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Type, Prevalence, and Significance of Core Promoter/Enhancer II Mutations in Hepatitis B Viruses from Immunosuppressed Patients with Severe Liver Disease

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Little is known about the functional significance of hepatitis B virus (HBV) sequence heterogeneity. Here we analyzed the type, frequency, and function of mutations in the core promoter/enhancer II region of HBV in immunosuppressed patients. The major HBV population in immunosuppressed patients with severe liver disease had deletions, insertions, and/or base changes in this region. Such mutations were not found in immunosuppressed patients with mild disease. Except for two mutations, all created a hepatocyte nuclear factor 1 (HNF1) binding site or a potential HNF3 binding site. Occasionally, known binding sites for C/EBP and HNF4 were additionally duplicated. Eleven mutated core promoter prototype sequences were functionally tested in the context of a wild-type genome by transfection in Huh7 cells. Despite the diversity of mutations tested, all decreased steady-state levels of pre-C mRNA drastically and increased those of the C mRNA/pregenomic RNA. This correlated with reduced levels of secreted hepatitis B e antigen and increased intracellular levels of core and Pol proteins and replicative HBV DNA intermediates. The levels of secreted HBV DNA-containing particles were also increased although most of the mutations reduced the levels of pre-S/S mRNA and pre-S1, and pre-S2 proteins as well as secretion of hepatitis B surface antigen. These data reveal a novel class of HBV variants with HNF1 binding sites in the core promoter which are characterized by a defect in hepatitis B e antigen expression, enhanced replication, and altered protein levels, all probably mediated by altered transcription factor binding. The phenotype of these variants and their prevalence only in immunosuppressed patients with severe liver disease may indicate that they play a role in pathogenesis.

The hepatitis B virus (HBV) genome contains four genes encoding all known proteins of this virus. The C gene codes for the core protein and the secreted hepatitis B e antigen (HBeAg); the S gene codes for the three envelope proteins, pre-S1, pre-S2, and S protein (hepatitis B surface antigen [HBsAg]); the Pol gene codes for a protein with several functions in replication (reverse transcriptase, DNA polymerase, RNase H, and primer for DNA synthesis); and the X gene codes for the X protein, which has transactivating properties. The expression of these viral gene products is regulated by four promoters directing the synthesis of 3.5-, 2.4-, 2.1-, and 0.7-kb mRNAs, respectively. All promoters are activated by two enhancers (I and II) (for a review, see reference 13). The core promoter, on which we focus here, regulates the replication of the virus, as the 3.5-kb C mRNA/pregenome not only serves for translation of the core and Pol proteins but also represents the template for reverse transcription (9, 46, 49). From the second core promoter transcript, the pre-C mRNA, only the HBeAg precursor is translated (9, 46). The core promoter is composed of a minimal or basic core promoter (BCP) sufficient to initiate transcription and of upstream regulatory sequences (URS) (31, 50, 54). A short TA-rich sequence in the BCP serves as both the initiator and TATA box for transcription initiation of the pre-C mRNA and C mRNA/pregenome, respectively (6). An important activating URS element is the alpha box, which binds hepatocyte nuclear factor 4 (HNF4), C/EBP, or other liver-specific transcription factors (19, 31, 45, 54, 56, 57, 61). In addition, binding sites for HNF3 and ubiquitous factors like Sp1 were identified in this promoter (22, 60). Unlike the pre-S1 promoter, the core promoter of wild-type HBV does not contain an HNF1 binding site (36). Enhancer II overlaps with the core promoter and consists of three major sequence elements: the activating alpha and beta boxes and an upstream negative regulatory element (30, 45, 52, 55–57, 61).

Because of its crucial role in the viral life cycle, naturally occurring sequence variation in the core promoter of HBV in patients is under intense investigation. Specific point mutations in the BCP were found in HBV from patients with fulminant hepatitis (20, 21, 23, 39). Similar mutations as well as different short deletions or insertions were found in viremic HBeAg-negative patients with chronic hepatitis B (21, 32, 33, 39, 41). Since for these patients no mutation in the C gene could explain the lack of HBeAg expression, it was speculated that promoter mutations may be responsible. Specific types of short deletions in the core promoter region were found in patients with extremely low-level viremia, in some cases without any serological marker for HBV infection (11, 14, 35, 44). Thus, a particular phenotype may be caused by specific mutations in the core promoter. However, this speculation has not previously been substantiated by experimental evidence.

Recently, we have identified dominant HBV populations with deletions in the C gene which accumulated and persisted in long-term-immunosuppressed patients with severe liver disease (15, 16). In this study, we examined whether the HBV genomes from these patients additionally contain mutations in the core promoter which may dysregulate viral gene expression

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and play a role in pathogenesis. Here we demonstrate that the major viral population in sera of long-term-immunosuppressed patients with severe liver disease represents a novel class of core promoter/enhancer II (Cp/EnII) variants with a particular functional phenotype. This phenotype may play a role in pathogenesis, as the variants were not found in the control group of immunosuppressed patients with mild or no liver disease.

MATERIALS AND METHODS

Patients and sera. Sera were from patients with HBsAg- and HBeAg-positive chronic hepatitis (for details, see Table 1). None of the patients was anti-HBe positive during the course of infection, and all were negative for markers of hepatitis C virus, hepatitis delta virus, and human immunodeficiency virus infection. Most patients were under long-term immunosuppression because of renal transplantation (described in detail in reference 15). Only patient J was not treated with immunosuppressive drugs.

Isolation of DNA from serum. From each serum sample, 300 μl was incubated at 65°C for 4 h in 20 mM Tris-HCl (pH 8.0)–10 mM EDTA–0.1% sodium dodecyl sulfate (SDS)–0.8 mg of proteinase K per ml. The DNA was then extracted with phenol and subsequently with chloroform and precipitated with ethanol by using 20 μg of tRNA as a carrier. The pellet was dissolved in 20 μl of H_2O.

Amplification and cloning of complete virion-encapsidated HBV genomes. HBV DNA isolated from serum was amplified as described previously (16) by PCR in 50 μl of buffer containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), 0.01% gelatin, 5 U of *Taq* DNA polymerase (Boehringer, Mannheim, Germany), and 1 μM (each) primers P1 and P2 (P1, 5′-CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCT GCCTAATCA-3′; P2, 5′-CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCA TGGTGCTGG-3′ [HBV-homologous sequences spanning nucleotides 1821 to 1841 and 1823 to 1806, respectively; heterologous nucleotides used for cloning are underlined]). A 45-μl reaction premix was heated to 80°C, and 5 μl of enzyme was then added. The amplification was performed for 20, 25, or 30 cycles at 94°C for 40 s, 60°C for 1 min, and 72°C for 4 min with an increment of 5 s per cycle in a Perkin-Elmer thermocycler (48 wells).

Analysis of the core promoter region of HBV DNA by PCR and sequencing. The amplified HBV genomes were diluted 1:100, and 1 μl thereof was used as a template to amplify the Cp/EnII region with primers P2 and P3 (P3 sequence, 5′-CGTTCCGACCGACCACGGGGCGC-3′ [HBV nucleotide sequence positions 1505 to 1527]) and, as a control, the amino-terminal region of the X gene with primers P4 (5′-CCATACTGCGGAACTCCTAG-3′ [positions 1266 to 1286]) and P5 (5′-CGGTGGTCTCCATGCGACGTGC-3′ [positions 1620 to 1599]). Fragments were separated in long (30-cm) 2% agarose gels, and their electrophoretic mobilities were compared with that of a fragment amplified from an HBV wild-type genome (12). Cp/EnII fragments homogeneous and identical in size to the reference genome were directly sequenced by using the amplified full-length HBV genomes and primer P3; all others were sequenced after cloning. For cloning, the amplified full-length HBV genomes were purified by phenol-chloroform (1:1) extraction, precipitated, dissolved in water, and digested with *Sst*I (cutting site in heterologous primer sequences) for at least 6 h. Fragments 3.2 kb in length were recovered from the agarose gel and cloned via the *Sst*I site into vector pUC19. One microliter of the bacterial overnight culture was used to amplify the complete HBV DNA insert in a 25-μl assay mixture with 0.1 μM primers P1 and P2. The length of the Cp/EnII region on the resulting 3.2-kb PCR fragments was analyzed by subgenomic PCR as described above. Fragments larger or smaller than wild-type length were grouped according to size, and clones representative of each size class and also clones of wild-type length were sequenced. Sequences in the core promoter region with similarity to HNF1 binding sites were searched for by using the *legame* software program (43).

Preparation of nuclear extracts. Nuclear extracts were prepared from Huh7 cells at 4°C essentially as described previously (1). Cells were washed with ice-cold phosphate-buffered saline (PBS), harvested in PBS, and pelleted for 5 min at 500 × *g*. The cells were resuspended in 5 volumes of buffer A (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, Complete proteinase inhibitor mix [Boehringer]), incubated for 10 min, and vortexed for 10 s. Nuclei were pelleted for 1 min at 10,000 × *g*, resuspended in 2 volumes of buffer C (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 420 mM NaCl, 25% glycerol, 0.5 mM DTT, Complete proteinase inhibitor mix) and incubated for 30 min with slow agitation. Nuclear debris was removed by centrifugation for 10 min at 10,000 × *g*, and the supernatant fraction was aliquoted, quickly frozen, and stored at −70°C. The protein concentration was determined by the Bradford procedure.

Gel mobility shift assay. All double-stranded oligonucleotides used terminated at both 5′ ends with a protruding AGCT sequence. The plus-strand oligonucleotide sequences without the 5′-protruding sequence were as follows: 1, 5′-AAT AGGTTAATCATTACTTTGTA-3′; 2, 5′-AGATAGTTAATCATTAGGAGG CT-3′; 3, 5′-ATTAGGTTAAATATTAGGAGGCT-3′; 4, 5′-ATTAGGTTAAA GGTTTATGTATT-3′; 5, 5′-TAAAGGTTTATGTATTAGGAGGC-3′; 6, 5′-A TTAGGTTAAAGGTCTTTGTATTAGGAGGC-3′; 7, 5′-ATTAGGTTAATG ATCTTTGTATT-3′; and 8, 5′-ATCTAGTTAATCATTACTTCCAA-3′.

