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Regulation of hepatitis B virus gene expression by its two enhancers

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ABSTRACT **Hepatitis B virus (HBV) infection causes acute and chronic hepatitis and is closely associated with the development of hepatocellular carcinoma. The principal site of HBV infection is liver, and HBV actively replicates in hepatocytes. Two regions of the HBV genome have been shown previously to display properties of a transcriptional enhancer. In this study, we show that either of the two HBV enhancers can activate all three major HBV promoters in several human hepatoma lines, and the cooperative action of the two enhancers ultimately affects overall activity of the three promoters. In addition, our data suggest that HBV gene expression may be differentially regulated by its enhancers. HBV infection causes chronic liver inflammation and hepatocyte regeneration. It has been proposed that progressive accumulation of mutations during the regenerative hyperplasia may lead to alterations in the differentiation state of hepatocytes. Thus, the development of two differentially regulated enhancers may reflect a strategy of HBV to replicate efficiently in less differentiated hepatocytes during hepatocyte regeneration or hepatocarcinogenesis.**

Hepatitis B virus (HBV) infection causes acute and chronic hepatitis, and chronic HBV infection is closely associated with the development of hepatocellular carcinoma (1–3). Although HBV replicates episomally in infected hepatocytes, HBV DNA has been found to be integrated into the genome of hepatoma tissues, and virus replication and gene expression are generally absent in those hepatoma cells (2). The presence of integrated HBV DNA in hepatocellular carcinoma has led to the hypothesis that viral integration may contribute to the process of hepatocarcinogenesis (4, 5). However, the mechanisms responsible for tumorigenesis and for the shutdown of HBV gene expression and replication in hepatocellular carcinoma remain unclear.

Three major classes of HBV-specific messages are detected in infected hepatocytes, and the 5′ ends of the RNAs are heterogeneous (Fig. 1) (7, 8): the 3.5-kilobase (kb) RNAs, which are slightly larger than the 3.2-kb unit length of HBV genome serve as the mRNAs for expression of the core protein and reverse transcriptase. Because these RNAs are the only species containing the full complement of viral genetic information, they also serve as templates for reverse transcription during HBV replication. Two other mRNAs are subgenomic in size: the 2.4-kb RNAs encode the large envelope protein HBV surface antigen, and the 2.1-kb RNAs encode the middle and major HBV surface antigens. Expression of these HBV-specific mRNAs is controlled by three different promoters in the HBV genome (8): the core promoter regulates expression of the 3.5-kb RNAs, whereas the preS promoter and the S promoter regulate expression of the 2.4- and 2.1-kb RNAs, respectively.

In human, the principal site of clinical pathology after HBV infection is the liver because HBV actively replicates only in hepatocytes. Consistent with this observation, the 3.5-kb genomic transcript has been detected primarily in well-differentiated human hepatoma cell lines transfected by the cloned HBV genome (9–11), suggesting that liver-specific factors are needed for efficient transcription of the genomic transcripts from the core promoter. Two regions of the HBV genome are known to display properties of a transcriptional enhancer (Fig. 1) (12–14). Enhancer 1 has been mapped to a position between the envelope and X open reading frames. Because activation of transcription by this enhancer is greater in several cultured hepatoma cells than in nonhepatic cells, it has been suggested that this enhancer is responsible for liver-specific gene expression of HBV (12). We have recently reported a second enhancer (enhancer 2) immediately upstream from the coding region of the core gene (14). Unlike enhancer 1, the activity of enhancer 2 is highly liver specific, functioning only in highly differentiated human hepatoma cells. Furthermore, enhancer 2 activity varies in different hepatoma lines, suggesting that this enhancer is regulated according to the differentiation state of the hepatoma line used. How and whether these two enhancers interact with each other to regulate liver-specific HBV gene expression remain to be understood. Because enhancers have been shown to play a pivotal role in the regulation of mammalian and viral gene expression and because HBV gene expression is tightly coupled to the step of reverse transcription in its replication cycle, an understanding of the mecha-

