Tissue Preferential Expression of the Hepatitis B Virus (HBV) Surface Antigen Gene in Two Lines of HBV Transgenic Mice

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Two transgenic mice were produced by microinjection of the entire hepatitis B virus (HBV) genome as a 3.2-kilobase *EcoRI* DNA fragment into one-cell embryos. Each animal contained a single, unique locus of HBV sequence. One founder animal, G7, contained a partially deleted HBV genome lacking both putative HBV surface antigen (HBsAg) promoters. The other animal, G26, contained greater-than-genome-length HBV sequences organized as a partial head-to-tail dimer. Both transgenic animals transmitted the HBV sequences in a Mendelian fashion, and all subsequent transgenic animals had detectable HBsAg in the serum. Expression of HBV sequences in tissues from G7- and G26-derived mice showed preferential expression of the 2.1-kilobase HBsAg RNA transcript in liver and kidney tissues by Northern (RNA) blot analysis. These data are consistent with the notion that HBV DNA contains *cis*-acting regulatory sequences which are responsible for the predominant expression of HBsAg transcripts in the liver and kidney of transgenic mice.

The genetic organization of HBV as deduced from the nucleotide sequence reveals at least four open reading frames (ORFs), S, X, C, and P (reviewed in reference 25). Two major viral RNA transcripts of 3.5 and 2.1 kilobases (kb) have been identified in HBV-infected human and chimpanzee liver (25). The 2.1-kb polyadenylated transcript encodes the surface antigen. This message has heterogeneous initiation sites, and two potential 5' promoters have been identified (7, 8, 19, 23). One putative promoter, which has been mapped to sequences upstream of the pre-S region, contains a "TATA" box at map position (mp) 2790. Although it is functional *in vitro*, no *in vivo* transcripts have been identified from this promoter (8, 19). Another promoterlike sequence, which shows some sequence homology to the late promoter of simian virus 40, has been identified within the pre-S region (7). This promoter appears to be just upstream from the start of the major 2.1-kb HBV surface antigen (HBsAg) transcript (7, 23). A viral enhancer has been identified in the region from mp 1,000 to mp 1,250 on the viral genome (21, 26). It resides within the ORF of the polymerase gene and is approximately 1,100 bp downstream and 500 bp upstream from initiation sites of the 2.1-kb and 3.5-kb transcripts, respectively. Although some reports indicate that the enhancer element is essential for activity of the core antigen transcript, others suggest it might also be required for HBsAg and X gene expression (21, 22, 27). The enhancer element also has been shown to confer enhanced expression of heterologous genes in liver-derived cells (18, 21, 28). In addition, a glucocorticoid responsive element has recently been identified in the HBV genome at mp 30-735 (28).

The predominant hepatotropic expression and life cycle of HBV may be due to either preferential viral attachment and penetration of human liver cells, regulatory liver-specific viral elements, or both. Recent reports differ on whether HBV endogenous regulatory sequences are recognized by

the mouse and, moreover, whether they can direct tissue-specific expression (3, 10). To study the molecular basis of HBV liver tropism and the pathologic conditions associated with HBV, we have constructed HBV-containing transgenic mice as a model system.

Transgenic mice were produced essentially as described by Brinster et al. (5). A cloned 3.2-kb *EcoRI* HBV DNA fragment was gel purified from the vector pAO1 and injected into the male pronucleus of one-cell embryos obtained from B6A/F₁ females (Jackson Labs) mated to CD1 males (Charles River) (12). After transfer of injected embryos into pseudopregnant females, 34 animals were born of which 2 (G7 [male] and G26 [female]) were identified as transgenic by Southern blot analysis of DNA obtained from distal tail segments. After G7 and G26 were mated to normal mice, G7 sired 30 offspring of which 20 (67%) were transgenic, whereas G26 produced 13 offspring of which 2 (15%) were transgenic. However, G26 F₁ transgenics transmitted HBV sequences to 11 of 22 (50%) offspring.

To assess whether the HBV transgenes were functional, serum samples from G7 and G26 and their F₁ transgenic offspring were assayed for the presence of HBsAg by using the commercial AUSRIA II solid-phase radioimmunoassay kit (Abbott Laboratories, North Chicago, Ill.). All HBV transgenic mice contained abundant serum HBsAg, whereas their nontransgenic littermates contained no serologically detectable HBsAg. Males had higher levels of HBsAg in serum than females ($P < 0.05$): G7 males, 2.0 ± 1.0 $\mu\text{g/ml}$; G7 females, 1.0 ± 0.4 $\mu\text{g/ml}$, and transgenics from G26 produced more HBsAg than G7 transgenics: G26 males, 13.8 ± 7.8 $\mu\text{g/ml}$; G26 females, 4.3 ± 2.0 $\mu\text{g/ml}$.

