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Liver-Specific Expression of Hepatitis B Virus Is Determined by the Combined Action of the Core Gene Promoter and the Enhancer

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The hepatitis B virus (HBV) enhancer and the core gene promoter regulate the expression of the core and polymerase genes, as well as of the 3.5-kilobase pregenomic RNA. RNA analysis and chloramphenicol acetyltransferase gene expression by plasmids carrying the HBV enhancer linked to the heterologous β-globin or simian virus 40 early promoter demonstrated that the HBV enhancer is 3- to 20-fold preferentially expressed in human liver cells. Core gene promoter activity was mapped to a 100-base-pair fragment which was shown to be sufficient for accurate initiation of transcription. The partial tissue specificity of this promoter was demonstrated by transient transfection into various cell lines with a plasmid containing the core gene promoter linked to the heterologous simian virus 40 enhancer. When the HBV core gene promoter was examined under the control of the HBV enhancer, there was high tissue specificity in that activity could be observed only in differentiated human liver cells. These results suggest that the strict tissue specificity of HBV gene expression is determined by the combinatorial action of these two elements.

Hepatitis B virus (HBV) is carried by an approximated 200 million people worldwide. It causes symptoms ranging from mild to severe hepatic injury and is found in high association with hepatocellular carcinoma. The viral genome consists of four open reading frames coding for the surface, core, and polymerase proteins and an X gene product. It also includes an enhancer element responsible for the transcriptional regulation of at least two of these genes (2, 3, 8, 22). Two major mRNA species have been detected in vivo. The 2.1-kilobase message codes for surface antigen, and the 3.5-kilobase pregenomic message, larger than genome-length RNA, is ultimately packaged into the viral nucleocapsid to serve as a template for DNA synthesis (7).

The hepatotropism of HBV extends beyond the ability of the virus to attach to and penetrate its host cell. Failed attempts to obtain viral particles by transfection of viral DNA into nonliver cells served to demonstrate the dependence of viral replication on a liver cell environment. Until now, the HBV enhancer has been attributed with containing a certain degree of tissue specificity (11, 22). However, a recent report has argued in favor of characterizing the enhancer as being cell type nonspecific (27). The core gene promoter has been directly implicated in regulating the synthesis not only of the core and e antigens but also of the 3.5-kilobase pregenomic message (6, 29) which is essential for viral replication. Furthermore, expression and replication of the HBV genome in HeLa cells, a human nonliver cell line, have been attained by the replacement of only the core gene promoter with the non-tissue-specific heterologous metallothionein IIA promoter (12). Taken together, these findings have led us to believe that the core gene promoter under the homologous HBV enhancer is largely responsible for the cell type-restricted replication of HBV.

We examined the tissue-specific activity of the HBV enhancer and its core gene promoter by measuring their effects on heterologous promoters and enhancers, respectively, in various cell lines, on the levels of both RNA and chloramphenicol acetyltransferase (CAT) gene expression. Our results demonstrate that the HBV enhancer and the core gene promoter, assayed separately, exhibit partial tissue specificity. The combination of both, however, showed restricted expression, being active strictly in differentiated hepatoma cell lines. We propose that restricted HBV gene expression is determined by the combinatorial action of its promoter(s) and the enhancer.

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MATERIALS AND METHODS

Enzymes and substrates. Restriction enzymes, calf intestinal alkaline phosphatase, T4 polynucleotide kinase, DNA ligase, and Klenow fragment of polymerase were purchased from New England BioLabs, Inc., or Pharmacia. Avian myeloblastosis virus reverse transcriptase was from Molecular Genetic Resources. ^{32}P-labeled deoxynucleoside triphosphates and [^{14}C]chloramphenicol were from New England Nuclear Corp. Acetyl coenzyme A was purchased from Sigma Chemical Co.

