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In Vivo Activity of the Hepatitis B Virus Core Promoter: Tissue Specificity and Temporal Regulation

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The contribution of the hepatitis B virus enhancers I and II in the regulation of the activity of the core and the X promoters was assessed in transgenic mice. Surprisingly, despite the presence of heterologous promoters linked 5′ of the X gene, the transgene expression is mostly due to core promoter (Cp) activity present in the X coding sequence. Moreover, the restriction of Cp activity to hepatic tissue required the combined action of both enhancers I and II, whereas the proximity of these two enhancers was insufficient to confer tissue specificity on Xp activity. Furthermore, the liver-specific activity of the Cp was developmentally regulated in an enhancer I-independent manner.

Hepatitis B virus (HBV), which belongs to the hepatotropic family *Hepadnaviridae*, replicates almost exclusively in the liver. This specificity might reflect both viral recognition of a specific receptor on the liver cell surface and the requirement for specific hepatocytic transcriptional factors for the transcription and replication of the virus. The transcription of HBV mRNAs is controlled by four different promoters—the core (Cp), the pre-S1, the pre-S2/S, and the X (Xp) promoters—which produce the 3.5-, 2.4-, 2.1-, and 0.8-kb RNAs, respectively. The polyadenylation of these RNAs occurs at the unique polyadenylation signal (PAS) located 100 bp downstream of the Cp. RNAs initiated at the Cp are efficiently polyadenylated only at the second pass of the RNA polymerase on the PAS, which allows the synthesis of transcripts longer than the genome size involved in the replication of the viral genome. Optimal expression of HBV transcripts from Cp and Xp requires two enhancers. Enhancer I, which is embedded within the Xp, requires both liver and ubiquitous nuclear factors for activity (6, 7, 14, 17). Although enhancer I displays preferential activity in hepatic cell lines (3, 7, 8, 11, 12, 18, 20), it is also functional in a variety of cell lines (5, 21). Therefore, the tissue specificity of this enhancer in vitro is still controversial. Enhancer II, which is associated with the Cp, binds multiple liver and ubiquitous factors (9, 13, 26, 28) and is highly specific for hepatic cell lines (22, 24, 25). As the 3.5-kb RNA serves as the template for the synthesis of genomic DNA, the synthesis of this transcript, which is governed by the Cp, is crucial for the viral life cycle of HBV. There is no evidence demonstrating that the association of the Cp with enhancer II, by itself, is sufficient for cell-type-restricted transcription of pregenomic RNAs in vivo. Furthermore, enhancer I does not seem to be indispensable for Cp activity in hepatic cell lines (10, 19, 26, 27). However, the elements that contribute to the liver-specific activity of the Cp in vivo have not yet been characterized.

In the present study, we report an analysis of the viral sequences contributing to the liver-specific activity and to liver-specific developmental regulation of the Cp in transgenic mice. (All animal experiments reported in this article were performed in accordance with French government regulations [Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture]).

The three constructs microinjected in C57BL/6xDBA2 hybrid embryos are depicted in Fig. 1. The *Acc*I-to-*Bgl*II fragment (nucleotides [nt] 826 to 1987) was excised from the pFC80 plasmid, which carries four copies of the HBVayw genome. This PEX fragment (1161 bp) contained the X coding sequence under the control of its own regulatory sequences (enhancer I and Xp). For the ATIII and the SVX constructs, the X coding sequence from the *Nco*I site to the *Bgl*II site (nt 1374 to 1987) was 3′ linked to either the 680 bp (*Sau*3A to *Sau*3A) of the hepatospecific antithrombin III (ATIII) regulatory sequences (16) or the simian virus 40 (SV40) early region promoter/enhancer (*Hpa*II to *Hin*dIII), respectively. Because of the genetic organization of HBV, enhancer II and Cp were present in all the transgenes. Five transgenic founders were obtained and further analyzed (Fig. 1).

