JOURNAL OF VIROLOGY, Feb. 1989, p. 579–583
0022-538X/89/020579-05$02.00/0
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Vol. 63, No. 2

Hepatitis B Virus (HBV) Promoters Are Regulated by the HBV Enhancer in a Tissue-Specific Manner

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Received 13 June 1988/Accepted 17 October 1988

The activities of the individual hepatitis B virus (HBV) promoters and the effects of the HBV enhancer on these promoters in several human cell types have been compared by measuring the activity and RNA levels of the linked reporter function chloramphenicol acetyltransferase. The relative promoter activities in the human HepG2 (liver), HeLa, and HS27 (fibroblast) cell lines are in the order precore > X > preS2 > preS1; thus, the promoters of the gene producing the largest quantity of viral proteins have relatively low activity. The juxtaposition of the HBV enhancer in either orientation increased the promoter activities only modestly (2- to 5-fold) in the nonliver cell lines, whereas it dramatically increased (20- to 100-fold) the promoter activities in the liver cell line. Thus, the HBV enhancer is especially active in liver cells. This may be one of the causes of hepatotrophicity of the virus.

The human hepatitis B virus (HBV) causes chronic liver disease and has been linked to hepatocellular carcinoma (13, 27). The DNA sequences of several subtypes of HBV have been determined (7, 8, 17, 18, 29). The virus has four promoters (3, 10, 15, 21, 23–26, 28): the precore promoter expresses the largest HBV transcript, which encodes the core antigen and probably the polymerase; the preS1 and preS2 promoters express transcripts which encode the different forms of the S antigen; and the X promoter transcribes the X gene, which encodes a protein that acts as a *trans*-activator.

An enhancer element has been detected upstream from the X promoter (21, 22). Its stimulatory effects on some of the HBV promoters have been reported (2, 20), but whether it acts in a tissue-specific fashion is still controversial (6, 12, 22, 30).

In the work described in this report, we have determined the relative strengths of the four HBV promoters and the effects of the HBV enhancer on the activities of the promoters. For these studies, the precore, preS1, and preS2 promoters of HBV were fused to the coding sequence for bacterial chloramphenicol acetyltransferase (CAT). The HBV enhancer also has been juxtaposed in both orientations 5′ to each of these promoters. The elements of the X promoter and the HBV enhancer have not been physically resolved and were treated as a single unit. The HBV enhancer was also tested on the herpes simplex virus thymidine kinase (*tk*) promoter to determine whether its effects were specific to HBV. All chimeric CAT constructs were tested for CAT activity and specific mRNA levels in a highly differentiated human liver cell line (HepG2) and in two human nonliver cell lines (HeLa and HS27 fibroblasts). The HBV enhancer exhibited greater activity (10- to 30-fold) in the liver cell line than in the nonliver cell lines.

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MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* HB101 (*recA*) was grown in standard LB medium (16) containing 50 μg of ampicillin per ml to select for recombinant plasmids. This strain was used for all recombinant DNA manipulations.

Mammalian cell lines. The HepG2 cell line is a highly differentiated line derived from a human hepatoma, HeLa is a well-established cell line from a human cervical carcinoma, and HS27 was derived from human foreskin. All cell lines were obtained from the Cell Culture Facility at the University of California at San Francisco. Mammalian cells were grown in Dulbecco modified Eagle medium–10% fetal calf serum containing penicillin and streptomycin each at a final concentration of 100 U/ml.

Materials. Restriction enzymes, polynucleotide kinase, and DNA polymerase (large fragment) were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany. The T4 DNA ligase and calf intestine alkaline phosphatase were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. The reverse transcriptase was obtained from Life Sciences, Inc., St. Petersburg, Fla. The [^{14}C]chloramphenicol for CAT assays and [α-^{32}P]dATP were purchased from Du Pont, NEN Research Products, Boston, Mass. Nonradioactive deoxynucleoside triphosphates were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. The plasmid carrying the Rous sarcoma virus long terminal repeat fused to β-galactosidase coding sequences (pRSV-β-gal) has been described earlier (1).

