The hepatitis B virus enhancer 1 element plays a fundamental role in the liver-specific regulation of hepatitis B virus gene expression. A central region of enhancer 1, the enhancer core domain, contains at least four conserved sequence motifs that are essential for enhancer 1 activity. In this study, we have investigated an essential motif within the core domain previously defined as footprint V (FPV). Transient transfection analyses demonstrate that FPV is capable of independently functioning in a liver-specific manner to activate transcription. Therefore, to further examine the liver-specific properties of enhancer 1 activity, we have carried out the biochemical purification and characterization of FPV binding activity from rat liver nuclei. This study has conclusively identified hepatocyte nuclear factor 3β (HNF-3β), a liver-enriched member of the HNF-3/forkhead gene family, as the predominant purified protein that interacts with the FPV motif. Moreover, a cellular protein(s) that copurified with HNF-3β specifically interacts with a novel sequence motif that partially overlaps FPV. Since this novel motif contains a palindrome sequence, we have tentatively referred to the protein(s) that binds to this site as palindromic-binding factor (PBF). Additional evidence indicates that HNF-3β and PBF cooperatively interact with enhancer 1. Therefore, this study supports the hypothesis that FPV-mediated enhancer activity involves a cooperative interplay between HNF-3β and at least one other enhancer 1-binding protein, PBF.

The human hepatitis B virus (HBV) predominantly infects hepatocytes, which is a prominent characteristic of hepadnaviruses. The majority of individuals that are chronically infected with HBV ultimately experience severe liver disease (1) and are at high risk for developing hepatocellular carcinoma (2, 3). To investigate the regulatory mechanisms that contribute to the hepatotropic nature of this virus, a considerable amount of work has focused on liver-specific aspects of HBV gene expression (for recent reviews, see Refs. 4 and 5). The HBV genome is a partially double-stranded circular DNA molecule that is composed of ~3200 base pairs (Fig. 1A). The compact nature of this small genome necessitates an extensively overlapping arrangement of the genetic information. Therefore, it is likely that a number of complex mechanisms regulate the temporal and differential expression of the viral RNAs. This regulation is mediated by the HBV promoter and enhancer elements (see Fig. 1A), which have been shown to exhibit liver-specific properties (4–8). Numerous studies have indicated that enhancers 1 and 2 play integral roles in regulating the expression of HBV genes (e.g. see Refs. 9–15). Although these enhancer elements are capable of functioning independently in a liver-specific manner (10, 15–21), the presence of both enhancers has been shown to markedly increase the overall level of transcriptional activation (12, 15). Furthermore, a recent analysis of transgenic mice has provided in vivo evidence suggesting that the liver-specific activity of the core/pregenomic promoter is dependent upon the combined activities of enhancers 1 and 2 (22). Taken together, these results support the hypothesis that HBV gene expression, in vivo, is regulated by a mechanism(s) involving a cooperative interplay of the viral enhancer elements.

The enhancer 1 element (Fig. 1B), which was first identified in 1985 (16, 23), spans nucleotides 970–1240 on the HBV genome (subtype adw). Work from a number of laboratories supports the conclusion that enhancer 1 plays an important role in regulating the activity of the HBV surface antigen (9, 10, 13, 15), core/pregenomic (10, 13–15, 18, 22, 24, 25), and X (11, 13, 14, 26) promoter elements. While it is clear that enhancer 1 exhibits liver-specific properties (e.g. see Refs. 10 and 16–18), enhancer 1 has also been shown to exhibit activity in dedifferentiated hepatocyte and non-hepatocyte cell types (4, 5, 7). The activities attributed to enhancer 1 in a broad spectrum of cell types are most likely facilitated by a complex array of liver-enriched as well as ubiquitous trans-acting cellular factors that are capable of specifically interacting with enhancer 1 (see Fig. 1B and Refs. 4–7). Although ubiquitous factors may activate enhancer 1 in non-hepatocytes, the activity of enhancer 1 within the HBV-infected liver is likely to be dependent upon cooperative interactions between multiple liver-enriched and ubiquitous factors (27–30).

A central region of enhancer 1 that is located at nucleotides 1080–1165, referred to here as the enhancer core domain (see Fig. 1B and Ref. 31), appears to serve as the predominant functional unit of this regulatory element. Additional contributions to enhancer 1 activity may be mediated by protein-binding motifs that are located 3’ of the core domain at nucleotides 1166–1240 (31–33, 35). Mutational analyses, which were carried out in the context of the transient transfection assay, have indicated that at least four distinct sequence motifs within the enhancer core domain are essential for enhancer 1 activity (29, 36)}
Purification of HBV Enhancer 1-binding Proteins

