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Differentiated Liver Cell Specificity of the Second Enhancer of Hepatitis B Virus

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Hepatitis B virus is a hepatotropic virus. Its replication and gene expression are mainly restricted to hepatocytes in the infective process. The viral gene expression thus provides a unique system with which to study the control of tissue-specific gene expression. We have previously reported the identification and characterization of the second enhancer (enhancer II) of hepatitis B virus. In this report, we further demonstrate that the minimal functional constituents of the second enhancer, box α and box β, display liver cell and differentiation state specificity. Moreover, box α exhibits the same liver cell and differentiation state specificity when functioning as an upstream regulator for the basal core promoter. Gel shift experiments reveal a unique box α-binding protein, protein a, which is present only in differentiated liver cells, where enhancer II is functional. The converse is true for another box α-binding protein, protein f, which is present only in poorly differentiated liver cells and nonliver cells. The simplest hypothesis that explains these results is that protein a activates and/or protein f suppresses the enhancer and upstream regulator functions. Although C/EBP is a candidate for a transcription factor that interacts with box α or box β, none of the binding factors identified in the gel shift assays, including protein a and protein f, is likely to be C/EBP because they differ from C/EBP in heat lability and sequence preference.

Eukaryotic gene expression is in large part regulated at the transcription level. Such regulation is governed by the constellation of *trans*-acting cellular factors that bind to specific *cis*-acting elements and act in either a positive or negative manner (25). Cell-type-specific gene activation, a primary determinant of cellular differentiation, represents a more complex type of interplay, as both constitutive and tissue-restricted *trans*-acting regulatory factors are involved (24). The differential sequence-specific recognition of these *cis*-acting elements in promoters and/or enhancers by their cognate factors provides a mechanistic basis for the tissue- and differentiation-specific regulation of gene expression. The study of model viral genes which display distinct tissue tropism can provide valuable insight into the intricate effects of cell-type-specific transcription regulation on differentiation.

Hepatitis B virus (HBV) is a hepatotropic virus. Although HBV DNA has been found in nonhepatic tissues in infected patients and in transgenic mice, HBV replication and maturation and, as a result, the pathological process are mainly restricted to hepatocytes (2, 5, 11, 12, 35). There are four promoters in HBV: two surface promoters, one core promoter, and one X promoter. These promoters produce messages that encode structural components of the virus and the polymerase that is essential for viral genome replication (4, 15, 23, 28, 29, 39, 41, 44). Moreover, the pregenomic RNA produced by the core promoter serves as a template for replication via reverse transcription (31, 33). So far, two enhancers have been found in the HBV genome. Enhancer I is located between the open reading frames of the surface and X genes and partially overlaps the X promoter (15, 32). Enhancer II is within the X open reading frame (40, 42). The latter has a unique bipartite structure: two minimal functional constituents, a 23-bp sequence called box α and a 12-bp sequence called box β, are both required for enhancer II function (43). Interestingly, either box α or box β in an upstream position can regulate the activity of the nearby core promoter (41).

Using DNA transfection to bypass viral entry into cells, we and others have demonstrated that the expression of HBV genes exhibits liver cell and differentiation state specificity, recapitulating what we observed in the infective process in vivo (6, 8, 17, 30, 34, 36, 39). Our previous studies show that only in the human hepatoma cell lines HepG2 and HuH-7, which have the feature of well-differentiated liver cells, does enhancer II have strong enhancing activity on the simian virus 40 (SV40) early promoter. In contrast, this is not seen in the poorly differentiated HA22T/VGH cells or the nonliver HeLa cells (42). These results also apply to the upstream regulatory effect on the basal core promoter (BCP) (41). These differentiated actions, therefore, may contribute at least in part to the observed hepatotropism of HBV. In this study, we further investigated the liver cell and differentiation state specificity of the minimal essential elements, box α and box β, of enhancer II. We also used gel shift and footprinting assays to study the interactions of different *trans*-acting factors with these sequence motifs. We found that HA22T/VGH and HeLa cells are missing one of the two box α-binding factors, protein a, which is abundant in HepG2 and HuH-7 cells. The first two cell lines, however, contain an additional binding protein, protein f. The possible involvement of these factors in the establishment of differentiation specificity is discussed below.

MATERIALS AND METHODS

Plasmid constructions. The HBV sequence used in this study is of the *adw* subtype. The numbering of the HBV sequence begins at the unique *Eco*RI site. All reporter plasmids used in transfection experiments contain a head-to-tail trimeric tandem repeat, referred to as A3, of a 237-bp

