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Functional Interaction of Nuclear Factors EF-C, HNF-4, and RXRα with Hepatitis B Virus Enhancer I

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Hepatitis B virus (HBV) enhancer I contains *cis*-acting elements that are both sufficient and essential for liver-specific enhancer function. The EF-C binding site was previously shown to be a key element in enhancer I. EF-C binding activity is evident in hepatic and nonhepatic cells. Although the EF-C binding site is required for efficient HBV enhancer I function, the EF-C site does not possess intrinsic enhancer activity when assayed in the absence of flanking elements. We have defined a novel region in HBV enhancer I, termed the GB element, that is adjacent to and functions in conjunction with the EF-C binding site. The GB element and EF-C site confer interdependent liver-specific enhancer activity in the absence of flanking HBV enhancer sequences. The nucleotide sequence of the GB element is similar to sequences of the DNA binding sites for members of the steroid receptor superfamily. Among these proteins, we demonstrate that HNF-4, RXR (retinoid X receptor), and COUP-TF bind to the GB element in vitro. HNF-4 transactivates a promoter linked to a multimerized GB/EF-C domain via the GB element in vivo in a manner that is dependent on the integrity of the adjacent EF-C binding site. RXRα also transactivates promoter expression via the GB element in vivo in response to retinoic acid but in a largely EF-C-independent manner. Finally, we show that COUP-TF antagonizes the activity of the GB element in human liver cells.

The hepatitis B virus (HBV) genome contains four overlapping reading frames that encode the core antigens (C, pre-C, and e), polymerase (P), the surface antigens (S, pre-S1, and pre-S2), and the X protein (14). These gene products are translated from genome- and subgenome-length mRNAs whose transcription is directed by four distinct promoter regions (core, pre-S2, pre-S1, and X promoters) (38). Two transcriptional enhancer regions (I and II) have been identified in the HBV genome. Both enhancers I and II exhibit greater activity in tissue culture cell lines of hepatic origin than in nonhepatic cells (19, 20, 41, 55, 58). Enhancer I is located within the P reading frame downstream of the S and upstream of the X reading frames (41, 44); the X promoter region overlaps enhancer I (16, 46, 47). Enhancer II is located upstream of and overlaps the core promoter region (53, 55–57). Although enhancers I and II function in conjunction with heterologous promoters, in the context of HBV promoters, enhancer I strongly stimulates the core and X region promoters and to a lesser extent activates the pre-S2 and pre-S1 promoter regions (2). Essentially nothing is known about how either enhancer I or enhancer II regulates HBV gene expression during the viral life cycle and in the course of viral pathogenesis in the liver.

Although enhancer I displays pronounced activity in liver cell lines, it has been reported that enhancer I also functions in cell lines of nonhepatic origin (10, 49). Consistent with this fact is the observation that among the numerous nuclear proteins that bind to enhancer I (Fig. 1), some are liver specific while others are ubiquitous (4, 9, 12, 28, 36, 37, 40, 47). Little is known about the functional relationship between the protein binding sites in HBV enhancer I that contribute to the enhancer activity observed in vivo. Much of what is known about the organization of eukaryotic enhancer regions comes from systematic studies of the simian virus 40 (SV40) enhancer; in that case, it was observed that individual *cis*-acting elements often did not have intrinsic enhancer function but rather functioned in conjunction with one or more adjacent *cis*-acting sequences (13, 34). The individual *cis*-acting elements have been referred to as enhansons, the basic unit of enhancer organization, and constitute the recognition sites for specific *trans*-acting proteins. Different types of enhansons, including (i) enhansons that have intrinsic enhancer activity, (ii) enhansons that function when dimerized or multimerized, and (iii) enhansons that function in combination with other enhanson elements, have been described for the SV40 enhancer.

We previously described a nuclear protein, termed EF-C, that is present in hepatic and nonhepatic cells and that binds to an important functional site in HBV enhancer I (also termed the EP site) (Fig. 1) (4, 35, 36, 40). EF-C binds to an inverted repeat sequence (5′-GTTGCYNGGCAAC-3′). While the EF-C binding site is among the key *cis*-acting elements essential for efficient enhancer I activity (36), an individual EF-C site does not have intrinsic enhancer activity, nor does it function when arranged in tandem copies (8). Therefore, the EF-C binding site appears to correspond to the third class of enhansons and likely functions in conjunction with an adjacent enhancer element(s) to contribute to enhancer I activity. In this report, we describe a novel element, termed GB, that is situated adjacent to the EF-C binding site in enhancer I that functions in conjunction with the EF-C site in vivo. The GB element and EF-C binding site function interdependently to confer enhancer activity in the absence of other HBV enhancer sequences in the human hepatoma cell line HepG2, but the GB/EF-C domain does not exhibit activity in nonhepatic cells. The nucleotide sequence of the GB element is similar to the sequences of binding sites of liver-enriched nuclear proteins HNF-4 (hepatocyte nuclear factor 4) (42) and RXRα (retinoid X receptor) (30) as well as the binding site for the ubiquitous nuclear protein termed COUP-TF (chicken ovalbumin upstream

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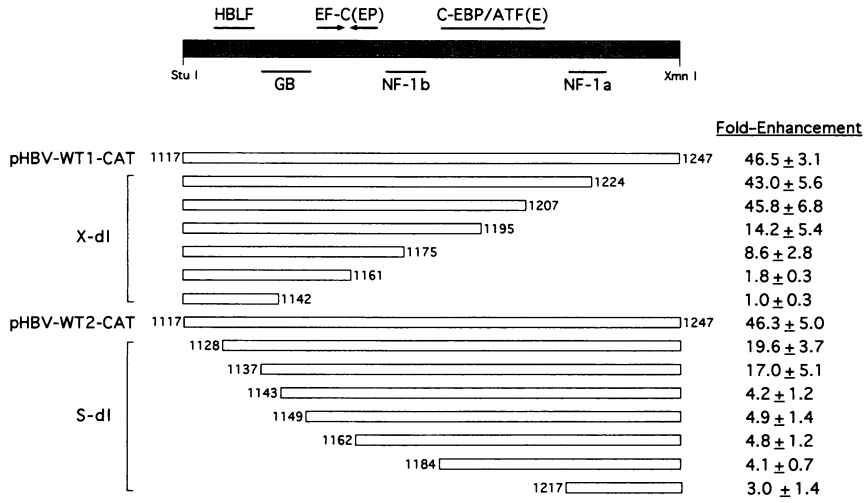


FIG. 1. Mutational analysis of HBV enhancer I. Shown at the top is a schematic view of the HBV enhancer I (*Stu*I-*Xmn*I fragment, nt 1117 to 1247) and the nuclear factors that have been found to bind to this region, including HBLF (47), EF-C (36), NF-1 (4), C/EBP (9), and ATF (28). The domains termed EP and E, as previously described, are indicated (4). Shown below the schematic are the deletion mutations described in the text. The top group of mutants (X-*dl* series) contain deletions that begin at the *Xmn*I site and progress leftward; each open bar represents the segment of the enhancer region that is intact with each mutant. Numbers represent the last nucleotides present on either side of each deletion. The bottom group of mutants (S-*dl* series) contain deletions that begin at the *Stu*I site and progress rightward. The mutant enhancer segments were introduced upstream of the SV40 early promoter in a CAT expression vector (see Fig. 3A) in an orientation such that the deletions always progressed into the enhancer region from 5′ upstream vector sequences. The X-*dl* series mutants were introduced in an inverted orientation relative to the schematic (i.e., *Xmn*I-*Stu*I/SV-CAT), while the S-*dl* series mutants were introduced in the orientation depicted in the figure (i.e., *Stu*I-*Xmn*I/SV-CAT). Each of the mutants in expression vectors was transfected into HepG2 liver cells, and CAT enzymatic activity was assayed from cellular extracts prepared 24 to 48 h after transfection. The fold enhancement, ± standard deviation, observed with each mutant (CAT activity of enhancer-containing/enhancerless vector ratio) is shown at the right and represents the average of six independent experiments.