FIG. 1. Schematic representations of the hepatitis B virus genome and enhancer 1 element. A, the numbered circle depicts the HBV genome. The numerical designations within the genome (0–3200 base pairs (bp)) are based on the adw2 subtype of HBV. S, C, P, and X represent the genes encoding the hepatitis B surface antigen, core antigen, polymerase, and X proteins, respectively. preS1, preS2, Cp, and Xp represent the promoter elements for the corresponding genes. The enhancer elements are designated as Enh 1 (spanning nucleotides 970–1240) and Enh 2 (spanning nucleotides 1636–1741). An, the single polyadenylation site utilized by all of the HBV RNAs. B, shown are the protein-binding sites on enhancer 1. Numerical designations correspond to nucleotide positions within the HBV genome. The thick bar denotes the enhancer core domain, which spans nucleotides 1080–1165. Previously defined footprint designations (38) are indicated above the corresponding protein-binding sites. The binding sites for trans-acting cellular factors are labeled accordingly. The circle with thin stripes represents the HBV RARE. The circle with thick stripes (FPVI) is a binding site for C/EBP, HNF-1, and OCT2. The gray circle (FPVII) is a binding site for C/EBP, NF-1, CREB/ATF2, and AP1. The question mark indicates the position of a binding site for an unknown cellular factor(s). RXRα-PPAR, retinoid X receptor α-peroxisome proliferator-activated receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor.

MATERIALS AND METHODS

Plasmids and Oligonucleotides—The following oligonucleotides were utilized throughout this study: (i) V (wild-type), which spans the FPV sequence motif (nucleotides 1124–1139) within enhancer 1, 5'-GATCCGGCTTCTCTAAACATGCATGAA-3'; (ii) V'- (point mutant), which contains the identical point mutations in the FPV motif previously used for functional analyses of enhancer 1 (34), 5'-GATCCGGCTTCTCTCAAGGACACACATGAA-3'; (iii) V'' (point mutant), 5'-GATCGGGCAAAAGAGTTGGCTTTG-3'; and (iv) TTR, which spans the HBV-3 binding site within the transthyretin promoter (45), 5'-GATCGGGCATGCTAACTACATCAAGGACT-3'. The wild-type (V) and mutant (V') oligonucleotides described above were used to generate clones containing multimerized binding sites. Double-stranded V and V' oligonucleotides were independently multimerized in the presence of DNA ligase. Multimers containing three and four copies were isolated and cloned into pGEM-3 to generate pGFPV3 (wild-type) and pGFPV4' (point mutant), respectively. DNA sequencing (Sequenase 1.0, U.S. Biochemical Corp.) was performed to confirm the orientation and copy number of the multimerized region. A DNA fragment containing the SV40 early promoter upstream of the firefly luciferase gene was excised from plasmid pUC4 (46) and subcloned into pGFPV3 and pGFPV4' to generate the reporter plasmids pGFPV3Luc (wild-type) and pGFPV4'Luc (point mutant), respectively. Plasmid pENSLuc contains the wild-type enhancer 1 (nucleotides 966–1308, HBV subtype adw1) located upstream of the SV40 early promoter (SV40Ep) and the luciferase reporter gene (34). Plasmids pSLuc2 and pSV2Luc (47) contain SV40Ep (enhancerless) and SV40Epenhancer located upstream of the luciferase gene, respectively.

Transient Transfection Assay—Transient transfection assays were performed using the human hepatoma cell line Huh-7 (48) and the human dedifferentiated liver cell line SK-Hep-1 (49). Cells were plated at a density of ~5 x 10^4 cells/60-mm dish and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and penicillin (75 units/ml) and streptomycin (50 units/ml) at 37 °C. Cell monolayers (~50% confluent) were transfected with 2 μg of a relevant luciferase reporter plasmid and 1 μg of a β-galactosidase expression plasmid (pCH10) using the calcium phosphate precipitation method (50). Four hours post-transfection, the plates were incubated with 2 ml of fresh medium. The cells were further incubated for 40 h, harvested, and then analyzed for luciferase (47) and β-galactosidase (50) expression.

Purification of HBV Enhancer 1-binding Proteins—Enhancer 1 binding activity was monitored throughout the purification procedure using the DNase I footprinting assay that is described below. Fresh livers were excised from female rats (Sprague-Dawley) and immediately placed in ice-cold saline buffer (pH 7.5) on ice. The isolation of rat liver nuclei and the subsequent preparation of nuclear extract were carried out as described previously (51). The remaining steps of the purification were conducted at 4 °C. Unless stated otherwise, all column chromatography was performed using the Pharmacia FPLC system. Prior to column chromatography, samples were clarified by centrifuga-
was fractionated by (NH4)2SO4 precipitation. The protein fraction was then desalted by gel filtration on a Sephacryl 100 column (Bio-Rad) that was initially equilibrated with buffer A (25 mM Tris-HCl (pH 7.9), 100 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM diethiothreitol (DTT), 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM pepstatin). Fraction II was then applied at 18 ml/h to a 15-ml heparin-Sepharose column that had been pre-equilibrated with buffer B (50 mM Tris-HCl (pH 7.9), 12.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM pepstatin) containing 0.2 M KCl. The column was washed with 25 ml of buffer B containing 0.2 M KCl, and protein fractions were then eluted with a 60-ml linear gradient of 0.2-0.6 M KCl in buffer B. Heparin-Sepharose fractions that exhibited FPV binding activity were pooled (Fraction III) and then dialyzed against buffer A containing 0.2 M KCl.