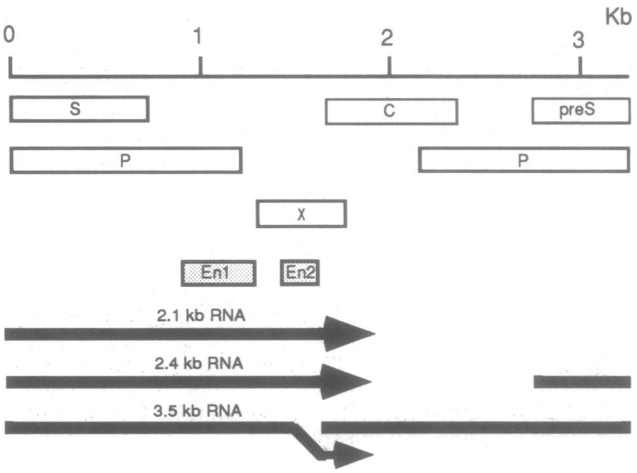

FIG. 1. Schematic diagram of HBV genome. Length of the HBV genome is indicated in kilobases (kb), numbering from the single *Xho* I site of subtype adr (6). The viral open reading frames are indicated as open boxes. Major viral transcripts are indicated as thick lines with arrowheads indicating direction of transcription and translation; the length of the transcripts is indicated. The two HBV enhancers are indicated as shaded boxes. En1, enhancer 1; En2, enhancer 2; S, HBV surface antigen; P, HBV polymerase; X, HBV X protein; and C, HBV core antigen.

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Abbreviations: HBV, hepatitis B virus; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase.

nisms for regulation of HBV enhancer activity may clarify the molecular basis for the absence of HBV replication and gene expression in hepatocellular carcinoma.

In this study, we show that the two HBV enhancers strongly affect the activity of all three major HBV promoters in human hepatoma cells and that the activity of HBV enhancers is differentially regulated, depending on the state of hepatocyte differentiation.

MATERIALS AND METHODS

Cell Lines, Transfection, and Chloramphenicol Acetyltransferase (CAT) Assays. All five cell lines used in this study, Hep3B, PLC/PRF/5, HepG2, Huh7, and Huh6, were derived from human hepatocellular carcinomas. Whereas PLC/PRF/5 contains multiple copies of the integrated HBV genome (15), Hep3B contains only a single copy of integrated HBV genome (16). Both were derived from patients with histories of HBV infection. HepG2, Huh7, and Huh6 cells were obtained from patients with no history of HBV infection, and no HBV DNA can be detected in these cells (17). Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO)/10% fetal calf serum at 37°C in 10% CO_2/90% air. Cells were transfected by the calcium phosphate precipitation method (18) with plasmids containing the bacterial CAT gene, and CAT assays were performed by the method of Sleigh (19).

Plasmid Construction. The adr serotype of HBV DNA (6) was used in this study. Construction of pTKCAT, pCTKCAT, pTKCATC1, pCCAT1, and pΔCCAT1 has been described (14). In plasmid pSCAT, a *Bgl* II–*Xho* I HBV fragment (nucleotides 2300–3214) spanning the putative HBV S promoter without the enhancers was inserted immediately upstream of the CAT gene. pE2SCAT contains a *Bam*HI–*Xho* I fragment (nucleotides 1272–3214) spanning the S promoter and enhancer 2. pESCAT-1 contains a *Rsa* I–*Xho* I HBV fragment (nucleotides 798–3214) spanning the S promoter and both HBV enhancers. pE1SCAT was derived from pESCAT-1 by deletion of the *Bam*HI–*Bgl* II fragment of HBV (nucleotides 1272–2300) spanning enhancer 2.

In plasmid pEpreSCAT-1, the CAT gene is controlled by a *Rsa* I–*Bst*XI fragment (nucleotides 798–2700) spanning the putative preS promoter and both HBV enhancers. ppreSCAT contains a *Bgl* II–*Bst*XI fragment (nucleotides 2300–2700) spanning the preS promoter without the enhancers. pE2preSCAT contains a *Bam*HI–*Bst*XI fragment (nucleotides 1272–2700) spanning the preS promoter and enhancer 2. pE1preSCAT was derived from pEpreSCAT-1 by deletion of the *Bam*HI–*Bgl* II fragment of HBV (nucleotides 1272–2300) spanning enhancer 2.

