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A Novel Method for Efficient Amplification of Whole Hepatitis B Virus Genomes Permits Rapid Functional Analysis and Reveals Deletion Mutants in Immunosuppressed Patients

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Current knowledge of hepatitis B virus (HBV) sequence heterogeneity is based mainly on sequencing of amplified subgenomic HBV fragments. Here, we describe a method which allows sensitive amplification and simplified functional analysis of full-length HBV genomes with or without prior cloning. By this method, a large number of HBV genomes were cloned from sera of six immunosuppressed kidney transplant patients. Two size classes of HBV genomes, one 3.2 kb and another about 2.0 kb in size, were found in all patients. The genome population from one serum sample was studied in detail by size analysis of subgenomic PCR fragments and sequencing. Regions with deletions and insertions were mapped in the C gene and pre-S region. Up to 100% of HBV genomes in all other immunosuppressed patients also had deletions in the C gene. Our results demonstrate the potential of the established method for the structural and functional characterization of heterogeneous populations of complete virion-encapsidated HBV DNAs and suggest that HBV genomes with C gene deletions can have a selective advantage in immunosuppressed patients.

Since the introduction of the PCR for amplification of viral DNA, investigation of the sequence heterogeneity of the hepatitis B virus (HBV) genome and its role in pathogenesis and viral protein expression has become a focus in hepadnavirus research. HBV mutants which cannot synthesize hepatitis B e antigen (HBeAg) because of mutations in the pre-C region have been studied in most detail (for a review, see reference 14). These variants were found to be dominant populations in asymptomatic chronic carriers and in patients with mild to severe chronic and fulminant hepatitis and often emerge during anti-HBeAg seroconversion and interferon therapy (10, 16). Variants with mutations in the immunodominant epitopes of the surface gene were also described and may escape immune elimination by neutralizing antibodies (for a review, see reference 27). Similar mechanisms may be responsible for the selection of variants which cannot express pre-S2 protein, one of the three envelope proteins (6, 20). Variants with mutations in all other genes and in regulatory regions of the HBV genome were also identified (for a review, see reference 13).

The biological and clinical significance and the possible role in viral pathogenesis of most of these variants are far from being clear. A major handicap in these studies is the lack of a sensitive and simple system for isolation and characterization of full-length genomes from complex virus populations. With only a few exceptions (15, 23, 24), most investigations published so far are based on an analysis of subgenomic fragments amplified by PCR. This method does not permit analysis of the complexity of mutations present on a single genomic molecule even when complete HBV genomes are reconstituted from subgenomic fragments by cloning (3, 11). Furthermore, the conventional method of isolation of full-length genomes by cloning of virion-encapsidated DNA after genome repair and linearization by a single-cutting restriction enzyme is only applicable for high-titered sera. Variants with mutations in the corresponding restriction sites are not identified by this approach. Clearly, sera with complex virus populations and a low titer of virus require a highly sensitive and simple method for full-length HBV genome isolation and characterization. Here we describe the establishment of such a method and its application for the characterization of HBV full-length and deletion mutants from sera of patients under immunosuppressive therapy.

MATERIALS AND METHODS

Patients and sera. Serum samples were from patients with hepatitis B surface antigen (HBsAg)-positive chronic hepatitis. Patients 1 to 6 were positive for HBeAg, had end-stage liver cirrhosis and were under immunosuppressive treatment because of kidney transplantation. Patients 7 and 8 were not treated with immunosuppressive drugs; the former was HBeAg positive and the latter had antibodies to HBeAg. Sera from patients A and B were used only for sensitivity testing and contained 170 and 100 pg of HBV DNA per ml, respectively, as previously determined by dot blot hybridization.

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, subsequently extracted with chloroform, and precipitated with ethanol with 20 μg of tRNA as a carrier. The pellet was dissolved in 20 μl of H_2O.

Amplification of complete virion-encapsidated HBV genomes. HBV DNA isolated from 15 μl of serum was amplified 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 dNTP, 0.01% gelatine, 5 U of *Taq* DNA polymerase (Boehringer Mannheim), and 1 μM primer P1-P2 (Fig. 1A) or primer P3-P4. Alternatively, HBV DNA from 15 μl each of sera A and B was amplified by the Expand High Fidelity assay (Boehringer Mannheim) containing 1.5 mM $MgCl_2$, 200 μM dNTP, 2.6 U of *Taq* DNA-*Pwo* DNA polymerase mix, and 0.3 μM primer P1-P2 in 50 μl of buffer. The reactions were carried out as a "hot start" PCR. A 45-μl reaction premix was heated to 80°C, and 5 μl of enzyme was then added. The amplification was performed for the appropriate number of cycles with denaturation at 94°C for 40 s, annealing at 60°C for 1.5 min, and elongation at 68°C for 3 min with an increment of 2 min after each 10 cycles in a Robocycler (Stratagene) or with denaturation at 94°C for 40 s, annealing after shifting to 60°C in 50 s and

