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Transcription of the S Gene in Transgenic Mice Is Associated with Hypomethylation at Specific Sites and with DNase I Sensitivity

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The methylation status of hepatitis B virus (HBV) DNA was investigated in different organs from two strains of transgenic mice (E36 and E11) expressing the hepatitis B surface antigen (HBsAg) gene specifically in the liver. Specific sites in the S gene were shown to be methylated in all the organs of adult mice except in the liver. These sites were methylated in 14-day-old fetal liver and were progressively demethylated during development and after birth. In one strain in which HBsAg expression is lost upon transmission by females, extensive de novo methylation of the transgene was detected in the livers and bodies of 14-day-old fetuses from transgenic females. The extent of methylation was such that activation of the gene was no longer possible. DNase I-hypersensitive sites were detected in the enhancer region of HBV in the liver of HBsAg-positive mice but not in HBsAg-negative progeny of E36 females. These data indicated that in two independent transgenic lines, HBV sequences are reproducibly activated in the developing liver along with cellular liver-specific genes and that transcription is associated with demethylation at specific sites in the S gene and with DNase hypersensitivity.

We and others have used transgenic mice to study the expression of hepatitis B virus (HBV) genes since this virus does not naturally infect mice. This virus has a strong tropism for the liver, but during natural infection it can be found in extrahepatic locations and in particular in kidney, pancreas, and blood cells. The current hypotheses concerning the regulation of HBV gene expression involve the action of regulatory sequences activated by ubiquitous and liver-specific factors (for a review, see reference 6). In addition, De-Medina et al. (2) suggested that inhibitors present in nonliver cells regulate the expression of the S gene. Regulation by glucocorticoids was demonstrated by Tur-Kaspa et al. (14), and the regulatory sequences were located in the S coding region.

We described previously two transgenic mouse strains (E36 and E11) expressing the HBV surface antigen (HBsAg) gene specifically in the liver (1). We also showed that S gene expression started at day 15 of development together with that of albumin and was regulated by glucocorticoids and sex steroids (5). In the E36 strain, HBsAg expression was systematically lost in the progeny of females (7). This was accompanied by de novo methylation of the viral and vector sequences and was not erased by subsequent passage through the male germ line.

The study of a large number of genes in transgenic mice has shown that the tissue-specific expression depends on *cis*-acting elements carried by the injected sequences but also on host-flanking sequences in the integrated state (for reviews, see references 11 and 15).

To understand the basis for the restricted pattern of expression in our transgenic mice, we determined the methylation status of the transgene during development and in different tissues of adult mice.

In the transgenic mouse strains E36 and E11, about one copy of the recombinant plasmid pAC2 integrated into the mouse genome (1). This plasmid contains the pBR322 sequences and the HBV genome except for the capsid gene (Fig. 1). In the two strains, the major 2.1-kilobase (kb) surface antigen mRNA is synthesized principally in the liver. The 0.9-kb mRNA encoding the X viral transactivator is found in large amounts in the testis together with other larger species still uncharacterized. One of them, about 3 kb long, hybridizes only to HBV and could be due to a readthrough of the 2.1-kb surface antigen mRNA or to transcription from a cryptic promoter in the plasmid sequences (unpublished observation).

To analyze the methylation status of pAC2 sequences, we used the restriction enzyme *Hpa*II which recognizes the site CCGG and which does not cut when the site is methylated as follows: CmCGG. The methylated *Hpa*II site, however, can be cut by the isoschizomere *Msp*I. We also used in some experiments the enzymes *Xho*I, which cuts the site CTCGAG but not the methylated site CTmCGAG, and *Hha*I, which cuts the site GCGC but not the methylated site GmCGC. HBV sequences in the pAC2 plasmid contain three *Hpa*II sites, site 1 in the S coding region, site 2 in the enhancer, and site 3 in the X region (Fig. 1). There is one *Xho*I site near the third ATG of the S coding region and seven *Hha*I sites, two in the pre-S coding region and five in the enhancer and X coding region (see Fig. 3).