RESULTS

Differential Regulation of HBV Enhancer Activity in Human Hepatoma Lines. To test the hypothesis that the activity of HBV enhancer 2 may be determined by the state of cell differentiation, we measured CAT activity after transfection of the plasmids shown in Fig. 2 into five human hepatoma lines—Hep3B, PLC/PRF/5, HepG2, Huh7, and Huh6 cells—which are known to express different subsets of liver-specific markers (17). The latter three cell lines have been shown to produce viral replicative intermediates and Dane particle-like structures after transfection with cloned HBV DNA (9–11), suggesting that they contain host factors required for HBV replication and gene expression. Plasmid pTKCAT contains the bacterial CAT gene controlled by the promoter of the herpes simplex virus TK gene (20). In pTKCATC1, HBV enhancer 2 is inserted downstream of the CAT gene. In pCTKCAT, HBV enhancer 1 is inserted immediately upstream of the TK promoter. These plasmids

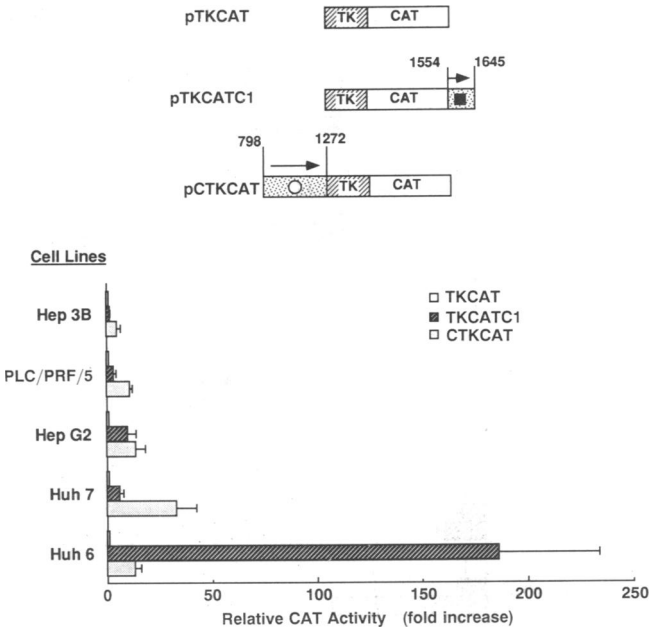

FIG. 2. Activation of CAT expression from thymidine kinase (TK) promoter in human hepatoma lines. The herpes TK promoter is represented by hatched boxes, and the CAT gene is represented by open boxes. The HBV enhancer-containing fragments are represented by dotted boxes with the filled square representing enhancer 2 and the open circle representing enhancer 1. Numbers over dotted boxes indicate the nucleotide number from the sequence of HBV DNA, and arrows indicate the orientation of each HBV fragment relative to the direction of transcription of HBV genes. Relative CAT activity of transfected cells with each plasmid is indicated on the abcissa. CAT activity of plasmid pTKCAT in each cell line is defined as 1. Each value of relative CAT activity is the mean of at least three independent experiments. Bars indicate the SD.

were introduced into each cell line by calcium phosphate coprecipitation, and CAT expression from each plasmid was determined and normalized to that of pTKCAT.

As shown in Fig. 2, HBV enhancer 1 in pCTKCAT stimulates TK promoter activity in all five human hepatoma lines tested. Its activity remains relatively constant, varying from 5-fold stimulation in Hep3B cells to 30-fold stimulation in Huh7 cells. In contrast, the activity of enhancer 2 in pTKCATC1 is highly variable in different hepatoma lines. In Huh6 cells, the TK promoter activity is stimulated >150-fold by enhancer 2. Its enhancer activity decreases >10-fold in Huh7 and HepG2 cells relative to that in Huh6 cells, and there is little or no enhancer activity in Hep3B and PLC/PRF/5 cells. To determine whether the different effect of enhancer 2 on the TK promoter may be due to its position and orientation in pTKCATC1, we also tested other pTKCAT plasmids containing enhancer 2 inserted at the opposite orientation relative to that in pTKCATC1 or inserted upstream of the TK promoter. Results similar to those of pTKCATC1 were obtained (data not shown). We interpret these results to indicate that HBV enhancer 2 activity is differentially regulated, depending on the differentiation state of the hepatoma line used.