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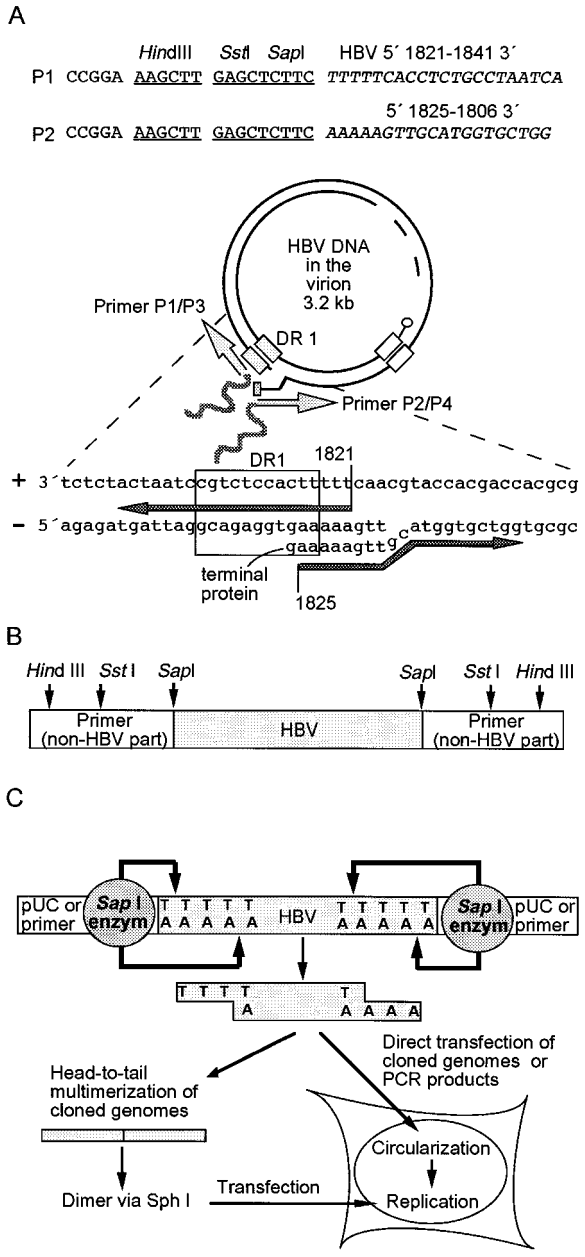

FIG. 1. Primers and the strategy of the method. (A) Sequences of the primers used for amplification of the whole HBV genome and primer binding sites at the triple-stranded region of the genome are shown. (B) Structure of the amplification product and restriction sites present in the non-HBV part of the primers which can be used for cloning of the PCR products. (C) Structure of the HBV genome which can be released from amplified HBV DNA directly or after cloning. The ligation of this genome allows the production of circular HBV DNA without nonhomologous sequences and without the need to introduce artificial mutations. Ligase-mediated polymerization will not result in head-to-head or tail-to-tail polymers but only in the production of head-to-tail HBV polymers because of the nonpalindromic sticky ends. From a dimer or polymer, a single-unit HBV genome can be released with any single-cutting restriction enzyme.

remaining at that temperature for 1 min, and elongation after shifting to 72°C in 15 s, and remaining at that temperature for 4 min with an increment of 5 s/cycle for elongation in a Perkin-Elmer thermocycler (48 wells).

Calculation of the error frequency. The error frequency (*f*), defined as the proportion of expected nucleotide errors in the amplified sequence, can be calculated from the error rate per nucleotide of the polymerase (*p*) and the number of effective amplification cycles (*n*) with the equation $f = n \times p/2$ (5). By substituting *n* with the equation $2^n = N_f/N_i$, where N_f is the estimated final copy number and N_i is the initial copy number, *f* was calculated as $\log(N_f/N_i) \times p/0.6$.

Cloning of PCR products. The PCR products generated with primers P1-P2 and HBV DNA from serum samples from patients 1 to 7 were purified by phenol-chloroform (1:1) extraction, precipitated, dissolved in water, and digested with *Sst*I for at least 6 h. Fragments 3.2 kb in length and fragments between 3.2 kb and 1 kb were recovered from the agarose gel and cloned separately into vector pUC19. The 3.2-kb fragments amplified with primers P3-P4 and HBV DNA from serum from patient 8 were gel purified, treated with T4 DNA polymerase, and cloned into vector pDIRECT (Clontech Inc.) according to the manufacturer's instructions.

Analysis of cloned HBV DNA by PCR and sequencing. One microliter of bacterial overnight culture was used to amplify the subgenomic fragments A to I (see Fig. 3, top) of the cloned HBV DNA in 25-μl assays with 0.1 μM primer combination P1-P2, P1-P11, P5-P12, P6-P13, P7-P14, P8-P15, P6-P15, P9-P16, P10-P2, or others not shown. The nucleotide positions of the primers (nomenclature is according to reference 9) on the HBV genome were as follows: P1 (1821 to 1841), CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTA ATCA; P2 (1823 to 1806), CCGGAAAGCTTGAGCTCTTCAAAAAGTTG CATGGTGCTGG; P3 (1825 to 1841), CTCGCTCGCCCAAATTTTCACC TCTGCCTAATCA; P4 (1823 to 1806), CTGGTTCGGCCCAAAAAGTTGC ATGGTGCTGG; P5 (1928 to 1950), GGAGCTACTGTGGAGTTACTCTC; P6 (2357 to 2380), GGCAGGTCCCCTAGAAGAAGAACT; P7 (2812 to 2832), GTGGGTCACCATATTCTTGGG; P8 (67 to 90), CTCCAGTTCAGGAACA GTAAACCC; P9 (634 to 656), ATTCCTATGGGAGTGGGCCTCAG; P10 (1266 to 1286), CCATACTGCGGAACTCCTAGC; P11 (2043 to 2021), CAAT GCTCAGGAGACTCTAAGGC; P12 (2400 to 2381), CTTCGTCTGCGAGG CGAGGG; P13 (2957 to 2935), TTGGGATTGAAGTCCCAATCTGG; P14 (202 to 179), CTGTAACACGAGAAGGGGTCCTAG; P15 (738 to 716), ATA ACTGAAAGCCAAACAGTGGG; P16 (1394 to 1372), GCAGCACAGCCTA GCAGCCATGG; P17 (2744 to 2767), CTATTTACACACTCTATGGAAGGC; P18 (3039 to 3061), GGGGTGGAGCCCTCAGGCTCAGG; P19 (2510 to 2487), AGGTACAGTAGAAGAATAAAGCCC.

Fragments smaller or larger than predicted from an HBV genome of subtype ayw (9) were partially sequenced with appropriate primers in the areas where deletions or insertions were previously roughly mapped by PCR and size analysis.