To determine the structural organization of the two HBV transgenes, genomic DNA from animals derived from G7 and G26 was analyzed by restriction analysis and Southern blotting as previously described (data not shown) (6). All 20 first-generation G7-derived transgenic animals contained a single 10.7-kb *EcoRI* hybridizing fragment, indicating that the HBV transgene was integrated at a single, stable locus. The 1.4-kb *BamHI* fragment containing the HBsAg coding sequences was intact. The 5' end of the HBV sequence in the

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G7 kindred mapped to the region between the deleted *EcoRI* site (mp 1) and the *Bam*HI site (mp 30) which was present. The 3' viral end was bounded by the *Bgl*II site (mp 2431) present in the distal portion of the core gene and the *Hind*II site (mp 2590), which was deleted. A partial restriction map of the G7 HBV transgene locus, deduced from Southern blot data, is shown in Fig. 1A.

A similar series of mapping experiments were performed on genomic DNA isolated from a second-generation transgenic offspring of G26. Digestion with *EcoRI* produced two fragments, 7.2 and 4.3 kb, attributable to a head-to-tail partial dimer configuration of the injected HBV sequence. The presence of a head-to-tail partial dimer was verified by *Bgl*II digestion, which yielded a 2.8-kb fragment, and *Taq*I digestion, which produced a 3.2-kb fragment containing a unit-length HBV genome. Confirmation that the HBV sequence in the G26 kindred was present at a single locus was demonstrated by the presence of a single band after digestion with either *Kpn*I or *Hind*III, neither of which cleaves the HBV genome. In addition, all G26-derived F_2 transgenics (F_1 transgenic $\times$ nontransgenic) contained the same 7.2- and 4.3-kb *EcoRI* hybridizing fragments. Since the 2.8-kb *Bgl*II fragment was present, whereas the 1.9-kb *Bam*HI fragment

was absent, the 5' end was localized to the region between the *Bam*HI site (mp 1403) and the *Bgl*II site (mp 2431). The 3' junction was localized to the region between the intact *Taq*I site (mp 2900) and the deleted *EcoRI* site (mp 1). A partial restriction map of the G26 HBV transgene locus is shown in Fig. 1B.

By restriction mapping, it was apparent that a portion of the HBV genome in the G7 kindred was deleted. Both putative promoters of the HBsAg message and a recently reported nuclear factor I binding site (20) were absent, whereas the viral enhancer, glucocorticoid responsive element, and polyadenylation signal were present (Fig. 1). The HBsAg coding region appeared intact. The polymerase ORF was interrupted, as was the proximal portion of the pre-S2 ORF, whereas the pre-S1 ORF was deleted. We could not determine whether the 3' end of the core gene was intact by restriction analysis and Southern blotting. Moreover, no viral polyadenylation signal could be present in transcripts initiating in the core or precore vicinity. In contrast, the G26 HBV transgene contained a greater-than-unit-length viral genome. Since the HBV sequence was in a partial head-to-tail dimer configuration, the pre-S1, pre-S2, and S gene ORFs were intact. Both putative HBsAg promoters were

FIG. 1. Genetic organization of the HBV transgene loci. (A) Restriction map of DNA sequences at the location of the HBV sequence in the G7 kindred. (B) Restriction map of the DNA sequences at the location of the HBV transgene in the G26 kindred. The HBV sequences are indicated by the hatched box. (C) Schematic representation of two HBV genomes in a head-to-tail arrangement with the HBV genome opened at the start of the core gene ORF. The position of the two *EcoRI* sites defining the injected fragment is indicated on the bold line. The various ORFs (C, pre-S1, pre-S2, S, X, P) are indicated below the bold line with horizontal arrows. Putative transcription initiation sites of the 2.1- and 3.5-kb RNAs are identified by vertical arrows. The polyadenylation signal is shown as an open triangle. Restriction endonuclease site abbreviations: *Taq*, *Taq*I; *HII*, *Hind*II; *Bgl*, *Bgl*II; *Pst*, *Pst*I; *Bam*, *Bam*HI.

present, as were the enhancer and polyadenylation signal. We could not determine whether the 5' end of the polymerase ORF was intact. At least one copy of the core ORF was intact, but no viral polyadenylation signal was present downstream of the intact core ORF.