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*BclI-Bam*HI fragment from the SV40 polyadenylation signal. A3 is placed 5′ of the promoter sequence of interest and has been shown to stop transcription readthrough from spurious upstream initiation (42). Plasmid pBCP-CAT contains the chloramphenicol acetyltransferase (CAT) reporter gene driven by the BCP, corresponding to nucleotides nt 1744 to 1851 of the HBV genome (41). Plasmids pα/BCP-CAT and pβ/BCP-CAT were constructed by insertion of annealed oligonucleotide sequences corresponding to the box α(5′GATCCATCGATCAAGGTCTTACATAAGAGGACTCTT3′ and 5′AAGAGTCCTCTTATGTAAGACCTTGATCGATG3′; underlined nucleotides indicate nt 1645 and 1669, respectively, of the HBV sequence) and box β (5′GCTACTTCAAAGACTGTGGTACCCATG3′ and 5′GGTACCACAGTCTTTGAAGTAGCTGCA3′; underlined nucleotides indicate nt 1705 and 1721 of HBV) oligonucleotides, respectively, in the position immediately upstream of the BCP of pA3BCP-CAT. Plasmid pSVpCAT contains the CAT reporter gene driven by the 188-bp SV40 early promoter. Plasmid pSVpCAT/αβ was constructed by the insertion of box α followed by box β downstream of the CAT gene of pA3SVpCAT.

Cell lines, transfections, and CAT assays. Three human hepatoma cell lines, the well-differentiated lines HuH-7 (26) and HepG2 (1) and the poorly differentiated line HA22T/VGH (7), as well as the human cervical carcinoma cell line HeLa, were cultured as previously described (41). Cells were transfected with plasmids containing the CAT gene by the calcium phosphate precipitation method. CAT assays were performed by the method of Gorman et al. (14). The amounts of protein samples in each experiment varied by no more than 5%. The CAT activity of each sample was normalized to the CAT activity of pSV2CAT, which was taken as 100%. If CAT activity was high, the cell lysate was serially diluted before the CAT assay (41–43).

Preparation of nuclear extracts and heparin-agarose fractionation of extracts. Nuclear extracts from HuH-7, HepG2, HA22T/VGH, and HeLa cells were prepared as previously described (9). Extracts were stored in small aliquots at −70°C after being quickly frozen under liquid nitrogen. The nuclear extract was fractionated at 4°C essentially as described by Lichtsteiner et al. (21), with a slight modification. Briefly, 25 mg of crude nuclear extract in 10 ml of nuclear dialysis buffer was loaded onto a 20-ml heparin-agarose column (heparin Ultrogel-A4R; 4 to 6 mg of heparin per ml; IBF, Garenne, France). The proteins bound to heparin were collected by step elution with nuclear dialysis buffer containing 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, or 1.0 M NaCl. For experiments in which nuclear extracts were subjected to a thermolability test, the 0.4 and 0.3 M NaCl fractions were heated (50°C for 5 min, 70°C for 5 min, or 90°C for 15 min) and then centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatants were collected for the DNase I footprinting and gel shift analyses.

DNase I footprinting and gel shift analyses. The end-labeled probes were generated via fill-in with [α-^{32}P]dATP (3,000 Ci/mmol; Amersham, Amersham, Buckinghamshire, England) at either terminus of a 450-bp *Bam*HI-*Hin*dIII fragment containing the HBV sequence from nt 1402 to 1851. Plasmids carrying this insert were cut with one enzyme, end labeled, cut with a second enzyme, and then subjected to agarose gel electrophoresis. The labeled fragments were eluted from the agarose gel with a Spin-X filtering unit (Costar, Cambridge, Mass.). The DNase I footprinting assay was performed as previously described (9). The crude or fractionated nuclear extracts were incubated with labeled DNA fragment for 30 min at 4°C. After addition of $MgCl_2$ and $CaCl_2$, the proper amount of DNase I was added to digest for 90 s at room temperature. The reactions were stopped by deproteination. The extracted DNA was analyzed on 4% polyacrylamide–8 M urea gels.

Annealed double-stranded oligonucleotides (200 ng) corresponding to the box α or box β sequence were end labeled with 80 U of polynucleotide kinase (Toyobo, Osaka, Japan) and 80 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham). The free [γ-^{32}P]ATP was removed from the labeled oligonucleotides with an Ultrafree-C3 filter unit (UFC3 LGC NB; Millipore, Bedford, Mass.). The gel shift assay was performed as previously described (43). Briefly, proteins (10 to 20 μg) were incubated in a 20-μl binding buffer containing 1 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia, Inc.) and 6 × 10^4 cpm of labeled DNA. The reaction mixtures were incubated at 30°C for 30 min, and protein-DNA complexes were resolved on 4% polyacrylamide gels. For competition experiments, 5-, 25-, 125-, and 625-fold molar excesses of unlabeled double-stranded oligonucleotide were preincubated with nuclear extracts on ice for 5 min before addition of probe. The oligonucleotides for double-stranded C/EBP consensus sequences were 5′GATCGAACATATTGCGCA

