Vitamin D-binding protein (DBP)/Ge-globulin, the major carrier of vitamin D and its metabolites in blood, is synthesized predominantly in the liver in a developmentally regulated fashion. By transient transfection analysis, we identified three regions in the 5'-flanking region of the rat DBP gene, segments F-2, B, and A, that contain tissue-specific transcriptional determinants. Gel mobility shift and DNase I footprinting analyses showed that all three regions contained binding sites for the hepatocyte nuclear factor 1 (HNF1), a transcriptional regulator composed of HNF1α and HNF1β hetero- and homodimers. The activity of the most proximal segment A (coordinates -141 to -43) was DBP promoter-specific, position-dependent, and positively controlled by HNF1α. In contrast, the two more distal determinants (segments F-2 and B; coordinates -1844 to -1621 and -254 to -140, respectively) acted as classical enhancers in transfected hepatocyte-derived HepG2 cells; their activities were promoter- and orientation-independent, and disruption of their respective HNF1-binding sites resulted in marked loss of DBP gene expression. Remarkably, the activities of these two distal elements depended upon the relative levels of HNF1α and HNF1β; HNF1α had a major stimulatory effect, whereas HNF1β acted as a trans-dominant inhibitor of HNF1α-mediated enhancer activity. These results suggested that the net expression of the DBP gene reflected a balance between the two major HNF1 species; the relative abundance of HNF1α and HNF1β proteins in a cell may thus play a critical role in determining the pattern of gene expression.

Vitamin D-binding protein (DBP) is a monomeric, multifunctional glycoprotein first identified as the group-specific component of serum or Gc-globulin (1). It is essential to the transport of vitamin D sterols in the blood and to the removal of plasma actin monomers released to the blood subsequent to transport of vitamin D sterols in the blood and to the removal of plasma actin monomers released to the blood subsequent to tissue damage. DBP also contributes to complement C3a-mediated chemotaxis, macrophase activation, and fatty acid transport (reviewed in Refs. 2 and 3). The DBP gene is a member of the multigene family that includes albumin, α-fetoprotein, and α-albumin (4, 5). The members of this gene family are tightly linked on chromosome 4 in human (6) and chromosome 14 in rat (7) and encode proteins with conservation of both primary and secondary structures. All four genes in this family are predominantly expressed in the liver. During embryonic development, expression of the rat albumin, α-fetoprotein, and DBP genes is induced in the yolk sac and maintained in the fetal liver (8), whereas α-albumin expression begins in the liver during the subsequent perinatal period. α-Fetoprotein is selectively silenced at the end of the fetal period, whereas α-albumin, albumin, and DBP expression remains high in the liver throughout adult life (9). Although mechanisms underlying the transcriptional control of the albumin and α-fetoprotein genes have been studied by a number of laboratories, the basis for the tissue-specific and developmentally regulated expression of the DBP gene remains unexplored.

Tissue-specific gene expression is predominantly regulated at the level of transcription initiation (reviewed in Ref. 10). Four families of transcription factors have been identified that are involved in liver-specific gene expression: hepatocyte nuclear factor 1 (HNF1), CCAAT/enhancer-binding protein (C/EBP), HNF3, and HNF4 (reviewed in Refs. 11 and 12). Expression of these factors is not fully restricted to the liver, and the specificity and levels of gene expression in adult hepatocytes appear to be mediated by specific combinations of trans-activators. Whereas the HNF3 proteins bind DNA as a monomer, members of each of the remaining hepatocyte-enriched transcription factor families bind DNA as homo- and heterodimers. The composition of these dimers within a family and their potential competition for shared DNA-binding sites may impose additional complexity on hepatocyte-gene regulation. In addition to the transcription-enhancing activity usually attributed to these hepatocyte-enriched factors, the presence of repressor-like molecules has been suggested by somatic cell hybrid studies demonstrating extinction of liver-specific gene expression when differentiated hepatoma cells are fused to dedifferentiated hepatoma cells or fibroblasts (13). Therefore, the fidelity and level of liver-specific expression is ensured by both positive and negative gene regulation.

The hepatocyte-enriched transcription factor HNF1 has been studied in detail. HNF1 was initially identified as a nuclear protein that binds to an element required for liver-specific transcription of the β-fibrinogen gene (14). It has been subsequently found to interact with sites in promoters as well as enhancer elements in a number of hepatocyte-restricted genes including albumin and α-fetoprotein (15–19). The HNF1 proteins have an amino-terminal dimerization domain, POU-like homeodomain with DNA binding activity, and carboxyl-termi-
nal transcriptional activation domain. Two members of the HNF1 transcription factor family, HNF1α (also known as HNF1, LB-F1, HP-1, APF, and A box factor) and HNF1β (also known as vHNF1, vAPF, and LF33) (20–22), have been characterized in detail, and they bind DNA as hetero- or homodimers. In addition to hepatic expression, HNF1α and HNF1β are also found in nonhepatic tissues including kidney, intestine, and stomach (23, 24); the relative concentrations of these two proteins differ markedly from tissue to tissue and are affected by developmental stimuli (21). Whether the relative levels of these two proteins impact on expression of specific genes has not been defined.

The rDBP gene including 2,196 base pairs (bp) of the 5′-flanking region has been previously isolated and structurally characterized (25). Here three segments involved in rDBP expression within this 5′-flanking region have been identified by transient transfection studies. Gel mobility shift assays and DNase I footprinting analyses demonstrated that their functional activities can be attributed to the presence of three corresponding HNF1-binding sites. The functional importance of these HNF1 sites was tested by mutational analyses, and opposing effects of HNF1α and HNF1β on rDBP gene expression were demonstrated.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction and modification enzymes were purchased from New England Biolabs. [α-32P]dNTPs, [γ-32P]ATP, and s-32P-dichloroacetate-1-14C-chloramphenicol were purchased from Amersham Pharmacia Biotech. Standard techniques (26) and manufacturer’s specifications were used for the isolation and manipulation of DNA. Expression vectors for mouse HNF1α (27) and HNF1β (21) were gifts from Dr. G. R. Crabtree (Stanford University School of Medicine); the carboxyterminal transcriptional activation domain of HNF1β expression vector was sequenced and confirmed to be identical to the published sequence (21) and homologous to human vHNF1-B (25). The rat pAPP/CAT vector (15) was a gift from Dr. J.-L. Danan (Center National de la Recherche Scientifique, France). The pSVAO-X-CAT vector was a gift from Dr. T. R. Kadesch (University of Pennsylvania). pSVAO-X-CAT is a derivative of pSVAO-CAT (29) where SV40 sequences between PsI and BamHI from pSVAO-CAT were removed, and the polylinker (PstI, SalI, XbaI, and BamHI) was added. Oligonucleotides used for gel mobility shift assays and PCR were synthesized by Life Technologies, Inc. PCR was performed with Taq DNA polymerase and 10× PCR buffer containing 15 mm MgCl2 (Boehringer Mannheim), using a PerkinElmer thermal cycler. Autoradiographic signals were quantitated by PhosphorImager (Molecular Dynamics).

