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Characterization and Genetic Analysis of Alternatively Spliced Transcripts of Hepatitis B Virus in Infected Human Liver Tissues and Transfected HepG2 Cells

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The transcriptional map of hepatitis B virus (HBV) has been expanded recently by the discovery of a singly spliced transcript in hepatoma cell lines transfected with cloned viral DNA and a doubly spliced one in naturally infected human liver tissues. By the use of reverse transcription and a subsequent polymerase chain reaction, the two spliced HBV RNAs were shown to be present in both types of cells. As further evidence, an HBV mutant was constructed and found to exclusively express the singly spliced RNA. This mutant was also used to quantitate the two spliced species in transfected HepG2 cells; they were found to be equally abundant, and each represented about 30% of the pregenomic RNA. The HBV mutant could still produce replication-competent HBV virions when transfected into HepG2 cells, indicating that the doubly spliced transcript, just like the singly spliced one, was not essential for HBV replication. However, the two abundant spliced HBV transcripts were detected in most naturally infected human liver tissues, suggesting that they may have biologic functions in vivo.

Hepatitis B virus (HBV) contains a genome consisting of an incomplete double-stranded circular DNA (7); however, it is one of the first viruses, other than retroviruses, that have been shown to replicate by reverse transcription (RT) (1, 7, 8). The organization of the HBV genome generally resembles that of retroviruses, and the HBV genome also has an open reading frame, *pol*, with sequences homologous to those of retroviral reverse transcriptase (9, 13, 21). The transcription of HBV produces three contiguous viral mRNAs for envelope (hepatitis B surface antigen), nucleocapsid (core antigen), and probably polymerase proteins (3, 7, 14, 23). It therefore differs from retroviruses in that splicing does not seem to be required to generate mRNA for viral structural proteins (22).

Spliced HBV transcripts, however, have recently been identified. By Northern (RNA) blot analysis, a 2.2-kb spliced HBV RNA species was found in some infected human liver tissues (17) and also in HBV DNA-transfected hepatoma cell lines (16, 19). Detailed characterization indicates that the 2.2-kb RNA is coterminal with the pregenomic RNA but that one intron of 1,217 bases is removed (16). Interestingly, we discovered another spliced HBV RNA of a similar size directly in infected human liver tissues (4). This species is also coterminal with the pregenomic RNA but is doubly spliced, with two introns totaling 1,298 bases. Sequence comparison indicates that the two spliced HBV RNAs share one common splicing donor site but have quite different coding capacities (4, 16).

The discovery of spliced HBV transcripts elicits interesting speculation about their possible translation products. Particular attention has been paid to the production of *pol*-related proteins (4, 12, 16, 19). However, it has been shown by genetic analysis that the singly spliced HBV RNA is not required for HBV replication in vitro (16). The significance of the doubly spliced species remains unclear.

Intriguingly, only one spliced species was observed in reports regarding HBV spliced transcripts (4, 16, 19). In one case, by the use of S1 nuclease mapping and genetic analysis of an HBV mutant, the doubly spliced transcript was not detected in an HBV DNA-transfected hepatoma cell line (16). In another case, Suzuki et al. used RT followed by a polymerase chain reaction (PCR), but the doubly spliced species was not observed (19). Such results were in contrast to those of our previous study, in which only the doubly spliced RNA was identified (4). Currently, it is not clear whether the discrepancy reflects differential HBV expression between naturally infected tissues and transfected cells or whether the two spliced species coexist in both systems but in different relative amounts. Therefore, it is important to systematically search for these two spliced transcripts and determine their relative levels in infected tissues and transfected cells.

In this study, we used a sensitive detection method based on RT of RNA followed by a PCR (11) to assay for the different RNA species in both naturally infected human liver tissues and transfected HepG2 cells. We will present evidence that both singly spliced and doubly spliced transcripts exist in both types of cells. Site-directed mutagenesis was used to generate an HBV mutant incapable of expressing the doubly spliced RNA; this mutant was used in transfection experiments to examine the significance of such a transcript on HBV replication in vitro.

MATERIALS AND METHODS

Plasmid construction. The plasmid used as the wild type, pHBV-48, contains a partial dimer of the HBV genome from nucleotides 2851 to 1280 cloned into the vector pGEM-3Z (Promega, Madison, Wis.). The single-stranded bacteriophage M13mp18-HBV(+) contains the plus strand of the full-length HBV genome (*Eco*RI-*Eco*RI) in the M13 vector

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mp18 (Boehringer, Mannheim, Federal Republic of Germany).

