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The Restricted Promoter Activity of the Liver Transcription Factor Hepatocyte Nuclear Factor 3β Involves a Cell-Specific Factor and Positive Autoactivation

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The transcription factor hepatocyte nuclear factor 3 (HNF-3) is involved in the coordinate expression of several liver genes. HNF-3 DNA binding activity is composed of three different liver proteins which recognize the same DNA site. The HNF-3 proteins (designated α, β, and γ) possess homology in the DNA binding domain and in several additional regions. To understand the cell-type-specific expression of HNF-3β, we have defined the regulatory sequences that elicit hepatoma-specific expression. Promoter activity requires −134 bp of HNF-3β proximal sequences and binds four nuclear proteins, including two ubiquitous factors. One of these promoter sites interacts with a novel cell-specific factor, LF-H3β, whose binding activity correlates with the HNF-3β tissue expression pattern. Furthermore, there is a binding site for the HNF-3 protein within its own promoter, suggesting that an autoactivation mechanism is involved in the establishment of HNF-3β expression. We propose that both the LF-H3β and HNF-3 sites play an important role in the cell-type-specific expression of the HNF-3β transcription factor.

Deciphering the mechanisms which regulate differential transcription of tissue-specific genes is of paramount importance in understanding the events governing mammalian development. Since tissue-specific gene expression is regulated at the transcriptional initiation step, an understanding of transcriptional control will provide insight into differentiation mechanisms (28). It has been determined that tissue-specific gene transcription is achieved through the recognition of promoter sites by transcription factors which display restricted cellular activity. The identification of genes responsible for developmental or homeotic mutations in *Drosophila melanogaster* and *Caenorhabditis elegans* has shown that sequential cascades of transcription factors are involved in the formation of body structures (39, 51). One component of this cascade scheme is temporal induction or repression of transcription factors by autoregulatory and cross-regulatory mechanisms (2, 26, 30). The existence of homologies between the homeotic proteins and mammalian transcription factors suggests that similar regulatory cascades are employed during mammalian development (22, 51). One approach to the identification of proteins included in the regulatory cascade of differentiation involves defining DNA regions and cognate factors which control the expression of tissue-specific transcription factors. Analyses of such transcription factors have revealed that autoregulatory loops play an important role in the onset of expression of the pituitary-specific Pit-1/GHF-1 (7, 44), the muscle-specific MyoD (56), and the erythroid-specific GATA-1 (57) factors.

The liver provides a system for analysis of the transcription of several functionally unrelated genes which are coordinately expressed at the same time during liver development. The functional dissection of DNA sequences controlling expression of these hepatocyte-specific genes has revealed similarities in the proteins which bind to their promoter regions (9, 10, 12, 23). The regulation of liver-specific genes involves the participation of hepatocyte transcription factors that are active in a minimal number of cell types (3, 9, 11, 23, 47, 60). The activity of these promoters requires a combination of several different liver transcription factors, which are intimately involved in maintaining hepatocyte-specific expression (5, 8, 20, 41, 42). In addition, negatively regulating *cis*-acting sequences can also play a role in the maintenance of cell-specific transcription patterns (25, 40, 50). The liver factors which have been cloned include liver factor B1/hepatocyte nuclear factor 1 (LF-B1/HNF-1), a member of the POU-homeobox gene family (1, 17), the leucine zipper dimerization family members C/EBP (36), DBP (48), and LAP (13), HNF-3, which shares homology with the *Drosophila* homeotic gene fork head (34, 35), and HNF-4, which belongs to the steroid hormone superfamily (53). The availability of cDNA clones encoding these proteins has allowed studies of the mechanisms used by this array of liver regulatory proteins to potentiate the activity of the basal transcriptional machinery (34, 35, 37, 49, 53). Thus, the identification of regulatory proteins controlling the onset of expression of these transcription factors in the liver will provide insight into the events leading to hepatocyte differentiation.

