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Developmental Regulation of Hepatitis B Surface Antigen Expression in Two Lines of Hepatitis B Virus Transgenic Mice

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Two lines of hepatitis B virus (HBV) transgenic mice, designated G7 and G26, show preferential expression of the 2.1-kilobase hepatitis B surface antigen (HBsAg) RNA transcript in liver and kidney tissues (R. D. Burk, J. A. DeLoia, M. K. ElAwady, and J. D. Gearhart, J. Virol. 62:649–654, 1988). This transcript was first identified in transgenic mice at gestational day 14 and was detected at similar or increased levels through birth and early development. However, in contrast to 2.1-kilobase HBsAg mRNA levels, HBsAg protein levels in serum decreased shortly after birth. Thereafter, serum HBsAg increased 100-fold to adult levels, with a corresponding 5- to 10-fold increase in HBsAg mRNA levels. In addition, adult males have higher levels of HBsAg in serum than females. HBsAg in serum in males was reduced approximately 50% by surgical castration and was restored to near-normal levels by testosterone supplementation. Since both transgenic lines show similar patterns of gene expression, we suggest that HBsAg gene expression is determined by viral regulatory elements in response to host factors. Whether tissue specificity, developmental regulation, and sexual dimorphism of expression of the exogenous HBV sequences were determined by single or multiple HBV regulatory elements remains to be determined.

Hepatitis B virus (HBV) infection is a major worldwide medical problem causing acute and chronic liver disease, a chronic carrier state, and a propensity to develop hepatocellular carcinoma (2). The outcome of infection is highly dependent on the developmental state of the infected host. Infection in the third trimester, perinatal period, or first year of life results in chronic infection in the majority of individuals (14, 17). Although there is no significant difference in the incidence of exposure of males and females to HBV, after puberty more males than females remain chronic carriers, and the incidence of hepatocellular carcinoma is 2- to 10-fold higher in males (18). Although the molecular basis of HBV-related disease in humans is poorly understood, recent advances in the molecular genetics of HBV have elucidated many interesting and novel features of the virus (reviewed in references 11 and 19). The nucleotide sequence of the HBV genome reveals at least four open reading frames: presurface and surface antigen (S), core antigen (C), polymerase (P), and X. Two major viral RNA transcripts of 3.5 and 2.1 kilobases (kb) have been identified in infected tissues which can encode the core and polymerase proteins and surface antigens, respectively. Multiple regulatory elements have been identified from in vitro studies, including putative pre-S, S, X, and C promoters (5, 6, 20); an enhancer element (16); a glucocorticoid-responsive element (21); and a polyadenylation signal (5, 6).

The relationship between viral regulatory elements and specific host factors appears to be important in the liver specificity of HBV gene expression (15). To develop an experimental system to study the host control of HBV gene expression and virus-induced liver disease, we have produced two lines of HBV transgenic mice, G7 and G26, which express the HBV 2.1-kb mRNA preferentially in the liver and kidneys (4). The exogenous HBV sequences that were introduced (transgenes) are inherited in a Mendelian fashion, and each has been bred to homozygosity. In this report, we describe the relationship between the developmental stage, hormonal status, gender, and gene dosage of the HBV sequences in transgenic animals and the levels of hepatitis B surface antigen (HBsAg) gene expression.

Transgenic mice were produced as previously described (3, 4). Approximately 200 copies of a complete adw HBV genome, linearized at the *Eco*RI site, were injected into the pronuclei of embryos collected from $B6AF_1$ females (Jackson Laboratory, Bar Harbor, Maine) which had been mated to CD-1 males (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Figure 1 depicts the HBV genome, displaying the four open reading frames, transcription start sites, regulatory elements, and the polyadenylation site. The HBV sequences integrated in the G7 and G26 transgenic lines are shown in schematic representation around the HBV genome. Both transgenes contain intact HBsAg and X open reading frames, the HBV enhancer, the glucocorticoid-responsive element, the X and core promoter regions, and the viral poly(A) site. The G7 HBV transgene lacks the putative viral promoter for the 2.1-kb surface antigen transcript. In addition, the structural organization of the G7 HBV transgene has been confirmed by cloning and sequence analysis (R. Burk, unpublished observations). The two HBV transgenes occupy unique chromosomal locations, on the basis of extensive restriction analyses by Southern blot hybridization and genetic crosses (R. Burk, unpublished observations).