Wild-type and mutant oligonucleotide affinity columns were prepared as described previously (52) using oligonucleotides V and 'V, respectively. Ligated oligonucleotides (~10 copies of V or V' ligated DNA strand) were covalently attached to CNBr-activated Sepharose 4B (Pharmacia Biotech Inc.) with a coupling efficiency of 85%, which resulted in a DNA mass of resin. The wild-type and mutant affinity resins (1 ml) were packed into separate columns (HR 5/5, Pharmacia Biotech Inc.) and stored with a solution containing 10 mM Tris-HCl (pH 7.6), 0.3 M NaCl, 1 mM EDTA, and 0.02% (w/v) NaN3. Prior to affinity chromatography, columns were equilibrated with buffer C (50 mM Tris-HCl (pH 7.9), 12.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.05% Nonidet P-40, and 1 mM leupeptin) containing 0.1 M KCl. Fraction III was mixed with 10 μg of specific competitor DNA (poly[d(C)]-Boehringer Mannheim) and incubated for 10 min on ice. The sample was then clarified as described above and applied to the wild-type affinity column (DNA affinityI) at 9 ml/h. The column was washed with 10 ml of buffer C containing 0.1 M KCl, and protein fractions were then eluted with a linear gradient of 0.2-10 M KCl in buffer C. Fractions containing FPV binding activity were pooled (Fraction IV) and then dialyzed against buffer C containing 0.1 M KCl. The remaining affinity chromatography steps were carried out in the absence of poly[d(C)]. Fraction IV was clarified and then applied to the mutant affinity column (DNA affinityII) as described above. Fractions containing PBF binding activity were pooled and then dialyzed against buffer D (25 mM Hepes (pH 7.9), 10 mM MgCl2, 0.1 mM KCl, 1 mM EDTA, 1 mM DTT, 25% glycerol, and 1 μM leupeptin). Fractions containing FPV binding activity were pooled (Fraction V) and directly applied to the wild-type affinity column (DNA affinityIII) as described above. Protein fractions eluted from DNA affinityIII that exhibited FPV binding activity were pooled (Fraction VI) and then dialyzed against buffer D containing 0.1 M KCl and 10% glycerol. These fractions were then subjected to the method of Bradford (53) using Bio-Rad protein standards. Samples utilized for protein-DNA interaction assays and immunoblot analyses were supplemented with carrier protein (bovine serum albumin, 1 mg/ml). Protein samples were rapidly frozen in a dry ice/ethanol bath prior to storage at -80°C.

Protein-DNA Interaction Assays—The DNase I footprinting assay was carried out essentially as described (54) in the presence of a DNA fragment spanning nucleotides 1074–1308 on the HBV genome (enhancer 1 probe). The enhancer 1 probe was end-labeled using T4 polynucleotide kinase (U. S. Biochemical Corp.) and [γ-32P]ATP (7000 Ci/mmm; ICN Pharmaceuticals, Inc.), ethanol-precipitated, purified from a 5% polyacrylamide gel, and then utilized for DNAase I footprinting analyses. DNase I digestion products were analyzed on 8% urea, 8% polyacrylamide gels. Following electrophoresis (2 h at 1500 V), which was carried out in 0.5 X TBE buffer at 4°C, gels were dried and then exposed to x-ray film (Kodak X-OMAT AR) for 40 h at -80°C. Purification of HBV Enhancer 1-binding Proteins

The DNase I footprinting assay was carried out using multiple tandem copies of the wild-type (pFPV3Luc) or point mutant (pFPV4Luc) FPV sequence motif located upstream of the SV40 early promoter and the luciferase reporter gene (Fig. 2A). When reporter plasmids were transfected into the human hepatoma cell line Huh-7, the expression of luciferase from plasmids pFPV3Luc and pFPV4Luc was induced by ~5- and 2-fold, respectively, relative to the control plasmid pSV2Luc2 (Fig. 2B). These results indicate that the wild-type FPV motif is capable of independently trans-activating luciferase reporter gene expression in a liver-derived cell line, while point mutations within this motif reduce FPV-mediated activity by ~2.5-fold. The luciferase expression mediated by the full-length enhancer (pENSLuc) was ~13-fold higher than that derived from pFPV3Luc. Therefore, although the FPV sequence motif is capable of independently activating transcription, these results are consistent with the hypothesis that the maximal level of enhancer 1 activity results from the combined activities of multiple sequence motifs within the enhancer 1 element. Furthermore, when transfection analyses were carried out in the dedifferentiated liver cell line SK-HeP-1 (Fig. 2C), luciferase expression derived from pFPV3Luc and pFPV4Luc was induced by ~1.7- and 1.5-fold, respectively. Therefore, the low level of activity associated with pFPV3Luc in SK-HeP-1 cells further supports the conclusion that the FPV sequence motif functions in a liver-specific manner. In addition, luciferase expression from the control plasmids pENSLuc and pSV2Luc was induced by ~10- and 60-fold, respectively. Activity derived from pENSLuc was most likely facilitated by ubiquitous trans-acting cellular factors. The high level of luciferase expression from pSV2Luc, which contains the SV40 enhancer, is consistent with previous work (47).

