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Genotype-Specific Synthesis and Secretion of Spliced Hepatitis B Virus Genomes in Hepatoma Cells

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Hepatitis B virus-infected patients frequently have viral particles with DNA derived from differently spliced RNA. Which factors influence the synthesis of these splice variants is unclear. We analysed the type of splice variants produced from different genotypes and determined whether they are secreted as efficiently as wild-type virus. We demonstrate production of a single splice variant from genotypes D, C, and E as dominant species in two hepatoma cell lines. The type of minor splice variants synthesised varied between genotypes but was identical in both hepatoma cell lines. A novel splice variant with a deletion in the core gene was identified for genotype D. Viral DNA from intracellular compared with extracellular viral particles was spliced approximately five times more often than wild-type-sized genomes. A variable amount of the major splice variant was also identified in sera from patients infected with genotypes A, D, and C. These data indicate genotype A-, C-, D-, and E- as well as hepatoma cell line-independent synthesis of a dominant single splice variant and argue for a biological function of the corresponding splice sites. This study clearly demonstrates the intracellular accumulation of viral particles containing spliced genomes and offers a tool for the investigation of underlying mechanisms.

INTRODUCTION

Substantial sequence heterogeneity of hepatitis B virus (HBV) genomes in viral particles isolated from sera and livers of infected patients becomes increasingly evident in recent studies (reviewed in Günther *et al.,* 1999). The sequence heterogeneity of HBV genomes is mainly based on the fact that synthesis of the partially double-stranded circular HBV DNA genome involves reverse transcription of an RNA pregenome that is mediated by the virus-encoded P protein (reviewed in Ganem, 1996; Nassal and Schaller, 1996; Nassal, 1999). Because reverse transcriptases lack proofreading activity and have an intrinsic property for template switches, point mutations and deletions/insertions are often created during this process. The sequence heterogeneity of HBV full-length genomes led to the classification of HBV into six different genotypes named genotype A to F with distinct prevalences in different geographical regions (reviewed in Pult *et al.,* 1998). The intergroup sequence divergence of the different HBV genotypes varies between about 8% and 14%, whereas the intragroup divergence is considerably smaller (about 1–4%) (Norder *et al.,* 1994). Whether the sequence heterogeneity of different HBV genotypes influences the viral life cycle or the outcome of an HBV infection is currently under investigation (Mayerat *et al.,* 1999). Genotype-independent point mutations, insertions, and deletions were identified in a large variety of so-called variants, and some of them were shown or are believed to be associated with a specific course of infection, vaccine, and antiviral therapy failure (reviewed in Ngui *et al.,* 1999; Günther *et al.,* 1999; Pult *et al.,* 1998).

One class of HBV genome variants, the so-called splice variants, derives from encapsidation and reverse transcription of spliced RNA pregenomes. Such variants were isolated from sera and liver biopsies of chronically infected patients as well as from transfected hepatoma cells (Terre *et al.,* 1991; Rosmorduc *et al.,* 1995; Günther *et al.,* 1997). The presence of a specific splice variant was reported to be closely associated with the chronic course of HBV infection and viral multiplication in vivo (Rosmorduc *et al.,* 1995). However, analysis of viral genomes with mutations in two frequently used splice sites revealed no effect on viral replication in transfected hepatoma cell lines (Su *et al.,* 1989; Wu *et al.,* 1991). Although splicing of the HBV pregenome appears not to be essential for viral replication *in vitro,* other aspects of the viral life cycle may be affected but have not been analysed so far. In fact, a spliced mRNA of the HBV-related duck hepatitis B virus (DHBV) has recently been shown to be functionally important for virus replication in infected primary duck hepatocytes and ducks but not for virus formation in cells transfected with viral genomes (Obert *et al.,* 1996). In contrast to HBV, in DHBV splicing appears to be much less complex because only two spliced RNAs were found so far (Obert *et al.,* 1996). In addition, none of these mRNAs are encapsidated and reverse transcribed due to the deletion of one or both known encapsidation signals on the pregenomes by these splicing events (Obert *et al.,* 1996). Therefore, it is questionable whether

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splicing has the same meaning in DHBV and HBV. For HBV, 11 smaller than full-length genomes with single, double, and triple splice deletions were isolated and characterized so far from viral particles of sera from chronically infected patients (Terre *et al.,* 1991; Rosmorduc *et al.,* 1995; Günther *et al.,* 1997). In addition, for the woodchuck hepatitis B virus (WHV), two differently spliced genomes were found in sera and livers of chronically infected woodchucks (Hantz *et al.,* 1992; Ogston *et al.,* 1992).

An argument for the possible biological importance of splicing in HBV is the conservation of eight of the nine known functional splice donor and acceptor sites in all known HBV genotypes (Günther *et al.,* 1997). However, the prevalence of viral particles with spliced genomes appears to vary from patient to patient (Günther *et al.,* 1997; Rosmorduc *et al.,* 1995). In addition, the ratio of splice variants to wild-type virus in sera and livers of HBV-infected patients as well as in WHV-infected woodchucks seems to be variable and to be higher in livers than in sera (Terre *et al.,* 1991; Hantz *et al.,* 1992). Whether the variable amounts of splice variants are due to host- and/or virus-specific factors is an open question. To address this question, we investigated *in vitro* under defined conditions which types of splice variants are synthesised from different genotypes and whether viral particles containing spliced genomes are as efficiently secreted as those containing wild-type genomes. To study the possible effects of host factors on HBV splice variant production, we representatively used two hepatoma cell lines that differ in both the genetic make-up and differentiation and analysed sera from patients infected with different genotypes.