Molecular cloning. All the recombinant DNA manipulations described for this work involved standard techniques (16). The plasmids pTE2ΔSN and pTE2ΔtkΔSN were similar. These two plasmids are pUC18-based subclones of plasmid pTE1, which has been described previously (5). Figure 1 shows the cloning scheme used to make the CAT constructs in this study. The 3′ border of the DNA fragments of the HBV promoter always excluded the HBV ATG codons known to signal an open reading frame. Thus, translation should be initiated at the correct ATG of the CAT coding sequence. The 5′ borders of the promoter fragments were extended as far as possible to include all potentially

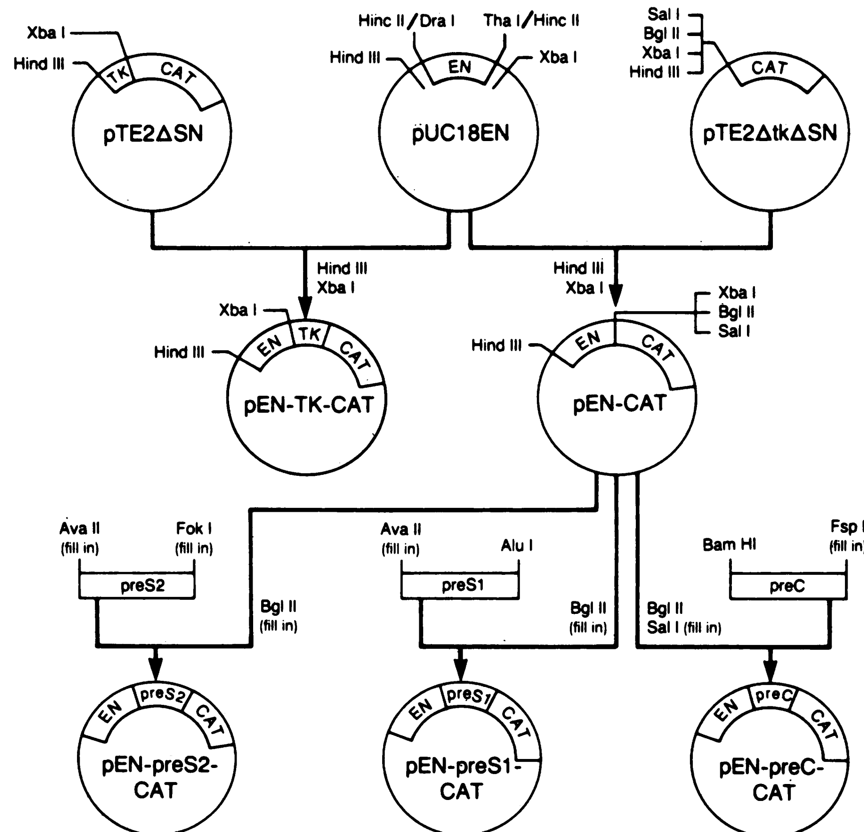

FIG. 1. Construction of CAT plasmids. The HBV enhancer (EN) was inserted into pUC18 in both orientations, although only one is shown in this figure. For all constructs shown (except pEN-preC-CAT), a second plasmid was made with EN in the reverse orientation.

active promoter elements but not the coding sequences of the upstream HBV gene.

For future cloning convenience, the entire HBV enhancer region (EN) and the overlapping X gene promoter (*Dra*I-*Tha*I fragment of 519 base pairs) were inserted into the *Hin*cII site of pUC18 in both orientations relative to the other polylinker sites. The HBV enhancer region was then excised from pUC18 by using *Hin*dIII and *Xba*I and ligated to pTE2ΔSN and to pTE2ΔtkΔSN (Fig. 1). The precore promoter contained in a 401-bp *Bam*HI-*Fsp*I fragment was cloned 5′ to the CAT coding sequence by using the *Bgl*II and *Sal*I sites in the polylinker of plasmid pEN-CAT. For this construction both the *Fsp*I and the *Sal*I sites were filled in with Klenow polymerase. The preS1 promoter, consisting of a 478-base-pair *Ava*II(filled-in)-*Alu*I fragment, was inserted 5′ to the CAT gene by using the *Bgl*II site (filled-in) in the polylinker of plasmid pEN-CAT. A 258-base-pair *Ava*II (filled-in)-*Fok*I fragment containing the preS2 promoter was cloned into the *Bgl*II site (filled-in) of plasmid pEN-CAT. Although Fig. 1 shows the manipulations described above, similar ligations were also carried out with pTE2ΔtkΔSN instead of pEN-CAT.