Purification of FPV Binding Activity from Rat Liver Nuclear Preparations—Previous work (11, 36-38) and the transfection studies described above suggest that the activity attributed to the FPV sequence motif is mediated by a liver-enriched trans-acting cellular factor(s). Therefore, we have addressed this issue by carrying out the biochemical purification of FPV binding activity from rat liver nuclei (see Materials and Methods for details) (Fig. 3). Rat liver nuclear extract (Fraction I) was initially fractionated by (NH4)2SO4 precipitation. The protein fraction...
that precipitated in the range of 0–50% saturated (NH₄)₂SO₄ (Fraction II) exhibited all of the detectable FPV binding activity (Fig. 4A). The DNase I footprint spanning the FPV sequence motif exhibits a characteristic DNase I-hypersensitive site at the approximate center of the protected region (compare lane 1 with lanes 2–5 and see Ref. 38). Fraction II was further fractionated on a heparin-Sepharose column (Fig. 4B). The heparin-Sepharose pool (Fraction III), which eluted between 0.33 and 0.44 M KCl, was then applied to the wild-type DNA affinity column (DNA affinityI). FPV binding activity predominantly eluted from DNA affinityI between 0.3 and 0.5 M KCl (Fig. 4C, lanes 3–5). Moreover, these fractions also contained a novel binding activity that produced a DNase I footprint partially overlapping FPIV and FPV (see lanes 4 and 5). Since this novel footprint spans a region containing an 8-base pair perfect palindrome, AAGGCCTT, the factor(s) that binds to this site has been tentatively designated as PBF. Additionally, DNA affinityI effectively segregated FPV binding activity from the other enhancer 1 binding activities that were present in Fraction III (compare lanes 2 and 4). The DNA affinityI pool (Fraction IV) also exhibited several DNase I-hypersensitive sites that were not associated with FPV. Fraction IV was then applied to the mutant DNA affinity column (DNA affinityII). While all of the detectable FPV binding activity flowed through DNA affinityII at 0.1 M KCl (data not shown), PBF binding activity effectively bound to this column and was eluted between 0.4 and 0.5 M KCl (discussed below). The DNA affinityII pool containing FPV binding activity (Fraction V) was then directly applied to the wild-type DNA affinity column (DNA affinityIII). The protein fractions that eluted between 0.5 and 0.6 M KCl, which contained the majority of the FPV binding activity (Fig. 4D, lanes 5 and 6), were pooled (Fraction VI) and processed as described above. DNase I footprinting analyses demonstrated that Fraction VI was completely devoid of detectable PBF (Fig. 4, compare C (lane 4) with D (lanes 5 and 6)) as well as other enhancer 1 binding activities (Fig. 4D, compare lane 2 with lanes 5 and 6).

**Fig. 2. Functional analysis of the FPV sequence motif.** Transient transfection analyses were carried out as described under “Materials and Methods.” Luciferase expression was normalized for transfection efficiency based upon the expression of β-galactosidase from the internal control plasmid pCH10. A, the wild-type (V) and mutant (V₉) FPV oligonucleotides were used to generate the reporter plasmids pFPV3Luc and pFPV4₉Luc, respectively, as described under “Materials and Methods.” pENSLuc and pSLuc2 served as control plasmids. B, transfections were carried out in Huh-7 cells. Luciferase activity is expressed as-fold induction over the base-line level of activity observed using reporter plasmid lacking enhancer sequence (pSLuc2). The means ± S.D. were derived from five independent experiments carried out in triplicate. C, transfections were carried out in SK-Hep-1 cells. The relevant reporter plasmids are indicated below the graph. Luciferase expression was determined as described above. The means ± S.D. were derived from three independent experiments carried out in triplicate.

**Fig. 3. Purification scheme for enhancer 1-binding proteins.** FPV and PBF binding activities were purified as described under “Materials and Methods.”
FIG. 4. Identification of purified fractions containing FPV binding activity. A, DNase I footprinting analysis of Fraction II. Samples were analyzed in the presence of the enhancer 1 probe (25,000 cpm; labeled at nucleotide 1074) and increasing amounts of Fraction II. Lanes 1–5 contained 0, 75, 100, 125, and 150 μg of protein. G/A, sequencing ladder. The thick bars on the right are aligned and labeled with the corresponding DNase I footprint, as described previously (38). The FPV sequence is shown (nucleotides 1124–1139) and is labeled with arrows indicating a 4-base pair direct repeat. Small open circles indicate DNase I-hypersensitive sites. B, graphic representation of the protein elution profile from heparin-Sepharose chromatography. Fraction II was loaded onto the heparin-Sepharose column, and proteins were eluted over the range of KCl concentration indicated by the dotted line. The solid line shows the ultraviolet absorbance profile (wavelength, λ = 280 nm). The thick bar represents the pooled fractions that exhibited FPV binding activity (Fraction III). C, DNase I footprinting analysis of Fraction IV. Samples were analyzed in the presence of the enhancer 1 probe and the following: lane 1, no protein; lane 2, 25 μg of Fraction III (L, load); lanes 3–7, ~50 ng of protein eluted from DNA affinity, at 0.3, 0.4, 0.5, 0.6, and 0.7 M KCl, respectively. The thick bars on the right are aligned and labeled with the corresponding DNase I footprint. The PBF-binding site is indicated. Small open circles indicate DNase I-hypersensitive sites. D, DNase I footprinting analysis of Fraction VI. Samples were analyzed in the presence of the enhancer 1 probe and the following: lane 1, no protein; lane 2, 25 μg of Fraction III as a positive control to show the full range of footprints on the enhancer; lanes 3–7, ~8 ng of protein eluted from DNA affinity, at 0.3, 0.4, 0.5, 0.6, and 0.7 M KCl, respectively. The thick bars on the right are aligned and labeled with the corresponding DNase I footprint. Small open circles indicate DNase I-hypersensitive sites.
Fractions II–VI were subsequently analyzed for purity by SDS-polyacrylamide gel electrophoresis (Fig. 5). Fraction VI contained one predominant protein that exhibited an apparent molecular mass of ~47,000 Da (lane 5). This fraction also contained several proteins that were barely detectable by the silver staining method. Table I provides a numerical summary of the purification described above. Specific activity determinations indicated that the FPV binding activity in Fraction VI was purified ~25,000-fold with respect to Fraction II.

