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# Differentiation-Specific Transcriptional Regulation of the Hepatitis B Virus Large Surface Antigen Gene in Human Hepatoma Cell Lines†

ANNEKE K. RANEY,[1] DAVID R. MILICH,[2] ANDREW J. EASTON,[3] AND ALAN McLACHLAN[1]*

*Departments of Molecular and Experimental Medicine[1] and Molecular Biology,[2] Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037, and Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom[3]*

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**The transcriptional activities of the four hepatitis B virus promoters were compared in three differentiated hepatoma cell lines, HepG2, Hep3B, and PLC/PRF/5; a dedifferentiated subline of HepG2, HepG2.1; a human cervical carcinoma cell line, HeLa S3; and a mouse fibroblast cell line, NIH 3T3. The plasmid constructs, which contain the complete hepatitis B virus genome directing the expression of the luciferase reporter gene, were analyzed by transient transfection assays. The relative orders of the levels of the transcriptional activities of the four promoters were similar in each of the cell lines. The major surface antigen and X-gene promoters displayed the highest activity levels, the core promoter activity level was less than or similar to the activity levels of these two promoters, and the large surface antigen promoter had the lowest activity level in all of the cell lines examined. The core promoter demonstrated an approximately 2- to 20-fold higher relative level of expression in the differentiated hepatoma cell lines, suggesting that this promoter might be preferentially active in these cells. The relative level of activity of the large surface antigen promoter in the differentiated hepatoma cell lines was approximately 5 to 90 times greater than that observed in the other cell lines, indicating that the activity of this promoter is highly specific for differentiation state and cell type. Deletion analysis of the large surface antigen promoter demonstrated that the sequence element responsible for the differentiation state-specific expression from this promoter is located between nucleotides 2719 and 2733 (−90 and −76). Within this sequence element is a binding site (GTTAATCATTACT) for the liver-specific transcription factor hepatocyte nuclear factor 1 (HNF1). This indicates that the preferential expression from the large surface antigen promoter in the differentiated hepatoma cell lines is probably mediated by HNF1 or an HNF1-related transcription factor.**

The genome of the hepatitis B virus (HBV) is a 3.2-kilobase (kb) partially double-stranded DNA molecule. In the virus particle, it is packaged in a 27-nm nucleocapsid composed of hepatitis B core antigen which is enveloped by a lipoprotein coat comprising cellular lipid and hepatitis B surface antigen (20, 56). The envelope of HBV contains three surface antigen polypeptides, the major, middle, and large polypeptides, which are produced from a single open reading frame by translation from three in-frame initiation codons (20, 24, 56). The major and middle surface antigen polypeptides are synthesized from abundant 2.1-kb RNAs which are transcribed from the major surface antigen promoter (20, 36). The large surface antigen polypeptide is synthesized from a minor 2.4-kb RNA which is transcribed from the large surface antigen promoter (20). The HBV genome contains two additional promoters, the X-gene and core promoters, which direct the transcription of 0.7- and 3.5-kb transcripts, respectively (25, 28, 29, 51, 57, 67). The X-gene product has been reported to transactivate transcription from several viral and cellular promoters (12, 47, 49, 52, 59–62, 65, 69). The 3.5-kb RNAs serve as the template for replication of the HBV genome by reverse transcription and encode the HBV nucleocapsid and polymerase polypeptides (4, 9, 64).

HBV infection is primarily restricted to hepatocytes. However, the reason for this highly restricted tropism is unknown. Replication of HBV may be restricted to hepatocytes for several reasons, including the absence of viral receptors on nonhepatic cells and the requirement for liver-specific transcription factors for expression from one or more of the HBV promoters. Characterization of the cell type specificity of the HBV enhancer and promoters has suggested that the enhancer sequence and the core promoter may display preferential activity in hepatoma cell lines (1, 25, 26, 29, 67), whereas the major surface antigen gene appears to be expressed efficiently in a range of cell types (15, 38, 50). It has been demonstrated that production of virus particles can be achieved by transfection of hepatoma cells with HBV genomic DNA (7, 45, 55, 58, 66). The requirement for hepatoma cells in the production of HBV and woodchuck HBV by this approach can be circumvented by expressing the pregenomic RNA from an exogenous promoter rather than from the core promoter (27, 44). This observation suggests that in transfection experiments, a block to virus production in nonhepatic cells is at the level of transcription from the core promoter. Similarly, for transgenic mice which synthesize HBV particles, it has been shown that the major HBV transcripts are synthesized predominantly in liver and kidney tissue. This also indicates that there is a tissue-specific transcriptional restriction in the expression of the HBV genes in this system (2, 18).

In this study, the transcriptional activities of the four HBV promoters were investigated in the context of the complete HBV genome to examine further the role of transcriptional regulation in the restricted tropism of the viral life cycle. This analysis indicated that the core promoter and, to a greater extent, the large surface antigen promoter were preferentially active in differentiated hepatoma cell lines.

* Corresponding author.

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The sequence elements involved in the preferential expression from the large surface antigen promoter in these cells were characterized and shown to contain a binding site for the liver-specific transcription factor hepatocyte nuclear factor 1 (HNF1) (13, 19, 30). In contrast, the relatively lower level of expression from the large surface antigen promoter in a dedifferentiated hepatoma cell line was independent of the HNF1 recognition sequence. These observations may explain the preferential activity of this promoter in the differentiated hepatoma cell lines.