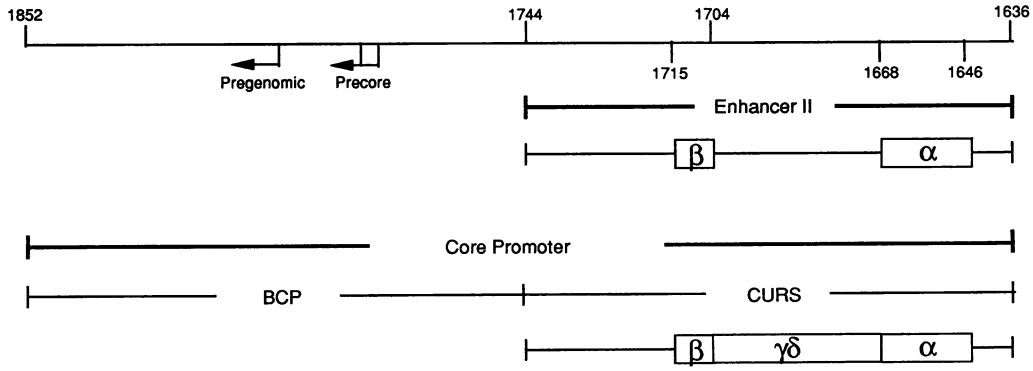

FIG. 1. Locations of enhancer II and core promoter. A schematic representation of the structure of HBV from nt 1636 to 1852 is shown. The arrows indicate the transcription start sites for the precore and pregenomic RNAs within the BCP. Enhancer II and its minimal essential sequence elements, box α (nt 1646 to 1668) and box β (nt 1704 to 1715), are demarcated at the top. The core promoter is composed of the BCP and its upstream regulatory sequence (CURS), and its structure is shown at the bottom. Deletion analysis reveals the presence of at least three functional constituents: box α, box γδ (nt 1672 to 1703), and box β. The first two are the most potent (41).

TABLE 1. Differentiated hepatoma cell specificity of enhancer function on the SV40 early promoter

Cell line	CAT activity (%)[a]		Induction (fold)[b]
	pSVpCAT	pSVpCAT/αβ	
HepG2	1.60	72.0	45
HuH-7	1.72	25.8	15
HA22T/VGH	1.11	1.67	1.5
HeLa	1.90	2.28	1.2

[a] Relative CAT activity was calculated as a percentage of that of pSV2CAT. The values are averages of four independent experiments. pSVpCAT contains the CAT gene driven by the SV40 early promoter. pSVpCAT/αβ has box α followed by box β downstream of the CAT gene of pSVpCAT. The oligonucleotides used for box α and box β are described in Materials and Methods.

[b] Fold induction was calculated relative to CAT activity expressed from constructs lacking box α and box β elements. The values are averages of four independent experiments.

ATACATTTCCCAAGT3′ and 5′ACTTGGGAAATGTATT GCGCAATATGTTC3′.

RESULTS

Differentiated hepatoma cell specificity of enhancer activity and upstream activator. We have previously (42) shown that enhancer II (Fig. 1) displays differentiated liver cell specificity in that it is active in such differentiated hepatoma cells as HepG2 and HuH-7 cells but not in poorly differentiated hepatoma cells such as HA22T/VGH cells or nonliver cells such as HeLa cells. It was subsequently demonstrated that enhancer II of HBV has a unique bipartite structure: a 23-bp sequence, box α, and a 12-bp sequence, box β, are both sufficient and necessary for enhancer II activity. Neither box α nor box β alone stimulates the activity of the SV40 early promoter, regardless of whether it is located in the upstream or downstream position. We were interested in whether these minimal functional constituents exhibit the same cell lineage and differentiation state specificity. To address this, box α and box β were cloned downstream of the SV40 early promoter and tested for their enhancer activities in four cell lines. It turns out that although there is negligible stimulation in HA22T/VGH and HeLa cells, there are 45- and 15-fold enhancements in HepG2 and HuH-7 cells, respectively (Table 1). The results indicate that these minimal essential elements, i.e., box α in conjunction with box β, retain the same differentiated liver cell specificity as does enhancer II in its entirety.

TABLE 2. Differentiated hepatoma cell specificity of upstream activating function on the BCP

Cell line	CAT activity (%)[a]			Induction (fold)[b]	
	pBCP-CAT	pα/BCP-CAT	pβ/BCP-CAT	Box α	Box β
HepG2	0.038	4.56	0.296	120	7.8
HuH-7	0.080	3.28	0.512	41	6.4
HA22T/VGH	0.150	0.615	2.40	4.1	16
HeLa	0.110	0.198	0.726	1.8	6.6

[a] Calculation of CAT activity is described in Table 1, footnote *a*. pBCP-CAT contains the CAT gene driven by the BCP. pα/BCP-CAT and pβ/BCP-CAT have box α and box β, respectively, immediately upstream of the BCP of pBCP-CAT. The oligonucleotides used for box α and box β are described in Materials and Methods.

[b] Calculated as described in Table 1, footnote *b*.

We have found that either box α or box β, especially box α, in the upstream position has stimulatory effects on the activity of the nearby BCP. We placed box α or box β upstream of the BCP and assayed their stimulatory activities in four cell lines. Box α activates the BCP strongly in HepG2 and HuH-7 cells (120- and 41-fold, respectively) but activates it only slightly in the HA22T/VGH and HeLa cells (4.1- and 1.8-fold, respectively). In contrast, box β has moderate activities in all cell lines tested (Table 2). This clearly establishes that box α has differentiated hepatoma cell specificity.