Biochemically Purified FPV Binding Activity Is Mediated by HNF-3β—We proceeded to analyze protein-DNA interactions between Fraction VI and the enhancer 1 probe (nucleotides 1074–1308 on the HBV genome) using the EMSA. To demonstrate that the binding activity observed in the EMSA was due to specific interactions with the FPV sequence motif, competition studies were carried out using unlabeled oligonucleotides V (wild-type) and V' (mutant) (Fig. 6A). In the absence of competitor DNA, two distinct complexes were formed between a protein(s) present in Fraction VI and the enhancer 1 probe (lane 2). When the assay was carried out in the presence of increasing amounts of oligonucleotide V (lanes 3–5), both complexes with the enhancer 1 probe were completely abrogated by a 125-fold molar excess of competitor (lane 5). Identical amounts of oligonucleotide V' had no effect upon the protein-DNA complexes (lanes 6–8). These results demonstrate that both protein-DNA complexes result from a specific interaction(s) with the FPV sequence motif. Moreover, this conclusion is consistent with DNase I footprinting analyses, which showed that FPV was the only region of enhancer 1 protected from DNase I digestion in the presence of Fraction VI (see Fig. 4D). Since recent studies suggested that the FPV sequence motif is recognized by one or more of the HNF-3 isoforms (36, 37), we investigated the possibility that Fraction VI contains HNF-3α, -β, and/or -γ. To address this issue, competition studies were carried out in the presence of an unlabeled oligonucleotide that spans the HNF-3-binding site within the transthyretin (TTR) promoter (Fig. 6B) (45). While increasing amounts of a nonspecific (NS) competitor had no effect upon the specific protein-DNA complexes (lanes 3–5), oligonucleotide TTR completely competed for both complexes by a 125-fold molar excess of competitor (lane 8). This result suggests that Fraction VI contains one or more of the HNF-3 isoforms. To determine which isoform(s) was present in Fraction VI, the EMSA was carried out in the presence of polyclonal antibodies directed against HNF-3α, -β, and -γ (Fig. 6C). While antibodies directed against the α-isoform (lane 3) and γ-isoform (lane 5) of HNF-3 had no effect upon the protein-DNA complexes, the antibody directed against HNF-3β “supershifted” both specific complexes into at least two markedly retarded complexes (lane 4). Normal rabbit serum (RS) did not affect the specific complexes (lane 6). Taken together, the results from the EMSA analyses support the conclusion that the FPV binding activity present in Fraction VI is mediated by HNF-3β.

Immunoblot experiments were carried out to further address the results shown in Fig. 6C. Bacterially expressed HNF-3α (36) and in vitro translated HNF-3α (37) have been shown to interact with the FPV sequence motif. Therefore, although the antibody directed against HNF-3α did not affect the protein-DNA complexes observed using the EMSA (Fig. 6C), we further examined Fraction VI for the presence of HNF-3α by immunoblot analysis (Fig. 7A). While crude rat liver nuclear extract contained an immunoreactive protein with an apparent molecular mass of 50,000 Da (lane 1), which is the known molecular mass of HNF-3α (58), the HNF-3α antibody did not immunoreact with Fraction VI (lane 3) or the PBF pool (lane 4). The immunoreactive 50,000-Da protein was detected in the heparin-Sepharose fraction (lane 2) on a longer exposure of the film shown in Fig. 7A, which indicates that HNF-3α was present in Fraction III. When immunoblotting was carried out using a

![Fig. 5. SDS-polyacrylamide gel electrophoresis analysis of purified fractions containing FPV binding activity.](https://www.jbc.org/)

TABLE I Purification of FPV binding activity

| Fraction         | Proteina | Specific activity b | Total activity b | Purification | Yield |
|------------------|----------|---------------------|------------------|--------------|-------|
|                  | mg       | units/ mg           | units            | -fold        | %     |
| I. Rat liver nuclear extract | 1123.0 | NDc | ND | 1 | 100 |
| II. (NH₄)₂SO₄ (0–50%) | 675.0 | 6.7 | 4502 | 1 | 100 |
| III. Heparin-Sepharose | 44.5 | 33.3 | 1483 | 5 | 33 |
| IV. DNA affinity, (wild-type) | 0.031 | 24,213.0 | 751 | 3630 | 16.7 |
| V. DNA affinity, (mutant) | 0.010 | 35,971.0 | 360 | 5393 | 8.0 |
| VI. DNA affinity, (wild-type) | 0.002 | 169,462.0 | 339 | 25,411 | 7.5 |