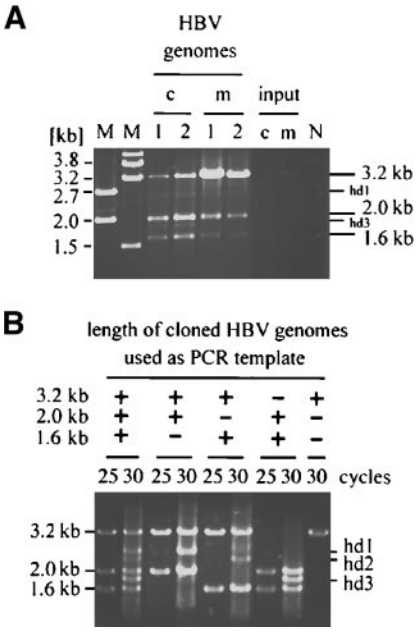

FIG. 1. (A) Gel electrophoretic analysis of HBV genomes derived from Huh7 cells transfected with plasmid pSM2, genotype D. Viral DNA isolated from viral nucleocapsids of the cytoplasm (c) or from virions of the cell culture medium (m) were amplified with primer pair P1/P2 for 25 cycles, separated in an agarose gel, and stained by ethidium bromide. Transfection was performed in doublets (lanes c1, c2, and m1, m2) and in independent experiments (data not shown). Input-DNA contamination was controlled by transfection with a replication incompetent HBV monomer containing construct (plasmid pHBV1). Lane M, DNA size marker; lane N, mock transfection with herring-sperm DNA. (B) Creation of HBV DNA heteroduplex molecules (marked as hd1, hd2, and hd3) by coamplification of wild-type length genomes and spliced genomes. Amplification was performed with primer pair P1/P2 for 25 and 30 cycles. Amplification products were separated in an agarose gel and stained by ethidium bromide.

RESULTS

Genome size analysis of HBV populations derived from genotype D-transfected Huh7 cells

To analyse the size heterogeneity of HBV genome populations that arises from a defined HBV genome sequence, Huh7 cells were transfected with HBV genotype D genomes. After transfection, the viral DNA was isolated from both viral nucleocapsids of the cytoplasm and viral particles released into the cell culture medium. Thereafter, a full-length HBV genome amplification of the DNA was performed using PCR primers that anneal to the nick region of the HBV genome as described previously (Günther *et al.,* 1995). The amplification products were electrophoretically separated in an agarose gel and stained by ethidium bromide. As expected, amplified HBV genomes of 3.2-kb wild-type length were detected in both the cytoplasm and the culture medium (Fig. 1A, lanes HBV genomes, c and m). In addition to the HBV genomes of wild-type length, four amplification products with a higher electrophoretic mobility were visible (Fig. 1A, lanes HBV genomes, c and m). Cloning and sequencing of these amplification products revealed that the major amplification products were 2.0 and 1.6 kb long, respectively; each represents defective HBV genomes containing single large internal deletions derived from spliced and reverse transcribed RNA pregenomes (Fig. 2, genomes designated sp1 and sp2). The additional shorter amplification products with a higher electrophoretic mobility than wild-type length were refractory to cloning (Fig. 1A, marked as hd1 and hd3). These bands are created artificially by heteroduplex (hd) formation of full-length and sp1 and sp2 HBV DNA, respectively, as shown by coamplification of the corresponding cloned HBV DNAs (Fig. 1B, 30 cycles, hd1, hd2, and hd3). Note that these heteroduplex molecules become increasingly visible after extensive amplification (Fig. 1B, compare lanes 25 and 30 cycles). Amplification of cloned HBV genomes of 3.2 kb did not result in bands corresponding to heteroduplex formation, which excludes the possibility that genomes with deletions were created artificially during PCR (Fig. 1B). Taken together, these data demonstrate that the dominant type of HBV genomes in viral particles from cytoplasm and culture medium of genotype D-transfected Huh7 cells are 3.2, 2.0, and 1.6 kb in size.