Regulation of HBV Core Promoter Activity. The regulation of HBV core promoter activity plays an essential role in the life cycle of this virus because this promoter directs the expression of the 3.5-kb HBV-specific mRNA (7, 8). The 3.5-kb mRNA not only encodes the HBV core protein and reverse transcriptase, but it also serves as an intermediate in the replication cycle of HBV. To determine how the two HBV enhancers affect HBV core promoter activity, we transfected the plasmids shown in Fig. 3 into the same five hepatoma lines described above, and CAT activity was

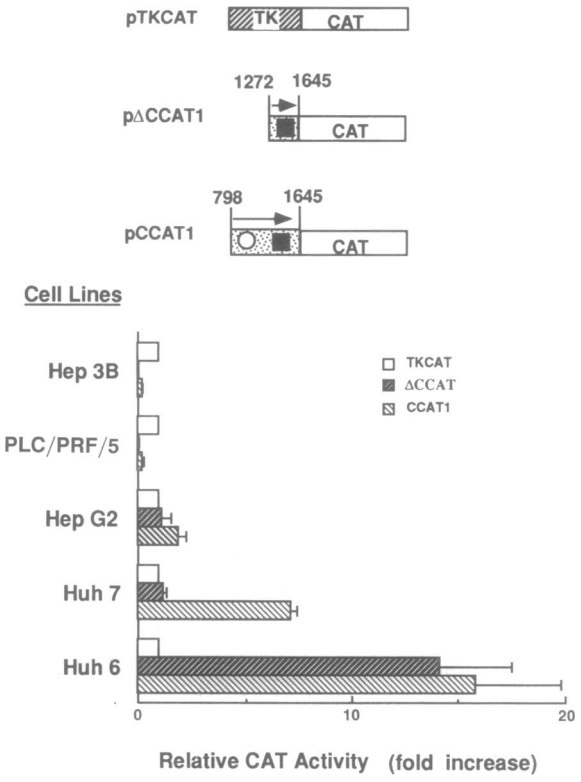

FIG. 3. Activation of HBV core promoter activity by the two HBV enhancers. CAT activity of plasmid pTKCAT in each cell line is defined as 1, and relative CAT activity is determined as described for Fig. 2.

determined and normalized to that of pTKCAT. pCCAT1 contains the CAT gene controlled by an 848-base pair (bp) HBV DNA fragment spanning both HBV enhancers and the putative promoter for the core gene. pΔCCAT1 is identical to pCCAT1, except that enhancer 1 is deleted.

The two plasmids containing the core promoter generate similar levels of CAT activity in Huh6 cells (Fig. 3), indicating that enhancer 1 is dispensable for core gene expression in this cell line. This conclusion is consistent with the results in Fig. 2, which show that enhancer 2 is at least 10-fold more active than enhancer 1 in activating the TK promoter in Huh6 cells. In contrast, the CAT activity of pΔCCAT1 is significantly decreased relative to that of pCCAT1 in the other four human hepatoma lines tested, suggesting that the activity of enhancer 1 is important for regulating core gene expression in those cells. However, the role of enhancer 2 in stimulating core promoter activity in these four hepatoma lines is not clear. Both HBV core promoter and enhancer 2 have been colocalized in a 90-bp *Hin*cII–*Rsa* I restriction fragment (nucleotides 1554–1645) (Fig. 3) (14). Because we have not yet been able to separate enhancer 2 activity from the core promoter activity, assessment of the effect of enhancer 2 alone on core promoter activity in these hepatoma cells is not possible. However, the results presented below suggest that enhancer 2 is indispensable for efficient HBV gene expression.

Regulation of HBV S and preS Promoter Activity. To determine whether the two HBV enhancers have any effect on other major HBV promoters and whether the two enhancers interact cooperatively to modulate HBV gene expression, we have constructed several plasmids containing the CAT gene linked to the S promoter with or without the HBV enhancers (Fig. 4). These plasmids were transfected into the five hepatoma lines, and CAT activity was determined and