Plasmids containing the reference ayw HBV genome. Plasmids pMCH and pSM2 containing a HBV monomer and a head-to-tail dimer of subtype ayw (9), respectively, cloned via the *Eco*RI cleavage site, were used. The plasmid pHBV-SapI also contains an HBV genome of subtype ayw (9) cloned by the novel method described in this communication (Fig. 1) by amplification of the HBV DNA from plasmid pSM2. In this experiment PCR was run with *UlTma* DNA polymerase (Perkin-Elmer) for six cycles only, and 400 ng of the plasmid pSM2 was used as a template to minimize the frequency of artificially created mutations. The plasmid pHBV-SapI-(40 cyc.) has been constructed in the same way by amplifying 10 fg of linearized pSM2 through 40 cycles with *Taq* DNA polymerase, applying the standard conditions as given above. The HBV insert of both plasmids is therefore flanked by the primer sequences P1 and P2 (Fig. 1A).

Preparation of full-length ayw HBV DNA genomes by PCR for direct transfection. Full-length HBV genomes of subtype ayw (9) devoid of vector sequences were prepared with *Taq* DNA polymerase (25 cycles) in several 50-μl tubes with primer P1-P2 and pSM2 as a template. The DNA was pooled and washed five times by centrifugation in Centricon-100 concentrator (Amicon) to remove residual dNTPs and primers. The DNA was then purified by phenol-chloroform (1:1) extraction, precipitated with ethanol, and quantified.

Transfection of HBV DNA. All cloned DNAs were purified by the Qiagen procedure. Linear HBV monomers were released from plasmid pMCH by cleavage with *Eco*RI and from pHBV-SapI and pHBV-SapI-(40 cyc.) with 0.5 U of *Sap*I per μg of DNA (New England Biolabs) for at least 12 h. For direct transfection of PCR products the HBV genomes were cleaved with *Sap*I to release the heterologous primer sequences and to create linear HBV monomers with *Sap*I sticky ends. Subsequently, digested DNA was purified by phenol-chloroform (1:1) extraction and ethanol precipitation. Transfection of 10 μg of DNA was carried out according to the calcium phosphate-precipitation method. HuH7 cells were plated at a density of 1.3×10^6 cells per 60-mm-diameter petri dish. The medium was changed 1 day after transfection, and cells were harvested 4 days later. Each construct was transfected at least three times. HBsAg and HBeAg were assayed by using commercially available kits (Abbott).

Purification of HBV DNA from intracellular core particles. The cells were washed once with ice-cold phosphate-buffered saline and lysed in 500 μl of lysis buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% Nonidet P-40) per 60-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 14,000 rpm (Eppendorf 5415C centrifuge). 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 the addition of EDTA to a final concentration of 25 mM. Proteins were digested with 0.5 mg of proteinase K per ml and 1% SDS for 2 h at 37°C. Nucleic acids were purified by phenol-chloroform (1:1) extraction and ethanol precipitation after the addition of 20 μg of tRNA.

Purification of HBV DNA from extracellular viral particles. The cell culture medium was clarified by centrifugation at 8,000 rpm (Sorvall SS34 rotor) for 30

min. Four milliliters of medium was laid on top of a 1-ml 20% sucrose cushion in TNE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 100 mM NaCl) and centrifuged at 45,000 rpm (Beckman SW50.1 rotor) for 4 h. 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.

Southern blot analysis of HBV DNA. DNAs isolated from cytoplasmic core particles and from extracellular viral particles were separated on a 1.5% agarose gel, blotted onto Hybond N nylon membranes (Amersham), and hybridized with a ^{32}P-labelled gel-purified full-length HBV fragment (10^6 cpm/ml; specific activity 1.6×10^8 cpm/μg). The probe was generated with a random-primed labelling kit (Amersham). The signals were detected by autoradiography.

RESULTS

Methodological considerations. When devising our method we had three major aims in mind: first, the amplification of virion- or core particle-encapsidated DNA representative of the whole viral population should be efficient; second, it should be possible and simple to characterize the genomes on the structural level with or without prior cloning; and third, functional analysis of the genomes in eukaryotic cells should not depend on the production of a head-to-tail tandem HBV genome and should also be possible without prior cloning.

The unusual genomic structure of virion-encapsidated HBV DNA restricts selection of PCR primers when aiming at an efficient amplification of full-length HBV genomes. The main reason for this is that neither of the two strands of the HBV genome is made up of covalently linked circular molecules. When elongation goes beyond both discontinuities (the nick and gap region [Fig. 1A]), amplification is approximately 1,000-fold less efficient than that of subgenomic fragments without discontinuities (12). To circumvent this problem, we have chosen primers for PCR which are located at the nick region (Fig. 1A) and do not require extension beyond the nick. Fortunately, these primer binding sites are highly conserved in all HBV genotypes.

When the amplified genomes are to be obtained as a single molecule devoid of heterologous sequences but with compatible ends which allow circularization for functional analysis, there is only one restriction enzyme (*Sap*I) which can be chosen. *Sap*I is a class II-S restriction enzyme with the recognition and the cleavage site spatially separated (Fig. 1C). When this cleavage site is appropriately engineered at the nick region of HBV with additional heterologous primer sequences, an HBV genome with compatible sticky ends and devoid of heterologous sequences is obtained after *Sap*I cleavage. A *Sap*I site is present only in 2 of the 39 published full-length HBV genomes, and therefore amplified full-length HBV molecules will only very rarely be cut into subgenomic fragments. In addition, head-to-tail tandemerization of HBV genomes by ligation, which can be useful for functional analysis, is facilitated because of the nonpalindromic *Sap*I sticky ends.

We reasoned that the introduction of two additional restriction enzyme cleavage sites (*Hin*dIII and *Sst*I) into the amplification primers (Fig. 2B) would be most appropriate for efficient cloning. A *Hin*dIII site is present in only 4 and an *Sst*I site is present in only 2 of 39 known full-length HBV genomes available from the National Center for Biotechnology Information sequence database, and none of these genomes contain both sites. The likelihood of internally cutting an HBV genome with an unknown sequence by *Hin*dIII or *Sst*I is therefore very low.