Double-stranded oligonucleotide 8 containing the HNF1 site of the pre-S1 promoter was labeled with [α-^{32}P]dCTP in a fill-in reaction with Klenow polymerase. Binding reactions were carried out in a 12-μl reaction volume containing 10 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.1 mM EDTA, 80 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, 0.75 μg of poly(dI-dC), 2 μg of sheared salmon sperm DNA, 9 μg of nuclear protein extract (3 μg/μl), and 0.02 to 1 pmol of double-stranded cold competitor oligonucleotides 1 to 7. After preincubation on ice for 5 min, 0.01 pmol of ^{32}P-labeled oligonucleotide 8 (0.2 ng, 10^5 cpm) was added, and the assay mixture was further incubated on ice for 20 min. For

TABLE 1. Clinical data for the patients

Patient[a]	Yr of serum sample	HBV infection (yr)	Immunosuppression therapy		Serum HBV DNA (ng/ml)	Diagnosis[b]	Clinical outcome
			Yr	Drugs[c]			
Renal transplant							
B	1993	13	13	Pred, Aza	>3	ESLD	Alive
C	1992	13	12	Pred, Aza, Cs	NA[d]	ESLD	Died from ESLD, 1993
D	1994	9	8	Pred, Aza, Cs	10	ESLD	Died from ESLD, 1994
F	1993	13	11	Pred, Aza	3	ESLD	Died from ESLD, 1993
G	1994	12	8	Pred, Aza, Cs	NA	ESLD	Alive
I	1992	14	11[e]	Pred, Aza, Cs	0.2	Active hepatitis, Ci[f]	Died from renal failure
K	1995	10	14	Pred, Aza	NA	Mild hepatitis	Alive
L	1995	7	5	Pred, Aza, Cs	NA	Mild hepatitis	Alive
M	1995	7	6	Aza, Cs	NA	Mild hepatitis	Alive
N	1994	10	7	Pred, Aza, Cs	NA	Mild hepatitis	Alive
O	1995	17	16	Pred, Aza	NA	Mild hepatitis	Alive
Nontransplanted							
J	1993	4	None	None	3	Exacerbation[g]	Unknown

[a] Designations for the patients are as in reference 15.
[b] Diagnostic criteria for end-stage liver disease (ESLD) were presence of ascites, increase of bilirubin, decrease of prothrombin time, and ultrasonographic signs of liver cirrhosis. Mild hepatitis was diagnosed if alanine aminotransferase (ALT) values were only slightly elevated and when clinical, biochemical, or ultrasonographic signs of liver cirrhosis were absent.
[c] Pred, prednisolone; Aza, azathioprine; Cs, cyclosporin.
[d] NA, not available.
[e] Immunosuppressive therapy was discontinued because of transplant failure in 1992.
[f] Ultrasonographic signs of liver cirrhosis (Ci); ALT, 250 U/liter.
[g] ALT, 1,200 U/liter.

supershift assays, 2 μl of anti-HNF1 rabbit antiserum pEX or pEP or 2 μl of unrelated anti-hepatitis B core rabbit antiserum was preincubated on ice for 15 min. Protein-DNA complexes were separated in 6% nondenaturating polyacrylamide gels in 0.25× Tris-borate-EDTA at 10 V/cm.

Construction of plasmids containing the Cp/EnII mutations within the context of a reference HBV genome. Eleven plasmid-integrated HBV genomes with different Cp/EnII mutations were cleaved with *Rsr*II at position 1571 and with *Sst*I at the primer P2-pUC19 boundary. As a wild-type control, a fragment of a genotype A reference genome (NCBI ID 59418) was amplified for 10 cycles with primers P2 and P3 with *UlTma*-polymerase (Perkin-Elmer) and treated similarly with *Rsr*II and *Sst*I. The *Rsr*II-*Sst*I fragments composing the complete Cp/EnII region were exchanged with the corresponding fragment of the pUC-based plasmid pHBV-SapI, which contains an HBV wild-type genome of genotype D (12) also flanked by the primers P1 and P2 (16). Correct insertion of the mutated Cp/EnII sequences in the resulting hybrid genomes was ascertained by sequencing.

Transfection of HBV DNA. Two independent transfection experiments were carried out as described previously (16) with different preparations of DNA. For the first experiment the DNAs were purified by the anion-exchange procedure (Genomed, Bad Oeynhausen, Germany), whereas for the second experiment the DNAs were additionally treated with RNase A and proteinase K (Boehringer) to remove traces of contaminating RNA or protein. Linear HBV monomers with *Sap*I sticky ends were released from all plasmids by cleavage with 1 U of *Sap*I (New England Biolabs) per μg of DNA for 12 h. Subsequently, digested DNA was purified by phenol-chloroform (1:1) extraction and ethanol precipitation. Transfection of 5 μg of cleaved DNA or pUC19 as mock control was carried out by the calcium phosphate precipitation method. Huh7 cells were plated at a density of 1.2×10^6 cells per 50-mm-diameter petri dish. The medium was changed 1 day after transfection, and cells were harvested 4 days later. Each construct was transfected into two and three dishes per transfection experiment, respectively. HBsAg and HBeAg were assayed by using commercially available kits (Auszyme Monoclonal and HBe [rDNA]; Abbott). Transfection efficiency was measured by cotransfection of 1 μg of reporter plasmid expressing secreted alkaline phosphatase (SEAP) and determination of SEAP enzymatic activity in the cell culture supernatant (8). SEAP values in the medium of cells transfected with all variants were equal to or up to 30% lower than those for both wild-type viruses (data not shown). SEAP activity was not used for normalization of the transfection efficiency, as SEAP expression may be influenced by the type of virus DNA transfected.

Purification of HBV DNA from intracellular core particles. Cells were washed twice with ice-cold PBS and lysed in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% Nonidet P-40) per 50-mm-diameter petri dish. The lysed cells were transferred to Eppendorf tubes, vortexed, and allowed to stand on ice for 15 min. Nuclei were pelleted by centrifugation for 1 min at 14 krpm in a tabletop centrifuge (Eppendorf 5415C). A fraction (50%) of the supernatant was adjusted to 10 mM $MgCl_2$ and treated with 100 μg of DNase I per ml for 30 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 25 mM. Proteins were digested with 0.5 mg of proteinase K per ml–1% SDS for 2 h at 37°C. Nucleic acids were purified by phenol-chloroform (1:1) extraction and ethanol precipitation after addition of 20 μg of tRNA.

Purification of HBV DNA from extracellular viral particles. The cell culture medium was clarified by centrifugation at 8 krpm for 30 min (Sorvall SS-34 rotor). Four milliliters of medium pooled from three dishes was layered on top of a 1-ml 20% sucrose cushion in 10 mM Tris-HCl (pH 7.4)–1 mM EDTA–100 mM NaCl and centrifuged at 45 krpm for 4 h (Beckmann SW50.1 rotor). The pellet was resuspended in 500 μl of buffer (50 mM Tris-HCl [pH 8.0]) and treated as described above for the isolation of HBV DNA from cytoplasmic core particles.

Preparation of total RNA. Cells were washed twice with ice-cold PBS and lysed in 5 ml of guanidinium lysis buffer (2 M guanidinium thiocyanate, 12 mM Na citrate, 0.2 M Na acetate, 50% [vol/vol] phenol [water saturated], 0.4% [vol/vol] β-mercaptoethanol) per 50-mm-diameter petri dish. After addition of 500 μl of chloroform and 15 min of incubation on ice, the lysates were centrifuged for 20 min at 11.5 krpm (Sorvall SS-34 rotor). The RNA in the aqueous phase was precipitated with isopropanol, pelleted by centrifugation, washed with ethanol, and dissolved in water.

Southern and Northern (RNA) blot analyses. DNAs isolated from cytoplasmic core particles and from extracellular viral particles were separated on a 1.5% agarose gel. Thirty micrograms of total RNA was separated in a 1.5% agarose–formaldehyde gel. DNAs and RNAs were blotted onto Hybond N nylon membranes (Amersham) and hybridized with a ^{32}P-labeled, gel-purified, full-length HBV fragment (10^6 cpm/ml; specific activity, 1.6×10^8 cpm/μg). The probe was generated by using a random-primed labeling kit (Amersham).

Primer extension analysis of core promoter transcripts. Primer P6 (nucleotides 1991 to 1973; 5′-GAAGATCTCGTACTGAAG-3′) was ^{32}P labeled at the 5′ end (specific activity, 2×10^6 cpm/pmol) by using polynucleotide kinase (U.S. Biochemicals). In 15 μl of buffer (10 mM Tris-HCl [pH 8.3], 1 mM EDTA, 150 mM KCl), 20 μg of total RNA was denatured at 85°C for 10 min and hybridized to 0.2 pmol of primer P6 (4×10^5 cpm) during a 2-h temperature ramp down to 45°C. The annealed primers were elongated for 1 h at 42°C with 5 U of avian myeloblastosis virus reverse transcriptase (Promega) in a 45-μl assay mixture containing 53 mM Tris-HCl (pH 8.3), 0.33 mM EDTA, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 μg of actinomycin D per ml, 5 mM dNTPs, and 1 U of RNasin (Promega) per μl. The reaction products were phenol-chloroform (1:1) extracted, precipitated with ethanol, washed twice with 70% ethanol, dissolved in loading buffer, and separated in a 6% polyacrylamide–7 M urea gel. As a length marker a plasmid containing two head-to-tail HBV genomes was sequenced with the ^{32}P-labeled primer P6 by using the Sequenase kit (U.S. Biochemicals).

Immunoblot analysis. Cells were washed twice with ice-cold PBS, collected in 1 ml of PBS–10 mM EDTA, and pelleted at 2 krpm in a tabletop centrifuge. The cell pellet was lysed in 5 volumes of SDS loading buffer, boiled for 5 min, sonicated, and centrifuged. For detection of core and pre-S proteins, an aliquot (20%) of the supernatant was separated in an SDS–17.5% polyacrylamide gel. The proteins were transferred in Towbin buffer to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif.) and detected by using a chemiluminescence kit (Boehringer) and the following antibodies: polyclonal rabbit anti-core diluted 1:5,000, monoclonal mouse anti-pre-S2 F52 (Immunotech, Marseilles, France) diluted 1:200, and horseradish peroxidase-coupled secondary antibodies (Caltag Laboratories, San Francisco, Calif.) diluted 1:10,000. After separation of cell lysates in a 10% gel and blotting to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), HBV polymerase was detected by using the enhanced chemiluminescence kit (Amersham) and a mouse monoclonal antibody produced against a recombinant HBV polymerase polypeptide (anti-Pol 3.12F 1:10) (47). For detection of pre-S2 proteins and polymerase, three rounds of incubation with primary and secondary antibodies were performed before detection by chemiluminescence.

Statistical analysis of signal intensity. Autoradiography was detected with Fuji BAS III imaging plates which were analyzed with the FUJIX BAS 2000 Bio-Imaging Analyzer (Fuji, Tokyo, Japan) and TINA software (Raytest, Straubenhardt, Germany). Chemiluminescence was detected with X-ray film, and the image was subsequently digitized with a scanner (Sharp JX 610). For clarity of presentation, the brightness and contrast of the images were adjusted with Corel Photo Paint software (Corel Corporation, Ottawa, Ontario, Canada). Quantitative analysis of signal intensities on the digitized autoradiography and chemiluminescence images was performed on a Pentium PC with TINA software (Raytest). For each experiment, the values obtained with a variant were expressed relative to the value obtained with each of the two wild-type genomes. Means and standard deviations of relative values from two or more experiments were calculated, and the value relative to that for one of the wild-type viruses is shown in the figures. For the analysis of the statistical significance of the differences between viruses with and without novel binding sites in the core promoter, a one-sample *t* test was used to test whether the mean of values relative to both wild-type values was <1 (for pre-C mRNA and the ratio of pre-S/S to pre-C/C mRNAs) or >1 (for C mRNA, core protein, and HBV DNA intermediates) for a specific mutant. HBeAg values for variant and wild-type viruses were compared directly by an unpaired *t* test. This analysis revealed a *P* value of <0.05 unless stated otherwise in the figure legends.