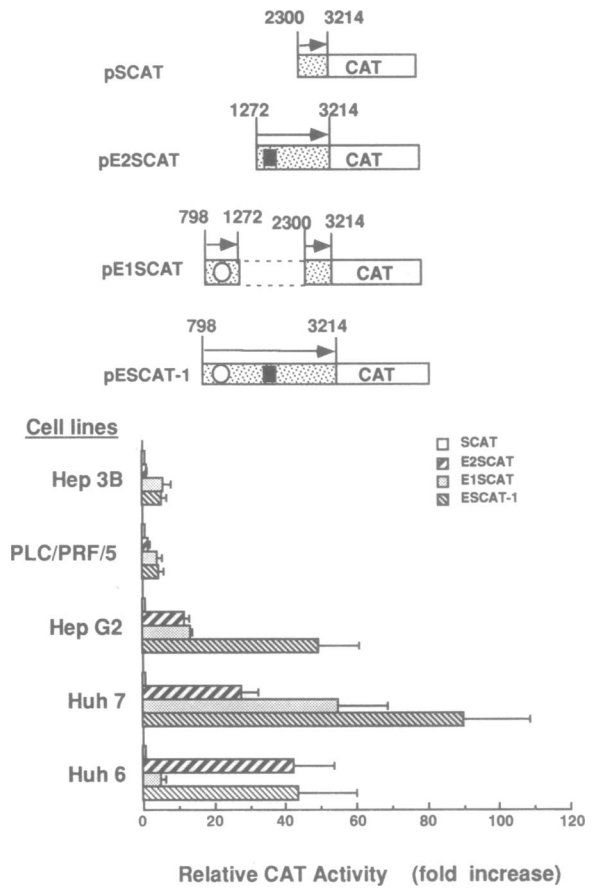

FIG. 4. Activation of HBV S promoter activity by the two HBV enhancers. Dotted lines in pE1SCAT represent deleted HBV sequences. CAT activity of plasmid pSCAT in each cell line is defined as 1, and relative CAT activity is determined as described for Fig. 2.

normalized to that of pSCAT, which contains the S promoter without the HBV enhancers (Fig. 4).

In Huh6 cells, S promoter activity is stimulated >40-fold by enhancer 2 (pE2SCAT), whereas it is stimulated only 5-fold by enhancer 1 (pE1SCAT). CAT activity produced by pESCAT-1 with both enhancers is comparable to that of pE2SCAT with enhancer 2 alone, suggesting that, like core promoter activity, enhancer 1 is dispensable for S promoter activity in this cell line. In Huh7 and HepG2 cells, either enhancer can stimulate S promoter activity. However, the most efficient CAT expression comes from pESCAT-1 (Fig. 4), indicating that the two HBV enhancers interact cooperatively to stimulate HBV S promoter activity in these two hepatoma lines. In PLC/PRF/5 and Hep3B cells, pE2SCAT generates levels of CAT activity similar to that of pSCAT, whereas enhancer 1 in pE1SCAT stimulates CAT expression ≈5-fold. pESCAT-1 fails to produce higher levels of CAT activity than pE1SCAT, consistent with the observation shown in Fig. 2 that enhancer 2 has no activity and enhancer 1 alone stimulates the promoter activity in PLC/PRF/5 and Hep3B cells.

The HBV fragment in pESCAT-1 spans the X gene and its promoter, whereas other S promoter-containing plasmids do not encode X protein, and X protein has been shown to transactivate HBV enhancer 1 (21, 22). To exclude the possibility that the CAT activity generated by transfection of pESCAT-1 is due to the effect of transactivation by the X gene product, we have inserted four extra nucleotides into the *Bam*HI site located in the coding region of the X gene, resulting in the generation of a frameshift mutation in the X protein-coding region of pESCAT-1. CAT expression from

this new plasmid is similar to that of pESCAT-1 in the five hepatoma lines used (data not shown). Thus, if the X gene protein is synthesized in pESCAT-1-transfected hepatoma cells, it does not appear to affect the S promoter activity significantly under the conditions described here.

Similar plasmids using the preS promoter instead of the S promoter to regulate CAT gene expression were constructed, and the results of transfection with these are shown in Fig. 5. In Huh6 cells, although enhancer 1 alone in pE1preSCAT stimulates the preS promoter only slightly, pEpreSCAT-1 with both enhancers generates the highest level of CAT expression, suggesting that it is the cooperative interaction of the two enhancers in this cell line that ensures efficient CAT expression from the preS promoter. In HepG2 and Huh7 cells, CAT expression from the preS promoter is stimulated to the maximum level by enhancer 1 with or without enhancer 2, suggesting that regulation of the preS promoter by the two HBV enhancers differs from that of the core or the S promoter. However, as for the S promoter, enhancer 2 alone fails to stimulate preS promoter activity in Hep3B and PLC/PRF/5 cells, and CAT expression from the preS promoter is solely dependent upon enhancer 1 activity. The effect of the two HBV enhancers on different promoters is summarized in Table 1. Together, these results indicate that the activity of all three major HBV promoters is strongly affected by the two HBV enhancers. However, the enhancer effect varies, depending on the nature of the hepatoma line used.