* Protein determinations were carried out using the Bradford assay (53) (Fractions I–III) and by scanning densitometric analysis of SDS-polyacrylamide gels stained with silver nitrate (Fractions IV–VI) as described under “Materials and Methods.”

b One unit is defined as the amount of protein required for 100% occupancy of the FPV sequence motif as determined by DNase I footprinting analyses.

c ND, not determined.
Figure 6. EMSA analysis of Fraction VI. Samples were analyzed on 3.5% polyacrylamide gels in the presence of the enhancer 1 probe (8000 cpm) as described under “Materials and Methods.” In A, lane 1 contained no protein, and lanes 2–8 contained 0.34 ng of protein (Fraction VI) incubated in the presence of the following competitor DNA: lane 2, no addition; lanes 3–5, 5, 25, and 125-fold molar excesses of oligonucleotide V (wild-type), respectively; lanes 6–8, 5, 25, and 125-fold molar excesses of oligonucleotide V mutant, respectively. Free probe (F) and bound protein-DNA complexes (B) are indicated. In B, lane 1 contained no protein, and lanes 2–8 contained 0.34 ng of protein (Fraction VI) incubated in the presence of the following competitor DNA: lane 2, no addition; lanes 3–5, 5, 25, and 125-fold molar excesses of oligonucleotide NS, respectively; lanes 6–8, 5, 25, and 125-fold molar excesses of oligonucleotide TTR, respectively. In C, lane 1 contained no protein, and lanes 2–6 contained 0.34 ng of protein (Fraction VI) incubated in the presence of the following: lane 2, no addition; lanes 3–5, samples incubated in the presence of polyclonal antibodies directed against HNF-3α, -β, and -γ, as indicated below the corresponding lanes; lane 6, a sample incubated in the presence of normal rabbit serum (RS). Supershifted ternary complexes composed of protein, DNA, and antibody (S) are indicated. Aggregated protein-DNA complexes were present in the loading well in the presence of HNF-3β antibodies only.

Figure 7. Immunoblot analysis of purified fractions. Samples were resolved on SDS-10% polyacrylamide gels and subjected to immunoblot analysis as described under “Materials and Methods.” A, immunoblot analysis was carried out using polyclonal antibodies directed against HNF-3α (1:5000 dilution). Lanes 1–4 contained the following protein samples: lane 1, 40 μg of rat liver nuclear extract (RLNE); lane 2, 25 μg of the heparin-Sepharose pool (Fraction III); lane 3, 0.034 μg (50 μl) of the DNA affinity II pool (Fraction VI); lane 4, 0.06 μg (50 μl) of the PBF pool. The immunoreactive 50-kDa protein present in rat liver nuclear extract, HNF-3α, is indicated. The migration position of the prestained molecular mass marker (ovalbumin, 53.2 kDa) is shown on the right. B, immunoblot analysis was carried out using a monoclonal antibody directed against HNF-3β (1:250 dilution). Lane 1 contained 1.3 ng of Fraction VI; lane 2 contained 15 ng of the PBF pool. The immunoreactive 47-kDa protein present in Fraction VI, HNF-3β, is indicated. Additional experiments indicated that an immunoreactive protein was not detected when 60 ng of the PBF pool was examined. The migration positions of prestained molecular mass markers (ovalbumin, 53.2 kDa; and carbonic anhydrase, 34.9 kDa) are shown on the right.

Monoclonal antibody directed against HNF-3β (Fig. 7B), an immunoreactive protein was detected in Fraction VI that exhibited an apparent molecular mass of 47,000 Da (lane 1). Therefore, since the known molecular mass of HNF-3β is 47,000 Da (59), this result supports the conclusion that the predominant 47,000 Da protein present in Fraction VI (Fig. 5) is identical to HNF-3β. Additionally, the HNF-3β monoclonal antibody did not immunoreact with the PBF pool (Fig. 7B, lane 2).

HNF-3β and PBF Cooperatively Interact with HBV Enhancer 1—As described above, PBF associated with DNA affinity, at low ionic strength and was subsequently eluted between 0.4 and 0.5 M KCl. The purification of PBF under these conditions is consistent with the presence of a region nearly identical to the PBF-binding motif on the ligated oligonucleotides that were conjugated to the wild-type and mutant affinity columns. The PBF pool was processed as described under “Materials and Methods.” Since all of the detectable FPV binding activity flowed through DNA affinity, at low ionic strength, this purification step effectively segregated these two binding activities. This conclusion is supported by DNase I footprinting analyses, which demonstrated that Fraction VI and the PBF pool were devoid of detectable PBF (see Fig. 1D, lanes 5 and 6) and FPV (Fig. 8A, lane 2) binding activities, respectively. The lack of FPV binding activity in the PBF pool is consistent with the absence of the FPV-hypersensitive site (lane 2). However, the
PBF pool exhibited the other DNase I-hypersensitive sites that were initially observed in the presence of Fraction V (see Fig. 4C). Moreover, DNase I footprinting demonstrated that the PBF-binding site overlapped the 5'-end of the FPV sequence motif by 4 base pairs.

