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The Transcriptional Activator Hepatocyte Nuclear Factor 6 Regulates Liver Gene Expression

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The hepatocyte nuclear factor 3α (HNF-3α), -3β, and -3γ proteins share homology in the winged-helix/*fork head* DNA binding domain and mediate hepatocyte-enriched transcription of numerous genes whose expression is necessary for organ function. In this work, we identify a liver-enriched transcription factor, HNF-6, which recognizes the −138 to −126 region of the HNF-3β promoter and binds the original HNF-3 site of the transthyretin promoter (−94 to −106). We show that HNF-6 and HNF-3 possess different DNA binding specificities by competition and methylation interference studies and are immunologically distinct. Site-directed mutagenesis of the HNF-6 sites in the HNF-3β and transthyretin promoters diminishes reporter gene expression, suggesting that HNF-6 activates transcription of these promoters. Using the HNF-6 binding sequence DHWATTGAYTWWD (where W = A or T, Y = T or C, H is not G, and D is not C) determined by sequence comparison and methylation interference, we predicted that HNF-6 will bind to 22 additional hepatocyte-enriched genes. Of these potential target genes, we selected seven of the HNF-6 binding sequences and demonstrated that they bind the HNF-6 protein. These include promoter sequences from α-2 urinary globulin, α-1 antitrypsin, cytochrome P-450 2C13, L-type 6-phosphofructo-2-kinase, mouse major urinary protein, tryptophan oxygenase, and α-fetoprotein genes. HNF-6 binding activity was also found in the intestinal epithelial cell line HT29, and potential HNF-6 binding sites were present in intestinal sucrase isomaltase, cdx-2 homeodomain protein, and intestinal fatty acid binding protein promoter regions. These studies suggest that HNF-6 may regulate hepatocyte-specific genes and may play a role in epithelial cell differentiation of gut endoderm via regulation of HNF-3β.

Cell-type-specific transcription is the first event, and perhaps one of the most crucial events, leading to expression of specific genes in differentiated cell types (16). Transthyretin (TTR) is a serum carrier protein expressed abundantly and specifically in only two organs, the liver and the choroid plexus (21). The regulatory regions necessary and sufficient for directing TTR expression to the liver have been determined by experiments in transgenic mice and consist of a 100-nucleotide enhancer located at −2 kb upstream and a proximal −150 to −90 bp promoter region (13, 83). These regulatory regions have served as a model for understanding cell-type-specific transcription in our laboratory (12, 13, 15, 59). Characterization of the factors binding to the TTR regulatory regions (Fig. 1) and those of other liver-enriched genes has resulted in the hypothesis that a number of factors converge to regulate liver-enriched gene expression (84).

The known liver-enriched transcription factors include members of the winged-helix/*fork head* DNA binding domain family (10) designated hepatocyte nuclear factor 3α (HNF-3α), -3β, and -3γ (38, 39); the steroid hormone receptor family members HNF-4 (73) and apolipoprotein AI (Apo AI) regulatory protein 1 (37); the POU homeodomain members HNF-1α and -1β (5, 20); the CCAAT enhancer binding protein (C/EBP) bZIP family members α, β, and δ (1, 8, 17, 35, 40, 41, 57, 82); and finally the proline and acidic amino acid-rich (PAR) bZIP family members albumin D-box binding protein (DBP) (49), vitellogenin binding protein (VBP) (19, 30), and hepatic leukemia factor (HLF) (26, 28).

Most of these factors, although not themselves liver specific, have been determined to regulate many liver-enriched genes and maintain their cell-type-specific expression by combinatorial interaction on their promoters (84). In the adult rodent, HNF-3β is expressed at low levels in the small and large intestines and lung and at higher levels in the liver and pancreas (34, 48). HNF-3 target genes in hepatocytes include the genes for the serum carrier proteins TTR, albumin, α-fetoprotein (AFP), Apo B, Apo AI, transferrin, and insulin-like growth factor binding protein 1 (4, 7, 13, 18, 23, 45, 78); the antiprotease α-1 anti-trypsin (α1AT); and the enzymes tyrosine aminotransferase (TAT), cholesterol 7α-hydroxylase, phosphoenolpyruvate carboxykinase (PEPCK), L-type 6-phosphofructo-2-kinase (PFK-2), aldolase B, and cytochrome P-450 2C6 (CYP2C6) (29, 47, 51, 61, 71); complement protein C (74); and the human hepatitis B virus (9, 52, 60). In support of HNF-3's role in regulating the expression of these hepatocyte-enriched genes, a hepatoma cell line which expresses a dominant-negative HNF-3 mutant extinguished transcription of several HNF-3 target genes (79). Furthermore, in vivo footprinting studies of the −10 kb albumin enhancer region have demonstrated that the HNF-3 proteins are involved in organizing the nucleosome architecture of the albumin enhancer in hepatocytes (46). Thus, the HNF-3 proteins not only contribute to transcriptional activation, but may also be required for the establishment of hepatocyte-specific accessibility within these genes' regulatory regions.

Mammalian HNF-3 (38, 39) and the *Drosophila* homeotic gene *fork head* (80) are prototypes of a large family of transcription factors that share homology in the winged-helix DNA binding domain and are involved in the differentiation of diverse cellular lineages (for review, see references 11 and 25). The HNF-3α and HNF-3β genes are expressed during the gastrulation stage of embryogenesis (3, 48, 65, 68) and are induced during retinoic acid differentiation of the F9 embry-

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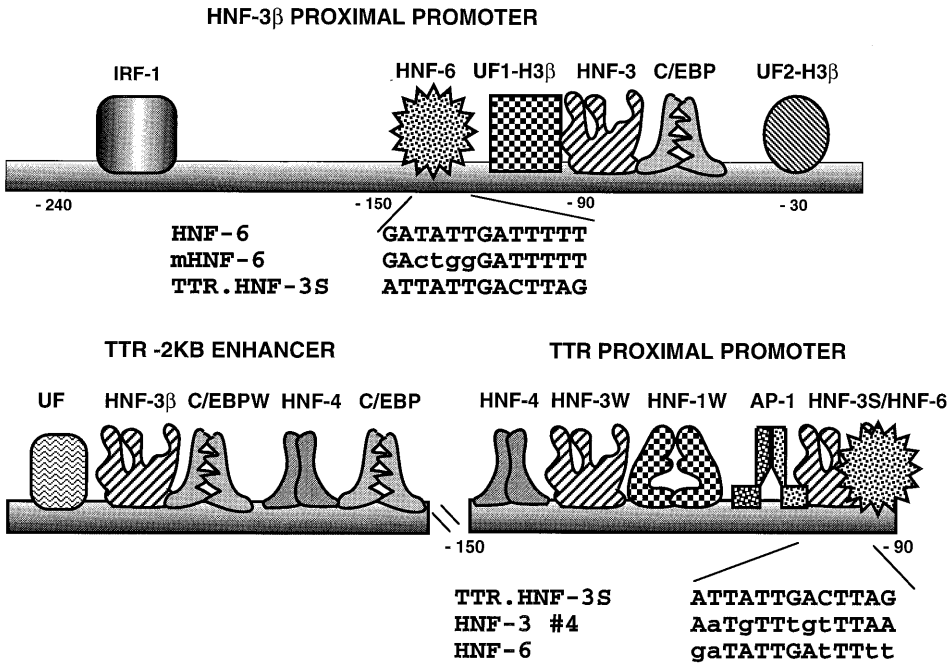


FIG. 1. Transcription factors binding to the TTR and HNF-3β regulatory regions. Schematically shown are the HNF-3β and TTR promoter constructs and their corresponding transcription factors. The TTR regulatory regions are bound by members of four different liver-enriched transcription factor families (HNF-1, HNF-3, HNF-4, and C/EBP proteins [13, 14]) and the growth factor-inducible AP-1 protein (59). The W or S indicates either weak- or strong-affinity binding sites. The TTR enhancer is also recognized by an uncharacterized ubiquitous factor (UF) and contains an HNF-3 binding site which is selectively recognized by the HNF-3β isoform (67). The HNF-3β promoter is regulated by two liver-enriched factors (HNF-3 and C/EBP) and three generally expressed factor binding sites (UF1-H3β and UF2-H3β and the interferon response factor-1 [IRF-1]) (54, 66). The −138 to −127 HNF-3β promoter sequence possesses homology with the TTR.HNF-3S site (−94 to −106). In this study, we identify a new liver-enriched transcription factor, HNF-6, that recognizes the −138 to −127 HNF-3β and the TTR.HNF-3S site promoter sequences.

onic carcinoma cell line (31, 62). Targeted disruption of the HNF-3β gene results in mouse embryos that are resorbed early in development after failing to form proper neurotube, node, somite, notochord, and definitive gut structures (2, 81). Furthermore, ectopic hindbrain expression of the HNF-3β gene in transgenic mice mediates the conversion of hindbrain to floorplate, as evidenced by activation of the endogenous HNF-3α and HNF-3β genes and other floorplate marker genes (69). Taken together, these embryonic studies indicate that the HNF-3 proteins play an integral regulatory role in cellular commitment events.

