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Binding of Nuclear Factors to Functional Domains of the Duck Hepatitis B Virus Enhancer

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We have analyzed the structures, relative organization, and activities of binding sites for nuclear factors in the duck hepatitis B virus (duck HBV) enhancer. DNase I footprinting analysis and mobility shift assays demonstrate that this enhancer of 192 bp contains at least three binding sites for transcription factors: one for hepatocyte-adipocyte C/EBP, a second for the liver-specific transactivator hepatocyte nuclear factor 1 HNF-1, and a third for a factor, called F3, which binds to a DNA sequence bearing some resemblance to that for the ubiquitous factor EF-C. Analysis of transcriptional activity reveals that oligonucleotides corresponding to the individual binding sites, inserted upstream from a heterologous promoter, display very weak enhancer activity, whereas the enhancer encompassing these three sites displays very high activity. Analysis of duck HBV enhancer mutants indicates that the deletion of any of these sites leads to a modification of transcriptional enhancer activity. The hepatocyte nuclear factor 1 binding site is crucial, since an internal deletion of 14 bp abolishes the activity. The C/EBP site can act as repressor, and the F3 site is required for full activity. Comparative analysis reveals that the nuclear factors are similar to those bound to the human HBV enhancer but that the organization of their binding sites in the duck HBV enhancer is different.

Duck hepatitis B virus (DHBV) is the most divergent member of the hepadnavirus group with respect to structure and genetic organization of the genome (23) as well as biological properties. The encapsidated DHBV molecules are mostly fully double-stranded DNA, whereas they are partially single stranded for the mammalian hepadnaviruses. DHBV lacks the transactivator X gene and infects in vivo not only the liver but also the kidney, pancreas, and spleen (17), with very few pathological consequences (15). In general, the DHBV genome does not integrate into the cellular genome. These characteristics of DHBV contrast sharply with those of human hepatitis B virus (HBV). Comparative study of the organization and function of *cis*-regulatory sequences that govern the genetic expression of DHBV and HBV should provide an understanding of their respective biological properties.

In HBV, two enhancers, enhancer I and enhancer II, have been characterized. Enhancer I, located in the DNA polymerase gene between nucleotides (nt) 991 and 1243, is active in a wide variety of tissues (11, 33). Several known nuclear factors have been shown to bind to this enhancer. Among them are AP1/*jun-fos*; C/EBP, which binds to several binding sites (11, 27); nuclear factor 1 (NF-1), which binds to three sites (3); and EF-C, which binds to only one site (26). Enhancer I is also the transactivation target of the X gene product (20, 32). Enhancer II, located in the X gene between nt 1645 and 1803, was recently identified and is functionally liver specific (34, 36). It binds the C/EBP-like proteins at two sites whose cooperation is indispensable for enhancer functions in vitro (37). Other binding sites, for transactivating factors such as hepatocyte nuclear factor 1 (HNF-1), have been located upstream of the major surface antigen promoter region (8, 28).

We have previously described the identification of a strong enhancer element upstream from the pregenomic RNA start site of the DHBV genome (10). We present here an analysis of the structures, relative organization, and functions of the binding sites of the DHBV enhancer for C/EBP, HNF-1, and a factor called F3 which bears some resemblance to the binding site for EF-C of HBV. We also compare the genetic organization of the DHBV enhancer with that of the HBV enhancer.

MATERIALS AND METHODS

Plasmid constructions. Plasmid DH23-1 contains the *Alu*I-*Nco*I 192-bp fragment (nt 2159 to 2351) upstream the thymidine kinase promoter followed by a chloramphenicol acetyltransferase (CAT) gene and the simian virus 40 polyadenylation signal of pBLCAT2 (22). DH07, DH05, and DH03 are pBLCAT2 containing oligonucleotides inserted upstream from the thymidine kinase promoter. DH07 was constructed with oligonucleotide F1 (5′-TCGACCACATG GCGCAATATCCCATATCCCGCGGG-3′), DH05 was constructed with oligonucleotide F2 (5′-GATCCGTCGACTT TAGCCAAGATAATGATTAAACCGCGGG-3′), and DH03 was constructed with oligonucleotide F3 (5′-TCGACCCACGT TGTCTCTTATCTGATTCAACTTTTCCGCGGG-3′). Consensus oligonucleotides C/EBP (5′-ATTCAATTGGGCAATC AG-3′) (1), HNF1 (5′-TGTGGTTAATGATCTACAGTTA-3′) (30), and EF-C (5′-CCCCGTTGCCCGGCAACGGGC-3′) (26) and their complementary strands were synthesized for competition experiments.

Plasmid DH23.1 was cut at either the *Hin*dIII or *Bam*HI site and digested with exonuclease BAL 31 in 12 mM $CaCl_2$–12 mM $MgCl_2$–0.2 M NaCl–20 mM Tris HCl (pH 8.0)–1 mM EDTA for 30 s or for 1 min at 37°C. Repair reactions with the Klenow fragment of DNA polymerase I were then performed. The extent of each deletion was examined by nucleotide sequencing, using a standard method. Plasmids DH2211, DH2295, and DH2277 were derived from plasmid DH23-1 after internal deletions from nt 2159 to 2210 (DH2211), 2351 to 2296 (DH2295), and 2277 to 2290 (DH2277).

Nuclear extracts. Nuclear extracts were prepared as previ-

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ously described (6). Briefly, cells were washed in phosphate-buffered saline and resuspended in 1 ml of 10 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid HEPES; pH 8.0)–50 mM NaCl–0.5 M sucrose–1 mM EDTA (pH 8.0)–0.5 mM spermidine–0.15 mM spermine. Nonidet P-40 was added at a final concentration of 0.1% to release the nuclei. Nuclei were recovered by centrifugation at 3,000 × *g* for 10 min at 4°C. Nuclear proteins were extracted by incubation for 45 min at 4°C with gentle rocking in 10 mM HEPES–370 mM NaCl in the presence of protease inhibitors (pepstatin, leupeptin, aprotinin, antipain, and chymostatin [5 μg/ml each], phenylmethylsulfonyl fluoride [1 mM], and benzamidine [2 mM]). Proteins were precipitated with ammonium sulfate (0.3 g/ml) for 16 h at 4°C, centrifugated at 30,000 × *g* for 20 min at 4°C, and dialyzed against 20 mM HEPES–60 mM KCl–1 mM dithiothreitol (DTT)–20% glycerol with the above-mentioned protease inhibitors for 3 h at 4°C. Protein concentration was calculated by the Bradford method (Bio-Rad).