Previous studies demonstrated that the major HBV-related transcript expressed from G7 and G26 transgenes corresponded to the 2.1-kb mRNA coding for HBsAg (4). This transcript was preferentially expressed in livers and, to a lesser degree, in kidneys of animals derived from both kindreds. To determine when the HBV sequences become transcriptionally active, total RNA was isolated from whole embryos at gestational day 12 (8 days before birth) and from livers from gestational day 14 (6 days before birth) through

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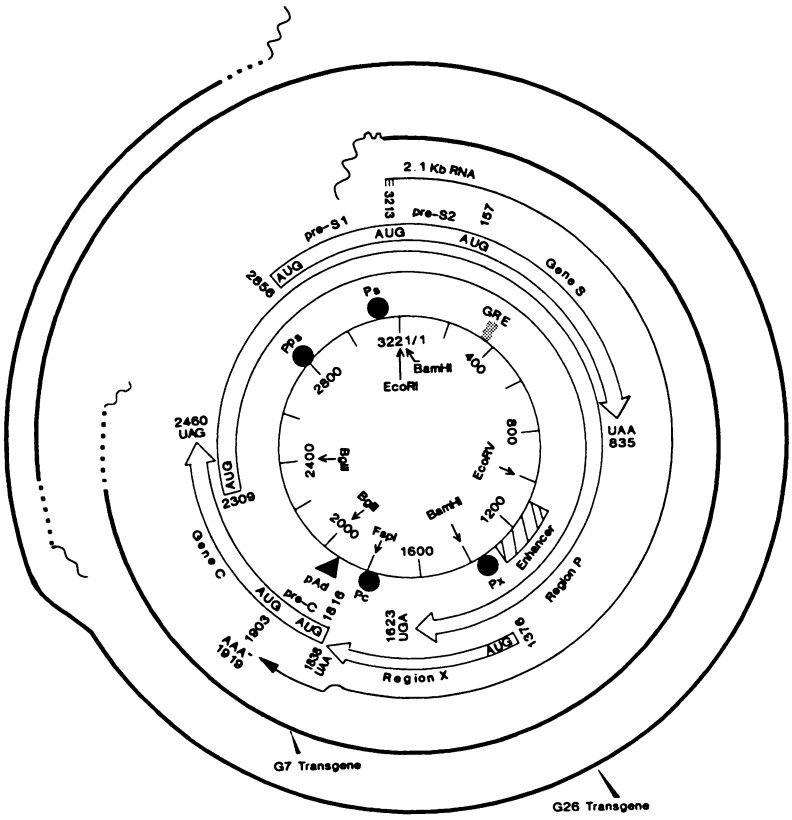

FIG. 1. HBV sequences present at the G7 and G26 HBV transgenes. A partial restriction map of an HBV adw genome derived from the sequence of Valenzuela et al. (22) is shown. The four open reading frames of the coding strand are shown (⇨), with the positions of the start and stop codons indicated. The 2.1-kb HBV mRNA, predominantly expressed in the transgenic mice, is shown (——→). The following HBV regulatory elements are indicated: putative promoters for the presurface (Pps), surface (Ps), X (Px), and core (Pc) transcripts; glucocorticoid-regulatory element (GRE); and polyadenylation site (pAd). The HBV sequences present in the G7 and G26 transgenic lines are indicated. Junction regions not precisely defined are indicated (– – –).

adulthood. RNA was isolated by the method of Chirgwin et al. (7), with minor modifications. Fresh tissue was homogenized in a 10-fold excess of buffer containing 5.0 M guanidium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1% anti-foam A, and 0.1 M beta-mercaptoethanol. After the addition of 4 volumes of a 5.7 M $CsCl_2$ solution, the sample was overlaid on a 5.7 M $CsCl_2$ cushion and centrifuged in an SW50.1 rotor for 18 h at 36,000 rpm. The RNA pellet was suspended in 10 mM Tris (pH 7.0)–5 mM EDTA–0.1% sodium dodecyl sulfate, phenol-chloroform extracted, and concentrated by ethanol precipitation. The HBV-related 2.1-kb transcript was barely detectable in RNA from whole embryos taken at gestational day 12 (Fig. 2, lanes −8; panels A and B correspond to RNA from G7- and G26-derived animals, respectively). However, by gestational day 14 (lanes −6), the 2.1-kb HBV message was abundant in fetal liver; this transcript was detected at similar or increased levels through birth and early development (Fig. 2, lanes −6 through +5). Adult liver had 5- to 10-fold-higher levels of the 2.1-kb transcript compared with fetal and neonatal livers (Fig. 2, lanes +90). G26 neonatal and adult livers contained higher levels of the 2.1-kb transcript than did comparable G7 tissues; however, no such difference was seen in fetal liver samples (compare Fig. 2A and B, lanes −6 to −3).