A point mutation mutant, pHBV-17, was constructed by use of a site-directed mutagenesis kit (Amersham International plc, Amersham, United Kingdom). An oligonucleotide covering the second splicing acceptor site but with a base substitution of T to G (see Fig. 4) was synthesized and hybridized to M13mp18-HBV(+). Construction and screening of the mutant were performed in accordance with the instructions of the manufacturer (20).

Transfection of HepG2 cells. Transfection of HepG2 cells with cloned HBV DNA was performed as described by Chang et al. (2). HepG2 cells grown in 10-cm petri dishes were transfected with 20 μg of plasmid DNA. Cells and media were collected for analysis.

RNA extraction and poly(A)$^+$ RNA isolation. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction (6) was used to obtain RNA from liver tissues or HepG2 cells. Poly(A)$^+$ RNA was obtained by selection through an oligo(dT)-cellulose column and stored in 70% ethanol at −20°C until use. Human liver tissues were obtained from hepatitis B surface antigen carriers undergoing a biopsy or surgical resection for hepatocellular carcinoma. The tissue samples were immediately placed in liquid nitrogen.

Synthesis of oligonucleotides. For site-directed mutagenesis, an oligonucleotide, 5′ TCCCCCGAGAAAATTGAG 3′, was synthesized (DNA synthesizer 381A; Applied Biosystems, Foster City, Calif.) and purified by the use of an OPC column (Applied Biosystems, Foster City, Calif.).

For PCR, three oligonucleotides were synthesized: primer C5, 5′ CCTTCTGACTTCTTTCC 3′ (nucleotides 58 to 74); primer C2, 5′ TCCCTCGCCTCGCAGA 3′ (nucleotides 480 to 495); and primer C3, 5′ GGGAAAGCCCTACGAA 3′ (nucleotides 1998 to 1983).

RT-PCR. RT was performed by use of a modification of the procedure of Kawasaki and Wang (11). One microgram of poly(A)$^+$ RNA was incubated at 42°C for 40 min in 1× PCR buffer (purchased from Cetus, Norwalk, Conn.)–1 mM deoxyribonucleotides–1 U of RNasin (Promega) per μl–100 pmol of hexanucleotide random primers (Pharmacia-LKB Biotechnology Inc., Piscataway, N.J.)–4 U of murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) per μl in a final volume of 50 μl. The cDNA products were heated at 95°C for 10 min to inactivate the enzyme. One-tenth volumes of the cDNA products were incubated in 1× PCR buffer–specific primer pairs (25 pmol each)–2.5 U of *Taq* polymerase (Promega) in a total volume of 50 μl. After being overlaid with 50 μl of mineral oil, the mixtures were subjected to 30 cycles of PCR in a thermal cycler (Perkin-Elmer, Norwalk, Conn.) as follows: 94°C for 1 min; 55°C for 1 min; and 74°C for 2 min. The RT-PCR products were analyzed by electrophoresis in either agarose gels or 8% polyacrylamide gels.

Asymmetrical RT-PCR and direct sequencing. To determine the nucleotide sequences of the RT-PCR products, we used asymmetrical PCR (8), which utilizes unequal concentrations of the two amplification primers to produce primarily a single-stranded DNA template. Double-stranded RT-PCR products were separated from primers in an 8% polyacrylamide gel and eluted. After ethanol precipitation, the products were resuspended in 10 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The RT-PCR products (2 to 5 μl) were incubated in 1× PCR buffer supplemented with deoxyribonucleotides (0.2 mM), 50 pmol of primer C3, 0.5 pmol of primer C2 (ratio of primers, 100:1), and 2.5 U of *Taq* polymerase. Asymmetrical PCR was carried out for 30 cycles as described above. DNA products were extracted once with phenol-chloroform and precipitated with ethanol in the presence of 2 M ammonium acetate. The products were sequenced directly by use of the T7 sequencing system (Promega) with oligonucleotide C2 as the primer.

Endogenous DNA polymerase reaction for HBV. To evaluate the effects of abolishing the doubly spliced transcript on HBV replication, we collected media from HepG2 cells transfected with either pHBV-48 or pHBV-17 at the sixth and ninth days posttransfection. The in vitro-produced HBV particles were collected by centrifugation and subjected to an endogenous polymerase reaction as previously described (2). Labeled HBV DNA was detected by autoradiography after electrophoresis in a 1.0% agarose gel.