RESULTS

Size analysis of Cp/EnII regions. HBV DNA was isolated from sera of chronically HBV-infected renal transplant patients with severe (Table 1, patients B, C, D, F, G, and I) or mild (Table 1, patients K, L, M, N, and O) liver disease and from a nontransplant patient with an exacerbation of chronic hepatitis (Table 1, patient J). Full-length viral genome amplification was performed as described recently (16). In order to visualize the size heterogeneity of the region constituting the Cp/EnII, this subgenomic region was amplified, size fractionated in long agarose gels, and compared with that amplified from a cloned wild-type genome (Fig. 1, right panel). As a control, the amino-terminal end of the X protein-coding region was also analyzed (Fig. 1, left panel). The Cp/EnII fragments of HBV from all immunosuppressed patients with severe liver disease showed size heterogeneity, with fragments larger and smaller than wild type, whereas the control fragments were uniform in size. Wild-type-sized Cp/EnII fragments were observed with HBV from all immunosuppressed patients with mild or no liver disease (data not shown). These data indicate that the size heterogeneity of subgenomic fragments is not a PCR artifact but reflects insertions and deletions in the Cp/EnII region.

In order to characterize the complexity and type of sequence changes of these heterogeneous virus populations in more detail, the complex mixture of the amplified HBV genomes was cloned. Individual Cp/EnII molecules were analyzed by sub-

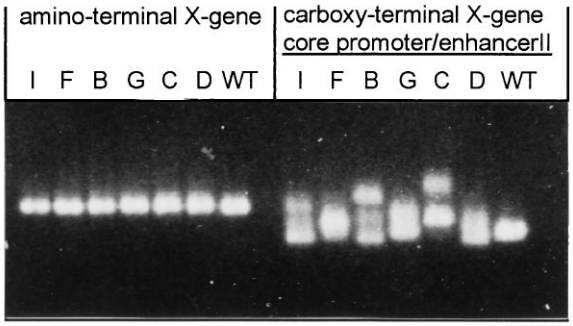

FIG. 1. Size analysis of an amplified amino-terminal X gene fragment (left panel) and of Cp/EnII fragments (right panel). HBV DNAs isolated from immunosuppressed patients I, F, B, G, C, and D with severe liver disease were amplified by PCR and separated on agarose gels. WT, fragments amplified from a cloned wild-type genome of genotype D (12). The material with slow electrophoretic mobility in the Cp/EnII lanes B and C probably does not represent fragments with large insertions but is generated by heteroduplex formation of Cp/EnII fragments with different lengths. Aberrant migration of heteroduplex DNA formed in PCRs was observed and analyzed previously (15, 58).

genomic PCR, size fractionation on agarose gels (resolution, 3-bp length difference), and sequencing. This analysis revealed that 95, 94, 100, 58, 91, 89, and 28% of the genomes from patients B, C, D, F, G, I, and J, respectively, contained insertions and/or deletions in the Cp/EnII (Fig. 2). The virus populations in most patients comprised several different types of Cp/EnII mutants (Fig. 2, patients B, C, F, G, I, and J). Taken together, the data indicate that most of the HBV genomes in the sera of the patients with severe liver disease contained insertions and/or deletions in the Cp/EnII region, whereas no length heterogeneity was found in the Cp/EnII fragments from five immunosuppressed patients with mild or no liver disease.

Evaluation of BCP sequences. Cp/EnII sequences representative of all types of mutants with insertions and/or deletions are shown in Fig. 3. In all variants a small segment in the BCP between nucleotide positions 1760 and 1780 was mutated (Fig. 2 and 3). In genomes from five different patients, nucleotides 1763 to 1770 were deleted (Fig. 2, patients B, D, F, I, and J; Fig. 3, sequences 1, 5, 7, 8, and 10). In one case, the region containing this deletion was duplicated (Fig. 2, patient I; Fig. 3, sequence 7). Only in patient B were two genomes with another type of deletion in this region found (Fig. 2, patient B; Fig. 3, sequence 9). Insertions in the BCP of 2, 3, or 15 nucleotides were found in HBV from patients G, F, and C, respectively (Fig. 2, patients G, F, and C; Fig. 3, sequences 4, 6, and 11). Mutations identical to those observed in HBV from patient G (Fig. 3, sequence 4) were found in genomes from patient C, but they were combined with two single-nucleotide deletions (Fig. 2, patient C). The BCP was highly rearranged in HBV genomes from patient G because of a BCP duplication in addition to a deletion, point mutations, and an insertion (Fig. 2, patient G; Fig. 3, sequence 3). Interestingly, two Sp1 binding sites, shown to be important for activity of the wild-type Cp/EnII region (59, 60), are removed by the deletion. Sequence analysis of the subpopulation of genomes without deletions or insertions revealed two types of point mutations in the BCP. A small fraction of genomes from patients C, F, G, and I had point mutations at positions 1762 and 1764 (Fig. 2, patients C, F, G, and I), whereas most of the genomes from patient J had, in addition to genotype B-specific sequence differences, point mutations at positions 1766 and 1768 not present in wild-type BCP sequences (Fig. 2, patient J; Fig. 3, sequence 2). Only one cloned HBV genome from patient B had no mutation in the BCP. While the directly determined Cp/EnII sequences from patients K, L, M, and N were completely identical to the genotype A consensus sequence (Fig. 3, sequence 12), those of the genomes from patient O had point mutations at positions 1762 and 1764 (Fig. 2, bottom).

Mutations in the BCP frequently create a novel HNF1 and rarely create a potential HNF3 transcription factor binding site. All mutations affected a binding site in the wild-type BCP (positions 1760 to 1780) for yet-unknown transcription factors (19, 31, 59) (Fig. 3). The focus of mutations in this region led us to examine whether the different types of mutations created new transcription factor binding sites. A search with the computer program *legame* (43) revealed that potential binding sites for HNF1 (42) were created in nearly all mutant BCPs (Fig. 4A). By using a gel mobility shift assay, the binding of HNF1 to these sites and their relative affinities were determined by measuring the abilities of the putative HNF1 sites to displace HNF1 from a high-affinity binding site present in the HBV pre-S1 promoter (5, 36, 43). In this assay the sequence motifs in variants 3 and 4 (from patients C and G) and 11 (from patient C), which are almost identical to the HNF1 site of the pre-S1 promoter (Fig. 4A), displayed an even higher affinity to HNF1 than the reference site (Fig. 4B and C). The 8-nucleotide deletion found in the variants from most patients (patients B, D, F, I, and J) also creates an HNF1 target but one with slightly lower affinity than the reference target (Fig. 4A, B, and C). The base changes at positions 1766 and 1768 (patient J) create two overlapping low-affinity HNF1 sites. In contrast, the wild-type BCP sequence (patients K, L, M, and N) does not bind HNF1 even after the introduction of point mutations at positions 1762 and 1764 as found in the genomes from patient O (Fig. 4A, B, and C).

Sequences 6 and 9 do not show similarity to a HNF1 binding site. Interestingly, however, a GTT insertion in sequence 6 creates similarity with the consensus sequence for the HNF3 binding site (7) (Fig. 4E). No similarity to HNF1 or HNF3 binding sites was evident in Cp/EnII sequence 9, which was found only once in the 50 fragments sequenced. Taken together, between 58 and 100% of the HBVs from the patients with severe liver disease contain functional HNF1 or potential HNF3 binding sites in the BCP, whereas such sites are not present in the BCPs of HBVs from patients with mild disease (Fig. 2).

Sequence changes in the alpha box, creating additional HNF4 and C/EBP binding sites. In addition to the mutations in the BCP, a sequence element important for the activity of enhancer II as well as the overlapping core promoter, the so-called alpha box (54–56), was completely or partially duplicated. This was observed in some genomes from four patients (Fig. 2, patients B, C, I, and J; Fig. 3, sequences 1, 9, 10, and 11). In these Cp/EnII sequences additional binding sites for liver-specific transcription factors, such as HNF4, C/EBP, and/or C/EBP-like factors (19, 31, 56, 57), are predictably created. In only two genomes from patients C and G were deletions found in the URS of the core promoter, in one case removing the alpha box and adjacent sequences and in the other case removing the negative regulatory element of enhancer II (30) (Fig. 2).

Effect of mutations on X gene expression. All mutations in the Cp/EnII region predictably affect the expression and function of the X protein. With two exceptions, only truncated X proteins can be expressed by the corresponding HBV genomes (Fig. 5). Truncations remove, totally or in part, the sequence homology with the Kunitz-type serine protease inhibitor domain, which was reported to be essential for the putative transactivation activity of X protein in vitro (2). In two of the