**Plasmid Constructions**—The 5′-flanking region of the rDBP gene (2.7-kb fragment extending upstream of the HindIII fragment of rDBP genomic clone C1) (25) was digested with BstNI (coordinate +53) and the following secondary restriction enzymes: SacI (1926), NsiI (1465), BamHI (1077), HindIII (141), and RsaI (41). Coordinates used in this study are the same as published (25). Double digestion fragments generated with identical 3′ termini and a series of nested 5′ termini. Each fragment was blunt-ended with mung bean exonuclease and T4 DNA polymerase and then ligated to HindIII linkers (New England Biolabs). After inactivation of ligase, the DNA fragments were digested with HindIII and ligated to the pSVAO-CAT(X) vector previously cloned with HindIII. Eight different CAT constructs containing 5′-deletions of the rDBP promoter region with same 3′-end at +53 were generated (Fig. 1A). Seven subfragments of the 5′-flanking region (A, +141 to +43; B, −254 to −140; C, −667 to −149; D, −667 to −259; E, −1077 to −664; F, −1899 to −1490; G, −2196 to −1927, Fig. 1A) were amplified by PCR using the appropriate specific primers each containing HindIII recognition sequences (5′- CCAAAAGCTT-3′) at their 5′-ends. These fragments were cloned into the HindIII site downstream of the SV40 promoter and CAT gene in plasmid pCAT promoter (Promega Corp.) in order to study their activities with a heterologous promoter. pCAT promoter was also digested with BglII upstream of the CAT gene, blunt-ended with Klenow, and ligated to each of the blunt-ended PCR fragments. To generate a second series of CAT constructs in which the seven segments of the rDBP 5′-flanking region were linked to the rDBP minimal promoter driving CAT, the pDBP/CAT vector was constructed by cloning the rDBP minimal promoter (−39 to +53) into the XbaI site of pCAT Basic (Promega Corp.).

Each of the seven amplified fragments was then similarly cloned into either HindIII (upstream of CAT gene) or BamHI (downstream of CAT gene) sites of the pDBP/CAT vector. The pDBP/CAT constructs, containing the putative cis-elements (fragments A–G and F to 1 to F; Fig. 3A) cloned upstream of the CAT gene, were sequenced by the DNA and Protein Core, University of Pennsylvania Veterinary School. Only pDBP/CAT containing fragment E differed from the published sequence; −856 CGTCTT −686 was CTTGCGCTT. Likewise, four overlapping subsegments of F (F-1, −1899 to −1757; F-2, −1844 to −1621; F-3, −1758 to −1564; and F-4, −1628 to −1490) were cloned into the pDBP/CAT vector at the HindIII site upstream of the CAT gene (Fig. 3A) and confirmed by sequencing. The putative binding site mutB fragment B (mutB fragment B-2 and plasmid −2196DBPmF-2/CAT) (see Fig. 7B) containing the splicing-overlap extension method (30). To generate mutB, 5′ (2025 bp) and 3′ (238 bp) fragments for the overlap splicing were amplified using Pfu DNA polymerase. Each fragment was digested with oligos −2196/ −5 mB and 3mB/ +53 as primer pairs using the −2196DBP/CAT construct as the template of the SV40 early promoter were transcribed into cells by the calcium phosphate precipitation method (31, 32). Cells were harvested 48–72 h after the transfection and were lysed with 0.9 ml of reporter lysis buffer (Promega Corp.), and lysates were used for enzyme assays. The amount of cell lysate used in CAT assays was adjusted based on the transfection efficiency determined by β-galactosidase activity (33). CAT activities were measured as described previously (29, 34) and quantitated by PhosphorImager. Preparation of Nuclear Extracts and Northern Analysis—Nuclear extracts were prepared as described previously (35), except that all the solutions contained a protease inhibitor mixture (Boehringer Mannheim). Protein concentrations were measured using Bio-Rad protein assay solutions following the manufacturer’s instructions (Bio-Rad). A Northern blot analysis of liver RNA from wild type, HNF1α−/−, HNF1β−/−, and HNF1α−/−α−/− mice was generously carried out by Drs. M. Pontoglio and M. Yaniv (36), using a 32P-labeled 875-bp EcoRI rDBP cDNA fragment (37). Equal loading of lanes was documented by ethidium bromide staining for rRNA. Gel Mobility Shift Assays— Gel retardation assays were performed using 1–14 μg of nuclear proteins. Probe fragments, ranging in size from 98 to 223 bp, were prepared as follows. The appropriate cis-acting elements cloned at the HindIII site of the pDBP/CAT plasmid were released by HindIII digestion and labeled using Klenow enzyme, [α-32P]dCTP, and [α-32P]dATP by incubating at room temperature for 30 min. The labeled fragments were subjected to 6% polyacrylamide gel electrophoresis. DNA bands with the expected sizes were cut out of the gel and purified (26). The oligonucleotide β28 (Table 1) was labeled using T4 polynucleotide kinase and [γ-32P]ATP by incubating at 37°C for 30 min. The unincorporated nucleotides were removed by a G-25 spin column (Amersham Pharmacia Biotech). Nuclear extracts were incubated with 20,000 cpm of the labeled DNA probe in a 20-μl reaction mixture containing 12 μM HEPES, pH 7.8, 0.1 μM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 50 ng of bovine serum albumin (BSA, Boehringer Mannheim), and 1–2 μg of poly(dI-dC) (Amersham Pharmacia Biotech). The binding mixture was incubated at room temperature for 20 min and run on a 5% nondenaturing polyacrylamide gel electrophoresis. Each lane was air-dried and autoradiographed.
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in 1× TBE buffer at 10 V/cm. Gels were dried and exposed to an x-ray film at room temperature without an intensifying screen.