RESULTS

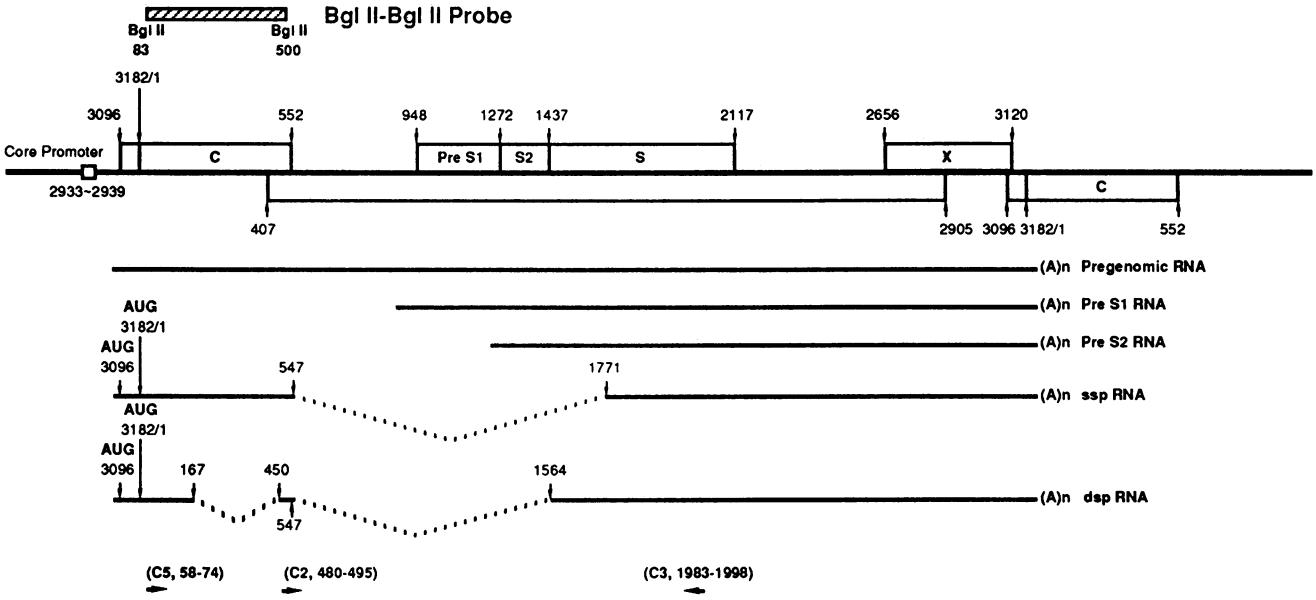
Demonstration of two spliced RNAs in infected liver tissues and transfected HepG2 cells. Previously, 2.2-kb spliced HBV transcripts had been detected in some infected liver tissues and in transfected HepG2 cells by Northern blot analysis (4, 16). However, methods such as Northern blotting and S1 nuclease mapping are not sensitive enough to determine whether the singly and doubly spliced species coexist (4, 19). We thus used the RT-PCR method to study this problem. RT-PCR not only is highly sensitive (11) but also can be used to differentiate the alternatively spliced transcripts, provided appropriate primers are chosen. Three oligonucleotides were thus synthesized as primers; their relative locations in the HBV genome are shown in Fig. 1. RT-PCR products from the two spliced RNAs primed by these oligonucleotides can be easily distinguished.

Poly(A)$^+$ RNAs extracted either from naturally infected human liver tissues or from HBV DNA-transfected HepG2 cells were subjected to RT-PCR with different pairs of primers. The RT-PCR products generated by the use of primers C2 and C3 are shown in Fig. 2A. Two fragments, corresponding to the sizes of products derived from the singly spliced (297-bp) and the doubly spliced (503-bp) HBV RNAs, were found. A DNA fragment corresponding to the RT-PCR product derived from the unspliced RNA was not apparent in this specific experiment, but it could be detected by Southern hybridization (data not shown).

Although the above-described experiment demonstrated the presence of two spliced HBV RNAs, it did not provide evidence for double splicing. Therefore, another pair of primers (primers C5 and C3) was used. The results (Fig. 2B) are consistent with those obtained with primers C2 and C3. Fragments of the sizes expected of RT-PCR products derived from both singly and doubly spliced RNAs (702 and 624 bp, respectively) were detected in HBV DNA-transfected cells (Fig. 2B, lane 2). To further characterize the RT-PCR products, we purified them, digested them with the restriction enzyme *Bgl*II, and analyzed them by electrophoresis in a 2% agarose gel. The digestion products (Fig. 2C, lane 2) were consistent with the presence of both spliced species (Fig. 1).

These results strongly suggest that both singly and doubly spliced HBV transcripts are present in infected liver tissues and transfected HepG2 cells.