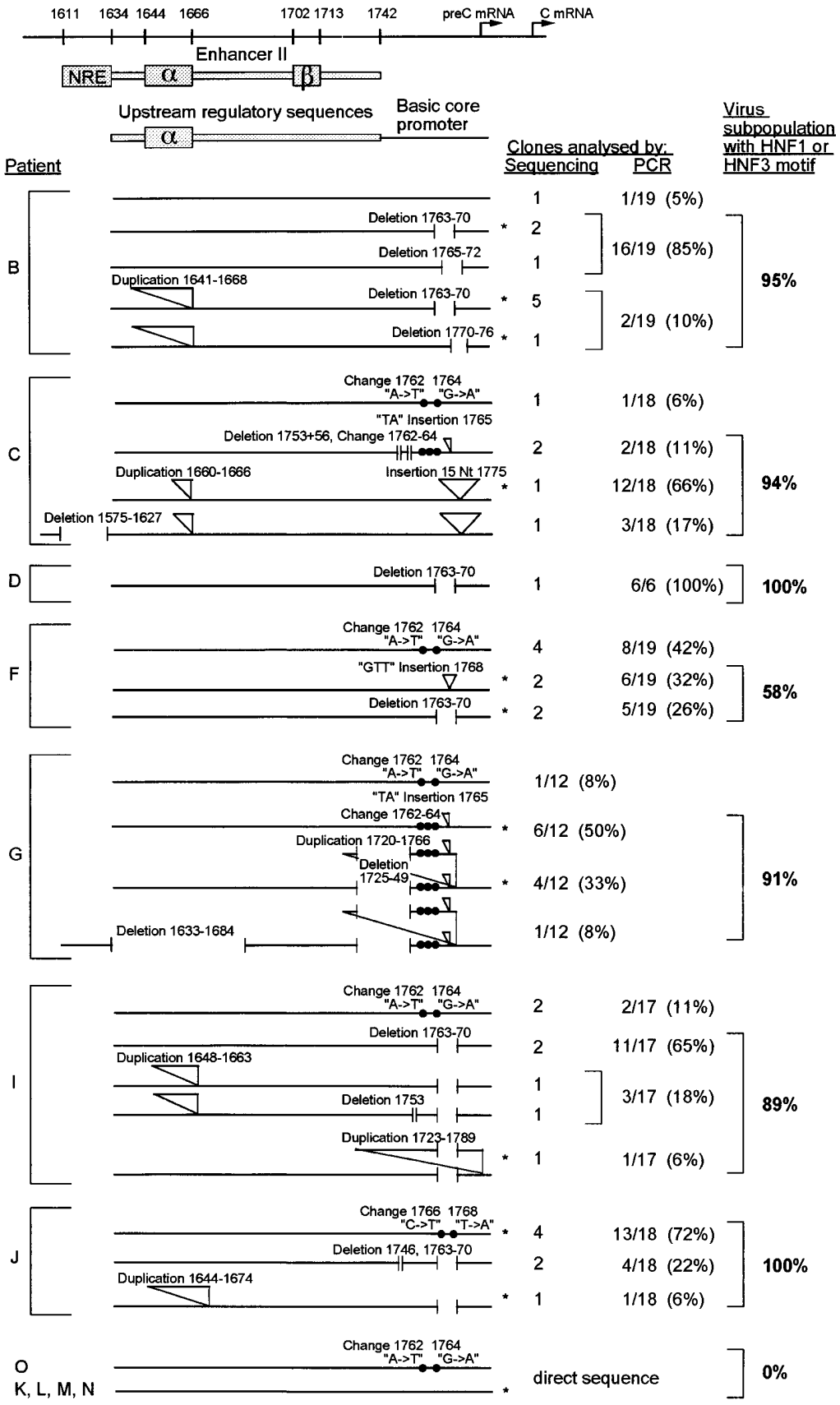

FIG. 2. Prevalence, type, and position of mutations in the Cp/EnII from patients with severe liver disease. At the top is a schematic presentation of the structure and functional elements of wild-type Cp/EnII, consisting of the alpha box (α), the beta box (β), and a negative regulatory element (NRE) (54, 56, 57). The frequency of mutated Cp/EnII regions in cloned HBV genomes was determined by subgenomic PCR and agarose gel electrophoresis (PCR) or sequencing and is indicated for each type of mutant. The proportion of genomes with HNF1 or putative HNF3 sites in relation to the total viral population is shown in boldface for each patient. Mutant Cp/EnII fragments marked by asterisks were functionally tested (detailed sequences are shown in Fig. 3).

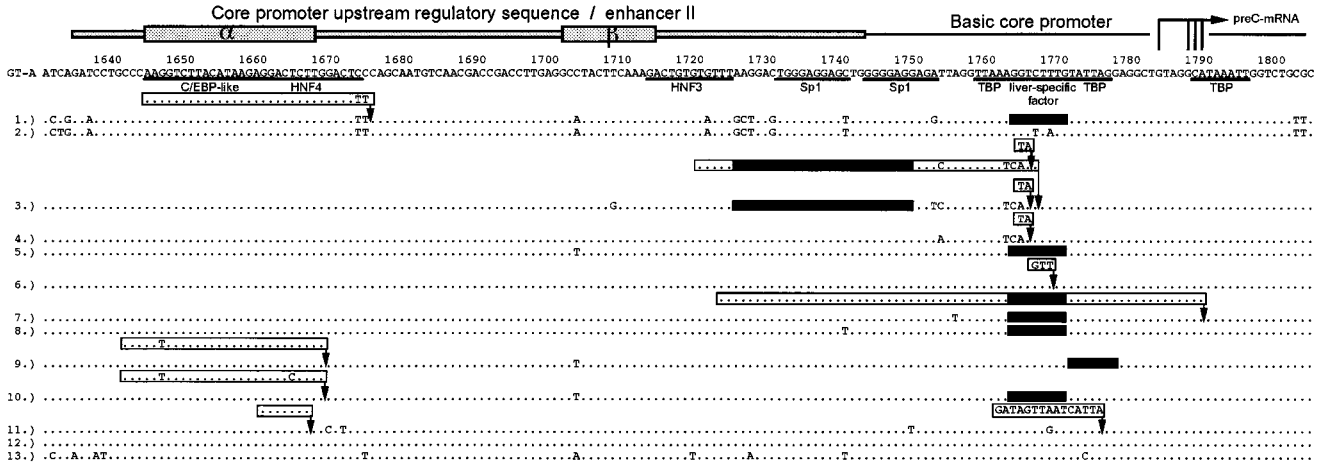

FIG. 3. Nucleotide sequences of 11 representative mutant Cp/EnII fragments and 2 genotype A- and D-specific wild-type Cp/EnII fragments used for functional analysis (sequences 1 to 11, 12, and 13, respectively). At the top is a schematic presentation of the structure and functional elements of wild-type Cp/EnII (54, 56, 57) and the pre-C mRNA transcription initiation sites as determined for wild-type sequences 12 and 13. Well- and less-well-characterized transcription factor binding sites (C/EBP or C/EBP-like, HNF3, HNF4, TBP, Sp1, and liver specific [6, 19, 22, 31, 56, 57, 59, 60]) are indicated and underlined. The sequences were aligned to the genotype A-specific consensus sequence of the Cp/EnII (GT-A). GT-A is based on six published sequences deposited in the NCBI database (no. 59455, 59418, 59416, 60429, 329633, and 260397). Sequences 1 and 2 were derived from patient J; 3 and 4 were derived from patient G; 5, 9, and 10 were derived from patient B; 6 and 8 were derived from patient F; 7 was derived from patient I; and 11 was derived from patient C. Sequence 12 is identical to the Cp/EnII sequences of HBV from patients K, L, M, and N. Deletions and insertions are indicated by filled and open boxes, respectively. The positions of insertions are marked by arrows.

Cp/EnII fragments, a full-length or overlength X protein could be expressed (Fig. 5, sequences 2 and 6), but a point mutation in this domain is again predicted to interfere with the transactivation function (2). Since a functional X protein has no effect on the replication and transcription of HBV in cultured hepatoma cells (4), analysis of the effects of the mutations on viral genome function as described below does not require coexpression of full-length X protein.

Functional analysis of the mutant Cp/EnII sequences in vitro. In order to determine the significance of the novel HNF1 binding sites and the other Cp/EnII mutations for virus transcription, protein expression, and replication, the mutant Cp/EnII regions were tested in the context of a genotype D reference genome (12) with known infectivity in vivo (48). As most Cp/EnII sequences of the genomes isolated from the immunosuppressed patients with mild disease were identical to the consensus sequence of genotype A (Fig. 3, row GT-A), the reference genome was also modified by insertion of the genotype A-specific Cp/EnII fragment. In total, three types of HBV full-length genomes were functionally tested by two independent transfection experiments with Huh7 hepatoma cells: genotype D with the various mutated Cp/EnII fragments, genotype D with the wild-type Cp/EnII of genotype A, and the wild-type genotype D genome (Fig. 3, sequences 3 to 11, 12, and 13, respectively).

Analysis of the pre-C mRNA and C mRNA/pregenome by primer extension. In order to determine whether the mutations lead to changes in the levels and initiation sites of these transcripts, primer extension analysis was performed (Fig. 6, top). For both wild-type genomes, 5′ ends of the pre-C mRNA and C mRNA/pregenome were mapped at positions 1783 to 1789 and 1816 to 1822, respectively, in a ratio of approximately 1:5. This is consistent with published mapping data (49, 51) and shows that genotype A- and D-specific point mutations do not significantly influence core promoter function. In contrast, compared with the wild-type genomes, significant and qualitatively similar changes were observed for all variants: the levels of pre-C mRNA dropped to very low levels (5 to 30%), and the levels of C mRNA/pregenomic RNA increased (130 to 750%) (Fig. 6, middle and bottom). The highest level of C mRNA/pregenome (750%) was observed with variant 3, which has two HNF1 sites but lacks the two Sp1 sites in the core promoter. This observation indicates that the presence of two HNF1 sites in this mutant more than compensates for the lack of the two Sp1 sites which are important for wild-type core promoter activity (59, 60). Medium-elevated levels of about 250% were found for variants 1, 2, 4, 7, 8, and 11, all of which have HNF1 binding sites. Marginally elevated C mRNA/pregenome levels were observed with variants 5 and 10 (130 to 150%). They share the same HNF1 site with the high-producer variants 1, 7, and 8 but differ in a point mutation at position 1703, which obviously interferes negatively with the stimulating effect of the HNF1 site (Fig. 7, left panel, C mRNA). The presence of a putative HNF3 binding site instead of an HNF1 site or duplication of the alpha box also elevated the levels of C mRNA (Fig. 6, variant 6, and Fig. 7, right panel, C mRNA, respectively). Taken together, the data indicate that all variants were characterized by enhanced levels of C mRNA/pregenome and reduced levels of pre-C mRNA. Both are presumably mediated by removal of wild-type transcription factor binding sites and/or creation of novel sites for HNF1, HNF3, C/EBP, and HNF4 in the BCP and alpha box region of the core promoter.