## MATERIALS AND METHODS

**Plasmid constructions.** The various steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (32). The HBV sequences in these constructions were derived from the plasmid pCP10, which contains two copies of the HBV genome (subtype *ayw*) cloned into the *Eco*RI site of pBR322 (17). The plasmid SpLUC (Fig. 1) was constructed by digesting pCP10 with *Xho*I, filling in the overhang with the Klenow fragment of *Escherichia coli* DNA polymerase, ligating *Hin*dIII linkers, digesting with *Hin*dIII, and cloning the 3.2-kilobase-pair HBV fragment into the *Hin*dIII site of the plasmid p19DLUC. p19DLUC was generated from p19LUC (63) by deletion of the polylinker sequences between the *Hin*dIII and *Sal*I sites and insertion of a *Hin*dIII linker at this position. This eliminated the polylinker *Sph*I site, including the nucleotides ATG. These nucleotides might have interfered with the translation of the luciferase gene product from the transcripts synthesized from the HBV promoters. The unique HBV *Xho*I site used in this construct is located 157 nucleotides 3′ to a predominant surface antigen gene transcription initiation site (5, 39). Therefore, the plasmid SpLUC contains one complete HBV genome (nucleotides 130 to 3182/1 to 133′) located immediately 5′ to the promoterless firefly luciferase (LUC) reporter gene such that the expression of the LUC gene is governed by the hepatitis B major surface antigen promoter. The designation 133′ has been used to indicate that nucleotides 130 to 133 are present twice in this plasmid and that nucleotides 130 to 133 and 130′ to 133′ are distal and proximal to the LUC open reading frame, respectively. The nucleotide sequences are designated by using coordinates derived from the GenBank genetic sequence data bank. Similarly, the plasmids XpLUC, CpLUC, and PS(1)pLUC (Fig. 1), which contain one complete HBV genome (nucleotides 1375 to 3182/1 to 1376′, 1805 to 3182/1 to 1804, and 2840 to 3182/1 to 2843′, respectively), were constructed such that the expression of the LUC gene was governed by the HBV X-gene, nucleocapsid, and large surface antigen promoters, respectively. The plasmid constructs containing the insertion and various deletions, the PS(1)pLUC series (see Fig. 3 and 4), were generated by appropriate restriction endonuclease or *Bal*31 nuclease digestions of HBV sequences and subsequent cloning steps similar to those described for SpLUC and PS(1)pLUC. All deletion breakpoints generated by *Bal*31 nuclease digestion were determined by dideoxynucleotide sequencing (42). The extent of the deleted nucleotide sequence is indicated in the plasmid designation with coordinates derived from the GenBank genetic sequence data bank.

**Cells, transfections, and enzyme assays.** The human hepatoblastoma cell lines HepG2 and HepG2.1 and the human hepatocellular carcinoma cell lines PLC/PRF/5 (Alexander cells) and Hep3B were grown in RPMI 1640 medium and

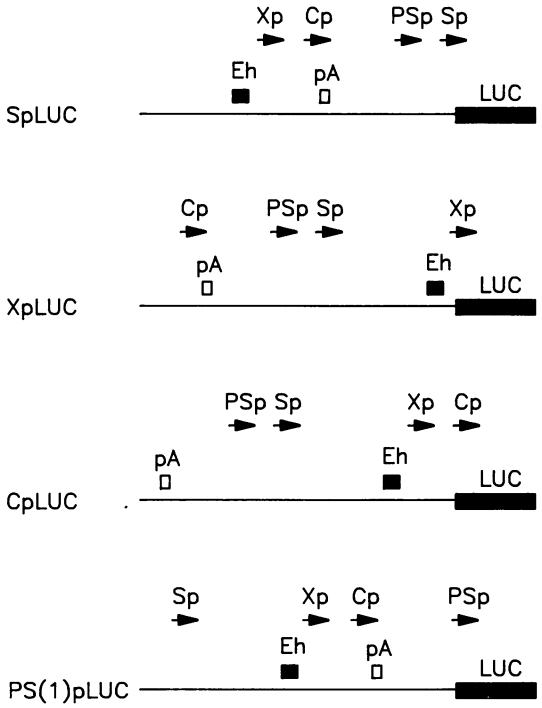



FIG. 1. Luciferase reporter gene constructs used to determine the relative transcriptional activities from the four HBV promoters. The SpLUC, XpLUC, CpLUC, and PS(1)pLUC constructs contain the complete HBV genome inserted into p19DLUC immediately 5′ to the luciferase reporter gene such that expression of the luciferase reporter gene is controlled by the major surface antigen, X-gene, nucleocapsid (core), and large surface antigen (pre-S1) promoters, respectively. Arrows indicate the position and direction of transcription from the HBV X-gene (Xp), core (Cp), pre-S1 (PSp), and major surface antigen (Sp) promoters. Boxes indicate the positions of the HBV enhancer sequence (Eh), the HBV polyadenylation recognition sequence (pA), and the luciferase (LUC) open reading frame. Horizontal lines indicate the HBV sequences present in the various luciferase constructs.