RT-PCR and direct sequencing to show the dominant spliced species and their splice junctions. Although the splice sites predicted by electrophoretic analysis of the RT-PCR products were consistent with those identified previously (4, 16), we felt that it was essential to confirm the results for two



	ssp RNA	dsp RNA	Unspliced RNA
RT-PCR Product (primer C5+C3)	702 bp (Bgl II digestion: 25, 418, 242 bp)	624 bp (Bgl II digestion: 25, 136, 449 bp)	1923 bp
RT-PCR Product (primer C2+C3)	297 bp	503 bp	1518 bp

FIG. 1. Transcriptional map of HBV and locations of oligonucleotide primers. The genome organization of HBV is shown at the top; open reading frames (ORFs) are indicated by open rectangles. The A of the first AUG of the core ORF is designated as nucleotide 1. The initiation and termination codons of each ORF are indicated. The bar at the top shows the position of a 417-bp *Bgl*II-*Bgl*II fragment which was used as a probe for Northern blots. Five HBV transcripts (pregenomic, pre-S1, pre-S2, singly spliced [ssp], and doubly spliced [dsp] RNAs) are depicted in the middle. The positions of oligonucleotides synthesized for use as primers in RT-PCR are also indicated. The sizes of RT-PCR products expected from relevant HBV RNAs and their *Bgl*II digestion products are shown at the bottom.

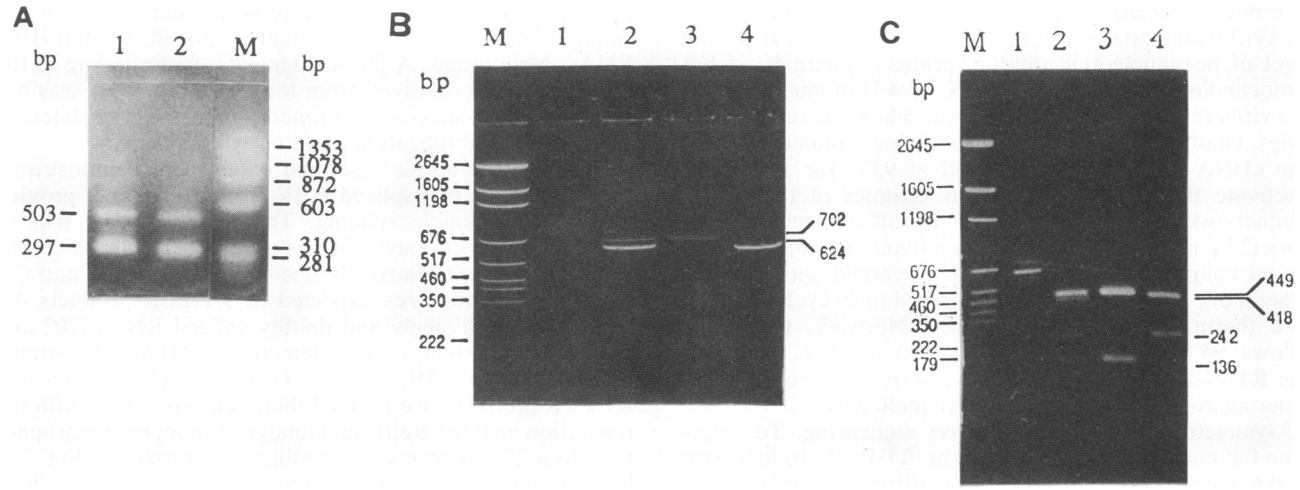


FIG. 2. Electrophoretic analysis of RT-PCR products from spliced HBV RNAs. (A) Poly(A)$^+$ RNAs were isolated from pHBV-48 DNA-transfected HepG2 cells (lane 1) or HBV-infected human liver tissues (lane 2) and amplified by RT-PCR with primers C2 and C3. The products were analyzed on a 1.5% agarose gel. Lane M contains markers prepared from ϕX174 DNA digested with *Hae*III. The sizes of marker DNAs and RT-PCR products are indicated. (B) RT-PCR products of RNAs from untransfected HepG2 cells (lane 1) and pHBV-48-transfected HepG2 cells (lane 2); primers C5 and C3 were used. RT-PCR products from a cDNA clone of the singly spliced RNA (lane 3) and from a cDNA clone of the doubly spliced RNA (lane 4) are also shown. Lane M contains pGEM DNA size markers (Promega). The sizes of RT-PCR products expected from spliced HBV RNAs are shown on the right. (C) Analysis of RT-PCR products from panel B. Shown are pGEM DNA size markers (lane M), undigested RT-PCR products from HBV DNA-transfected HepG2 cells (lane 1), *Bgl*II-digested RT-PCR products from transfected cells (lane 2), and *Bgl*II-digested RT-PCR products from cDNA clones corresponding to doubly (lane 3) and singly (lane 4) spliced RNAs. The sizes of *Bgl*II-digested products expected are shown on the right.