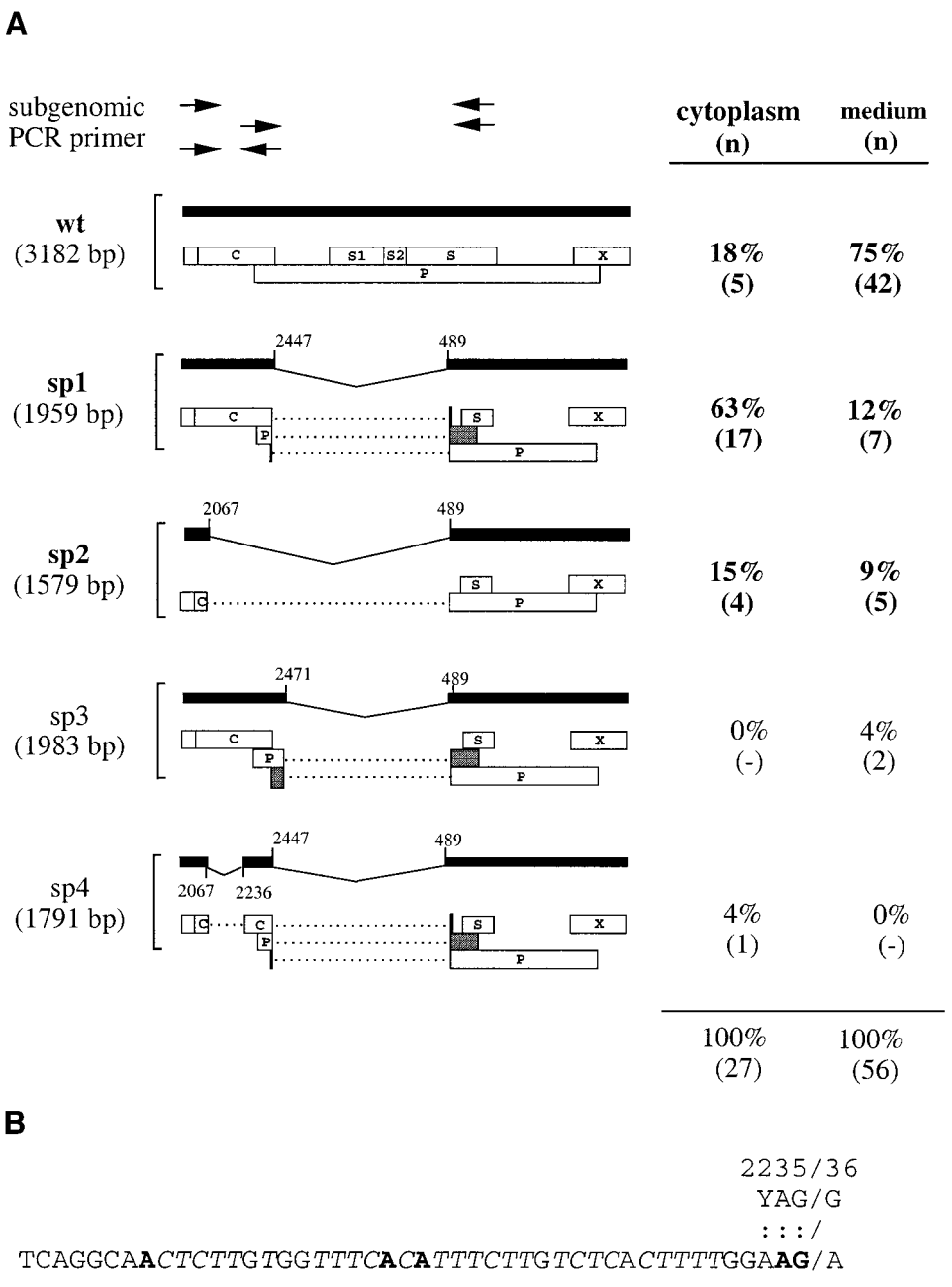

FIG. 2. (A) Structure and length of HBV genomes synthesized in Huh7 cells transfected by plasmid pSM2, genotype D. Wild-type length (wt) and spliced (sp1 to sp4) genomes isolated from viral nucleocapsids of the cytoplasm or secreted virions of the cell culture medium. Nucleotide positions are given for the exon boundaries. Known viral protein coding regions in each type of genome are depicted by capital letters (C, S1, S2, S, P, and X), whereas open reading frames of unknown significance are gray. The percentage of the five different HBV genomes in the total number of HBV clones (n) obtained from viral particles of the cytoplasm and culture medium is given. (B) Sequence of the new HBV splice acceptor site at position 2236 and the corresponding putative branch sites and pyrimidine stretch potentially regulating its function. Letters in bold denote invariable nucleotides in the consensus sequences for the splice acceptor (YAG/G) and putative branch sites (YNYYRAY) (Mount, 1982; Zeitlin and Efstratiadis, 1984; Jackson, 1991). Nucleotides of the polypyrimidine stretch are in italics.

Structural analysis by cloning and sequencing of HBV genomes derived from genotype D-transfected Huh7 cells

To characterize the amplification products, a detailed structural analysis was performed by cloning of the entire amplified viral DNA obtained from the cytoplasm and the culture medium. The HBV DNA length of all recombinants received was then first determined by PCR screening using the full-length HBV genome primers. Thus 27 and 56 recombinants obtained with the cytoplasm- and culture medium-derived viral DNA, respectively, were analysed. This screening revealed that 5 of 27 (18%, cytoplasm) and 42 of 56 (75%, medium) plasmids contained full-length HBV genomes (Fig. 2A), whereas

the remaining recombinants all contained HBV DNA smaller than wild-type size (Fig. 2A). The latter type of genomes were further analysed by subgenomic PCRs using primer pairs binding outside of previously described intronic sequences (Günther *et al.,* 1997, and references therein) of the HBV genome (Fig. 2A, top). This PCR screening indicated four types of HBV genomes smaller than wild-type length that all have deletions upstream of nucleotide position 690 (data not shown). To precisely identify the type and location of the deletions in these defective genomes, a representative recombinant of each size class was characterized by sequencing. The sequence analysis revealed that all four classes of smaller-than-full-length HBV genomes are derived from spliced and reverse-transcribed HBV pregenomic RNAs (Fig. 2A, spliced genomes, sp1 to sp4). Three of the four genome classes (sp1 to sp3) had a single splice deletion, and one (sp4) had two splice deletions. Spliced genomes sp1 to sp3 have been previously isolated from sera of patients (Terre *et al.,* 1991; Rosmorduc *et al.,* 1995; Günther *et al.,* 1997). The corresponding splicing events involve splice donor sites at positions 2067, 2447, and 2471 and the common splice acceptor site at position 489 (Fig. 2A). No equivalent of splice genome sp4 has so far been identified in the sera or livers of infected patients. One of the deletions in spliced genome sp4 is identical to that of genome sp1. The other splice deletion is due to the use of a novel splice acceptor site at position 2236 and of the identical splice donor site used by spliced genome sp2 (Figs. 2A, sp4, and 2B). This splicing event results in a 56-amino-acid in-frame deletion of the viral core protein and leaves the polymerase protein coding region untouched.

From the number and type of recombinants analysed, it appears that the ratio of full-length and spliced genomes isolated from intracellular and extracellular viral particles is drastically different. Provided the type of genomes obtained by amplification and cloning fairly accurately reflects the ratio of their presence in the intracellular and extracellular viral population, the following conclusion can be made. The two major singly spliced genomes sp1 and sp2 are more prevalent in the viral particles isolated from the cytoplasm than are those from the culture medium, whereas the reverse is true for the full-length HBV genomes (Fig. 2A). Accordingly, the major spliced genomes appear to represent about 20% of the genome population isolated from the culture medium and about 80% of the cytoplasmatic genome population (Fig. 2A). In contrast, spliced genomes sp3 and sp4 appear to be very rarely synthesized. A conclusion about their absolute prevalence and possible differences between cytoplasm and cell culture medium is not possible because of the few clones obtained.