10% fetal bovine serum at 37°C in 5% $CO_2$-air. The human cervical carcinoma cell line HeLa S3 and the mouse fibroblast cell line NIH 3T3 were grown in Dulbecco modified Eagle medium containing 4.5 mg of glucose per ml and 10% calf serum at 37°C in 5% $CO_2$-air. Transfections were performed as previously described (33). The transfected DNA mixture comprised 15 μg of a LUC plasmid and 1.5 μg of pSV2CAT (21) or pMTCAT, which served as an internal control for transfection efficiency. pSV2CAT and pMTCAT direct the expression of the chloramphenicol acetyltransferase (CAT) gene by using the simian virus 40 (SV40) early promoter and the mouse metallothionein promoter, respectively. Cell extracts were prepared 40 to 48 h after transfection and assayed for luciferase and CAT activity as previously described (16). For the biochemical analysis of the cell lines, cell extracts were prepared by suspending approximately $5 \times 10^6$ cells in 100 μl of 0.1 M $KPO_4$, pH 7.3, and freeze-thawing the cells three times. The whole cell extract was centrifuged at 20,000 × *g* for 30 min, and the supernatant was removed for enzyme analysis. The γ-glutamyltransferase assays were performed on 10 μl of cell extract with a Kodak Ektachem DT60 analyzer (Eastman Kodak Co., Rochester, N.Y.).

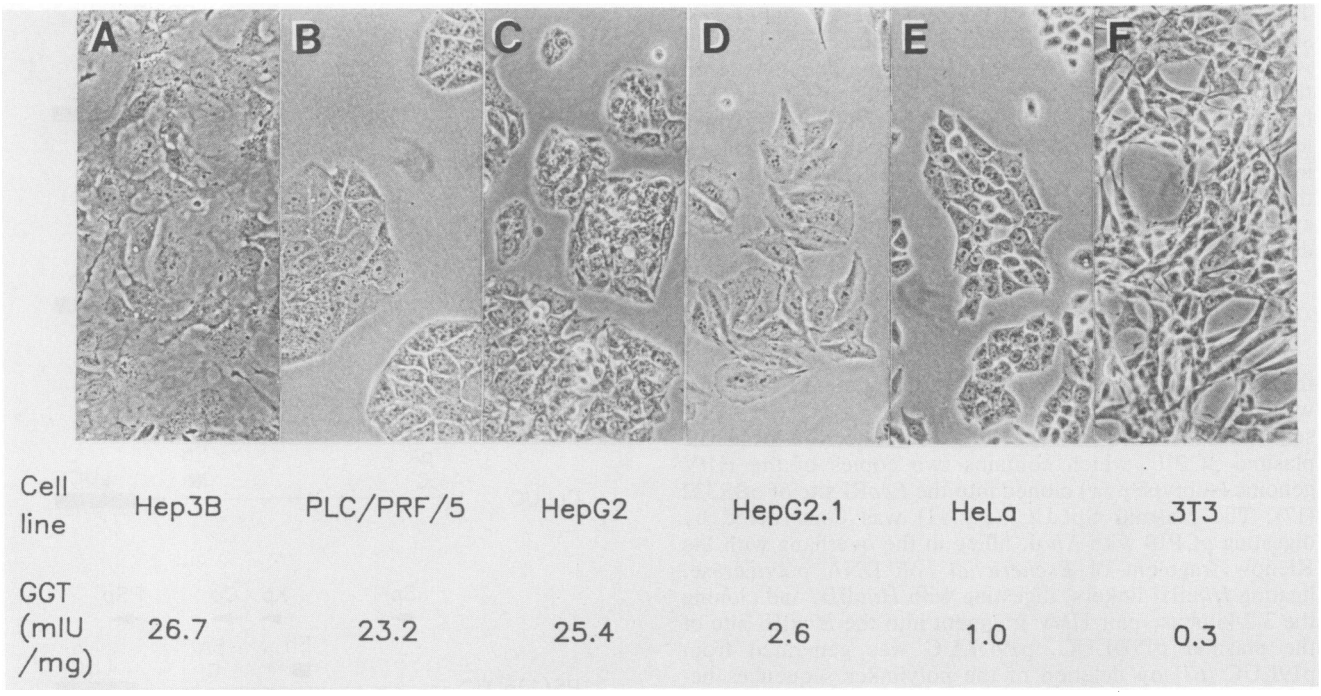



FIG. 2. Phase-contrast photomicrographs of living cultures of Hep3B (A), PLC/PRF/5 (B), HepG2 (C), HepG2.1 (D), HeLa S3 (E), and NIH 3T3 (F) cells. The γ-glutamyltransferase (GGT) specific activities (in milli-international units [mIU] per milligram of protein) in extracts from these cells are indicated.

## RESULTS

**Morphological and biochemical characterization of cell lines.** The appearances of the cells used in the transfection analyses were examined (Fig. 2). The differentiated hepatoma cell lines Hep3B, PLC/PRF/5, and HepG2 displayed hepatocytelike regular epithelial morphology. In contrast, the dedifferentiated HepG2.1 hepatoma cell line, which arose spontaneously from the HepG2 cell line and was identified on the basis of its morphological phenotype, showed an irregular, flat shape (Fig. 2). The conversion of the HepG2 cell line to the HepG2.1 cell line occurred with the majority of the cells in the population showing a progressively less differentiated morphology with extended time in culture. The appearance of the Hep3B and PLC/PRF/5 cells did not change under the same culture conditions. The HeLa S3 and the NIH 3T3 cells displayed typical epithelial and fibroblast morphologies, respectively. Biochemical evidence supporting the origin of the differentiated hepatoma cell lines was obtained by measuring the activity of the enzyme γ-glutamyltransferase (40) (Fig. 2). This enzyme activity was high in the differentiated hepatoma cell lines, very low in the nonhepatoma cell lines, and somewhat higher in the dedifferentiated hepatoma cell line HepG2.1 (Fig. 2). This result demonstrated a biochemical difference, in addition to the morphological difference, between the HepG2 and the HepG2.1 cell lines.