promoter transcription factor) (51). HNF-4, RXRα, and COUP-TF are all members of the steroid/thyroid nuclear receptor superfamily (3, 11). We demonstrate that HNF-4 and COUP-TF expressed in vitro bind to the GB element. Furthermore, antisera directed against HNF-4, RXRα, and COUP-TF recognize distinct DNA-protein complexes that form with the GB element in nuclear extracts prepared from HepG2 cells. Additionally, HNF-4 and RXRα transactivate a promoter linked to a multimerized GB/EF-C domain in vivo specifically through the GB element. Transcriptional activation of the GB element by HNF-4, but not RXRα, required the integrity of the adjacent EF-C binding site. In contrast, COUP-TF antagonized transcriptional activation mediated by the GB/EF-C domain. These data indicate that multiple transcription factors may activate or repress HBV enhancer I activity via the same DNA element, GB. These results imply that different environmental stimuli may affect enhancer I activity and the production of virus in infected liver.

MATERIALS AND METHODS

Plasmid DNAs, oligonucleotides, and probes. Deletion mutations in the HBV *Stu*I-*Xmn*I enhancer I DNA fragment (nucleotides [nt] 1117 to 1247, HBV subtype ayw; Fig. 1) were generated from two parental plasmids, pHBV-WT1-CAT and pHBV-WT2-CAT, that contain the enhancer region in either orientation relative to the SV40 early promoter (nt 1117 to 1247/SV-CAT and nt 1247 to 1117/SV-CAT, respectively) in the expression vector pSV-dlOP-CAT (36). Parental plasmids were linearized, and progressive deletions were generated by standard procedures using exonuclease III and nuclease S1. *Xba*I linkers were ligated at the deletion endpoints, and the extent of each deletion was determined by DNA sequencing. HBV enhancer region point mutations were generated by site-directed oligonucleotide mutagenesis as described by Kunkel (24).

The WT-GB/EF-C, GB/EFC-X, GB dpm2, and GB dpm3 35-bp oligonucleotides are shown in Fig. 3B. Direct, tetrameric copies of each oligonucleotide were introduced upstream of the SV40 early promoter in vector pSV-dlOP-CAT and upstream of the E1A promoter in vector pE1A-CAT-ENH$^-$ (5). A synthetic duplex oligonucleotide corresponding to the CRBPII RXRE site (5′-TCGACCAGGTC ACAGGTCACAGGTCACAGTTCAAC-3′ [31]) was inserted into the E1A-CAT vector. Synthetic duplex oligonucleotides corresponding to the following monomeric sites were inserted into the *Sal*I-*Xho*I sites of a pUC9 derivative (35): WT-GB/EF-C, GB/EFC-X, GB dpm2, and GB dpm3 (see Fig. 3B), APF1 (5′-TCGAGCAGGTGACCTTTGCCCA GCGC-3′ [42]), and βRARE (5′-TCGACGTAGGGTTCACC GAAAGTTCACTCC-3′ [43]). The α1-AT site (5′-AATTC CAGTGGACTTAGCCCC-3′ [42]) was inserted into the *Eco*RI-*Xmn*I sites in pUC19. These recombinant plasmids were used as a source of probe and competitor DNAs. The fragments of interest were excised by digestion with either *Hin*dIII-*Eco*RI or *Sal*I-*Xho*I, gel purified, and quantitated by ethidium bromide staining on a polyacrylamide gel in comparison with known quantities of a standard DNA marker. About 40 ng of a DNA fragment typically was used to prepare a ^{32}P-labeled probe by incorporating [α-^{32}P]dATP for *Eco*RI-*Hin*dIII DNA fragments and [α-^{32}P]dATP and [α-^{32}P]dCTP for *Sal*I-*Xho*I DNA fragments by using Klenow

DNA polymerase, yielding specific activities of ~20,000 cpm/fmol.

pf7, a pBS-SK(−) based plasmid (Stratagene) that contains the HNF-4 cDNA (a gift from F. Sladek and J. Darnell) (42), and pFL-COUP-TF-1, a pGEM-7Zf(+)-based plasmid (Promega) that contains the COUP-TF-1 cDNA (a gift from M.-J. Tsai and B. O'Malley) (7), were used as template DNAs for in vitro transcription by T3 RNA polymerase and SP6 RNA polymerase, respectively. In vitro translations were performed by using rabbit reticulocyte lysates (Promega) as recommended by the manufacturer. Mammalian cDNA expression vectors pLEN-4S and pLEN-4A (a gift from F. Sladek and J. Darnell) contain the rat liver HNF-4 cDNA in the sense (4S) and antisense (4A) orientations, respectively, as previously described (42). The RXRα cDNA expression vector, pCMX-RXRα (a gift from K. Umesono and R. M. Evans), was previously described (22). The COUP-TF-1 cDNA expression vector, pLEN-C-TF, was constructed by inserting the *Bam*HI-*Xba*I COUP-TF-1 cDNA fragment from pFL-COUP-TF-1 into the *Bam*HI site of the pLEN expression vector (42).

Cells and transfection. HepG2 cells were grown in minimal essential medium containing 10% fetal bovine serum. HeLa S3, CV-1, MOP-8, and 293 cells were grown in Dulbecco modified essential medium containing 10% defined, supplemented calf serum (HyClone). Plasmid DNA transfections were performed by the calcium phosphate precipitation procedure (15). Cells were split and plated the day before transfection. The following day, HepG2 cells were transfected with 1 μg of a chloramphenicol acetyltransferase (CAT) reporter plasmid DNA and 19 μg of salmon sperm DNA per 100-mm-diameter dish. HeLa, CV-1, MOP-8, and 293 cells were transfected with 2 μg of a CAT reporter plasmid DNA and 19 μg of salmon sperm DNA per 100-mm-diameter dish. The cells were incubated for 12 to 16 h with the calcium phosphate precipitate and then washed with Tris-buffered saline solution (TS), TS containing 3 mM EGTA, and TS. Fresh medium was then added, and total cell extracts were prepared 24 to 48 h later. CAT enzymatic activity in cellular extracts was assayed as previously described (15). CAT activity was quantitated by liquid scintillation counting of nonacetylated and acetylated forms of chloramphenicol excised from the chromatogram. Four or more independent experiments were quantitated, and values are presented as fold enhancement (enhancer/enhancerless activity).

Transient cotransfection assays were done with HepG2 cells transfected with various amounts (0, 2, 5, 8, and 10 μg) of either pLEN-C-TF (COUP-TF-1 cDNA expression vector) or pLEN-4A (antisense HNF-4 cDNA expression vector), 1 μg of a CAT reporter plasmid, and 15 μg of salmon sperm DNA per 100-mm-diameter dish. HeLa cells were transfected with 1 μg of pLEN-4S (HNF-4 cDNA expression vector), 2 μg of a CAT reporter plasmid, and 19 μg of salmon sperm DNA per 100-mm-diameter dish or with 0.8 μg of pCMX-RXRα, 2 μg of a CAT reporter plasmid, and 19 μg of salmon sperm DNA per 100-mm-diameter dish. Transient cotransfection assays were performed essentially as described for single-plasmid transfections with one exception. Fresh medium added to HeLa cells transfected with pCMX-RXRα contained calf serum that was previously treated with 5% dextran-coated charcoal and either contained or lacked 10 μM all-*trans* retinoic acid (RA). Levels of CAT expression in cotransfected cells were measured from three or more separate experiments.