DNase I footprinting analysis of the box α-box β region with crude nuclear extracts of four different cell lines. One of the simplest hypotheses to explain the cell lineage and differentiation state specificity of box α and box β is either that an essential *trans*-acting factor(s) that implements the activation is present in differentiated liver cells or that a negative factor(s) that silences the expression is present in poorly differentiated liver cells or nonliver cells. The two possibilities are, of course, not mutually exclusive. To address this hypothesis, DNase I footprinting analyses to detect binding of box α and box β were performed. As shown in Fig. 2, protection of both box α and box β is seen with nuclear

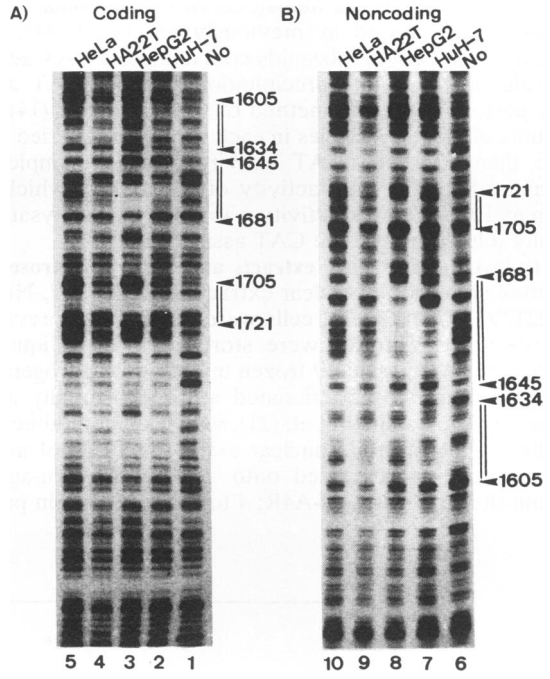

FIG. 2. DNase I footprinting analysis of the core promoter region of HBV with crude nuclear extracts of HuH-7, HepG2, HA22T/VGH, and HeLa cells. The footprinting analysis was performed by using a *Bam*HI-*Hin*dIII fragment containing nt 1402 to 1851 of HBV. Either the coding or noncoding strand was end labeled with the Klenow fragment of *Escherichia coli* DNA polymerase. The protected regions of HepG2 are indicated by double lines on the right, with numbers referring to nucleotide positions within the HBV genome and arrowheads indicating the DNase I-hypersensitive sites. (A) Footprinting analysis of the coding strand. The end-labeled probe was incubated with no protein (lane 1) or with 120 μg of protein from crude nuclear extracts of the cell lines indicated above lanes 2 to 5. (B) Footprinting analysis of the noncoding strand. The end-labeled probe was incubated with no protein (lane 6) or with 120 μg of protein from crude nuclear extracts of the cell lines indicated above lanes 7 to 10.

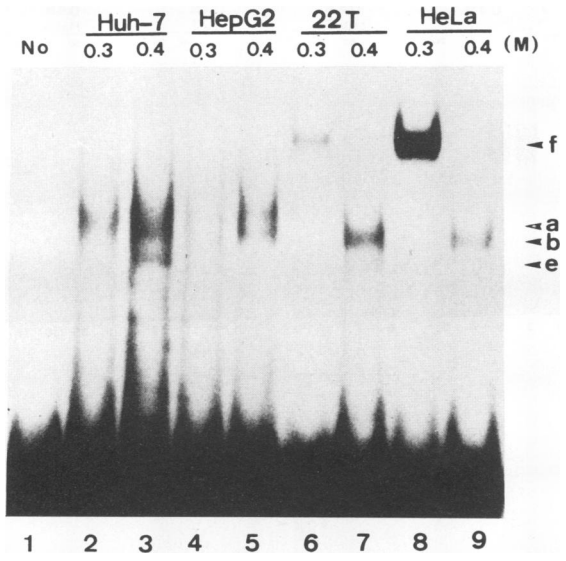

FIG. 3. Gel shift analysis of box α-binding proteins in four cell lines. Double-stranded oligonucleotides corresponding to the box α sequence were end labeled with polynucleotide kinase and incubated with proteins of fractionated 0.3 and 0.4 M NaCl nuclear extracts of HuH-7, HepG2, HA22T/VGH (22T), and HeLa cells at 30°C for 30 min. After incubation, the DNA-protein complexes were resolved on a native 4% polyacrylamide gel. Lanes: 1, no protein; 2 to 9, 10 μg of the indicated salt eluents of the heparin-agarose column from the indicated cell lines. Four shifted bands, a, b, e, and f, are indicated.

extracts from all four cell lines. The observation that the patterns found in all cell lines are not completely identical suggests that the binding proteins for either box α or box β are likely to be different.