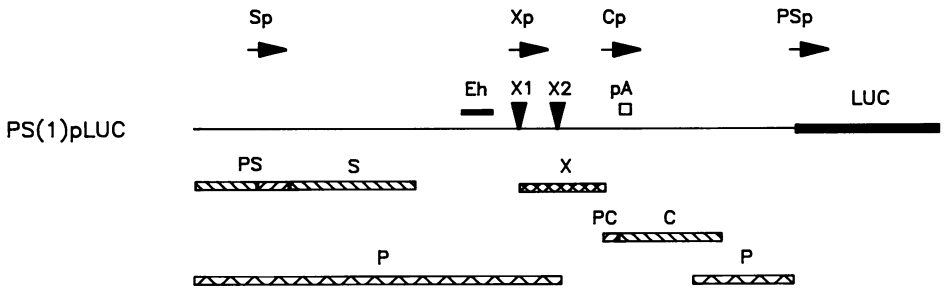
**Relative activities of the four HBV promoters.** The relative transcriptional activities of the four HBV promoters in the differentiated hepatoma cell lines HepG2, Hep3B, and PLC/PRF/5, the dedifferentiated hepatoma cell line HepG2.1, and the nonhepatoma cell lines HeLa S3 and NIH 3T3 were examined. The SpLUC, XpLUC, CpLUC, and PS(1)pLUC constructs (Fig. 1) were transfected into these cell lines, and variations in transfection efficiencies were normalized by determining the number of light units produced by the luciferase activity and dividing it by the percentage of chloramphenicol which was acetylated by the CAT activity in the same cell extract. The relative activities were then determined by calculating the ratios of the normalized luciferase activities observed after transfection of XpLUC, CpLUC, and PS(1)pLUC to the normalized luciferase activity observed after transfection of SpLUC. The internal control in these transfection experiments was pSV2CAT, and the observed CAT activities were similar in the extracts prepared from the various cell lines.

The relative activity levels of the four HBV promoters in these cell lines were compared, and the order of the activity levels of the four HBV promoters, Sp $\simeq$ Xp $\geq$ Cp > PS(1)p, is similar in each of the cell lines examined (Table 1). It is also apparent that all four HBV promoters have some level of activity in each cell line. In each case, the PS(1)p was the weakest promoter, suggesting that, at least in the case of expression in these cell lines, the large surface antigen polypeptide is not likely to be synthesized in excess over the major surface antigen polypeptide. Therefore, inhibition of secretion of surface antigen is unlikely to occur when the expression of the HBV genes is being controlled by the endogenous promoters. This contrasts with the situation in

TABLE 1. Relative activity levels of HBV promoters

| Promoter | Relative level of activity[a] with cell line: | | | | | |
|---|---|---|---|---|---|---|
| | Hep3B | PLC/PRF/5 | HepG2 | HepG2.1 | HeLa | NIH 3T3 |
| SpLUC | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| XpLUC | 2.02 | 1.33 | 0.66 | 0.65 | 2.44 | 0.88 |
| CpLUC | 0.34 | 1.35 | 0.90 | 0.15 | 0.20 | 0.068 |
| PS(1)pLUC | 0.078 | 0.016 | 0.046 | 0.0019 | 0.0028 | 0.0009 |

[a] Activity levels of HBV promoters are reported relative to the activity level of the major surface antigen promoter in each cell line. The internal control used to correct for transfection efficiencies was pSV2CAT.




Hep3B

| Transfected DNA | LUC (Light units) | CAT (% acetylation) | LUC/CAT ratio | Relative activity |
|---|---|---|---|---|
| PS(1)pLUC/pMTCAT | 1714 | 9.7 | 177 | 1.00 |
| PS(1)pX1LUC/pMTCAT | 1322 | 11.9 | 111 | 0.63 |
| PS(1)pX2LUC/pMTCAT | 1787 | 13.8 | 129 | 0.73 |
| PS(1)pLUC/pSV2CAT | 3167 | 12.8 | 247 | 1.00 |
| PS(1)pX1LUC/pSV2CAT | 880 | 5.6 | 157 | 0.64 |
| PS(1)pX2LUC/pSV2CAT | 2123 | 9.3 | 228 | 0.92 |