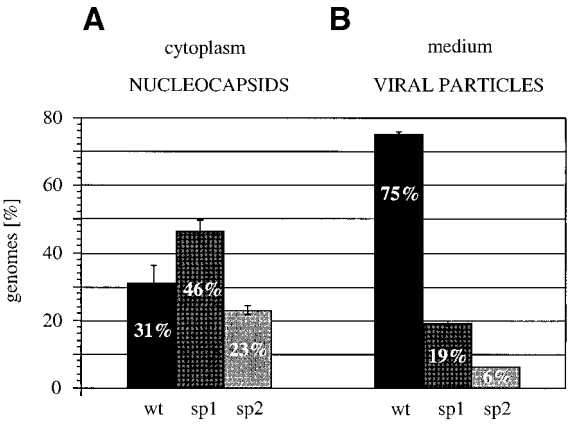

FIG. 3. Ratio of wild-type length (wt) and the predominant spliced genomes (sp1 and sp2) isolated from viral particles from cytoplasm and cell culture medium of genotype D transfected Huh7 cells. The signal intensity of the 3.2-, 2.0-, and 1.6-kb ethidium bromide-stained bands in Fig. 1A was determined densitometrically, and the corresponding percentage of the different viral genomes was calculated by setting the total HBV genome population to 100%.

Determination of the ratio between wild-type length and spliced HBV genomes by semiquantitative analysis

A higher prevalence of smaller-than-full-length HBV genomes in infected liver compared with the serum of individual patients was reported previously (Sommer *et al.,* 1997) and is suggested by studies by Terre *et al.* (see Fig. 3 in Terre *et al.,* 1991). These findings, as well as our semiquantitative results obtained by characterisation of the recombinants (Fig. 2A), prompted us to analyse whether spliced genomes are indeed more prevalent in viral particles isolated from the cytoplasm of transfected Huh7 cells. This should be evident in a semiquantitative analysis of amplification products after separation by agarose gel electrophoresis and ethidium bromide staining (Fig. 1A). This type of analysis is meaningful because we used a high concentration of template molecules, leading to reproducible banding patterns, a minimum number of amplification cycles, and a *Taq*/*Pwo*-DNA-polymerase mixture. All of these safeguards guarantee similar amplification efficiency of HBV genomes that vary in size as experimentally determined previously through the use of defined mixtures of cloned wild-type and spliced genomes as templates (Sommer *et al.,* 1997). With densitometric scanning of the corresponding ethidium bromide-stained bands, we found that the total HBV DNA population in the cytoplasm consisted of about 31% full-length HBV genomes, of 46% sp1 genomes, and of 23% sp2 genomes (Fig. 3A). In contrast, in the culture medium, the full-length genomes represented 75%, the sp1 genomes represented 19%, and the sp2 genomes represented 6% of the total genome population (Fig. 3B). These values are similar to those deduced from the type and number of HBV recombinants (see above). Amplifi-

cation of HBV DNA from cytoplasmic and cell culture-derived particles obtained by transfection of a replication-incompetent wild-type-length HBV genome resulted only in a very weak 3.2-kb band when using DNA from the culture medium (Fig. 1A). This indicates that very little input DNA from the culture medium and none from the cytoplasm are amplified, suggesting a rather accurate evaluation of the ratios of newly produced viral DNA in the cytoplasm and culture medium by the method used. Taken together, our semiquantitative analysis of amplification products on agarose gels and of recombinants revealed an approximately five times higher ratio of wild type length to singly spliced genomes in extracellular versus intracellular viral populations. Therefore, it appears that nucleocapsids containing wild-type-length genomes are preferentially enveloped or released from the cells, whereas those containing spliced genomes accumulate in the cytoplasm.

Analysis of HBV genotype- and cell line-dependent size heterogeneity of viral genome populations

To analyse whether viral genomes of different genotypes and/or host factors such as the genetics and differentiation status of hepatoma cell lines influence the size heterogeneity and splicing pattern of HBV genomes, we transfected Huh7 and HepG2 cells with viral genomes of genotypes D, C, and E. The three genotypes that we tested diverge by sequence by more than 8% (genotype D to E, 8.4%; D to C, 10.9%; and C to E, 11.0%). After transfection of Huh7 and HepG2 cells with genomes of genotype E, C, and D, respectively, the corresponding *de novo* produced HBV genome populations were isolated from the intracellular viral nucleocapsids. Amplification with the full-length genome primers and analysis of the products on agarose gels showed similar ratios of full-length and sp1 genomes obtained from both cell lines and with all three genotypes. However, there were clear differences in bands corresponding to genomes smaller than spliced genome sp1 (data not shown). These differences were identical and reproducible in both cell lines (data not shown), arguing that they are due to genotype-specific sequence differences but not to cell type-specific factors. To analyse the type of the genotype-dependent genomes smaller than spliced genome sp1, we performed subgenomic PCRs with primers that should detect all types of known spliced genomes (Fig. 4, top). As expected, each of the three HBV genotypes resulted in a specific and slightly different banding pattern. This pattern did not differ between both cell lines (Fig. 4A), confirming genotype-specific genome size differences independent of the cell line used. The use of primers P3 and P4, which amplify DNA fragments from all known spliced genomes, consistently amplified a fragment of 829 bp. This corresponds to spliced genome sp1 as indicated by the comigration of the product amplified from a cloned sp1 genome (Fig. 4A, lane sp1). Because spliced genome sp3 differs from sp1 by only 24 nucleotides, this was confirmed by an additional subgenomic PCR with primers P4 and P5 (Fig. 4B, lanes sp1 and sp3). Primers P3 and P4 resulted in amplification products of 449 bp when using DNA from genotype E and D but not from genotype C. This fragment corresponds to spliced genome sp2 as evident from comigration of the same fragments amplified from a cloned sp2 genome (Fig. 4A, lane sp2). The use of primers P3 and P6, which resulted with genotype D-derived DNA only in products with the size predicted for genomes without a