Proteins translated from pre-C and C mRNAs. In order to investigate whether higher C mRNA and lower pre-C mRNA levels lead to a corresponding change in protein expression, we measured the amounts of intracellular core and Pol proteins and of secreted HBeAg, which are translated from these RNAs (9, 46). Consistent with the RNA analysis, all variants secreted 50 to 90% less HBeAg into the cell culture supernatant than the wild-type genomes (compare Fig. 8 with Fig. 6, middle). A moderate correlation between the steady-state levels of the C mRNA and core protein levels was also found. Variants with greatly elevated C mRNA levels expressed 150 to 250% more core protein than the wild-type genomes, whereas little or no increase of core protein expression was seen with variants with slightly or marginally elevated C mRNA levels (compare Fig. 9 with Fig. 6, bottom). A very good correlation was found between the C mRNA levels and the amount of intracellular Pol

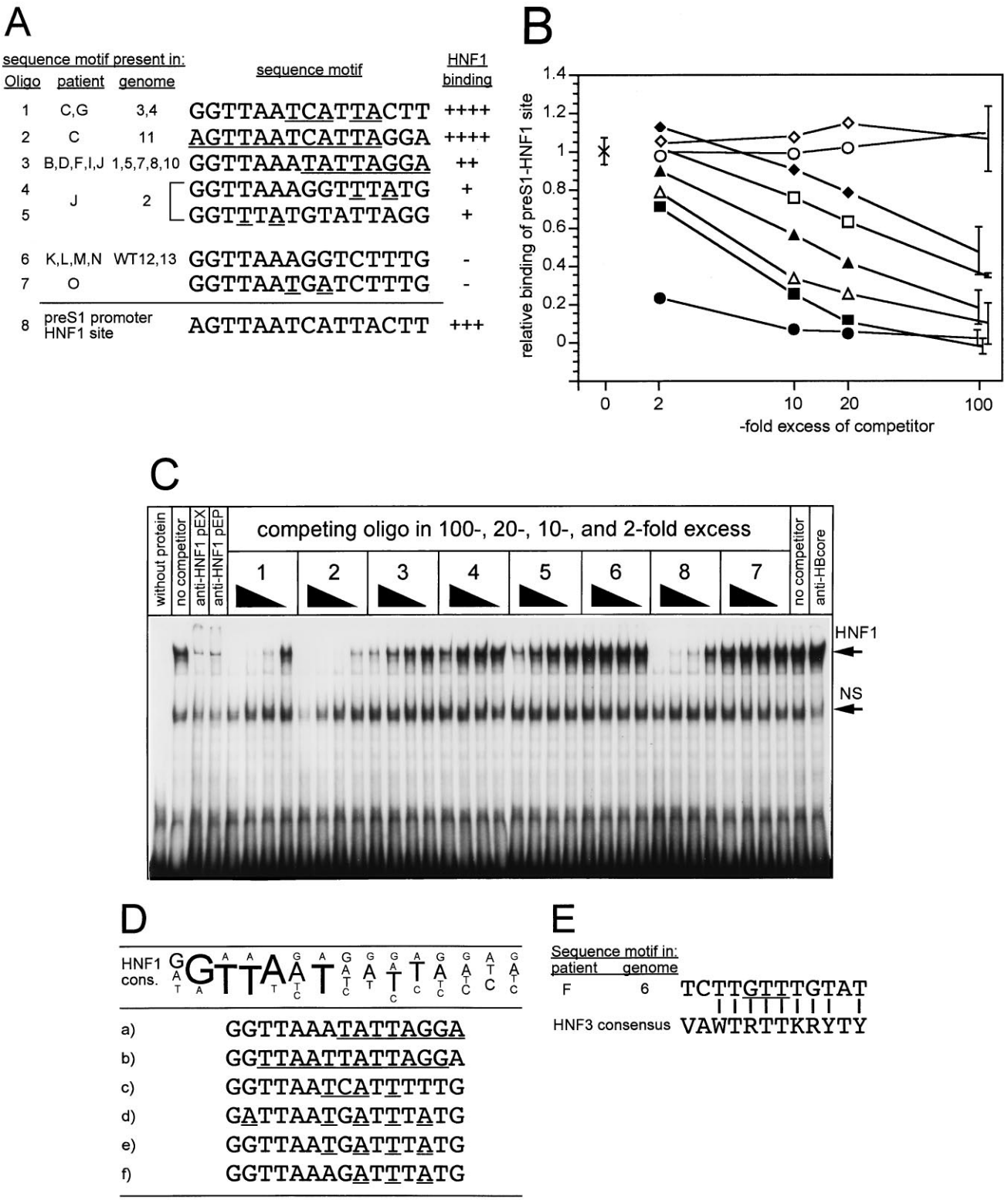

FIG. 4. (A) Sequence motifs in the oligonucleotides (Oligo) used for gel mobility shift assay and the origins of the sequences. Changes in comparison to the wild-type BCP sequence are underlined. The sequences of oligonucleotides 4 and 5 are derived from the same HBV genome with two overlapping HNF1 sites (position 8 in oligonucleotide 4 corresponds to position 1 in oligonucleotide 5). The relative HNF1 binding affinity as estimated from the analysis shown in panel B is indicated (++++, very high affinity; +, low affinity). (B) Quantitative analysis of the gel mobility shift assays. The fold excesses of cold competitor oligonucleotides 1 (■), 2 (●), 3 (▲), 4 (◆), 5 (□), 6 (○), 7 (◇), and 8 (△) and the amount of residual HNF1 protein-oligonucleotide 8 complex relative to that found in the absence of competitor (×) are indicated. The means from two experiments and the standard deviations for the 100-fold excesses are given. (C) Gel mobility shift assays with the ^{32}P-labeled oligonucleotide 8 (pre-S1 promoter HNF1 site [5]) complexed with HNF1 from Huh7 nuclear extracts. Complex formation was subjected to competition with various amounts of unlabeled oligonucleotides 1 to 7. The supershift of the complex with anti-HNF1 but not with an unrelated antiserum (anti-HBcore) demonstrates the specificity of the HNF1 complex formation. A faster-migrating band is not specific (NS). (D) Putative HNF1 sites in previously described HBV variants. Changes in comparison to the wild-type BCP sequence are underlined. Variants of type a have a deletion of nucleotides 1763 to 1770 (25, 26, 38); variant b has an 11-nucleotide insertion at position 1777 (25); and variants c (39), d (20), e (39), and f (32, 33) have point mutations at positions 1757, 1762, 1763, 1764, 1766, and/or 1768. For comparison, the consensus sequence of the HNF1 binding site (42) is shown at the top. (E) The putative HNF3 binding site in variant 6 is aligned with the HNF3 consensus binding site (7).

```
        100       110       120       130       140       150
GT-A RTLGLPAMSTTDLEAYFKDCVFKDWEELGEEIRLKVFVLGGCRHKLVCAPAPCNFFTSA
 1.) .....*
 2.) .....S.............AE...........Y..........S.........
 3.) ..............C.....SLG*
 4.) ..............................N..IITLY*
 5.) ..................................NIRRL*
                                       L
                                       |
 6.) ------------------------------------------------------------
 7.) ..............................W.NIRRL*
 8.) ..................................NIRRL*
 9.) ...AQCLT*
10.) ...AQCLT*
11.) ...DSCIPSNVNDRP*
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FIG. 5. Predicted C-terminal amino acid sequences of the X proteins expressed from variants 1 to 11. Sequences were aligned to the genotype A-specific consensus sequence of the X protein (GT-A). Asterisks indicate stop codons. The domain important for transactivation in vitro (2) is boxed.

protein, with up to eight times more Pol protein than observed with the wild type (compare Fig. 10 with Fig. 6, bottom). These data indicate that the altered levels of transcripts result in a corresponding change in the steady-state levels of HBeAg and core and Pol proteins.

Variants produce more intracellular replicative intermediates. Three components required for replication, i.e., the core protein, polymerase, and pregenomic RNA (13), are produced in larger amounts by the variants than by the wild-type genomes. We wondered whether this results in more intracellular replicative HBV DNA intermediates. By Southern blot analysis, up to eightfold-higher levels of replicative intermediates were detected in Huh7 cells transfected with the variants (Fig. 11). These levels correlated very well with those of pregenomic RNA and Pol protein and correlated moderately with those of the core protein (compare Fig. 11, bottom, with Fig. 6, 10, and 9, bottom, respectively). These data show that all core promoter mutations ultimately result in enhanced replication which is probably an indirect effect of the enhanced synthesis of all components required for replication.

Expression of pre-S/S mRNAs and envelope proteins. The known effects of enhancer II on all viral promoters (40, 55, 61) prompted us to test whether the levels of envelope proteins and the corresponding transcripts are affected by the mutations in Cp/EnII. Northern blot analysis revealed that only the variants with the highest levels of replication had pre-S/S transcript levels higher than (variant 3) or equal to (variants 2, 4, and 11) those of the wild-type genomes. The remaining variants had lower levels (Fig. 12). With the exception of variant 6, all variants had a lower ratio of pre-S/S to pre-C/C mRNAs than the wild-type genomes (Fig. 12, bottom). The transcript levels correlated rather well with the amounts of secreted HBsAg (compare Fig. 13 with Fig. 12, middle), whereas this was much less obvious for the intracellular levels of the pre-S proteins (compare Fig. 14 with Fig. 12, middle). These data suggest that HNF1 sites in Cp/EnII not only modulate core promoter activity but often also suppress the activity of the pre-S/S gene promoters, which results in reduced levels of pre-S/S mRNAs and secreted and intracellular envelope proteins.