In the E36 strain, transmission of the transgene by males only produces mice that express HBsAg and which are called H+. Mice receiving the transgene from a female contain the transgene but do not express HBsAg and are called Hm.

We first analyzed the progeny of HBsAg-positive males mated to normal females. DNA was purified from frozen tissue samples as described previously (1). The samples were pulverized in liquid nitrogen and incubated for 17 h in lysis buffer (10 mM Tris hydrochloride [pH 8], 10 mM NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate) with 50 μg of proteinase K per ml at 37°C. Proteins were extracted with phenol. DNA (15 μg) was digested overnight with 60 U of restriction enzymes in the buffer recommended by the manufacturers. The completeness of digestion was checked by migration of 0.5 μg on an agarose minigel. The DNA was then subjected to electrophoresis in a 1 to 1.2% agarose gel and transferred to a nylon membrane (N-Hybond; Amer-

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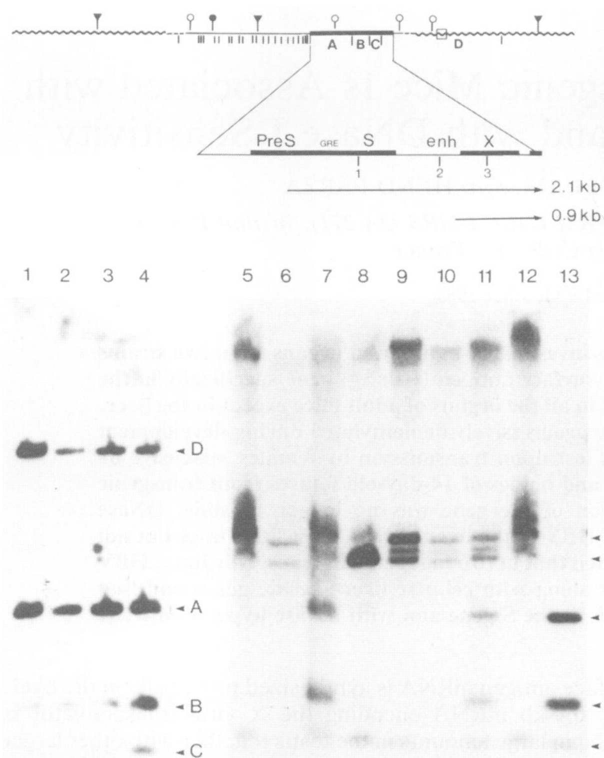

FIG. 1. Methylation status of HBV sequences in different organs of an E36 male and a female. Top: Organization of HBV and mouse flanking sequences. The pre-S, S, and X coding regions are represented by dark lines. GRE is the glucocorticoid element, and enh is the enhancer element. The thin lines are pBR322 sequences. Mouse sequences flanking the transgene are represented by a wavy line. The box represents the 4-12 probe described by Hadchouel et al. (7). Bars below the diagram represent *Hpa*II sites. *Eco*RI (○), *Pst*I (●), and *Pvu*II (▼) sites are shown. The two viral transcripts, 2.1-kb S mRNA and 0.9-kb X mRNA, are synthesized in the liver and the testis, respectively. Bottom: Southern blot analysis of *Msp*I- and *Hpa*II-digested DNA from different organs of a male (lanes 1 to 8) and a female (lanes 9 to 12). The total HBV genome was used as the probe. *Msp*I digestion of intestine (lane 1), heart (lane 2), liver (lane 3), testis (lane 4). *Hpa*II digestion of intestine (lanes 5 and 9), heart (lanes 6 and 10), liver (lanes 7 and 11), testis (lane 8), ovary (lane 12). *Msp*I-digested HBV DNA (13) was used as a size marker. On the side are indicated bands A, B, C, and D corresponding to total digestion of the transgene and the 2- and 2.4-kb fragments corresponding to partial *Hpa*II digestion.