Construction of plasmids. Cloning procedures were carried out essentially as described previously (15). Plasmids with inserts were sequenced by a double-stranded DNA-sequencing procedure (Promega Biotec). The HBV subtype and nucleotide numbering system is that described by Valenzuela et al. (26). For construction of p321ESR, p321ESF, and p321EFR, the vector used contained the *Hin*dIII fragment of the pCAT 0 plasmid (21) cloned into the *Hin*dIII site of plasmid pSP64 (Promega Biotec). As a result, the vector contained the pSP64 polylinker upstream of the CAT gene, making it ideal for cloning and testing putative promoter regions (see Fig. 2). p321E was constructed by cloning the simian virus 40 (SV40) *Stu*I-*Pvu*II enhancer region (nucleotides [nt] 5190 to 270) into the *Eco*RV site of the pBR322 region within the p321 vector. p321ESR was constructed by cloning the HBV *Stu*I-*Rsa*I (nt 1705 to 1852) fragment of HBV into the *Sma*I site within the polylinker of p321E (see Fig. 2). p321SR was constructed by inserting the HBV *Stu*I-*Rsa*I fragment into the p321 vector lacking the SV40 enhancer region.

The HBV *Stu*I-*Rsa*I fragment was cut at the *Fsp*I site (nt 1805), and the resulting two fragments were ligated to the *Sma*I-cut p321E vector. p321ESF contained the HBV *Stu*I-*Fsp*I (nt 1705 to 1805) fragment cloned into the *Sma*I site of

p321E (see Fig. 2). p321EFR contained the HBV *Fsp*I-*Rsa*I (nt 1806 to 1852) fragment cloned into the *Sma*I site on p321E (see Fig. 2).

pHFCAT was constructed by inserting the HBV *Hpa*I-*Fsp*I (nt 964 to 1853) fragment upstream of the CAT gene in pSVoCAT, an enhancerless promoterless plasmid which had previously been shown to possess no CAT activity (10). The *Hpa*I-*Fsp*I fragment was ligated with *Bgl*II linkers prior to its insertion into the *Bgl*II site of pSVoCAT (10).

Cells and CAT assay. Cells were cultured in Dulbecco modified Eagle minimal essential medium (GIBCO Laboratories) containing penicillin (100 U/ml) and streptomycin (100 μg/ml), with 10% fetal calf serum for Alexander (PLC/PRF/5), Hep-G2, and Hep-SK cells or with 10% calf serum for HeLa and CV-1 cells. The cells were seeded 1 to 3 days prior to transfection. At the time of transfection, the cells were 40 to 80% confluent.

CAT assays were performed 36 to 60 h after transfection by a method described previously (10), and the experiments were performed several times for each cell line.

Cytoplasmic RNA preparation from mammalian cells and RNA analysis. Cytoplasmic RNA was extracted from cells by the Nonidet P-40 method as described previously, with slight modifications (15). Cells were washed once with phosphate-buffered saline and once with magnesium buffer (150 mM NaCl–1.5 mM MgCl–10 mM Tris hydrochloride [pH 7.5]). Two packed-cell volumes of lysis buffer (magnesium buffer and 1% Nonidet P-40) were added, and after strong vortexing, cells were centrifuged for 15 min at 500 × *g*. Supernatants were transferred to new tubes and were mixed with equal volumes of urea buffer (10 mM Tris hydrochloride [pH 7.5]–1% sodium dodecyl sulfate (SDS)–0.35 M NaCl–10 mM EDTA–7 M urea). RNA was extracted once with equal volumes of phenol and chloroform and twice with chloroform and was precipitated with ethanol. For the RNase protection assay, α- and β-globin antisense α-^{32}P-labeled RNA probes were generated and used according to a procedure published elsewhere (16). For the primer extension analysis, 150 ng of single-stranded 20-mer CAT primer (28) was end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol) as described previously (15). A 100-μg sample of total RNA was annealed to approximately 2 × 10^6 cpm of a 20-mer end-labeled CAT primer in 120 μl of 2× clone turnaround buffer (250 mM NaCl–25 mM Tris [pH 7.5]) for 1 h at 55°C. After the reaction, 120 μl of 2× solution 1 (1 M NaCl–20 mM Tris hydrochloride [pH 7.5]–0.2% SDS) and 600 μl of 1× solution 1 (0.5 M NaCl–10 mM Tris hydrochloride [pH 7.8]–0.1% SDS) were added. The primer-template mixture was vortexed and applied to oligo(dT)-cellulose columns prepared in polypropylene pipetteman tips (Gilson). Columns were washed with approximately 3 ml of solution 1 and then with 1 to 2 ml of solution 2 (0.1 M NaCl–10 mM Tris hydrochloride [pH 7.5]–0.1% SDS). The poly(A)$^+$ fraction was eluted with 600 μl of solution 3 (10 mM Tris hydrochloride [pH 7.5]–0.05% SDS) together with linear tRNA (10 μg/ml). The eluted material was ethanol precipitated with 0.3 M sodium acetate and was counted in a gamma counter. To primer-template-dried pellet, 1.5 μl of a 3 mM mixture of deoxynucleoside triphosphates, 4 U of reverse transcriptase, and H_2O were added to a final volume of 15 μl. The reaction experiment was carried out for 40 min at 42°C and was stopped with 5 μl of formamide loading buffer (Promega Biotec). The hybrid was denatured by being boiled for 3 min and was run on a 5% polyacrylamide sequencing gel.