As all transgenes contained two promoters and the HBV weak polyadenylation signal, we expected to detect different species of RNAs. The theoretical lengths of these different RNAs are indicated in Fig. 2. Total RNAs prepared from many tissues were subjected to Northern (RNA) blot analyses. As expected, the Northern blot (Fig. 3) showed that more than one species of RNA was produced. In parallel we performed RNase T_2 mapping with different riboprobes specific for Cp, Xp, or the ATIII promoter. The Cp probe, which covers the Cp and the PAS (Fig. 1), produced four protected fragments (Fig. 4). Protected fragments 1 and 2 correspond to transcripts initiated either at the 5′ promoter or at the Cp of the preceding transgene in the tandem array (Fig. 2). The largest protected fragment (fragment 1; 267 nt in SV28 and AX mice and 257 nt in PEX mice) corresponds to the full protected probe and detected read-through events. Protected fragment 2 (210 to 212 nt) corresponds to the probe protected at its 3′ end and digested at a site corresponding to PAS at its 5′ end (Fig. 1). This fragment detects polyadenylation events (Fig. 2). Protected fragment 3 (189 nt) corresponds to transcripts of unknown origin. Protected fragment 4 (Cp in Fig. 2, 4, and 5) corresponds to transcripts initiated from Cp and polyadenylated after one or two read-through events (174 to 179 nt in the SV28 and AX transgenic lines and 158 to 165 nt in the PEX

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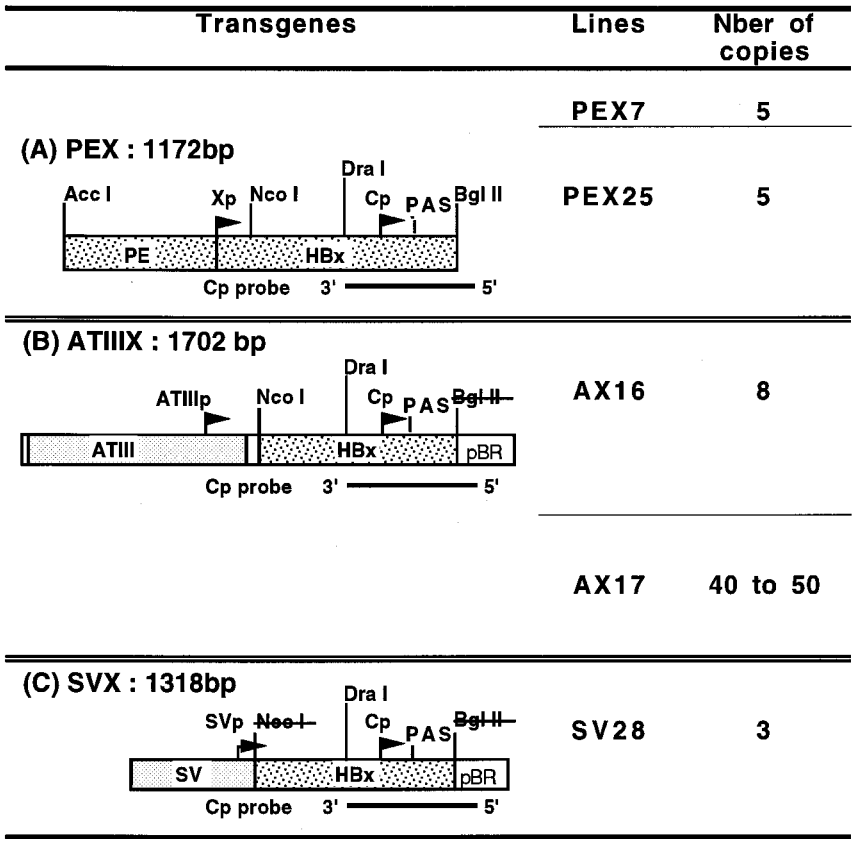