Because of the close proximity of the HNF-3β- and PBF-binding sites, we further examined the DNA binding properties of Fraction VI and the PBF pool. As shown by DNase I footprinting analysis (Fig. 8B), low to moderate levels of DNase I footprinting activity were observed when each binding activity was examined independently (HNF-3β, lanes 2 and 3; and PBF, lanes 4 and 5). When Fraction VI and the PBF pool were combined at the smallest amount of protein that was utilized for this assay (2 μl of each preparation), the DNase I footprinting activity was markedly increased at both binding sites (compare lanes 2 and 4 with lane 6). A 2-fold increase in the amount of protein (4 μl of each preparation) resulted in nearly maximal footprinting activity throughout the PBF/HNF-3β-binding region (lane 7), while the binding activity was considerably lower when 6 μl of each preparation was examined independently.
The increased binding activity in the presence of HNF-3β and PBF is further indicated by the presence of the strong DNase I-hypersensitive site located 5' of the PBF-binding site (compare Fig. 8B (lanes 6 and 7) with Fig. 4C (lanes 3-5)). The EMSA was then utilized as an alternative approach to examine this issue (Fig. 8C). A large amount of Fraction VI was used to demonstrate the migration positions of specific protein-DNA complexes containing HNF-3β (lane 2; refer to Fig. 6A). At smaller amounts of Fraction VI, complexes containing HNF-3β were barely detected (Fig. 8C, lanes 4-6). When identical amounts of Fraction VI were analyzed in the presence of a constant amount of the PBF pool, protein-DNA complexes were markedly increased in a protein concentration-dependent manner (lanes 7-9). In addition to an elevated level of HNF-3β-specific complexes (compare lanes 6 and 9), two distinct complexes associated with the PBF pool were markedly increased (compare lane 3 with lanes 7-9). These results are consistent with the DNase I footprinting analysis shown in Fig. 8B, which indicated that footprinting activity was increased at both binding sites when HNF-3β and PBF were analyzed concurrently. Taken together, these binding studies support the conclusion that HNF-3β and PBF cooperatively interact with enhancer 1. Fig. 9 summarizes the overlapping components of the enhancer core domain, which reflects our results from the biochemical purification and DNA binding analyses of HNF-3β and PBF.

**DISCUSSION**

The HBV enhancer 1 element plays a crucial role in the overall liver-specific regulation of HBV gene expression. To study the mechanisms that dictate enhancer 1 activity, we have further investigated a central region of enhancer 1 that is referred to as the enhancer core domain. This work has concentrated on biochemical and functional analyses of the FPV sequence motif (11, 31, 34, 36-38), which is centrally located in the core domain within an extensively overlapping cluster of protein-binding sites (Fig. 9). While previous work indicated that the FPV motif is inactive when it is present as a mono-meric element (11, 29), we have shown that multiple tandem copies of FPV function in a liver-specific manner to activate transcription (Fig. 2). FPV-mediated transcriptional activation was markedly reduced in the presence of an FPV multimer that contained point mutations, which is consistent with previous functional analyses of the full-length enhancer 1 containing various point mutations within the FPV motif (34, 36, 37). Taken together, these findings support the following conclusions. 1) The FPV sequence motif is capable of independently activating transcription in liver-derived cells, and 2) FPV contributes to the liver-specific activity of enhancer 1 by serving as a binding site for a liver-enriched transcriptional activator(s). Furthermore, our transient transfection analyses strongly correlate with the results from the biochemical purification and DNA binding studies presented here. Oligonucleotides V and V' were used to prepare the FPV multimer clones (pFPV3Luc and pFPV4'Luc) and the oligonucleotide affinity columns and were utilized for EMSA competition studies. We have determined that FPV binding activity is mediated by the liver-enriched nuclear factor HNF-3β, which was purified by DNA affinity chromatography (see Figs. 4 and 6). Since HNF-3β failed to interact with the mutant affinity column, the low level of activity exhibited by pFPV4'Luc in Huh-7 cells (Fig. 2B) was most likely due to the absence of an interaction between HNF-3β and the FPV motif. This possibility is further supported by the EMSA analyses, which showed that oligonucleotide V' was unable to compete for HNF-3β binding in the presence of the wild-type enhancer 1 probe (Fig. 6A). Therefore, this work supports the hypothesis that HNF-3β contributes to the activation of enhancer 1 in vivo by binding to the FPV motif.