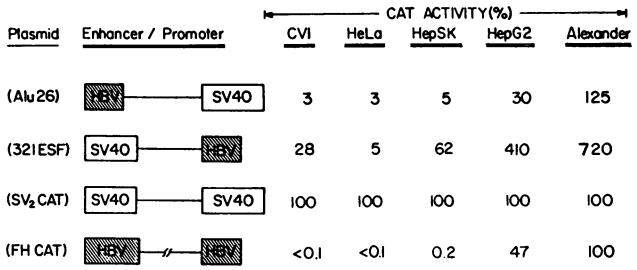

FIG. 1. Tissue-specific expression of the core gene promoter. CAT gene expression directed by the core gene promoter under the heterologous SV40 enhancer, by the SV40 promoter under the HBV enhancer, and by the core gene promoter under the homologous HBV enhancer is shown. Cells from the various cell lines indicated were transiently transfected with p321ESF, pAlu26, or pHFCAT. The results are summarized as percent activity relative to that of the positive control, pSV_2CAT, which was considered 100%. The results presented are an average of several experiments.

RESULTS

Tissue specificity of the HBV enhancer. We have previously demonstrated that sequences of HBV positioned upstream of the putative core gene promoter exhibit characteristics of an enhancer (22). This enhancer appears to be more active in Alexander cells than in monkey CV-1 cells (22). To extend these studies, we tested the effect of the HBV enhancer on two heterologous promoters by using different experimental approaches. In the first set of experiments, the HBV enhancer element (nt 964 to 1231) was inserted upstream from the SV40 early promoter which was linked to the bacterial CAT gene. This plasmid, pAlu26, was transfected into various cell lines and was assayed for CAT activity. The HBV enhancer showed only 3 to 5% activity relative to that of the SV40 enhancer when assayed in CV-1, HeLa, or nondifferentiated human liver Hep-SK cells (Fig. 1). When assayed in differentiated human liver cells (Alexander and Hep-G2), the HBV enhancer gave a high activity of 30 to 125% relative to that of the SV_2CAT control. Since both pSV_2CAT and pAlu26 contain the same SV40 promoter, the encoded CAT mRNAs are identical in structure. Thus, CAT analysis correlates to transcriptional efficiency rather than to translational efficiency or RNA stability. Next, the enhancer (nt 1043 to 1266) was linked to the β-globin promoter (18) (plasmid pEN223) and was assayed in various cell lines. RNA mapping was used to test for enhancer activity. A plasmid ($pSVHP\alpha_2$) (25) carrying the α-globin sequences under the control of the SV40 enhancer-promoter was used as an internal control. Plasmids $pSVHP\alpha_2$ and pEN223 were cotransfected into various cell lines and probed by the RNase protection procedure. Fig. 2 shows the effect of the HBV enhancer on transcription from the β-globin promoter. After standardization to the $pSVHP\alpha_2$ internal control, it was clear that the HBV enhancer was about threefold more efficient in the differentiated Alexander, Hep-3B, and Hep-G2 liver cell lines than in the HeLa nonliver cell line and the nondifferentiated Hep-SK liver cell line. Together, these experiments demonstrate that the HBV enhancer linked to the SV40 or β-globin promoter is at least 3- to 20-fold more active in differentiated human liver cells.

