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Regulation of Transcription from the Hepatitis B Virus Large Surface Antigen Promoter by Hepatocyte Nuclear Factor 3†

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The influence of hepatocyte nuclear factor 3 (HNF3) on the level of transcriptional activity from the four hepatitis B virus promoters was investigated by transient-transfection analysis in the dedifferentiated hepatoma cell line, HepG2.1. It was found that the large surface antigen promoter and, to a much lesser extent, the nucleocapsid promoter were transactivated in the presence of HNF3. DNase I footprinting analysis demonstrated that purified recombinant HNF3α protects one region of the large surface antigen promoter. Gel retardation analysis showed that a double-stranded oligonucleotide containing this HNF3-binding site formed a specific complex with DNA-binding proteins in the differentiated hepatoma cell lines, Huh7 and HepG2. The complex formed with Huh7 cell extract comigrated with exogenously expressed HNF3β in HeLa S3 extracts and was specifically inhibited from forming by the addition of HNF3β antiserum. The promoter element which appears to mediate the HNF3 transactivation was functionally mapped by mutational analysis to a region between nucleotides −65 and −54 relative to the transcriptional start site. This regulatory sequence is within the region protected from DNase I digestion by HNF3α and contains 10 of 12 nucleotides homologous to the HNF3-binding-site consensus sequence. A synthetic promoter construct containing this HNF3-binding site was able to mediate transactivation by HNF3β. These and previous results suggest that the hepatitis B virus large surface antigen promoter is regulated by at least two liver-enriched transcription factors, HNF1 and HNF3, which together may contribute to the differentiated liver cell type specificity of this promoter.

The 3.2-kb hepatitis B virus (HBV) DNA genome is transcribed in the nucleus of the infected hepatocyte or transfected hepatoma cell line to produce 3.5-, 2.4-, 2.1-, and 0.7-kb transcripts (6, 7, 18, 47, 53). These transcripts encode the nucleocapsid and polymerase polypeptides, the large surface antigen polypeptide, the middle and major surface antigen polypeptides, and the X gene-encoded protein(s), respectively (14, 34). The envelope of HBV is composed primarily of the major surface antigen, but it also contains small quantities of the large and middle surface antigen polypeptides (16, 45, 46). The large surface antigen polypeptide represents a critical component of the virus envelope, because it is absolutely required for the correct envelopment of the HBV nucleocapsid at the membrane of the endoplasmic reticulum (5, 50). The relative abundances of the three envelope polypeptides must be regulated such that an excess of the large surface antigen polypeptide is not synthesized relative to the middle and major surface antigen polypeptides, because overexpression of the large surface antigen polypeptide blocks the secretion of surface antigen particles and virions which accumulate in the lumen of the endoplasmic reticulum (5, 24, 29). It appears that a major regulatory step in determining the relative abundances of the surface antigen polypeptides is at the level of transcription. The 2.4-kb transcript is observed at a much lower level than the 2.1-kb transcript in infected hepatocytes or transfected hepatoma cell lines (3, 7, 12, 41, 47–49, 52, 53). Therefore, it is of considerable interest to determine the molecular mechanisms controlling the expression of each of the HBV RNAs with a view to understanding how these transcripts are coordinately regulated. Additionally, it is apparent that HBV transcription is limited largely to cells of hepatic origin, indicating that at least some of the transcription factors involved in the expression of the HBV genes are liver enriched in distribution.

Previously, we examined the relative transcriptional activities from the four HBV promoters in several cell lines and determined the minimal promoter elements for each gene (31, 32, 36–38, 54–56). It was determined that the large surface antigen and, to a lesser degree, the nucleocapsid promoter exhibited higher relative transcriptional activities in differentiated hepatoma cell lines than in dedifferentiated hepatoma or nonhepatoma cell lines (36). This suggested that these two promoters may have greater specificity for transcriptional activity in differentiated hepatoma cell lines than the major surface antigen or X gene promoters. Subsequently, it was shown that the highly liver-enriched transcription factor hepatocyte nuclear factor 1 (HNF1) plays a major role in the regulation of transcription from the large surface antigen promoter (8, 32, 36) but not the other HBV promoters. From these studies, it appeared that the total transcriptional activity of the large surface antigen promoter required only the HNF1 and TATA-binding protein recognition sites (31). However, careful examination of the effect of clustered point mutations in the region between the HNF1- and TATA-binding protein-binding sites suggested that an additional regulatory element might exist in this promoter (31). As the effect of this regulatory element was observed only in the differentiated hepatoma cell line, Huh7, it appeared likely that this regulatory element might bind a liver-enriched transcription factor. Examination of this regulatory element revealed a sequence displaying a high degree of homology to the binding site for the liver-enriched transcription factor HNF3 (27).

In the present analysis, the influence of HNF3 on the transcriptional activity from the four HBV promoters was examined. It was found that the large surface antigen promoter and,

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to a lesser extent, the nucleocapsid promoter were transactivated in the presence of HNF3. The promoter element which appears to mediate the HNF3 transactivation was functionally mapped by mutational analysis to a region between nucleotides −65 and −54 relative to the transcriptional start site. This region is included within the previously identified regulatory element located between the HNF1- and TATA-binding protein-binding sites, which was identified in Huh7 cells (31). This regulatory element contains 10 of 12 nucleotides homologous to the HNF3-binding-site consensus sequence reported previously (27). It was shown to bind specifically to purified recombinant HNF3α protein and the HNF3β protein present in the differentiated hepatoma cell line, Huh7. These results suggest that the HBV large surface antigen promoter is regulated by at least two liver-enriched transcription factors, HNF1 and HNF3, both of which are likely to contribute to the apparent differentiated liver cell type specificity of this promoter.