Our laboratory has analyzed the HNF-3β promoter in order to identify the transcription factors responsible for enriched expression of this gene in the liver. We initially found that at least five different proteins bind to the proximal −245 nucleotides (Fig. 1) (54, 66). Two of these transcription factors are widely expressed and thus have been designated ubiquitous factor-1–HNF-3β (UF1-H3β) and UF2-H3β. Two other binding sites in the HNF-3β promoter bind liver-enriched proteins, including HNF-3 itself (an autoregulatory site) and C/EBP or PAR bZIP family members. Finally there is an interferon regulatory factor 1 (IRF-1) binding site that mediates responsiveness to gamma interferon just as the C/EBP site mediates responsiveness to interleukins 1 and 6 (66).

In this work, we identify a liver factor, HNF-6, which recognizes transcriptionally important sequences in the HNF-3β (−138 to −126 bp) and TTR (−94 to −106 bp) promoter regions (13). Our results suggest that the HNF-6 and HNF-3 proteins possess distinct DNA recognition and immunological properties and that the HNF-6 protein complex formation is not inhibited by binding sites for the other known liver-enriched transcription factors. We demonstrate that HNF-6 binding activity is restricted to nuclear extracts prepared from the liver and the intestinal epithelial HT-29 cell line. We used the HNF-6 consensus to identify 22 additional potential liver-enriched target genes and several genes expressed in intestinal epithelium. We selected seven of these putative HNF-6 binding sites and demonstrated that HNF-6 binds to these promoter sequences and thus identified these hepatocyte-enriched genes as potential targets for HNF-6 regulation. One of these HNF-6 binding sites was the L-type PFK-2 promoter site IV (LP4), which was previously shown to bind a liver factor that is inhibited by the TTR.HNF-3 site, but that was not disrupted by HNF-3 antibodies (44). Our studies extend the observations of Lemaigre et al. (44) by demonstrating that HNF-6 is important not only for the regulation of a wide variety of genes expressed in hepatocytes and, putatively, intestinal epithelium, but also for the inception of gut endoderm-derived organs via activation of the HNF-3β protein.

MATERIALS AND METHODS

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed with polyacrylamide gel-purified, double-stranded oligonucleotides (DNA International) that were radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) and rat tissue nuclear extracts (58, 59). Briefly, the binding reaction mixture was incubated at room temperature for 30 min and consisted of a 20-μl reaction mix containing 1 ng of ^{32}P end-labeled double-stranded oligonucleotide, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.9]), 4% Ficoll, 2 mM $MgCl_2$, 40 mM KCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 4 μg of poly(dI-dC):poly(dI-dC) (Pharmacia), 4 μg of salmon testes DNA (Sigma), and 4 μg of nuclear extract. After this incubation period, the binding reaction mixture was then loaded and electrophoresed on a nondenaturing 9% polyacrylamide gel to separate the protein-DNA complexes from unbound DNA probe. A 200-fold molar excess of unlabeled oligonucleotide was included in the reaction when competition experiments were performed unless otherwise indicated in the figure legend. The

relevant sequences of the oligonucleotides used are given in the figures, and their references are as follows: the winged-helix DNA recognition sites HNF-3/*fork head* homolog-1#3 (HFH-1#3) and HNF-3#4 isolated via in vitro DNA binding site selection (53), the strong-affinity HNF-3 site from the TTR promoter (TTR.HNF-3S; −111 to −88 bp) (13), the HNF-3 site from the HNF-3β promoter (HNF-3β; −97 to −83 bp) (54), the HNF-3β isoform-specific binding site from the TTR enhancer (TTR-2) (67), the weak-affinity HNF-3 site from the TTR promoter (TTR.HNF-3W; −150 to −130) (13), the HNF-3 site (−485 to −457 bp) from the HNF-3α promoter (56), the HNF-3 site (−125 to −104 bp) in the CC10 promoter (70), the HNF-1 site from the β-fibrinogen promoter (5), the HNF-4 site from the Apo CIII promoter (73), the C/EBP site from the hemopexin promoter (57), the HNF-3α promoter sites −128 to −108 and −2 to +31 (56), the GATA-6 binding site oligonucleotide (a gift from John B. E. Burch) (42), and Hox A5 binding sites generated from in vitro DNA binding site selection (55). HNF-3β protein was generated via in vitro transcription and translation of the full-length HNF-3β cDNA cloned into pGEM1 with the TNT rabbit reticulocyte lysate kit (Promega). For the antibody supershift analyses, antibody-protein complexes were formed for 10 min, followed by the addition of DNA probe and further incubation for 30 min prior to gel electrophoresis as described previously (59).

Construction of site-directed mutants, transfections, and CAT assays. Site-directed mutagenesis was performed via flanking oligonucleotide PCR amplification of mutant oligonucleotide-generated PCR products (24). Mutations were confirmed by dideoxynucleotide sequencing. Human hepatoma HepG2 cells were maintained as described previously (58) and transfected by lipofection as per the manufacturer's protocol (Gibco) with 10 μl of lipofectin per plate and with a cytomegalovirus promoter-driven reporter β-galactosidase construct to normalize for transfection efficiency. Cells were harvested 48 h later for determination of cytoplasmic chloramphenicol acetyltransferase (CAT) enzyme levels with ^{14}C-chloramphenicol (ICN) and *n*-butyryl coenzyme A (Pharmacia) substrates for determination of product formation via liquid scintillation counting (58) of the xylene-extracted *n*-butyryl chloramphenicol. Experiments were performed more than three times to generate meaningful average values and standard deviations.

Methylation interference assays. Oligonucleotides to be analyzed for methylation interference assays were treated with kinase with [γ-^{32}P]ATP on one strand and then annealed to the unlabeled complementary DNA strand. Partial methylation of guanine nucleotides in these oligonucleotides was accomplished with 1 μl of dimethylsulfate in 200 μl of reaction buffer (50 mM sodium cacodylate, 0.1 mM EDTA) as described previously (54). Uniquely end-labeled oligonucleotide binding sites were mixed with rat liver nuclear extract under conditions identical to those used for the EMSA. The protein-DNA mix was electrophoresed on a native 7.5% polyacrylamide gel, and the bound and free oligonucleotides were eluted. Methylated guanine residues in the HNF-6 site which disrupt protein binding were absent in the protein-bound probe and thus were not cleaved by piperidine. The methylated DNA was cleaved with 0.1 M piperidine at 90°C for 30 min, and after three cycles of freeze-drying to remove piperidine from the pellets, 5% of the DNA was counted via liquid scintillation so that equally radioactive quantities of DNA could be electrophoresed on a 14% acrylamide–8 M urea gel. An additional aliquot of labeled oligonucleotide was treated with formic acid prior to piperidine cleavage for the G-plus-A reaction mixture, which was also loaded on the gel as a nucleotide sequence marker. In order to examine HNF-3 binding to the TTR site, the −146 to −122 oligonucleotide from the HNF-3β promoter was used to compete for HNF-6 binding activity. Conversely, to examine HNF-6 binding, the HFH-1#3 site was used to compete for HNF-3 binding.

RESULTS

The liver-enriched HNF-6 protein binds the HNF-3β promoter and possesses DNA recognition properties distinct from HNF-3. In the course of elucidating the factors regulating the HNF-3β promoter, we performed computer-assisted searches of the promoter sequences for a variety of transcription factor binding sites, including the consensus HNF-3 binding sequences. The computer search indicated that there was a putative HNF-3 site at −138 to −127 of the HNF-3β promoter (Fig. 1), which resembled the HNF-3 binding site derived from the TTR promoter (ATTATTGACTTAG). This HNF-3β promoter site is also similar to a subset of HNF-3 binding sites derived from liver-enriched promoters (AWTRTTKRYTY; where R = A or G, W = A or T; K = G or T, and Y = T or C), but it does not adhere to the HNF-3 binding sequence consensus obtained from in vitro binding site selection (RW WTRTTTRYTY) (53).