HBV DNA-containing particles in the culture medium. The increased levels of core protein and replicative intermediates and the frequent decrease in viral envelope protein synthesis seen with the variants raised the question whether these changes influence the amount of HBV DNA-containing virus particles in the culture medium. In order to study this, viral particles in the supernatant from the transfected Huh7 cells were pelleted by ultracentrifugation, and the particle-associated HBV DNA was analyzed by Southern blotting. Compared with the wild-type genomes, 50 to 400% more particle-associ-

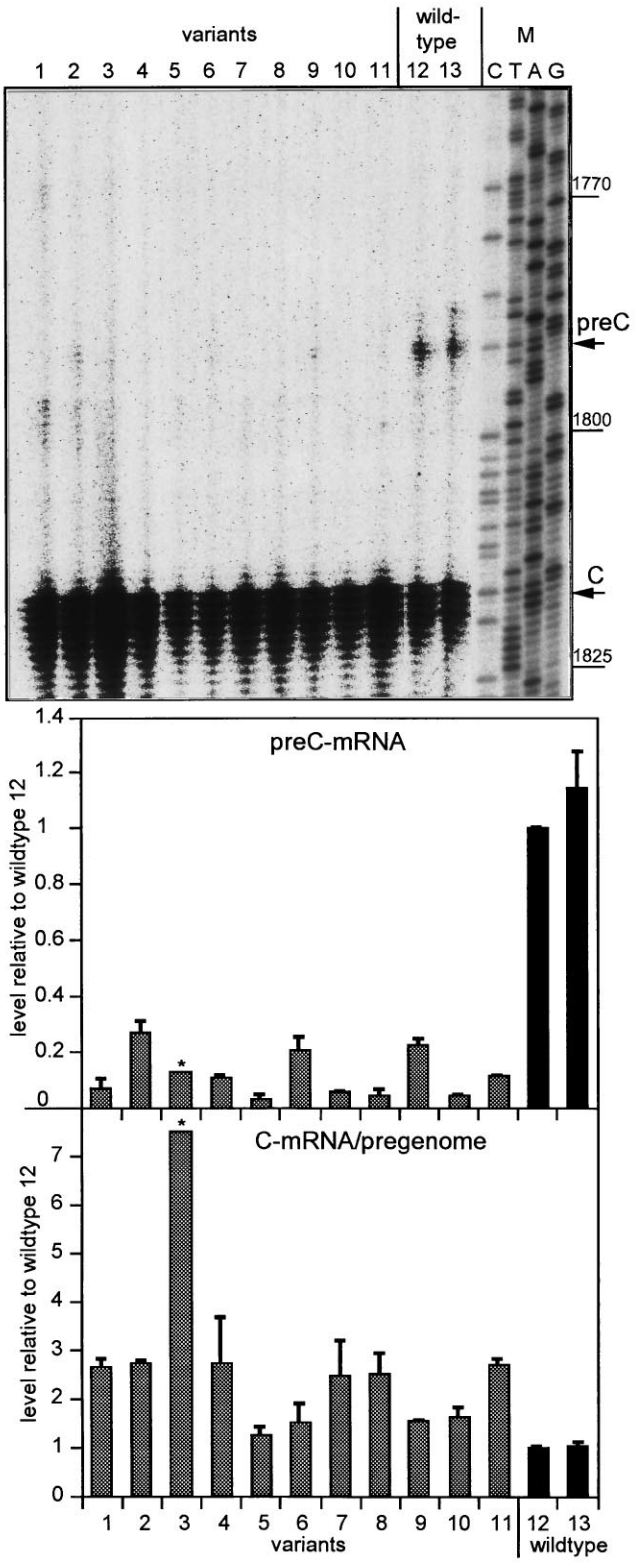

FIG. 6. Primer extension analysis of pre-C mRNA and C mRNA/pregenome 5′ ends and quantitative evaluation. The signals of the pre-C mRNA and C/pregenomic RNA observed in two independent primer extension reactions (top) (one reaction is shown) were quantitatively evaluated (middle and bottom, respectively). An asterisk indicates that the experiment with this variant was performed only once. RNA isolated from the mock-transfected cells yielded no signal (data not shown). M, fragments from a sequence reaction as a length marker, with nucleotide positions indicated.

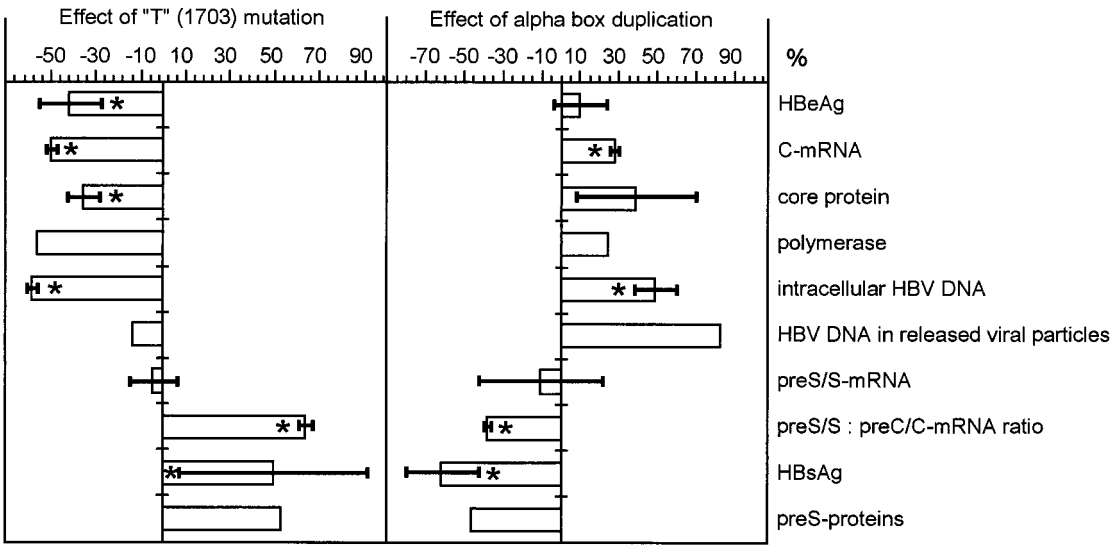

FIG. 7. Comparative analysis of variants 5 and 8 and variants 10 and 5. Variant 5 was compared with variant 8 to elucidate the effects of a C-to-T change in position 1703 (left panel). The two variants differ only by this and a second genotype D-specific mutation (Fig. 3). The genotype D-specific mutation has no functional consequence, since there were no significant differences in any of the parameters measured for the genomes with genotype A- and D-specific wild-type Cp/EnII sequences. Variant 10 was compared with variant 5 to analyze the effects of the alpha box duplication (right panel), as they differ only by duplication of nucleotides 1641 to 1668. The differences between the values obtained for the variant with the mutation (5 and 10) and the variant without the mutation (8 and 5) are expressed relative to the value obtained for the variant without the mutation (0%). If more than one value was available, the mean and standard deviation are indicated. The statistical significance of the differences between two variants was evaluated as described for the differences between variant and wild-type virus (see Materials and Methods). A P value of <0.05 is indicated by an asterisk.

ated HBV DNA was secreted or released from cells harboring variant HBVs (Fig. 15). Interestingly, the levels correlated only roughly with the levels of intracellular HBV DNA as a marker for mature core particles but not with the intracellular level of envelope proteins (compare Fig. 15, bottom, with Fig. 11 and 14, bottom, respectively). These data suggest that the variant HBV DNA-transfected cells secrete or release more HBV DNA-containing particles than wild-type viruses.

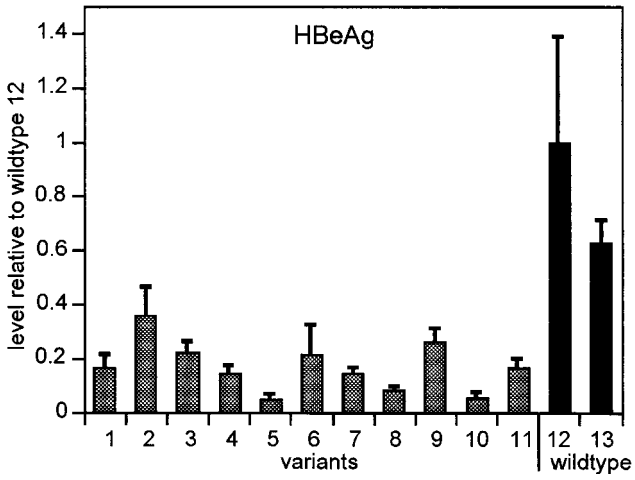

FIG. 8. HBeAg values as determined by radioimmunoassay in the cell culture supernatants from five dishes of two independent transfection experiments. The values were corrected for background with the mock transfection values (approximately 150 cpm). A level of 1.0 corresponds to approximately 18,000 cpm for undiluted supernatant.

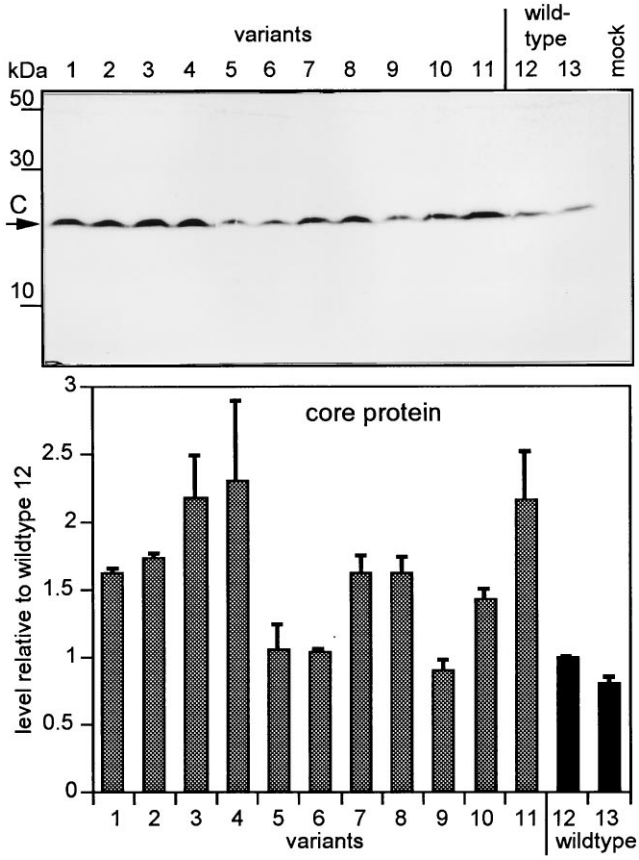

FIG. 9. (Top) Immunoblot analysis of intracellular core protein. The position of the 21-kDa core protein (C) is marked. (Bottom) The core protein signals obtained with two independent immunoblot analyses were quantitatively evaluated (for variants 5, 6, and 9 only, the P values are >0.05).

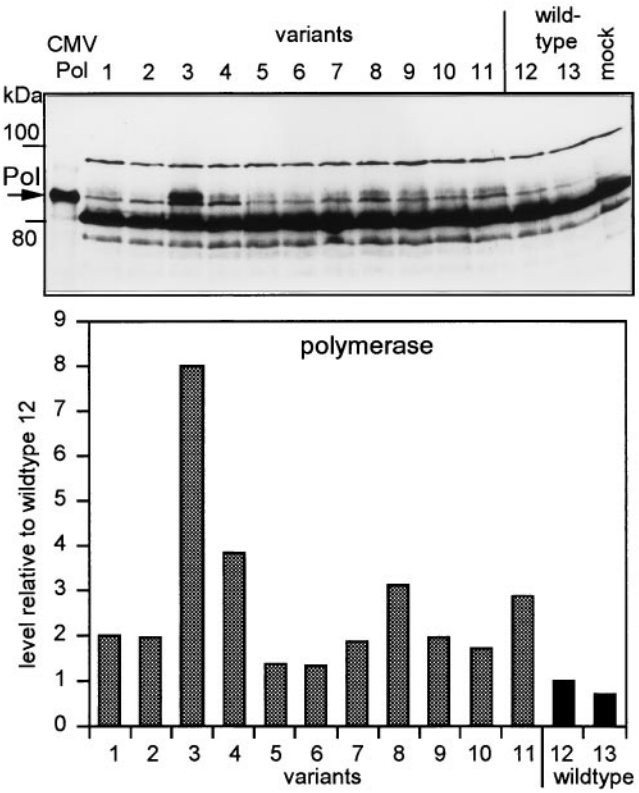

FIG. 10. (Top) Immunoblot analysis of intracellular Pol protein. The position of the Pol protein is indicated. As a positive control, a lysate of cells transfected with a plasmid expressing the Pol gene under control of the cytomegalovirus promoter was analyzed (lane CMV Pol). No Pol-specific signal was observed after transfection of a defective genome which can express all viral proteins except Pol and pre-S1 proteins (data not shown). Only one Pol-specific band is seen with cells transfected with the CMV-Pol expression construct, whereas a closely spaced double band is visible with extracts from cells transfected with full-length HBV genomes. The reason for this difference is unclear. (Bottom) The Pol-specific signals were quantitatively evaluated.

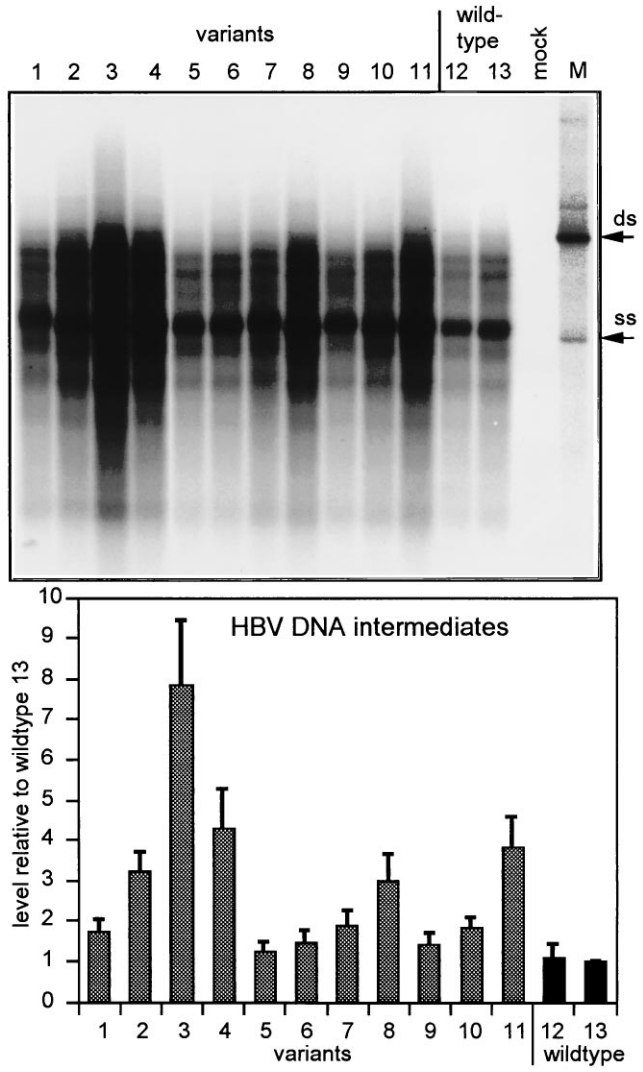

FIG. 11. (Top) Southern blot analysis of intracellular replicative HBV intermediates. M, marker lane loaded with DNA generated by asymmetric PCR with primers P1 and P2 (1:25) and pHBV-SapI as a template. ds and ss, amplified 3.2-kb double- and single-stranded HBV DNAs, respectively. (Bottom) The HBV DNA signals obtained with three DNA preparations from two independent transfection experiments were quantitatively evaluated (for variant 5 only, the *P* value is >0.05).

DISCUSSION

We have identified Cp/EnII variants as major HBV populations in long-term-immunosuppressed patients with severe liver disease, whereas these variants were not detectable in those with mild disease. Different types of deletions, insertions, and/or point mutations were found in the BCP and the alpha box. Despite this diversity, common to almost all these variants was that the mutations created binding sites for the hepatocyte-enriched transcription factor HNF1, and in one case perhaps HNF3, and affected HBV gene expression and replication in similar ways. In particular, the mutations caused low steady-state levels of pre-C mRNA, HBeAg, and often also pre-S/S transcripts and envelope proteins. In contrast, the levels of C mRNA/pregenome, core protein, Pol protein, replicative intermediates, and HBV DNA-containing viral particles in the culture medium were increased. This indicates that HNF1 variants have a phenotype which is drastically different from that of wild-type virus.