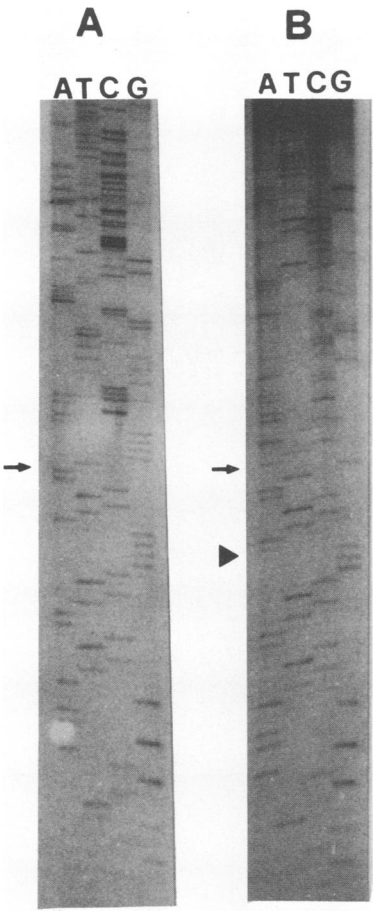


FIG. 3. Direct sequencing of RT-PCR products from spliced HBV transcripts. Shown are autoradiograms of nucleotide sequence reactions, primed by C2, of RT-PCR products derived from the spliced HBV RNAs. The RT-PCR products were asymmetrically amplified with primers C3 and C2 prior to sequence analysis. Shown are the sequences of RT-PCR products derived from the doubly spliced RNA from naturally infected liver cells (A) and the singly spliced RNA from HBV DNA-transfected HepG2 cells (B). The splice junctions are indicated by arrows. The strain of HBV in the naturally infected liver tissue was different from that used to transfect HepG2 cells, as shown by the arrowhead in panel B, which marks a sequence variation.

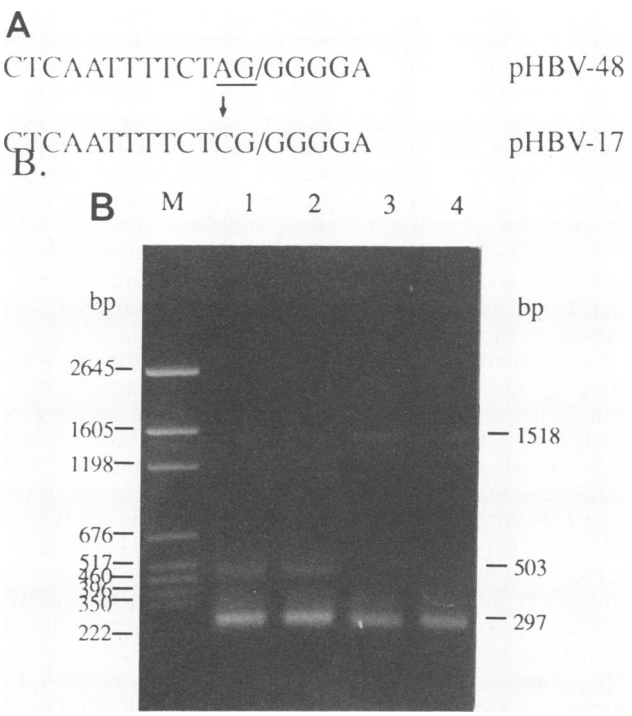


FIG. 4. HBV mutant that does not express the doubly spliced transcripts. (A) Shown is the strategy for in situ mutagenesis. The conserved AG dinucleotide (underlined) bracketing the 3′ end of the second intron of the doubly spliced RNA was changed to CG. (B) Gel electrophoresis of RT-PCR products, primed by C2 and C3, from HepG2 cells transfected with either wild-type or mutated HBV DNA. Lane M contains molecular size markers (λ DNA digested with *Hin*dIII). Lanes 1 and 2 show RT-PCR products of poly(A)$^+$ RNAs from HepG2 cells transfected with wild-type HBV DNA (pHBV-48) at days 2 and 6, respectively. Lanes 3 and 4 show RT-PCR products of poly(A)$^+$ RNAs from HepG2 cells transfected with mutated HBV DNA (pHBV-17) at days 2 and 6, respectively. The sizes of the products expected from doubly spliced (503-bp), singly spliced (297-bp), and unspliced (1,518-bp) HBV RNAs are indicated on the right. The RT-PCR products of the doubly spliced RNA were absent in HepG2 cells transfected with mutant pHBV-17.

reasons. First, it was necessary to determine whether the splice sites obtained from sequence analysis of a limited number of cDNA clones were correct. Second, the possibility of reverse transcriptase artifacts or other alternatively spliced transcripts resulting in RT-PCR products of comparable sizes had to be excluded.