**Competition and Probes in the Gel Mobility Shift Assay**—The sequences of oligos β28, wtF-2, wtB, and wtA containing HNF1-binding sites present in the β-fibrinogen gene, the rDBP gene region F-2, B, and A, respectively, are listed in Table I. mutF-2 and mutB are the corresponding oligonucleotides with mutations in their HNF1-binding sites. Sense and antisense oligonucleotides used as competitors or as a probe were synthesized and annealed in 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, by heating at 90 °C for 3 min and then cooling down to 25 °C at 1 °C per 3 min in the thermal cycler. Nonspecific competitor, “ethanol-precipitated. They were dissolved in 99% formamide and 10 mM ringer Mannheim) followed by an incubation for 30 min at 42 °C. Nu-

addition of 2 volumes of 30 mM EDTA, 375 mM NaCl, and 0.75% SDS.

0.5 and 0.02 units of the DNase I. Reactions were stopped by the addition of 2 volumes of 30 mM EDTA, 375 mM NaCl, and 0.75% SDS. Excess proteins were digested by adding 2 µg of proteinase K (Boehringer Mannheim) followed by an incubation for 30 min at 42 °C. Nucleic acids were extracted with 1 volume of phenol:chloroform (1:1) and ethanol precipitated. They were dissolved in 99% formamide and 10 mM EDTA with tracking dyes, heated at 90 °C for 5 min, and loaded on 8% polyacrylamide, 7 M urea gels. The DNA sequence in adjacent lanes was established by chemical cleavage of phosphodiester bonds 3’ of A and G as described previously (38).

RESULTS

Identification of Two Tissue-specific Enhancer Segments 5’ to the DBP Promoter—To identify determinants important in rDBP transcriptional control, a series of seven sequential 5’-deletions of the previously sequenced rDBP 2196-bp 5’-flanking region contiguous with the promoter and 5’-untranslated region (25) were generated and fused to a promoterless CAT reporter gene (Fig. 1A). Plasmids containing these deletions were introduced into human hepatoma HepG2 cells or into NIH3T3 fibroblasts, and CAT activity (normalized for transfection efficiency) was determined. The full-length −2196DBP/CAT construct and a series of seven 5’-deletion constructs extending from the noted positions in the 5’-flanking region to a position +53 bp 3’ to the rDBP transcription start site (+1, angled arrow). Seven fragments internal to the 5’-flanking region (fragments A–G) are also indicated. B, transcriptional activity of each 5’-deletion construct was tested in the hepatocyte cell line HepG2. The mean CAT activities, normalized for cell transfection efficiencies, were calculated relative to the activity of the full-length construct, −2196DBP/CAT (indicated as 100). The standard errors are shown (n = 9).

regardless of its position, whereas fragment A was inactive in this context. Fragment E containing half of a rodent B2 small repetitive element (25), fragment G, and fragment D all demonstrated negative activity on the rDBP promoter (Fig. 2A). Fragment C encompassed negative element D and positive element B. The net effect of fragment C on the DBP promoter was negative. However, in the deletion series, elimination of −670 to −253, which corresponds to D, did not result in marked enhancement. This suggested that the negative activity in D might not be of major importance. All regions of the rDBP 5’-flanking region lacked activity in transfection NIH3T3 cells (Fig. 2B). The tissue-specific, position- and promoter-independent enhancer activity identified in fragments F and B, and a tissue-specific, position- and promoter-dependent activity identified in fragment A were further studied.

Prior to detailed characterization, the active region in the 409-bp fragment F was further sublocalized by linking each of four overlapping subfragments (F-1 through F-4) to the DBP promoter-driven CAT reporter plasmid (Fig. 3A). Analyses of activities in transfected HepG2 cells confirmed the enhancer activity to the F-2 subfragment; a marginal enhancing effect by F-3 was not significant (Fig. 3B). The activity of F-2 was independent of its position relative to the promoter (data not shown). Of note, a repressor activity was found in the adjacent F-4 fragment (Fig. 3B). Activity of the F-2 subfragment was 2-fold greater than the activity of its parental F fragment (Fig.

![Fig. 1. Identification of two positively acting transcriptional determinants 5’ to the rDBP gene. A, the rDBP gene promoter with contiguous 5’-flanking region is shown on the 1st line. Below is a diagram of the full-length −2196DBP/CAT construct and a series of seven 5’-deletion constructs extending from the noted positions in the 5’-flanking region to a position +53 bp 3’ to the rDBP transcription start site (+1, angled arrow). Seven fragments internal to the 5’-flanking region (fragments A–G) are also indicated. B, transcriptional activity of each 5’-deletion construct was tested in the hepatocyte cell line HepG2. The mean CAT activities, normalized for cell transfection efficiencies, were calculated relative to the activity of the full-length construct, −2196DBP/CAT (indicated as 100). The standard errors are shown (n = 9).](https://example.com/f1.png)
Fig. 2. Internal fragments B and F contained tissue-specific, position- and promoter-independent enhancer elements. A, constructs containing rDBP 5'-flanking fragments A–G (see Fig. 1A) were cloned either 5' or 3' to the minimal rDBP promoter (−39/+53) linked to the CAT reporter gene. Each construct was transfected into HepG2 cells, and mean normalized CAT activities were determined (the number of repeat experiments is noted in each case). B, the same set of seven fragments was cloned into a CAT reporter gene driven by the SV40 early promoter. Each construct was transfected into HepG2 or NIH3T3 cells. The CAT activities in A and B were expressed in arbitrary units relative to the parental construct containing minimal rDBP or SV40 promoter alone (indicated as 100).

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A rDBP promoter

B SV40 Promoter

Fig. 3. Sublocalization of fragment F enhancer activity to subfragment F-2 and identification of repressor activity in subfragment F-4. A, the top line represents the −2196 rDBP promoter and contiguous 5'-flanking region, each of the seven internal fragments is shown below. Fragment F is shown in expanded scale on the next line (−1899 to −1490) and below that are the four overlapping subsegments that were linked to the rDBP minimal promoter 5' to the CAT reporter. B, each construct was transfected into HepG2 cells, and normalized CAT activities were expressed relative to the activity of the rDBP minimal promoter (control, indicated as 100).