MATERIALS AND METHODS

Plasmid constructions. The steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (39). HBV DNA sequences in these constructions were derived from plasmid pCP10, which contains two copies of the HBV genome (subtype *ayw*) cloned into the *Eco*RI site of pBR322 (11). The firefly luciferase (LUC) reporter gene in these constructions was derived from plasmid p19DLUC (36). Plasmid PS1pLUC contains one complete HBV genome located directly 5′ to the promoterless LUC reporter gene such that the expression of the LUC gene is governed by the hepatitis B virus large surface antigen promoter (36). Similarly, the plasmids SpLUC, XpLUC, and CpLUC contain one complete HBV genome located directly 5′ to the promoterless LUC reporter gene such that the expression of the LUC gene is governed by the HBV major surface antigen, X gene, and nucleocapsid promoters, respectively. Details of the construction of these plasmids and the large surface antigen promoter deletion constructs PS1pΔ2840-2425LUC and PS1pΔ2840-2707LUC have been described previously (36).

Plasmids M1LUC, M2LUC, M3LUC, M4LUC, M5LUC, and M6LUC contain clustered point mutations which were generated by a two-step PCR method with appropriate mutant oligonucleotides to make the desired mutation within the HBV sequence (21). The PCR-generated mutant large surface antigen promoter fragments were subsequently cloned into the LUC vector p19DLUC by standard procedures (39). The M1LUC, M2LUC, M3LUC, M4LUC, and M6LUC plasmids are derivatives of plasmid PS1pΔ2840-2707LUC and contain clustered mutations of 7 to 10 nucleotides in the minimal large surface antigen promoter (see Fig. 3A). Plasmid M5LUC is derived from the PS1pΔ2840-2425LUC construct and contains a 13-nucleotide mutation within the HNF1-binding site (see Fig. 3A). The sequences of the fragments produced by PCR were verified by dideoxynucleotide sequencing (40).

Plasmids pHBVTATALUC, HNF3(1)TATALUC, HNF3(2)TATALUC, and HNF3(3)TATALUC were constructed by inserting synthetic double-stranded oligonucleotides into sites in the polylinker of p19DLUC. pHBVTATALUC was constructed by inserting a double-stranded oligonucleotide containing the large surface antigen promoter TATA-box element, produced by annealing the oligonucleotides CTATATTATATAAGAGAGAAGCT and TCTCTCTTATATAATATAGGTAC (spanning HBV coordinates 2773 to 2791), into the *Sac*I and *Kpn*I sites of p19DLUC in the same orientation as the TATA-box element occurs in the HBV genome (31). HNF3(1)TATALUC was made by inserting one copy of the double-stranded oligonucleotide containing the large surface antigen promoter HNF3-binding site (PH; see Fig. 5), produced by annealing the oligonucleotides TCGACACTATTTACACACTCTATG and TCGACATAGAGTG TGTAAATAGTG (spanning HBV coordinates 2742 to 2763), into the unique *Sal*I site of pHBVTATALUC. HNF3(2)TATALUC and HNF3(3)TATALUC were constructed in a similar manner to HNF3(1)TATALUC and contained two and three copies, respectively, of the large surface antigen promoter HNF3-binding-site double-stranded oligonucleotide. The sequence of each construct was verified by dideoxynucleotide sequencing (40).

The pCMVHNF3α and pCMVHNF3β vectors express HNF3α and HNF3β polypeptides from the HNF3α and HNF3β cDNAs, respectively, using the cytomegalovirus immediate-early promoter (20, 28). The pCMV vector, which lacks a cDNA insert, was generated by digestion of pCMVHNF3α with *Eco*RI and subsequent ligation.

The pGEXHNF3α vector expresses a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli* DH5α which includes the complete HNF3α polypeptide sequence. The pGEXHNF3α vector was generated by cloning the 1.6-kbp HNF3α cDNA *Eco*RI fragment into the *Eco*RI site of the expression vector, pGEX-2T (43). Expression and subsequent affinity purification of the GST-HNF3α fusion protein with glutathione-agarose was performed as described previously (43).

TABLE 1. Relative activity of HBV promoters in HepG2.1 cells with and without exogenously expressed HNF3 polypeptide

Promoter	Relative activity[a]			Fold induction	
	−HNF3	+HNF3α	+HNF3β	HNF3α	HNF3β
SpLUC	1.00	1.45	1.87	1.5	1.9
XpLUC	1.11	1.22	1.24	1.1	1.1
CpLUC	0.33	0.51	1.04	1.6	3.2
PS1pLUC	0.0042	0.12	0.36	28.6	85.7

[a] The activities of the HBV promoters are reported relative to the activity of the major surface antigen promoter (Sp) in the absence of exogenously expressed HNF3. Xp, X gene promoter; Cp, nucleocapsid promoter; PS1p, large surface antigen promoter. The expression vectors used were pCMV (−HNF3), pCMV-HNF3α (+HNF3α), and pCMVHNF3β (+HNF3β).

Cells and transfections. The human hepatoma cell lines Huh7 (26), HepG2 (1), and HepG2.1 (36) were grown in RPMI 1640 medium plus 10% fetal bovine serum at 37°C in 5% CO_2 in air. The human cervical carcinoma cell line HeLa S3 (30) was grown in Dulbecco's modified Eagle's medium plus 4.5 mg of glucose per ml and 10% calf serum at 37°C in 5% CO_2 in air. Transfections were performed as previously described (15, 44). The transfected DNA mixture comprised 15 μg of a LUC plasmid and 1.5 μg of pCMVβ, which served as an internal control for transfection efficiency. pCMVβ directs the expression of the *E. coli* β-galactosidase gene by using the cytomegalovirus immediate-early promoter (Clontech). When appropriate, the DNA mixture also included 1.5 μg of the HNF3α or HNF3β expression vector, pCMVHNF3α or pCMVHNF3β, respectively, or the control expression vector, pCMV. Cell extracts were prepared 40 to 48 h after transfection and assayed for LUC activity essentially as previously described (10). The extracts were measured for β-galactosidase activity with a Galacto-Light kit (Tropix, Inc.) as specified by the manufacturer.