We synthesized an oligonucleotide to the −146 to −122 bp region of the HNF-3β promoter and used it to perform

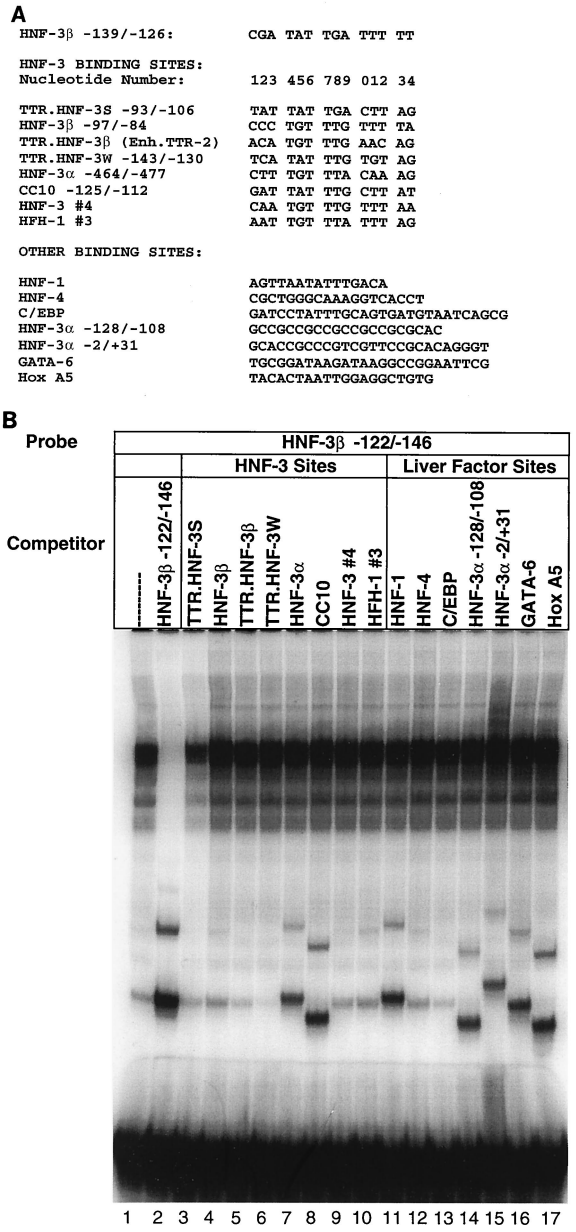


FIG. 2. None of the binding sites for liver-enriched factors diminish protein-DNA complex formation with the HNF-3β −146 to −122 promoter site. (A) Relevant sequences of oligonucleotides used in EMSA competition experiments are listed and are described in Materials and Methods. Shown is the sequence of the HNF-3β −146 to −122 binding site and HNF-3 binding sites derived from various promoters and in vitro DNA binding site selection which were used in our competition studies (B). Also shown are binding sequences and two HNF-3α promoter binding sites (56) which are recognized by other liver-enriched transcription factors. (B) Competition studies with binding sites for other liver-enriched factors in an EMSA indicate that none of them diminish protein-DNA complex formation with the HNF-3β −146 to −122 promoter site. Included are six different HNF-3 sites from various promoters (lanes 3 to 8) and two that were generated via binding site selection (lanes 9 and 10). Also sites that bind the well-characterized liver factors HNF-1, HNF-4, C/EBP, GATA-6, and Hox A5 and two HNF-3α promoter sites binding uncharacterized liver factors (lanes 11 to 17) were examined in competition EMSA.

EMSAs with rat liver nuclear extract in order to determine if HNF-3 proteins were binding to this site (Fig. 2). The HNF-3β −146 to −122 oligonucleotide formed specific protein-DNA complexes with liver nuclear extracts which were inhibited by a

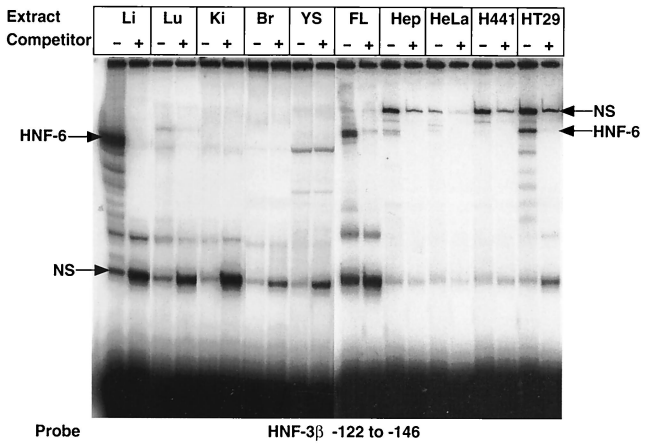


FIG. 3. HNF-6 binding activity is seen only in the liver and liver cell lines. Results from an EMSA with nuclear extracts prepared from rat organs or tissue culture cells and the HNF-3β −146 to −122 promoter site are shown. Nuclear extract from several different rat organs, including liver (Li), lung (Lu), kidney (Ki), brain (Br), yolk sac (YS), and fetal liver (FL), and several cell lines, including the human HepG2 hepatoma, cervical carcinoma epithelial HeLa, bronchiolar epithelial Clara cell line H441, and intestinal epithelial HT29 cell line, was bound to the HNF-3β −146 to −122 promoter site and subjected to EMSA. Lanes with a + indicate self-competition. Because only nuclear extracts from liver, fetal liver, HepG2, and HT29 cells contain binding activity to the HNF-3β −146 to −122 promoter site, we designate this binding activity as HNF-6. Less-abundant specific HNF-6 complexes are also found in lung nuclear extracts.

100-fold excess of unlabeled HNF-3β −146 to −122 oligonucleotide (Fig. 2B, lanes 1 and 2). Competition studies with oligonucleotides corresponding to a variety of HNF-3 binding sites (sequences given in Fig. 2A) suggested that the HNF-3β −146 to −122 protein possessed DNA binding properties that were distinct from those of the HNF-3 proteins (Fig. 2B, lanes 4 to 10). Protein complex formation was not inhibited by HNF-3 binding sites derived from either promoter regions (Fig. 2, lanes 3 to 8) or in vitro binding site selection (53), even though the latter recognize multiple winged-helix family members (Fig. 2B, lanes 9 and 10). Competition with binding sites for other liver-enriched transcription factors, which include HNF-1, HNF-4, C/EBP family members, GATA-6, HoxA5, and two binding sites from the HNF-3α promoter region, also did not disrupt protein complex formation with the HNF-3β −146 to −122 site (Fig. 2B, lanes 11 to 17). These studies suggest that a protein which possesses binding properties that are distinct from other known liver-enriched factors recognizes the HNF-3β −146 to −122 promoter binding site.

While we had determined that the HNF-3β −146 to −122 binding activity is present in liver, we had not determined whether it is also present in other organs. EMSA was performed with the HNF-3β −146 to −122 oligonucleotide and nuclear extract generated from rat liver, lung, kidney, brain, yolk sac, and fetal liver (17 days postcoitum) and also from human hepatoma HepG2 cells, cervical epithelial carcinoma HeLa cells, the pulmonary Clara cell line H441, and the intestinal epithelial cell line HT29 (Fig. 3). All of these nuclear extracts contained active protein capable of binding several ubiquitous binding sites as demonstrated by EMSA (data not shown). The HNF-3β −146 to −122 sequences formed specific protein-DNA complexes with nuclear extract prepared from the liver, the fetal liver, HepG2 cells, and, to a relatively lesser extent, the lungs (Fig. 3). Specific complexes were also observed with the HNF-3β −146 to −122 site and nuclear extracts from HT29 cells (Fig. 3), an intestinal epithelial cell line which also expresses the HNF-3α and -3β proteins (data not shown). HeLa cells also contained proteins which formed complexes with the HNF-3β −146 to −122 oligonucleotide, but these complexes possessed mobility and intensity different from those of other cell or tissue types (Fig. 3). Previous studies demonstrated that liver and lung nuclear extracts form abundant HNF-3α and -3β protein-DNA complexes (53), but these protein binding patterns differ from those observed with the HNF-3β −146 to −122 binding site. Since the liver-enriched protein recognizing the HNF-3β −146 to −122 sequence is distinct from other known liver transcription factors, we have designated this binding protein as HNF-6.