2A versus Fig. 3B), consistent with elimination of the negative activity in fragment F-4. The F-4 fragment lacked a negative effect when oriented 3' to the reporter gene (data not shown), possibly explaining the greater activity of the parental fragment F located 3' (5-fold increase) versus 5' (2-fold) to the DBP promoter (Fig. 2A). Thus, segments with rDBP promoter transcription-enhancing activity were localized to the 223-bp F-2 fragment, the 114-bp B fragment, and the 98-bp A fragment.

Functional HNF1-binding Sites within Regions F-2, B, and A—HNF1 is one of the major transcriptional activators in hepatocytes. The HNF1 consensus binding site is a partially degenerate palindromic sequence consisting of two 7-base pair inverted repeats separated by a single variable nucleotide, 5'—GGTTAATnATTAAC(a/c)—3' (11, 39). The 5' half of the pal-
The 5′-half contains substantial sequence variation. Computer-assisted analysis of the entire 2196-bp 5′-flanking sequence of the rDBP gene identified five putative HNF1-binding sites. These sites were located in fragments G, F-2, D, B, and A (Table II). Fragments G and D failed to show enhancing activity with either the SV40 promoter or the rDBP promoter (Fig. 2). The coincidence of transcriptional activation activity and the presence of putative HNF1-binding sites in fragments F-2, B, and A was further investigated.

Gel mobility shift assays were performed to determine whether HNF1 could bind to regions F-2, B, and A. When incubated with HepG2 nuclear extract, fragment F-2 formed two predominant retarded complexes on the native gel (Fig. 4A; the bracketed doublet and single band). Both complexes were sensitive to self-competition by F-2 but were not competed by nonspecific competitor β-lac. F-2 was divided into two nonoverlapping halves, F-2a and F-2b (Fig. 4B). F-2b, which contains the putative HNF1-binding site, inhibited formation of both complexes. A 28-mer containing a known functional HNF1-binding site present in the β-fibrinogen gene promoter (β28) selectively competed only the more slowly migrating complex identifying this as the HNF1 complex. The identity of the faster migrating band was not determined in this study. F-2a showed partial competition for the slower migrating complex; this effect was variable and not further pursued. NIH3T3 nuclear extract did not form any specific retarded complexes with fragment F-2. The DNA-protein doublet formed by fragment B and HepG2 nuclear extracts were specifically inhibited by F-2 and F-2b, suggesting that the same proteins that bound to F-2 were involved in formation of the B fragment complexes. The B fragment complexes were also competed by β28 confirming that they involved HNF1. Binding of HNF1 to fragment A was similarly confirmed by efficient competition of the retarded band by F-2, F-2b, and β28. These mobility shift studies demonstrated that HNF1 bound in vitro to fragments F-2, B, and A.

To delineate precise protein-binding sites in fragments F-2, B, and A, DNAse I footprinting was performed on end-labeled coding strands of each fragment (Fig. 5). HepG2 nuclear extracts protected a 16-bp region (21645 to 21661) in fragment F-2 (left panel) corresponding to the HNF1 site. This was a weak footprint (see below). A somewhat larger 29-bp region (2186 to 2158) was protected on fragment B extending 10 bp 3′ from the HNF1 consensus site (middle panel) to include the half-palindrome of an adjacent predicted glucocorticoid response element consensus sequence (see Table II). A 23-bp region (−65 to −43) including the HNF1-binding site in fragment A was also specifically protected by HepG2 nuclear extracts (right panel). Specificity controls including NIH3T3 nuclear extracts and BSA did not result in protection (Fig. 5 and data not shown). The relative positions of HNF1 consensus sites from the 5′-end of each footprint were similar in all fragments. The footprint on the F-2 fragment appeared weaker...
in intensity, smaller in size, and shortened at its 3'-end compared with that on fragments B and A.

**HNF1 Bound to Fragment B with Higher Affinity Than to Fragments F-2 or A—**A modified competition binding study was carried out to extend the observation that the HNF1 footprints on fragments F-2, B, and A differed in relative intensities. Double-stranded 25-mer oligonucleotides containing the HNF1-binding sequences from each fragment (wtF-2, wtB, and wtA, respectively; Table I) were synthesized and used as competitors in a gel mobility shift assay with the 32P-labeled wtA, respectively; Table I) were synthesized and used as competitors in a gel mobility shift assay with the 32P-labeled β28 HNF1 oligonucleotide as a probe. The purity and amounts of each of the annealed oligonucleotides used as cold competitors were verified by native polyacrylamide gel electrophoresis after 32P-end labeling (data not shown). Incubation of β28 with HepG2 nuclear extract generated a set of retarded bands consistent with the presence of HNF1 in the extract (Fig. 6, 1st lane) (40). Assignment of the major bands was aided by analysis of complexes formed from nuclear extracts of NIH3T3 cells transfected with HNF1α and/or HNF1β expression vectors as follows: the HNF1α homodimers formed the two most slowly migrating bands, and HNF1β homodimers formed the most rapid complex, and HNF1αβ heterodimers were intermediate. The predominance of the HNF1α bands was consistent with the predominance of this HNF1 isoform in HepG2 cells. Competition for the 32P-β28 complexes was carried out with 1-, 4-, and 16-fold molar excess of unlabeled wtF-2, wtB, and wtA oligonucleotides, and signal intensities of the residual bands were quantified. These data revealed that an equimolar excess of wtF-2, wtB, or wtA reduced the labeled HNF1α homodimer complex signal by 12, 87, and 29%, respectively. This result ordered the relative affinities of each HNF1-binding site for HNF1α homodimer binding as follows: fragment B >> fragment A >> fragment F-2. The lowest affinity of fragment F-2 was consistent with its weaker footprint.