To demonstrate the translation of HBV sequences into protein during fetal development, the presence of HBsAg protein during prenatal development was determined. HBsAg levels were determined by a solid-phase radioimmunoassay (AUSRIA II; Abbott Laboratories, North Chicago, Ill.) in serum and organ homogenates. Sample values were compared with a standard curve derived from serial dilutions of purified HBsAg, kindly provided by Merck Sharp & Dohme Research Laboratories, West Point, Pa. Serum samples were obtained from retro-orbital plexus phlebotomies in adult mice or by decapitation in neonatal mice. Organ homogenates were prepared from prenatal animals in a 10-fold excess (volume to weight) of 50 mM Tris hydrochloride, pH 7.5, containing 50 μg of ampicillin per ml at 4°C. Total protein was determined by the assay of Lowry et al. (12). HBsAg was detected at low levels in homogenates prepared from both G7- and G26-derived whole embryos at gestational day 12. By gestational day 15, liver homogenates from G7 fetuses expressed 3.6 ng of HBsAg per ml (0.1% of total protein), whereas liver homogenates from G26 expressed 7.8 ng of HBsAg per ml (0.2% of total protein). Thus, HBV sequences were both transcribed and translated during prenatal development.

Postnatal expression of HBsAg was ascertained by quantitating HBsAg protein in sera from postnatal and adult mice. Multiple animals were assayed at each time point to normalize for individual variation. Postnatal expression of HBsAg in the sera of G26-derived animals is depicted in Fig. 3. Surface antigen was abundant in sera collected from new-

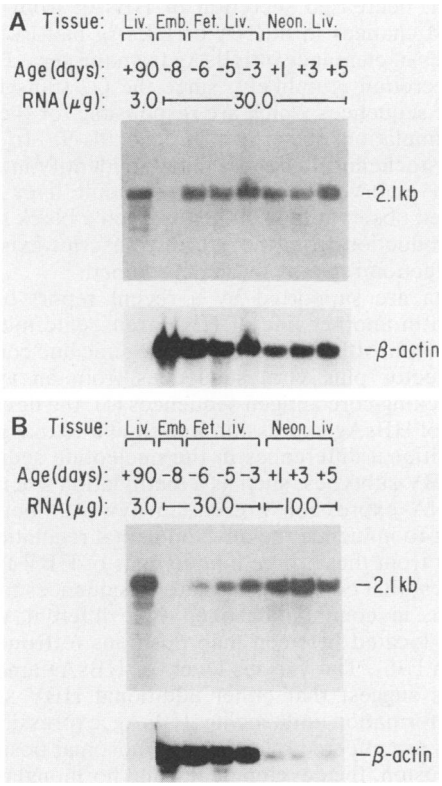

FIG. 2. Prenatal and perinatal expression of the 2.1-kb HBsAg mRNA in homozygous transgenic mice. Total RNA was collected from whole embryos at gestational day 12 and from livers at later stages. RNA was analyzed by Northern blot hybridization, using a ^{32}P-radiolabeled HBV DNA probe. The amount of RNA present in each lane is indicated. The ages of mice are shown; prenatal ages −8, −6, −5, and −3 days from birth correspond to gestational days 12, 14, 15, and 17, respectively. The tissue sources of RNA were adult liver (Liv.), whole embryos (Emb.), fetal liver (Fet. Liv.), and neonatal liver (Neon. Liv.). The HBV probe was eluted from the filters, which were subsequently hybridized with a mouse β-actin probe as a control. (A) Results for animals of the G7 kindred. (B) Results for G26 animals.