Whole-cell extracts, gel retardation analysis, and DNase I footprinting. Whole-cell extracts were prepared from Huh7, HepG2, HepG2.1, and HeLa S3 cells by a rapid micropreparation technique as described previously (2). HeLa S3 cells were transfected with 15 μg of the expression vectors encoding HNF3α or HNF3β 40 to 48 h before preparation of the whole-cell extracts.

Gel retardation analysis was performed essentially as described previously (32). One nanogram of ^{32}P-labeled double-stranded large surface antigen promoter HNF3-binding-site oligonucleotide (see above) was incubated with 9 μg of whole-cell extract prior to 4% polyacrylamide gel electrophoresis and autoradiography. When gel retardation competition analysis was performed, the cell extract was preincubated with 1 μg of cold double-stranded competitor oligonucleotide for 15 min prior to the addition of the ^{32}P-labeled double-stranded oligonucleotide. Gel retardation analysis with antibodies was performed essentially as described previously (22). The cell extract was incubated with 2 μl of antiserum specific for the HNF3α, HNF3β, or HNF3γ polypeptide (20) for 1 h prior to the addition of the ^{32}P-labeled double-stranded oligonucleotide.

The DNase I footprinting assay was performed as described previously (4, 33, 37). The reaction mixtures contained 1 to 5 ng of end-labeled DNA fragment in a 100-μl volume containing 10 mM Tris hydrochloride (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% (vol/vol) glycerol, and purified recombinant GST-HNFα or GST polypeptide. Binding was carried out for 15 min at 0°C and then for 2 min at room temperature, after which 100 μl of 5 mM $CaCl_2$–10 mM $MgCl_2$ containing DNase I was added at room temperature. The reaction mixture was incubated for 2 min, and the reaction was stopped by the addition of 200 μl of 1% (wt/vol) sodium dodecyl sulfate–20 mM EDTA–200 mM NaCl containing 125 μg of tRNA per ml. The mixture was precipitated with ethanol and analyzed by 6% urea–acrylamide sequencing gel electrophoresis and autoradiography.

RESULTS

Influence of HNF3 on transcription from the four HBV promoters. The influence of the liver-enriched transcription factor HNF3 on the transcriptional activities of the four HBV promoters in the dedifferentiated hepatoma cell line HepG2.1 was measured by transient-transfection analysis. Expression of the HNF3α polypeptide appears to increase the level of transcription from the large surface antigen promoter approximately 29-fold, whereas it does not appear to affect the activity from the other HBV promoters (Table 1). Similarly, the transcriptional activity from the large surface antigen promoter is increased by the expression of the HNF3β polypeptide approximately 86-fold, and little, if any, effect is observed on the activity from the other HBV promoters (Table 1). The activity

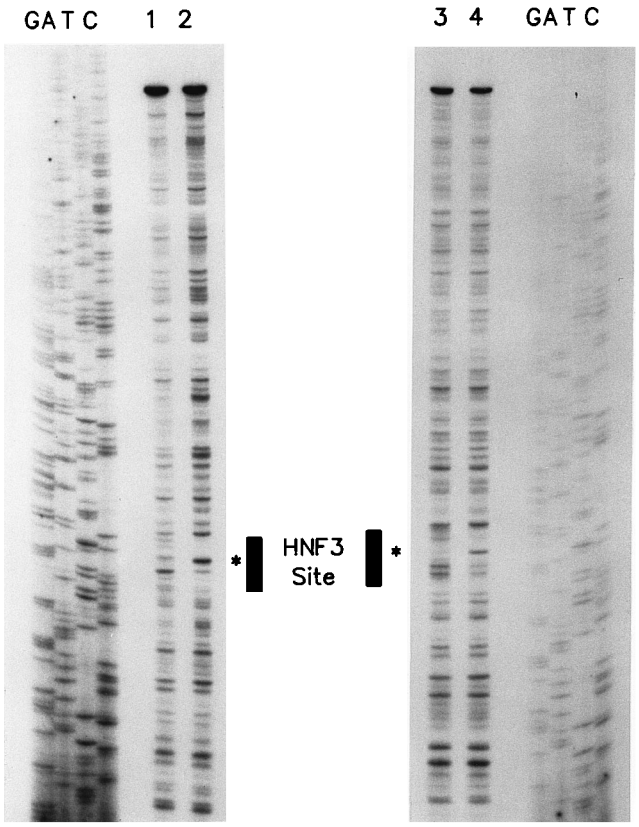

FIG. 1. DNase I footprinting analysis of the short (plus) (lanes 1 and 2) and long (minus) (lanes 3 and 4) strands of the HBV large surface antigen promoter. A 423-nucleotide HBV DNA fragment from −379 to +44 was 3′ end labeled at +44 (lanes 1 and 2) or 5′ end labeled at +44 (lanes 3 and 4) and incubated in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of purified recombinant GST-HNF3α. An asterisk indicates the DNase I-hypersensitive sites within the HNF3 footprints. An unrelated DNA sequence (GATC) is adjacent to lanes 1 and 4 and was used as a size standard to locate the HNF3-binding site.

of the nucleocapsid, or core, promoter (CpLUC) may be slightly influenced by the expression of HNF3β in this system, because it is increased approximately threefold. Although all of the full-length promoter constructs used in this analysis contained the same sequence elements, it appears that only the large surface antigen and, perhaps to a small degree, the nucleocapsid promoters are influenced by the expression of the HNF3 proteins.