**Functional Importance of HNF1 Binding for rDBP Gene Expression—**The above results suggested that HNF1 binds to the F-2 and B enhancer fragments as well as to the rDBP gene promoter (fragment A), although with different affinities. To determine the relative functional importance of HNF1 binding to the F-2 or B enhancer elements, each of the two respective HNF1 sites was disrupted individually or in combination, and the effect on expression was determined (Fig. 7). The HNF1-binding sites were disrupted by introducing base substitutions into the more conserved 5'-half of the HNF1 palindromic sequence, and loss of HNF1 binding was confirmed by gel mobility shift assays (Fig. 7A). The mutF-2 and mutB substitutions were then introduced at their respective positions in the −2196DBP/CAT construct, and the activities of the resultant genes were tested by transfection into HepG2 cells (Fig. 7B). Mutation of the HNF1-binding site in region F-2 or B reduced rDBP promoter activity to 23 and 7% of wild type −2196DBP/CAT expression, respectively (Fig. 7B). When both F-2 and B were mutated, the DBP promoter activity was further decreased to 1% of wild type. These data demonstrated that the HNF1-binding sites in the F-2 and B segments mediated positive enhancer function in HepG2 cells, the higher affinity B site having a greater effect on gene expression than the F-2 site. This enhancing activity was most likely mediated

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**Table I**

| Oligonucleotide name | Sequence |
|---------------------|----------|
| −2196               | 5'-CCCAAGCTTGAATTCTGTGCAAGTA-3' |
| +53                 | 5'-CCCAAGCTTGGAGGATCTCAG-3' |
| 5'-mB               | 5'-TAATCTTCTCCTTTGACCTGGGAA-3' |
| 3'-mB               | 5'-CAAGACAGTTTAAACCTTCCCA-3' |
| 5'-mF-2             | 5'-TAGCTAAGTTGCTGTGCAGACAGAG-3' |
| 3'-mF-2             | 5'-TCAAGCATAGGCAATTTTAAGG-3' |
| wtF-2*              | 5'-CTCTGACACAGACCTCCATAACGACAGAG-3' |
| wtB*                | 5'-AGTGACAAAAGGTATACATCAGACACGAC-3' |
| wtA*                | 5'-TGTCAGAGATTAATTAATCAGACAGAG-3' |
| mutF-2#             | 5'-GTCTGACAGACCTCCATAACGACAGAG-3' |
| mutB#               | 5'-AGTGACAAAAGGTATACATCAGACACGAC-3' |

* The sequence of the footprinted region is shown in italics and the conserved half of the putative HNF1-binding site is indicated in bold.

# The mutated sequence in each HNF1-binding site is shown in bold.
by HNF1α homodimers because they are the predominant form of HNF1 in the HepG2 cells (see above; Fig. 6). Furthermore, the levels of transcriptional enhancement measured when these two sites were functioning in concert was greater than their additive effects, suggesting synergistic interaction.

The above data demonstrated a major contribution of the HNF1 sites to transcriptional enhancement of DBP gene expression in tissue culture cells. To determine whether HNF1a also has an enhancing effect on DBP expression in vivo, Northern analysis for DBP mRNA levels was carried out on mRNA from the livers of mice with targeted disruption of the endogenous HNF1α gene (36). The level of DBP mRNA in the livers of the homozygous null mice (HNF1α−/−) was reduced to 50% that of the heterozygotes (HNF1α+/−) and wild type littermates (data not shown). The magnitude of this decrease, similar to that previously reported for expression of the albumin and

FIG. 6. HNF1 bound to fragment B with higher affinity than to fragment F-2 or A. The affinities of HNF1 for fragments F-2, B, and A were compared using an electrophoretic mobility shift-based competition assay. End-labeled probe, β28, was incubated with HepG2 nuclear extracts, and competition for complex formation was carried out with unlabeled oligonucleotides containing the corresponding HNF1-binding sites in fragments wtF-2, wtB, and wtA (Table I). Binding reactions included each of the three oligonucleotides at 1-, 4-, or 16-fold molar excess over β28 (indicated above respective lanes). Positive controls for the migration of the HNF1α homodimer and the HNF1β homodimer were generated by using nuclear extracts from NIH3T3 cells transfected with an HNF1α or HNF1β expression vector. The position of the HNF1α/HNF1β heterodimer was deduced from the appearance of the intermediate band in cells expressing both HNF1α and HNF1β.

FIG. 7. Mutation of the HNF1-binding sequences in fragments F-2 and/or B disrupted transcription from the rDBP promoter. A, end-labeled fragments F-2 and B were used as a probe in an electrophoretic mobility shift assay with HepG2 nuclear extracts. In each case competition was performed with either the wild type sequence of the HNF1-binding sites in fragments F-2 and B (wtF-2 and wtB) or with HNF1-mutated sequences (mutF-2 and mutB). The sequences of the oligonucleotides are shown below the autoradiograph. The right and left palindrome halves are underlined, and the mutated sequences are shown in bold. B, the diagram shows the −2196DBP/CAT with the three mutations introduced. These constructs (5 μg) were transfected into HepG2 cells. The normalized CAT activities are shown relative to −2196DBP/CAT (indicated as 100).

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-2196DBP/CAT(µg) 5.0 5.0 5.0 5.0 5.0
HNF-1α (µg) - 2.0 2.0 2.0 -
HNF-1β (µg) - - 0.5 1.0 2.0

| NIH3T3 | | | | |
|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 |

**Fig. 8.** HNF1α and HNF1β had distinct effects on rDBP transcription. The -2196DBP/CAT construct was co-transfected in NIH3T3 cells with the indicated concentrations of HNF1α and/or HNF1β expression vectors. A representative CAT assay is shown, and mean CAT activities of three experiments are indicated below the autoradiograph.

β-fibrinogen genes (36), confirmed the importance of HNF1α to DBP gene expression in the liver. These data, along with the mutation studies, documented the functional importance of HNF1α in enhancement of DBP gene expression.

HNF1α and HNF1β Displayed Opposing Effects on Transcriptional Activation of the rDBP Gene—HNF1α and HNF1β share conserved amino-terminal DNA-binding domains but diverge in their carboxyl-terminal transcription activation domains (21). HNF1α is the predominant isoform in hepatocytes and the HepG2 cell line, and it mediates an enhancement of DBP gene transcription. HNF1β is the predominant isoform in kidney and several other organs (21). The functional impact of each of these two HNF1 isoforms on DBP gene expression was further explored by expressing each of these proteins individually or together in NIH3T3 cells along with the wild type -2196DBP/CAT reporter gene. NIH3T3 cells have no endogenous HNF1α activity and were unable to transcribe the -2196DBP/CAT gene (Fig. 8, lane 1). Gel mobility shift assays demonstrated that nuclear extracts prepared from NIH3T3 cells transfected with either the HNF1α and/or HNF1β expression vectors formed new DNA-protein complex(es) with the β28 HNF1-binding site oligomer (Fig. 6). When co-transfected with an HNF1α expression vector, the -2196DBP/CAT gene was expressed in the fibroblasts (Fig. 8, lane 2). A positive, although much less intense, response was also detected when the HNF1β expression vector was co-transfected (Fig. 8, lane 5). Unexpectedly, co-expression of HNF1β with HNF1α was not additive in transcriptional enhancement, but instead HNF1β decreased HNF1α enhancement in a dose-dependent manner (Fig. 8, lanes 2–4). Thus each of the two isoforms of HNF1 had a positive effect on rDBP gene expression independently but differed in the potency of transgene activation. In addition, the two HNF1 isoforms appeared to act antagonistically when co-expressed.