HNF-6 is immunologically distinct from the HNF-3 proteins and recognizes the TTR HNF-3S binding site. Unlike other high-affinity HNF-3 binding sites, the TTR promoter sequence (TTR.HNF-3S; −111 to −88) was able to partially compete for HNF-6 binding activity to the HNF-3β −146 to −122 site (Fig. 2, lane 3). This suggested that the HNF-6 protein may also recognize the TTR.HNF-3S site. In order to further characterize the relative binding affinities of HNF-6 for these sites, we performed competition studies with the HNF-3β −146 to −122 probe and liver nuclear extract, which included increasing amounts of each of the unlabeled oligonucleotide competitors (10-, 100-, and 300-fold). This competition study (Fig. 4A) demonstrated that while the HNF-3β −146 to −122 site could inhibit its own complex formation with as little as a 10-fold molar excess, a 300-fold molar excess of unlabeled TTR.HNF-3S competitor DNA was required to diminish complex formation with the HNF-3β −146 to −122 site. Furthermore, mutations which alter the sequence of the HNF-3β −146 to −122 binding site in the region of homology with the TTR.HNF-3S sequence (Fig. 1) render it incapable of inhibiting HNF-6 complex formation (Fig. 4A, mut.HNF-3β). These DNA binding studies suggest that HNF-6 has a higher relative affinity for its site in the −146 to −122 region of the HNF-3β promoter than for the TTR.HNF-3S site.

In order to determine if HNF-6 protein is immunologically distinct from the HNF-3 proteins, antibodies to the HNF-3α, -3β and -3γ isoforms were included in an EMSA to supershift their respective complexes (Fig. 4B and data not shown). Inclusion of each of the HNF-3 isoform-specific antisera in the EMSA did not disrupt complex formation with the HNF-3β −146 to −122 sequence (Fig. 4B, lanes 5 to 7 and data not shown). These results provide further support that the HNF-3 and HNF-6 binding activities are the result of distinct proteins. To demonstrate the position of the HNF-6 complex, the TTR.HNF-3S oligonucleotide was also labeled and used to perform EMSA with all of the same competitors and antibodies (Fig. 4B, right panel). Inhibition of the TTR.HNF-3S protein complexes by the HFH-1#3 site eliminated the formation of the HNF-3 bands but failed to inhibit the HNF-6 protein complex (Fig. 4B, indicated by the white dot in lane 11). The HNF-6 protein complex was clearly inhibited from the TTR.HNF-3S sequence by the HNF-3β −146 to −122 oligonucleotide, which did not inhibit the formation of the HNF-3 protein complexes (Fig. 4B, lane 10). HNF-3 isoform-specific antisera did not disrupt HNF-6 protein-DNA complex formation, providing further evidence that HNF-6 is immunologically distinct from the HNF-3α, -3β, and -3γ proteins (Fig. 4B, lanes 12, 13, and 14).

The HNF-6 binding site is essential for HNF-3β and TTR promoter activity. While the preceding EMSA and antibody supershift experiments demonstrate HNF-6 protein recognition of two different promoters, they do not predict an effect of HNF-6 on promoter activity. We performed site-directed mutagenesis of the HNF-6 site in the HNF-3β promoter and of

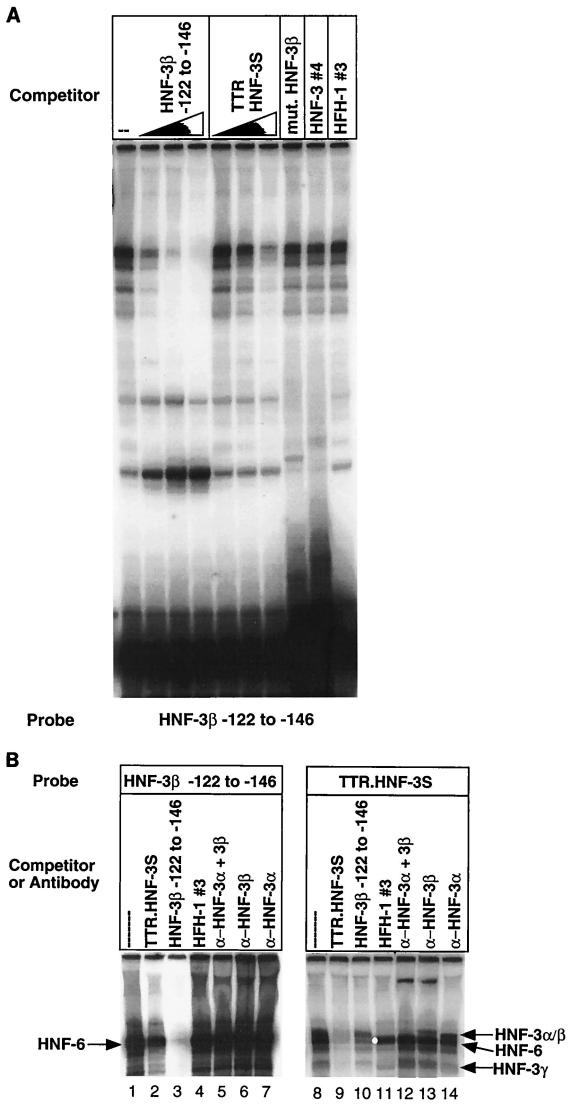


FIG. 4. HNF-6 is immunologically distinct from HNF-3 and recognizes the TTR HNF-3S site. (A) HNF-6 has a higher affinity for the HNF-3β promoter site than the TTR.HNF-3S site. Rat liver nuclear extract was incubated with the HNF-6 site in the HNF-3β promoter and subjected to EMSA in the presence of 10-, 100-, or 300-fold of unlabelled self-competing (−122 to −146) or cross-competing (TTR.HNF-3S; −111 to −88) oligonucleotide. Also included in this EMSA is competition with two of the sites used for site-directed mutagenesis (mut.HNF-3β and HNF-3 4 [Fig. 1]), which do not bind the HNF-6 protein. (B) HNF-3 antibody supershift demonstrates that HNF-6 protein is distinct from HNF-3. EMSAs were performed with rat liver nuclear extract and labeled HNF-6 binding site from the HNF-3β promoter (−122 to −146) or the HNF-3/HNF-6 binding site from the TTR promoter (TTR.HNF-3S site). Included are competitions with self (lanes 3 and 9), cross competitions (lanes 2 and 10), and competitions with the HFH-1#3 site, which binds multiple winged-helix family members (lanes 4 and 11). While self-competition of the TTR.HNF-3S site (lane 9) eliminates all protein-DNA complex formation, competition with the HNF-6 site from the HNF-3β promoter (lane 10) disrupts primarily HNF-6 binding, and competition with the HFH-1#3 binding site (lane 11) conversely disrupts all of the protein-DNA complexes, except for that of HNF-6 (white dot). Addition of affinity-purified specific antibody for either HNF-3α (lanes 7 and 14), -3β (lanes 6 and 13), or both (lanes 5 and 12) or HNF-3γ antisera (data not shown) demonstrated that HNF-6 is distinct from HNF-3. Antibody specific for HNF-3α and -3β (lanes 12, 13 and 14) supershifted complexes formed by their respective proteins but not those by the HNF-6 protein.