The approach taken was to determine the nucleotide sequences of the RT-PCR products primed by oligonucleotides C2 and C3. To do this, we performed direct sequencing with primer C2 of single-stranded DNAs produced by asymmetrical PCR. The sequences of the RT-PCR products from the doubly spliced RNA are shown in Fig. 3A. The nucleotide sequence at the splice junction (TCTCAAT/GGGGAA) was identical to that of a cDNA clone obtained from infected liver tissues (4). The sequence at the splice junction of the singly spliced RNA (TCTCAAT/GATCAT) (Fig. 3B) was identical to that of a cDNA clone from transfected HepG2 cells (16, 19).

The nucleotide sequence of the splice junction in the core region could not be determined by direct sequencing of RT-PCR products because of technical difficulties. Instead, it was determined by sequencing eight independent clones of the 136-bp *Bgl*II fragment, which was generated as shown in Fig. 2C, lane 2. The results (data not shown) confirmed that the splice junction was identical to that previously found (4).

Genetic evidence for the coexistence of two spliced RNAs in transfected HepG2 cells. The above-described experiments revealed the coexistence of two previously identified spliced HBV RNAs in infected liver tissues and transfected HepG2 cells. To finally confirm the presence of the doubly spliced RNA, however, we found it necessary to obtain direct genetic evidence. To this end, we performed site-directed mutagenesis to change the AG splicing acceptor site of the second intron of the doubly spliced RNA to CG. Such a mutation would likely abolish processing of the doubly spliced RNA but should not affect that of the singly spliced species. The substitution would not change the amino acids of either the P or the S proteins.

The mutated HBV DNA (pHBV-17) was transfected into HepG2 cells, and poly(A)$^+$ RNA was isolated at days 2 and

6 posttransfection and subjected to RT-PCR with primers C3 and C2. The RT-PCR products expected from the two spliced species were found in cells transfected with wild-type HBV DNA (Fig. 4B, lanes 1 and 2). In contrast, the band representing the RT-PCR products from the doubly spliced RNA was absent in cells transfected with mutated HBV DNA (Fig. 4B, lanes 3 and 4). Furthermore, the band representing the RT-PCR products from unspliced pregenomic RNA (1,518 bp) became prominent.

Analysis of the HBV mutant (pHBV-17) clearly demonstrated that two spliced HBV transcripts are present in transfected cells and can be genetically manipulated to be expressed in an independent manner.

Determination of the relative abundance of the two spliced HBV RNAs in transfected HepG2 cells. In an attempt to determine the amounts of the two spliced species, we analyzed by Northern blotting poly(A)$^+$ RNAs isolated from cells transfected with either wild-type or mutated HBV DNA; the probe was a *Bgl*II-*Bgl*II fragment which hybridized only to pregenomic and spliced RNA species (Fig. 1). Although two RNA species were detected in both types of cells, the hybridization signal of the 2.2-kb species appeared to be less intense in cells transfected with mutated HBV DNA (Fig. 5A, lanes 1 and 2). Subsequent quantitation by densitometer scanning clearly showed the difference (Fig. 5B). These transfection experiments were repeated four times, with quantitation each time by densitometry. The relative signal strengths of the different RNAs were calculated and standardized against those of the pregenomic 3.6-kb RNAs; they were as follows: the relative abundance of the combination of both singly and doubly spliced transcripts in wild-type-transfected cells was 0.79 ± 0.23, whereas that of the singly spliced RNA in mutant-transfected cells was 0.31 ± 0.10. We conclude that splicing takes place rather frequently in transfected HepG2 cells and that the two processed transcripts exist in comparable amounts.