Analysis of the box α- and box β-binding proteins present in four cell lines by gel shift assay. In earlier studies, we have demonstrated the existence of gel shifting activities from fractionated nuclear extracts of HepG2 cells. Two shifted bands, a and b, are detected when the box α oligonucleotide is used as the probe, while bands c and d are formed when the box β oligonucleotide is used. These DNA-binding proteins, being candidate transcription factors that mediate the enhancer function, display distinct sequence preferences and heat labilities (43). It is not clear, however, whether the same DNA-binding activities are present in these different cell lines. To assess this, a gel shift assay was performed. The labeled box α and box β oligonucleotides were incubated with different fractions of nuclear extracts eluted from the heparin-agarose column. As shown in Fig. 3, when box α is used as the probe, a shifting complex that migrates to the same position as band b is present in the 0.4 M NaCl fraction of all four cell lines. Three apparently different shifting activities are observed. A band shift identical to band a is present only in the 0.4 M NaCl fraction of both HepG2 and HuH-7 nuclear extracts. A shifting activity that produces band f is present instead in the 0.3 M NaCl fraction of both the HA22T/VGH and HeLa nuclear extracts. Binding activity f is much more abundant in the HeLa nuclear extract than in the HA22T/VGH nuclear extract. Another shifting activity yielding band e is present in the 0.4 M NaCl fraction of the HuH-7 nuclear extract but not in that of HepG2. These results are summarized in Table 3.

Two box β shifting bands designated c and d are detected by incubation with the 0.3 M NaCl fractions of the nuclear extracts from HuH-7, HepG2, and HA22T/VGH cells, while only band c is observed with the 0.3 M NaCl fraction from HeLa cells (Fig. 4 and Table 4). No other gel shift activity in response to either box α or box β is detected from any other fraction of nuclear extracts of these four cell lines.

The observation that shifting complexes of identical mobilities are detected with fractionated nuclear extracts from different cell lines suggests that these DNA-binding proteins may be the same. To further address this, the heat sensitivity and the sequence preference of these binding proteins were examined. For heat sensitivity, the fractionated nuclear extracts were first treated at a given temperature and then tested for their intactness by the gel shift assay. As for the sequence preference experiments, oligonucleotides of similar but not identical sequences were tested for their abilities to disrupt the formation of the previously identified shifting complexes. In view of the weak homology exhibited by box α (CAAGGTCTTACATAAGAGGACTCTT, from nt 1645 to 1669) and box β (CTACTTCAAAGACTGTG, from nt 1705 to 1721) to the C/EBP consensus sequence (ATTGCGCA AT) (abbreviated C/EBPpp) (3, 10, 13, 19), all three oligonucleotides were chosen for the competition study.

TABLE 3. Box α-binding proteins of four cell lines

NaCl concn and cell line	Relative position	Temp sensitivity[a]				Sequence specificity
		37°C	50°C	70°C	90°C	
0.4 M						
HuH-7	a	++	++	−	−	Box α >> box β ~ C/EBPpp
	b	++	++	−	−	Box α >> box β ~ C/EBPpp
	e	++	++	++	++	ND[b]
HepG2	a	++	++	−	−	Box α >> box β ~ C/EBPpp
	b	+++	++	−	−	ND
HA22T/VGH	b	++	++	−	−	Box α >> box β ~ C/EBPpp
HeLa	b	++	++	−	−	Box α >> box β ~ C/EBPpp
0.3 M						
HA22T/VGH	f	++	+	−	−	Box α > box β ~ C/EBPpp
HeLa	f	++	+	−	−	Box α > box β ~ C/EBPpp

[a] Temperature sensitivity was shown by the relative intensities of shifting bands. The number of plus signs indicates the abundance of binding proteins, while a minus sign indicates the absence of binding proteins.
[b] ND, not determined.

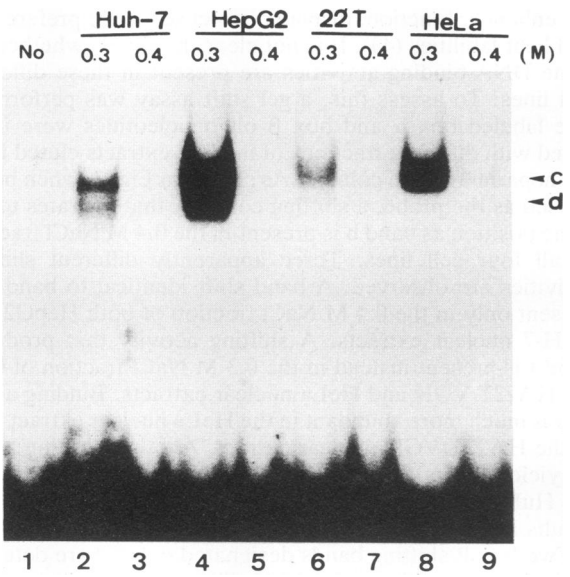

FIG. 4. Gel shift analysis of box β-binding proteins in four cell lines. Double-stranded oligonucleotides corresponding to the box β sequence were labeled as probes. Other experimental details are as described in the legend to Fig. 3. Lanes: 1, no protein; 2 to 9, 10 μg of the indicated salt eluents of the heparin-agarose column from the indicated cell lines. Two shifted bands, c and d, are indicated.