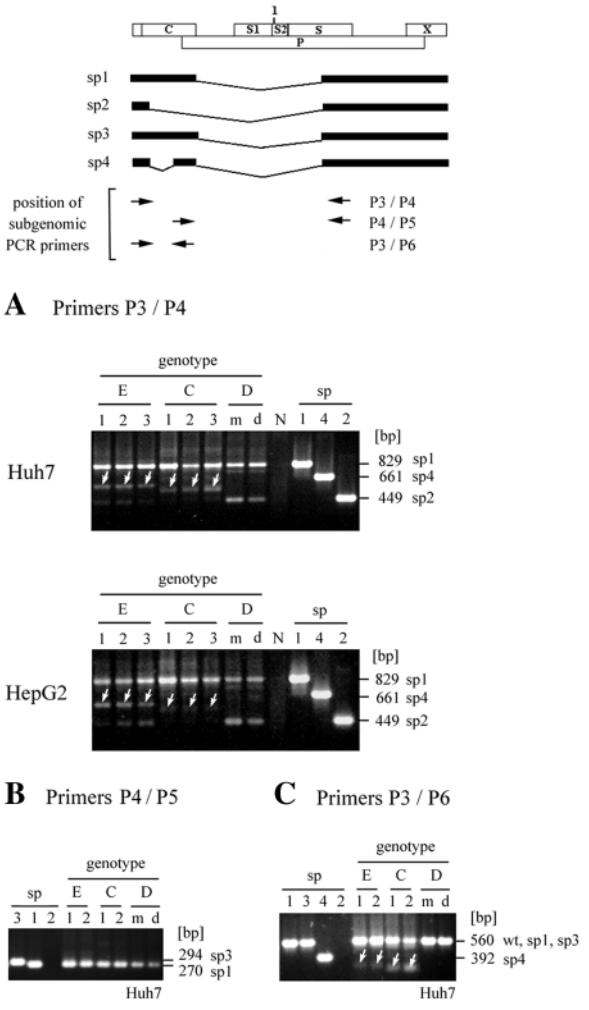

FIG. 4. Agarose gel analysis of smaller-than-full-length genomes produced by genotypes E, C, and D and amplified by subgenomic PCRs. Top, Schematic outline of the structure of the four spliced genomes identified for genotype D and type of primer pairs used for amplification. (A–C) Amplification product obtained with the various primer pairs and DNA present in Huh7 and HepG2 cells transfected (Fugene Reagent) with three different genomes (lanes 1–3) of genotypes E and C or monomeric (m) and dimeric (d) genomes of genotype D. Lanes sp denote amplification products obtained with cloned spliced genomes sp1 to sp4. DNA fragments that probably correspond to splice variants of unknown structure are marked by arrows. In lanes N, amplification products obtained with DNA from mock-transfected cells (herring-sperm DNA) were loaded.

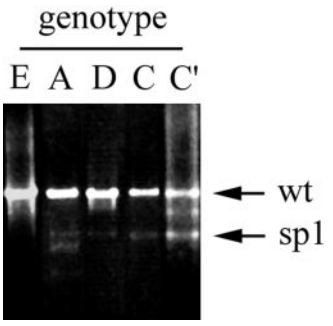

FIG. 5. Gel electrophoretic analysis of HBV genomes derived from patients chronically infected with different HBV genotypes. Viral DNA isolated from patient sera was amplified with primer P1/2, separated in an agarose gel, and stained by ethidium bromide. Lanes loaded with viral DNA of genotypes A, D, C, and E are depicted by large letters. The highest level of spliced genomes sp1 relative to nonspliced genomes (wt) we detected by screening of sera is shown in lane C′ and stems from a patient infected with HBV genotype C.

deletion in this region and in no product with spliced genome sp2, supports this conclusion (Fig. 4C, lanes sp2 and genotype D, m and d). Genotype E- and C-specific amplification products of about 600 and 550 bp, respectively, were obtained with primers P3 and P4 (Fig. 4A, bands marked with arrows). The origin of these bands is not clear, but the size of the amplification products obtained with primer pairs P3/4 and P3/6 (Fig. 4C, about 350 and 330 bp) suggests that they are derived from genomes that have at least two deletions, one of them occurring in the core region (Fig. 4C, lanes genotype E and C). Because these two bands also did not comigrate with an amplification product obtained from cloned spliced genome sp4, we have to assume there is a splice in the core region that is different from that in the core region of sp4 (Figs. 4A and 4C). Taken together, these data demonstrate that HBV genomes of all three genotypes tested produce the singly spliced genome sp1 with similar efficiency, whereas other smaller-than-full-length genomes are not common to all genotypes. In addition, the genotype-specific synthesis of spliced genomes was independent of the cell line used.

To also provide evidence for the *in vivo* synthesis of the dominant spliced genome sp1 from different genotypes, we analysed sera of patients chronically infected with genotypes A, C, D, and E as evident from sequencing of the amplified HBV DNA (data not shown). For identification of spliced genomes in these sera, the viral DNA was amplified by PCR, separated by agarose gel electrophoresis, and stained by ethidium bromide. Bands corresponding in size to spliced genomes sp1 (and verified by cloning and sequencing, data not shown) could thus be visualized with viral DNA from sera of patients infected by HBV of genotypes A, D, and C (Fig. 5; for representative examples, see lanes A, D, and C). However, by screening several patients infected with these genotypes, we did not find in all sera spliced genomes (data not shown), indicating that the fraction of spliced genomes in the total virus population is highly variable and often below the detection limit of our method. Interestingly, we noted even variation of the relative amount of sp1 genomes during the chronic course of infection, as exemplarily shown for viral DNA of genotype C isolated from two sera that were taken at different time points from the same patient (Fig. 5, compare lanes C and C′). In addition, the testing of 13 samples of sera from patients infected with HBV of genotype E did not result in visible bands corresponding to sp1 genomes (Fig. 5; see lane genotype E for a representative example). Therefore, analysis of additional sera from genotype E-infected patients is necessary to determine whether genotype E produces *in vivo* either no sp1 genomes or much less than other genotypes. However, the highly variable level of sp1 genomes in HBV genotype A-, D-, and C-containing sera, which ranged from nondetectable up to at least 20% as roughly estimated from the signal intensity of the ethidium bromide-stained bands, and the demonstration that *in vitro* genotype E produces sp1 genomes as efficiently as other genotypes (Fig. 4), argue against a principle defect of sp1 genome synthesis by genotype E *in vivo*.