Nuclear extract preparation and electrophoretic mobility shift assays. HepG2 cell nuclear extracts were prepared according to the method of Shapiro et al. (39). HepG2 cell monolayers were rinsed once with cold phosphate-buffered saline solution (PBS) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and the cells were detached by treatment with PBS-PMSF containing 10 mM EDTA. The cell suspension was collected and centrifuged, and the cell pellet was rinsed three times with PBS-PMSF before the preparation of nuclear extracts.

Electrophoretic mobility shift assays were performed in a final volume of 12.5 μl containing 3.5 μg of HepG2 nuclear extract, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4), 30 mM KCl, 1.0 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 4% Ficoll, 160 ng of poly(dI-dC) per μl, 13.0 ng of single-stranded salmon sperm DNA per μl, and 40,000 cpm of probe (~2.0 fmol). The DNA binding reactions were performed at room temperature for 45 to 60 min, and the resulting DNA-protein complexes were resolved by electrophoresis on 4% (30:1) polyacrylamide gels run in 0.5× TBE (25 mM Tris [pH 8.3], 25 mM borate, 0.5 mM EDTA) at 200 V at 4°C. In mobility shift assays in which antiserum was used, the HNF-4 antipeptide antiserum (a gift from F. Sladek and J. Darnell) (42) and COUP-TF antiserum (a gift from M.-J. Tsai and B. O'Malley) (51) were diluted 1:10, and the RXRα (a gift from R. M. Evans) antiserum was diluted 1:5 in PBS–3% bovine serum albumin–0.2% sodium azide; 1 μl of the diluted antiserum was added to the DNA binding reactions after 30 min of incubation, and the incubation continued for an additional 45 min. For mobility shift assays performed with in vitro-synthesized HNF-4 and COUP-TF proteins, 5 μl of rabbit reticulocyte extract programmed with in vitro-synthesized transcripts encoding either HNF-4 or COUP-TF was assayed in a 12.5-μl DNA binding reaction volume. DNA binding reaction conditions were as described above except that 40 ng of poly(dI-dC) per μl, 6.0 ng of single-stranded salmon sperm DNA per μl, and 100,000 cpm of probe (~5.0 fmol) were used.

RESULTS

Definition of a novel element in HBV enhancer I. Although the EF-C binding site is a critical functional component of HBV enhancer I, it lacks intrinsic enhancer activity (8). It seemed likely, therefore, that the EF-C site functioned in conjunction with another element(s) in enhancer I. To determine what HBV sequences might function in conjunction with the EF-C binding site, two sets of deletion mutations were generated. Mutations that progressed unidirectionally either from the *Stu*I site at nt 1117 or from the *Xmn*I site at nt 1247 were introduced in enhancer I (Fig. 1, S-*dl* and X-*dl* mutants). The enhancer mutants were introduced upstream of the SV40 early promoter in a CAT expression vector in an orientation such that the deletions always progressed from 5′ flanking vector sequences into the HBV enhancer region. This was done to avoid any spacing effects of the deletions on the enhancer relative to the promoter region. The wild-type parental vectors, pHBV-WT1-CAT (orientation *Xmn*I-*Stu*I/SV-CAT) and pHBV-WT2-CAT (orientation *Stu*I-*Xmn*I/SV-CAT), and deletion mutant derivatives were transfected into the human hepatoma cell line HepG2. CAT expression was measured and is presented as fold enhancement relative to the level of expression derived from the enhancerless parental plasmid (Fig. 1). The wild-type HBV enhancer, in either orientation, stimulated CAT expression greater than 45-fold. Analysis of the deletions progressing

from the *Xmn*I site showed that the deletion of the region containing the NF-1a site (X-*dl*1224 and X-*dl*1207) did not reduce enhancer activity. Additional deletion of the E domain (X-*dl*1195) resulted in a ~3-fold decrease in enhancer activity relative to the wild type. An enhancer fragment that contained the EF-C and HBLF binding sites (X-*dl*1175) stimulated CAT expression ~9-fold. This level of enhancement was greatly reduced when the EF-C site was mutated (X-*dl*1161). Activity was abolished with deletion of the region immediately to the left of the EF-C binding site but not including the HBLF binding site (X-*dl*1142). Analysis of the deletions progressing from the *Stu*I site showed that deletion of the HBLF binding site (S-*dl*1128 and S-*dl*1137) resulted in a ~2.5-fold decrease in enhancer activity relative to the wild type. A distinct element, termed GB, adjacent to the EF-C binding site was identified by subsequent mutations. A ~5-fold reduction in enhancer activity was observed when the region between the HBLF and EF-C binding sites was deleted (S-*dl*1143 and S-*dl*1149). Additional deletion of the EF-C and NF-1b binding sites and the E domain (S-*dl*1162, S-*dl*1184, and S-*dl*1217) did not result in any further change in enhancer activity. We conclude from these results that a distinct enhancer element, termed GB, that contributes to efficient enhancer I activity is located between the HBLF and EF-C binding sites. Substantial enhancer activity was observed with mutant X-*dl*1175. This mutant fragment contained only the EF-C and HBLF binding sites and the GB element. The EF-C site did not contribute to the activity of the NF-1a and NF-1b binding sites and the E domain.

To examine the roles of the GB element and EF-C binding site in the context of the intact HBV enhancer region, point mutations were introduced into these sites (see Fig. 3B) and assayed as described above. The mutations included (i) a 2-bp substitution in the GB element (GB dpm2), (ii) a 4-bp substitution in the EF-C site (EFC-X) that disrupts EF-C binding (data not shown), and (iii) a double mutant (GB dpm2/EFC-X). These mutations were introduced into the SV40 expression vector in the intact HBV enhancer (nt 1117 to 1247) in both orientations. The results of enhancer activity assays in HepG2 cells are shown in Fig. 2. Mutation of the GB element (GB dpm2) dramatically reduced HBV enhancer activity (12- to 18-fold). Mutation of the EF-C binding site (EFC-X) also resulted in a significant decrease in enhancer activity, but to a slightly lesser extent (six- to eightfold). The HBV enhancer containing the double mutation (dpm2/EFC-X) was reduced to a similar extent. These results confirm the importance of the GB element and EF-C binding site for enhancer I activity and strengthen the supposition that these sites function in conjunction with one another.

The GB element and EF-C binding site display activity independent of adjacent HBV sequences. The results of the analyses presented above suggest that the GB element and EF-C binding site could constitute a functional enhancer domain. To test this idea directly, we synthesized 35-bp oligonucleotides that contain the wild-type GB element and EF-C binding site (Fig. 3B, WT-GB/EF-C). In addition, individual point mutations were introduced into these sites (Fig. 3B, GB/EFC-X, GB dpm2, and GB dpm3). The oligonucleotides were ligated to generate four direct tandem copies and were introduced into CAT expression plasmids upstream of the SV40 early promoter and upstream of the adenovirus E1A promoter (Fig. 3A). The activities of the wild-type and mutant expression plasmids were analyzed by transfection of HepG2 cells (Fig. 3C). A monomeric wild-type GB/EF-C oligonucleotide minimally enhanced expression of the SV40 early promoter, while a dimeric copy of the