Identification of the HNF3 transcription factor-binding site in the large surface antigen promoter. Previous mutational analysis of the large surface antigen promoter had indicated that a regulatory element identified in the differentiated hepatoma cell line Huh7 was located between the HNF1- and TATA-binding protein-binding sites (31). A region of the identified regulatory sequence element was homologous at 10 of 12 positions to the HNF3 consensus sequence (see Fig. 5) (27). To determine if this sequence in the large surface antigen promoter is an HNF3-binding site, DNase I footprinting analysis with a purified recombinant GST-HNF3α polypeptide was performed (Fig. 1). The footprint spanned the promoter region from −62 to −47 on the plus strand and from −67 to −51 on the minus strand (see Fig. 5). The 12-nucleotide region homologous to the consensus HNF3-binding site was located within the footprinted region on the minus strand. On the plus strand, the GST-HNF3α polypeptide protected nucleotides spanning most of the HNF3 consensus sequence, as well as seven nucleotides downstream of this recognition element. DNase I-hypersensitive sites characteristic of HNF3 footprints (23, 25) are located within the protected regions of the plus and minus strands (Fig. 1). No other GST-HNF3α protected regions were apparent in the large surface antigen promoter, which suggests that the sequence homologous to the consensus HNF3-binding site is the most likely element to mediate the transcriptional effects of HNF3 on the large surface antigen promoter.

Characterization of the DNA-binding proteins from Huh7 cells which interact with the HBV large surface antigen promoter HNF3-binding site. DNase I footprinting analysis has demonstrated that purified recombinant GST-HNF3α binds a regulatory element in the large surface antigen promoter which has high homology to the HNF3 consensus sequence. However, this analysis does not indicate the nature of the factor(s) interacting with this regulatory element in hepatoma cell lines. Consequently, a double-stranded oligonucleotide spanning the large surface antigen promoter region protected by the GST-HNF3α polypeptide was prepared to examine the DNA-binding proteins in cell extracts which associate with this site in a gel retardation analysis (Fig. 2). The large surface antigen promoter HNF3-binding-site oligonucleotide (PH; see Fig. 5) was examined for its ability to bind factors in Huh7 extracts (Fig. 2A). To determine the specificity of the interaction between the factor(s) and the HNF3-binding-site oligonucleotide, several unlabeled double-stranded oligonucleotides were used as competitors in the gel retardation analysis. Unlabeled double-stranded oligonucleotides representing a nucleocapsid promoter Sp1-binding site (CpC), a control oligonucleotide (CpD), an unknown liver factor-binding site (CpF), an X gene promoter region (X), and the large surface antigen promoter HNF1-binding site (HNF1) failed to compete efficiently for the factor(s) binding the HNF3 oligonucleotide. The unlabeled large surface antigen promoter HNF3-binding-site oligonucleotide (PH) and the nucleocapsid promoter oligonucleotide CpE, which contains 11 of 12 nucleotides homologous to the HNF3 consensus sequence, efficiently competed with the ^{32}P-labeled PH oligonucleotide for binding the factor(s) in Huh7 extract. These results suggest that the DNA-binding protein is specific for the HNF3-binding site.

Several cell line extracts were compared in the gel retardation analysis to examine the cell line specificity of the factor(s) (Fig. 2B). The gel retardation complexes were also compared with the complexes formed by the exogenous expression of HNF3 polypeptides. Specific gel retardation complexes were formed with extracts from the differentiated hepatoma cell lines Huh7 and HepG2 which had similar migration properties to the exogenously expressed HNF3β in HeLa S3 cells. The dedifferentiated hepatoma cell line, HepG2.1, and the nonhepatoma cell line, HeLa S3, did not form readily detectable DNA-protein complexes. These results suggest that the differentiated hepatoma cell lines express a factor which may be the same or similar to the HNF3β polypeptide, whereas the dedifferentiated hepatoma and nonhepatoma cell lines do not produce a factor which binds the HNF3 oligonucleotide in sufficient quantities to be detected in the gel retardation analysis. This is consistent with the liver-enriched distribution of the HNF3 polypeptides (19, 20, 51). The mobility of the exogenously expressed HNF3α polypeptide-DNA complex is slightly lower than that of the HNF3β polypeptide, consistent with previously reported gel retardation analyses (19, 20).

Gel retardation inhibition analysis was also performed with appropriate antisera to confirm the specificity of the gel retardation complexes (Fig. 2C). HeLa S3 cell extract containing exogenously expressed HNF3α or HNF3β polypeptide formed complexes which were inhibited by the addition of the corre-