Mapping of the HBV core gene promoter. To map the core gene promoter, a 150-base-pair (bp) fragment situated upstream of the core gene open reading frame was cloned downstream from the SV40 enhancer and upstream of the promoterless CAT gene to produce plasmid p321ESR (Fig.

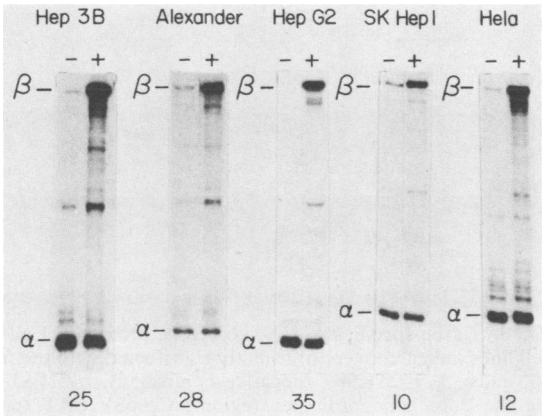

FIG. 2. HBV enhancer activity in transfected cell lines. EN223 was constructed by cloning the HBV *Eco*RV-*Sau*3A enhancer fragment (nt 1043 to 1266) upstream of the β-globin gene and promoter. pEN223 was cotransfected with pSVHPα$_2$, which contains the SV40 enhancer and the α-globin gene, into different cell lines. After 48 h, cytoplasmic RNA was prepared, and a sample was assayed for the amount of β- and α-globin transcripts. The transfected cell lines are indicated. The correctly inititiated transcript of globins (α and β) are shown. Lanes: −, control of transfection by using the enhancerless β-globin plasmid; +, activity of the pEN223 plasmid. In each lane, activity was taken as the ratio of β-globin activity to α-globin activity. The number below each panel represents the ratio of the activity of pEN223 to that of the enhancerless plasmid. Each number represents the mean from three independent assays.

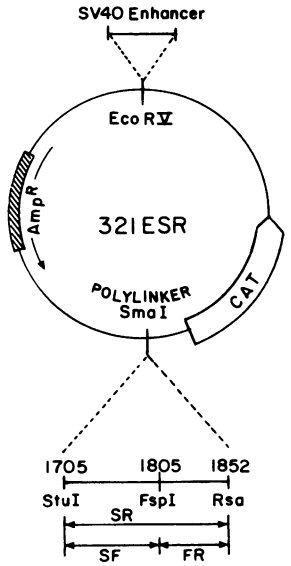

FIG. 3. Construction of plasmids p321ESR, p321ESF, and p321ERF. p321ESF was constructed first by cloning the SV40 enhancer into the *Eco*RV site of the p321 vector to form p321E. The HBV *Stu*I-*Rsa*I (SR) fragment (nt 1705 to 1852), containing the putative core gene promoter region, was cloned into the *Sma*I site within the polylinker of p321E, upstream of the CAT gene, to form p321ESR. The *Stu*I-*Rsa*I HBV fragment was cut into the smaller *Stu*I-*Fsp*I (SF) and *Fsp*I-*Rsa*I (FR) fragments, each of which was cloned separately into p321E to form p321ESF and p321EFR, respectively. The same fragments were also cloned into the same site in the opposite orientation to form p321ESF$^-$ and p321EFR$^-$, to be used as controls.