DNase I footprinting assays. Binding reactions were performed in 20 mM HEPES (pH 7.6)–150 mM NaCl–10 mM $MgCl_2$–10 mM spermidine–0.1 mM EDTA–2 mM DTT with 20,000 cpm of labeled [^{32}P]DNA, 1 μg of poly(dI-dC) · poly(dI-dC), and 60 μg of nuclear protein. After incubation in ice for 10 min, DNase I (2 μg to 200 ng) was added for 1 min at 20°C. The reaction was stopped by addition of an equal volume of 0.2 M NaCl–20 mM EDTA–1% sodium dodecyl sulfate solution. DNA was extracted with phenol-chloroform and chloroform, ethanol precipitated, and run on a 6% denaturing polyacrylamide gel. Dried gels were autoradiographed.

Mobility shift assay. The probes used for DNA-binding gel electrophoresis were derived from plasmids DH03, DH05, and DH07 by cutting the polylinker at the 5'-end *Hin*dIII site (10) and then end labeling with Klenow polymerase before cutting in the polylinker at the *Sac*II site. Fragments F1 (nt 2178 to 2200), F2 (nt 2271 to 2294), and F3 (nt 2294 to 2323), corresponding respectively to the C/EBP, HNF-1, and EF-C putative binding sites from the DHBV enhancer, were then gel purified. Nuclear extracts (5 μg of proteins) were incubated in 20 μl of binding buffer (7.5 mM HEPES [pH 8.0], 35 mM NaCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 1 mM DTT, 7.5% glycerol) containing 2 μg of poly(dI-dC) · poly(dI-dC). The ^{32}P-labeled probe (0.5 ng) was then added with various competitor DNA fragments (2.5 to 50 ng as indicated), and the mixture was incubated for 20 min at room temperature and then loaded directly into a 5% nondenaturing acrylamide gel (0.5× Tris-borate-EDTA, 10 V/cm) before drying and autoradiographic analysis.

Supershift assay. Nuclear extracts of rat liver (6 μg) and HNF-1 consensus site or anti-HNF-1 antibodies were incubated at room temperature for 10 min with 20 μl of a binding buffer consisting in 20 mM HEPES (pH 8), 20% glycerol, 150 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 4 mM spermine, 4 mM $MgCl_2$, and 1 μg of salmon sperm DNA with 1 μl of anti-HNF-1 antibodies or preimmune serum. The F2 oligonucleotide probe was added, and the mixture was incubated for 10 min at room temperature and then directly loaded onto a nondenaturing acrylamide gel.

Cell lines. HepG2 cells were grown in minimal essential medium (GIBCO-BRL) supplemented with 10% fetal calf serum (Flow Laboratories) and antibiotics (penicillin and streptomycin).

Transfection and CAT assays. The calcium phosphate coprecipitation technique was used for transfection of cells by the method of Chen and Okayama (9). Cells were seeded at 5 × 10^5 cells per 100-mm-diameter plate in minimal essential medium supplemented with 10% fetal calf serum. Ten micrograms of the indicated CAT construction was transfected with carrier DNA to a final quantity of 20 μg. After 12 h of incubation, cells were washed and incubated in minimal essential medium supplemented with 10% fetal calf serum for 48 h. Cells were then harvested and lysed in 250 mM Tris (pH 8.0) containing 0.05% sodium dodecyl sulfate; CAT assays were performed as described previously (10, 16). Briefly, one-fourth of the extract was incubated with 0.1 μCi of [^{14}C]chloramphenicol at 37°C for 45 min. The acetylated forms of [^{14}C]chloramphenicol were separated on a thin-layer chromatography plate before counting in a scintillation counter. CAT activities were calculated by expressing the values of acetylated forms as a percentage of the total radioactivity associated with the [^{14}C]chloramphenicol substrate. Each experiment was repeated at least three times.

RESULTS

Identification of three binding sites for nuclear factors from HepG2 cells in the DHBV enhancer. The DHBV enhancer has been previously mapped between nt 2159 and 2351 upstream from the pregenomic RNA start site of the DHBV genome (10). In hepatic cells, this 192-bp enhancer element potentiates a marked increased activity from the heterologous thymidine kinase promoter in an orientation-independent manner and at proximal as well as distal locations (10, 31). To better define the function of this enhancer, we searched for regulating transcriptional factors that would bind to the DHBV enhancer region by DNase I footprinting assays. For this purpose, crude nuclear extracts from HepG2 cells were incubated with the 330-bp *Alu*I-*Taq*I (nt 2159 to 2489) DNA fragment which contains the 192-bp enhancer element. This fragment was end labeled on either the coding or noncoding strand. Complexes were digested with DNase I, and the products were analyzed on a 6% acrylamide denaturing gel.

As shown in Fig. 1, three protected regions, F1 (nt 2185 to 2192), F2 (nt 2272 to 2294), and F3 (nt 2296 to 2321), were observed. F1 and F2 were detected on the coding strand, and F3 was detected on the noncoding strand.

Computer analysis of the nucleotide sequence of each protected region reveals that the F1 site contains a sequence, 5'-TGGCGCAAT-3', perfectly matching the C/EBP (2) consensus site T(T/G)NNGC(A/C/G)A(T/G) (Fig. 2). The F2 site (5'-AGATAATGATTAAAC-3') matches the HNF-1 (29) consensus site (G/A)GTTAATNATTAAC(C/A) except for the nt 3 and 14, where adenosine is in place of a thymidine and a cytosine, respectively. The F3 site bears only some resemblance to the EF-C binding site, harboring two GTTG and CAAC inverted repeats but with a spacing of 14 bp instead of 5 bp in the C element of polyomavirus or HBV (26).

From these experiments, we conclude that the DHBV enhancer contains at least three binding sites for HepG2 nuclear factors: F1, related to C/EBP; F2, related to HNF-1; and F3, related to a factor which binds to a DNA sequence bearing some resemblance to that for EF-C.