DISCUSSION

In this study, we analysed in hepatoma cells the type of spliced HBV genomes produced intracellularly and shed into the culture medium, as well as possible effects exerted on splicing by genotype-specific sequence differences and the type of cell line used. We demonstrate that one defective genome with a single splice is produced as the most dominant subpopulation of smaller-than-wild-type-length genomes by genotypes D, C, and E *in vitro* and by genotypes A, C, and D *in vivo*. None of the other defective genomes are produced consistently by all of these genotypes. The synthesis of spliced and defective genomes was shown to be independent of the cell line used. Furthermore, we identified a novel splice variant that increases the number of known functional HBV splice acceptor sites from four to five. We also found that the percentage of spliced genomes of the total genome population is higher in intracellular than in extracellular viral particles. Taken together, our data argue for a role of the genotype-independent major splice donor and acceptor site, but not of minor sites, in the life cycle of the virus. Moreover, our data demonstrate complex HBV splicing events as well as intracellular accumulation of spliced genomes in hepatoma cells similar to reports on *in vivo* situations. Our findings imply that hepatoma cells are useful tools for the investigation of basic aspects of HBV splicing, which is so far a largely neglected field of research.

The genotype D-, E-, and C-independent dominant spliced genome contains a deletion due to a splicing

event between nucleotide positions 2447 and 489. The same spliced genome was identified previously as a major viral subpopulation in sera and liver biopsy samples of several chronically infected patients (Terre *et al.,* 1991; Rosmorduc *et al.,* 1995; Günther *et al.,* 1997). In one of these reports, this spliced genome was obviously produced *in vivo* from genotype A (Günther *et al.,* 1997). Our data confirmed this and showed in addition the presence of spliced genome sp1, also in sera of patients chronically infected with genotypes C and D. Therefore, this splicing event is common at least for four different genotypes, which further supports the likely role of the corresponding splice sites for the biology of HBV. However, our data obtained with human sera also strongly suggest that *in vivo* host factors and possibly also the sequence heterogeneity of the viral populations can significantly affect the steady-state levels of spliced genome-containing viral particles. Identification of these factors and knowledge about the biological function of this splice event will probably depend on the use of animal systems and infection experiments with splice site knockout mutant viruses for the following reasons. A mutation of the splice acceptor site at nucleotide position 489 was previously shown not to affect viral replication *in vitro* (Su *et al.,* 1989). However, the same was observed with a splice site knockout mutant of DHBV, but the mutation had a significant effect on infection *in vivo* (Obert *et al.,* 1996). Similar to the dominant spliced mRNA of DHBV, the major spliced HBV mRNA lacking intron 2447/489 can represent up to 30% of the pre-C/C transcripts as revealed in transfected hepatoma cells, in the liver of chronically infected patients, and in HBV transgenic mice (Su *et al.,* 1989; Suzuki *et al.,* 1989; Choo *et al.,* 1991; Kajino *et al.,* 1991; Terre *et al.,* 1991; Wu *et al.,* 1991). Consistent with these findings, our study has shown for genotype D that approximately 50% of all intracellular genomes represent the genotype-independent major spliced genome. Unlike in HBV, which has only one encapsidation signal, the dominant spliced mRNA of DHBV is not encapsidated due to the deletion of one of the two encapsidation signals by the splicing event, which prevents synthesis of the corresponding spliced genomes. Therefore, if the major DHBV and HBV splice events have a similar function, it is conceivable that it is not the synthesis of spliced genomes of HBV, which is biologically relevant, but the corresponding mRNAs and/or splice sites. In support of this speculation is a recent report that suggests a function of the major splice acceptor site and upstream sequences in posttranscriptional export of viral transcripts (Putlitz *et al.,* 1999). Therefore, the recognition of splice sites and upstream sequences by the splice apparatus, but not the splicing per se, may play a role in the viral life cycle. On the other hand, the proteins synthesized from the spliced transcripts may have a functional role in the viral life cycle and the course of infection. Consistent with this speculation, the major HBV spliced genome sp1 transfected into hepatoma cells was previously shown to lead to increased levels of X transcripts, large amounts of secreted HBeAg, and intracellular accumulation of core protein compared with cells transfected with wild-type genomes (Rosmorduc *et al.,* 1995). In addition, a novel viral protein translated from the sp1 spliced mRNA was recently detected in liver biopsy samples of patients with HBV-related cirrhosis and was shown to induce apoptosis *in vitro* (Soussan *et al.,* 2000). These and our findings, as well as the more frequent detection of the spliced genomes in chronically compared with acutely infected patients, support the previous suggestion that splicing may contribute to the pathogenicity and/or persistence of HBV (Rosmorduc *et al.,* 1995; Soussan *et al.,* 2000).

Genotype-specific differences in the synthesis of genomes smaller than wild-type length became apparent in our study, which implies that some minor splice sites are functional or highly active only in specific genotypes. The most striking example is represented by genotype C, which showed no common minor spliced genome with genotypes D and E, except for the dominant spliced genome sp1 (Fig. 4A, lanes genotype C). These differences in splicing between genotypes D/E and C cannot be due to mutations of the known 10 splice donor and acceptor sites because all, except one, are conserved as evident from sequence inspection (data not shown and Günther *et al.,* 1997). The exception concerns the splice donor site at nucleotide position 2087, which is exclusively present in genotype C but mutated in genotypes D and E (Günther *et al.,* 1997). This may imply that the unique amplification products obtained with genomes of genotype C and primer pairs 3/4 and 3/6 (Figs. 4A and 4C) may correspond to doubly spliced genomes derived from mRNAs that were spliced at this unique donor site combined with other acceptor sites. The structure of a doubly spliced mRNA present in liver infected with genomes of genotype C and in transfected hepatoma cells as reported previously (Chen *et al.,* 1989; Wu *et al.,* 1991) and the size of the amplification products obtained are consistent with this speculation or use of the very proximal splice donor site 2067. Because no evidence for the production of sp2 genomes, which are synthesized from genotypes D and E, was obtained for genotype C, the splice donor site at position 2067 appears to be nonfunctional in genotype C in combination with the splice acceptor site at position 489. This may be due to mutations in the corresponding branch site and/or polypyrimidine stretch necessary for splicing, as supported by the fact that genotype C sequences differ more from genotype D and E (about 11.0%) than D differs from E (about 8%) (our data, and Norder *et al.,* 1994). Swapping experiments with fragments of different genotypes and mutational analysis will be necessary to confirm these assumptions.