3). This *Stu*I-*Rsa*I fragment, spanning nt 1705 to 1852, included the 5′ ends of the HBV pregenomic RNA (20, 30) as well as 90 bp of upstream sequences. Plasmids p321ESF and p321ERF contained subfragments of this 150-bp fragment (spanning nt 1705 to 1805 and 1806 to 1852, respectively). These plasmids were transfected into Hep-SK cells, and the CAT activity was measured (Fig. 4). Initially, core gene promoter activity was mapped to the 150-bp *Stu*I-*Rsa*I fragment (Fig. 4). Subsequently, it was shown that the entire activity resides within the smaller *Stu*I-*Fsp*I fragment, which was later used for further characterization of the promoter (see Fig. 6). p321ESR encoded high CAT activity, comparable to that of pSV$_2$CAT, while the enhancerless p321SR and promoterless p321E gave only background levels of CAT activity. Plasmids p321ERF and p321ESF$^-$, the latter containing the *Stu*I-*Fsp*I fragment in the opposite orientation, were inactive (not shown). These results demonstrate that the core gene promoter is localized within the 100-bp HBV *Stu*I-*Fsp*I (nt 1705 to 1805) fragment and that its activity is enhancer dependent.

Primer extension analysis was used to map the 5′ ends of the core transcripts directed by the *Stu*I-*Fsp*I fragment. RNA from p321ESF-transfected cells was extracted 60 h after transfection and hybridized to a ^{32}P-end-labeled 20-mer CAT oligonucleotide (28). Subsequently, poly(A)$^+$ RNA annealed to the end-labeled primer was selected on an oligo(dT) column and was extended by reverse transcriptase. Two extension products were observed (Fig. 5), a major band of 103 bp and a minor band of 74 bp, corresponding to initiation sites in HBV at nt 1792 ± 3 and 1821 ± 3, respectively. These results demonstrate that the *Stu*I-*Fsp*I fragment (nt 1705 to 1805) contains all the sequences necessary to accurately initiate core messages. RNA from cells transfected with pSV$_2$CAT, used as a positive control, gave four bands corresponding to the expected initiation sites, characteristic of the SV40 early promoter (19). Cells transfected with p321EFR, containing the HBV *Fsp*I-*Rsa*I fragment (nt 1806 to 1852), did not produce CAT-specific RNA. These results are in accordance with the results obtained by CAT assays indicating that in our system CAT activity reflects levels of RNA.

Tissue specificity of the core gene promoter. Plasmid p321ESF, containing the 100-bp core promoter fragment linked to the heterologous SV40 enhancer, was used to study the tissue specificity of the HBV core gene promoter. p321ESF$^-$ (containing the *Stu*I-*Fsp*I fragment in the nonsense orientation) and pSV$_2$CAT were used as negative and positive controls, respectively. CAT activity of p321ESF

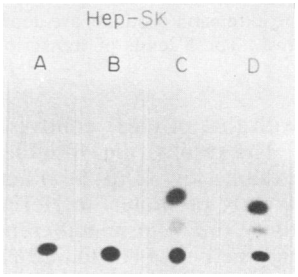

FIG. 4. Expression of CAT activity by the putative promoter region in Hep-SK cells transiently transfected with the following plasmids: A, p321SR; B, p321E; C, p321ESR; D, pSV$_2$CAT. After 60 h, the extracts were prepared and assayed for CAT activity as described in Materials and Methods. Activities are given relative to that of the positive control, pSV$_2$CAT, which was considered 100%.