Transfection of mammalian cells. Mammalian cells were transfected by calcium phosphate coprecipitation (9); 10 μg of test plasmid DNA was used with HeLa cells and HS27 cells, and 10 μg of test plasmid DNA plus 5 μg of pRSV-β-gal plasmid DNA was used with HepG2 cells. The plasmid DNAs intended for transfections were prepared by alkaline lysis followed by two successive CsCl gradient purifications as described previously (16).

Enzyme assays. The transfected cells were incubated for 48 h, at which time homogenates were prepared and CAT assays were done as described previously (31). Either 50 μg of protein or 100 μg of protein was assayed for 1, 2, or 5 h, depending on the amount of CAT activity in each extract. The spots on the chromatograms corresponding to the unacetylated and the monoacetate forms of chloramphenicol were cut out, and the radioactivity was quantitated by scintillation counting. The enzymatic activity of β-galactosidase was quantitated by standard methodology (1, 11), except that the phosphate buffer was at pH 8. For each transfection of the HepG2 cell line, CAT activity was normalized to β-galactosidase activity as a control for the efficiency of transfection. For the HeLa and the HS27 cells, each transfection was done in triplicate to account for variations in the transfection efficiency. The percent CAT conversion shown for each construct in HeLa and HS27 cells is the mean of triplicate values, and the standard error was less than 30% of the mean value. For each of the three cell lines, CAT activity from pTE2ΔSN was set equal to 1 so that comparison within a cell line was simplified.

RNA analysis. Total RNA from at least 10^8 cells per transfection was prepared by the guanidinium-CsCl method (4). Three to six transfections were set up for each construct, and the cell extracts were pooled to isolate the RNA, thus averaging out differences in transfection efficiencies. Total RNA was separated on 1.5% agarose–formaldehyde gels (14) at a maximum rate of 5 V/cm. RNA (20 μg) was loaded for each sample, except pEN-TK-CAT and pEN-preC-CAT (8 and 10 μg, respectively), so that all densitometry measure-

TABLE 1. HBV promoter-enhancer activities in liver and nonliver cells

Construction	% CAT conversion			Fold enhancement
	Without enhancer	With enhancer	With enhancer[a]	
HepG2 cells[b]				
TK-CAT	1[c]	19	20	20
preC-CAT	0.35	22	NA[d]	60
preS2-CAT	0.05	2.9	2.9	58
preS1-CAT	0.02	1.9	2.0	98
X-CAT	NA	8.8	NA	
HeLa cells				
TK-CAT	1[c]	2.2	2.5	2
preC-CAT	0.1	0.5	NA	5
preS2-CAT	0.002	0.004	0.004	2
preS1-CAT	ND[e]	0.004	0.004	
X-CAT	NA	0.5	NA	
HS27 cells				
TK-CAT	1[c]	2.2	1.8	2
preC-CAT	0.07	0.2	NA	3
preS2-CAT	0.01	0.03	0.03	3
preS1-CAT	ND	0.02	0.01	
X-CAT	NA	0.1	NA	

[a] The HBV enhancer was inserted in the 3'-to-5' orientation with respect to the X promoter.
[b] Values for the HepG2 cell line are normalized to β-galactosidase activity.
[c] The actual value represented by 1 in the HepG2 cell line is 10 times greater than the values represented by 1 in the HeLa and the HS27 cell lines.
[d] Construct was not available.
[e] CAT activity was not detectable.

ments could be made from one autoradiogram. The RNA was transferred to GeneScreen (Du Pont, NEN) for at least 48 h. The probe was the 1.65-kilobase *Hin*dIII-*Bam*HI fragment containing the CAT coding sequences which had been nick translated with [α-^{32}P]dCTP by a standard method (19). Autoradiography was done at −70°C with intensifying screens for at least 24 h.