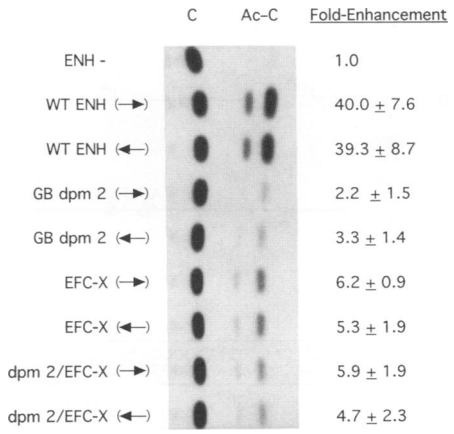
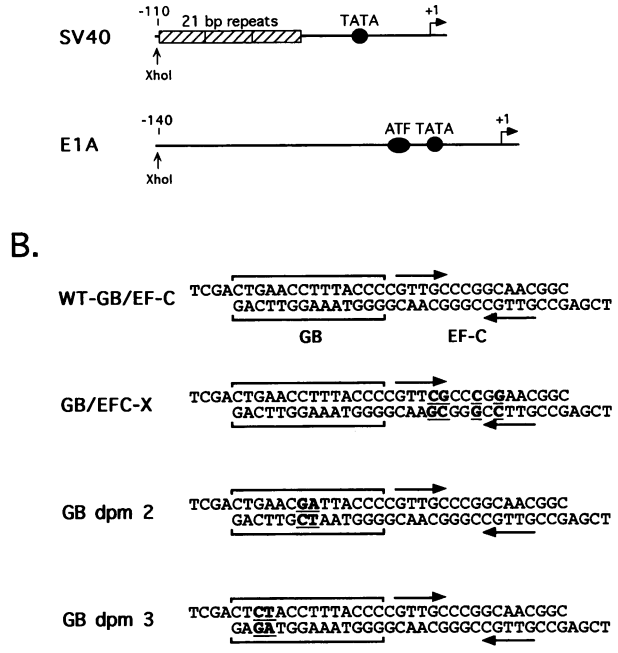


FIG. 2. Analysis of point mutants in HBV enhancer I. Point mutations in the GB site, the EF-C site, and both sites (see Fig. 3B for mutations) were introduced into the context of the *Xmn*I-*Stu*I enhancer I fragment. The wild-type (WT) and mutant enhancer (ENH) regions were introduced upstream of the SV40 early promoter, in both orientations as indicated by the arrows, in the SV-CAT expression vector, and CAT activity was assayed following transfection of HepG2 cells as described in the legend to Fig. 1. The fold enhancement, ± standard deviation, observed with each mutant expression vector is shown at the right and represents the average of five independent experiments. The autoradiogram represents a typical CAT assay performed with extracts from cells transfected with the wild-type and mutant vectors; C and Ac-C represent the unacetylated and acetylated forms of [^{14}C]chloramphenicol.

oligonucleotide stimulated expression ~9-fold (data not shown). The tetrameric wild-type GB/EF-C oligonucleotide enhanced expression of the SV40 early promoter ~23-fold and expression of the adenovirus E1A promoter >90-fold. Mutation of the EF-C binding site (GB/EFC-X) resulted in a five- to eightfold reduction in enhancer activity. Enhancer activity was abolished when mutations were introduced into the GB element (GB dpm2 and GB dpm3). The activity of the wild-type reporter vector, WT-GB/EFC-SV-CAT, was also tested in HeLa, MOP-8, 293, and CV-1 cells but did not exhibit any enhancer activity relative to the enhancerless vector in these cells (data not shown). We conclude from these results that the GB element and EF-C binding site have intrinsic enhancer activity independent of adjacent HBV sequences. Full activity of this domain required the integrity of both elements. Residual activity was observed when the EF-C site, but not the GB element, was mutated. Of several cell lines tested, GB/EF-C enhancer activity was observed only in HepG2 cells, a differentiated human hepatoma cell line (1).

Nuclear factors HNF-4, RXRα, and COUP-TF bind to the GB element. The nucleotide sequence of the GB element is similar to sequences of the binding sites for nuclear proteins HNF-4, COUP-TF, and RXRα. Shown in Fig. 4 is a sequence comparison between the GB element, the consensus sequence for HNF-4 (42), and binding sites for COUP-TF (52), RARα (RA receptor α), and RXRα (25). The extent of GB sequence identity with these sites is indicated by the underlined nucleotides. The GB element exhibits an imperfect, hexameric repeat that is separated by a 1-bp spacer (arrows under GB site) that is characteristic of an RXR binding site (31). A mobility shift assay using a radiolabeled GB/EFC-X oligonucleotide probe and nuclear extract pre-



C.

	Fold-enhancement	
	SV vector	E1A vector
WT-GB/EF-C	22.6 ± 7.3	91.5 ± 25.5
GB/EFC-X	4.6 ± 1.2	11.0 ± 3.5
GB dpm 2	0.46 ± 0.11	N.D.
GB dpm 3	0.35 ± 0.14	1.5 ± 0.30

FIG. 3. Evidence that the HBV GB element and EF-C binding site function in concert. (A) Schematic view of the CAT expression vectors used in transient expression assays. The SV40 vector contains a unique *Xho*I site for oligonucleotide insertion immediately upstream of the SV40 early promoter which carries the 21-bp repeats, the TATA box region, and the transcription initiation site fused to the CAT gene (vector pSV-dlOP-CAT [36]). The E1A vector contains a unique *Xho*I site for oligonucleotide insertion ~140 nt upstream of the E1A transcription initiation site (vector E1A-CAT [5]). The E1A promoter regions contains a TATA box and upstream binding site for ATF. (B) Sequence of the wild-type and mutant 35-nt GB/EF-C oligonucleotides. WT-GB/EF-C carries the wild-type HBV enhancer region sequence between nt 1137 and 1167 with cohesive *Sal*I-*Xho*I ends. GB/EFC-X contains four point mutations in the EF-C site, as indicated by the underlined and bold letters, that disrupt EF-C binding. GB dpm2 and GB dpm3 contain two point mutations in the GB site, as indicated by the underlined and bold letters. (C) CAT activity with expression vectors carrying the wild-type and mutant oligonucleotides. Four tandem direct copies of each oligonucleotide shown in panel B were inserted at the unique *Xho*I sites in the SV40 and E1A-CAT expression vectors shown in panel A. The expression vectors were transfected into HepG2 cells, and CAT activity was assayed as described in the legend to Fig. 1. The fold-enhancement, ± standard deviation, observed with each expression vector represents the average of four independent experiments. The basal levels of expression of the two enhancerless parental vectors were comparably low (within twofold of each other; data not shown). N.D., not determined.

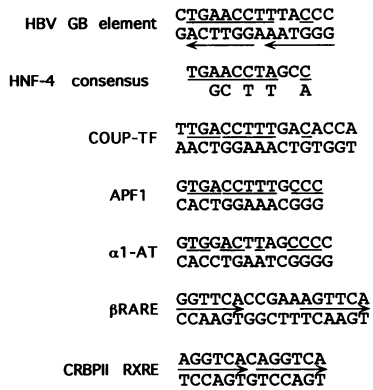


FIG. 4. Sequence similarity between the HBV GB element and other nuclear protein binding sites. The sequence of the HBV GB element is shown at the top. Shown below are the sequences of binding sites for transcription factors described in the text, including HNF-4 (42), COUP-TF (52), an HNF-4 and COUP-TF recognition site in the ApoCIII promoter region termed the APF1 site (42), an HNF-4 recognition site in the alpha-1 antitrypsin promoter region termed α1-AT (42), an RAR binding site (βRARE [43]), and an RXR binding site in the CRBPII promoter region (CRBPII RXRE [31]). The extent of sequence similarity between the HBV GB site and the HNF-4 and COUP-TF sites listed is indicated by the underlined nucleotides. The repeated sequence motifs in the RAR and RXR binding sites are indicated by arrows under the sequences, and the similarity with the HBV GB element is indicated by the reverse arrows under the GB sequence.

pared from HepG2 cells was performed (Fig. 5). A mutated EF-C site was used with this probe, since EF-C-specific complexes interfered with the visualization of complexes that formed with the GB element (data not shown). Three distinct complexes (I, II, and III) were evident with the GB element probe (Fig. 5A, lane 9). These complexes were compared with complexes that formed with both an APF1 probe (that binds HNF-4 and COUP-TF) and an α1-AT probe (that binds HNF-4) (32, 42). Complexes were observed with the APF1 and α1-AT sites that migrated at similar positions compared with the complexes observed with the GB element (Fig. 5A, lanes 1 and 5). In competition binding assays, a 50-fold molar excess of the APF1 site competed efficiently for complexes I, II, and III that formed with the GB/EFC-X probe. A 50-fold molar excess of the α1-AT site, however, did not compete for complex I and competed less efficiently for complexes II and III compared with the APF1 site (data not shown). From these results, the complexes that formed with the GB/EFC-X probe appear to be related, if not identical, to those that formed with the APF1 site (HNF-4 and COUP-TF binding site) and the α1-AT site (HNF-4 binding site).