FIG. 1. Structure of the microinjected sequences and characterization of the transgenic lines. PE, HBV enhancer I and Xp; SV, SV40 early regulatory sequences; HBx, HBV X coding sequence. The putative initiation start sites are indicated (arrowheads). The Cp-specific riboprobe used in RNase T_2 mapping is shown below each transgene. The *Dra*I site corresponds to the 3′ end of the probe. Sites that were modified are crossed out.

line). Detection of the fourth protected fragment reflects the Cp activity. The size of this fourth protected fragment allowed us to deduce the transcription initiation site for the Cp, which is in agreement with a previous report (23). This Cp activity was also demonstrated by primer extension with specific oligonucleotides (data not shown). The detection of the first, second, and also the fourth protected fragments reflected an efficient read-through of the PAS. The first and second protected fragments were detected in all the analyzed tissues. This result is consistent with previous data concerning HBV replication, which indicated that the read-through of the PAS may occur in different cell types (15). RNase mapping analysis never detected any transcripts corresponding to an initiation at the Cp without read-through of the PAS 100 bp downstream (115-nt protected fragment), in agreement with the report of Cherrington et al. (4). As shown in Fig. 4 and Table 1, in both AX lines the Cp was active in the liver, lungs, kidneys, stomach, and intestine. In the AX16 line, it was also active in the testis. In the SV28 line, the Cp was active in the same organs as in the AX lines and in the spleen and thymus. In contrast, this promoter was active only in the liver of the PEX7 transgenic line. In the other tissues, as well as in the testis tissue of the AX17 mice, this promoter was inactive. No transcripts initiated from the Cp were detected in the liver of the PEX25 founder (data not shown), suggesting that the age of the animals may be important for this promoter activity. We next studied the use of the 5′ promoter (i.e., SV, ATIII, or X) by RNase T_2, primer extension, and Northern blot analysis. These experiments indicated that, in the SV28 line, the SV promoter was active in the kidneys and spleen. In the PEX lines, the Xp was active in all tissues tested (liver, lung, kidneys, intestine, stomach, spleen, thymus, heart, brain, testis, and tongue), whereas the ATIII promoter in the AX lines was inactive except in the testis tissue of AX17 mice (data not shown). These results are summarized in Table 1.

The Cp activity is detectable only in the liver of the PEX7 line, in which enhancer I is present, whereas it is active in various tissues of the SV28 and AX lines, which lack this enhancer (Table 1). The results presented here suggest that the Cp associated with enhancer II is ubiquitous and that enhancer I, in combination with enhancer II, is responsible for the liver-restricted activity of the Cp. In the SV28 mice, the large pattern of activity of the Cp could be due to an influence of the SV40 enhancer. However, the tissue distribution of the Cp activity in the SV28 mice is similar to that in the AX lines, in which the 5′ promoter should be specific for the liver. The wide tissue distribution of the Cp activity suggests that these 5′ sequences (ATIII or SV40) do not have any influence and that the ubiquitous activity is due to the core gene regulatory sequences. Furthermore, X transcripts synthesized from the Xp are detected in all tested tissues from PEX transgenic mice in which enhancers I and II are present (Table 1). These results suggest that, in vivo, the association of these enhancers has no influence on Xp tissue specificity.

These observations correlate well with functional analyses of the Cp, which showed a combinatorial effect of the core gene