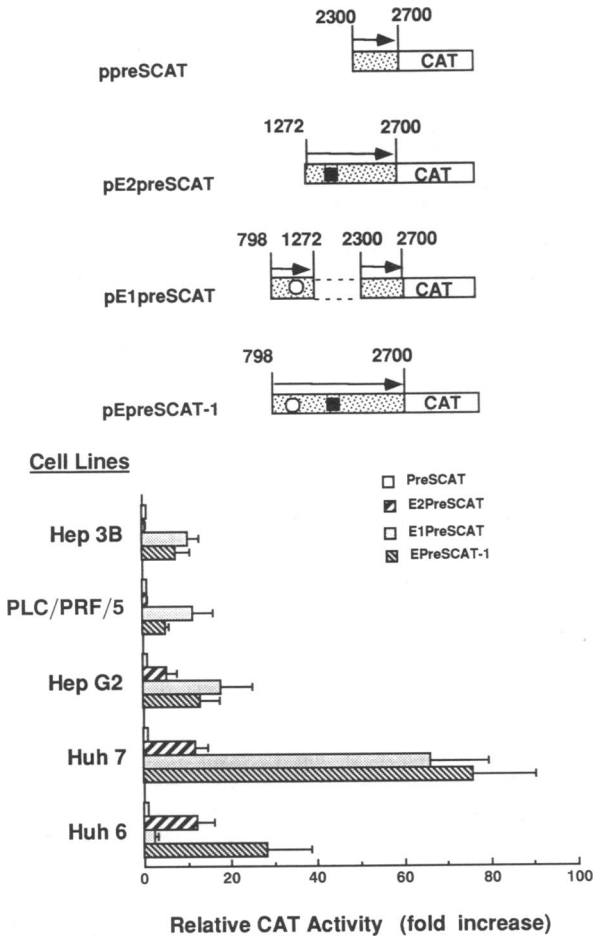

FIG. 5. Activation of HBV preS promoter activity by the two HBV enhancers. Dotted lines in pE1preSCAT represent deleted HBV sequences. CAT activity of the plasmid ppreSCAT in each cell line is defined as 1, and the relative CAT activity is determined as described for Fig. 2.

Table 1. HBV enhancer activity on different promoters

Promoter	Enhancer	HBV enhancer activity, -fold induction				
		Hep3B	PLC/PRF/5	HepG2	Huh7	Huh6
TK	1	5	11	14	33	13
	2	2	3	10	6	186
Core	2	<0.1	0.1	1	1	14
	1 + 2	0.2	0.2	2	7	16
S	1	6	4	14	55	5
	2	1	2	12	28	42
	1 + 2	6	5	50	90	44
preS	1	11	12	19	66	2
	2	1	1	6	12	12
	1 + 2	8	5	14	76	28

DISCUSSION

Although HBV DNA has been detected in nonhepatic tissues (2), HBV infection is restricted primarily to hepatocytes. After transfection with the cloned HBV genome, virions can be detected only in well-differentiated human hepatoma cells, suggesting that replication and gene expression of HBV may depend on liver-specific factors (9–11). Because enhancement of transcription by the two HBV enhancers is greater in several cultured hepatoma lines than in nonhepatic cells, the activity of the two HBV enhancers has been suggested to be responsible, at least partially, for the observed hepatotropism of HBV (12, 14). Our studies show that either of the two HBV enhancers can, indeed, enhance activity of all three major HBV promoters in the human hepatoma cells tested and that the cooperative action of the two enhancers ultimately affects overall activity of the three promoters. There are some exceptions, however. For example, enhancer 1 is dispensable for the core or the S promoter activity in Huh6 cells, but this enhancer plays an important role in the regulation of the preS promoter activity in this cell line (Table 1). In contrast, enhancer 2 has little effect on preS promoter activity in Huh7 and HepG2 cells in the presence of enhancer 1, although enhancer 2 cooperates with enhancer 1 to stimulate S promoter activity in these two cell lines (Table 1). Sequence analysis indicates that the preS promoter contains a sequence element 5′-GTTAATCATTACT-3′, which binds the liver-specific transcription factor, hepatocyte nuclear factor 1 (23). Deletion of this sequence element has been shown to result in a major reduction of preS promoter activity in HepG2 cells (24). This sequence element, however, is absent from the core and the S promoter. The interplay between hepatocyte nuclear factor 1 and the other transcription factors that recognize the HBV enhancer elements may account for the feature of the regulation of the preS promoter distinct from the S or the core promoter. Together, these results demonstrate that the functions of the two HBV enhancers are not redundant and suggest that proper coordination of the two enhancer activities is needed to ensure efficient HBV gene expression from its three major promoters in different human hepatoma lines.