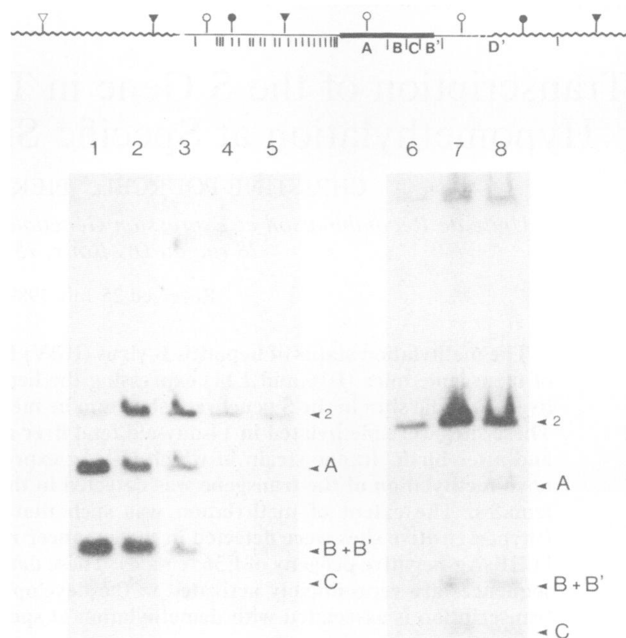

FIG. 2. Methylation status of HBV sequences in different organs of an E11 male and a female. The diagram is as described in the legend to Fig. 1. B' is an HBV-pBR band comigrating with band B. D' is the fragment of mouse DNA flanking the transgene on one side. DNA extracted from organs of an adult male (lanes 1 to 5) and an adult female (lanes 6 to 8) was restricted with *Msp*I (lane 1) or *Hpa*II (lanes 2 to 8) and hybridized with an HBV probe. Lanes: 1, 2, and 8, liver samples; 3, testis; 4 and 6, intestine; 5, sperm; 7, ovary.

sham Corp., Arlington Heights, Ill.). The prehybridization and hybridization were performed at 42°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as detailed in Babinet et al. (1). The complete HBV DNA used as a probe was prepared from plasmid pCP10 (3).

With *Msp*I, four bands were observed upon hybridization with an HBV probe in all the organs tested (Fig. 1, lanes 1 to 4). Bands A, B, and C correspond to HBV fragments of 1,286, 637, and 415 base pairs (bp), respectively. The fragment corresponding to the mouse sequences flanking HBV on the right was detected in band D.

With *Hpa*II, three different patterns of methylation were observed in the different organs. Band C was always seen in equal amounts in all the organs tested, showing that sites 2 and 3 were hypomethylated. The HBV bands A and B appeared strongly in the liver only (Fig. 1, lanes 7 and 11). In the other organs tested, including sperm (data not shown), these bands were always very faint or absent, showing that the *Hpa*II site 1 in the S coding region was methylated. In all the organs, additional bands were always produced by *Hpa*II digestion, owing to methylation of at least one-half of the sites. There were essentially two bands, one of about 2 kb corresponding to A plus B and one of 2.3 kb which hybridizes to pBR322 but not to the X region (data not shown). This band is due to partial methylation in the plasmid sequences. It is absent in the testis, which may be related to the presence of a cryptic promoter responsible for the synthesis of large RNAs in this organ only. The ovary DNA was not different from that of the other organs, but we could not analyze the oocytes for technical reasons. Bands B and D were never detected, although band C was produced, showing that the first site in the mouse DNA flanking the transgene was always methylated in adult tissues. These different patterns were repeatedly observed in three other animals tested. Digestion with *Hha*I gave similar results. The sites located upstream of the S coding region were also hypermethylated in nonexpressing organs, and in the testis, the sites of the plasmid region upstream of HBV were hypomethylated (data not shown). The *Xho*I site located upstream of the S ATG was resistant to digestion in all the organs except the liver.