The Negative Effect of HNF1β on HNF1α-induced DBP Gene Transcription Was Mediated by the F-2 and B-binding Sites—The fragments of the DBP promoter and 5′-flanking region that mediated the positive and/or negative effects of the two HNF1 isoforms were determined by co-transfecting HNF1α or HNF1β expression vectors into HepG2 cells along with the wild type -2196DBP/CAT construct or with the constructs containing the defined HNF1-binding site mutations. The levels of HNF1α in HepG2 cells are lower than in adult liver, and transcription of the HNF1α expression vector has been demonstrated previously to result in enhancement of co-transfected albumin or α-fetoprotein gene expression (22). With the increased levels of HNF1α generated by the expression vector, -2196DBP/CAT expression increased 2-fold in the HepG2 cells (Fig. 9A, panel i). HNF1α also modulated the expression of the -2196DBP/CAT constructs containing the mutations of the HNF1-binding sequences at the F-2 site, B site, or both (Fig. 9A, panels ii, iii, and iv). Disruption of the F-2 site (-2196DBPmF-2/CAT) decreased base-line expression to 20% of the wild type construct, and overexpression of HNF1α returned expression to full wild type levels (Fig. 9A, panel ii). In contrast, loss of the B site (-2196DBPmB/CAT) resulted in a more substantial loss of expression to 8% of the wild type construct and overexpression of HNF1α only increased levels to 25% of wild type (Fig. 9A, panel iii). Combined loss of the A and B site (-2196DBPmF-2&B/CAT) further depressed base-line expression to 5% of wild type, and the ability of HNF1α overexpression to enhance expression was as limited as that seen with the single B site mutation (Fig. 9A, panel iv). These results demonstrated the following: (a) that HNF1α was a positive transcription factor for rDBP gene expression, (b) that HNF1-binding sites B and F could each mediate a positive response to HNF1α, and (c) that site B was the main mediator of overall HNF1α enhancing activity. It was further inferred that site A could mediate a positive response by HNF1α based upon residual positive activity in the double mutant (Fig. 9A, panel iv), but this was not rigorously proven.

In contrast to the positive enhancing effect of HNF1α, HNF1β mediated a dose-dependent trans-dominant repressor activity in transfected HepG2 cells (Fig. 9B). The level of -2196DBP/CAT gene activity was decreased by 90% at the highest level of HNF1β provided (Fig. 9B, panel i). This contrasted with the known positive HNF1β effect on the rat α-fetoprotein gene (Fig. 9B, panel i, inset, and see Ref. 15). Co-transfection of HNF1β with each of the three HNF1 mutant constructs demonstrated that the dominant-negative effect of HNF1β could be mediated by the F-2 and B sites. HNF1β markedly decreased the expression of -2196DBPmF-2/CAT at all levels of co-transfection (Fig. 9B, panel ii). In contrast, expression of the -2196DBPmB/CAT gene was slightly enhanced at low amounts of HNF1β, whereas at higher levels HNF1β exerted a negative effect (Fig. 9B, panel iii). The lack of a negative effect by HNF1β on the expression of -2196DBPmF-2&B/CAT (Fig. 9B, panel iv) excluded the site A as a negative-effect mediator. Thus HNF1-binding sites F-2 and B mediated both positive and negative effects on rDBP gene transcription, reflecting the relative levels of the two HNF1 isoforms HNF1α and HNF1β. The B site was the major effector of both the enhancer and trans-dominant repressor activity.

**DISCUSSION**

This study demonstrated multiple roles for HNF1 in transcription of the vitamin D-binding protein gene. Three functional HNF1-binding sites were identified within the proximal 2 kilobase pairs of the rDBP 5′-flanking region. These three sites differed in the details of HNF1 binding and in their impact on rDBP expression. Moreover, the sites mediated unique transcriptional responses to alterations in the balance of HNF1α and HNF1β subunits. The profiles of HNF1 binding and activity for the rDBP gene were distinct from those previously reported for the closely related albumin and α-fetoprotein genes. The overall impact of these three binding sites is likely to underlie the distinct tissue and developmental pattern of rDBP gene expression.

Fragment A (HNF1-binding site; -65 to -41) appeared to constitute part of the rDBP promoter. It was active only when
directly linked to the DBP transcription start site and adjacent basal promoter region (first 40 bases of 5'-flanking region). DNA sequence alignments of the promoters of the albumin, α-fetoprotein, and DBP genes showed conservation of this proximal HNF1 site in all these genes and in species ranging from human to rat, mouse, and Xenopus laevis (41). In the cases of albumin and α-fetoprotein, the proximal HNF1 site was necessary for activity of the respective promoters (42, 43), and in both cases the proximal HNF1 site mediated a positive effect on gene expression. From these previous data in closely related genes and the residual activity in response to HNF1 by the DBP double mutant, a positive regulatory role for the footprinted site in DBP fragment A could be inferred. These data suggested that the proximal HNF1 site is a highly conserved positive element for hepatic gene expression within the multi-gene family.

The two 5' HNF1-binding sites located in fragments F-2 and B behaved like classical enhancer elements. The F-2 and B fragments both increased the activity of homologous and heterologous promoters in a position-independent manner in transfected HepG2 cells (Figs. 1–3). Individual mutation of the HNF1-binding sites at these two positions severely decreased DBP gene expression in HepG2 cells, and the two sites also appeared to act in a synergistic fashion (Fig. 7). Individually, loss of the B site had a more profound effect on the expression of the DBP promoter in HepG2 cells than loss of the F-2 site (Fig. 7). The data were consistent with a predominant role of site B in enhancing expression; the decrease in expression in HepG2 cells due to loss of the F-2 site could be fully compensated by an increase of HNF1a levels acting via the intact B site, whereas loss of the B site could not be compensated in the reciprocal manner (Fig. 9A, panels ii and iii). Thus regions F-2 and B both mediated HNF1-dependent enhancement of DBP promoter activity.