The doubly spliced transcript is dispensable for in vitro HBV replication, but the two spliced HBV transcripts are present in most infected liver tissues. As the doubly spliced species was not expressed in HepG2 cells transfected with pHBV-17, the system was ideal for examining whether this RNA species is important for HBV production in vitro. Culture media collected at the sixth and ninth days after transfection were assayed for the presence of HBV particles by the endogenous DNA polymerase reaction (2, 10). HBV particles isolated from either pHBV-17 (mutant)-transfected HepG2 cells (Fig. 6A, lanes 2 and 4), pHBV-48 (wild-type)-transfected HepG2 cells (Fig. 6A, lanes 1 and 3), or patient serum (Fig. 6A, lane 5) were all able to generate the expected 3.4-kb products. Therefore, we conclude that the doubly spliced HBV transcript is dispensable for HBV production in vitro.

Although the doubly spliced transcript is not essential for replication, it may have some biological function. For example, the viral X protein and hepatitis B e antigen are not essential for HBV replication, but they do have significant biological roles (15, 24). An example of a nonessential RNA transcript that plays a significant role in viral pathogenesis is the human immunodeficiency virus *nef* transcript (5). Therefore, we felt that it was important to determine whether the spliced transcripts were present during natural HBV infection, as they might play some role in vivo. Previous studies with Northern blotting found spliced transcripts (singly or doubly) in only 10 to 20% of naturally infected samples (17). To better detect the spliced transcripts in natural HBV infection, we used the RT-PCR method. Poly(A)$^+$ RNAs

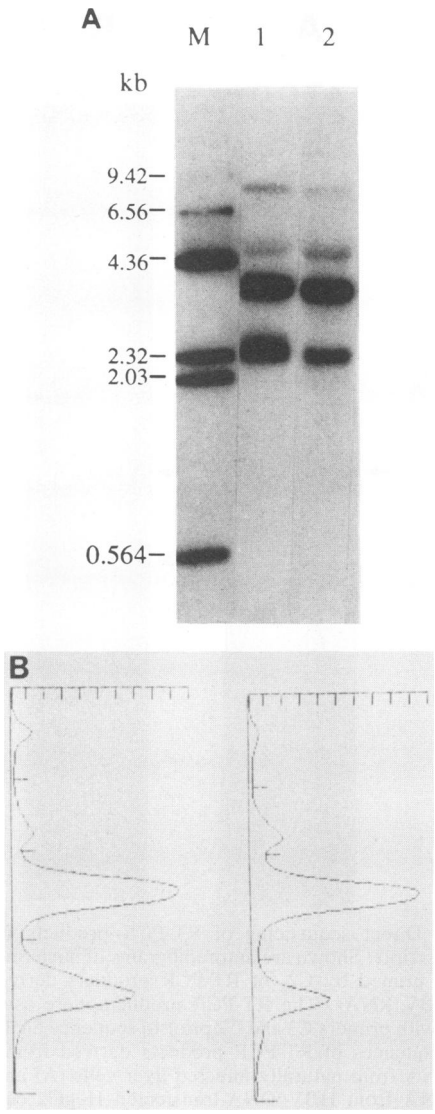


FIG. 5. Quantitation of spliced HBV transcripts in transfected HepG2 cells. (A) Poly(A)$^+$ RNAs were prepared from wild-type HBV DNA (pHBV-48, lane 1)- or mutated HBV DNA (pHBV-17, lane 2)-transfected HepG2 cells on day 2 and subjected to gel electrophoresis, Northern blotting, and hybridization with a 417-bp *Bgl*II-*Bgl*II fragment (Fig. 1). Lane M contains DNA size markers. Locations of the 3.6-kb pregenomic RNAs (arrowhead) and the spliced RNAs (arrow) are indicated. (B) Densitometer tracings of lane 1 (left) and lane 2 (right) of panel A.

were extracted from nontumor liver tissues of hepatitis B surface antigen carriers and subjected to RT-PCR with primers C2 and C3. The two spliced RNAs were found in all 13 infected human liver tissues (Fig. 6B).

DISCUSSION

In this study, it was clearly demonstrated that alternatively spliced transcripts of HBV coexist in both transfected HepG2 cells and infected human liver tissues. The results were supported not only by the presence of specific RT-PCR products of each spliced transcript but also by definite genetic evidence. Therefore, our study (4) and other studies