RESULTS

Relative promoter activities. Table 1, column 1, shows the relative CAT activities of the promoter-CAT constructs in the HepG2, HeLa, and HS27 cell lines. The order of the promoter strength in all cell lines is TK ≥ precore > preS2 > preS1 (no unenhanced X promoter constructs were available). The CAT activity for pTE2ΔSN in the HepG2 cell line was approximately 10-fold higher than the activity in the HeLa and the HS27 cell lines. This is probably due to differences in transfection efficiencies between the cell lines.

Although the relative promoter order was the same in all three cell lines, quantitative differences between promoters were apparent. For example, in HepG2, HeLa, and HS27 cells, the activity ratios of the TK to the core promoters were 3, 10 and 14, respectively, and the ratios of precore to preS2 promoters were 7, 50, and 7, respectively. These quantitative differences between promoter activities in the three cell lines were evident after experimental variation such as transfection efficiency had been accounted for.

The enhancer effect is maximal in liver cells. The presence of the HBV enhancer resulted in increased CAT activity for each promoter tested in all three cell lines (Table 1, column 2). However, the enhancer effect was markedly greater in the liver cell line. The enhancer increased CAT gene expression only 2- to 5-fold in the HeLa and HS27 cell lines, whereas the effect was 20- to 100-fold in the HepG2 cell line. The enhancer was at least 10-fold more effective for each promoter tested in the HepG2 cell line than in the HeLa and HS27 cell lines (Table 1, column 4). For the TK, preS1, and preS2 promoters, the enhancer exerted the same effect independent of orientation (Table 1, column 3).

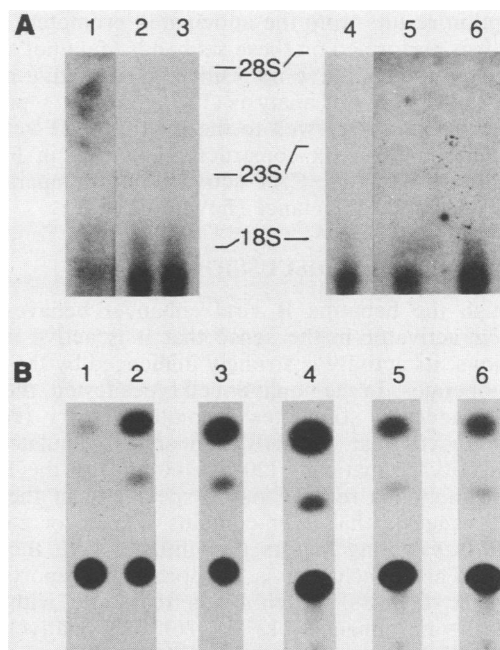

FIG. 2. (A) Northern blot of total RNA from HepG2 cells. Total RNA was isolated from HepG2 cells transfected with the following CAT constructs: pTE2ΔSN (lane 1), pEN-TK-CAT (lane 2), pEN-CAT (lane 3), pEN-preC-CAT (lane 4), pEN-preS1-CAT (lane 5), and pEN-preS2-CAT (lane 6). The probe was the ^{32}P-labeled 1.65-kilobase *Hin*dIII-*Bam*HI fragment from pTEΔSN containing the CAT structural gene. The positions of rRNA markers are shown by arrows in the center of the figure. (B) Cat assays in HepG2 cells. For each sample, 50 μg of protein was assayed. Lanes: 1, pTE2ΔSN; 2, pEN-TK-CAT; 3, pEN-CAT; 4, pEN-preC-CAT; 5, pEN-preS1-CAT; 6, pEN-preS2-CAT.