The distinct effects of the two major forms of HNF1, HNF1α, and HNF1β on DBP promoter action were unanticipated. Although both forms enhanced expression of −2196DBP/CAT in fibroblasts, the action of HNF1β was only 5% that of HNF1α (Fig. 8). More surprisingly, co-transfection of the minimally active HNF1β with the highly active HNF1α resulted in a dose-dependent loss of HNF1α-mediated transcriptional enhancement. HNF1β appeared to exert the same antagonistic effect on the HNF1α enhancement in the HepG2 cells, suppressing expression of the −2196DBP/CAT reporter by over 10-fold (Fig. 9B, panel i). The ability of HNF1β to enhance expression from the rDBP promoter in the NIH3T3 fibroblasts but to suppress expression in the HepG2 hepatocytes suggested that the suppressive effect was due to direct interference with preexisting HNF1α-mediated enhancement. This dominant-negative effect of HNF1β on HNF1α enhancement was mediated through both F-2 and B sites (Fig. 9B, panels ii and iii). Thus, in the context of the rDBP promoter, the two 5' HNF1-binding sites, F-2 and B, were each capable of mediating opposing effects of HNF1α and HNF1β on rDBP gene transcription.

Although fragments F-2, B, and A each bound HNF1, the details of these interactions were different. The footprints at the HNF1 sites were distinct as were the affinities for HNF1α as measured in vitro by the competition assay. The relative affinities of the three sites may parallel their distinct HNF1 footprint patterns as both properties probably reflect differences in the less conserved 3'-half of the HNF1-binding site as well as the sequences surrounding the respective sites. The B site, with a 28-bp footprint, bound HNF1 more avidly than the A or F-2 sites that had 22- or 16-bp footprints, respectively. The DNase I footprints generated on fragments F-2 and A by the HepG2 nuclear extracts included only the HNF1 consensus sequences. This does not rule out the possibility of other transcription factor(s) binding to these regions because binding conditions might not be optimal for the association of additional factors or the amount of such factors might not be sufficient in HepG2 cells for detection by DNase I footprinting analysis. For example, HepG2 cells have been shown to contain at least an order of magnitude less C/EBP than adult liver, and a C/EBP site of the hepatitis B virus enhancer is not footprinted by HepG2 nuclear extracts (44). The HNF1 footprint in fragment A had an 11/13 base identity to the α-fetoprotein enhancer referred to as the “AT-rich motif” (5'-ATTTAATAT-TACA-3'). A 306-kDa protein, AT motif-binding factor-1 (ATBF1) has been identified to bind to this motif more efficiently than HNF1 (45, 46). By co-transfection studies ATBF1 selectively suppresses the activity of the α-fetoprotein enhancer and promoter regions containing functional AT-rich motifs, apparently by com-
The putative HNF1-binding sites in region G, F-2, D, B, and A are shown in bold. Sequences that match the HNF1 consensus are shown in capital letters, and the divergent nucleic acids are shown in lowercase letters. The region protected by HepG2 nuclear extracts in DNase I footprinting experiments are underlined. The putative glucocorticoid response element in fragments B is shown in italics.

| Fragment | HNF1 consensus sequence, GGTTAATNATAC(A/C) |
|----------|-------------------------------------------|
| G        | −2075 TAAAAATATACAAATACGTATAATTCTTGACATCGTTGCCTCT −2030 |
| F-2      | −1671 GACTCTGTGCTGCA/GGTTAAATCTTAGCAGGAGATTGTTG −1630 |
| D        | −550 ATGGATCTAGAGAACGGTTAATCCTAGGCTCTGCTC −509 |
| B        | −193 CCCAGTCAGCAC/GGTTAATACGTAAATACGACCTGCAGCTGCTC −152 |
| A        | −76 CTGGCTTGTGCA/GGTTAATACGTAAATACGACCTGCAGCTGCTC −35 |

The protected region in fragment B was larger than that of fragment F-2 and A, extending 10 nucleotides downstream from the HNF1 consensus site. A sequence similar to a glucocorticoid response element (GRE: 5′-GGTACAnnnTGT-TCT-3′ (47)) was detected 3′ to the HNF1-binding site of fragment B (5′-CTCCCCAGCTGCTC-3′; Table II). The extended protection toward this putative GRE and previous reports showing positive regulation of DBP by glucocorticoid (48) suggested that glucocorticoid receptors might also bind to this region, and composite response elements containing GREs have been previously observed (49). The protected region of fragment B also included the sequence 5′-CTCTCC-3′, which is found at the 3′ boundary of many HNF1 sites and has been shown to be important for enhancing the activity of the human prothrombin gene (16). Either of these elements, if functional, may contribute to the higher affinity of the B fragment for HNF1, perhaps by stabilizing the complex.

In this study, HNF1β showed a trans-dominant negative effect on HNF1α-stimulated activity of the DBP promoter. This was mediated by an apparent interference with the positive effects of HNF1α at the same sites. The human vHNF1-C isoform in which the activation domain has been interrupted by alternative splicing was previously identified as a trans-dominant repressor as well (28). In contrast, HNF1β has been demonstrated to have a positive effect on some binding sites such as those in albumin and α-fetoprotein, or more commonly no functional effect at all, such as in the β-fibrinogen promoter (21), the sucrose-isomaltase promoter (50), and the CYP2E1 promoter (51). The HNF1β expression vector used in the present study had been cloned from mouse (21), and DNA sequence analysis of this clone and comparison to published sequences confirmed that it was the murine counterpart of the full-length human vHNF1-B, clearly distinguishing it from vHNF1-C (data not shown). Previously, HNF1β has been inferred to mediate a negative feedback loop on its own expression, but this effect was indirect, via down-regulation of HNF4 (52, 53). Several additional reports have documented direct trans-dominant repressor activity by HNF4 as well as C/EBP (54–57). None of these reports appear to relate to the presently described effect of HNF1β.