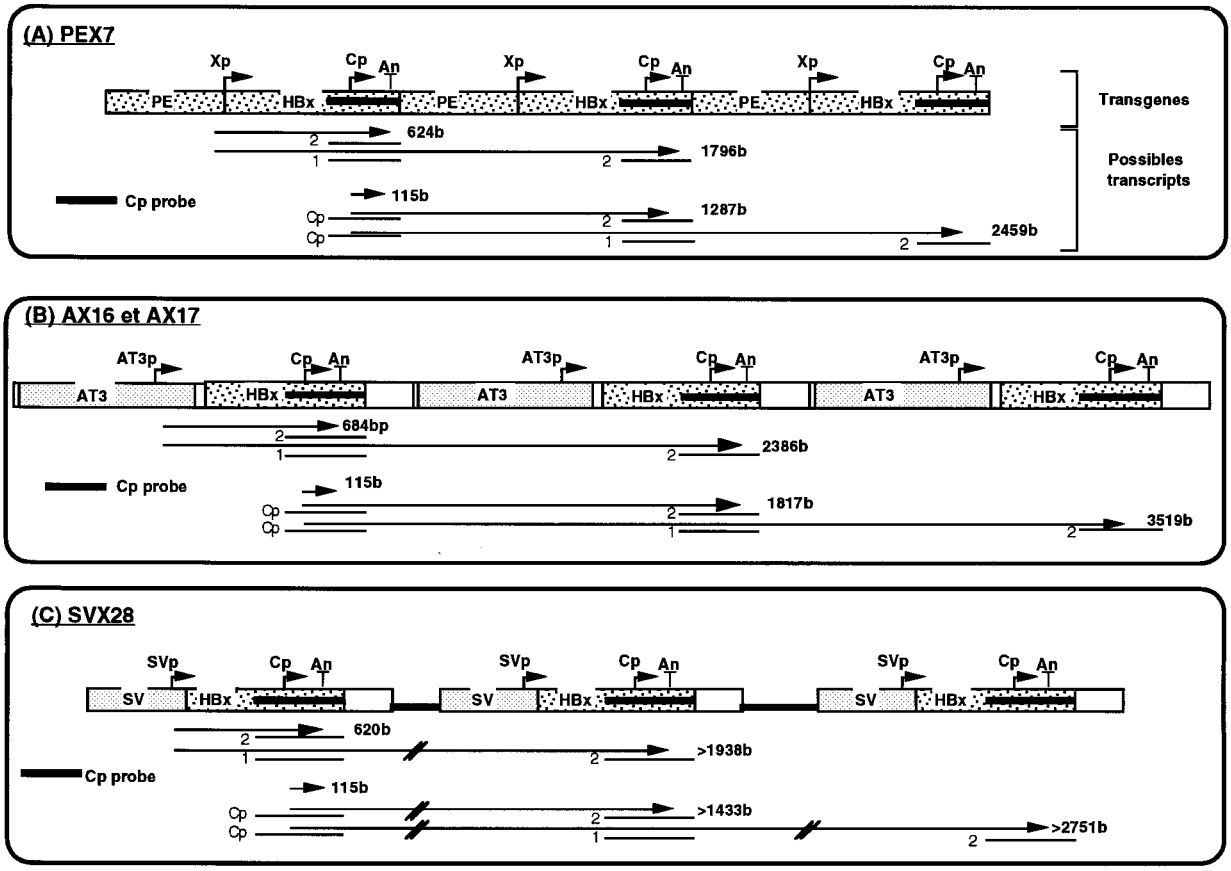

FIG. 2. Transgene integrations and theoretical lengths of RNAs. Cp probe, Cp-specific riboprobe used for RNase T_2 mapping analysis (Fig. 4 and 5). 1, 2, and Cp, hybridizations of the Cp-specific probe on the different RNAs, corresponding to the protected fragments described in the legend to Fig. 4. PE, X gene regulatory region (enhancer I and Xp); AT3p, ATIII promoter; SV, SV40 early regulatory sequences; HBx, HBV X coding sequence; b, bases; An, polyadenylation site.

regulatory sequences and enhancer I on tissue specificity (10, 26) and a more efficient effect of enhancer I on the Cp than on the other HBV promoters (1).