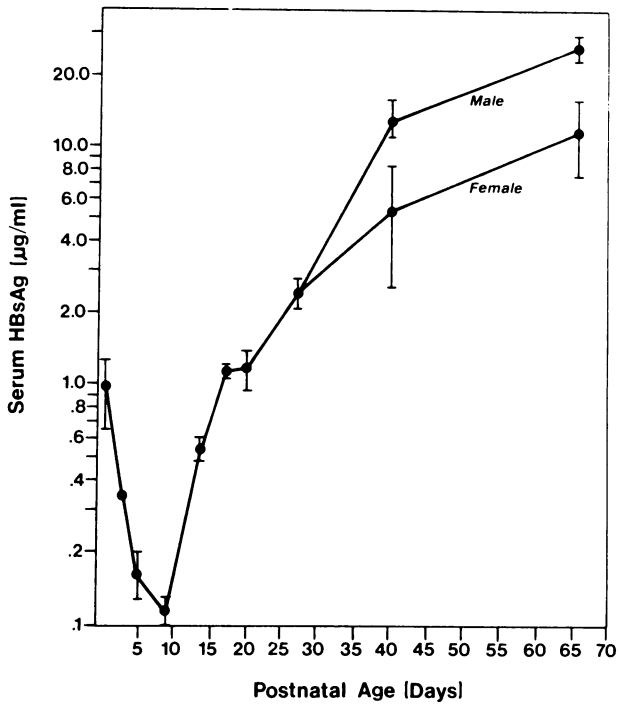

FIG. 3. Postnatal expression of HBsAg in the G26 line. Serum samples were collected from animals from birth (day 1) through adulthood and assayed for HBsAg, using the AUSRIA II kit (Abbott). Each point on the graph represents the average of HBsAg levels from at least three animals. The standard deviation is represented by the vertical bars. The sex was recorded for the prepubescent animals, but since no differences existed in HBsAg levels between males and females, they were grouped together. The G7 line showed a similar pattern of HBsAg expression, albeit at lower levels.

born animals. Within a few days of birth, levels of HBsAg dropped nearly 10-fold to between 0.1 and 0.2 μg per ml of serum and thereafter rose sharply. Whereas prepubescent mice showed no gender differences in levels of HBsAg in serum, the mature animals displayed sexual dimorphism. A similar pattern of postnatal expression of HBsAg in sera of animals derived from G7 was observed, albeit the levels were two- to fivefold lower. The amount of HBsAg found in sera of adult animals over 3 months of age is shown in Table 1. The expression of HBsAg was greater in male than in female animals from both kindreds. Gene dosage also had an effect on the level of HBsAg. The average concentration of HBsAg in the sera of G7 homozygotes was approximately twice that of heterozygotes, and a similar dosage effect was observed for homozygous and heterozygous G26 transgenic lines (Table 1).

To investigate the basis of the sexual dimorphism of HBsAg levels in sera of adult mice, we examined the possible role of the male hormone, testosterone, by castration and hormone supplementation. Serum samples were drawn prior to and 2 weeks after surgical castration from five male G7 and six male G26 homozygous transgenic mice. The average reductions of circulating HBsAg were 55.8 ± 5.2% and 55.5 ± 5.2% for these G7 and G26 animals, respectively. Furthermore, these levels remained low or dropped even further over time. To document further the role of testosterone on the levels of HBsAg in males, 11 male G7 heterozygotes were either surgically castrated (n = 6) or sham castrated (n = 5). Four castrated and three sham-castrated animals were supplemented with a surgically implanted testosterone (10 mg)-releasing pellet (Innovative Research of America) placed subcutaneously along the dorsum; the

TABLE 1. Levels of HBsAg in the sera of adult transgenic mice[a]

Line	Genotype[b]	Sex	n	Avg HBsAg level ± SD (μg/ml)
G7	+/−	M	25	7.0 ± 2.0
		F	21	3.4 ± 1.6
	+/+	M	15	14.3 ± 6.4
		F	14	8.4 ± 6.3
G26	+/−	M	10	23.5 ± 6.7
		F	10	10.8 ± 4.5
	+/+	M	7	67.0 ± 26.8
		F	7	36.0 ± 16.6

[a] Animals were 3 to 16 months in age. All values were obtained with the AUSRIA II radioimmunoassay kit (Abbott).

[b] +/−, Heterozygote for the HBV transgene; +/+, homozygote for the transgene.

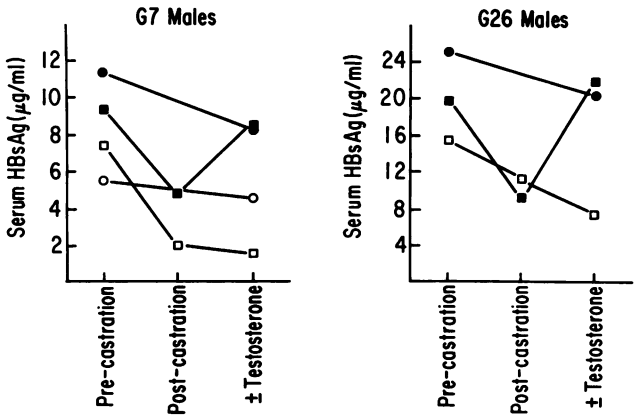

FIG. 4. Influence of hormonal manipulation on HBsAg expression in HBV homozygous transgenic males. Six G7 transgenic males were surgically castrated; four received testosterone pellets (■) and two received pellets without testosterone (□). Five animals were sham operated; three received testosterone (●) and two received pellets without testosterone (○). Animals were bled prior to sham or actual castration, at the time of pellet implantation, and 2 weeks after implantation. A similar set of experiments was performed on G26 transgenic males: five were castrated with testosterone supplementation (■), two were castrated without testosterone (□), and three were sham castrated with testosterone (●).