Prevalence and selection of Cp/EnII mutations. A large variety of core promoter mutants have already been identified by others (3, 11, 14, 20, 21, 23, 25, 26, 32, 33, 35, 38, 39, 41, 44). However, in the previous papers it was not reported whether the mutations create new transcription factor binding sites, nor was their functional significance tested. We found that most of the core promoter variants described so far are characterized primarily by A-to-T and G-to-A point mutations at positions 1762 and 1764, respectively, which do not create an HNF1 binding site (Fig. 4A, B, and C). However, the core promoters of viral subpopulations in some previous studies have such motifs. They are created by insertions, deletions, or point mutations similar or identical to those described here (3, 20, 21, 25, 26, 32, 33, 38, 39, 41) (Fig. 4D, variants a to f). Thus, these types of core promoter variants are not specific for immunosuppressed patients but also occur in asymptomatic and symptomatic chronic carriers (3, 21, 26, 32, 33, 39, 41) and in patients with fulminant hepatitis (20, 21, 25, 39) (Fig. 4D, variants a, c, d, and e).

Consistent with our results, dominant virus populations with HNF1 sequence motifs in the core promoter were previously found in an immunosuppressed liver transplant patient (25) (Fig. 4D, variant b) and in an immunocompromised child (38) (Fig. 4D, variant a). Therefore, it is conceivable that variants

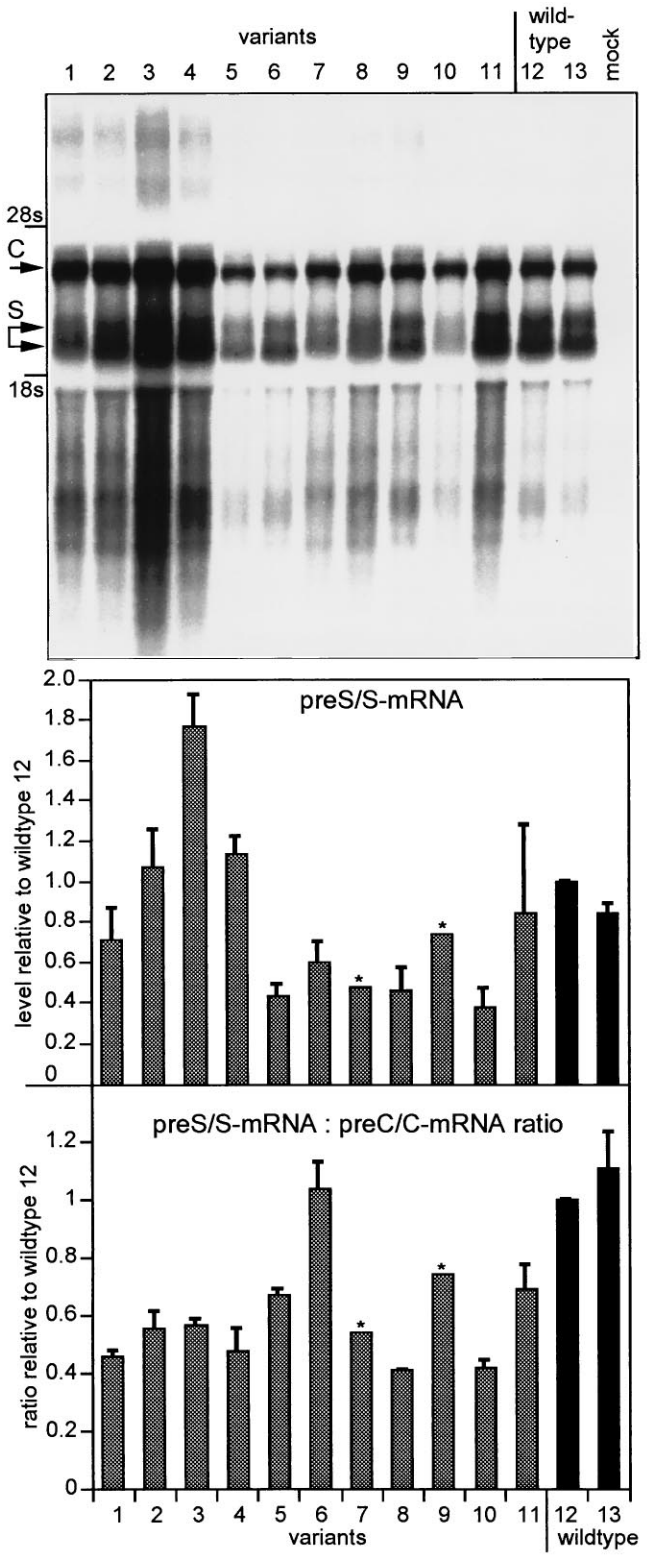

FIG. 12. (Top) Northern blot analysis of HBV transcripts. The positions of the rRNAs, the 3.5-kb pre-C mRNA and C mRNA/pregenome, and the 2.1- and 2.4-kb pre-S/S mRNAs are indicated (18s/28s, C, and S, respectively). (Middle) The pre-S/S mRNA signals from two independent transfection experiments were quantitatively evaluated. (Bottom) Calculated ratios of pre-S/S to pre-C/C mRNAs (for variant 6 only, the *P* value is >0.05). An asterisk indicates that the experiment with this variant was performed only once.

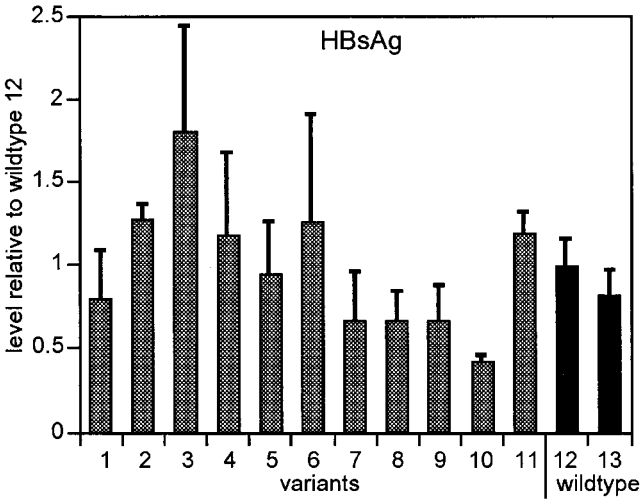

FIG. 13. HBsAg values as determined by enzyme immunoassay in the cell culture supernatants from five dishes from two independent transfection experiments. The values were corrected for background with the mock transfection values. A level of 1.0 corresponds to an optical density of approximately 1 unit for supernatant diluted 1:100.

with HNF1 binding sites in the core promoter have a selective advantage if the immune system is suppressed. Consistent with this speculation, Laskus et al. (25) have shown accumulation of such variants during immunosuppressive therapy (Fig. 4D, variant b). JC polyomavirus mutants containing insertions and deletions in regulatory sequences were also found preferentially in immunosuppressed patients (24, 53). This and our

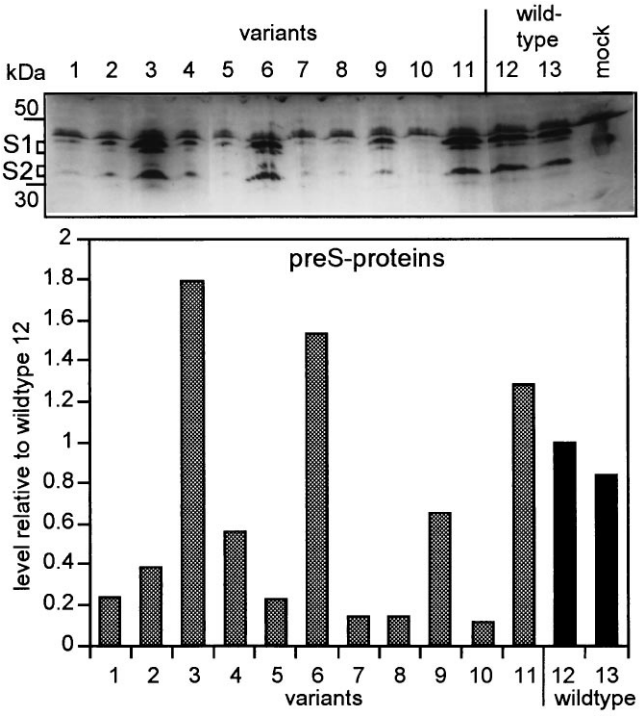

FIG. 14. (Top) Immunoblot analysis of intracellular pre-S proteins. The positions of the 33- and 36-kDa pre-S2 proteins and the 39- and 42-kDa pre-S1 proteins are marked (positions S2 and S1, respectively). (Bottom) The pre-S protein signals were quantitatively evaluated.

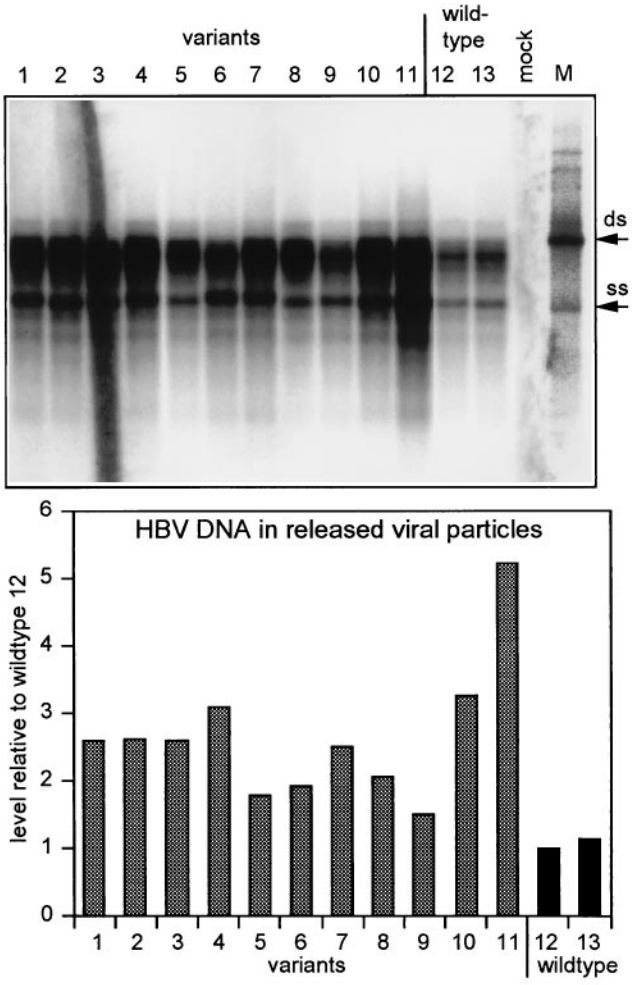

FIG. 15. (Top) Southern blot of HBV DNA in viral particles secreted or released from transfected Huh7 cells. M, marker lane loaded with DNA generated by asymmetric PCR with primers P1 and P2 (1:25) and pHBV-SapI as a template. ds and ss, amplified 3.2-kb double- and single-stranded HBV DNAs, respectively. (Bottom) The HBV DNA signals were quantitatively evaluated.

observations indicate that the emergence of viruses with changes in regulatory sequences may be a more general phenomenon in immunocompromised patients.

Our findings that the variants replicate more efficiently and express more core and Pol proteins may provide a potential explanation for their selection in an immunocompromised host. Core and Pol proteins are potent targets for cytotoxic T lymphocytes (34, 37). Therefore, enhanced expression of these proteins may increase recognition of the variant-infected cells by corresponding cytotoxic T lymphocytes, which may lead to specific cytolysis or noncytolytic suppression of viral replication. In immunocompetent hosts this may cause selective elimination of the variants, whereas in immunocompromised patients they would have a selective advantage, as they will not be selectively eliminated and can overgrow wild-type virus because of their enhanced replication competence.

On the other hand, HNF1 variants could also be selected by the antiviral immune response because of their reduced HBeAg or envelope protein expression. For the patients studied here we consider this an unlikely possibility, as none of them had ever developed an anti-HBe or anti-HBs response during the course of infection. In favor of our speculation that the HNF1 variants were selected because of immunosuppression is also the fact that such variants (Fig. 4D, variant a) are selectively eliminated during alpha interferon-induced seroconversion to anti-HBe (26), whereas pre-C stop and start codon variants are frequently selected under these conditions (17).

Pathogenic relevance of Cp/EnII mutations. An important question concerns the potential role of the Cp/EnII mutations in the pathogenesis of the severe liver disease in the patients studied. We have recently reported that progression of liver disease in our patients was associated with the persistence and accumulation of variants with deletions in the C gene (15, 16). Here we have shown that in the same patients the dominant virus population has a novel HNF1 or a putative HNF3 binding site in the Cp/EnII, whereas such sites again were absent from HBV in immunosuppressed patients with mild liver disease. Since the two groups of patients do not differ in other relevant parameters (Table 1) (15), this suggests a role of the mutants in pathogenesis. Since most of the mutant genomes contain both C gene deletions and Cp/EnII mutations (18), it is likely that in the livers of patients with severe disease, large amounts of defective core and nonencapsidated Pol proteins are expressed. The high-level viremia found in most of these patients (Table 1) indicates a high level of virus replication in the liver and little negative interference by defective core and X proteins. The lower pre-S/S protein levels produced by the BCP mutants and the treatment with immunosuppressive drugs may further contribute to intracellular accumulation of these proteins (27). This could lead to cytopathic effects, as observed for duck HBV pre-S protein variants, which also showed increased intracellular core protein levels (28). Taken together, this and our previous study (15) suggest that the combination of C gene deletions with Cp/EnII mutations could be important for the development of severe liver disease in immunosuppressed patients.