Gel shift analysis of DNA fragments containing the F1, F2, and F3 sites. Mobility gel shift assays were performed to study the specificity and nature of the binding activity to sites F1, F2, and F3. For this purpose, double-stranded oligonucleotides, corresponding to the F1, F2, and F3 protected regions observed by the footprinting assays, were synthesized. Oligonucleotides F1, F2, and F3 were end

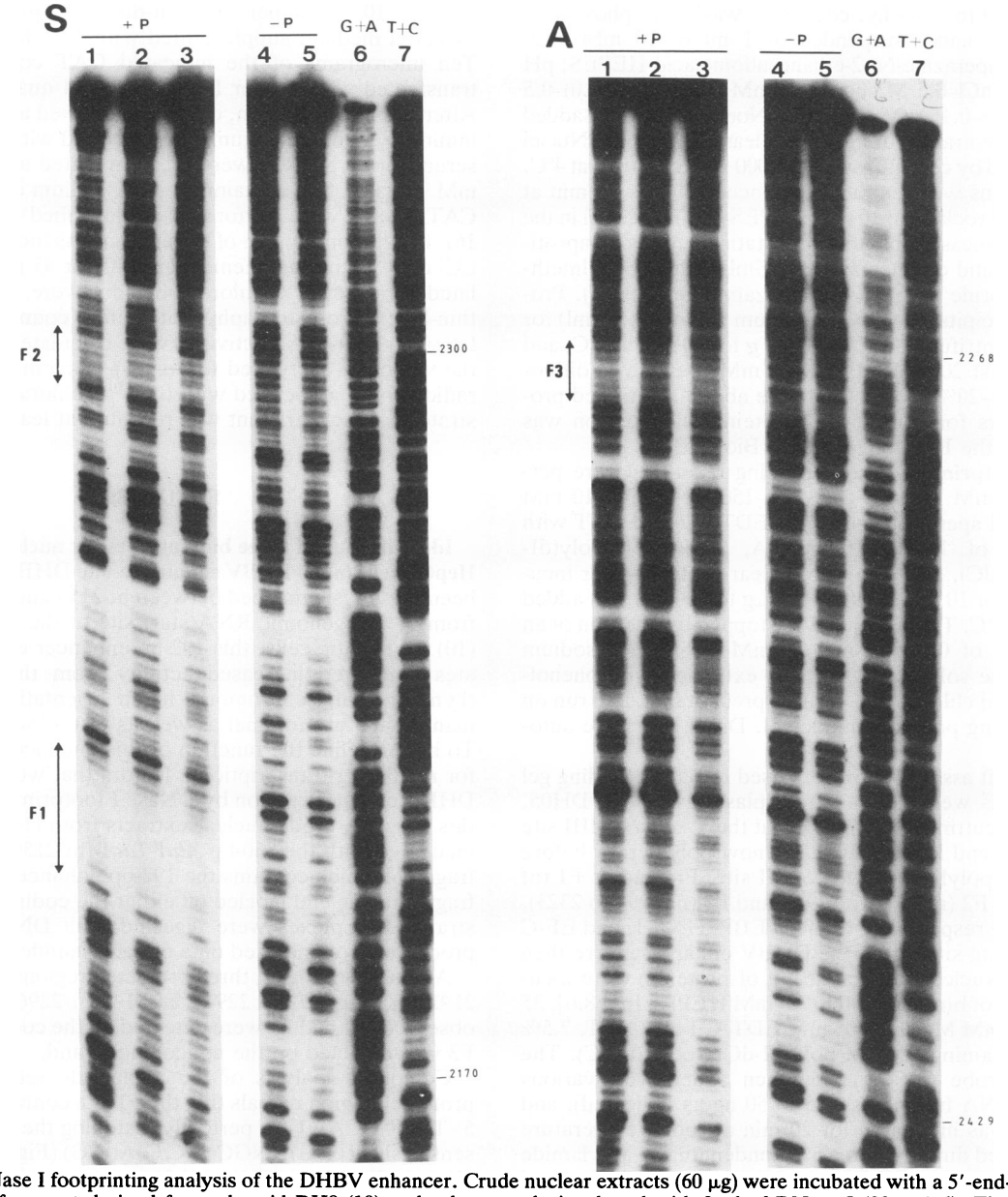

FIG. 1. DNase I footprinting analysis of the DHBV enhancer. Crude nuclear extracts (60 μg) were incubated with a 5'-end-labeled 330-bp *Hin*dIII-*Bgl*II fragment derived from plasmid DH8 (10) and subsequently incubated with 2 μl of DNase I (20 μg/ml). The samples were analyzed by 5% polyacrylamide–urea gel electrophoresis together with a Maxam-Gilbert sequencing reaction (lanes G+A and T+C). The most obvious protected sites (F1, F2, and F3) are marked. S, sense strand; A, antisense strand.

labeled, incubated with HepG2 nuclear extracts in the presence or absence of cold (unlabeled) competitor DNA, and run on a nondenaturing 5% acrylamide gel.

Typical results of autoradiography are shown in Fig. 3. Each oligonucleotide derived from the F1, F2, or F3 site shows several complexes. With the F1 probe, two complexes with equal intensity are detected (Figure 3A, lane 2). With the F2 probe, two complexes are detected; one is strong and slow migrating, the other is slight and fast migrating (Fig. 3B, lane 2). With the F3 probe, three complexes are detected (Fig. 3C, lane 2).

To test binding specificity, competition experiments using 5- to 100-fold molar excesses of cold competitors were performed.