Several mechanisms might be considered to explain the observed negative regulation by HNF1β on DBP gene expression observed in the current report. It was unlikely that the negative effect of HNF1β on the DBP promoter resulted from squelching of the general transcriptional machinery or from direct transcriptional down-regulation of HNF1α expression because such mechanisms would be expected to affect the expression from HNF1α-dependent α-fetoprotein promoter similarly, and this was not the case (Fig. 9B, panel i, inset). It is feasible that overexpression of HNF1β may alter the transcriptional activity of HNF1α by sequestration of cofactors such as the 11-kDa dimerization cofactor of HNF1 (DcoH) required for the positive activity of HNF1α homodimers via stabilization of dimer binding (58, 59). Since DcoH can also bind HNF1β, HNF1β might sequester DcoH and decrease the transcriptional activity of HNF1α homodimer by destabilizing it. This mode of inhibition of HNF1α, however, would also be expected to affect α-fetoprotein expression and thus appears unlikely. Direct competition of a nonfunctional or less functional HNF1β homodimer or αβ heterodimer with an active HNF1α homodimer for DNA binding at the DBP enhancer sites might be the most simple mechanism for this observed gene-specific trans-dominant repression. A differential ability of the competing dimers to assemble at the various promoters (DBP versus α-fetoprotein) might explain the disparate effect on these two genes.

The trans-dominant effect of HNF1β on the DBP promoter activity was different than its effect on the albumin as well as the α-fetoprotein promoter. The enhancement of the α-fetoprotein promoter by HNF1β is greater than by HNF1α, whereas HNF1β and HNF1α have comparable enhancing effects on the albumin promoter (15). However the positive effect of HNF1β on the extended −2196DBP promoter is only 5% that of HNF1α in cotransfected NIH3T3 cells (Fig. 8), and there is no enhancement by HNF1β on the promoter containing the HNF1 A site alone (Fig. 9B, panel iv). Moreover, HNF1β abolished the HNF1α-mediated enhancement of DBP promoter activity (Fig. 7), but transcriptional activation by HNF1α on the albumin promoter is unaffected by HNF1β (22). Finally, the same level of HNF1β that decreased DBP promoter activity to 10% in transfected HepG2 cells resulted in a 4-fold increase in expression from the α-fetoprotein promoter (Fig. 9B, panel i, inset). The basis for the distinct, gene-specific effects of HNF1β on these various promoters may reflect several potential mechanisms. It has been shown that the composition of core promoters can differentially regulate the transcriptional response to a given transcription factor (60, 61). For example, tumor suppressor p53 protein specifically represses the activity of TATA box-dependent promoters, but it has no effect on promoters directed by a pyrimidine-rich initiator element (62). Various TATA sequences have been reported to vary in their responses to different upstream regulators (63). In this regard, it is interesting to note that the DBP promoter does not contain a classic TATA box, instead it has the sequence TGTG, whereas a classic TATA is present in the promoters of the α-fetoprotein and albumin genes (25). This duality of the transcriptional activity of HNF1β on various promoters may depend upon protein conformation that can be influenced by the nature of the DNA-binding site and/or may trigger differential interactions with distinct cofactors.

The functional importance of HNF1α in DBP expression was supported by the 50% decrease in expression of the endogenous DBP gene in the liver of HNF1α−/− mice. This decrease was remarkably similar to that observed for the albumin and β-fibrinogen genes. As shown by the fibroblast co-transfection ex-

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3 Y.-H. Song and N. E. Cooke, unpublished observations.
periments (Fig. 8), HNF1β directly enhanced DBP promoter function in an environment lacking HNF1α although this enhancement was only 5% of the effect observed with an equivalent amount of HNF1α. Therefore in the HNF1α-deficient mice, the net effect of HNF1β on DBP expression might be positive and sustain a minor level of DBP expression. The doubling of the HNF1β mRNA level in the livers of the HNF1α-deficient mice (36) might further accentuate this positive effect. The fact that the endogenous mouse DBP gene was still expressed in liver at appreciable levels may also reflect control by other, as yet unidentified, transcription factors acting on the DBP gene.

The partial loss of endogenous mouse DBP gene expression in HNF1α−/− mice is thus consistent with the finding in cell culture that HNF1β homodimers can support DBP promoter activity in the absence of HNF1α.

A model to explain liver-restricted expression of the DBP gene can be proposed based upon the observation that the DBP gene is oppositely regulated by HNF1α and HNF1β and on the different tissue distributions of HNF1α, HNF1β, and DCoH. Gel mobility shift assays showed that HNF1α homodimers are more highly represented than the HNF1α/HNF1β heterodimers in the adult liver, whereas the heterodimers are more abundant than both types of homodimers in the kidney where DBP is expressed at much lower levels (21). Like HNF1, the DCoH gene is also expressed in a tissue-specific way, being most abundant in liver and kidney with less detected in intestine and stomach (58). Although HNF1α in the kidney can be stabilized by DCoH, the trans-dominant negative activity of HNF1β present at high levels in this organ (21) may overcome the positive effect of low levels of HNF1α and suppress DBP gene expression. Therefore, high level expression of DCoH and HNF1α compared with HNF1β would result in expression in the liver, and the reversal of this balance would result in a relative repression of DBP expression in the kidney. Although this model fits the presently available data, it is no doubt overly simplistic and does not take into account the expected contributions and/or interactions with other transcription factors involved in rDBP gene expression in vivo.

The 5′-flanking region of the rDBP gene was studied to determine the basis for its liver-specific expression. As was the case for other members of the DBP multigene family, HNF1 was identified to be a major regulator of DBP gene expression. Importantly, the mechanism of DBP gene regulation was clearly unique; the relative abundance of HNF1α and HNF1β played a critical role in determining the pattern of DBP gene expression in a genespecific fashion. Based on these findings it is reasonable to predict that this reciprocal regulation by the two subunits of HNF1 may contribute to expression patterns of certain other genes in HNF1-containing cell types and that the mechanism(s) that mediate their opposing effects can now be investigated.

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36. Perou, M., Barra, J.,值得一提的是，基因HNF1β的表达在肝脏中明显高于肾脏，而基因HNF1α的表达则在肾脏中占主导地位。这种差异的产生可能是由于肝脏和肾脏中HNF1α和HNF1β的表达水平不同。在肝脏中，HNF1α的表达水平远高于HNF1β，而在肾脏中，HNF1β的表达水平远高于HNF1α。因此，肝脏中DBP的表达水平受到HNF1α和HNF1β的共同调节，而肾脏中DBP的表达水平则主要由HNF1β调节。这表明HNF1α和HNF1β在基因表达调控中具有不同的作用和机制。