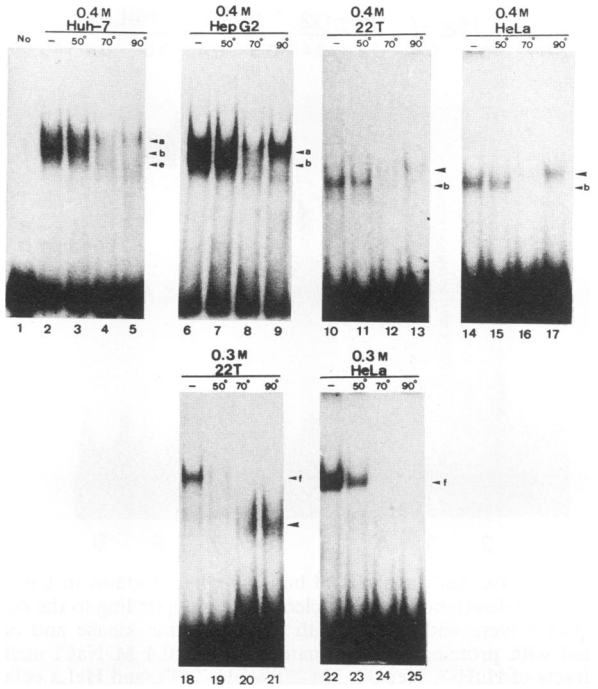

FIG. 5. Heat sensitivities of box α-binding proteins in four cell lines. End-labeled box α was incubated with 10 μg of protein of the indicated salt eluents from the indicated cell lines without heat treatment (lanes 2, 6, 10, 14, 18, and 22) or with heat treatment at 50°C for 5 min (lanes 3, 7, 11, 15, 19, and 23), 70°C for 5 min (lanes 4, 8, 12, 16, 20, and 24), or 90°C for 15 min (lanes 5, 9, 13, 17, 21, and 25). Lane 1, control (no protein). Other experimental details are as described in the legend to Fig. 3. The shifted bands, a, b, e, and f, are indicated with arrowheads. Another shifted band that appeared during 90°C treatment is indicated with larger arrowheads without letters.

As shown in Fig. 5, the box α-binding proteins that are present in bands a and b are moderately heat resistant, since both can withstand heat treatment at 50 but not at 70°C for 5 min. A novel band that migrates to the same position as band a emerges only after heat treatment in all four cell lines. This band is most likely a spurious heat-resistant binding protein. With respect to the sequence preference for the box α-binding proteins, unlabeled box α, box β, and C/EBPpp were added at 5-, 25-, 125-, and 625-fold molar excesses as competitors in the gel shift assay. Complex b can be inhibited only by the box α sequence but is slightly affected by box β and C/EBPpp at a 625-fold molar excess in the HuH-7, HA22T/VGH, and HeLa nuclear extracts (Fig. 6). This is also the case for the HepG2 cells (data not shown; a 0.5 M NaCl fraction containing protein a only is used in Fig. 6). Taken together, the same gel shift position, heat lability, and relative sequence preference strongly suggest that the binding proteins in complexes b (referred to as proteins b) from all four cell lines are identical. Similarly, proteins a in complexes a from the HepG2 and HuH-7 cells are probably identical and exhibit the same sequence preference as protein b. Members of the third group of box α-binding proteins, proteins f in complexes f, which are present only in HA22T/VGH and HeLa cell extract, are probably identical as well because both are destroyed when heated at 50°C for 5 min and exhibit the sequence preference box α > box β ~ C/EBPpp (Fig. 7).

As for the box β-binding proteins in the shifted complexes c and d, proteins c and protein d are both heat labile, as they are destroyed by heat treatment at 50°C for 5 min (Fig. 8).

TABLE 4. Box β-binding proteins of four cell lines

Cell line[a]	Relative position	Temp sensitivity[b]				Sequence specificity
		37°C	50°C	70°C	90°C	
HuH-7	c	++	–	–	–	C/EBPpp ~ box β > box α
	d	++	+	–	–	ND[c]
HepG2	c	+++	–	–	–	C/EBPpp ~ box β > box α
	d	++	+	–	–	C/EBPpp ~ box β > box α
HA22T/VGH	c	+++	–	–	–	C/EBPpp ~ box β > box α
	d	++	+	–	–	ND
HeLa	c	+++	+	–	–	C/EBPpp ~ box β > box α

[a] Data are for the 0.3 M NaCl fractions of nuclear extracts of the indicated cell lines.
[b] See Table 3, footnote *a*.
[c] ND, not determined.