Amplification of virion-derived HBV DNA. *Taq* DNA polymerase introduces point and frameshift mutations (5) into the amplified genomes, and this may interfere with a structural and functional analysis. The number of misincorporations can be dramatically reduced when a thermostable polymerase with proofreading activity is used alone or as a mixture with *Taq* DNA polymerase (2). Unfortunately, several of these polymerases (Vent DNA polymerase [New England Biolabs], *UlTma* DNA polymerase [Perkin-Elmer], and *Pfu* DNA polymerase [Stratagene]) were found to be at least 1,000-fold less efficient in amplification than *Taq* DNA polymerase (data not shown). Therefore, we used the Expand High Fidelity enzyme mix (Boehringer Mannheim) containing *Taq* and *Pwo* DNA polymerases which has the very low error rate of 8.3×10^{-6} as determined experimentally (8). Applying reaction conditions nearly identical to those of the fidelity assay (8), as little as 1 fg of virion HBV DNA, corresponding to approximately 300 viral genomes, resulted in a visible band of 3.2 kb on an ethidium bromide-stained gel when 40 cycles were performed (Fig. 2, lanes Taq + Pwo A3 and B3). The error frequency for both reactions (1 μg of estimated final reaction product) was calculated to be 1.2×10^{-4} or one error per 8.3 kb. Thus, even under the most extreme amplification conditions used here, the majority of HBV genomes should not have artificially introduced mutations.

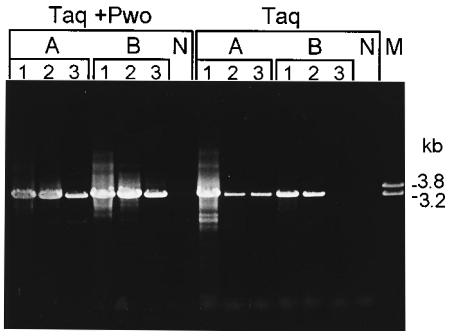

FIG. 2. Agarose gel analysis of HBV genomes amplified by the new method in a Robocycler by using 40 cycles. For the experiment 100, 10, and 1 fg of virion HBV DNA (lanes 1, 2, and 3, respectively) corresponding to approximately 30,000, 3,000, and 300 HBV genomes isolated from two serum samples (A and B) were amplified with *Taq* DNA polymerase alone (Taq) or in combination with *Pwo* DNA polymerase (Taq + Pwo). Lanes N, the negative control; lane M, DNA size markers.

The method was applied to amplify HBV DNA isolated from sera of eight patients with the primers shown in Fig. 1A. After 20 to 35 cycles of amplification (with *Taq* DNA polymerase, since proven high-fidelity PCR systems were not yet available at the beginning of the study) and separation of the products on agarose gels, a major 3.2-kb and several minor bands predominantly in the range between 1.5 and 2.2 kb, and similar to those seen for serum A could be visualized by ethidium bromide staining (Fig. 2, lane Taq A1).

Cloning of PCR products. For cloning, the PCR products were cut with *Sst*I in the heterologous primer sequences and separated on agarose gels, and the 3.2-kb fragments and fragments smaller than 3.2 kb were recovered by gel elution and cloned into vector pUC19. Cloning a small amount of the 3.2-kb PCR fragments derived from patients 1 to 7 yielded between 6 and 300 positive clones. Of 175 colonies screened, 122 contained a 3.2-kb insert as determined by PCR with primers P1 and P2 (five representative examples are shown in Fig. 3A, lanes 1 to 5). All 122 plasmids were analyzed in addition by an HBV core gene-specific PCR with primers P1-P19 or P1-P12 or by sequencing. All plasmids were found to contain HBV DNA inserts as evidenced by positive PCR assays or HBV-specific sequences (data not shown).

On a theoretical basis, up to 60,000 full-length genomic clones can thus be obtained from the material of a single PCR. We also wondered whether PCR products smaller than 3.2 kb