In addition to the previously described five splice donor and four splice acceptor sites known to remove

intronic sequences from the viral RNA pregenome (Chen *et al.,* 1989; Su *et al.,* 1989; Suzuki *et al.,* 1989, 1990; Choo *et al.,* 1991; Wu *et al.,* 1991; Günther *et al.,* 1997), we identified a new functional splice acceptor site at position 2236 in genotype D genomes. In the corresponding spliced genome, the splice acceptor site at position 2236 is used in combination with the splice donor site at position 2067 and results in an in-frame deletion of the viral core gene. This splice event may contribute to the accumulation of C-gene deletion variants similar as observed in long-term immunosuppressed patients and shown to be associated with progressive liver disease (Günther *et al.,* 1996; Preikschat *et al.,* 1999).

An interesting finding of our study was that *in vitro,* the ratio between wild-type and spliced genomes differed dramatically between viral particles isolated from the intracellular compartment and those from the extracellular space. The intracellular genome population consisted predominantly of spliced HBV genomes, whereas extracellular particles contained predominantly wild-type length genomes. An accumulation of encapsidated spliced genomes in the cytoplasm implies that these defective genomes are efficiently *trans*-complemented by P-protein derived from wild-type HBV genomes. The fact that for propagation spliced genomes need to be and can be *trans*-complemented by viral P-proteins was shown by cotransfection of spliced genomes with defective wild-type length genomes that lack a functional encapsidation signal (Rosmorduc *et al.,* 1995). However, *in vitro* the P-protein has also been shown to act primarily in *cis;* that is, pregenomic RNAs from which P-protein is synthesized are preferentially encapsidated (Bartenschlager *et al.,* 1990). Therefore, the similar high amount of spliced genomes in sera of chronic carriers and in cell culture media of transfected hepatoma cells (our data and Terre *et al.,* 1991; Rosmorduc *et al.,* 1995; Günther *et al.,* 1997) suggests that *in vivo* and *in vitro,* the P-protein is expressed in excess from wild-type genomes and allows efficient packaging of spliced pregenomic RNA.

The intracellular accumulation of viral nucleocapsids containing spliced genomes strongly suggests that compared with those containing spliced genomes, nucleocapsids containing wild-type length genomes are preferentially enveloped or released from the cell. This is probably similar *in vivo* as suggested by a report demonstrating the accumulation of the major spliced genome sp1 compared with wild-type length genomes in liver samples of chronically infected patients (Terre *et al.,* 1991). Further support for this conclusion comes from an independent study that revealed a higher relative amount of smaller-than-full-length HBV genomes in the infected livers than in the corresponding sera (Sommer *et al.,* 1997). In addition, spliced genomes of the WHV were detected less prevalently in sera compared with the liver biopsy samples of chronically infected woodchucks (Hantz *et al.,* 1992). These findings and our results raise the question as to the mechanism leading to preferential intracellular accumulation of viral particles with spliced genomes. Previous studies with DHBV and HBV have revealed a strong bias in favor of the export of viral particles with genomes that have completed minus-strand synthesis and at least initiated plus-strand synthesis (Gerelsaikhan *et al.,* 1996; Wei *et al.,* 1996). Because the successful amplification of spliced genomes by the PCR technique that was used requires the presence of completed DNA minus strands in the viral particles but not synthesis of DNA plus strands, it is conceivable that spliced RNAs are reverse-transcribed into DNA minus strands only. Therefore, the corresponding nucleocapsids may accumulate intracellularly because they cannot mature to an extent that leads to the appearance of a signal on the surface needed for efficient envelopment and subsequent secretion. Alternatively and more likely, DNA plus-strand synthesis of spliced DNA minus strands may not be hampered, and it is a difference in the structure of the spliced DNA that hinders maturation of the viral particles.

One of the possible differences between full-length and spliced HBV DNA-containing nucleocapsids in envelope acquisition may be phosphorylation of the core proteins. This could lead to enhanced attachment of the nucleocapsid to the nuclear pore (Kann *et al.,* 1999), possibly resulting in enhanced nuclear import of the spliced genomes, and its conversion into cccDNA. Thus spliced RNAs without the contribution of the splicing apparatus may be synthesized from this cccDNA. This may in part explain the previously published indications for a higher prevalence of spliced genomes in chronic HBV carriers compared with acutely infected patients (Rosmorduc *et al.,* 1995) provided the accumulation of spliced DNA in the form of nuclear cccDNA takes some time. One consequence of nuclear entry and repair of spliced DNA genomes could be the accumulation of the corresponding cccDNA. Provided this type of cccDNA accumulates to a similar level and has a similar pathogenic effect as enhanced levels of cccDNA made from full-length HBV DNA, as suggested from experiments performed with a pre-S mutant of DHBV (Summers *et al.,* 1990; Lenhoff *et al.,* 1999), spliced genomes may indirectly also contribute to hepatopathogenicity. Testing of these hypothesis will require the use of primary human hepatocytes or the woodchuck model because cccDNA formation in hepatoma cells is very inefficient and does not occur in transgenic mice (Bock *et al.,* 1994; reviewed in: Chisari, 1995). In addition, a direct hepatopathogenic effect by the intracellular accumulation of spliced genomes sp1 is conceivable because it may lead to an enhanced expression of the recently described splice-generated viral protein (HSBP), which can induce cell apoptosis (Soussan *et al.,* 2000).