HBV enhancer 1 is active in all human hepatoma lines we have examined. This enhancer has previously been shown to contain overlapping binding sites for multiple transcription factors—including NF-1, C/EBP, AP-1, CREB, and ATF (25, 26)—and the synergistic action of these factors may account for the observed activity of this enhancer in liver cells. The ubiquitous distribution of some of these transcriptional factors may also explain why enhancer 1 not only functions in human hepatoma cells but also functions in several cell lines derived from nonliver cells (12, 13, 25). In contrast, enhancer 2 activity is strictly liver specific (14), and its activity is highly variable in the different hepatoma lines used. Enhancer 2 is extremely active in Huh6 cells but is inactive in PLC/PRF/5 and Hep3B cells, regardless of the

promoter used. If the different hepatoma lines represent hepatocytes arrested at different stages of differentiation, our data suggest that HBV gene expression may be differentially regulated by its enhancers. HBV infection is known to cause chronic liver inflammation and lead to hepatocyte regeneration. It has been proposed that progressive accumulation of mutations during this regenerative hyperplasia may alter the differentiation state of hepatocytes (27). The presence of two separate, differentially regulated enhancers within its small genome may reflect a strategy of HBV to continue to replicate efficiently in less differentiated hepatocytes during hepatocyte regeneration or hepatocarcinogenesis.

It is interesting that the three HBV promoters in conjunction with both enhancers function more efficiently in HepG2, Huh7, and Huh6 cells than in Hep3B and PLC/PRF/5 cells. This situation is especially obvious in the regulation of the core promoter activity. CAT expression from pCCAT1 is at least 50-fold more efficient in Huh6 cells than in Hep3B or PLC/PRF/5 cells (Fig. 3). This result is consistent with the previous observation that viral particles can be produced from the cloned HBV DNA transfected into HepG2, Huh7, or Huh6 cells (9–11). However, the relative efficiency of virus production in these cell lines has not been compared directly. The results shown in Fig. 3 indicate that pCCAT1 generates more CAT activity in Huh7 cells than in HepG2 cells, suggesting that transcription of the 3.5-kb HBV genomic RNA from the core promoter may be more efficient in Huh7 cells than that in HepG2 cells. Yaginuma *et al.* (11) have reported that the efficiency of producing HBV-specific transcripts was higher in Huh7 cells than in HepG2 cells when the cloned HBV genome was transfected into these two cell lines. The data reported here are consistent with their observation.

Although both PLC/PRF/5 and Hep3B cells have been found to contain integrated HBV DNA, they do not synthesize detectable core antigen or DNA polymerase, nor do they produce HBV virion. The failure to produce core antigen or DNA polymerase has been attributed to rearrangement of the integrated HBV DNA (28). However, our results show that the core promoter functions poorly in these two cell lines, even in transient-transfection assays, suggesting that the absence of core gene expression and HBV replication in these two lines may be the result of inefficient transcription from the core promoter. Although inefficient HBV promoter activity in these two hepatoma lines containing integrated HBV DNA could simply be a coincidence, an alternative explanation suggested by our data is that hepatoma lines containing integrated HBV DNA may represent hepatocytes arrested at specific differentiation states unable to support HBV replication and gene expression efficiently. Although HBV replicates episomally in infected hepatocytes, HBV DNA is frequently integrated into host chromosomes in primary hepatocellular carcinoma. This observation has led to the hypothesis that viral integration may contribute to the process of hepatocarcinogenesis. Our demonstration of the inefficient core promoter activity in hepatoma lines containing the integrated HBV DNA suggests that hepatocyte dedifferentiation during hepatocarcinogenesis could lead to shutdown of the core promoter activity, allowing the nonreplicative, extrachromosomal forms of HBV DNA to serve as a substrate for HBV integration into host chromosomes. Additional genetic changes associated with the integration events may then contribute to the eventual malignant transformation of the infected hepatocytes (5). It should be feasible to test this hypothesis further by analyzing the core promoter activity in other human hepatoma lines containing the integrated HBV DNA.

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