To determine whether the HepG2-derived complexes that formed with the GB element contained HNF-4, COUP-TF, and RXRα, antisera directed against these nuclear proteins were used in mobility shift assays (Fig. 5). The addition of an antipeptide antibody directed against HNF-4 to these binding reactions specifically reduced the mobility of (supershifted) a majority of GB/EFC-X complexes II and III (Fig. 5A, lane 10). This antiserum also supershifted complexes that formed with the APF1 and α1-AT sites that migrated at a similar position with GB/EFC-X-associated complexes II and III (Fig. 5A, lanes 2 and 6). The addition of a COUP-TF-specific antiserum to DNA binding reactions containing the APF1, α1-AT, and GB/EFC-X sites resulted in the

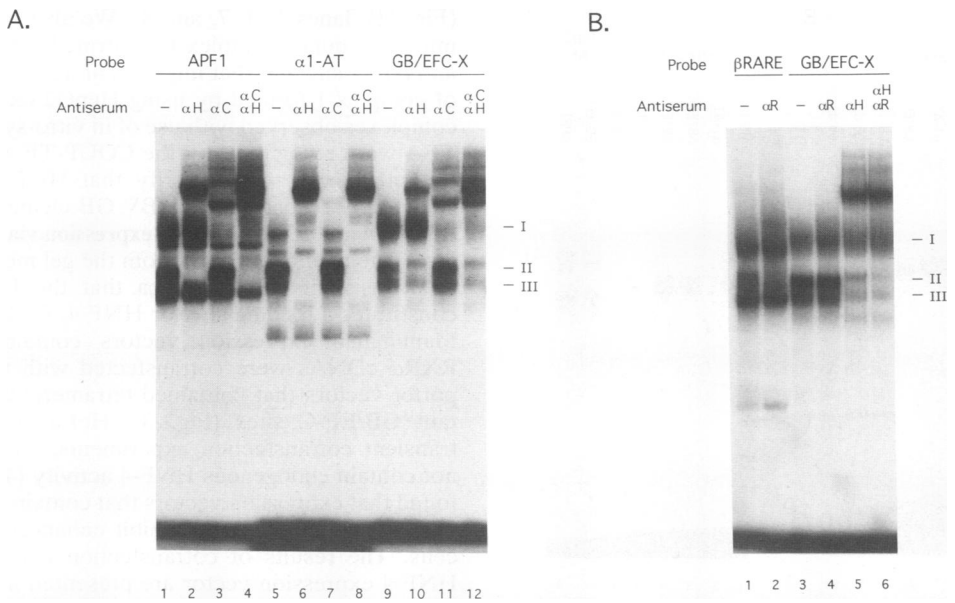


FIG. 5. Binding of HNF-4, COUP-TF, and RXR in HepG2 cell nuclear extract to the HBV GB element. (A) Binding of HNF-4 and COUP-TF to the GB element. Mobility shift assays were performed with nuclear extract prepared from HepG2 cells and the APF1 probe (lanes 1 to 4), the α1-AT probe (lanes 5 to 8), and the GB/EFC-X probe (lanes 9 to 12). Binding reaction mixtures incubated with the different probes minus the addition of antisera are shown in lanes 1, 5, and 9. Lanes 2, 6, and 10 are binding reactions that contained an HNF-4-specific antiserum (αH antiserum); lanes 3, 7, and 11 are binding reactions that contained a COUP-TF-specific antiserum (αC antiserum); lanes 4, 8, and 12 are binding reactions that contained both antisera. GB-specific complexes I, II, and III described in the text are indicated at the right. Complexes supershifted by the different antibodies are evident above complexes I, II, and III. (B) Binding of RXR to the GB element. Mobility shift assays were performed with nuclear extract prepared from HepG2 cells and a binding site probe for RAR and RXR (βRARE; lanes 1 and 2) (22, 31) and the GB/EFC-X probe (lanes 3 to 6). Binding reaction mixtures incubated with the different probes minus the addition of antiserum are shown in lanes 1 and 3. Lanes 2 and 4 are binding reactions that contained an RXR-specific antiserum (αR antiserum); lane 5 is a binding reaction that contained an HNF-4-specific antiserum (αH antiserum); lane 6 is a binding reaction that contained both antisera. GB-specific complexes I, II, and III are indicated at the right.

supershifting of the majority of complex I that formed with the APF1 and GB/EFC-X, and to a minor extent the α1-AT, probes (Fig. 5A, lanes 3, 7, and 11). In the presence of both HNF-4 and COUP-TF antisera, GB/EFC-X complexes I, II, and III were all supershifted, although it was evident that additional complexes that migrated at the position corresponding to complexes II and III were not recognized by these antibodies (Fig. 5A, lane 12). These complexes (termed II* and III*) were also evident when only the HNF-4 antiserum was added to the binding reaction with the GB/EFC-X probe (Fig. 5A, lane 10) and were still evident in antibody titration experiments when excess antibody was added to the binding reactions (data not shown). We conclude that nuclear proteins related to or identical to HNF-4 and COUP-TF bind specifically to the HBV GB element. Furthermore, there may be different proteins that recognize the GB element and result in the formation of complexes II* and III*.

A potential binding protein that could be associated with complex II* or III* is RXRα. To analyze this possibility, an RXRα peptide-specific antiserum was used in mobility shift assays (Fig. 5B). To visualize the effect that this antibody had on GB/EFC-X-specific complexes II* and III*, the HNF-4 antiserum was also included in the binding reaction to resolve the overlapping HNF-4-related complexes. The addition of both HNF-4- and RXRα-specific antisera to the DNA binding reaction resulted in a diminution in complex II*, and a faintly supershifted complex was observed (Fig. 5B, lane 5 versus lane 6). This antiserum had a similar effect on a complex that formed with a βRARE probe (that binds RAR/RXR [22, 31]) that migrated at the same relative position as did GB/EFC-X complex II* (Fig. 5B, lanes 1 versus lane 2). Therefore, it seems likely that RXRα is in complex II*.