Transcriptional changes due to Cp/EnII mutations. The mutations in the BCP most likely result in up- and downregulation of transcription initiation of the C mRNA/pregenome and pre-C mRNAs, respectively. Alternative mechanisms, such as posttranscriptional mechanisms, are unlikely, as both RNAs contain the same mutations, which can hardly change the RNA half-life differently. A direct effect of the mutations on the replication of the virus is also unlikely, since none of the viral proteins and known sequence motifs involved in RNA packaging and DNA plus- and minus-strand synthesis is affected by the mutations. The effects of the mutations in the BCP on promoter activity are surprising when compared with results from previously reported core promoter studies. First, all mutations introduced by linker scanning into exactly the same BCP region where mutations were found naturally lowered core promoter activity because of the loss of liver-specific transcription factor binding sites (19, 31, 59). Second, deletion of this BCP region or introduction of point mutations only marginally changed the ratio of pre-C to C mRNA transcription initiation (6). In contrast to these reports, the mutant BCPs reported here show even higher activities than wild-type BCPs despite the presumed loss of transcription factor binding sites and show a drastically different ratio of pre-C to C mRNA 5′ ends. Thus, the BCPs with naturally occurring mutations have a function qualitatively different from that of BCPs from wild-type genomes. This is probably due to the binding of new transcription factors, such as HNF1 or HNF3, which are expressed in the differentiated hepatoma Huh7 cells used in this study (references 5 and 22 and this study). It is tempting to speculate that qualitative and quantitative changes in transcription factors in the cirrhotic livers of the immunosup-

pressed patients contribute to the selection of the genomes with particular Cp/EnII mutations. In contrast to HBV, in duck HBV and woodchuck hepatitis virus, most of the transcriptional activity of the wild-type Cp/EnII-homologous regions is conferred by HNF1 binding sites (10, 29). It is currently unclear why this difference between human and animal hepadnaviruses exists.

The effects of the BCP mutations on the promoter activity differ quantitatively from variant to variant. One reason for this observation is probably that five different HNF1 binding sites are present in the variants (Fig. 4A), two with high affinity one with medium affinity, and two overlapping sites with low affinity. The duplication of HNF1 sites in two of the variants (variants 3 and 7) is another reason. Furthermore, mutations in the URS seem to enhance or downregulate the positive effect of the HNF1 sites. Comparison of the transcript, protein, and replication levels of variants 5 and 8 (Fig. 7) strongly indicates that a T at position 1703 negatively influences the stimulatory effect of the HNF1 site. This mutation is not present in any of the published genomes in GenBank. The location of this mutation in an important regulatory element of enhancer II, the so-called beta box (55–57), is in agreement with this conclusion. Variants 5 and 10 differ only by duplication of positions 1641 to 1668. This sequence covers the so-called alpha box, to which HNF4, C/EBP, or similar transcription factors bind (19, 31, 45, 54, 56, 57, 61). It represents the second essential element of enhancer II and is important for core promoter activity (54, 56, 57). A comparison of the transcript, protein, and replication levels of these two variants (Fig. 7) indicates that this duplication increases the levels of the C mRNA, HBeAg, core protein, Pol protein, and intra- and extracellular particle-associated HBV DNA and decreases the ratio of pre-S/S to pre-C/C mRNAs and the HBsAg and pre-S protein levels. These data and the lack of an increase in pre-S/S transcript levels indicate that duplication of the alpha box upregulates core promoter activity but does not strengthen enhancer II function with regard to the pre-S/S gene promoters. Only variant 6 has a putative HNF3 site instead of an HNF1 site in the BCP region and also shows a moderate decrease and increase of pre-C and C mRNAs, respectively. Moreover, other types of mutations may also produce the variant-specific phenotype, as exemplified by variant 9, which has neither an HNF1 site nor a putative HNF3 site.

Binding of the TATA-box binding protein (TBP) to TA-rich sequences located at either end of the BCP sequence from position 1760 to 1780 (6) could be important for pre-C mRNA transcription initiation. The binding of HNF1 to this region may compete here with TBP binding, whereas it may enhance binding of TBP at the pre-C mRNA transcription initiation site (6) located a few nucleotides downstream (Fig. 3). Each of these effects offers an explanation for the downregulation of pre-C mRNA transcription initiation. Decreased pre-C mRNA transcription is most likely responsible for the lack of HBeAg in viremic sera of some patients infected with HBV which do not have precore mutations to prevent HBeAg synthesis (21, 32, 33, 39, 41) but contain putative HNF1 sites in the BCP (Fig. 4D, variants b, e, and f).

The reason for the downregulation of the pre-S/S mRNA levels by most mutations in the BCP is less obvious. This is mainly due to the fact that little is known about the mechanisms of enhancer II-pre-S/S promoter interaction (40, 55, 61). The opposing effect of the mutations on the core and pre-S/S promoters shows that the enhancer function is altered by mutations in the nearby BCP region. It has previously been speculated that the same alpha box-binding proteins simultaneously enhance the pre-S/S promoters and regulate BCP activity (57). If this is true, the function of alpha box-binding proteins may well change if they interact with HNF1 instead of with the transcription factors bound to the wild-type BCP. Taken together the data indicate that the novel phenotype identified in this study seems to be mediated mainly by binding of HNF1, HNF3, or other transcription factors to the mutant BCPs and/or by the lack of transcription factor binding sites present in wild-type BCP.

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