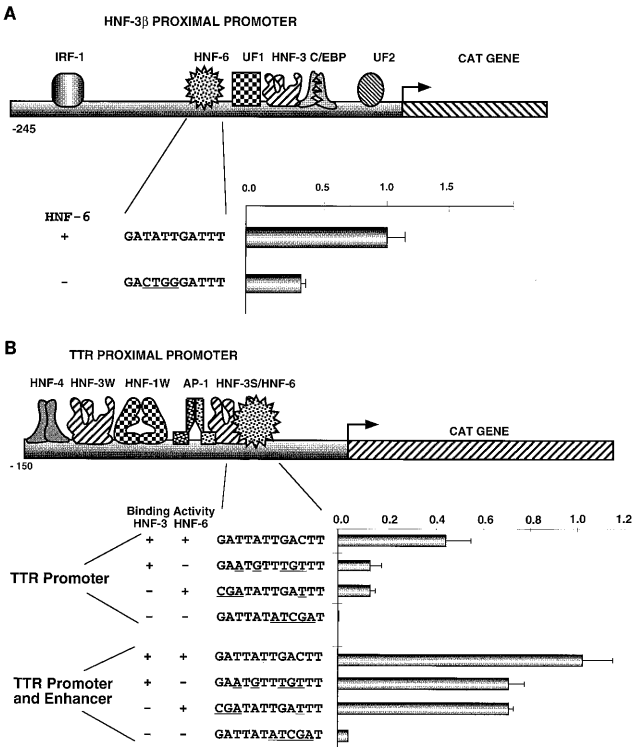


FIG. 5. HNF-6 contributes to TTR and HNF-3 promoter activity. (A) Targeted disruption of the HNF-6 site in the HNF-3β promoter reduces promoter activity. A −245 HNF-3β promoter construct containing a mutated HNF-6 binding site (Fig. 4A, mut.HNF-3β) driving the expression of the CAT gene was generated via site-directed mutagenesis (schematically shown). Both the mutant and wild-type constructs were transfected separately into the hepatoma HepG2 cell line for analysis of promoter activity as described in Materials and Methods. The −245 HNF-3β promoter construct with the mutated HNF-6 binding site retained 37% of the activity of the wild type, suggesting that HNF-6 is a transcriptional activator of the HNF-3β promoter. (B) Targeted disruption of the TTR.HNF-3S site to one that binds either HNF-3 or HNF-6 alone or that binds neither protein results in decreased TTR promoter activity. The wild-type TTR.HNF-3S site (GATTATTGACTT) binds both HNF-3 and HNF-6 proteins. Site-directed mutagenesis was performed to generate TTR promoter CAT constructs (schematically shown) which bind either HNF-3 only (HNF-3#4; Fig. 2 and 4; GAATGTTTGTTT) or HNF-6 only (HNF-3β, −139 to −128 bp; Fig. 2 and 4; CGATATTGATTT), or neither transcription factor (GATTATATCG AT; mutant 11 [12]). TTR mutant promoter constructs with or without the TTR enhancer were transfected into HepG2 cells and analyzed for promoter expression via CAT enzyme activity (see Materials and Methods).

the HNF-3/HNF-6 site in the TTR promoter. The HNF-6 binding sequence in the HNF-3β promoter was altered by four nucleotides (TATT to CTGG) that are required for HNF-6 binding (Fig. 4A, mut. HNF-3β). We compared wild-type and HNF-6 mutant −245 HNF-3β promoter activity by transfection of these promoter CAT constructs into HepG2 cells and then quantitation of CAT enzyme activity in the cytoplasmic protein extracts prepared 48 h later. These transfection experiments showed that disruption of the HNF-6 site resulted in a 63% reduction of HNF-3β promoter activity, suggesting that the HNF-6 protein activated HNF-3β gene transcription (Fig. 5A).

In order to assess the contribution of HNF-6 or HNF-3 recognition of the TTR.HNF-3S sequence (−94 to −106) to TTR promoter activity, we used site-directed mutagenesis to alter its protein binding properties. We changed the TTR.HNF-3S promoter sequence such that it will bind only HNF-6 by conversion to the HNF-6 site in the HNF-3β promoter or only HNF-3 by conversion to the HNF-3#4 sequence

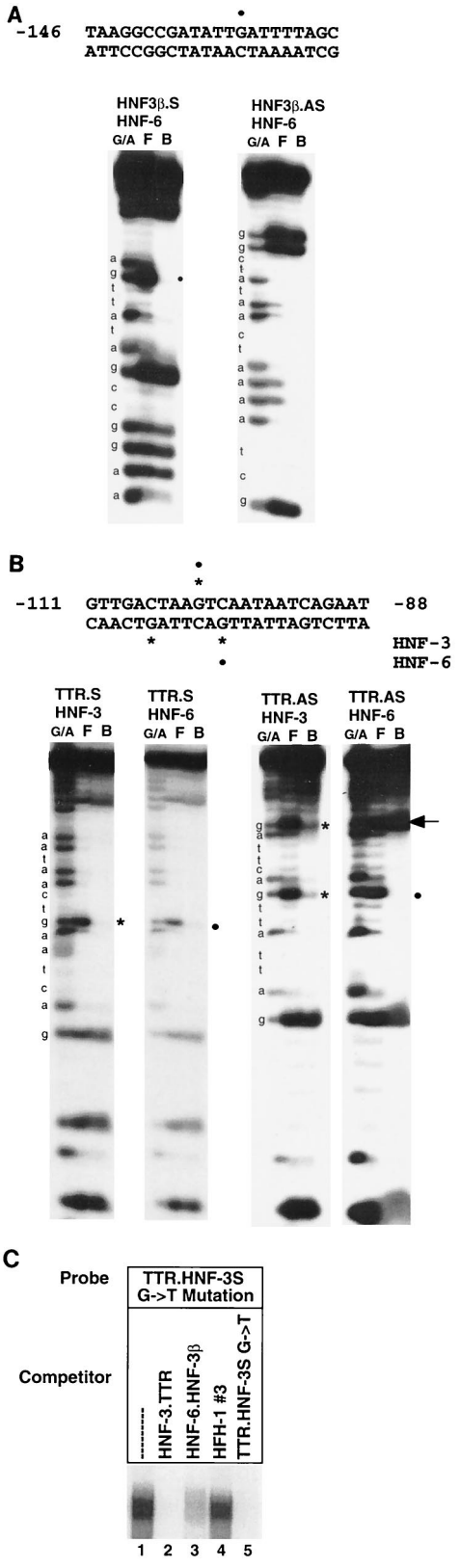


FIG. 6. Methylation interference studies identify differences in nucleotides required for HNF-3 and HNF-6 binding. (A) A sequence spanning 12 nucleotides is necessary for HNF-6 to bind to its HNF-3β promoter site. Methylation interference was performed with the −146 to −122 region of the HNF-3β promoter methylated with dimethylsulfate and then allowed to bind protein as for an EMSA. The protein-bound (B) and free (F) oligonucleotide was eluted from the gel, and then the methyl guanosine was cleaved with piperidine and run on a denaturing acrylamide gel. As a sequence marker, the G-plus-A cleavage lane was loaded. Nucleotides determined to contact protein via comparison of the free probe and probe bound to protein are identified at the top of the figure by a dot. (B) HNF-3 protein binding to the TTR promoter requires one more 5′ guanine nucleotide at −106 which is not contacted by HNF-6. Methylation interference was performed for the TTR promoter as described for the HNF-3β promoter site with the additional modification of using either unlabelled HFH-1#3 (HNF-3) or HNF-6 (−146 to −122; HNF-3β) oligonucleotide competition to isolate only HNF-3 or the HNF-6 protein–DNA complex. Asterisks indicate nucleotides necessary for HNF-3 binding, while dots indicate those necessary for HNF-6. The only difference noted between the two binding patterns was with the guanine nucleotide at the −106 position (arrow) of the antisense TTR promoter strand that was necessary for HNF-3 binding but not for HNF-6 binding. (C) Conversion of the guanine nucleotide at position −106 to a thymine reduces affinity for the HNF-3 protein. EMSA analysis was performed with rat liver nuclear extract and the TTR.HNF-3S promoter site with one changed nucleotide (G to T at position −106; HNF-3.TTR G→T). Diminished competition of the HFH-1#3 site with the mutant oligonucleotide indicates its lower affinity for HNF-3 proteins.

(53) (Fig. 2). We also engineered mutations which completely altered the TTR.HNF-3S such that it was no longer recognized by either HNF-3 or HNF-6 proteins. HepG2 transfection of TTR promoter constructs which replace the TTR HNF-3S site with sequences that bound either HNF-3 or HNF-6 resulted in a 75% decrease in TTR promoter activity (Fig. 5B). When these mutations are tested in the context of the TTR enhancer region, a less severe 30% reduction in TTR promoter activity is observed. Mutations which disrupt recognition of both HNF-3 and HNF-6 proteins completely eliminated TTR promoter activity regardless of the presence of the TTR enhancer region (Fig. 5B). These data suggest that recognition by both HNF-3 and HNF-6 is required for full activity of the TTR promoter region.

High-affinity HNF-3 binding of the TTR promoter requires a −106 guanine residue which is not contacted by the HNF-6 protein. In order to determine which nucleotides in the −146 to −122 site of the HNF-3β promoter were being contacted by the HNF-6 protein, we performed a methylation interference assay with EMSA to separate protein-bound and free oligonucleotide probe. Within a 12-nucleotide region flanked by guanine residues which are not required for HNF-6 recognition, methylation of a guanine residue (Fig. 6A, −133 bp, dots indicate interference) prohibited binding, and therefore this band is absent in the protein-bound DNA (lane B) compared with the free DNA probe (lane F). These methylation interference studies defined a 12-nucleotide region in the HNF-3β promoter that is required for HNF-6 protein recognition (AT ATTGATTT).