Enhancer I has a different effect on tissue specificity that

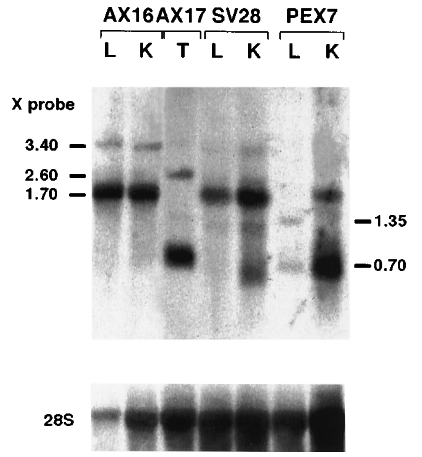

FIG. 3. Northern blot analysis of X-specific transcripts. Total RNAs from the different transgenic lines (10 μg [AX16 liver] or 40 μg [other samples]), extracted from various tissues, were fractionated in denaturing conditions on a 1.5% agarose gel and transferred to a nylon membrane. The membrane was then hybridized with a ^{32}P-labelled X-specific probe. L, liver; K, kidney; T, testis.

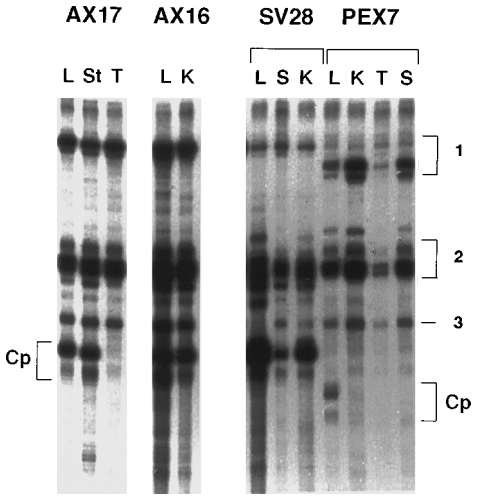

FIG. 4. RNase T_2 mapping analysis of X transcripts. The specific Cp probe (Fig. 3) was hybridized with 10-μg (AX16 liver) or 20-μg (other tissues) samples of total RNAs from the different transgenic lines. We detected four types of protected fragments: 1, fully protected probe by internal hybridization corresponding to transcripts initiated from the 5′ promoter or the Cp of the preceding transgene and with one or more read-through; 2, 5′ digested probe corresponding to the 3′ end of transcripts initiated at the 5′ promoter or the Cp of the preceding transgene; 3, protection not yet elucidated; and Cp, 3′ digested probe corresponding to the 5′ end of the transcripts and specific for the Cp activity (Fig. 2). L, liver; St, stomach; T, testis; K, kidney; S, spleen.

TABLE 1. Promoter activities in transgenic lines

Mice (age) and promoter	Activity										
	Liver	Lung	Kidney	Intestine	Stomach	Spleen	Thymus	Testis	Heart	Brain	Tongue
AX16 (2 wk)											
Cp	+	+	+	+	+	−	−	+	−	−	ND[a]
ATIIIp	−	−	−	−	−	−	−	−	−	−	ND
AX17[b]											
Cp	+	+	+	+	+	−	−	−	−	ND	ND
ATIIIp	−	−	−	−	−	−	−	+	−	ND	ND
SV28 (3 wk)											
Cp	+	+	+	+	+	+	+	ND	−	−	−
SVp	−	−	+	−	−	+	−	ND	−	−	−
PEX7 (3 wk)											
Cp	+	−	−	−	−	−	−	ND	−	−	−
Xp	+	+	+	+	+	+	+	ND	+	+	+
PEX25 (13 mo)											
Cp	−	−	−	−	−	−	−	ND	−	−	−
Xp	+	+	+	+	+	+	+	ND	+	+	+

[a] ND, not done.
[b] Testis tissue, 6-week-old mice; other tissues, 1-week-old mice.

depends on the promoter. This was already suggested by Guo et al., who showed that the ubiquitous HSV thymidine kinase promoter associated with enhancer I is active only in hepatic cell lines whereas the Xp associated with enhancer I is active in hepatic as well as in nonhepatic cell lines (8). The molecular basis for this variable effect is unknown, but it could depend on the combination of the transcriptional factors bound to enhancers I and II that could interact specifically with proteins bound to the Cp but not with proteins bound to the Xp.