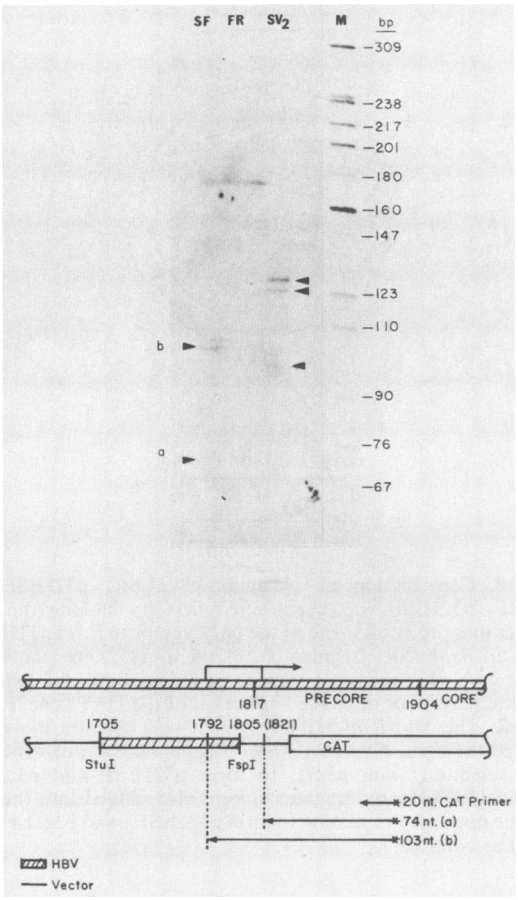

FIG. 5. Mapping of initiation sites of RNA transcribed from the core gene promoter region. Hep-SK cells were transiently transfected with several different plasmids. At 60 h later, RNA was extracted by the Nonidet P-40 method of cytoplasmic RNA preparation. A 20-μg sample of total RNA was hybridized to 30 ng of end-labeled (1.5×10^6 cpm) 20-mer CAT primer. The poly(A)$^+$ primer-annealed RNA fraction, which was purified on an oligo(dT)-cellulose column, was extended with reverse transcriptase as described in Materials and Methods and was loaded onto a 5% denaturing polyacrylamide gel. Lanes: SF, p321ESF; FR, p321EFR; SV_2, pSV_2CAT; M, pBR322 DNA digested with *Hpa*II as a size marker. In lane SF, arrow a indicates a 74-nt extension product, while arrow b indicates a 103-nt extension product in p321ESF. In lane SV_2, arrows indicate the extension products in the positive control, pSV_2CAT. Below is a schematic representation of the primer extension products. The p321ESF vector is aligned to the corresponding sequences in the HBV genome above. The 20-mer CAT primer and the extension products are depicted underneath by the horizontal arrows. The 5′ ends of transcripts are indicated by dotted lines.

was compared with that of these controls in several transfected cell lines. The results (Fig. 6) indicate that the core gene promoter exhibits low (4 to 28%) activity (relative to that of the SV40 early promoter) in HeLa and CV-1 cells, intermediate activity (62%) in nondifferentiated liver Hep-SK cells, and high activity (410 to 720%) in Hep-G2 and Alexander cells. These differences indicate that the core gene promoter linked to the heterologous SV40 enhancer exhibits a preference for differentiated human liver cells.

The HBV enhancer-core gene promoter complex is highly tissue specific. To measure the tissue-preferential activity of the core gene promoter under the control of its homologous

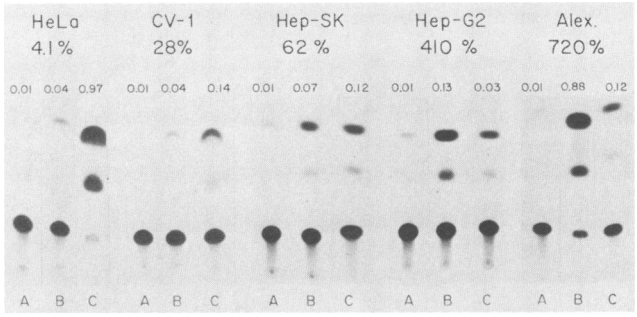

FIG. 6. Tissue specificity of the core gene promoter. Cells from the cell lines indicated were transiently transfected with the following plasmids: A, p321ESF$^-$ (negative control); B, p321ESF (containing the core gene promoter region); C, pSV_2CAT (positive control). Cell extracts were prepared 60 h after transfection and were assayed for CAT activity. Indicated above each panel is the absolute conversion of chloramphenicol to its acetylated forms. The activity of the core gene promoter (p321ESF) is indicated as percent conversion relative to that of pSV_2CAT. Alex, Alexander.