Further verification that HNF-4 and COUP-TF specifically bind the GB element was demonstrated by using HNF-4 and COUP-TF-1 proteins synthesized in vitro. The in vitro-translated products were used for binding reactions containing the following radiolabeled probes: APF1, α1-AT, WT-GB/EF-C, GB/EFC-X, GB dpm2, and GB dpm3. The results of mobility shift assays are shown in Fig. 6. In vitro-synthesized HNF-4 bound to the WT-GB/EF-C and GB/EFC-X probes but not to the probes with point mutations in the GB element (GB dpm2 and GB dpm3; Fig. 6A, lanes 6 to 9). HNF-4 bound to the APF1 and α1-AT probes more efficiently than to the HBV GB site (Fig. 6A, lanes 4 and 5). The major HNF-4 complex that formed with the different probes migrated at approximately the same position as did complex II that formed with these probes in HepG2 cell nuclear extract (Fig. 6A, lanes 1 to 3). The observed DNA-protein complexes were supershifted with use of the HNF-4 antipeptide antiserum (data not shown). In vitro-synthesized COUP-TF-1 efficiently bound to the WT-GB/EF-C, GB/EFC-X, and APF1 probes and bound very poorly to the α1-AT site (Fig. 6B, lanes 1 to 4). In vitro-synthesized COUP-TF-1 did not bind to the GB dpm2 and GB dpm3 probe DNAs (Fig. 6B, lanes 5 and 6). The major COUP-TF-1 complexes that formed with in vitro-translated COUP-TF-1 and the different probes migrated at a position similar to that of complex II formed by using HepG2 cell nuclear extract

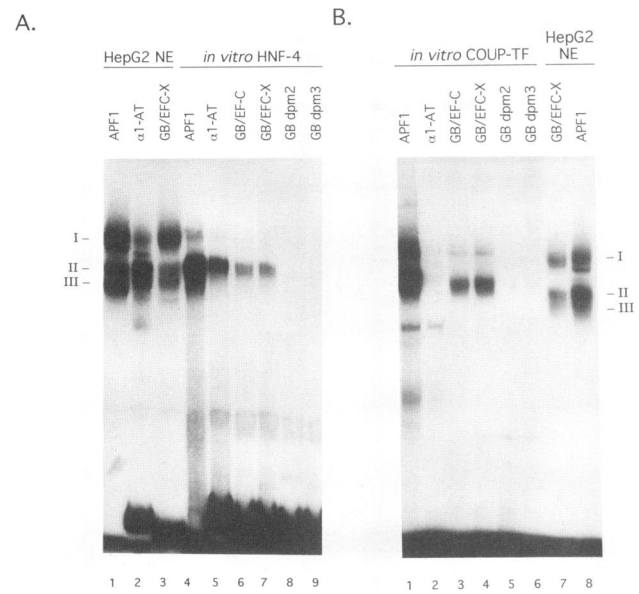


FIG. 6. Binding of in vitro-synthesized HNF-4 and COUP-TF to the HBV GB element. (A) Binding of HNF-4 to the GB element. HNF-4, produced by in vitro transcription and translation in a reticulocyte lysate, was used in binding reactions with the APF1 and α1-AT probes (lanes 4 and 5) and the wild-type and mutant GB/EF-C probes (GB/EF-C, GB/EFC-X, GB dpm2, and GB dpm3; lanes 6 to 9). Lanes 1 to 3 display binding reactions with the APF1, α1-AT, and GB/EFC-X probes in HepG2 cell nuclear extract (NE). Complexes I, II, and III are indicated on the left. (B) Binding of COUP-TF to the GB element. COUP-TF, produced by in vitro transcription and translation in a reticulocyte lysate, was used in binding reactions with the APF1 and α1-AT probes (lanes 1 and 2) and the wild-type and mutant GB/EF-C probes (GB/EF-C, GB/EFC-X, GB dpm2, and GB dpm3; lanes 3 to 6). Lanes 7 and 8 display binding reactions with the GB/EFC-X and APF1 probes in HepG2 cell nuclear extract. Complexes I, II, and III are indicated on the right.

(Fig. 6B, lanes 1, 3, 7, and 8). We also observed a slower-migrating minor complex that formed with the GB element and APF1 site and that migrated at a position similar to that of complex I formed by using HepG2 extract. Both of the complexes observed with use of in vitro-synthesized COUP-TF-1 were recognized by the COUP-TF antibody (data not shown). These results verify that HNF-4 and COUP-TF specifically recognize the HBV GB element.

HNF-4 and RXRα activate expression via the GB element in vivo. The results obtained from the gel mobility shift assays are consistent with the idea that the HBV GB element constitutes a binding site for HNF-4, COUP-TF, and RXR. Mammalian expression vectors containing HNF-4 and RXRα cDNAs were cotransfected with the E1A-CAT reporter vectors that contained tetrameric wild-type and mutant GB/EF-C sites (Fig. 3). HeLa cells were used in transient cotransfection experiments, since these cells do not contain endogenous HNF-4 activity (42). Moreover, we found that expression vectors that contain the WT-GB/EF-C oligonucleotides did not exhibit enhancer activity in HeLa cells. The results of cotransfection experiments with the HNF-4 expression vector are presented in Fig. 7A. HNF-4 stimulated expression of the WT-GB/EF-C vector ~6-fold relative the enhancerless parental vector, E1A-CAT-ENH$^-$. In contrast, no enhanced expression by HNF-4 was observed with vectors containing the mutant GB element or EF-C binding site (Fig. 7A, E1A+GB/EFC-X, E1A+GB dpm2, and E1A+GB dpm3). In all cases, cotransfections that were done with an expression vector that contains an HNF-4 antisense cDNA insert did not affect CAT expression (data not shown). We conclude that HNF-4 transactivates expression via the HBV GB/EF-C domain dependent on the integrity of both the GB element and EF-C binding site.

Similar cotransfection experiments were done in HeLa cells by using an RXRα expression vector in the absence and

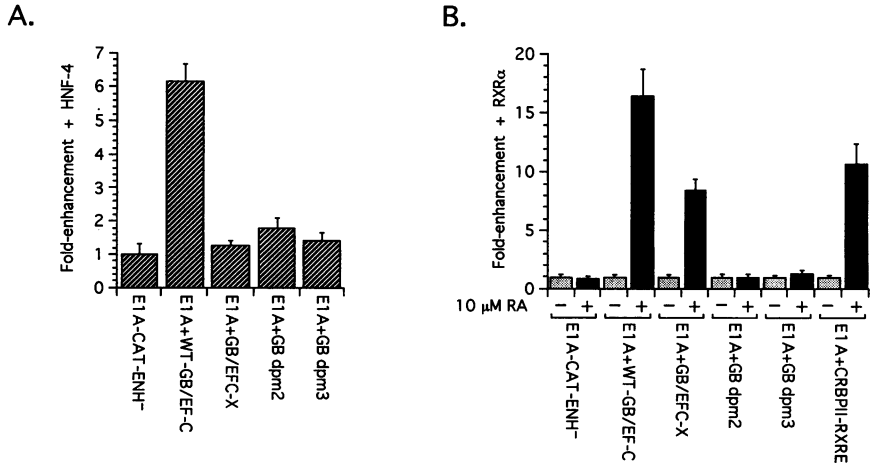


FIG. 7. Evidence that HNF-4 and RXRα transactivate the GB/EF-C domain in vivo. (A) Transactivation by HNF-4. E1A-CAT expression vectors containing wild-type and mutant GB/EF-C multimerized sites (Fig. 3) were cotransfected into HeLa cells with an HNF-4 expression vector. The level of CAT expression observed with the enhancerless vector, E1A-CAT-ENH$^-$, is set at the value of 1.0, and the relative levels of expression by the vectors carrying the different GB/EF-C sites are presented as fold induction relative to the level for the enhancerless vector. (B) Transactivation by RXRα. E1A-CAT expression vectors containing wild-type and mutant GB/EF-C multimerized sites or the CRBPII RXR response element (CRBPII RXRE) were cotransfected into HeLa cells with an RXRα expression vector. Following transfection, fresh medium was added to the cells that either lacked (−) or contained (+) 10 μM all-*trans* RA. The level of CAT expression observed with the enhancerless vector, E1A-CAT-ENH$^-$, with medium lacking RA is set at the value of 1.0, and the relative levels of expression by the vectors carrying the different GB/EF-C sites, without or with RA, are presented as the fold induction relative to the level for the enhancerless vector; standard deviations are indicated by error bars. The results represent the averages of four (A) and three (B) independent experiments.