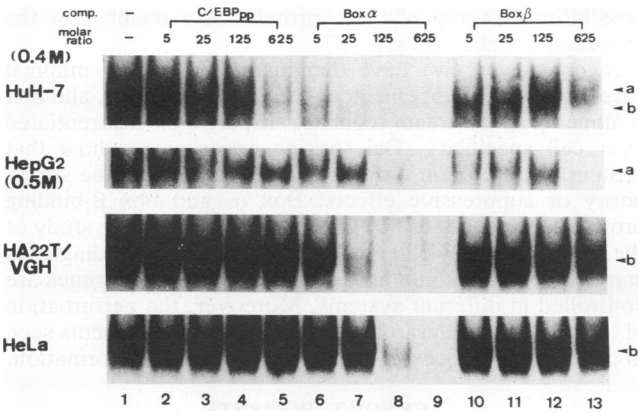

FIG. 6. Relative efficiencies of competition by box α, box β, and C/EBP consensus sequences for box α-binding proteins a and b. End-labeled box α was incubated with 10 μg of protein of the 0.4 M NaCl fraction of HuH-7, HA22T/VGH, and HeLa cells as well as the 0.5 M NaCl fraction of HepG2 cells. Lanes: 1, no competitor; 2 to 5, unlabeled C/EBPpp; 6 to 9, box α; 10 to 13, box β. The molar excess of each competitor in the gel shift assay is indicated above each lane. Two shifted bands, a and b, are indicated on the right.

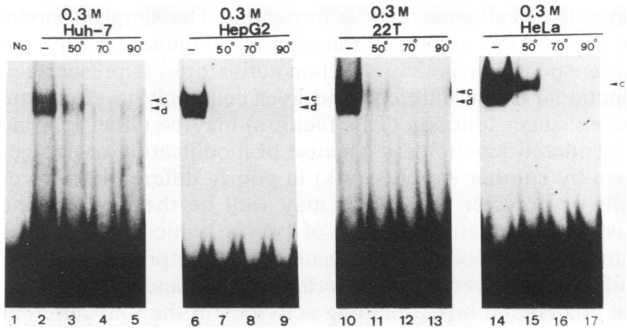

FIG. 8. Heat sensitivities of box β-binding proteins in four cell lines. End-labeled box β was incubated with 10 μg of protein of the 0.3 M NaCl fractions from the indicated cell lines without heat treatment (lanes 2, 6, 10, and 14) or with heat treatment at 50°C for 5 min (lanes 3, 7, 11, and 15), 70°C for 5 min (lanes 4, 8, 12, and 16), or 90°C for 15 min (lanes 5, 9, 13, and 17). Lane 1, control (no protein). Other experimental details are as described in the legend to Fig. 3. The shifted bands, c and d, are indicated with arrowheads.

Moreover, both display the sequence preference C/EBP ~ box β > box α (Fig. 9). These results support the conclusion that the proteins c and d from the different cell lines are probably identical.

DISCUSSION

Specialized higher eukaryotic cells differ from each other by virtue of the proteins that they make. Most of the control of gene expression that underlies these differences operates at the level of transcription. The interaction of various *trans*-acting factors with their cognate specific *cis*-acting elements is the major point at which such regulation is exerted. The unique hepatotropism displayed by HBV in the infective cycle suggests a stringent requirement for the liver-specific factors for viral gene expression. It is hoped that an insight into such transcriptional control in HBV as a model system will shed some light on how other liver-specific genes are regulated.

We have previously reported the identification and characterization of the second enhancer (enhancer II) of HBV. Enhancer II activates such homologous promoters as SPI, SPII, and XP and such heterologous promoters as the SV40 early promoter in a position- and orientation-independent manner (42). Further analysis reveals that enhancer II has a unique bipartite structure in that two interacting minimal essential elements, box α and box β, are both sufficient and necessary for the enhancer function. Interestingly, box α or box β alone stimulates the nearby downstream BCP (41). Box α stands out as a strong regulator, while box β has a moderate level of activity.

Both enhancer II and the core upstream regulator exhibit differentiated liver cell specificity. When tested in transient-transfection experiments, both are functional in well-differentiated liver cells such as HepG2 and HuH-7 cells but not in poorly differentiated liver cells such as HA22T/VGH cells or nonliver cells such as HeLa cells. In this study, we have demonstrated that box α in conjunction with box β retains not only the enhancer II function but also well-differentiated liver cell specificity. Furthermore, box α, as an upstream regulator by itself, also displays well-differentiated liver cell specificity. Box β, in contrast, has moderate upstream regulator activity in both liver and nonliver cells.

The observation that the two minimal essential elements of the second enhancer, box α and box β, display differen-

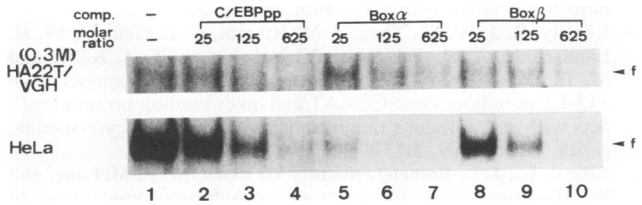

FIG. 7. Relative efficiencies of competition by box α, box β, and C/EBP consensus sequences for box α-binding protein f. End-labeled box α was incubated with 10 μg of the 0.3 M NaCl fractions of HA22T/VGH and HeLa cells in the absence of competitor (lane 1) and in the presence of unlabeled C/EBPpp (lanes 2 to 4), box α (lanes 5 to 7), and box β (lanes 8 to 10) competitors at the indicated molar excesses in a gel shift assay. One shifted band, f, is indicated on the right.