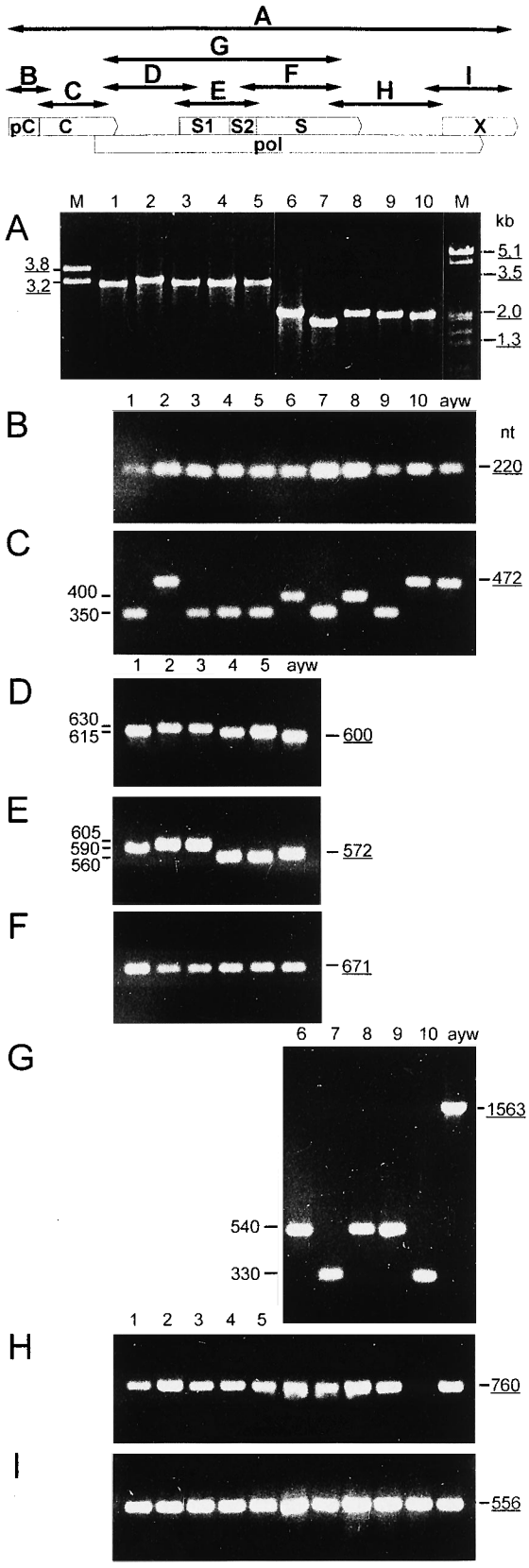

FIG. 3. Structural analysis of 10 cloned HBV genomes derived from kidney transplant patient 1. Subgenomic fragments of five genomes, approximately 3.2 kb in length (lanes 1 to 5), and of five smaller genomes about 2.0 kb in length (lanes 6 to 10) were amplified by PCR with nine different combinations of primers (A to I; positions on the genome are schematically shown at the top) and analyzed by agarose gel electrophoresis. All fragments of precisely known length are underlined (lanes M and ayw). In panels B to I, the underlined numbers indicate the size of the subgenomic fragments of the reference ayw HBV genome (9). The lengths of the shorter and longer fragments from the patient-derived HBV genomes were estimated by polygonal regression curves calculated from three neighboring bands of a DNA size marker (standard III, Boehringer Mannheim). No HBV DNA was amplified in panel H, lane 10, because of a deletion of the primer binding sequence. pC, pre-C region; C, C gene; S1, pre-S1 region; S2, pre-S2 region; pol, polymerase gene; S, S gene; X, X gene.

which were often visible on the gel correspond to HBV genomes with deletions or to PCR artifacts. Therefore, we also cloned PCR fragments smaller than 3.2 kb after elution from the gel (size fraction, 3.0 to 1.0 kb). This experiment yielded between 2 and 63 clones per sample. Of 59 plasmids tested from five patients by PCR with primers P1 and P2, 57 contained inserts ranging in length from 1.5 kb to 2.2 kb (representative examples are shown in Fig. 3A, lanes 6 to 10). Additional PCR analysis with core and S gene-specific primers revealed that all of these clones contain HBV DNA.

Structural analysis of the amplified and cloned viral genomes. Ten cloned genomes isolated from the immunosuppressed renal transplant patient 1, five of which were 3.2 kb in length and five of which were between 1.5 and 2.2 kb in length, were further analyzed by amplification of partially overlapping subgenomic fragments spanning the whole genome (the location of the fragments is schematically shown in Fig. 3, top). In addition, five genomes 3.2 kb in length from a nonimmunosuppressed carrier (patient 8) were tested accordingly. No deletions or insertions were found in the genomes of this patient, as identical fragment sizes were obtained with the HBV ayw reference genome (data not shown). However, one or more fragments slightly or substantially smaller or larger than predicted were observed with all HBV genomes from the renal transplant patient (Fig. 3C to E, and G). PCR was negative with one primer pair used for one of the genomes (Fig. 3H, lane 10) because of the deletion of the primer binding site. Taken together, these results suggested that all genomes contain deletions, insertions, or combinations of both.

To confirm and study these results in more detail, the corresponding regions of seven genomes were sequenced with primers P5, P6, P17, and P18 (for the location of these primers with respect to the HBV genome, see Fig. 4, top). Sequencing revealed that five of the seven genomes contain in-frame deletions in the middle of the core gene (Fig. 4, genomes 1, 4 to 6, and 9). In addition, in two of the deletion variants (Fig. 4, genomes 4 and 5) 30 nucleotides were deleted in the pre-S region, including the translational start codon for the pre-S2 envelope protein. Three of the genomes (Fig. 4, genomes 6, 9, and 10) showed large deletions of amino-terminal sequences of the P gene, including most or all of the pre-S sequences and, in one case, part of the HBs-coding region. The large deletions in genomes 6 and 9 correspond exactly to sequences removed in a spliced HBV transcript (4, 28). The deletion breakpoints in genome 10 do not show any similarity to splice donor and acceptor consensus sequences. One genome (Fig. 4, genome 2) had a 33-bp insert at the amino terminus of the pre-S1 region nearly identical to that first described for an HBV genome of adw subtype (25). A similar 33-bp insertion is also found in other non-ayw HBV genomes. Interestingly, a truncated version of this insert (18 nucleotides), so far not observed in any other full-length HBV genome, is present in three genomes (Fig. 4, genomes 1, 4, and 5). Taken together, these data show that most HBV genomes in the serum of this patient have deletions and/or insertions. The absence of these variants in

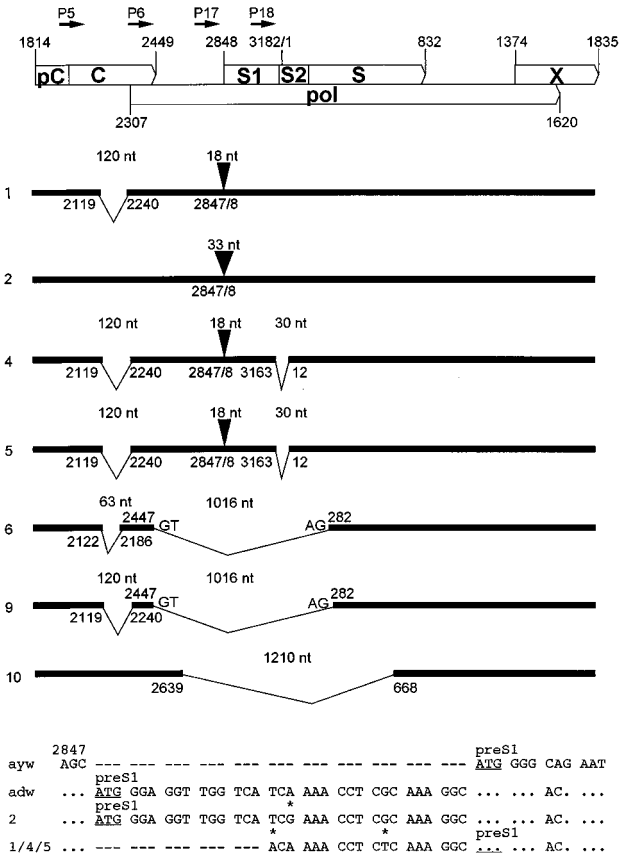

FIG. 4. Location of insertions and deletions in seven HBV genomes from virion DNA isolated from the serum of kidney transplant patient 1. After determining the approximate positions of insertions (arrowheads) and deletions (interruptions) by PCR with the primers shown in Fig. 3 and others not shown, the precise positions were defined by sequencing. Nucleotide positions flanking deletions and insertions are given according to the nomenclature of the HBV reference genome (9). Sequence insertions observed at the 5′ end of the pre-S region of HBV genomes 1, 2, 4, and 5 are listed at the bottom. For comparison, the sequences of the reference ayw genome (9) and an adw genome (25) are given. Dashed lines indicate sequences not present, dots represent identical sequences, stars pinpoint nucleotide changes, and potential or known translational start codons are underlined. Abbreviations are as defined in the legend to Fig. 3.

serum of another patient strongly argues against the theoretical possibility that these variants represent a PCR artifact.