As shown in Fig. 3A, the two complexes observed with the F1 probe are dissociated equally well when the cold F1 competitor is added. With the F2 probe, only the slow-migrating, strong complex is displaced when the cold F2 competitor is added. It appears that the faster-migrating complex is not relevant to the DHBV enhancer, since this DNA sequence does not inhibit the formation of the faster-migrating F2 complex. Each complex observed with the F3 probe is competed for when the cold F3 competitor is added. The slow-migrating complex (open arrow in Fig. 3C) is less sensitive to the competitor. The F1 probe is competed against with the cold F1 but not the cold F2 or F3 competitor (Fig. 3A); the F2 probe is competed against with the cold F2 but not the F1 or F3 competitor (Fig. 3B); the F3 probe is

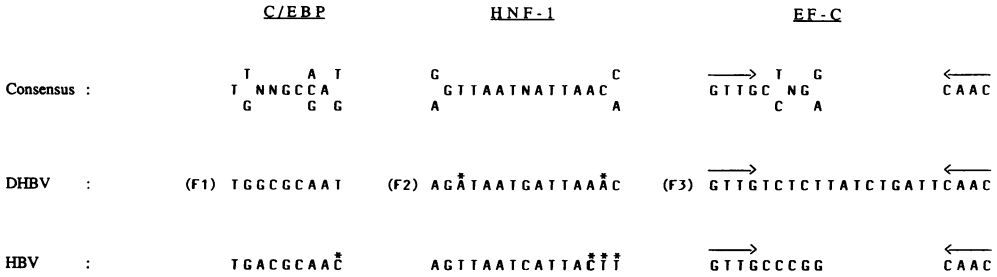

FIG. 2. Comparison of nucleotide sequences between the binding sites of nuclear factors in the DHBV and HBV genomes. Astericks indicate nucleotides differences with the consensus sequences; arrows indicate inverted repeats.

competed against with the cold F3 but not the F1 or F2 competitor (Fig. 3C).

We conclude from these experiments that the binding of HepG2 nuclear factors to the protected regions F1, F2, and F3 is specific.

To verify that the nuclear factors bound to each individually protected region also bind to the whole enhancer, we performed competition experiments using either the F1, F2, or F3 labeled probe and the 192-bp enhancer fragment (nt 2159 to 2351) as the cold competitor. Figure 4 shows that each binding site is competed for by the 192-bp enhancer fragment at a 20- to 100-fold molar excess. These results indicate that each binding site is fully operational in the whole enhancer.

Examination of each of the protected regions reveals that the F1 site contains a sequence matching the C/EBP consensus site. The F2 site matches the HNF-1 binding site from the rat albumin proximal element, and the F3 site shares homology only with the two GTTG and CAAC inverted repeats of the EF-C binding site. To identify formally each of these putative binding sites and to compare them with related sites in the HBV genome, we used mobility shift assays with competitor oligonucleotides derived from the consensus site for C/EBP as described by Bakker and Parker (2) and from the well-studied rat albumin proximal element for HNF-1 (30) and from EF-C (nt 1147 to 1170) (26).

As shown in Fig. 4A the C/EBP oligonucleotide at a 100-fold molar excess competes, although not completely, for HepG2 nuclear factors with the F1-derived labeled oligonucleotide (compare lane 2 with lanes 7 to 9). The heat stability of the factors involved in the two complexes is evident (Fig. 4A, lane 3), although treatment at 95°C for 15 min slightly decreases the binding capacity of the protein. This observation is a good indication of the presence of C/EBP (7, 37). However, it is possible that a protein other than C/EBP can bind to this site, since there are several transcription factors which can recognize the C/EBP binding site.

The HNF-1 oligonucleotide from the rat albumin proximal element completely competes, even at a fivefold molar

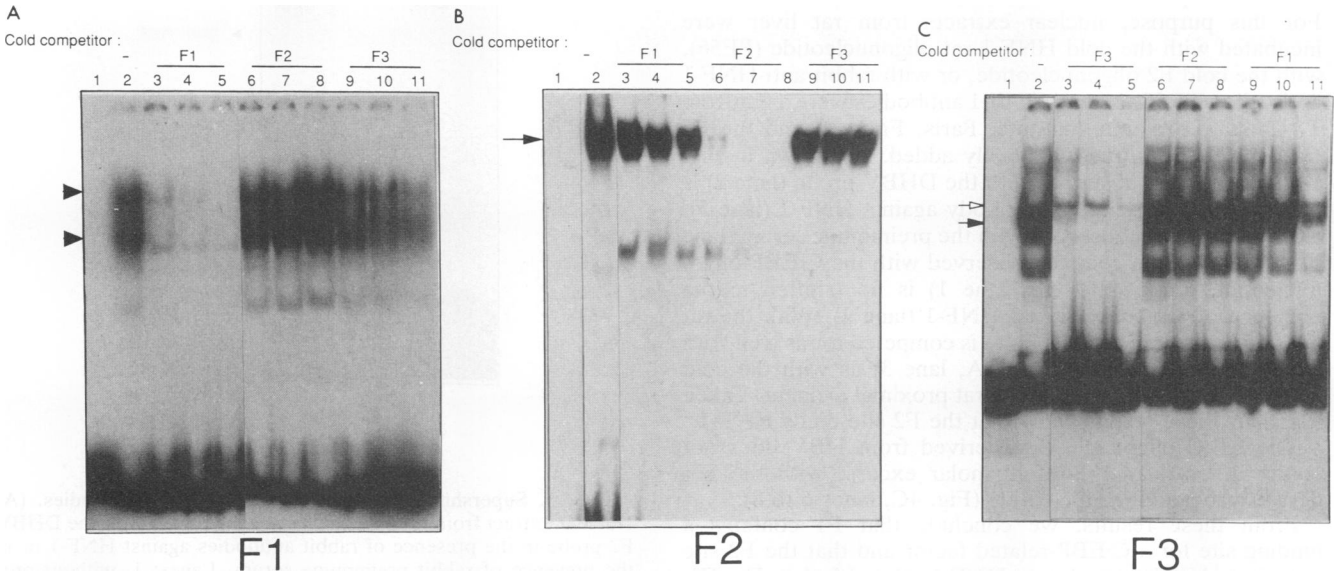

FIG. 3. Binding of nuclear extracts to oligonucleotides encompassing regions F1, F2, and F3. HepG2 nuclear extracts (5 μg) were incubated at room temperature with 0.5 ng of oligonucleotide F1, F2, or F3 (for nucleotide sequences, see Materials and Methods). Increasing amounts of cold competing oligonucleotides were added to the reaction mixtures as follows: lanes 3, 6, and 9, 5-fold molar excess; lanes 4, 7, and 10, 25-fold molar excess; lanes 5, 8, and 11, 100-fold molar excess. Lanes 1, without proteins; lanes 2, with proteins and without competitors (−). Arrowheads and arrows point to the specific strong complexes. Note that all of these complexes are already displaced with a fivefold molar excess of their own cold competitors. Samples were submitted to electrophoresis in a 5% nondenaturing acrylamide gel; after drying, the gel was autoradiographed.