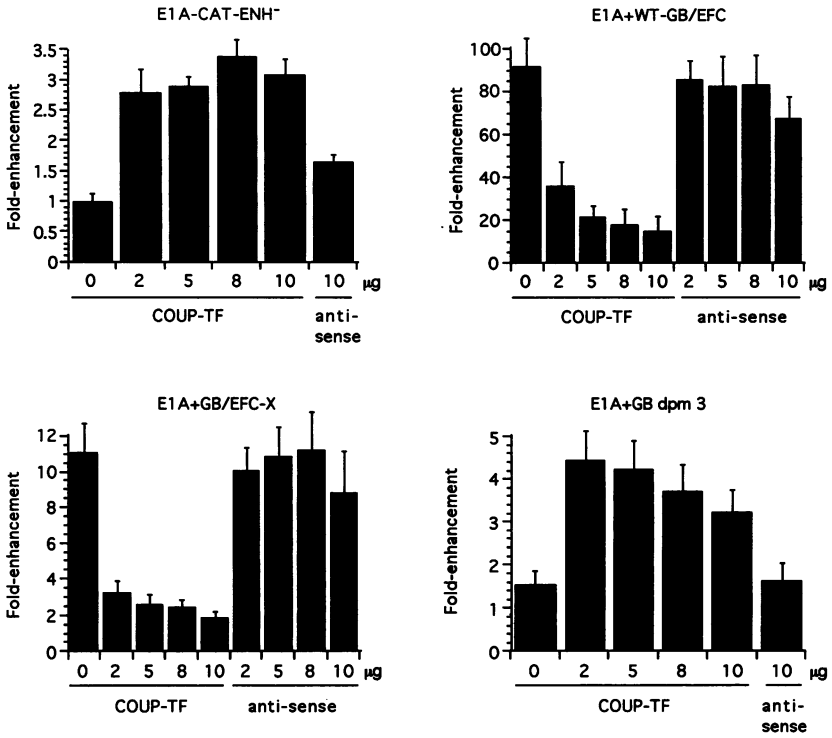


FIG. 8. Evidence that COUP-TF antagonizes the activity of the GB/EF-C domain in vivo. E1A-CAT expression vectors lacking an enhancer or containing wild-type or mutant multimerized GB/EF-C sites (Fig. 3) were cotransfected with increasing concentrations of a COUP-TF expression vector (0 to 10 µg) or the same expression vector but containing an HNF-4 antisense mRNA insertion (anti-sense). The level of CAT activity observed with the enhancerless vector, E1A-CAT-ENH⁻, without a cotransfecting plasmid (0 µg) is set at a value of 1.0, and the relative levels of expression by the vectors carrying the different GB/EF-C sites, without and with different amounts of a cotransfected expression plasmid, are presented as the fold induction relative to the level for the enhancerless vector; standard deviations are indicated by error bars. The results represent the averages of three independent experiments.

presence of 10 µM all-*trans* RA (Fig. 7B). The panel of E1A-CAT vectors all displayed a similar low level of expression in the presence of the RXRα expression vector but in the absence of RA (Fig. 7B, − columns). However, in the presence of the RXRα expression vector and RA, the E1A vectors containing the WT-GB/EF-C and GB/EFC-X oligonucleotides were induced ~16- and ~8-fold, respectively. Similarly, a CAT vector that contains the CRBPII RXR response element in the context of the E1A promoter was induced ~10-fold. The vectors containing mutations in the GB element (E1A+GB dpm2 and E1A+GB dpm3) were not induced by RXRα expression and RA. These results show that the GB element can function as an RXRα response element. Transcriptional activation by RXRα was quite evident, although less efficient, in the absence of the EF-C site.

COUP-TF antagonizes GB/EF-C-mediated activation in HepG2 cells. It has previously been reported that COUP-TF antagonizes transcriptional activation by HNF-4, RXRα, and other nuclear receptors that share binding sites with COUP-TF in transient expression assays (7, 21, 32, 45). Presumably COUP-TF competes for occupation of the binding sites recognized by the positive-acting factors. This raised the possibility that COUP-TF could similarly affect transcriptional activation mediated by the GB/EF-C domain. Therefore, a COUP-TF-1 expression vector was cotransfected with the wild-type and mutant E1A-CAT reporter vectors into HepG2 cells. The COUP-TF-1 expression vector was titrated with a fixed concentration of each E1A-CAT vector in the cotransfection assays. To control for any background effect of the expression vector in these assays, the homologous expression vector containing the antisense HNF-4 cDNA was used (Fig. 8). In the presence of 2 µg of the COUP-TF-1 expression vector, the enhancer activity of the WT-GB/EF-C domain was reduced 2.6-fold and showed decreasing activity with increasing amounts of the cotransfected COUP-TF-1 vector. Similar results were obtained with use of the E1A-CAT vector containing the multimeric GB/EFC-X oligonucleotide. Increasing amounts of the COUP-TF-1 expression vector marginally stimulated the activity of the parental vector E1A-CAT-ENH⁻ and the GB dpm3 mutant expression vector. With all of the E1A-CAT vectors, increasing amounts of an expression vector carrying the HNF-4 antisense cDNA did not substantially affect expression in comparison with the COUP-TF expression vector. We conclude that COUP-TF antagonizes the activity of the GB element in HepG2 cells.

DISCUSSION

We previously reported the binding of enhancer factor EF-C to a site in HBV enhancer I that is important for enhancer activity in vivo (36). A single EF-C binding site or multimerized EF-C sites, however, lack intrinsic enhancer activity (8) (Fig. 3C, GB dpm2 and dpm3), indicating that EF-C likely functions in combination with other distinct elements in the HBV enhancer. In this report, we have demonstrated that substantial enhancer activity is obtained

with a minimal enhancer fragment that contains the EF-C and HBLF binding sites and the GB element (Fig. 1). Furthermore, when an oligonucleotide containing only the GB element and EF-C binding site (GB/EF-C domain) was multimerized, significant enhancer activity was observed independent of adjacent HBV enhancer sequences (Fig. 3). Not only are the GB element and EF-C site critical for enhancer activity, these sites function synergistically in HepG2 cells. In addition, the EF-C binding site does not function with all protein binding sites in the HBV enhancer, since the EF-C site did not contribute to the activity of the NF-1a and NF-1b binding sites and the E domain (Fig. 1). The function of the EF-C binding site with the GB element and HBLF binding site is consistent with previously reported results demonstrating the enhancer activity of DNA fragments containing the EF-C site (FP III), the HBLF site (FP V), and the GB element (47). Our results are also consistent with the results of Huan and Siddiqui, who recently demonstrated that the region corresponding to the GB element is important for enhancer I activity and for responsiveness to RA (18). Although EF-C has been described as a ubiquitous nuclear protein (35), our results show that the GB/EF-C domain displayed enhancer activity in the human hepatoma cell line HepG2 but not in the nonhepatic cell lines analyzed (HeLa, CV-1, 293, and MOP-8). In a nonhepatic cell background, however, the transient expression of the liver-enriched protein HNF-4 restored the enhancer activity of the GB/EF-C domain via the GB element (Fig. 7A). This result demonstrates that the GB element accounts for the liver specificity of enhancer activity generated by the GB/EF-C domain. These results also suggest that the EF-C protein does not require any hepatic cell-specific modification for activity. Similarly, the EF-C binding site in the polyomavirus enhancer is an important component for enhancer activity in MOP-8 cells, indicating that EF-C of nonhepatic origin is functional (6). It is therefore likely that a significant portion of the activity of HBV enhancer I observed in HepG2 cells is the result of interaction of liver-specific nuclear factors with the GB element (and presumably the HBLF site) in conjunction with the ubiquitous nuclear protein EF-C. It is not clear how the EF-C site functions to stimulate the activity of HBV enhancer I. EF-C binds to its site in a very stable manner (half-life greater than 1 h; unpublished results); therefore, bound EF-C could stabilize the interaction of another enhancer factor to an adjacent recognition site. However, our data do not support the idea that EF-C cooperatively interacts with any of the proteins that bind to the GB element (unpublished data). Alternatively, the interaction of EF-C with an adjacent enhancer-binding protein may be required to form a functional transcriptional activation complex. Last, bound EF-C could exclude the binding of proteins that function as negative regulators of transcription.