HepG2.1

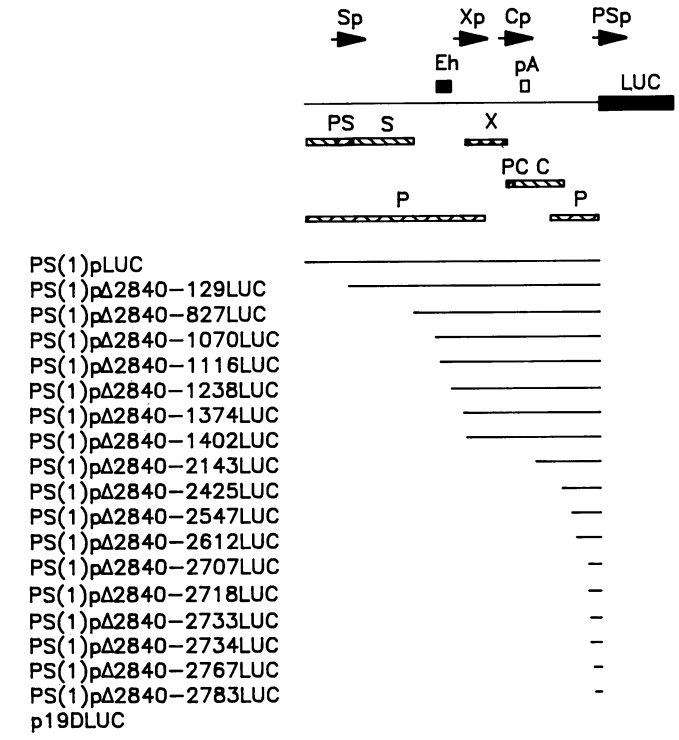
| Transfected DNA | LUC (Light units) | CAT (% acetylation) | LUC/CAT ratio | Relative activity |
|---|---|---|---|---|
| PS(1)pLUC/pMTCAT | 335 | 3.5 | 95.7 | 1.00 |
| PS(1)pX1LUC/pMTCAT | 386 | 10.3 | 37.5 | 0.39 |
| PS(1)pX2LUC/pMTCAT | 236 | 5.8 | 40.7 | 0.43 |
| PS(1)pLUC/pSV2CAT | 373 | 13.7 | 27.2 | 1.00 |
| PS(1)pX1LUC/pSV2CAT | 427 | 26.5 | 16.1 | 0.59 |
| PS(1)pX2LUC/pSV2CAT | 264 | 34.9 | 7.6 | 0.28 |

FIG. 3. Influence of the X-gene open reading frame on the expression of the large surface antigen, SV40 early, and mouse metallothionein promoters in Hep3B and HepG2.1 cells. Arrows, boxes, and line designations are as described for Fig. 1. Hatched boxes indicate the positions of the presurface antigen (PS) open reading frame (ORF), surface antigen ORF (S), X-gene ORF (X), precore ORF (PC), core ORF (C), and polymerase ORF (P). The arrowheads designated X1 and X2 indicate the locations of the 4-nucleotide deletion and 11-nucleotide insertions in the PS(1)pX1LUC and PS(1)pX2LUC constructs, respectively. LUC, Luciferase; CAT, chloramphenicol acetyltransferase.

which the synthesis of the large surface antigen polypeptide is controlled by a strong exogenous promoter (10, 11, 33, 35, 37, 53). In addition, the relative level of activity of the PS(1)p in the differentiated hepatoma cell lines was approximately 5 to 90 times that observed in the dedifferentiated hepatoma cell line and the nonhepatic cell lines. This suggests that this promoter may have a greater specificity for transcriptional activity in differentiated cells of hepatic origin than the other HBV promoters. As a consequence, it is possible that the level of synthesis of the large surface antigen, an essential component of the virion (24), represents a limiting step in Dane particle production in cell culture. This could explain why virus production in transfection experiments has been readily detectable only with differentiated hepatoma cell lines (7, 45, 48, 55, 58, 66). The Cp demonstrated a slightly higher relative level of activity in the differentiated hepatoma cell lines, approximately 2- to 20-fold, as compared with the dedifferentiated hepatoma cell line and the nonhepatic cell lines, suggesting that this promoter might also be preferentially utilized in differentiated hepatoma cell lines.

**Influence of the X gene on the large surface antigen promoter.** It has been reported that the product of the X gene can transactivate the SV40 enhancer and early promoter (49, 52, 60, 69), whereas there have been conflicting results concerning the effect of the X-gene product on the transcriptional activities from HBV genes (12, 49, 52, 60). Since several of the constructs used in this study have the potential either to code for the X-gene product or to be transactivated by it, this possibility was examined. The construct PS(1)pLUC (Fig. 3), which contains the intact X gene and the regulatory sequence elements necessary for its expression, was modified either by deleting 4 nucleotides (coordinates 1375 to 1378, representing part of the *Nco*I site), including the initiation codon for the X gene, or by inserting 11 nucleotides into the middle of the X-gene open reading frame (the insertion of a *Sac*I linker into the *Rsr*II site at coordinate 1577). Both of these modifications, which generated plasmids PS(1)pX1LUC and PS(1)pX2LUC, respectively, were designed to generate derivatives of PS(1)pLUC which could not produce the product of the X gene. The absolute and relative activity levels of the large surface antigen promoter, the SV40 early promoter, and the mouse metallothionein promoter were compared in transient cotransfection assays with either Hep3B or HepG2.1 cells. If the X-gene product was being synthesized by the PS(1)pLUC construct, it did not appear to influence substantially the activity of any of the promoters examined in this system (Fig. 3). The results suggest that if the product of the X gene influences the activity of any of the promoters examined, it transactivates the large surface antigen promoter by less than threefold, as the relative activity of this promoter is slightly reduced in constructs in which the X-gene product cannot be synthesized.