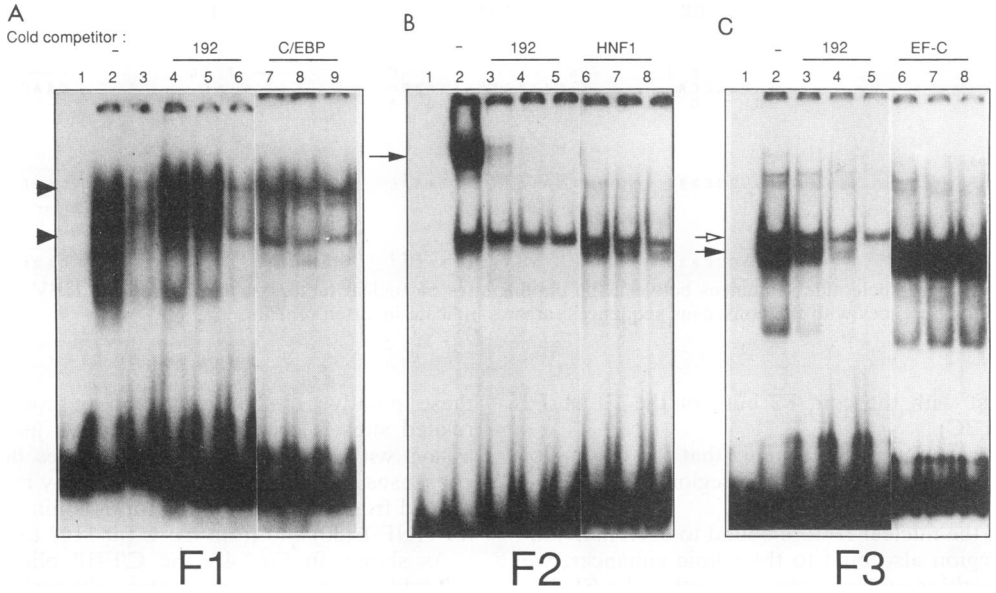

FIG. 4. Competition analysis of the factors bound to the F1, F2, and F3 sites by gel shift experiments. HepG2 nuclear extracts (5 μg) were incubated at room temperature with 0.5 ng of oligonucleotide F1, F2, or F3. Assays used increasing amounts of cold competitor DNA molecules corresponding to the 192-nt *Alu*I-*Nco*I DHBV enhancer fragment (192) or to oligonucleotides encompassing the consensus sequence for the C/EBP binding site (C/EBP), the HNF-1 binding site in the rat albumin promoter (HNF1), and the EF-C binding site in HBV enhancer I (EF-C). (A) Lanes: 1, without protein; 2, with proteins and without competitors (−); 3, nuclear extracts treated for 15 min at 95°C before incubation without competitors; 4 and 7, 5-fold molar excess; 5 and 8, 25-fold molar excess; 6 and 9, 100-fold molar excess. (B and C) Lanes: 1, without proteins; 2, with proteins and without competitor (−); 3 and 6, 5-fold molar excess; 4 and 7, 25-fold molar excess; 5 and 8, 100-fold molar excess.

excess, with the F2-derived labeled oligonucleotide (Fig. 4B, lanes 6 to 8). Since the F2 factor is a crucial element for DHBV enhancer function (see below), supershifting experiments were performed to formally identified F2 as HNF-1. For this purpose, nuclear extracts from rat liver were incubated with the cold HNF-1 rat oligonucleotide (PE56), with the cold F2 oligonucleotide, or with rabbit anti-HNF-1 antibodies (PE56 and anti-HNF-1 antibodies were a gift from T. Chouard, Pasteur Institute, Paris, France), and the F2 oligonucleotide probe was finally added. As shown in Fig. 5A, the complex observed with the DHBV probe (lane 2) is shifted in the presence of antibody against HNF-1 (lane 5). Such a shift is not observed with the preimmune serum (lane 6). Moreover, the complex observed with the C/EBP oligonucleotide probe (Fig. 5B, lane 1) is not shifted in the presence of antibody against HNF-1 (lane 2). With the rat liver nuclear extract, the F2 site is competed for as well with the cold F2 competitor (Fig. 5A, lane 3) as with the cold HNF-1 consensus site from the rat proximal element. Taken together, these results show that the F2 site binds HNF-1.

The EF-C oligonucleotide derived from HBV does not compete, even at a 100-fold molar excess, with the F3-derived labeled oligonucleotide (Fig. 4C, lanes 6 to 8).

From these results, we conclude that F1 contains a binding site for a C/EBP-related factor and that the F2 site contains a binding site for an HNF-1-related factor. For F3, we observed a complete absence of competition of the F3 site with the EF-C oligonucleotides; therefore, although the DNA sequence bears some resemblance to that of EF-C, it is unlikely that F3 is related to EF-C. It must be noted that the F3 probe is competed against with the F3 cold competitor as well as with the cold 192-bp DHBV enhancer fragment.

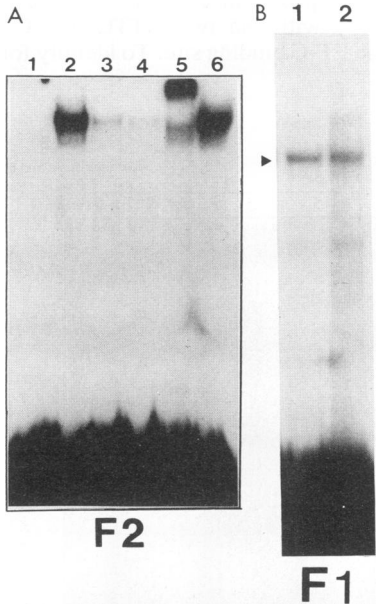

FIG. 5. Supershifting assays using anti-HNF-1 antibodies. (A) Nuclear extract from rat liver (6 μg) was incubated with the DHBV F2 probe in the presence of rabbit antibodies against HNF-1 or in the presence of rabbit preimmune serum. Lanes: 1, without proteins; 2, with proteins; 3, with proteins and a 20-fold molar excess of cold F2 competitor; 4, with a 20-fold molar excess of cold PE57 rat oligonucleotide competitor; 5, with 1 μl of rabbit antibodies against HNF-1; 6, with 1 μl of rabbit preimmune serum. (B) Nuclear extract from rat liver (6 μg) was incubated with the DHBV F1 probe. Lanes: 1, with proteins; 2, with proteins and 1 μl of rabbit antibodies against HNF-1.