remaining animals were implanted with a placebo pellet lacking testosterone. Animals were bled prior to sham or actual castration, at the time of pellet implantation, and 2 weeks after implantation. Sham-operated animals had only the first and last bleedings. The hormonal effect of castration and testosterone supplementation was determined by the measurement of testosterone (Coat-A-Count radioimmunoassay kit; Diagnostics Products Corp.) in the second and third bleedings (postcastration and supplementation). All castrated animals had barely detectable testosterone levels, whereas testosterone-supplemented animals had levels comparable to those of normal adult male mice. The results of the manipulations of testosterone levels described above on HBsAg are shown in Fig. 4 (left panel). Castrated G7 males showed a marked decrease in HBsAg levels in their sera which returned to near-normal values with testosterone supplementation. Castrated males without supplementation continued to show reduced levels of HBsAg in their sera. Sham-castrated animals with or without hormone supplementation showed no significant change in HBsAg levels. Similar results were obtained in experiments performed with G26 heterozygous males (Fig. 4, right panel).

In this report, we describe the characterization of HBsAg expression in two lines of HBV transgenic mice constructed with an adw HBV genome. Previously, we had shown that the liver and, to a lesser extent, the kidneys were the major organs expressing the 2.1-kb HBsAg mRNA. The 2.1-kb message first appears in the liver of the developing embryo at gestational day 14, and by day 15, HBsAg protein is detectable in the embryonic liver. At birth there are high levels of HBsAg, which fall 5- to 10-fold within the first week. Thereafter, HBsAg levels increase to adult levels, with correspondingly higher steady-state levels of 2.1-kb HBsAg transcript. Interestingly, during the perinatal period mRNA levels do not decrease concomitantly with protein levels, suggesting a posttranscriptional level of gene regulation. The mechanism of the uncoupling of HBsAg message and protein levels is unclear. Possible explanations include a block in translation, decreased secretion of HBsAg from the liver, age-related changes in mRNA or HBsAg half-lives, or different times of clearance of HBsAg from the sera. Decreased HBsAg secretion is unlikely, since the G7 transgene lacks the pre-S1 sequences which are responsible for the intracellular accumulation of surface antigen (8, 9). In addition, immunohistochemical analysis failed to identify intracellular HBsAg in embryonic, neonatal, or adult liver (DeLoia, unpublished observations). Whether such a block in HBsAg protein production from the 2.1-kb transcript exists during natural infection remains to be determined.

Our data are supported by a recent report of HBsAg expression in another line of HBV transgenic mice, designated E36 (10). Although the E36 transgenic line contains the plasmid vector plus viral sequences from an ayw HBV subtype lacking core antigen sequences (1), the developmental profile of HBsAg expression was similar to that presented here. Additional differences in the nucleotide sequences of the two HBV subtypes, such as a methylation site regulating 2.1-kb RNA expression present in ayw but not in adw, appear not to influence the developmental regulation. Thus, the results from these three unique lines of HBV transgenic mice suggest that *cis*-acting regulatory sequences for HBsAg expression, in common between two different viral subtypes, are located between map positions 6 (from G7) and 1980 (from E36). The various levels of HBsAg among these three lines suggest that either additional HBV sequences contain information influencing HBsAg expression or the transgenes are affected by their chromosomal positions.

In conclusion, the developmental and hormonal regulation of HBV gene expression are similar to the regulation of specific host genes expressed in the liver. Potentially, similar mechanisms of gene regulation may operate through homologous or related elements. We hypothesize that the HBV genome has either evolved or acquired DNA regulatory elements similar to those present in liver genes. Interestingly, Miller and Robinson (13) have suggested that the HBV enhancer and X open reading frame region may be of recent cellular origin. Whether the HBV transgene developmental expression, tissue specificity, and sexual dimorphism are the result of a single or multiple regulatory elements remains to be determined.

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