We next wanted to determine whether the HNF-3 and HNF-6 proteins utilized identical sequences to recognize the TTR.HNF-3S binding site. Because the TTR.HNF-3S site binds both HNF-3 and HNF-6 proteins and these complexes migrate at the same position on the gel (Fig. 4B), it is not possible to separate these protein-DNA complexes. In order to isolate each of these protein-DNA complexes separately, we included either unlabeled HNF-3β −146 to −122 (HNF-6 binding site) or HFH-1#3 (HNF-3 binding site) oligonucleotide to compete for the formation of one of these complexes (Fig. 4B, lanes 10 and 11). Although these proteins required similar nucleotides for binding, these methylation interference studies identified a guanine residue at position −106 on the lower strand of the TTR.HNF-3S site, which was required for HNF-3 (Fig. 6B) binding but was not contacted by the HNF-6 protein (Fig. 6B). The results therefore suggest that these proteins recognize TTR.HNF-3S through slightly different nucleotides.

In order to determine the importance of this −106 guanine nucleotide for HNF-3 binding, we generated a mutant oligonucleotide that replaced the guanine residue with a thymine residue. EMSAs with liver nuclear extracts and the mutated TTR.HNF-3S site were performed to examine its binding properties to the HNF-3 and HNF-6 proteins (Fig. 6C). Our studies indicate that the G-to-T nucleotide change in the TTR.HNF-3S site resulted in reduced DNA binding affinity for HNF-3 protein but not for the HNF-6 protein. The HNF-3β promoter site completely inhibited HNF-6 complex formation with the G-to-T mutant TTR.HNF-3S site, but the remaining HNF-3 complexes with the G-to-T mutant TTR.HNF-3S site were diminished compared with those with the wild-type TTR.HNF-3S probe (compare Fig. 4B, lane 10, and Fig. 6C, lane 3). Furthermore, recombinant HNF-3β protein exhibited reduced affinity for the G-to-T mutant TTR.HNF-3S site compared with the wild-type binding site (Fig. 7A), and the winged-helix binding site HFH-1#3 no longer competed for complex formation (Fig. 6C, lane 4). These results indicate that the guanine nucleotide at position −106 in the TTR.HNF-3 sequence is important for high-affinity HNF-3 binding.

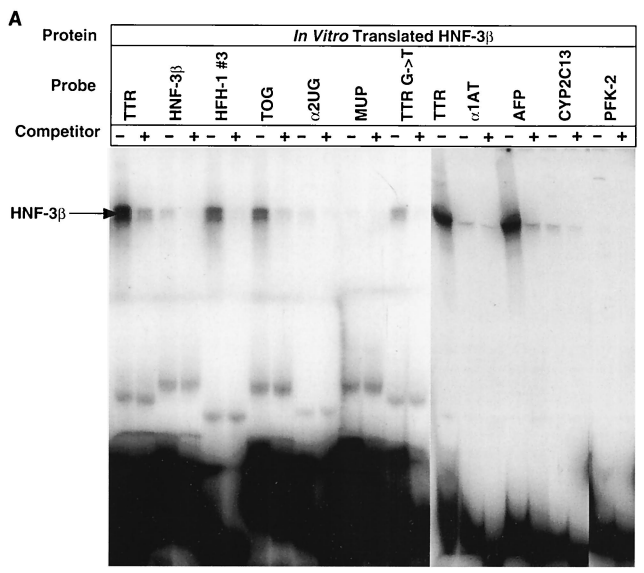
Computer-assisted searches predict other genes regulated by HNF-6. The methylation interference results suggested that the HNF-6 binding sequence TATTGAYTTW could be used to search for putative HNF-6 target genes. Twenty-two potential target genes containing HNF-6 binding sites were found among 110 hepatocyte-enriched promoter regions examined (Fig. 7D). We selected seven of these putative HNF-6 binding sites for further investigation via EMSA with recombinant HNF-3β protein and liver nuclear extracts (Fig. 7B). These liver-enriched promoter regions include the rat tryptophan oxygenase (TOG; −220 to −208), α-2 urinary globulin (α2UG; −196 to −183), AFP (−6091 to −6103), PFK-2 (−200 to −212), cytochrome P-450 enzyme 2C13 (CYP2C13; −53 to −41), mouse α1AT (−195 to −183), and major urinary protein (MUP; −120 to −132) (relevant sequences given in Fig. 7D). DNA binding assays with recombinant HNF-3β and equivalently labeled radioactive probes demonstrated that two of these seven sites bound HNF-3β. The HNF-6 binding site derived from the AFP enhancer region was capable of HNF-3 recognition as strong as that of the TTR site (HNF-3S −94 to −106), while the TOG promoter site bound HNF-3β with a moderate relative affinity (Fig. 7A). EMSA analysis with labeled sites binding both HNF-3 and HNF-6 (the TTR promoter site; −94 to −106), HNF-6 only (the HNF-3β promoter site; −138 to −127), or HNF-3 only (HFH-1#3), and competition with each of the putative HNF-6 binding sites confirms that only two of these sites compete for HNF-3 binding (Fig. 7B). Both the AFP and TOG promoter sites did compete with the HFH-1#3 probe and also competed quite readily with the TTR probe (Fig. 7B, lanes 7 and 11), confirming that they indeed bind HNF-3, as demonstrated by recognition with recombinant HNF-3β protein (Fig. 7A). None of the other putative HNF-6 sites competed significantly for HNF-3 binding with either the TTR or HFH-1#3 probes. When the HNF-6 site from the HNF-3β promoter was used as the probe, all of the HNF-6 binding sites competed for complex formation with various affinities (Fig. 7B). The CYP2C13, MUP, and TOG promoter sites were effective competitors for HNF-6 recognition of the HNF-3β site and exhibited nearly identical abilities to inhibit complex formation as HNF-3β self-competition (Fig. 7B, compare lanes 3, 8, 9, and 11). The TTR, α1AT, PFK-2, and α2UG sites exhibited moderate affinity for HNF-6 recognition (Fig. 7B, compare lanes 2, 5, 6, and 10), whereas AFP was a poor competitor for HNF-6 binding (Fig. 7B, lane 7).

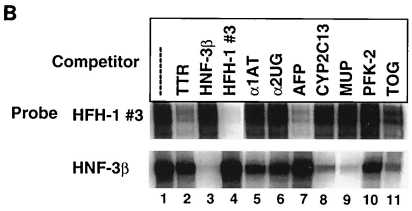
Each of the seven putative HNF-6 sites formed a complex with rat liver nuclear extract (Fig. 7C, lane 1) that was inhibited by itself (lane 6) and the unlabeled HNF-6 site from the HNF-3β promoter (lane 3), indicating that they bound HNF-6 specifically. The TTR site (Fig. 7C, lane 2), which possessed moderate affinity for the HNF-6 protein, was an effective competitor with the weaker-affinity HNF-6 sites (PFK-2, α2UG, α1AT, and AFP). Only the AFP enhancer site is recognized by both HNF-3 and HNF-6 proteins and behaved like the TTR.HNF-3S site (Fig. 4B). The HNF-3β site competed for HNF-6 recognition of the AFP site, leaving the HNF-3 complexes intact (Fig. 7C, lane 3), whereas the HFH-1#3 site competed for HNF-3 recognition of the AFP sequences, leaving the HNF-6 protein-DNA complex intact (Fig. 7C, lane 4). None of the other HNF-6 binding sites were inhibited by the HFH-1#3 sequence, confirming the results with recombinant HNF-3β protein by demonstrating that they possess weak binding affinity for HNF-3 protein (Fig. 7C, lane 3). These EMSA results confirm the findings of Fig. 7A and B and are summarized in Fig. 7D, which lists these HNF-6 binding sites along with other putative HNF-6 binding sequences from promoters of hepatocyte- and intestinal epithelium-specific genes. These studies suggest that the HNF-6 protein may regulate the expression of several important hepatocyte- and intestinal epithelium-enriched target genes.