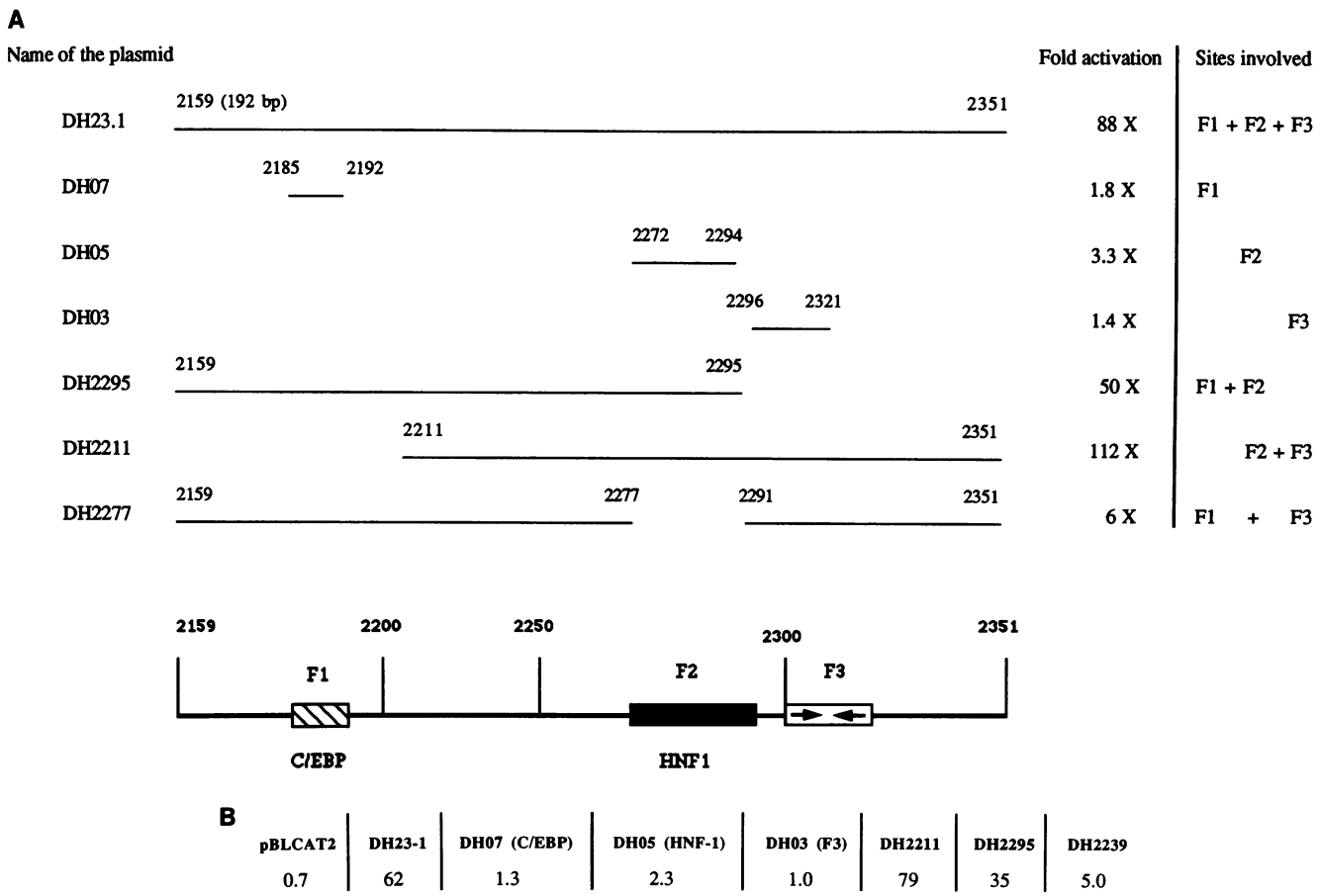

FIG. 6. (A) Contribution of each binding site for transactivating factors to the activity of the DHBV enhancer. The drawing is a schematic representation of the structures and activities of deletion mutants of the DHBV enhancer. The 192-bp DHBV enhancer was digested with exonuclease BAL 31. DNA fragments obtained were subcloned into pBLCAT2 vectors after the ends were made blunt. Activities of the resulting clones were analyzed by transfection into HepG2 cells. The fold increase or decrease in each construct's activity compared with that of the 192-bp DHBV enhancer is indicated. Corresponding binding sites for nuclear factors are indicated at the bottom. Arrows indicate inverted repeats. Fold activation corresponds to the CAT activity of a plasmid divided by the CAT activity of the 192-bp enhancer. The percentage of CAT activity of each plasmid is shown in panel B. (B) CAT activities of C/EBP, HNF-1, and F3 DNA sites and of deletion products of the enhancer. HepG2 cells were transfected with different constructs for 16 h and then allowed to express the CAT gene for another 24 h. CAT activity was measured as described in Materials and Methods. DH23-1 contains the 192-bp enhancer. DH07, DH05, and DH03 contain the C/EBP, HNF-1, and F3 binding sites, respectively. The clone number indicates the deletions in the 5′ end for DH2211 and in the 3′ end for DH2295 and a central deletion for DH2277. See panel A for a physical map. Results are expressed as percent acetylation of [^{14}C]chloramphenicol.

Transcriptional activity of the C/EBP, HNF-1, and F3 binding sites. Each of the oligonucleotides F1, F2, and F3 may constitute an enhanson (12, 14). To correlate the binding of these nuclear factors with DHBV enhancer function, we tested the enhancing activity of each of these three binding sites. For this purpose, each oligonucleotides was cloned in the sense orientation in the polylinker of plasmid pBLCAT2. Plasmids DH07, DH05, and DH03 contain a C/EBP-related binding site (F1), an HNF-1-related binding site (F2), and binding site F3, respectively. These clones were used to transfect the human hepatoblastoma cell line HepG2, and the amount of CAT activity found in extracts of these cells was determined (Fig. 6). For the three clones, very low activity is detected. In sharp contrast, the entire DHBV enhancer element of 192 bp (plasmid DH23-1) shows an 88-fold-higher activity. This result indicates that each factor exhibits a poor transactivating efficiency.