To determine which tissues were expressing HBV sequences in animals derived from G7 and G26, RNA was isolated from tissues and organs by the procedure of Chirgwin et al. (9). Using total RNA, a Northern (RNA) blot experiment was performed and probed with the 3.2-kb isolated HBV DNA fragment labeled to high specific activity by random primer extension (24) (Fig. 2). Transcription of HBV sequences has been assessed in multiple animals from each transgenic kindred with similar results. Representative examples are presented below. Animal G7-61, a 3-month-old, male, first-generation G7 offspring, showed strong hybridization of a 2.1-kb transcript in liver tissues with a similarly sized band of less intensity present in kidney and in stomach tissues (Fig. 2A). The 2.1-kb transcript was not seen in spleen, testes, pancreas, small intestine, heart, or

brain. Integrity of RNA samples was assessed from the presence of intact 28S and 18S RNA bands, and the blots were reprobed with a beta-actin probe (Fig. 2A, bottom). Although the 28S and 18S bands were intact in RNA from the pancreas, failure to hybridize with the beta-actin probe indicated the RNA was slightly degraded. RNA extracted from tissues of a G26-derived, F₂, 3-month-old female was similarly assayed for HBV transcripts by Northern blot analysis (Fig. 2B). A very strong signal was detected in RNA from liver and kidney tissues. The major transcript detected was 2.1 kb, similar in size to the hybridization signal seen in liver tissue of the transgenic offspring from kindred G7 (G7-61). Hybridization of a 2.1-kb transcript was also seen in pancreas, lung, stomach, small intestine, and brain, but these signals were at significantly lower abundance than those seen in the liver and kidney. The RNAs were subsequently hybridized with beta-actin as a control (Fig. 2B, bottom).

The 5' ends of the 2.1-kb HBsAg transcripts were mapped by primer extension to determine whether the authentic HBsAg promoter was used in G26 and to investigate the nature of the 5' ends in G7, since the native HBsAg promoters were absent. Poly(A)⁺ RNA was isolated from the liver by oligo(dT)-cellulose chromatography of total RNA prepared by the method of Auffray and Rouillon (1, 2).

Primer extension analysis and RNA sequencing were performed with minor modifications of the methods of Geliebter et al. (14). A 10-μg sample of poly(A)⁺ RNA was mixed with 10 ng of ³²P-end-labeled single-stranded oligonucleotide primers A (mp 156-176) or B (mp 91-109) and heated at 80°C for 5 min in 15 μl of annealing buffer (250 mM KCl, 10 mM Tris [pH 8.3], 1 mM EDTA). The primer and mRNA were then allowed to anneal for 1 h at 50°C. A 2-μl sample of the RNA primer-containing solution was added to 3.3 μl of transcription buffer (24 mM Tris [pH 8.4], 16 mM MgCl₂, 8 mM dithiothreitol, 0.4 mM dATP, 0.4 mM dCTP, 0.8 mM dGTP, 0.4 mM dTTP, 100 μg of actinomycin D per ml, and 1,500 U of avian myeloblastosis virus reverse transcriptase [Life Sciences, St. Petersburg, Fla.] per ml) plus either no or one dideoxynucleoside triphosphate (0.8 mM ddATP, 0.15 mM ddCTP, 0.8 mM ddGTP, 0.8 mM ddTTP) and incubated at 50°C for 1 h. The reaction was stopped by adding 2 μl of loading buffer and boiled for 3 min. Samples of 4 μl from each reaction were run in an 8.0% polyacrylamide-7.0 M urea sequencing gel. The gel was transferred to blotting paper, dried under vacuum, and exposed to XAR film at -70°C. Figure 3A shows the results obtained by using the 21-bp primer A. To align the primer extension products, a sequence ladder was generated from the HBsAg mRNA by using the same primer in the presence of dideoxynucleotides. Heterogeneous 5' ends were observed in the lanes containing the extended products from the liver RNA of both animals. The major 5' termini detected in liver RNA from G7 and G26 are indicated by arrows. To corroborate and further map the HBsAg mRNA transcription initiation sites, primer B was utilized in a similar primer extension experiment (Fig. 3B). The major 5' termini of the mRNA isolated from the liver of G26 were (in order according to the strength of their signal, followed by their map position): (1) 3219, 3221/0; (2) 9, 10; (3) 3199; and (4) 7 (see Fig. 3A). The 5' ends of the HBsAg mRNA from the liver of a G7 animal, listed in a similar manner, were: (1) 17; (2) 21, 22; and (3) cellular sequence. Furthermore, the 5' viral-cell junction of the G7 transgene was identified from a number of experiments (Fig. 3B, right side). Integration of HBV sequences occurred at mp 6. Thus, as found with restriction mapping and Southern blotting, the *Eco*RI site was deleted,