We then analyzed the methylation status of HBV in different tissues of E11 mice (Fig. 2). In this strain, the progeny of both males and females express HBsAg when receiving the transgene. Restriction with *Msp*I produced the same bands A, B, and C as in E36 mice (Fig. 2, lane 1). An additional pBR-HBV fragment of 634 bp (band B') comi-

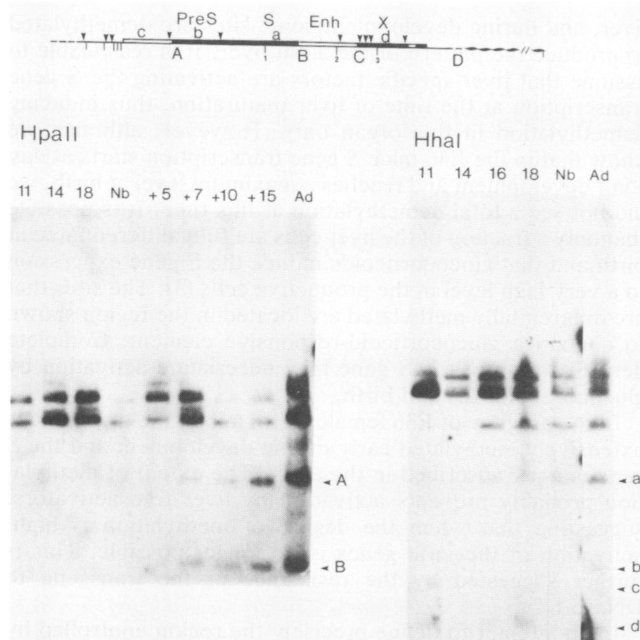

FIG. 3. Methylation of HBV sequences during development and in the first weeks of age. The diagram is as described in the legend to Fig. 1. The *Hha*I sites in HBV and in the plasmid sequences upstream are represented by arrowheads. *Hpa*II digested DNA from an 11-day-old embryo and from the livers of 14- and 18-day-old fetuses, newborns (Nb), mice 5, 7, 10, and 15 days after birth, and adults (Ad). Band C is absent from this blot but was seen in all the samples in other experiments. *Hha*I digested DNA from total embryo and liver samples as above. The positions of the *Hpa*II bands A and B and the *Hha*I bands a, b, c, and d, detected by an HBV probe, are shown on the side.

grated with the 637-bp HBV band B. D′ is the fragment flanking the transgene on the right. It contains a small fragment of plasmid and was not detected with an HBV probe. Upon restriction with *Hpa*II, band A was found in the liver of both a male and a female (lanes 2 and 8), in the testis (lane 3), and faintly in sperm (lane 5). In all the other organs tested (only intestine and ovary are shown in Fig. 2, lanes 4, 6, and 7), band A was absent or faint. Band A + B was found in all the organs, but the 2.3-kb band was never detected.

Since the S region is methylated in sperm DNA of both E36 and E11 mice, this suggests that demethylation must take place during development to produce the pattern of adult mice. We thus analyzed the methylation status of the transgene in the total fetus at 11 days and in the liver starting at day 14 of development after liver gene expression is activated.

An E36 transgenic male was mated to normal females, and the fetuses were recovered at different times of development. DNA was prepared from total bodies and from extraembryonic tissues at 11 days of development and from liver at 14, 16, and 18 days of development. Liver DNA was also prepared at birth and 5, 7, 10, and 15 days after birth. Digestions with *Msp*I and *Hpa*II were performed, and the pattern of methylation was compared with that of adult liver DNA. Upon *Msp*I digestion, a similar pattern was found in all the samples tested (data not shown). *Hpa*II digestion of 11-day-old embryo DNA and of DNA extracted from liver samples at 14 and 18 days of development produced the pattern of sperm, with no A and B bands (Fig. 3). These bands were only detected at birth, and the intensity increased to reach the adult level around 10 days after birth. Although the amount of DNA varied from one lane to the other, it was possible to evaluate the relative intensity of each band in one sample. Band D was detected faintly at 11 and 14 days of development.

Digestion of liver DNA with *Hha*I showed a slight demethylation of the S region upstream of the S gene (reflected by the presence of bands a, b, and c) from day 14 to birth, but it was still more methylated than in the adult liver (Fig. 3, right).