Activity of DHBV enhancer mutants with deletions in the C/EBP, HNF-1, and F3 binding sites. To test whether there could be a synergy between these factors that would account for the very high activity of the DHBV enhancer, in contrast with the weak activity of each individual binding site, we deleted each binding site of the 192-bp enhancer. For this purpose, deletions at chosen sites, using exonuclease BAL 31, were performed. The extent of each deletion was examined by sequence analysis. Among the deletion mutants, we retained three that were interesting with respect to deletion of the F1, F2, or F3 binding site (Fig. 6A). The C/EBP, HNF-1, and F3 binding sites were deleted in mutants DH2211, DH2277, and DH2295, respectively.

The activities of these deletion mutants are shown in Fig. 6. In HepG2 cells, the activities of plasmids DH2211, DH2277, and DH2295 varied and were quite different from the activity of the wild-type enhancer (plasmid DH23-1). The enhancer activity was increased (from 62 to 79%) when the C/EBP binding site was deleted (plasmid DH2211) and

decreased from 62 to 35% when the F3 binding site was deleted (plasmid DH2295). The main difference concerns DH2277, which has an internal 14-bp deletion of the HNF-1 binding site and in which the activity was almost completely abolished (from 62 to 5%).

From these experiments, we conclude that the HNF-1 binding site is crucial for the enhancer activity and that sequences including the C/EBP and F3 sites modulate the activity. The C/EBP site seems to act as an inhibitory element, and the F3 site is required for full activity.

DISCUSSION

We analyzed the fine structure of the enhancer upstream from the pregenomic RNA start site of the DHBV genome and demonstrated that this strong enhancer of 192 bp contains at least three binding sites, one for C/EBP, a second for HNF-1, and a third for F3, a factor which bears some resemblance to EF-C.

C/EBP binds to enhancer sequences of several viral enhancers and CAAT sequences (18). It is involved in transcriptional control of the albumin gene in hepatocytes and of adipocyte-specific genes (5). The specific consensus sequence T(T/G)NNGC(A/C/G)A(T/G) for C/EBP is also recognized by several proteins, such as IL-6-DBP, Ig/EBP-1, and NF-IL-6. These factors belong to the same multigenic family, since their basic and leucine zipper regions are very homologous (1, 19). C/EBP can form a dimer with any of these factors or with the recently discovered proteins CRP1 and CRP3 (35). The combination of these factors may result in the formation of transcriptional complexes with different properties as suggested for C/EBP-β and C/EBP-δ, which are differentially expressed during adipocyte differentiation (5).

In HBV, C/EBP binds to two sites (nt 1180 to 1204) in enhancer I and can function as a transcriptional activator at low concentrations and as a repressor at high concentrations. This binding seems to proceed in a hierarchic and cooperative manner and in some circumstances might involve other sites within enhancer I (11). More recently, it was shown that enhancer II contains nucleotide sequences, box α and box β, related to the C/EBP binding site (37).

In the DHBV enhancer, we found by DNase I footprinting assays that the protected region F1 is 100% homologous to the C/EBP binding site. A synthesized oligonucleotide corresponding to this C/EBP sequence binds specifically to nuclear extracts of the HepG2 cells, and two DNA-protein complexes are observed in mobility shift assays (Fig. 3 and 4). The heat stability of the factors involved in the two complexes is high. Figure 4A, lane 3, shows two DNA-protein complexes, observed after treatment at 95°C for 15 min (7, 37). Given the fact that the F1 site is 100% homologous to the C/EBP consensus site and that the C/EBP oligonucleotide competes, although not completely, with F1, the recognized site on F1 likely binds C/EBP. The presence of two bands in mobility shift assays could involve oligomerization of either the same factor or two factors bound to the DHBV C/EBP binding site. It is possible that the binding factors for the two related sites are slightly different. It must be noted that the sequence from HBV does not match exactly the C/EBP consensus sequence [5′-T(T/G)NNGC(A/C/G)A(T/G)-3′], unlike the sequence from DHBV. These factors have an inhibitory effect on the enhancer, since deletion of the C/EBP site results in an increased activity in HepG2 cells (Fig. 6).

HNF-1 is a liver-specific transactivating factor belonging to the class of homeoproteins that have a dimerization domain and a subregion homologous to the POU domain present in Pit 1/GHF, Oct 1, and Oct 2 factors (30). HNF-1 was described as a DNA-binding protein in rat, human, and murine liver. It interacts with enhancer elements of genes, such as those encoding albumin, α- and β-fibrinogen, α1-antitrypsin, α-fetoprotein, pyruvate kinase, transerythrin, and aldolase B, the expression of which is confined to the liver (4). For most of these genes, the HNF-1 binding sites are essential to ensure liver-specific transcription. However, recent experiments showed that even if HNF-1 is necessary, it cannot by itself force the high level of expression of genes in nonhepatic tissues such as kidney, where it is also present (30). Therefore, it seems that the regulation of genes controlled by HNF-1 must involve other factors.

In DHBV, the HNF-1 binding site (5′-AGATAATGAT TAAAC-3′) matches the HNF-1 consensus site [(G/A)GT TAATNATTAAC(C/A)]. Competition experiments revealed that the HNF-1 oligonucleotide from the prototype rat albumin proximal element completely competes, even at a fivefold molar excess, with the DHBV HNF-1 binding site. Moreover, supershift experiments using rabbit antibodies against HNF-1 strongly suggest that HNF-1 indeed binds to the F2 site in the DHBV enhancer. An internal deletion of 14 bp in the HNF-1 site of the DHBV enhancer abolishes almost completely the transcriptional activity. However, the HNF-1 binding site has a very weak transcriptional activity. This finding suggests that the presence of HNF-1 alone cannot influence the basic intrinsic very low activity of the thymidine kinase promoter and that HNF-1 needs other factors to be fully operational and ensure the very high activity of the DHBV enhancer.