PCR analysis of the HBc-coding region of cloned genomes isolated from five additional immunosuppressed renal transplant patients. In order to test whether HBV genomes with core gene deletions are commonly found in immunosuppressed patients, HBV DNA from sera of a further five immunosuppressed patients with end-stage liver cirrhosis was studied. A total of 105 amplified and cloned genomes isolated from patients 1 to 6 were analyzed by a core gene-specific PCR and fragment length analysis as shown in Fig. 4. In all six patients, between 13 and 100% of the genomes contained deletions in the HBc-coding region (Table 1). This analysis indicates that deletions in the core gene may be characteristic for immunosuppressed patients with liver cirrhosis.

Functional analysis of linear monomeric HBV genomes with *Sap*I sticky ends. So far, only monomeric linear full-length HBV genomes with *Eco*RI sticky ends or head-to-tail tandemerized HBV genomes were used for the transfection of eukaryotic cells and were shown to lead to virus production. If

TABLE 1. Analysis of 105 cloned HBV genomes isolated from the serum of six renal transplant patients

Patient	No. of HBV genomes[a]:	
	Analyzed	With core gene deletions (%)
1	20	19 (95)
2	18	12 (67)
3	18	4 (22)
4	20	16 (80)
5	23	3 (13)
6	6	6 (100)

[a] HBV genomes were cloned as described in Materials and Methods and analyzed by a core gene-specific PCR with primers P1-P19 or P1-P12 and by size determination of the PCR fragments on agarose gels.

monomeric HBV genomes with *Sap*I sticky ends generated by PCR and subsequent *Sap*I cleavage could be used as well, testing of the biological characteristics of the many HBV variants would be much facilitated. This would allow the testing of a heterogeneous mixture of amplified HBV genomes even without prior cloning and would not require recloning of the plasmid-integrated HBV inserts or tandemerization. To test this, human hepatoma HuH7 cells were transfected with monomeric HBV genomes created by PCR and digested with *Sap*I. In addition, cells were transfected with HBV genomes released by *Sap*I digestion from plasmids pHBV-SapI and pHBV-SapI-(40 cyc.) into which a HBV genome of subtype ayw (9) was previously cloned after amplification through 6 or 40 cycles, respectively, by the new method. For comparison and as a control, the same genome was transfected in linear monomeric form with *Eco*RI ends (released from plasmid pMCH by *Eco*RI digestion) and as a head-to-tail tandem in plasmid-integrated form (pSM2). For all these genomes, replicative intermediates from intracellular core particles and the viral antigens HBsAg and HBeAg in the cell culture supernatant were determined.

By Southern analysis, similar types and amounts of intracellular replicative intermediates were observed after transfection of these HBV genomes (Fig. 5). In all lanes a smear characteristic of HBV replicative intermediates was observed, extending from the position of single-stranded HBV DNA of minus-strand polarity (Fig. 5, position ss) up to the position of open circular HBV DNA (Fig. 5, position oc). Discrete bands for single-stranded and open circular HBV DNA were seen in some transfection experiments with *Eco*RI- or *Sap*I-generated monomeric HBV DNA (Fig. 5, lanes Eco-10, Eco-5, Sap-10, and Sap-5). For unknown reasons a substantial fraction of the input linear double-stranded HBV DNA remained intact, although the cytoplasmic fraction was intensively predigested with DNase I before HBV DNA extraction from core particles. The tandemerized ayw HBV genome in plasmid-integrated form (Fig. 5, lane pSM2) produced significantly more replicative intermediates than the monomeric forms of the same genome. Thus, tandemerization of HBV genomes may be required for HBV variants with severely reduced replication efficiency. Analysis of viral DNA in the cell culture supernatant of the HBV DNA-transfected cells (Fig. 6) showed full-length open circular and incomplete HBV genomes. This was evident for cells transfected with linear monomeric HBV genomes with *Sap*I and *Eco*RI ends and with the tandem of the ayw HBV genome (Fig. 6). In conclusion, all three replication-competent genomes led to the secretion of viral particles, and there was no significant difference between HBV genomes with *Sap*I or *Eco*RI sticky ends.

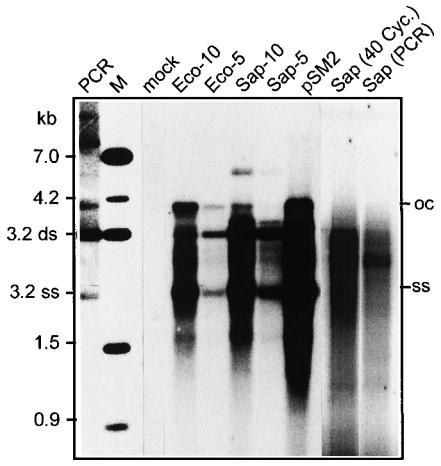

FIG. 5. Southern blot analysis of HBV replicative intermediates. Lanes correspond to DNA extracted from viral core particles derived from HuH7 cells which were transfected with linear monomeric HBV DNA with *Eco*RI or *Sap*I sticky ends released from 10 and 5 μg of pMCH (lanes Eco-10 and Eco-5), 10 and 5 μg of pHBV-SapI (lanes Sap-10 and Sap-5), 10 μg of pHBV-SapI-(40 cyc.) [lane Sap (40 Cyc.)], 10 μg of PCR products [lane Sap (PCR)], 10 μg of circular dimeric HBV DNA (lane pSM2), and 10 μg of calf thymus DNA as a mock control (lane mock). The lane denoted PCR was loaded with DNA generated by asymmetric PCR with primer P1-P2 (1:25) and pHBV-SapI as a template. In this lane the bands correspond from top to bottom to open circular, linear, and covalently closed circular forms of the plasmid, amplified double-stranded (ds) HBV DNA, and amplified single-stranded (3.2 ss) minus-strand HBV DNA. The positions of the open circular (oc) and the single-stranded (ss) forms of the HBV DNA genome are indicated. A minor band in lane Sap-10 at a position of approximately 6.0 kb corresponds to linear pHBV-SapI plasmid. Lane M, DNA size markers.