For the E11 strain, samples were recovered from the liver, body, and placenta at 14 days of development and the DNA was analyzed after *Hpa*II restriction (data not shown). In the body and the liver, band A was faint compared with that of the adult liver and testis, showing that the methylation status was still that of the sperm.

In progeny of E36 females, the transgene and flanking mouse sequences were found to be methylated over a large region in total fetus at 11 days and in liver, body, and yolk sac at 14 days of development (data not shown) and, to a lesser extent, in the placenta. To further analyze the structure of the transgene and the adjacent mouse sequences, we determined the DNase I sensitivity of the region in different tissue samples. We either purified nuclei and treated them with DNase I or used tissues that had been spontaneously degraded by internal DNases during thawing from −80 to 0°C. In this latter case, the detection of regions of hypersensitivity was very efficient. Nuclei were prepared from either fresh or frozen tissues in reticulocyte standard buffer and 0.5% Nonidet P-40 as described previously (16). They were digested at 37°C in reticulocyte standard buffer at a DNA concentration of 1 mg/ml with 0.1 DNase I U/μg of DNA (Sigma Chemical Co., St. Louis, Mo.) for variable times. The reaction was stopped with 10 mM EDTA. The DNA was then extracted and analyzed as described above.

Figure 4 shows some of the results obtained with E36 samples from H+ and Hm mice. For each experiment, the filters were hybridized with HBV, pBR322, and mouse probe 4-12 (probe 4-12 is a 270-bp mouse genomic fragment described in reference 7). After digestion with DNase, the DNA was restricted with *Pvu*II, which produces a 9-kb fragment hybridizing to the three probes and a 5-kb fragment hybridizing to pBR only. In the livers of HBsAg-positive E36 mice treated with DNase, three new bands were detected with an HBV probe, one of them usually of a stronger intensity. One site of hypersensitivity was positioned at 1,100 bp (in the enhancer region) and a weaker site was about 200 bp downstream. In some experiments, an additional site was detected in the pBR322 sequences. The localization of the sites was confirmed by restriction with two other restriction enzymes, *Eco*RI and *Pst*I (data not shown). The region was also DNase sensitive to some extent in kidney and intestine (data not shown) but was resistant in testis and spleen. The same sites were detected in the livers of E11 mice but not in the testis (data not shown). In the livers of Hm mice, degradation by increasing amounts of DNase I did not lead to the production of distinct bands, showing that the transgene was in a closed chromatin configuration (Fig. 4, lanes Hm).

Developmental and tissue-specific regulation of gene expression involves the interaction between *cis*-acting elements and the corresponding cellular transactivators. Results from different laboratories have suggested that the access to the regulatory sequences of certain genes is controlled by their chromatin configuration, which is re-