**Characterization of the large surface antigen promoter elements.** The absence of preferential expression from the large surface antigen promoter in the dedifferentiated hepatoma cell line HepG2.1 is consistent with the loss of liver-specific properties in this cell line. To examine this observation further, the analysis of the transcriptional regulatory elements that control the expression of the HBV large




| | Relative luciferase activity | | | |
|---|---|---|---|---|
| | Hep3B | Al | HepG2 | HepG2.1 |
| PS(1)pLUC | 1.00 | 1.00 | 1.00 | 1.00 |
| PS(1)pΔ2840–129LUC | 0.84 | 2.32 | 1.51 | 0.58 |
| PS(1)pΔ2840–827LUC | 1.61 | 6.05 | 2.68 | 2.05 |
| PS(1)pΔ2840–1070LUC | 3.55 | 8.30 | 4.60 | 2.44 |
| PS(1)pΔ2840–1116LUC | 1.44 | 4.18 | 2.32 | 0.92 |
| PS(1)pΔ2840–1238LUC | 0.28 | 3.04 | 3.67 | 3.93 |
| PS(1)pΔ2840–1374LUC | 0.28 | 4.30 | 2.36 | 0.80 |
| PS(1)pΔ2840–1402LUC | 0.24 | 2.81 | 2.52 | 0.83 |
| PS(1)pΔ2840–2143LUC | 0.14 | 2.44 | 0.40 | 0.21 |
| PS(1)pΔ2840–2425LUC | 0.41 | 0.62 | 1.69 | 0.35 |
| PS(1)pΔ2840–2547LUC | 0.56 | 5.31 | 1.76 | 0.15 |
| PS(1)pΔ2840–2612LUC | 0.42 | 1.55 | 2.50 | 0.64 |
| PS(1)pΔ2840–2707LUC | 1.11 | 3.83 | 2.64 | 0.58 |
| PS(1)pΔ2840–2718LUC | 1.06 | 0.70 | 1.40 | 0.67 |
| PS(1)pΔ2840–2733LUC | 0.05 | 0.13 | 0.04 | 0.84 |
| PS(1)pΔ2840–2734LUC | 0.02 | 0.12 | 0.14 | 0.36 |
| PS(1)pΔ2840–2767LUC | 0.01 | 0.04 | 0.01 | 0.14 |
| PS(1)pΔ2840–2783LUC | 0.00 | 0.00 | 0.00 | 0.01 |
| p19DLUC | 0.00 | 0.00 | 0.00 | 0.00 |

FIG. 4. Deletion analysis of the HBV large surface antigen promoter in differentiated and dedifferentiated hepatoma cell lines. Al (Alexander cells) indicates the PLC/PRF/5 cell line. Arrows, boxes, and line designations are as described for Fig. 3. The horizontal lines indicate the HBV sequences present in the various PS(1)pΔLUC series plasmids. The plasmid PS(1)pLUC contains the HBV sequences from nucleotides 2840 to 3182/1 to 2843′ (nucleotide sequences are designated by using coordinates derived from the GenBank genetic sequence data bank). The designation 2843′ has been used to indicate that the nucleotides 2840 to 2843 are repeated in this plasmid and that the nucleotides 2840 to 2843 and 2840′ to 2843′ are distal and proximal to the *LUC* open reading frame, respectively. The HBV sequences deleted from the various plasmids are designated by nucleotide coordinates. The internal control used to correct for transfection efficiencies was pSV2CAT.

surface antigen gene was performed by using transient expression assays in the human hepatoma cell lines Hep3B, PLC/PRF/5, HepG2, and HepG2.1. A series of promoter deletions was constructed and used to direct the expression of the luciferase reporter gene (Fig. 4). The initial plasmid, PS(1)pLUC (Fig. 1 and 4), contains the complete sequence of the HBV genome. A series of 5′ deletions of the HBV genome was tested for its effect on the transcriptional activity of the large surface antigen promoter. In the differentiated hepatoma cell lines, Hep3B, PLC/PRF/5, and HepG2, transcriptional activity was retained when sequences upstream of −90 [plasmid PS(1)pΔ2840-2718LUC] were deleted, indicating that a minimal promoter element exists between −90 and +35 (coordinates 2719 to 2843) (Fig. 4 and 5). Sequences distal to −90, including the HBV enhancer, major surface antigen, X-gene, and nucleocapsid promoters, do not appear to influence expression greatly from the large surface antigen promoter. A possible excep-