A determination of the amounts of HBsAg and HBeAg secreted into the culture medium of the transfected cells revealed similar levels of both antigens expressed from HBV genomes with *Sap*I ends or *Eco*RI ends and whether PCR-generated or plasmid-derived HBV DNA was used (Table 2). The measurement of HBeAg in cell culture supernatants after ultracentrifugation revealed a reduction in the amount of antigenic material by more than 50%. Therefore, approximately half of the immunoreactive material probably represents core particles that were released from the cells. This is also consistent with the type of replicative intermediates identified in viral particles pelleted from the supernatant (Fig. 6). Compared with the monomeric HBV genomes, similar amounts of HBsAg and HBeAg were found in the culture medium from cells transfected with the dimeric form of the HBV ayw genome (Table 2), although significantly higher levels of viral particle-encapsidated DNA were seen by Southern blotting (Fig. 5 and 6).

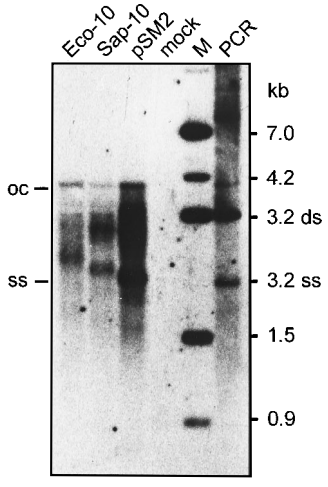

FIG. 6. Southern blot analysis of HBV DNA derived from virions and core particles released from HuH7 cells transfected with 10 μg of *Eco*RI-linearized pMCH (lane Eco-10), 10 μg of *Sap*I-digested pHBV-SapI (lane Sap-10), 10 μg of HBV dimer pSM2 (lane pSM2), and 10 μg of calf thymus DNA (lane mock). The lanes denoted with PCR and M were loaded with DNA as described in the legend to Fig. 5. Abbreviations are as defined in the legend to Fig. 5.

Three major conclusions can be drawn from these experiments. First, linear monomeric HBV genomes with *Sap*I ends can initiate a full replication cycle and lead to viral antigen expression as efficiently as those with *Eco*RI ends. Second, PCR-generated HBV genomes with *Sap*I ends can be used directly for transfection with or without prior cloning. Third, tandemerized HBV genomes may be more efficient in the production of replicative intermediates and the secretion of viral DNA-containing particles but are similarly efficient in the production of secreted viral proteins.

DISCUSSION

A novel method was described which allows sensitive amplification and cloning of full-length virion-encapsidated HBV genomes. Evidence was presented which indicates that the biological function of these genomes can be tested by transfection with or without prior cloning and does not require tandemerization. By this method a large number of full-length HBV genomes was cloned from eight serum samples, and two size classes of viral genomes, approximately 3.2 and 1.5 to 2.2 kb in length, were observed. Detailed analysis of some of these genomes obtained from serum of an immunosuppressed patient revealed deletions and insertions in almost all genomes. Deletions in the C gene appear to be characteristic for a substantial fraction of HBV genomes in immunosuppressed patients with end-stage liver cirrhosis. These data demonstrate the great potential of the new method for the structural and functional analysis of heterogeneous HBV populations.

A prerequisite for an evaluation of the role of HBV variants for the establishment of chronic infection, immune escape, and pathogenesis is a detailed knowledge of the complexity of HBV sequence heterogeneity. With currently available techniques sequence heterogeneity in subgenomic regions can be determined, but the combinations of mutations on single full-length genomic molecules cannot. Reconstitution of full-length genomes from amplified subgenomic fragments by cloning is very tedious and may result in the generation of HBV variants which do not exist in vivo. Conventional cloning and sequencing of full-length HBV genomes can only be achieved with relatively high-titered sera. HBV DNA which does not contain the appropriate restriction cleavage site and genomes from defective viruses with only single-stranded DNA will be cloned less efficiently or not at all. The method described here circumvents most of these problems. A large number of full-length HBV genomes can be isolated and cloned even from very low-titered sera independently of the restriction sites present on the genome, and the reconstitution of subgenomic fragments is not required. The large number of genomes that can thus be obtained provides a unique opportunity to uncover the combination of mutations in many individual genomic molecules and to determine the complexity of complete virion-encapsidated functional and defective molecules. However, specific HBV variants may also escape detection when the novel method described here is used. All genomes which have

TABLE 2. HBsAg and HBeAg secreted by transfected cells

Transfected DNA[a]		Level of HBsAg by EIA (OD_{492}) at dilution[b]:		HBeAg by RIA (cpm)[c]
		1:10	1:100	
Type	Amt (μg) in first expt (second expt)	First expt (second expt)	First expt (second expt)	First expt (second expt)
Eco	10 (10)	>2.20 (>2.20)	0.50 (0.43)	17,519 (6,879)
Eco[d]	10	0.04	0.01	7,757
Eco	5	1.52	0.17	9,646
Sap	10 (10)	>2.20 (>2.20)	0.40 (0.69)	18,451 (9,365)
Sap[d]	10	0.02	0.03	8,554
Sap	5	1.04	0.06	7,732
pSM2	10 (10)	>2.20 (>2.20)	0.20 (0.53)	16,502 (7,581)
pSM2[d]	10	0.02	0.01	8,168
Sap (40 cyc.)	6 (10)	>2.20 (>2.20)	0.99 (1.43)	16,560 (15,324)
Sap (PCR)	6 (10)	>2.20 (>2.20)	0.34 (0.84)	11,444 (8,933)
Sap (PCR)	6	>2.20	0.50	10,200
Mock	10 (10)	0.02 (0.03)	0.01 (0.03)	403 (357)