The mice used in this study were first designed to study the effect of the in vivo expression of the X protein. Histopathological examination of transgenic mice has not shown serious liver damage in any of the mice, although a functional X protein is expressed, at least in AX16 transgenic mice (2). Since none of our mice presented any liver pathology, we hypothesized that this could be due to a defect in the expression of the transgene in the liver. We were therefore interested in the time course of transgene expression in different tissues of the AX and PEX transgenic lines. In the PEX7 transgenic line, RNase mapping analysis with core and X-promoter probes showed that the number of transcripts initiated from the Cp declined more rapidly over time, to become undetectable in 17-week-old mice, than the number of transcripts initiated from the Xp (Fig. 5A, lanes 1 to 5 versus lanes 6 to 10). In the AX16 (Fig. 5B) and AX17 (data not shown) transgenic lines, the results of RNase mapping indicated that the Cp also became rapidly inactive in a liver-restricted manner (from 8 days for the AX17 line to 8 weeks for the AX16 line) (lanes 11 to 16), whereas this promoter remained active in other tissues up until several months (lanes 17 to 21 and data not shown). Taken together, these results suggest that the developmental extinction of the Cp in vivo was independent of enhancer I but was dependent on the hepatic cell type. This observation led us to hypothesize either that Cp activity is dependent on the proliferative status of hepatocytes or that certain transcriptional factors crucial to Cp activity are absent in quiescent hepatocytes. In conclusion, the Cp appears to be tightly regulated in vivo at various levels, with liver-specific activity being exclusively dependent on enhancer I and developmental regulation being independent.

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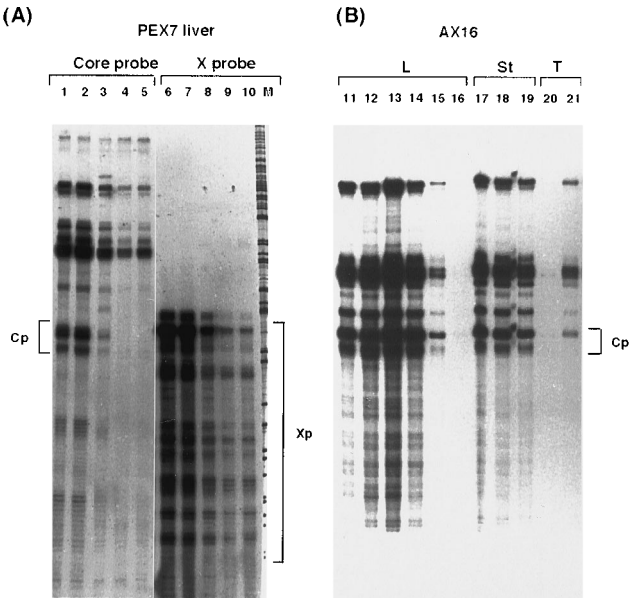

FIG. 5. Analysis of Cp activity during liver development. (A) Twenty micrograms of total RNA from the liver tissue of PEX7 transgenic mice was analyzed by RNase T_2 mapping using the Cp-specific riboprobe or the Xp-specific riboprobe (Fig. 2). Lanes 1, 2, 6, and 7, 3-week-old mice, lanes 3 and 8, 6-week-old mice; lanes 4, 5, 9, and 10, 17-week-old mice; lane M, molecular weight markers. (B) Ten or 15 μg of total RNA from an AX16 mouse tissue was subjected to RNase T_2 mapping with the Cp probe. Lanes 11 to 16, 2.5-day-old, 1-week-old, 2-week-old, 6-week-old, 4.5-month-old, and 18-month-old mice, respectively; lanes 17 to 19, 2-week-old, 6-week-old, and 18-month-old mice, respectively; lane 20, 8-day-old mouse; lane 21, 18-month-old mouse. L, liver; St, stomach; T, testis. Cp, protected fragments corresponding to transcripts initiated from Cp; Xp, protected fragments corresponding to multisite-initiated transcripts from the Xp.

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