EF-C is a ubiquitous factor present in nuclear extracts of murine and human cells. It binds to the C element of the polyomavirus enhancer and to HBV enhancer I (25, 26). The binding site contains two inverted repeats (GTTG and CAAC) separated by five nucleotides. Mutagenesis studies indicate that EF-C is probably stabilized by dimerization (26). The F3 binding site of DHBV shares homology with the EF-C consensus sequence, harboring the two inverted repeats but with a different spacing. This spacing has been shown to be essential for HBV enhancer function. Competition experiments in mobility shift assays indicate that the F3 site is competed for completely with a whole DHBV enhancer fragment but not at all with the EF-C of the HBV enhancer. Ostapchuck et al. (26) have shown that the HBV EF-C sequences do not compete for the HBV enhancer I after insertion of nucleotides between the two inverted repeats. Therefore, it is possible that another factor binds to the DHBV F3 binding site. Indeed, in the case of hormone receptors, it has been reported that the same sequence of inverted repeats binds different factors according to the length of the spacing (24). Deletion of the F3 site of the DHBV enhancer leads to a decrease in activity of 50%. As for the C/EBP and HNF-1 sites, the F3 site has a very weak transcriptional activity.

The DHBV enhancer and HBV enhancer I are located in the coding region of the DNA polymerase gene. At least two and perhaps three binding sites, which we describe here for the DHBV enhancer, have been described for HBV. The main difference between DHBV and HBV concerns the number of binding sites and their organization (Fig. 7).

Concerning the number of binding sites in the DHBV enhancer, we found a single binding site for each nuclear factor. The C/EBP and HNF-1 sites are on the same coding strand, and the F3 site is located on the noncoding strand. In

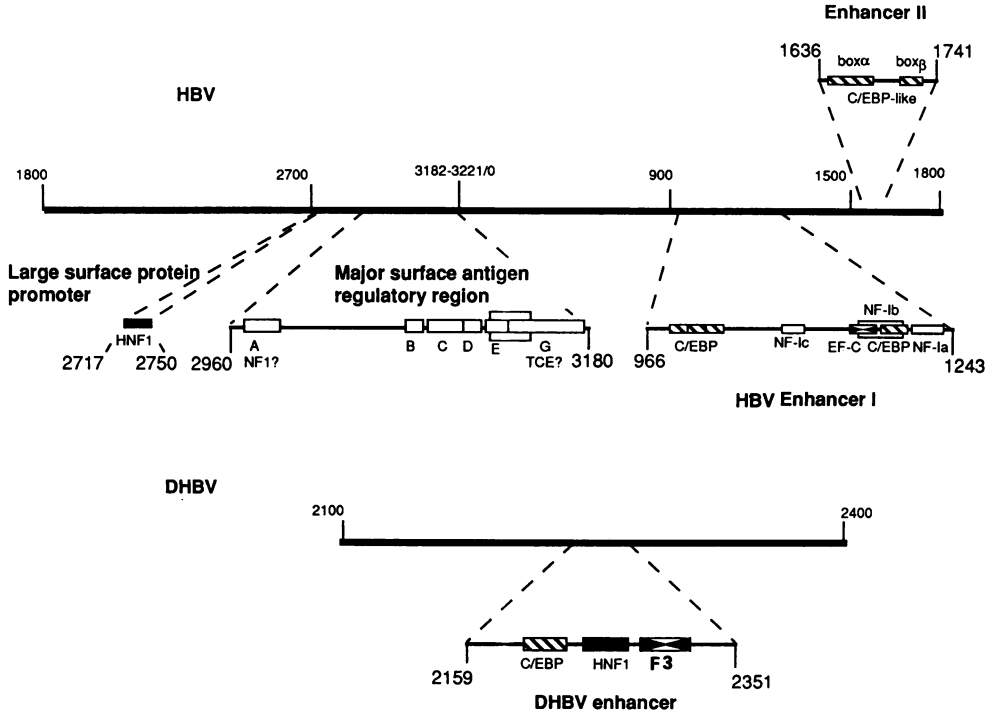

FIG. 7. Organization of nuclear factor binding sites in the DHBV and HBV genomes. Heavy lines represent the genomes and lengths in nucleotides. Regions are depicted according the indicated references as follows: HBV enhancer I (11, 26), enhancer II (36), the large surface protein promoter (8, 28), and the major surface antigen regulatory region (29).

the HBV enhancer I, at least two binding sites for C/EBP, three for NF-1, and one for EF-C have been described. It must be noted that the HNF-1 binding site for HBV is located upstream of the S promoter and therefore very distant (≈1,000 nt) from enhancer I, whereas it is located within the enhancer between the C/EBP and F3 binding sites for DHBV.

It has been reported for HBV that C/EBP can bind to multiple sites and acts as an activator at low concentrations or as a repressor at high concentrations (11). In this study, we have not tested the concentration effect of C/EBP and therefore cannot exclude the presence of other C/EBP binding sites which can counterbalance or reverse the activity of the DHBV enhancer. Furthermore, we cannot exclude the binding of other factors involving specific experimental conditions for their detection.

Concerning the organization of the binding sites, in DHBV, the C/EBP site is located at the 5′ end of the enhancer between nt 2185 and 2192, HNF-1 site occupies the central region at nt 2272 to 2294 and, the F3 site is located at nt 2296 to 2321 in the 3′ end. Thus, these three sites in DHBV do not overlap. In HBV, one C/EBP site is located between the EF-C and NF-1a sites, and the NF-1b site overlaps the C/EBP and EF-C sites. Figure 7 summarizes this organization.

In conclusion, we have demonstrated that the DHBV enhancer contains at least three binding sites for nuclear factors: one for C/EBP, a second for HNF-1, and a third for a factor called F3. The deletion of any of these sites leads to a decrease in transcriptional enhancer activity. The mechanism of activation seems to occur via a synergy between HNF-1, which plays a central role, and F3 and C/EBP. The F3 site modulates positively, since it is required for full activity, and the C/EBP site modulates negatively, as its deletion increases transcriptional activity. The factors that bind to the HBV and DHBV sites are similar, but the HBV and DHBV enhancers differ in genetic organization. This difference may contribute to the differential genetic expression and biological properties of HBV and DHBV.

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