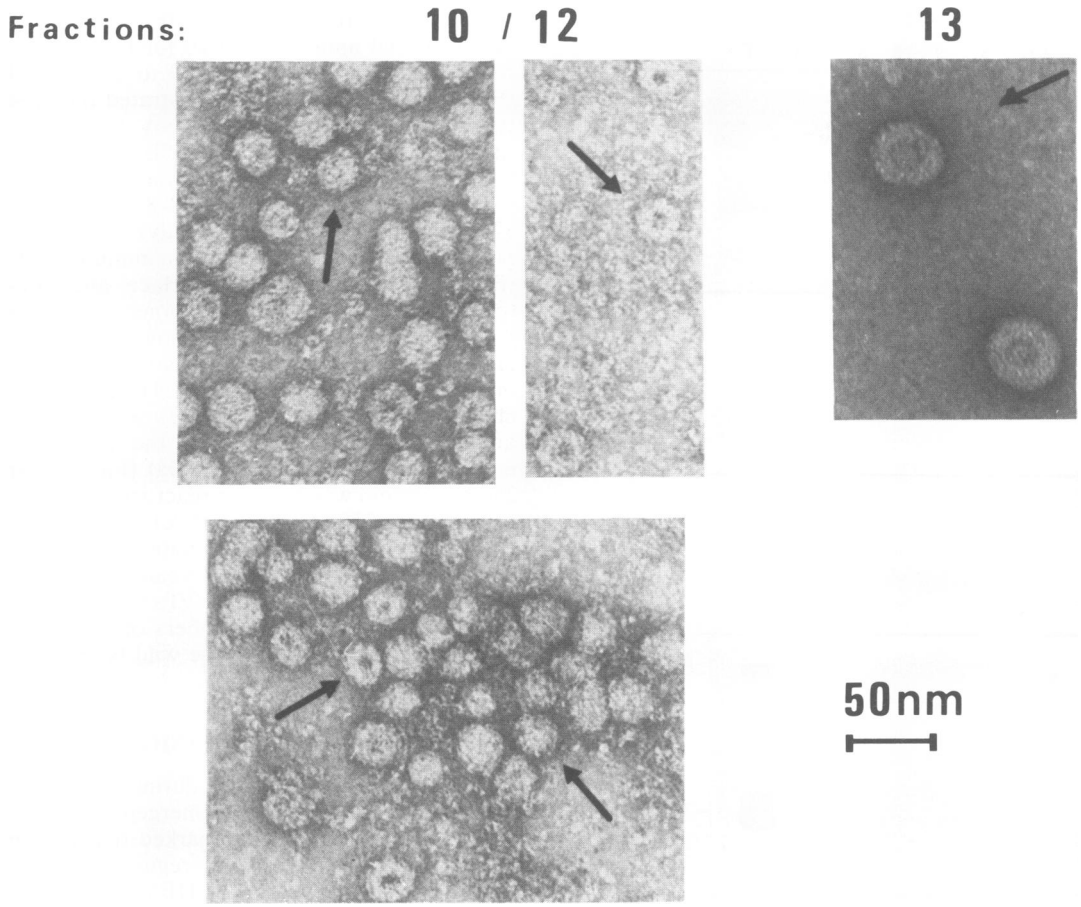

FIG. 7. Electron-microscopic examination of fractions 10/12 and 13 from B1-89 (Fig. 5A). In fraction 10/12, arrows indicate full and empty 30-nm particles. In fraction 13, 42-nm Dane particles are shown.

and pre-S mutated sequences were both present on the same viral DNA molecule.

Pre-S codes for immunogenic viral envelope proteins which are probably important targets in viral clearance and in the prevention of infection (15, 24). B- and T-cell antigenic determinants have been mapped in the pre-S1, pre-S2, and S regions (1, 9, 14, 30), whose sequences were modified by the mutations we identified (Fig. 1). By contrast, it is interesting that the pre-S2-specific sequence carrying the receptor for polymerized human serum albumin (R-pHSA) recognized by the MAb F124 (22) and region 21-47 of the pre-S1 domain involved in the recognition of HBV by hepatocyte receptors recognized by the MAb F35.25 (23) were expressed on the surface of the mutant HBV molecules we describe.