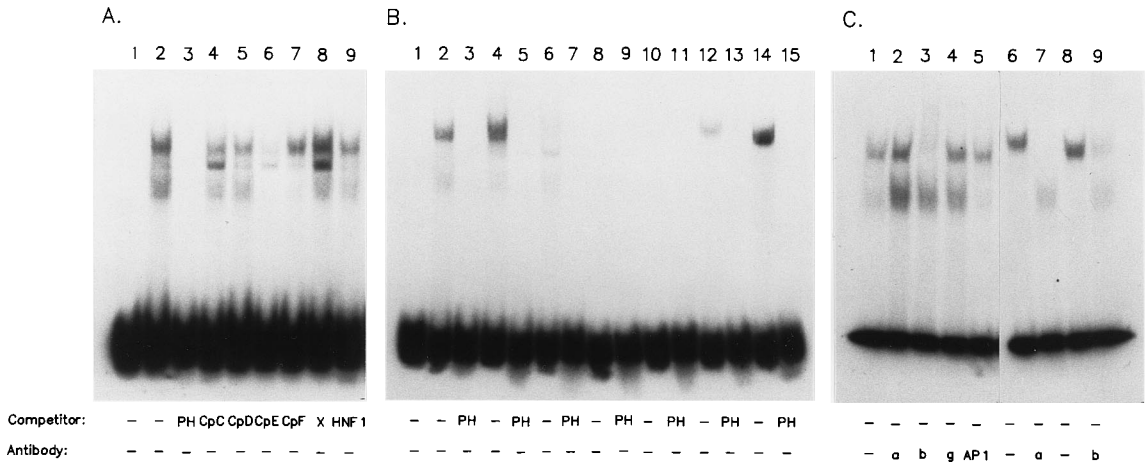

FIG. 2. Gel retardation and complex inhibition analysis of the HBV large surface antigen promoter HNF3-binding site. The ^{32}P-labeled, double-stranded oligonucleotide PH (see Fig. 5) was used for this analysis. (A) Analysis of the complexes formed with the DNA-binding proteins present in Huh7 cell extracts. Unlabeled double-stranded oligonucleotides were used as competitor DNAs to demonstrate the specificity of the observed complexes. PH, large surface antigen promoter HNF3-binding site; CpC, nucleocapsid promoter Sp1-binding site (56); CpD, nucleocapsid promoter control oligonucleotide (56); CpE, nucleocapsid promoter liver-specific factor-binding site with homology to the HNF3 recognition sequence (54); CpF, nucleocapsid promoter liver-specific binding site (54); X, X-gene promoter region oligonucleotide (54); HNF1, large surface antigen promoter HNF1-binding site (32). (B) Analysis of the complexes formed with the DNA-binding proteins present in untransfected Huh7 cells (lanes 2 and 3), HepG2 cells (lanes 4 and 5), HepG2.1 cells (lanes 6 and 7), HeLa S3 cells (lanes 8 and 9), HeLa S3 cells transfected with pCMV (lanes 10 and 11), HeLa S3 cells transfected with pCMVHNF3α (lanes 12 and 13), and HeLa S3 cells transfected with pCMVHNF3β (lanes 14 and 15). Cell extract was omitted from lane 1. The unlabeled, competitor PH oligonucleotide was included in lanes 3, 5, 7, 9, 11, 13, and 15. (C) Analysis of the inhibition of complex formation by anti-HNF3α antiserum (a) (lanes 2 and 7), anti-HNF3β antiserum (b) (lanes 3 and 9), anti-HNF3γ antiserum (g) (lane 4), and anti-AP1 antibodies (AP1) (lane 5). Antibody was omitted from lanes 1, 6, and 8. The extracts analyzed were from Huh7 cells (lanes 1 to 5), HeLa S3 cells transfected with pCMVHNF3α (lanes 6 and 7), and HeLa S3 cells transfected with pCMVHNF3β (lanes 8 and 9).

sponding antiserum against HNF3α or HNF3β (20). The formation of the specific complex between the Huh7 extract and the PH oligonucleotide was inhibited only by the HNF3β antiserum, not by the HNF3α antiserum, the HNF3γ antiserum, or the unrelated control polyclonal antipeptide c-Jun/AP1 antibody. The Huh7 DNA-protein complex also comigrated with the exogenously expressed HNF3β in the HeLa S3 cell extract. The results of the gel retardation inhibition analysis and the migration properties of the complex formed with the Huh7 extract suggest that the factor present in Huh7 cells which binds the large surface antigen promoter HNF3-binding site is predominantly HNF3β.

HNF3 transactivates expression from the HBV large surface antigen promoter through the HNF3-binding site. In an attempt to define the functional HNF3-binding site in the large surface antigen promoter, a series of clustered point mutations was introduced into the region of the minimal promoter and tested for transcriptional activity in the HepG2.1 transient-transfection assay (Fig. 3). The large surface antigen promoter clustered point mutation constructs were transfected into HepG2.1 cells in the presence of the negative control vector pCMV (−HNF3) or the expression vector pCMVHNF3α or pCMVHNF3β. In the absence of the expression vector (−HNF3), none of the clustered point mutations appeared to modulate greatly the transcriptional activity of the large surface antigen promoter. In the presence of HNF3α, the full-length large surface antigen promoter construct (PS1pLUC) and the deletion constructs (PS1pΔ2840-2425LUC and PS1pΔ2840-2707LUC) were transactivated approximately 13-fold (Fig. 3B). The mutations M1, M4, and M5 did not appear to affect the level of induction by HNF3α very much, as the levels of induction were approximately 8- to 10-fold in the constructs containing these mutations. The region of the promoter which appeared to mediate the transcriptional transactivation by HNF3 (−75 to −42) was partially mutated in each of the constructs M1LUC, M2LUC, and M3LUC. The M1 mutation did not have a large effect on the inducibility of the promoter by HNF3α, which suggests that the region from −73 to −64 is not involved in the transactivation by HNF3α. The M2 mutation, however, resulted in a loss of induction by HNF3α to 2.5-fold, approximately 5-fold less than the induction of the full-length and parental deletion used for these mutations (constructs PS1pLUC and PS1pΔ2840-2707LUC). The M2 mutation alters the HBV sequence which contains homology to the consensus HNF3-binding sequence (27). Between nucleotides −65 and −54 (CACTATTTACAC), 10 of 12 bases match the consensus HNF3-binding-site sequence (VAWTRTTKRYTY, where K is G or T, R is A or G, V is A, C, or G, W is A or T, and Y is C or T). The M2 mutation results in a match of only 7 of the 12 conserved nucleotides. This alteration probably accounts for the observed reduction of transcriptional transactivation from the M2LUC promoter sequence by the HNF3α polypeptide. The M3 mutation also resulted in the partial loss of transcriptional transactivation by HNF3α, although not as much as the M2 mutation. It is possible that the sequence altered by the M3 mutation, which is adjacent to the HNF3-binding site, is involved in the HNF3 DNA-protein interaction because of its proximity to the HNF3 recognition sequence and consequently affects transcriptional transactivation from the large surface antigen promoter by HNF3 transcription factors. The M6 mutation, which alters the TATA-box sequence, also affects the induction by HNF3α, reducing it to 2.5-fold. The elimination of the TATA-box sequence may interfere with the ability of HNF3α to recruit elements of the basic transcription machinery, which are needed for transcriptional induction by HNF3α, to the TATA box.