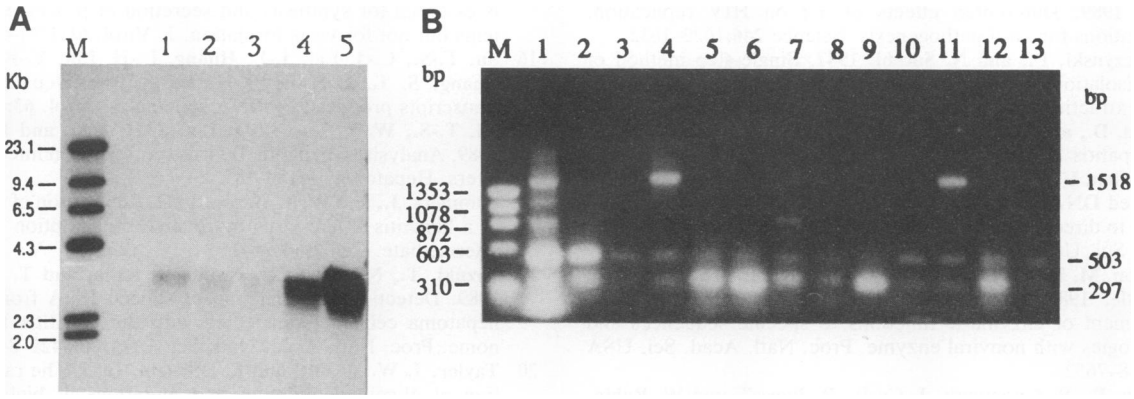


FIG. 6. Evidence that the doubly spliced HBV transcript is nonessential for HBV replication in vitro but is expressed in most naturally infected liver tissues. (A) HBV particles were collected from culture media of HepG2 cells transfected with pHBV-48 (lanes 1 and 3) or pHBV-17 (lanes 2 and 4) at days 6 (lanes 1 and 2) and 9 (lanes 3 and 4) after transfection and subjected to endogenous polymerase reactions. The labeled HBV DNAs were extracted and examined by agarose gel electrophoresis. Lane M contains size markers. Lane 5 shows the reaction products of HBV virions isolated from the plasma of a patient known to be an HBV carrier. (B) RT-PCR products, primed by C2 and C3, from 13 human liver tissues persistently infected with HBV. Poly(A)$^+$ RNAs were obtained from all 13 liver tissues, and RT-PCR was performed. The resulting products were separated on an agarose gel. Lane M contains DNA markers. Lanes 1 to 13 represent RT-PCR products from the 13 independent liver tissues. The sizes of the products expected from singly spliced (297-bp), doubly spliced (503-bp), and unspliced (1,518-bp) HBV RNAs are shown on the right.

(16, 19) in which only one of the two spliced HBV RNAs was identified probably represented a partial characterization of the splicing process. The reason why a previous study with a similar RT-PCR method failed to detect the doubly spliced species (19) could be the choice of different primers or reaction conditions that resulted in the unfavorable synthesis of larger products. (A similar situation was encountered in this study, in which the largest RT-PCR product of the unspliced pregenomic RNA was the least frequently obtained.) The necessity of using multiple sets of primers to obtain consistent results for RT-PCR products was shown by this study.

The presence of the two spliced HBV transcripts in infected tissues and transfected cells leads us to speculate about their possible biological function in the HBV life cycle. Results of genetic analyses from this study and a previous one (16) indicated that neither spliced transcript was essential for viral replication, arguing against the previous conjecture that they might encode the HBV polymerase protein (4, 16).

At this stage, the biological implication of splicing in the HBV life cycle remains obscure. However, although the two spliced HBV transcripts are not essential for viral production, their presence and relative abundance appear to be unusual for a virus which so efficiently uses its genome (7). In addition, the fact that the two spliced transcripts were present in all infected human liver tissues tested in this study suggests that they may play certain roles in vivo.

Is there any possibility to be explored? One speculation about the biological functions of the spliced transcripts is that they may be related to the regulation of HBV production and assembly. There are two stages at which such regulation could be exerted. At the level of transcription, the diversion of pregenomic RNAs to spliced ones would reduce the amounts available for packaging into virions. From this point of view, it could be relevant that the endogenous polymerase reaction products of mutant pHBV-17 resulted in stronger signals than did those of wild-type pHBV-48 (Fig. 6A, lanes 3 and 4). In addition, the RT-PCR products of the pregenomic RNA appeared to be more prominent in mutant pHBV-17 (Fig. 4B). At the level of viral assembly, the putative translation products from the spliced RNAs could generate novel core, hepatitis B surface antigen, or core-S fusion proteins (4, 16). Such defective viral products might interfere with the assembly of normal viral core or S proteins into mature virions. Thus, the production of HBV could be negatively regulated at the level of splicing. In this respect, spliced transcripts might behave as the nonessential negative regulator (*nef*) of human immunodeficiency virus (5), although probably through a different mechanism. The possibilities are interesting, and studies to further examine the possible negative regulatory activities of the doubly spliced RNA are under way.

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