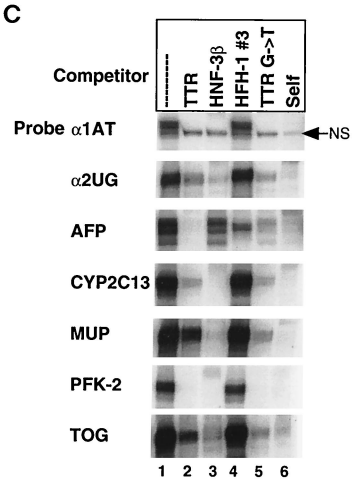
DISCUSSION

HNF-3β is among the first liver-enriched factors to be expressed during formation of the definitive endoderm (3, 48, 65, 68). Mouse embryos containing targeted disruption of the HNF-3β gene are defective in the invagination of gut endoderm and in the formation of node, notochord, and neurotube (2, 81, 86). HNF-3β expression is limited to hepatocytes, pancreatic acinar cells, and intestinal and bronchiolar epithelium in the adult rodent (27, 34, 48, 86). We have previously shown that the hepatocyte-enriched activity of the HNF-3β promoter involves recognition by the bZIP C/EBP and PAR bZIP transcription factor family and autoactivation by the HNF-3 proteins (54, 66). In this paper, we identify a third liver-enriched protein, HNF-6, which recognizes the HNF-3β −138 to −127 region and is critical for activation of the HNF-3β promoter. Although we initially chose to examine the −146 to −122 region of the HNF-3β promoter because of its similarity to the HNF-3 site in the TTR promoter, our data suggest that the HNF-6 and HNF-3 proteins possess distinct DNA recognition and immunological properties. HNF-6 protein possesses DNA binding properties that are also distinct from those of the other known liver-enriched transcription factors (C/EBP, HNF-1, HNF-4, GATA-6, and HoxA5). Furthermore, we show that the HNF-6 protein binds to 7 other hepatocyte-enriched promoters, and putative HNF-6 binding sites are contained in 15 additional liver-enriched promoters (Fig. 7D). As shown with the HNF-3 isoforms, the HNF-6 protein may collaborate with other liver-enriched factors to coordinately regulate hepatocyte-specific gene transcription.

Perhaps the most compelling evidence that HNF-3 and HNF-6 are distinct proteins is the fact that they recognize unique binding sites as evidenced by both EMSA and the methylation interference assay. Two different HNF-3 consensus sequences have been established by binding site selection and compilation of known sites. The first consensus, AWTRT TKRYTY (where R = A or G, W = A or T, K = G or T, and Y = T or C), was compiled via analysis of sites in liver-enriched promoters (53). The second consensus, RWWTRTTTRYTY, which has a TRTTTR (type II) core as opposed to an RTTK (type I) core, was generated by in vitro binding site selection







D

HNF-6 Binding Sites:

Gene	Access.	Position	Sequence	Binding Activity HNF-6	Binding Activity HNF-3
nucleotide number:			123 456 789 012 3		
m.α1-AT		-195/-184	TCC ATT GAT TTA G	+	-
r.PFK-2	M26251	-200/-212	GAA ATT GAT TTC A	+	-
r.α2UG	X05612	-196/-183	ATT ATT GAT AAA A	+	-
m.MUP	M17814	-120/-132	TTT ATT GAT TAG A	+	-
r.CYP2C13	X79810	-53/-41	AAT ATT GAT TCT G	+	-
r.HNF-3β	U50407	-138/-126	GAT ATT GAT TTT T	+	-
r.TOG	X05145	-220/-208	TCT ATT GAT TTA T	+	+
m.TTR G->T		-94/-106	ATT ATT GAC TTA T	+	+
m.TTR	M19524	-94/-106	ATT ATT GAC TTA G	+	++
r.AFP	M18351	-6091/-6103	TTT ATT GAC TTT G	+	++

Putative HNF-6 Binding Sites (known HNF-3 binders indicated):

nucleotide number:			123 456 789 012 3	
h.ApoB	M19808	+892/+904	AAT ACT GAC TTT A	+
m.α1-AT		-378/-366	AAT ATT GAC TTT G	+
r.α2-MAC1	M23567	-442/-454	GGT ATT GAC TTT A	+
h.ADH-2	M24308	-150/-162	AAT ATT GAC TTC C	
r.CYP2C13	X79810	-161/-173	TAT ATT GAC TTA A	
r.PEPCK	K02299	-247/-259	TTG ATT GAC TAA A	
r.Cerp.	M80529	-483/-471	TTT ATT GAC TAC T	
r.Arginase	M17924	-231/-243	ACA ATT GAC AAT T	
h.RBP	X02775	-552/-540	AAT ATT GAC CAG A	
h.thrombin	M17262	-4749/-4737	GAA ATT GAC TTA T	
r.carn. PT	X68100	-353/-365	AAC ATT GAT GTA G	
r.albumin	X76456	+898/+910	TTG ATT GAT TGA T	
h.OTCase	D00221	-314/-302	TCA ATT GAT TTT G	
m.cystatin C	U10098	-645/-657	TAA ATT GAT CTT G	
h.β-Fibrin.	X05018	-470/-458	ACT ATT GAT TTT A	
h.Apo AI	J00098	+3691/+3703	AAC ATT GAT CAA G	
m.SI	S40076	-25/-37	TAT ATT GAC TAC T	
r.Fabpi	M18080	-645/-633	TAG ATT GAT TTG A	
r.Fabpi	M18080	-623/-635	TTT ATT GAT ATC A	
m.Cdx-2	U00454	+2079/+2067	ATT ATT GAC TGC G	
m.Cdx-2	U00454	+3353/+3365	GAT ATT GAT ATT C	
h.Carb. An.	M77176	-845/-857	CCT ATT GAT TTG A	
HNF-6 Consensus:			DHW ATT GAY TWW D		

FIG. 7. Identification of putative hepatocyte- or intestinal epithelium-specific HNF-6 target genes. (A) Determination of HNF-3 binding to various putative HNF-6 sites. On the basis of the results of the methylation interference assay, we used an HNF-6 consensus binding sequence to search a computer database of promoters for genes that are expressed in the liver. Oligonucleotides corresponding to several of the promoter sequences found in the search were synthesized and tested by EMSA with in vitro-translated HNF-3β protein. The binding sites include the TTR (TTR.HNF-3S; −111 to −88), HNF-3β (HNF-3β; −146 to −122 bp), TOG, α2UG, mouse MUP, α1AT, AFP, CYP2C13, and PFK-2 promoters. The winged-helix HFH-1#3 binding site was included as a positive control. The TTR, AFP, and TOG promoter sites, along with the positive control, are the only sites binding the HNF-3β protein. (B) EMSA with labeled probes binding HNF-6 (HNF-3β; −146 to −122 bp) or HNF-3 (HFH-1#3) and competition with rat liver nuclear extract and each of the putative HNF-6 binding sites used in panel A. (C) EMSA with each of the putative HNF-6 sites as probe and rat liver nuclear extract. Self lanes included homologous unlabeled oligonucleotide as a competitor. NS, nonspecific band. (D) Summary of HNF-6 binding affinity and identification of putative target genes. Shown are the name of the gene, the GenBank accession number, the position in the promoter, and the sequence recognized by HNF-6. Summarized from EMSA are the binding capabilities for HNF-6 protein: −, no binding; +, binding. Relative binding affinities with recombinant HNF-3 protein: −, no binding; +, low-level binding; ++, strong binding. The letters m, r, and h are abbreviations for mouse, rat, and human genes, respectively. Other abbreviations are as follows: r.α-2 MAC, α-2 macroglobulin; r.Cerp, ceruloplasmin; r.carn. PT, carnitine palmitoyltransferase; h.β-fibrin, β-fibrinogen; h.ADH-2, class I alcohol dehydrogenase β-1 subunit; h.OTCase, ornithine transcarbamylase; and h.RBP, retinol binding protein. Rat intestinal fatty acid binding protein (r.Fabpi) (72), mouse cdx-2 homeodomain (32) and sucrase-isomaltase (m.SI) (77), and human carbonic anhydrase II (h.carb. An.) are expressed in intestinal and/or colonic epithelium. Nucleotide abbreviations of the HNF-6 consensus are as follows: D is not C, H is not G, W = A or T, and Y = C or T.

(53). A type II consensus core was identified in the TAT enhancer, and the protein recognizing this site was designated as HNF-5 (63), although later studies demonstrated that HNF-3 recognized this TAT sequence (51). We demonstrate here that HNF-6 binds to sites similar to the type I (RTTK) but not type II (TRTTTR) class of HNF-3 consensus site via recognition of a DHWATTGAYTWWD (where H is not G, W = A or T, Y = T or C, and D is not C) sequence (Fig. 7D).