Although the magnitude of induction when using the HNF3β expression vector was greater than when using the HNF3α expression vector, the clustered point mutations affected the transcriptional activity of the constructs similarly. The transcriptional activity of the full-length promoter (con-

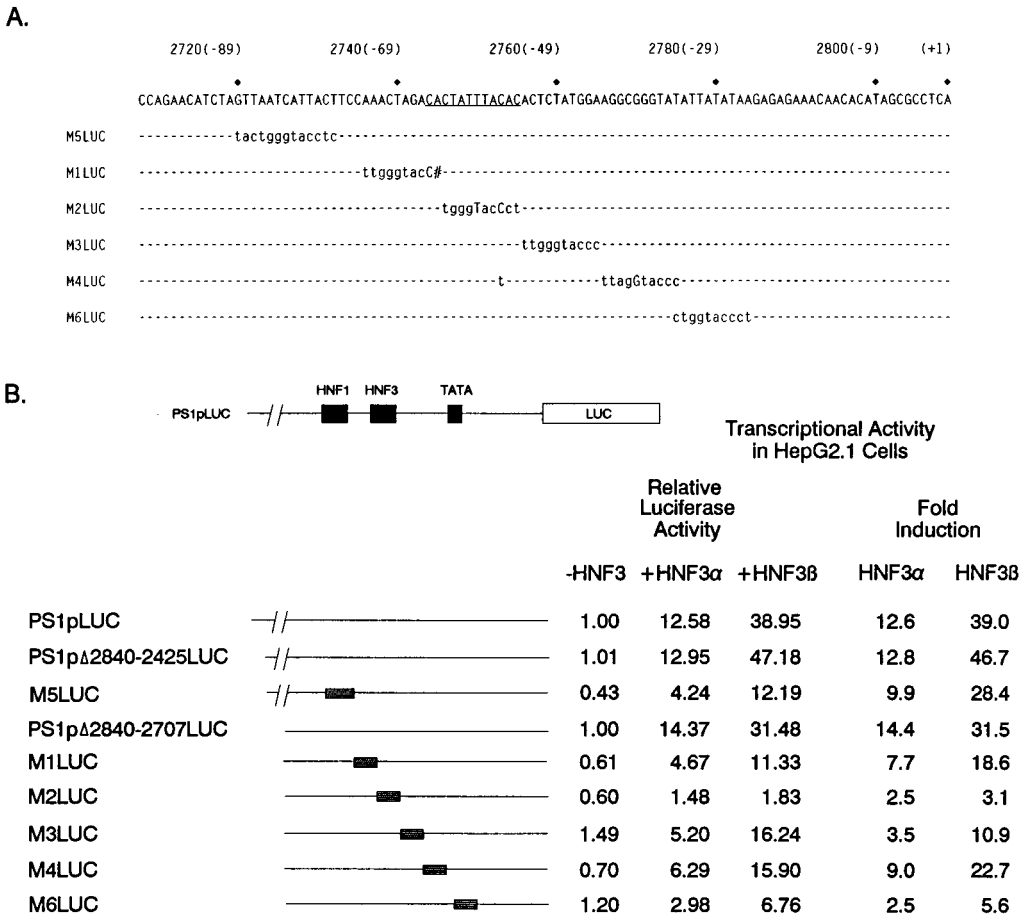

FIG. 3. Clustered point mutational analysis of the minimal large surface antigen promoter. (A) Sequence (subtype *ayw*) of the HBV large surface antigen promoter region from −101 to +1 (13). Coordinates are derived from the GenBank database, and their positions relative to the transcription initiation site (+1) are shown in parentheses. The region of homology to the HNF3 recognition sequence is underlined. The sequence of the clustered point mutation is shown for each mutant construct. The nucleotides which differ from the HBV sequence are indicated in lowercase letters, and unchanged nucleotides are indicated in capital letters in the mutated sequence and by dashes in the remaining minimal promoter sequence. (B) Relative activities of the clustered point mutation constructs in HepG2.1 cells in the presence (+HNF3α and +HNF3β) or absence (−HNF3) of exogenously expressed HNF3. The transcriptional activities are reported relative to the activity of the large surface antigen promoter (PS1pLUC) in the absence of exogenously expressed HNF3. Plasmid PS1pLUC contains the complete HBV genome upstream of the LUC open reading frame such that expression of LUC is directed by the large surface antigen promoter. The horizontal lines indicate the HBV sequences present in the various large surface antigen promoter plasmids. The HBV sequences deleted from the various plasmids are designated by nucleotide coordinates derived from the GenBank sequence database. Solid boxes indicate the positions of HNF1, HNF3, and TATA-box binding elements. The open box indicates the position of the LUC open reading frame. The locations of the M1, M2, M3, M4, M5, and M6 mutations are indicated by stippled boxes. The internal control used to correct for transfection efficiencies was pCMVβ.