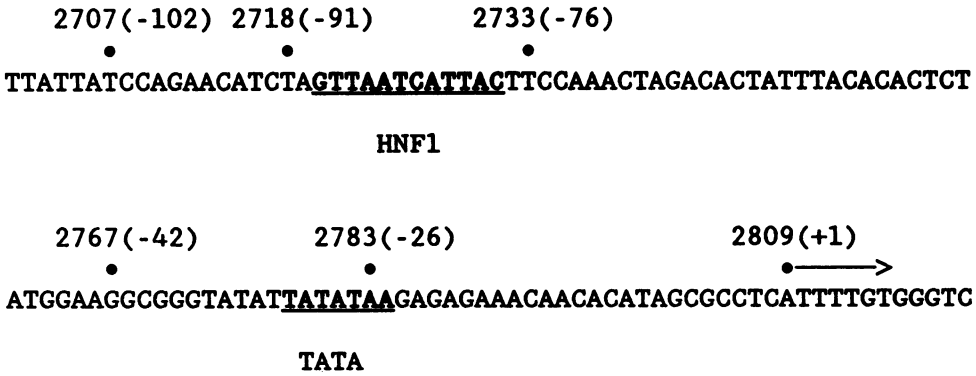



FIG. 5. Sequence of the HBV large surface antigen promoter region (subtype *ayw*). The numbered nucleotides (•) indicate the breakpoints of the functionally significant deletions. The numbers in parentheses indicate the locations of the breakpoints relative to the HBV large surface antigen transcription initiation site (46, 66), which is designated by an arrow. The underlined sequences represent the HNF1 consensus sequence and TATA box sequence homologies.

tion to this observation may be the influence of the enhancer sequences (coordinates 1117 to 1238) on the activity of the large surface antigen promoter in Hep3B cells. It appears that in this cell line, the presence of these sequences results in an approximately fivefold increase in transcription from this promoter.

In the differentiated hepatoma cell lines, deletion of the large surface antigen promoter sequences upstream of −75 (Fig. 4 and 5) results in the loss of 80 to 95% of the transcriptional activity from this promoter [plasmid PS(1)pΔ2840-2733LUC]. This region contains a sequence (GTTAATCATTACT) homologous to the consensus recognition sequence (GTTAATNATTAAC) for the transcription factor HNF1, which has been shown to bind to this element in the large surface antigen promoter (13). Since HNF1 has a role in the expression of several liver-specific genes (14, 23, 31, 34, 43), it seems likely that the loss of binding of this factor or a related factor is responsible for the observed loss of transcriptional activity associated with this deletion in the differentiated hepatoma cell lines examined. Since HNF1 is a highly liver-specific transcription factor (19, 30), it is possible that the restricted cell type expression of the HNF1 gene contributes to the tissue tropism of the hepatitis B virus. In addition, these observations may explain the preferential expression from the large surface antigen promoter in differentiated hepatoma cell lines (Table 1). Deletion of an additional 34 nucleotides resulted in an almost complete loss of transcriptional activity from the large surface antigen promoter [plasmid PS(1)pΔ2840-2767LUC] in the differentiated hepatoma cell lines. In contrast, the expression from the large surface antigen promoter in the dedifferentiated hepatoma cell line, HepG2.1, showed essentially no dependence on the HNF1-binding sequence, as transcriptional activity remained when sequences upstream of −75 [plasmid PS(1)pΔ2840-2733LUC] were deleted. This defines a minimal functional promoter element between −75 and +35 (coordinates 2734 to 2843) in this dedifferentiated cell line. Complete loss of transcriptional activity is not observed in the dedifferentiated hepatoma cell line until the large surface antigen TATA box sequence (−25 to −31) is partially deleted [plasmid PS(1)pΔ2840-2783LUC]. These results suggest that the loss of the hepatocytelike morphology correlates with the loss of a functional HNF1 or HNF1-related transcription factor. This is reflected in the relative activity levels and in the sequence elements involved in the regulation of the large surface antigen promoter in differentiated versus dedifferentiated hepatoma cell lines (Table 1; Fig. 4 and 5). A similar situation has been reported for the sequence elements involved in the regulation of the expression of the rat β-fibrinogen gene in the rat hepatoma cell line Fao and in the dedifferentiated cell line FaoflC2, which is derived from the Fao cell line (3).

## DISCUSSION

HBV is a human hepatotropic virus which has a strict host range, infecting only humans and chimpanzees. In cell culture, HBV has been shown to infect primary human hepatocytes, but it has not been possible to infect tissue culture cell lines (22, 41). For these reasons, it has been difficult to investigate the factors responsible for the tropism of HBV. It is possible that tissue-specific transcriptional regulation of expression of HBV RNAs might be responsible, in part, for the observed viral tropism. Analysis of the transcriptional properties of the HBV enhancer and core promoter suggests that these regulatory elements are preferentially active in cells of hepatic origin (1, 25, 26, 29, 67). In transgenic mice containing complete copies of the HBV genome integrated into their genomes, it has been observed that transcription of the 2.1- and the 3.5-kb HBV mRNAs occurs primarily in the liver and the kidney (2, 18). These observations suggest that transcriptional regulation may be an important factor in determining the tissues capable of synthesizing HBV particles.