FIG. 2. Expression of HBV transgene sequences in different tissues of transgenic mice. A 10-μg sample of total RNA from each tissue was run in a Northern blot. Expression of HBV RNA was detected primarily as a 2.1-kb band. The tissues analyzed were spleen, liver (Liv.; Liv.-1 and Liv.-2 are two different samples from the same animal); testes, kidney (Kid.), pancreas (Pancr.), small intestine (Sm. Int.), stomach (Stom.), heart (Hrt.), brain (Brn.), large intestine (Lrg. Int.), peritoneum (Perit.), and lung. Sizes were estimated from the positions of the 28S (4.85-kb) and 18S (1.85-kb) RNAs. (A) Northern blot of tissues from a first-generation male of the G7 kindred (G7-61). (B) Northern blot of RNA isolated from various tissues and organs of a transgenic, second-generation animal of the G26 kindred (G26-19); liver RNA from a G7 animal (G7-61) is included for comparison. Blots were reprobed with a beta-actin probe, and signals from different tissues are shown at the bottom of each panel.

FIG. 3. Primer extension analyses of HBsAg mRNAs in transgenic liver tissues. (A) The 21-bp oligonucleotide probe "A" (mp 156-176) was 5'-end labeled and hybridized to poly(A)⁺ RNA isolated from the liver of a G26-derived or G7-derived animal. Extension with reverse transcriptase was carried out, and the products were characterized by electrophoresis in a 7 M urea-8% polyacrylamide gel as described in the text. The RNA from the liver of a G26 animal was totally extended (lane marked [-]) or extended in the presence of dideoxynucleoside triphosphates to establish a sequence ladder for accurate placement of the 5' ends. The lanes labeled T, G, C, or A correspond to the sequence of the plus strand of HBV. The numbers in parentheses indicate map position of the HBV sequence ladder derived from the HBsAg mRNA of G26. The arrows on the left side of the figure correspond to the 5' ends of G26 RNA, labeled according to the intensity of their signal. The arrows on the right correspond to the major 5' ends of G7 RNA, labeled according to their signal intensity. (B) The 19-bp oligonucleotide probe "B" (mp 91-109) was used as described above. The sequence to the left corresponds to the nucleotide sequence determined for the HBsAg mRNA from G26 as determined from three independent experiments. The numbered arrows indicate the nucleotide positions of the HBsAg 5' ends according to their signal intensity and are shown in relation to the sequence and not the position of the band in the gel, as displayed in panel A. The numbers in parentheses correspond to the map position on the HBV genome. The ATG in the box is the start site of the pre-S2-HBsAg protein. The open bracket indicates the reformed *EcoRI* site. The sequence to the right of the figure corresponds to the nucleotide sequence derived from G7 HBsAg mRNA from three independent experiments. The capital letters correspond to HBV sequences, and the lowercase letters correspond to cellular sequences. The junction region is indicated by a horizontal line.

as were the promoter sequences upstream of the *EcoRI* site. The four major HBsAg mRNA 5' ends identified in transcripts from the livers of G26 animals are in excellent agreement with the major HBsAg 5' ends previously identified both in infected liver and in transfected mouse cells (7, 23).