The nucleotide sequence of the GB element is similar to sequences of the binding sites of HNF-4, COUP-TF, RXR, and other members of the steroid/thyroid nuclear receptor superfamily (Fig. 4) (25, 31, 42, 51). This sequence is highly conserved in the genomes of different human HBV subtypes, but unrelated sequences are found at the comparable positions in the ground squirrel hepatitis B virus (GSHV) and woodchuck hepatitis B virus (WHV) genomes (Fig. 9). In contrast, the adjacent EF-C site is conserved in the human as well as ground squirrel and woodchuck HBV genomes (Fig. 9), and EF-C binds to the GSHV sequence efficiently (36). This finding suggests that EF-C likely functions in conjunction with alternative enhancer elements in the context of the GSHV, and possibly WHV, enhancer regions. DNA-protein complexes that formed with the GB element in HepG2 cell nuclear extract (complexes I, II, and III; Fig. 5) were recognized by antisera directed against HNF-4, COUP-TF, and RXRα, indicating that all three proteins are present in HepG2 cells and are capable of binding to the GB element. In addition, in vitro-synthesized HNF-4 and COUP-TF-1 specifically bound to the GB element (Fig. 6). The mobility of the complex formed by in vitro-synthesized HNF-4 was similar to that of complex II formed with nuclear extract from HepG2 cells that was recognized by the anti-HNF-4 antiserum. In vitro-synthesized COUP-TF-1, however, formed a predominant complex that migrated with HepG2-GB complex II and only a minor complex that migrated with HepG2-GB complex I that was recognized by the anti-COUP-TF antiserum. Two classes of COUP-TF binding activities have been described in HeLa cell extracts. Lower- and higher-molecular-weight COUP-TF activities that form, respectively, faster (C1) and slower (C2) electrophoretic DNA-protein complexes have been identified (50, 51). The inconsistency in mobility of GB complexes formed by in vitro-synthesized COUP-TF-1- and COUP-TF-related complexes formed in HepG2 cell nuclear extract may, therefore, reflect these distinct COUP-TF activities. In vitro-synthesized COUP-TF-1 (the lower-molecular-weight activity) may form a faster-migrating complex, and COUP-TF in HepG2 cells may represent the higher-molecular-weight activity and form the slower-migrating complex at the position of complex I. Alternatively, COUP-TF associates with a second protein, termed S300-II (48), that could be present in HepG2 extracts to generate the slower electrophoretic complex I; only low levels of this activity may be present in reticulocyte lysates to account for the minor, slower-migrating COUP-GB complex formed with use of in vitro-synthesized COUP-TF-1.

Our data demonstrate that RXR is present in nuclear extracts prepared from HepG2 cells and binds to the GB element (Fig. 6B). However, we did not observe the binding of in vitro-synthesized RXRα to the GB element or to the CRBPII RXR response element in DNA binding reactions performed in the absence or presence of all-*trans* RA (data not shown). This was not surprising, since it was previously observed that in vitro-synthesized RXRα binds poorly to the

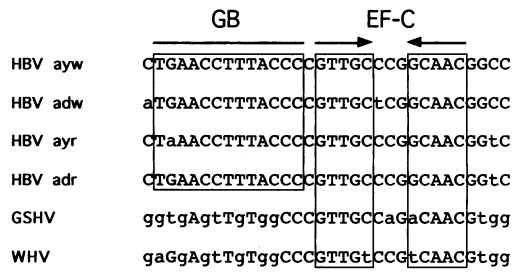


FIG. 9. Sequence comparison of the GB/EF-C domain among HBV genomes. The sequence of the GB element and EF-C binding site of HBV strain ayw is shown at the top. Shown below are the sequences present in the comparable positions in the genomes of HBV strains adw, ayr, and adr and in the GSHV and WHV genomes. The GB element and EF-C binding site are indicated. The boxes represent regions of sequence conservation between the different genomes. Uppercase letters correspond to sequences in the different viruses that are identical to the sequence in the HBV ayw genome; lowercase letters correspond to sequences that differ from the HBV ayw sequence.

CRBPII binding site (22, 60). Huan and Siddiqui, using high concentrations of bacterially expressed full-length RXRα, recently demonstrated that RXRα binds specifically to HBV enhancer I (to a site that corresponds to the GB element) (18). Recently, it was shown that the addition of 9-*cis* RA (a naturally occurring high-affinity ligand for RXRα [17, 27]) to a DNA binding reaction containing in vitro-synthesized RXRα induced the formation of RXRα homodimers and augmented the efficiency of binding to the CRBPII RXR binding site (60). We have not tested the effect of 9-*cis* RA on the binding of RXRα to the GB site.

The GB element and EF-C binding site function interdependently for enhancer I activity in HepG2 cells (Fig. 2 and 3). This functional synergy was also observed when HNF-4 was expressed in HeLa cells by using vectors containing multimeric wild-type and mutant GB/EF-C domains (Fig. 7A). In contrast, RXRα transactivation of the GB element in these assays was largely independent of the EF-C site (Fig. 7B). These results suggest that HNF-4 may represent the activity that is primarily responsible for HBV enhancer I function in HepG2 cells. It remains unknown which of the multiple activities that are capable of binding to the GB element normally function in the context of HBV enhancer I in infected liver. Northern (RNA) blot and in situ hybridization analyses demonstrated that HNF-4 and RXRα are expressed at higher levels in normal liver than in other tissues (29, 42). In addition to forming stable homodimers, RXRα heterodimerizes with RAR, the thyroid hormone receptor, the vitamin D receptor, the peroxisome proliferator activation receptor, COUP-TF-1, and ARP-1 (COUP-TF-2) (21–23, 26, 50, 54, 59). Our data do not distinguish whether RXRα homodimers or heterodimers are involved in transactivation of the HBV enhancer in vivo. Lastly, HepG2 cells contain a COUP-TF-related activity that may influence HBV enhancer I activity (Fig. 5). Overexpression of COUP-TF-1 in HepG2 cells antagonized GB/EF-C-mediated enhancer activity (Fig. 8). This observation is in accordance with previous studies demonstrating that COUP-TF antagonizes transcriptional stimulation by HNF-4, RXRα, and other nuclear receptors that share binding sites (7, 21, 32, 45, 54). The observed COUP-TF antagonism can be explained by invoking a previously proposed mechanism (32) in which COUP-TF competes with the positive-acting proteins(s) (e.g., HNF-4) for DNA binding.

A central question is how the binding of HNF-4, COUP-TF, or RXRα to the GB element enhancer I may affect the regulation of the various HBV promoters during the course of acute and chronic infection. Our findings indicate that multiple nuclear proteins binding to the GB element may function in both activation and repression of transcription. The fact that a diverse group of proteins bind to the GB element suggests that HBV enhancer I activity and thus viral gene expression may be influenced by different environmental stimuli. Indeed, our data demonstrate that the GB site functions as an RXRα response element whereby transcriptional stimulation may be controlled by RA. In addition, ligands for HNF-4 and COUP-TF have not been identified (33, 42, 51), which raises the possibility of alternative transcriptional effects induced by ligand signalling.

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