enhancer, plasmid pHFCAT was constructed. This plasmid comprised the *Hpa*I-*Fsp*I fragment (nt 964 to 1805) which included the HBV enhancer and the core gene promoter upstream of the CAT gene (Fig. 7). Although the pHFCAT construct included the upstream X promoter, RNA initiating from this promoter should not be expressed at the level of CAT activity due to interfering downstream AUGs (14). Figure 1 summarizes the tissue-specific activities of the HBV enhancer and the core gene promoter assayed in combination either with each other or with the heterologous SV40 elements. The HBV enhancer and the core gene promoter, when assayed alone, show low activity in nondifferentiated liver cells or nonliver cells and higher activity in differentiated human liver cell lines. The combination of the HBV enhancer with the core gene promoter (pHFCAT) resulted in strict tissue specificity in that CAT expression was restricted to differentiated human liver (Alexander and Hep-G2) cells only.

DISCUSSION

We have previously demonstrated that the HBV enhancer showed high activity in Alexander cells and low activity in

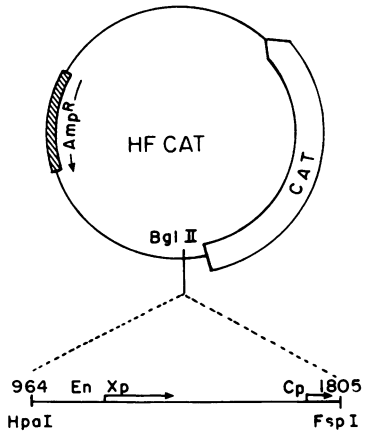

FIG. 7. Construction of plasmid pHFCAT by cloning the HBV *Hpa*I-*Fsp*I (nt 964 to 1805) region into the *Bgl*II site, upstream of the CAT gene in the enhancerless and promoterless plasmid pSVoCAT (10). The *Hpa*I-*Fsp*I fragment contains the following HBV elements: enhancer (En), X gene promoter (Xp), and core gene promoter (Cp).

CV-1 cells (22). These results suggested that the HBV enhancer may, at least in part, confer the tissue-specific expression of HBV genes. Similar results were also reported by another group (20). Recently, contradictory results have made tissue specificity of the HBV enhancer questionable (5, 27). This controversy could be resolved by considering the different constructs used in these studies, since some contained the core gene promoter while others did not. Here, we analyzed the tissue-specific expression of the HBV enhancer element and the core gene promoter linked to heterologous regulatory elements. We report that the HBV enhancer and core gene promoter each exhibit some tissue specificity. However, when they were linked together, expression was restricted to human liver cells.

When the HBV enhancer was linked to the heterologous SV40 early-gene promoter, induction of expression in differentiated liver cells was 5- to 20-fold higher than in other cells. The same tissue-specific pattern of expression was demonstrated when the HBV enhancer was linked to the β-globin gene promoter. In the latter situation, the HBV enhancer effected only a threefold higher expression of globin RNA in differentiated cell lines. The small difference between the amount of induction observed in the CAT assays and that seen in the RNA analysis may reflect the different relative activities of the two heterologous promoters used in these studies. While the SV40 promoter was shown to be non-tissue-specific (10), β-globin promoter activity may fluctuate with different cell types, which may explain the discrepancies between the results of these experiments. It was therefore of interest to examine the tissue-specific expression of the HBV enhancer-core gene promoter complex.

Although the 5′ ends of the core and pregenomic HBV mRNA were previously mapped in HBV (20, 30) and other hepadnaviruses (6, 17), the HBV core gene promoter was never mapped, nor is its full expanse known. We were able to map the core gene promoter to within a 100 bp-region spanning nt 1705 to 1805 of the HBV genome. This 100-bp region was shown to possess promoter activity under the heterologous SV40 enhancer by driving expression of a reporter CAT gene. By primer extension analysis, this core gene promoter initiated transcription at sites correlating to the 5′ ends of the core messages (20, 30). We mapped two initiation sites corresponding to nt 1792 ± 3 and 1821 ± 3 of HBV. Transcription from these sites in vivo would encode the pre-C and core-initiated proteins, respectively. Recently, we have mapped the same sites by an in vitro transcription system (manuscript in preparation). The results of both CAT and primer extension analyses shown here refute a previous work which found that HBV sequences downstream of nt 1806 are essential for core gene promoter activity (20).