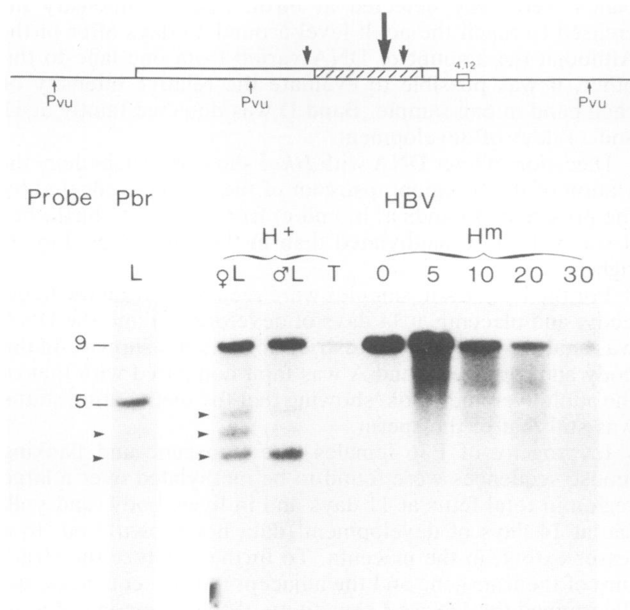

FIG. 4. DNase I sensitivity of HBV sequences in the E36 strain. Top: The diagram shows the *Pvu*II sites in the transgene and the flanking mouse sequences. Open bars are plasmid and hatched bars are HBV sequences. The DNase-hypersensitive sites are indicated by arrows. Bottom: Nuclei were prepared from liver (L) or testis (T) of an HBsAg-positive (H+) mouse and from liver of an HBsAg-negative (Hm) mouse. The H+ samples were digested for 20 min as described in Materials and Methods. The Hm liver sample was digested for 0, 5, 10, 20, and 30 min. The DNA was then purified and restricted with *Pvu*II. The probes are pBR322 and HBV. The arrowheads on the side represent the bands generated by cutting at a hypersensitive site.

flected by the methylation status of the DNA and its sensitivity to DNases (9). In several instances, tissue-specific genes were shown to be hypomethylated and hypersensitive to DNase I in the expressing organs only. It is thought that after inactivation of genes that should not be expressed at a certain time of development, methylation may help maintain this state. However, if specific factors interact with the regulatory sequences to activate the gene, demethylation takes place (8–17). Methylation may also be the way by which certain regions of the chromosomes are differentially imprinted during gametogenesis (13).

We asked whether, in our transgenic mice, there is a relationship between the tissue specificity of the viral gene expression and the methylation status of the transgene and whether hypermethylation prevent transcription.

A total of 11 restriction sites inactivated by mCG methylation were analyzed, 4 of them located in the pre-S and S coding regions. The results showed that the sites in the S region are hypomethylated only in the livers of the E11 and E36 strains where the S gene is transcribed and in the testis of E11 strains. In this latter organ, large transcripts covering the S region were found. Thus, although we saw a relationship between S gene expression in the liver and hypomethylation, the results suggest that this is not the only requirement for gene expression and that transactivators are necessary.

In the sperm, the S gene is more methylated than in the liver, and during development some sites are demethylated to produce the pattern of the adult liver. It is reasonable to assume that liver-specific factors are activating the S gene transcription at the time of liver maturation, thus inducing demethylation in this organ only. However, although we know that in the E36 mice S gene transcription starts at day 15 of development and reaches a maximum level at birth, we did not see a total demethylation at this time. It is possible that only a fraction of the liver cells are fully differentiated at birth and that glucocorticoids induce the S gene expression to a very high level in the productive cells (5). The sites that are differentially methylated are located in the region shown to contain a glucocorticoid-responsive element. Complete demethylation of the S gene may necessitate activation by glucocorticoids around birth.

In the progeny of E36 females (Hm mice), the transgene is extensively methylated early during development and the S gene is not transcribed in the adult. The extent of methylation probably prevents activation by liver transactivators, suggesting that when the degree of methylation is high, activation of the viral genes is no longer possible. This is further suggested by the resistance of the transgene to DNase I.

In an attempt to define precisely the region controlled by methylation, we are presently analyzing the S gene expression in transfected cells after in vitro methylation of HBV DNA. We have already found that methylation of only *Hpa*II sites or only *Hha*I sites is not sufficient to prevent transcription in cell culture (unpublished observations). There are other CG in the S region which do not belong to a restriction site and which are probably involved in the methylation.

We have obtained other transgenic mice in which HBV sequences were totally methylated. The founder mice and their progeny never expressed HBsAg (4). In these cases and in the case of Hm mice, the transgene expression has been probably negatively influenced by the surrounding mouse sequences. In addition, we have found that in tumorous cells derived from HBsAg-positive E36 mice, S gene expression is lost and de novo methylation is observed (12; unpublished observations). These different observations suggest that hypermethylation of the viral sequences follows inactivation of the viral gene expression.

In many hepatocarcinomas developing in a chronically infected liver, HBV DNA is integrated and the viral genes are no longer transcribed. De novo methylation is often detected (10) and may help maintaining the inactivation of the core and S gene expression, thus preventing the destruction of the cell by the immune response.

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