[a] The designations of the transfected DNAs are identical to and explained in the legend to Fig. 5. All DNAs were transfected in two separate experiments.
[b] Levels of HBsAg secreted from transfected HuH7 cells were detected by enzyme immunoassay (EIA) at an optical density at 492 nm (OD_{492}).
[c] Levels of HBeAg secreted from transfected HuH7 cells were detected by radioimmunoassay (RIA).
[d] The cell culture media were cleared by ultracentrifugation to differentiate between core particle-associated HBe and HBc antigenity and nonparticulate HBeAg.

mutations in the sequences of PCR primer binding sites will not or much less efficiently be amplified. Since only 3 of 39 full-length HBV sequences in the National Center for Biotechnology Information sequence data bank contained up to three nucleotide mismatches, this is a minor problem which can be circumvented by the use of appropriate primers. The same is true for HBV variants with point mutations in the translational start codon of the pre-C region located at the nick region (10, 18, 19). The only way to prevent the escape of variants with mutations in the primer binding site is to amplify and sequence a corresponding subfragment with appropriate primers. For the HBV DNA in the few serum samples studied here we have done this and have not found evidence for mutation in this region (data not shown). However, even if all the possible point mutations are taken into account with different PCR primers, some variants with deletions in the nick region extending into the X gene (for a review, see reference 13) cannot be amplified with the set of primers described here.

Another potential problem of the new method concerns artificial mutations which are introduced during the PCR. The frequency of the misincorporation of nucleotides by *Taq* DNA polymerase is influenced by many parameters (5). We have taken this into account by optimization of the reaction conditions. For example, the number of misincorporations was kept low by determining experimentally for each DNA sample the minimum number of cycles needed for the amplification of full-length genomic HBV DNA and by using very low concentrations of free Mg^{2+} (0.7 mM). A genome cloned after 40 cycles of amplification by *Taq* DNA polymerase [pHBV-SapI-(40 cyc.)], as well as a pool of HBV genomes amplified for 25 cycles, was shown to produce levels of viral antigens and replicative intermediates similar to those produced by conventionally prepared HBV DNA. Thus, the few mutations presumably introduced by *Taq* DNA polymerase did not detectably interfere with the biological function of these amplified genomes.

To further decrease the number of PCR-mediated artificial mutations we applied very recently described new PCR conditions which combine the high amplification efficiency of *Taq* DNA polymerase with the low error rate of a thermostable polymerase with proofreading activity (2, 8). Using this system, we have achieved an even higher sensitivity of the amplification reaction than with *Taq* polymerase alone. From the experimentally determined error rate of this system (8), statistically only one mutation is expected in one of three HBV genomes amplified from a few hundred template molecules.

The potential of the new method to characterize the structure of virion-encapsidated HBV genomes by a simple PCR assay was demonstrated for immunosuppressed renal transplant patients. Interestingly, almost all viral genomes (19 of 20 genomes tested) in the serum of one of the patients studied in most detail contained deletions in the core gene. In addition, the high viremia in this patient and the fact that all the C gene deletions are in frame may indicate that these deletions do not interfere with viral production or even enhance virus production. This hypothesis can now be tested experimentally. Analysis of a large number of genomes from five additional immunosuppressed patients showed that C gene deletions may be characteristic for this subgroup of HBV carriers. A detailed analysis of the clinical course of these patients revealed end-stage liver cirrhosis with a bad prognosis. Interestingly, similar C gene deletions have so far been found mainly in patients with chronic active hepatitis (1, 21, 26). It was therefore speculated that core proteins with deletions may alter the immune recognition and be important for T-cell-mediated liver damage. Since our patients were under continuous immunosuppressive therapy, immune-mediated selection of the C gene deletion variants and a role in immunopathogenesis are unlikely. Our observations and the severe courses of chronic hepatitis associated with liver cirrhosis observed in HBV-infected renal transplant patients in general (17) are more compatible with the assumption of a direct cytopathic effect of these mutants. One selection mechanism for these variants which would be independent from the immune system could be an enhanced P protein expression by C gene deletion variants, which could result in an increased viral production. This is conceivable because the J and C AUGs, which both hamper P protein translation (7), are not functional in the C gene deletion mutants, whereas the translation initiation codon for the P protein is not affected.

Another interesting finding of our study is the high proportion of HBV genomes in the size range between 1.5 to 2.2 kb in all serum samples of the patients studied. According to the intensity of the staining signals and the number of clones obtained, they can represent a substantial fraction of HBV ge-

nomes in sera. PCR and sequence analysis revealed for five of these genomes large deletions spanning sequences from the C-terminal end of the C gene up to the middle of the S gene. The larger deletions in genomes 6 and 9 (Fig. 4) are reminiscent of previous studies which showed similar genomes created by encapsidation and reverse transcription of spliced pregenomic RNAs (22). In these cases, sequences between positions 2447 and 489 were spliced. In addition, pregenomic transcripts with a double splice removing sequences 2447 to 282 and 2067 to 2350 of the C gene were described (4, 28). In genomes 6 and 9, the lack of sequence 2447 to 282 is presumably due to the first of these splicing events. The second splicing event within the C gene did not take place in genomes 6 and 9, possibly because of a partial deletion of the intron sequences by other mechanisms. Alternatively, our observation may also indicate the existence of pregenomic mRNA with a single splice removing nucleotides 2447 to 282.

The newly developed method will greatly facilitate isolation and functional analysis of new HBV variants and will allow the study of interactions and selection mechanisms even in very heterogeneous viral genome populations.

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