MATERIAL AND METHODS

Full-length amplification and cloning of HBV genomes

The full-length amplification of isolated viral DNA was performed in a Robocycler (Stratagene, La Jolla, CA) as described previously (Günther *et al.,* 1995) by using the cycle number indicated and the Expand High Fidelity PCR System (Roche Diagnostics). The reaction mixture consisted of 50 μl of buffer containing 1.5 mM $MgCl_2$, 200 μM dNTPs, 2.6 U of *Taq* and *Pwo* DNA polymerase mixture, and 0.3 μM concentration of each primer. The primers contain sequences for restriction sites within heterologous sequences at the 5′-end (P1: 1821–1841 5′-CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTAATCA; P2: 1823–1841 5′-CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCATGGTGCTGG; *Sst*I and *Sap*I restriction sites are underlined) and hybridize at the nick region of the genome. The position of the primer corresponds to the nomenclature of HBV genotype D subtype ayw with numbering starting in the middle of the *Eco*RI site (Galibert *et al.,* 1979).

For cloning of the amplified HBV genomes, the PCR products were purified by phenol–chloroform extraction, precipitated, dissolved in water, and digested in the appropriate buffer with *Sst*I. The restriction fragments were purified by using the QIAquick PCR Purification Kit (Qiagen) and cloned into vector pUC19.

Isolation of DNA from sera of patients

DNA was isolated from 100 μl of serum by using the QIAamp Blood Kit (Qiagen). The DNA was eluted from the spin column in a volume of 50 μl. The isolated DNA was stored at −20°C until use.

Plasmids containing HBV reference genomes of different genotypes

Plasmid pSM2 contains an *Eco*RI head-to-tail dimer of genotype D, subtype ayw (Galibert *et al.,* 1979), which was cloned via *Eco*RI restriction sites into plasmid pMa5-8 (Stanssens *et al.,* 1989). pHBV1 contains a monomer of genotype D subtype ayw amplified by the full-length amplification technique and cloned via *Sst*I into vector pUC19 (Sommer *et al.,* 1997).

For isolation of genomes derived from genotypes E and C, DNAs isolated from sera of two chronically infected patients were amplified by the full-length amplification technique for 20 cycles, cloned into vector pUC19, and sequenced. Sequence alignment of the cloned full-length genomes by BLAST search (MacVector 6.0.1) to HBV genomes in the NCBI database revealed the highest homology of pHBVC1, pHBVC2, and pHBVC3 with a genome of genotype C (98% homology to HBV genome AF068756, Monkongdee *et al.,* 1998) and of pHBVE1, pHBVE2, and pHBVE3 with a genome of genotype E (98% homology to HBV genome HHVBBAS, Norder *et al.,* 1994). The three newly isolated HBV genomes of genotype C diverge from each other by less than 6-point mutations, and those of HBV genotype E diverge by less than 15-point mutations.

Analysis of amplified HBV genomes by subgenomic PCR and sequencing

For subgenomic PCRs, the purified viral DNAs or HBV DNAs in plasmid integrated form were amplified by running 40 cycles with denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and elongation at 72°C for 1 min. The PCRs were performed in a 25-μl assay containing 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs, 1 U of *Taq* DNA polymerase, and 0.2 μM concentration of each primer using combination P3/4 (P3: 1821–1841 5′-TTTTTCACCTCTGCCTAATCA; P4: 690–669 5′-AATGGCACTAGTAAACTGAGCC), P4/5 (P5: 2359–2384 5′-CAGGTCCCCTAGAAGAAGAACTCCCT), and P3/6 (P6: 2380–2359 5′-AGTTCTTCTTCTAGGGGACCTG). The positions of the primers correspond to the nomenclature of HBV genotype D subtype ayw (Galibert *et al.,* 1979).

The HBV DNA inserts of the recombinants were sequenced by using pUC19-specific reverse and forward primers hybridizing to sequences flanking the cloned fragments and by internally hybridizing HBV genome primers (477–455 5′-GGACAAACGGGCAACATACCTTG; 968–990 5′-GGCCTATTGATTGGAAAGTATGTC; 1121–1100 5′-AGAAAGGCCTTGTAAGTTGGCG; 2357–2380 5′-GGCAGGTCCCCTAGAAGAAGAACT) using the SequiTherm Sequencing Kit (Epicentre Technologies) and an automated sequencer (LICOR).

Transfection of HBV DNA into human hepatoma cell lines

The linear HBV monomer was released from the vector by *Sap*I digestion and transfected by calcium phosphate precipitation into the hepatoma cell lines Huh7 and HepG2 as described previously (Günther *et al.,* 1998). Plasmid pSM2 was transfected directly. Where indicated, the HBV DNA was alternatively transfected by using the Fugene 6 Transfection Reagent (Roche Diagnostics) according to the protocol of the supplier. The cells were harvested 5 days (calcium phosphate precipitation) or 4 days (Fugene Reagent) after transfection.

Purification of intracellular and extracellular HBV DNA produced by transfected hepatoma cells

The cells were washed twice with PBS and lysed in 500 μl of lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1% Nonidet P-40) 60-mm diameter petri dish for 15 min. Nuclei were pelleted by centrifugation for 1 min at 14,000 rpm (Eppendorf centrifuge). The supernatant was adjusted to 10 mM $MgCl_2$ and treated with 100 μg/ml of DNase I (Roche Diagnostics) 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 for 2 h at 37°C in 1% SDS with 0.5 mg/ml of Proteinase K (Roche Diagnostics). The nucleic acids were purified by phenol–chloroform (1:1) and chloroform extraction before ethanol precipitation in presence of 12 μg/ml of glycogen (Roche Diagnostics) added as a carrier. The pellet was resuspended in water and stored at −20°C until use.

For purification of HBV DNA from extracellular viral particles, the cell culture medium was clarified by centrifugation (Sorvall SS34 rotor, 8000 rpm) for 30 min. Three milliliters of the medium was laid on the top of a 1-ml 20% sucrose cushion in TNE (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl) and ultracentrifuged (Beckman SW50.1 rotor, 45,000 rpm). The pellet was resuspended in 500 μl of 50 mM Tris–HCl (pH 8.0), and the DNA was extracted as described above.

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