Mapping the core gene promoter to a defined 100-bp region allowed us to analyze the core gene promoter for tissue-specific expression. We demonstrated that under the control of the heterologous SV40 enhancer, the promoter expresses low activity in nonliver CV-1 and HeLa cells, intermediate activity in Hep-SK cells, and high activity in Hep-G2 and Alexander cells. Since Hep-SK cells were found to contain low levels of albumin and phenotypically resemble fibroblasts in culture, these cells were concluded to be nondifferentiated liver cells (A. Ben-Zeev, personal communication). Therefore, a plausible explanation for the intermediate activity observed in these cells is that they may contain some liver-specific transcriptional factors which are synthesized at an early stage of liver cell differentiation. However, Hep-G2 cells, which are more highly differentiated, might contain additional factors which enhance core promoter activity. Alexander cells are different from other differentiated hepatoma cell lines in that they contain integrated HBV sequences. It follows, then, that these sequences may be either directly or indirectly transactivating the core gene promoter in the cells, allowing for an even higher induction of transcription. Alternatively, the Alexander cell line, being associated with HBV, may represent a cell line which was naturally selected for higher HBV expression. This, too, provides an explanation for the high promoter activity in these cells. The pattern of cell type preference seen here is not unique to the core gene promoter and to the HBV enhancer, since it has also been observed in the case of the surface promoter (4, 8).

pHFCAT contains the core gene promoter under the homologous HBV enhancer. This combination proved to be totally tissue specific in that absolutely no activity was observed in the poorly differentiated Hep-SK cells. Hep-G2 and Alexander cells, on the other hand, provided an environment which permitted high CAT expression. Although the HBV enhancer-promoter complex exhibited high tissue specificity, the combined activity, at least in Alexander cells, was somewhat lower than expected. The core promoter and HBV enhancer, when assayed alone, showed 720 and 125% relative activity, respectively (Fig. 1). When combined, however, activity was only 100 rather than 845%, the expected result of an additive effect of the two elements. This may suggest that additional regulatory signals are present between the enhancer and core gene promoter. In fact, deletion analysis which removed the intervening sequences resulted in core gene promoter activation (data not shown). We propose several reasons which may account for the down-regulation of core promoter activity by these sequences. (i) A negative element may be present which regulates promoter activity. A similar type of regulation has recently been described for the HBV surface promoter, in which a negative element was responsible for decreasing promoter activity in certain cell types (4). (ii) Transcription from the upstream X promoter (24) may affect core activity by a mechanism termed promoter occlusion (1, 13). By activation of this promoter, down-regulation of the downstream core promoter could result. (iii) A polymerase III transcript, spanning HBV nt 1635 to 954, has been previously identified (23). The region encoding this transcript is present within the *Hpa*I-*Fsp*I construct used in our studies and may regulate the expression of the core gene promoter as has been previously suggested (23). (iv) The pHFCAT construct contains almost the entire open reading frame, encoding a carboxy-terminally truncated X protein which may play some regulatory role in this construct.

The high tissue specificity of the core gene promoter-enhancer combination, as shown here, by regulating production of the pregenomic message in liver cells, may contribute to the restricted replicative potential of the virus in differentiated liver cells and consequently to the high expression of its genes in those cells. The recently achieved expression and replication of the HBV genome in HeLa cells (a human, nonliver cell line) by replacement of the core gene promoter with the metallothionein IIA promoter (12) underscore the tissue-specific constraints imposed on HBV by the presence of the enhancer-core gene promoter combination. Synergistic action has already been described in the case of the immunoglobulin enhancer and promoter (9). Here, however, we show a combinatorial effect of an enhancer and a promoter on tissue specificity. This mechanism may have

wider implications in determining the tissue-specific expression of a large number of other genes.

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