While our work was in progress, two independent studies suggested that one or more of the HNF-3 isoforms interact with the FPV motif (36, 37). For these analyses, the previously cloned genes for the α-, β-, and γ-isomers of HNF-3 were used to express the corresponding proteins in bacteria or by in vitro translation. While the study by Chen et al. (36) indicated that all three isoforms of HNF-3 interact with an oligonucleotide containing the FPV motif, the protein-DNA binding analyses reported by Ori and Shaul (37) exclusively showed that recombinant HNF-3α binds to FPV. Moreover, since these binding studies did not examine whether HNF-3 interacts with the full-length enhancer 1 element in the context of a complex mixture of nuclear proteins, it was unclear whether HNF-3 would preferentially interact with the FPV motif under such experimental conditions. Therefore, to address this critical issue and to conclusively identify the trans-acting factor(s) that interacts with the FPV motif, we carried out the biochemical purification of FPV binding activity from rat liver nuclei. Our results clearly demonstrate that HNF-3β preferentially interacts with the FPV motif in the presence of the heparin-Sepharose pool (Fraction IIIC), which is composed of a population of proteins that is highly enriched with nuclear DNA-binding proteins. While HNF-3α was detected in Fraction IIIC, this isoform did not copurify with HNF-3β on DNA affinity (see Figs. 4C and 6C). Therefore, HNF-3β preferentially interacts with the FPV motif in the presence of HNF-3α, which suggests that HNF-3β is the physiologically relevant isoform of HNF-3 that regulates enhancer 1 through the FPV sequence motif. This finding is especially striking in light of previous work (58), which demonstrated that HNF-3α and -β derived from rat liver nuclear extract copurify from a heparin-agarose column under experimental conditions that were comparable to those used in this study. While it may be argued that our purification procedure resulted in a substantial loss of HNF-3α prior to and/or during heparin-Sepharose chromatography, DNase I footprinting analyses did not detect a second pool of FPV binding activity at any stage of the purification (data not shown). In addition, HNF-3γ was not detected in Fraction IV (Fig. 6C), which
serves the hypothesis that HNF-3β plays an important regulatory role during HBV gene expression by contributing to the liver-specific activity of the HBV enhancer 1 element. In addition to modulating enhancer 1 activity, HNF-3β may specifically affect other HBV transcriptional regulatory elements. In support of this notion, a recent study provided evidence indicating that HNF-3β contributes to the regulation of the HBV nucleocapsid promoter (73). Furthermore, it is possible that HNF-3β indirectly affects the HBV life cycle by modulating the expression of one or more liver-enriched proteins that may function to positively or negatively influence the viral infectious process. In this respect, HNF-3β has been shown to autoregulate its own expression (74), which may be relevant to the regulatory mechanisms that govern liver-specific aspects of HBV gene expression.

This work has led to the identification of a novel protein-binding site within enhancer 1 that partially overlaps FPIV and FPV. The DNA sequence of this binding site was not closely related or identical to any previously identified transcription factor-binding site according to analyses of the transcription factor data base (75, 76). The factor(s) that interacts with this binding site, PBF, was revealed during the purification and analysis of FPV binding activity (see Figs. 4C and 8A). While PBF binding activity was detected in the absence of HNF-3β (Fig. 8A), our study supports the conclusion that PBF and HNF-3β cooperatively interact with enhancer 1 when both binding activities are present (see Fig. 8, B and C). To our knowledge, this is the first evidence suggesting that HNF-3β activates transcription by cooperatively interacting with another cellular factor(s). Further studies will be necessary to determine whether this putative regulatory mechanism is generally utilized by HNF-3β to modulate the tissue-specific expression of cellular genes. Transcriptional activation of the serum albumin gene involves a cooperative interaction between HNF-3α and NF-1 on the albumin enhancer element (60), which suggests that all of the HNF-3 isoforms may be capable of utilizing a comparable activation mechanism(s).

The activation of the HBV enhancer 1 by a cooperative interplay between HNF-3β and PBF is consistent with previous reports that have implicated the involvement of cooperativity in the regulation of enhancer 1 activity (27–30). Although we have demonstrated that the FPV sequence motif is capable of independently activating transcription (Fig. 2), the maximal level of enhancer 1 activity appears to be dependent upon the combined activities of multiple sequence motifs within enhancer 1 (e.g. see Refs. 29 and 31–37). As described in the Introduction, previous mutational analyses have suggested that all of the binding sites located within the enhancer core domain are essential for enhancer 1 activity. However, it is clear from these studies that when one site is mutated, the other sites are unable to substitute for the altered site and reconstitute the wild-type level of enhancer 1 activity. Therefore, we hypothesize that the entire enhancer core domain is essential for enhancer 1 activity, which appears to rely on multiple cooperative interactions. This cooperativity may occur at several levels, including interactions within the core domain as well as a cooperative interplay between factors that interact with both enhancers 1 and 2. The complexity of this regulation is further exemplified by the involvement of an extracellular signaling pathway(s) that is mediated through the RARE within enhancer 1 (29, 34, 39). Furthermore, previous cotransfection studies carried out in the presence of an HNF-3β expression plasmid demonstrated that HNF-3β-mediated enhancer activity was not detected in Hepa cells, yet activity was observed in the dedifferentiated liver cell line SK-Hep-1 (37). This study suggests that HNF-3β activates enhancer 1 in conjunction with another enhancer 1-binding factor(s) that is present in SK-Hep-1 cells but is not expressed in a non-liver-derived cell line. Therefore, the results presented here suggest that PBF may function in this capacity. Moreover, since the PBF-binding site partially overlaps the FPV sequence motif (Fig. 4C), PBF may also contribute to enhancer 1 activity by engaging in a cooperative interaction with NF-1. These intriguing possibilities are currently under investigation to further our understanding of the regulatory mechanisms that control HBV gene expression.

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