A methylation interference assay of a site recognizing both the HNF-3 and HNF-6 proteins demonstrates that HNF-3 binds a sequence extending one nucleotide more 5′ than HNF-6's binding site. Previous binding site selection studies in our laboratory had not indicated that the identity of this 5′ nucleotide was critical for HNF-3 binding, but our conversion of a guanine at this site to a thymine demonstrates decreased binding activity (Fig. 6C). The methylation interference studies shift the HNF-3 recognition sequence by two nucleotides from what was originally reported with in vitro binding site selection, RWWTRTTTRYTY (53). Inspection of a number of HNF-3 binding sequences obtained from in vitro binding site selection and promoter targets (Fig. 2 and 7D and data not shown) suggest that nucleotides WR are preferred in positions 13 and 14. This finding is consistent with the consensus extension proposed by Roux et al. (64).

Characterization of the binding site for HNF-6 has made detection of other HNF-6 sites possible. Seven additional genes expressed primarily and abundantly in liver identified by such a search are TOG, α2UG, AFP, α1AT, PFK-2, CYP2C13, and mouse MUP. Three target genes coding for TTR, AFP, and TOG contain sites binding both HNF-3 and HNF-6, while the remainder only bind HNF-6. Thus, it is apparent that two categories of HNF-6 binding sites have evolved: those that bind HNF-6 only, and those that bind both HNF-3 and HNF-6. Comparison of sequences from both categories suggests that the critical nucleotides determining whether a site will bind HNF-6 only or both HNF-3 and HNF-6 are position 13 of the HNF-6 consensus where HNF-3 prefers a purine, and position 9 where HNF-3 prefers a C residue. HNF-3 also prefers either a TTT or TTA in positions 10 to 12 (Fig. 7D).

Our studies suggest the presence of HNF-6 binding activity in nuclear extracts derived from tissues and cell lines which express the HNF-3β gene. Abundant HNF-6 binding activity is found in adult and embryonic liver tissue and the human hepatoma HepG2 cell line (Fig. 3A). We also detected high levels of binding to the HNF-6 site in extracts for the intestinal epithelial HT29 cells and demonstrated that the HT29 cell line expresses the HNF-3α and HNF-3β proteins (Fig. 3 and data not shown). In support of this expression pattern, putative HNF-6 binding sites were found in transcriptionally important regions of the sucrase-isomaltase (SI) (77) and intestinal fatty acid binding protein (Fabpi) genes (72). Furthermore, the cdx-2 homeodomain transcription factor, which is critical for cell-specific gene regulation and differentiation of intestinal epithelium (75, 76), contains two putative HNF-6 binding sites in its genes. The binding of HNF-6 to the HNF-3β promoter and putatively the cdx-2 gene and several other intestinal promoter regions (Fig. 7D), suggests that HNF-6 may participate in cellular differentiation of intestinal epithelium. Moreover, recent immunohistochemistry studies of adult mouse lungs with HNF-3β-specific antisera have allowed the detection of low expression levels of HNF-3β protein in both bronchiolar and alveolar epithelium (27, 86). We also detected specific HNF-6 binding activity in adult lung nuclear extracts, but it is substantially reduced in comparison with liver nuclear extracts. In contrast, the pulmonary Clara cell line H441 and cervical carcinoma HeLa cell line do not express the HNF-3β gene (70), and nuclear extracts prepared from these cell lines lack detectable HNF-6 binding activity (Fig. 3A). Both the expression pattern of HNF-6 and its requirement for HNF-3β promoter activity provide evidence for HNF-6's role in regulating HNF-3β gene transcription.

Analysis of promoters regulating liver-enriched transcription factors suggests that expression in hepatocytes is maintained via cross-regulation of the promoter by one or more unrelated liver-enriched transcription factors. A cross-talk regulatory loop was discovered during the analysis of the HNF-1 and HNF-4 promoters in which each of these factors regulated the transcription of the other promoter (36, 85). Our current study extends these results by identification of the HNF-6 protein, which not only regulates transcription of hepatocyte-enriched genes but also cross-regulates the expression of the HNF-3β gene. HNF-6 binding activity was detected in embryonic liver nuclear extracts, and it may therefore participate in HNF-3β gene activation during hepatocyte differentiation with the C/EBPβ and the PAR bZIP VBP proteins (30, 50) and an HNF-3 autoregulatory site (54, 66). These studies suggest a role for the HNF-6 protein in the commitment and maintenance of the hepatocyte-specific differentiation and gene expression.

Given the large number of genes that are likely to be regulated by HNF-6, it is not surprising that this factor has been noted previously by other laboratories characterizing liver-enriched promoters. For example, a liver-enriched factor binding to a transcriptionally important L-type PFK-2 promoter site IV (LP4) was identified, which is inhibited by the TTR.HNF-3 site but was not disrupted by HNF-3 antibodies (44). The oligonucleotide taken from the PFK-2 promoter in our work (Fig. 7D) is identical to the LP4 promoter site oligonucleotide, suggesting that this site is recognized by the HNF-6 protein. Although the name LP4 was designated by this group in 1993, we suggest that HNF-6 is a more appropriate name, considering the large number of hepatocyte-enriched promoter regions that bind this protein.

With regard to the MUP promoter, the site which we have identified as binding HNF-6 lies directly adjacent to an NF-1 site and partially overlaps a growth hormone-responsive element (33). Homology of this MUP site to the growth hormone-responsive Stat 5 binding site of spi2.1 (serine protease inhibitor) suggests that perhaps HNF-6 is the growth hormone-responsive Stat 5 transcription factor (6). Analysis of the spi2.1 oligonucleotide shows that it differs from the HNF-6 consensus at only three nucleotides (CTAcTaATcCATGT [indicated in lowercase]); this nucleotide difference is sufficient to prevent HNF-6 complex formation in the EMSA (data not shown). Further evidence that HNF-6 and Stat 5 are distinct stems from the data that HNF-6 is active in wild-type liver, while Stat 5 requires hormonal induction to bind.

Another HNF-6 target, the cytochrome P-450 enzyme CYP2C13, is also growth hormone responsive. This catalyst of testosterone hydroxylation is constitutively expressed in male rat livers and transcriptionally repressed by growth hormone in female rats. In their examination of the CYP2C13 gene promoter, Legraverend et al. (43) characterize two of the sites that we identify as HNF-6 targets. The proximal site (−53 to −41), or site A, we demonstrate to bind HNF-6 (Fig. 7B and C), while the more upstream site (−173 to −161), or site C, is on our list of putative HNF-6 sites (Fig. 7D). On the basis of cotransfection of HNF-3 isoforms with CYP2C13 promoter constructs, Legraverend et al. concluded that CYP2C13 promoter site A (−54 to −41) binds HNF-3 (43). Our work demonstrates that the CYP2C13 promoter site A binds HNF-6 but not HNF-3, so we postulate that the transcriptional activation

seen with HNF-3 cotransfection is due to the HNF-3 site that they identify near the −54 to −41 HNF-6 site. This study further demonstrates that neither of the HNF-6 sites in the CYP2C13 promoter is altered in the footprint when nuclear extracts from the livers of growth hormone-stimulated rats are compared with those of the control group.

The HNF-6 site in the AFP enhancer has previously been shown to bind HNF-3 by Groupp et al. (22), who suggested that there are other proteins recognizing this site. In sequence, binding affinity, and relative contribution to activity, the HNF-3/HNF-6 site of the AFP enhancer is similar to that of HNF-3S site (−94 to −106) of the TTR promoter region (Fig. 4B and 7C). We predict that five other sites known to bind HNF-3 will also bind HNF-6 given their close homology to the TTR and AFP sites. These HNF-3 binding sites are from the promoter regions of PEPCK (29), Apo B (7), α1AT (13), α-2 macroglobulin, and cdx-2.

In summary, we have identified a liver-enriched transcriptional activator, HNF-6, which is critical for HNF-3β and TTR promoter activation. We developed an HNF-6 consensus sequence and used it to identify 22 putative hepatocyte-enriched target genes. We selected seven of these putative HNF-6 binding sites and demonstrated via EMSA that they were authentic HNF-6 binding sites. Furthermore, we demonstrated that intestinal epithelial HT29 cells contain HNF-6 and HNF-3β binding activity, and we identified several putative HNF-6 target genes in the intestinal epithelial cells, including the cdx-2 homeodomain protein. These results imply that HNF-6 may be important for activation of HNF-3β expression in hepatocytes and, putatively, intestinal epithelium and may also have a role in the coordinate regulation of cell-type-specific genes in these organs.

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