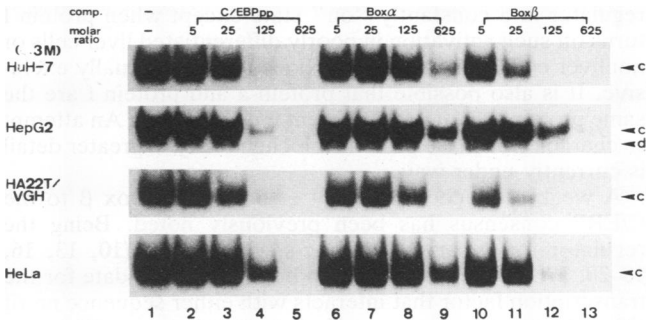

FIG. 9. Relative efficiencies of competition by box α, box β, and C/EBP consensus sequences for box β-binding proteins c and d. End-labeled box β was incubated with 10 μg of the 0.3 M NaCl fractions from the indicated cell lines in the absence of competitor (lane 1) and in the presence of unlabeled C/EBPpp (lanes 2 to 5), box α (lanes 6 to 9), and box β (lanes 10 to 13) competitors at the indicated molar excesses in a gel shift assay. Two shifted bands, c and d, are indicated on the right.

tiated liver cell specificity is intriguing. The simplest model to explain the enhancer function in conjunction with its tissue specificity is that a certain activator(s) is present and functional in the differentiated liver cells only to effectuate the enhancer function. This factor(s) may be either missing or rendered functionless because of modification or antagonism by another suppressor(s) in poorly differentiated liver cells or nonliver cells. This may well be the case for the upstream regulator function of box α, which exhibits the same tissue specificity as enhancer II. Footprinting and gel shift analyses were thus performed to examine and compare the box α- and box β-binding activities in the four different cell lines. The footprinting experiments reveal that protection over this region is observed with the nuclear extracts from all four cell lines, indicating that both sequence motifs are bound with nuclear factors despite their different functional statuses. These nuclear factors are most likely not all identical, as the footprint patterns are not all identical. These differences are borne out in the gel shift analysis. First, protein c appears to be present in all four cell lines tested. Its ubiquitous presence makes it the most likely candidate for the transcription factor that mediates the upstream activation by box β observed in all four cell lines. Second, box α-binding protein a is present in well-differentiated liver cells such as HepG2 and HuH-7 cells but not in poorly differentiated liver cells such as HA22T/VGH cells or nonliver cells such as HeLa cells. The converse is true, however, for a different factor, protein f. This correlates well with the stimulatory activities of box α as either the upstream activator or the enhancer in these four cell lines.

Box α not only can function as an upstream regulator for the BCP but also can serve as an indispensable part of the second enhancer. The presence of dual functions in a mere 23-bp sequence element and the same cell and differentiation state specificities exhibited by both suggests that the same factor(s) binds to this sequence motif and participates in both functions. In fact, detailed mutational analysis of box α reveals the same sequence requirement for both enhancer and upstream regulator functions, further lending support to this notion (unpublished data). It is likely that protein a, whose presence parallels the functional status of both the upstream regulator and enhancer II, effectuates both stimulations in cooperation with other box α- and box β-binding proteins. Of equal likelihood is that protein b is the positive regulator that can lock the enhancer and the upstream regulator in a constantly "on" state except when protein f turns off such activation in poorly differentiated liver cells or nonliver cells. These two situations are not mutually exclusive. It is also possible that protein a and protein f are the same protein, albeit with different modifications. An attempt to characterize these proteins biochemically in greater detail is currently under way.

A weak sequence homology of box α and box β to the C/EBP consensus has been previously noted. Being the regulator for a variety of liver-specific genes (10, 13, 16, 18–20, 27, 38), C/EBP seems to be a good candidate for the transcription factor that interacts with either sequence motif (22). This is, however, not likely to be the case. First and foremost, C/EBP is present at a very low level in HepG2 cells (13), where we observe the greatest effect of both the enhancer and the upstream activator. Second, on the basis of sequence preference and heat sensitivity, proteins a, b, c, and d are distinct from C/EBP. In this study, we tested the fifth binding protein, protein f, and showed that it cannot be C/EBP either. However, we cannot completely rule out the possibility that any of these proteins is a member of the extended C/EBP family (3, 37).

In conclusion, we have demonstrated that the minimal essential elements of enhancer II, box α and box β, and box α alone as an upstream regulator display well-differentiated liver cell specificity. Gel shifting experiments show that protein a and protein f are candidates to mediate the stimulatory or suppressive effects. Box α- and box β-binding proteins appear not to be C/EBP. We hope that the study of the interaction between these factors and their binding sites can help us understand how other lineage-specific genes are controlled in different systems. Moreover, the perturbation of this regulatory hierarchy may underlie derangements seen in pathological processes such as oncogenic transformation.

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