In contrast to the report of Chisari et al. (10), which suggested that the HBV may not carry strong regulatory sequences recognized by the mouse, we detected abundant expression of HBV from native HBV sequences in both our transgenic lines. Whereas Babinet et al. (3) found liver-specific HBV expression in two of six HBV transgenic mice, we saw a broader pattern of tissue expression, most notably including kidney in both our transgenic lines. The abundant expression of HBV sequences in liver and kidney tissues of adult transgenic mice suggests that the HBV regulatory elements can utilize *trans*-acting factors produced not only in liver but also in kidney. A similar pattern of liver and

kidney expression is seen in experimentally infected Pekin ducks with duck hepatitis B virus (17). Moreover, congenitally infected Pekin ducks also showed viral replication in liver and pancreas, which is of interest since we also noted significant levels of HBV transcription in the pancreas of G26-derived animals (see Fig. 2B). We also noted higher levels of HBsAg in serum of male mice compared with female mice, as similarly reported by Babinet et al. (3) for their E36 line. In contrast to the complete HBV expression (two of two) in our transgenic mice, the low rate of HBV expression in the HBV transgenic offspring observed in these two previous reports may be due to such factors as inhibition by adjoining plasmid sequences, integration of HBV DNA into an unfavorable location in the mouse genome, or differences in the HBV sequences.

One observation noted in the G7 transgenic line was that the liver-preferential expression of the 2.1-kb HBsAg transcript occurred in the absence of the putative HBsAg

promoters. The deletion of the HBsAg promoters was documented by restriction mapping and Southern hybridization and by sequence analysis of the HBsAg mRNA, which indicated the 5' viral junction occurred at mp 6 (see Fig. 3B). The ATG translation initiation codon of the HBsAg protein, at mp 158, is predicted to be intact, and the 2.1-kb transcripts should contain sufficient information to code for an intact P24^s or GP24^s surface antigen protein (25). Studies are in progress to characterize the HBV-related proteins.

The demonstration of abundant HBV transcripts in the liver tissues from two independent kindreds of transgenic mice supports the notion that the HBV genome contains regulatory elements which are recognized by hepatic *trans*-acting factors. Such hepatic expression may be related to the hepatotropism and hepatic pathology seen with HBV infection in humans. The observation that the putative 5' HBsAg promoters were not required for the liver-preferential expression of the 2.1-kb HBsAg transcript in the G7 line suggests that the liver specificity of HBV is likely to be determined by sequences 3' to the HBsAg mRNA start sites. *cis*-Regulatory sequences that contribute to the tissue-specific expression of a variety of eucaryotic genes have been identified 5' of, 3' of, and within the respective promoters of the genes studied, as well as within the body of the gene (4, 11, 13, 15, 16). These regulatory elements in many instances would be considered enhancer elements. However, in the case of the mouse immunoglobulin *kappa* gene, the 5' promoter sequences have been shown to direct the preferential expression in B-lymphoid cells, independent of the gene enhancer (13, 16). Whether the HBsAg promoters can function in specific cell types independent of the HBV enhancer remains to be determined.

One candidate element within the HBV genome likely to direct the relative tissue specificity is the enhancer element located within the P ORF, distal to the S ORF, and included in the transcript coding for HBsAg (21, 26). Recent reports indicate that this element is most active in liver-derived cells (18, 19). The data derived from the transcription of HBsAg mRNA in G26 and G7 liver tissue suggest that a native enhancer element within the HBV genome influences the expression of HBsAg in liver tissue. In addition, similar patterns of developmental expression of HBsAg are detected in animals derived from both kindreds, further indicating that HBV *cis*-regulatory elements are responsible for the regulated expression of HBV in these transgenic animals (J. DeLoia et al., manuscript in preparation). We have not ruled out the possibility that the G7-derived mouse liver HBsAg transcripts utilized a 5' cellular promoter potentially under the regulation of the HBV enhancer. Alternatively, the abundance of the HBsAg 2.1-kb RNA transcripts in the liver may reflect a combination of transcription and message stabilization. We have shown that in G7, lacking the native HBsAg promoters, we still detected heterogeneous 5' ends in close proximity to the normal 5' HBsAg ends detected in G26, which contains the intact native HBsAg putative promoters.

In conclusion, we have presented data showing that the HBV genome contains *cis*-regulatory sequences influencing the expression of the 2.1-kb HBsAg transcripts in liver tissues of transgenic mice. When the putative HBsAg promoters were deleted, we detected liver-preferential expression of the 2.1-kb HBsAg transcripts. This suggests that these promoters are not required for the liver-preferential expression of the HBsAg transcripts detected in G7-derived animals. These results raise interesting questions pertaining to the 5' sequence requirements of HBsAg RNA initiation

and the interaction of the distally located HBV enhancer with upstream sequences, especially since HBsAg RNA is a non-TATA-initiated message with naturally occurring heterogeneous 5' ends (7, 23).

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