The modifications observed in the pre-C/C region might also be important. Indeed, although the role of HBeAg is not known, it has been suggested that it could be involved in modulating the immune response to HBV (28). HBcAg epitopes are targets for T-cell cytotoxicity (25). Thus, all these mutations, including those in the pre-C/C and pre-S regions, would probably impair the clearance of these viral forms by the immune system.

The availability of serial serum samples from the same chronically infected individual allowed us to observe the kinetics of appearance of the defective HBV. During the first 2 years of follow-up, only the wild-type HBV was identified, whereas analysis of serum samples obtained 2, 5, and 6 years later showed the gradual emergence of and takeover by the mutated HBV forms. Several lines of evidence indicate that this phenomenon was not due to superinfection with another HBV strain. First, the variant forms also belong to the adw2 subgroup. Second, 60% of the mutations detected in the wild-type molecules were also found in the mutated molecules. These data indicate that the defective HBV emerged during the replication of the wild-type HBV.

Sucrose gradient analysis indicated that the mutated HBV DNA was present in circulating VPs positive for both pre-S1 and pre-S2 epitopes. By immunological and morphological analysis of circulating VPs and their polypeptides, we also obtained evidence that the pre-C/C and pre-S mutations were associated with the appearance of size-modified translation products and VPs. The most interesting results of the protein analysis were (i) that the pre-S mutations did not eliminate surface-specific epitopes that were expressed on the pre-S2 and pre-S1 sequences and that were critical for HBV infectivity and (ii) that the insertion in the N-terminal region of the C ORF probably results in the expression of an abnormal core-specific protein of 25 kDa. The 25-kDa modified translation product of the pre-C/C genes could lead to the defective synthesis of core (HBcAg) particles and thus to spherical VPs (diameter, 30 nm) visualized by electron microscopy. The serum HBeAg titer remained relatively stable throughout the follow-up period. However, because of the stop codon identified in the pre-C region, these

mutated HBV DNA molecules are incapable of HBeAg synthesis. There are three possible explanations for this apparent paradox. First, the rearrangement of the pre-C/C coding region could generate a size-modified HBeAg-positive core protein. Second, the mutated HBV strain might coexist with a very small amount of wild-type HBV. HBeAg may thus have been synthesized by the wild-type HBV DNA molecules. This seems unlikely, however, since the HBeAg titer did not change in 1989 when the proportion of the wild-type particles fell drastically. Third, HBeAg might be synthesized from the rearranged molecules with intact pre-C/C and modified pre-S/S sequences that we detected. Regarding pre-S1, because of its requirement for virion assembly and export, the pre-S1 mutation bearing the stop codon should have been complemented to be expressed and secreted. Complementation is probably due to recombinant molecules containing an intact pre-S, since the amount of wild-type molecules is very small.

It has been shown that truncated S protein may acquire a transactivating effect on cellular genes such as c-*myc* (11). Furthermore, rearrangements in pre-S/S genes can lead to the accumulation of covalently closed circular HBV DNA forms in an in vitro system (31). This viral DNA is a template for the synthesis of pregenomic RNA and might be involved in the persistence of viral infection. Therefore, in addition to modifying the immunogenicity, the size-modified viral proteins we detected might have a different effect on the host cell.

The generation of defective virus might be linked only to ongoing viral replication. However, host factors, mainly the immune response to the viral epitopes, as well as intrinsic properties of the mutated HBV strain, also select forms capable of escaping immune regulation. In this context, it is interesting that our patient underwent two unsuccessful therapeutic trials between 1985 and 1987; it is plausible that antiviral therapy favored selection of preexisting mutated viral DNA capable of escaping the immune host response.

Several different HBV DNA forms were evidenced in our patient, including a wild-type molecule and distinct mutated molecules. Cloning and expression of each should allow their respective potential in the course of HBV infection to be evaluated. Indeed, detailed molecular analysis of HBV strains such as those identified here should help to identify epitopes critical for viral clearance and prevention of infection.

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