Relative enhancer promoter activities. As was the case for the unenhanced promoters, the enhancer-promoter regulatory units displayed activity in the order TK ≥ precore ≥ X > preS2 > preS1. However, the relative strengths of the regulatory units were quantitatively different in the various cells. In HepG2, HeLa, and HS27 cells in the presence of enhancer, the ratios of TK to precore activity were 1, 4, and 10, respectively, and the ratios of precore to preS2 activity were 7, 100, and 6, respectively. Thus, the relative activities of the unenhanced promoters and the enhancer-promoter units were similar for each cell type.

Confirmatory mRNA analysis. Figure 2A shows a Northern (RNA) blot analysis of the mRNA in HepG2 cells. Each lane contains RNA from several transfection experiments performed with individual CAT constructs. The CAT mRNAs produced from the TK promoter (lane 1) and the enhancer-TK promoter unit (lane 2) comigrate; thus, transcription is initiated at the TK promoter and not within the HBV enhancer. The mRNAs in lanes 1 to 3 are ca. 1.64 kilobases, and those in lanes 4 to 6 are ca. 1.71 kilobases; these lengths are expected if transcription is driven by the anticipated promoter. In addition, the upper portion of the blot has no detectable mRNA bands (even with 10-day exposures; data not shown). This confirms that all detectable

transcription results from the anticipated promoter. Densitometry was performed on these six bands (data not shown) to determine their relative intensities. The relative mRNA levels deduced from this analysis (TK > precore > preS2 > preS1) correspond very well to the relative CAT activities observed from these six constructs. The data in Fig. 2B confirm that the relative CAT activities are comparable to the relative mRNA abundance shown in Fig. 2A.

DISCUSSION

Although the hepatitis B viral enhancer behaves as a classic *cis* activator in the sense that it is active in both orientations, its activity is strongly influenced by the cell in which it operates. In the nonliver cell types tested, the HBV enhancer modestly stimulates promoter activity (two- to fivefold). In contrast, the HBV enhancer stimulates promoter activity dramatically (20- to 100-fold) in the HepG2 liver cell line. This relative tissue specificity of the HBV enhancer suggests that liver contains specific or elevated levels of *trans*-acting factors that interact with the HBV enhancer. This conclusion is supported by reports of a liver-specific factor(s) which binds to a site within the hepatitis B viral enhancer (12, 21, 26). The relatively high activity of the enhancer in liver cells may be one of the explanations for the strong hepatotrophicity of HBV.

The HBV enhancer exerts its effects despite the presence of the X promoter in the constructs. This enhancer-promoter unit appears to be orientation independent, since it stimulates promoter activity equally in either orientation. Moreover, the sizes of the CAT mRNAs suggest that transcription initiates from the anticipated downstream promoter. Thus, if the X promoter is active in this context, its transcription must terminate prior to the CAT coding sequences. Consequently, it appears that the presence of the X promoter does not confound the activity of the enhancer.

The individual promoter strengths have been measured in the absence and presence of the HBV enhancer. When tested without the enhancer, the promoters exhibited strengths in the order TK > precore > preS2 > preS1. When tested with the enhancer, the order was TK ≥ precore ≥ X > preS2 > preS1. All promoters were more active in liver cells, possibly owing to the transfection efficiency; however, the relative activities of the various promoters were different for each cell type. This suggests cell-specific variation in the factors interacting with the promoters.

Since the enhancer-promoter constructs have the same relative activities as the unenhanced promoter constructs, the enhancer and promoters seem to function independently (noninteractively).

It is a paradox that the two strongest HBV promoters are the precore and the X promoters, whereas the S antigen constitutes the majority of the assembled viral products (2, 4). This suggests that other factors influence the relative activity of the promoters. A selective action of the HBV X protein on S gene expression could explain these data. In this manner, the functions of the virus could be temporally phased during its life cycle by the *trans*-activation mechanism.

ACKNOWLEDGMENTS

We thank D. N. Standring, M. D. Walker, and D. Melloul for helpful discussions and Leslie Spector for preparation of the manuscript.

T.K.A. was supported by grant PF2834 from the American Cancer Society. This work was supported by Public Health Service grant AI19744 from the National Institutes of Health.

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