In this study, the cell type specificity of the four HBV promoters was examined in the context of the complete viral genome. The complete viral genome was located upstream of the luciferase reporter gene such that the major surface antigen, X-gene, nucleocapsid, and large surface antigen promoters directed the expression of the reporter gene in the constructs SpLUC, XpLUC, CpLUC and PS(1)pLUC, respectively (Fig. 1). These constructs were designed to examine the activity of the four HBV promoters in the context of the complete HBV genome, in which transcriptional interference resulting from the activity of more than one promoter on a single HBV molecule might occur, and in the presence of all of the possible *cis*-acting regulatory sequence elements which might influence the activities of the HBV promoters. Characterization of the relative promoter activities indicated that the major surface antigen and X-gene promoters were the strongest in all of the cell lines examined. The core promoter had an activity level similar to those of the major surface antigen and X-gene promoters in differentiated hepatoma cells and a somewhat lower activity level in dedifferentiated hepatoma and nonhepatic cells. The large surface antigen promoter was the weakest promoter in all of the cell lines examined. However, like the core promoter, it demonstrated a higher relative activity level compared with the major surface antigen promoter in the differentiated hepatoma cell lines. These results suggest that there are factors in differentiated hepatoma cell lines which act specifically on the core and large surface antigen promoters to enhance their activity in these particular cells. The order and relative promoter strengths observed were different from those reported previously, and therefore it appears that the nature of the promoter construct may influence the relative promoter strengths (1). The relative promoter strengths observed in this study are consistent with the levels of HBV transcripts observed in infected liver tissue and differentiated hepatoma cell lines producing viral particles (6, 7, 54, 55, 58, 66, 68). This suggests that the constructs SpLUC, CpLUC, and PS(1)pLUC demonstrate a transcriptional activity profile similar to that which occurs during viral particle production in cell culture. The high activity level of the X-gene promoter does not correspond with the absence or relatively low abundance of the 0.7-kb transcript observed in infected liver tissue and differentiated hepatoma cell lines producing viral particles (6, 7, 54, 55, 58, 66, 68). This suggests that the 0.7-kb transcript may be relatively unstable compared with the luciferase transcript expressed by the XpLUC construct or that some aspect of the X-gene promoter regulation has been lost in these transient transfection experiments.

The observation that the relative activity level of the large surface antigen promoter was 5 to 90 times higher in differentiated hepatoma cell lines than in the dedifferentiated hepatoma cell line and the nonhepatic cell lines suggested that there were factors in differentiated hepatoma cells which specifically increase transcription from this promoter (Table 1). In an attempt to characterize the regulatory sequence elements in the HBV genome which mediated this effect, a deletion analysis of the large surface antigen pro-

moter in differentiated and dedifferentiated hepatoma cell lines was performed (Fig. 4). Since the initial construct, PS(1)pLUC, contained the complete X gene and the regulatory sequences necessary for its expression, the possible influence of this gene product on the large surface antigen promoter and the promoters directing the expression of the internal controls, the SV40 early promoter and the mouse metallothionein promoter, was characterized (Fig. 3). The constructs PS(1)pX1LUC and PS(1)pX2LUC contain modifications which prevent the production of the X-gene product by these constructs. Analysis of these constructs indicated that the influence of the X-gene product, if it is produced by the PS(1)pLUC construct, on the activity of each of the promoters examined appears to be minimal in Hep3B and HepG2.1 cells. The effect of deleting upstream sequences from the large surface antigen promoter [plasmids PS(1)pLUC to PS(1)pΔ2840-2718LUC] supports this observation, as deletion of the complete X-gene region does not significantly affect the transcriptional activity of the large surface antigen promoter in the hepatoma cell lines examined (Fig. 4). This deletion analysis suggested that the enhancer sequence (coordinates 1117 to 1238) increased the level of transcriptional activity from the large surface antigen promoter approximately fivefold in Hep3B cells but not in the other hepatoma cell lines.

The major reduction in transcriptional activity from the large surface antigen promoter in differentiated hepatoma cell lines was observed when the sequence element between −90 and −76 relative to the transcriptional initiation site (coordinate 2809) (46, 66) was deleted (Fig. 4 and 5). This region of the large surface antigen promoter contains a sequence element (GTTAATCATTACT) which binds the liver-specific transcription factor HNF1 (13) and is completely conserved in all of the sequenced HBV genomes. In the dedifferentiated hepatoma cell line HepG2.1, this sequence element does not contribute to the transcriptional activity of the large surface antigen promoter (Fig. 4). Since the relative activity level of the large surface antigen promoter is approximately 8 to 40 times higher in the differentiated hepatoma cell lines than in the dedifferentiated hepatoma cell line (Table 1) and deletion of the HNF1-binding site reduces the activity of this promoter about 5- to 20-fold in the differentiated hepatoma cell lines (Fig. 4), the simplest explanation for these observations is that the dedifferentiated hepatoma cell line lacks the transcriptionally functional HNF1 polypeptide necessary to increase transcription from this promoter. This explanation is also consistent with the similar observation that the level of expression of the β-fibrinogen gene in a differentiated rat hepatoma cell line, Fao, is approximately fivefold greater than that observed in a dedifferentiated rat hepatoma cell line, FaoflC2. The activity of the β-fibrinogen promoter and the morphology of the rat hepatoma cell lines correlated with the presence of the normal and variant forms of HNF1 in these cells (3). A similar situation may also exist in the differentiated hepatoma cell line HepG2 and the dedifferentiated hepatoma cell line HepG2.1, accounting for the preferential expression of the large surface antigen promoter in the differentiated hepatoma cell lines. The dependence of the activity of the large surface antigen promoter on the liver-specific transcription factor HNF1 may also partially account for the hepatotropism of HBV. The large surface antigen is an essential component of the virus envelope (24), and therefore, in a situation where this polypeptide is inefficiently produced, virus synthesis may be limited. Recently, the dependence of the expression of the large surface antigen promoter on the HNF1-binding site has been reported (8), further supporting the role of the HNF1 transcription factor in the regulation of the expression of this HBV gene in differentiated hepatoma cells.

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