struct PS1pLUC) was induced 39-fold by HNF3β, and the transcriptional activity of the promoter sequence from −383 to +35 (construct PS1pΔ2840-2425LUC) was induced 47-fold. The M5 mutation, altering the HNF1-binding site, resulted in transcriptional transactivation of 28-fold, and transcription from the deletion which served as the parental construct for the remaining clustered point mutations (construct PS1pΔ2840-2707LUC) was induced 32-fold by HNF3β. The M1 and M4 mutations did not affect the transcriptional inducibility by HNF3β to a large extent. The M2 mutation, which alters the HBV sequence containing homology to the HNF3-binding site, reduced the transactivation by HNF3β by more than 10-fold, to 3-fold. The M3 mutation had an approximately threefold effect, reducing the transcriptional transactivation by HNF3β to 11-fold. The TATA-box mutation also reduced transcriptional induction from the large surface antigen promoter by approximately fivefold. The mutation which appears to have the greatest effect on the ability of either HNF3α or HNF3β to transactivate the large surface antigen promoter is M2. The M2LUC sequence from −65 to −54 is CATgGgTacCcT (where the capital letters represent homology to the consensus sequence). This sequence retains conservation of 7 of the 12 nucleotides contained in the consensus HNF3-binding site, but the 5 mutated nucleotides appear largely to prevent transcriptional transactivation of the large surface antigen promoter by HNF3. The results of the mutational analysis using the transient-transfection assay are consistent and suggest that the element in the large surface antigen promoter most responsible for mediating the HNF3 transcriptional transactivation lies in the promoter region between −65 and −54.

Transactivation of transcription by the HNF3β transcription factor mediated through a synthetic large surface antigen promoter HNF3-binding site. Mutational analysis of the large surface antigen promoter suggested that the functional regulatory element responsible for mediating HNF3 transcriptional transactivation is the site located between nucleotides −65 and −54, which is highly homologous to the HNF3 consensus sequence. The DNase I footprinting and gel retardation analyses

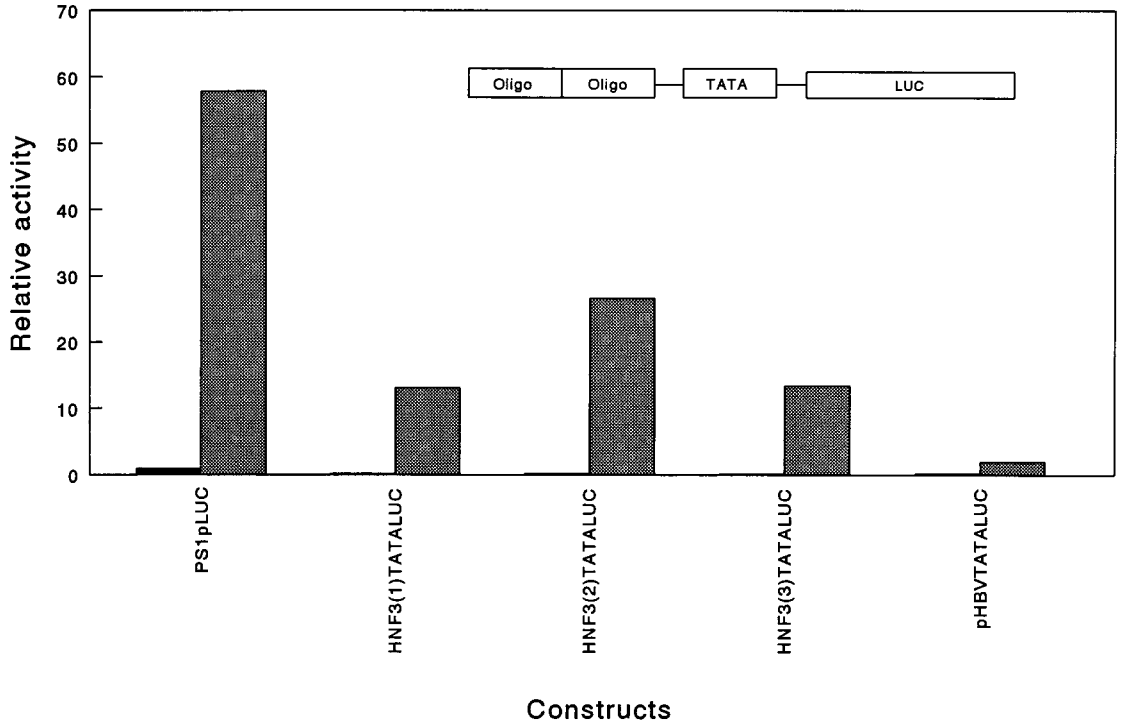

FIG. 4. Functional analysis of the HBV large surface antigen promoter HNF3-binding site. The constructs examined contain the PH double-stranded oligonucleotide (see Fig. 5) cloned into the minimal promoter construct, pHBVTATALUC (see insert) (31). The constructs HNF3(1)TATALUC, HNF3(2)TATALUC, and HNF3(3)TATALUC contain one, two, and three copies, respectively, of the PH double-stranded oligonucleotide cloned into pHBVTATALUC. The relative activities of the constructs in the absence (−HNF3β) (solid box) or presence (+HNF3β) (stippled box) of the expression vector pCMVHNF3β in HepG2.1 cells were examined. The transcriptional activities are reported relative to the PS1pLUC plasmid, which is designated as having a relative activity of 1.0. The internal control used to correct for transfection efficiencies was pCMVβ.

verified that HNF3 was able to bind this element. To determine whether the large surface antigen promoter HNF3-binding site (PH; see Fig. 5) could mediate transcriptional transactivation by HNF3 in the context of a TATA-box element, synthetic promoter elements containing these regulatory elements upstream of the LUC open reading frame were tested for their transcriptional activities in HepG2.1 cells in the absence (−HNF3β) or presence (+HNF3β) of the HNF3β expression vector (Fig. 4). The activities were shown relative to the activity of the full-length large surface antigen promoter (construct PS1pLUC) in the absence of exogenously expressed HNF3β (−HNF3β). The level of transcription from this promoter was increased approximately 58-fold in the presence of the HNF3β expression vector. In the absence of the HNF3β expression vector, the constructs containing the large surface antigen promoter HNF3 oligonucleotide had no transcriptional activity. When the HNF3β polypeptide was exogenously expressed, the synthetic constructs containing either one, two, or three HNF3 oligonucleotides had transcriptional activities of 13, 27, and 13, respectively, relative to the full-length promoter construct in the absence of the expression vector. Although these relative activities were not as high as that of the large surface antigen promoter in the presence of HNF3β, the synthetic oligonucleotide constructs were clearly responsive to the expression of HNF3β. In contrast, an unrelated synthetic oligonucleotide construct containing Sp1 transcription factor-binding sites cloned into pHBVTATALUC did not show transcriptional transactivation by exogenously expressed HNF3β (35). Therefore, the oligonucleotide containing the large surface antigen promoter HNF3-binding site (PH; Fig. 5) appears to be a functional site which can mediate specific transcriptional transactivation by HNF3β, at least in the context of a TATA-box sequence.

DISCUSSION

HBV transcripts have been shown to be expressed predominantly in the livers of transgenic mice containing the complete viral genome (3, 18). This indicates that a major determinant in HBV tropism is at the level of gene expression, although it is likely that there are additional factors which contribute to the liver specificity of HBV replication, including the tissue distribution of the HBV receptor. From these observations, it appears likely that liver-enriched transcription factors might be important in the regulation of HBV RNA synthesis. This contention is supported by the observation that C/EBP, HNF1, and HNF4 are transcription factors which can regulate HBV transcription.

The observation that the level of transcription from the large surface antigen promoter can be modulated by HNF3 has several implications regarding HBV transcriptional regulation. The fact that the liver-enriched transcription factors of the HNF3 family regulate the expression of the large surface antigen promoter further explains the high level of liver-specific transcription from this promoter (20, 51). Previously, it had been demonstrated that the large surface antigen promoter is regulated by the liver-enriched transcription factor HNF1 (8, 32, 36). Therefore, the two transcription factors which have been demonstrated to regulate the level of transcription from the large surface antigen promoter are both highly abundant in the liver but not in other tissues (51). The combinatorial action

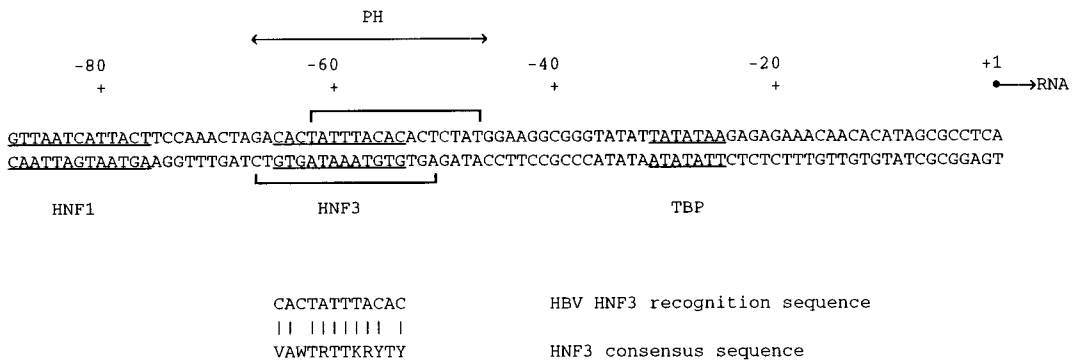

FIG. 5. Nucleotide sequence of the HBV large surface antigen promoter region (subtype *ayw*) (13). The nucleotide coordinates are derived from the GenBank database, and their positions relative to the transcription initiation site (+1; nucleotide coordinate 2809) are shown (42, 52). The underlined sequences represent the HNF1-binding site (HNF1), the region of homology to the HNF3 recognition sequence (HNF3), and the TATA-binding protein recognition sequence (TBP). The sequence of the oligonucleotide PH is indicated. The brackets over the top and under the bottom lines of sequence span the regions on the plus (short) and minus (long) strands, respectively, protected from DNase I digestion by purified recombinant GST-HNF3α. The HBV large surface antigen promoter HNF3 recognition sequence is shown, and the nucleotides homologous to the HNF3 consensus sequence (27) are indicated (K is G or T, R is A or G, V is A, C, or G, W is A or T, and Y is C or T).

of these two factors presumably explains the highly restricted expression of the 2.4-kb HBV transcript to the liver.

It is likely that the level of synthesis of the various HBV RNAs is coordinately regulated so that the correct levels of viral products are produced to permit viral biosynthesis to occur efficiently. In this regard, the observation that an important regulatory element (CpE) of the nucleocapsid promoter (54) also binds HNF3 transcription factors (Fig. 2) (17) suggests that this family of liver-enriched transcription factors might also serve a role in coordinately regulating the level of the 3.5- and 2.4-kb viral RNAs. However, it is clear that the magnitude of the effect of HNF3 on the level of transcription from the large surface antigen and nucleocapsid promoters is very different, at least in the transient-transfection analysis in HepG2.1 cells (Table 1). Recently, it has been observed that HBV enhancer I possesses an HNF3-binding site (9, 35). It appears that the HNF3 site in the enhancer I sequence can modulate the level of transcription in the context of the herpes simplex virus thymidine kinase promoter (9). However, in our study, exogenously expressed HNF3 does not stimulate transcription from any of the HBV promoters in the context of the complete viral genome by interacting with the enhancer I sequence, except possibly the nucleocapsid promoter. In contrast, HNF3 dramatically increases the level of transcription from the large surface antigen promoter in the absence of enhancer I sequences (Table 1; Fig. 3). This suggests that the transcriptional potential of the enhancer I HNF3 site can be observed only in transient-transfection analysis when the enhancer I sequence is removed from the context of the HBV genome. Despite the fact that the HNF3 transcription factors appear to influence the level of expression from the HBV promoters differentially in transient-transfection analysis, it seems likely that these transcription factors play some role in the coordinate regulation of the level of expression from the enhancer I/X gene, nucleocapsid, and large surface antigen promoters during viral infection.

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