The transcription factor HNF-3 was first discovered during functional analysis of the transthyretin (TTR) promoter and was subsequently shown to participate in the expression of several other liver genes (9). A recombinant HNF-3α cDNA clone which bound the HNF-3 target sequence was isolated (34). The HNF-3 family was further expanded by the identification of two additional genes, HNF-3β and -γ, that activated transcription through the HNF-3 binding site (35). The existence of these three HNF-3 genes corresponds to the multiple HNF-3 protein–DNA complexes which occur in liver nuclear extracts. The HNF-3 gene family possesses homology both in the DNA binding domain and in the amino and carboxyl termini. The HNF-3 gene also shares similar homology with a *Drosophila* homeotic gene, fork head, which suggests that the HNF-3 proteins may play an important role in liver development (35, 58, 59). In addition to being expressed in the liver, the HNF-3α and -β mRNAs are

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found in the lung, and HNF-3γ mRNA is detectable in the testis. The basis for this restricted expression pattern is at the level of transcriptional initiation (35, 60). Thus, the analysis of the HNF-3β promoter region will allow us to identify the regulatory proteins that are responsible for its cell-specific expression pattern.

In this paper, we report on isolation of an HNF-3β genomic clone and identification of regulatory proteins which control tissue-specific transcription of the HNF-3β gene. We defined four protein binding sites within a minimal 134-bp promoter region that contribute to HNF-3β transcriptional activity. They include two nuclear proteins that demonstrate DNA binding activity in a variety of tissue extracts. A third binding protein may be responsible for the onset of HNF-3β expression in liver, since its activity is restricted to liver and lung nuclear extracts (protein designated LF-H3β). In addition, the HNF-3β promoter region contains a binding site for the HNF-3 protein, thus suggesting that an autoregulatory loop is involved in the establishment and maintenance of HNF-3β transcription. We propose a model in which these requisite cell-specific factors (LF-H3β and HNF-3) play a pivotal role in the cell-type-specific transcription of the HNF-3β gene.

MATERIALS AND METHODS

Isolation and characterization of HNF-3β genomic clones. An *Eco*RI-*Pvu*II restriction fragment that contained the 5′ portion of the HNF-3β cDNA (nucleotides 1 to 470) was made radioactive by random hexadeoxynucleotide labeling (16) and used as a hybridization probe to screen 10^6 recombinant phage. This screening yielded 10 positive clones from a rat genomic library propagated in the λ Dash II vector (Sprague-Dawley male; Stratagene). Recombinant λ DNA was prepared from agarose plate lysates, inserts were isolated from low-melting-point agarose after *Not*I digestion, and cloned fragments were propagated in pGem-5 (Promega), using *Escherichia coli* DH5α (52). The HNF-3β clones were characterized by restriction enzyme digestion and Southern blot hybridization with radioactively labeled oligonucleotides and restriction fragments spanning the length of the HNF-3β cDNA. This analysis revealed that the HNF-3β gene was relatively small (about 4 kb) and contained only two introns. To further characterize the intron-exon genomic structure, we used the polymerase chain reaction (PCR) and HNF-3β oligonucleotides to amplify and clone specific regions from the genomic plasmids. PCR amplification of genomic subclones was performed with the *Taq* DNA polymerase (Promega), the oligonucleotides indicated below, and buffers provided by the manufacturer, using 30 cycles at an annealing temperature of 48°C. This yielded PCR fragments that contained a portion of two HNF-3β exons and a single intron region, which were subsequently cloned by using the restriction sites at the 5′ end of the oligonucleotides (underlined below). We determined the DNA sequences (24) of these PCR products, and this provided the sequence of the intron-exon boundaries (Fig. 1). The PCR oligonucleotides for first intron are CAC GAATTCTGAGGCCTCCCTGGGAC (sense; nucleotides 126 to 144) and TTCCATCTTCACGGCTCCCAGCATAC (antisense; nucleotides 214 to 189). Those for second intron are GCGGATCCATGGAAGGGCACGAGCCATCC (sense; amino acids 7 to 13) and GCGGATCCTTAGGCGC CCGCGCCCGGGGACAT (antisense; amino acids 86 to 80).

The location of the HNF-3β 5′ end was determined by primer extension analysis using a labeled antisense oligonu-

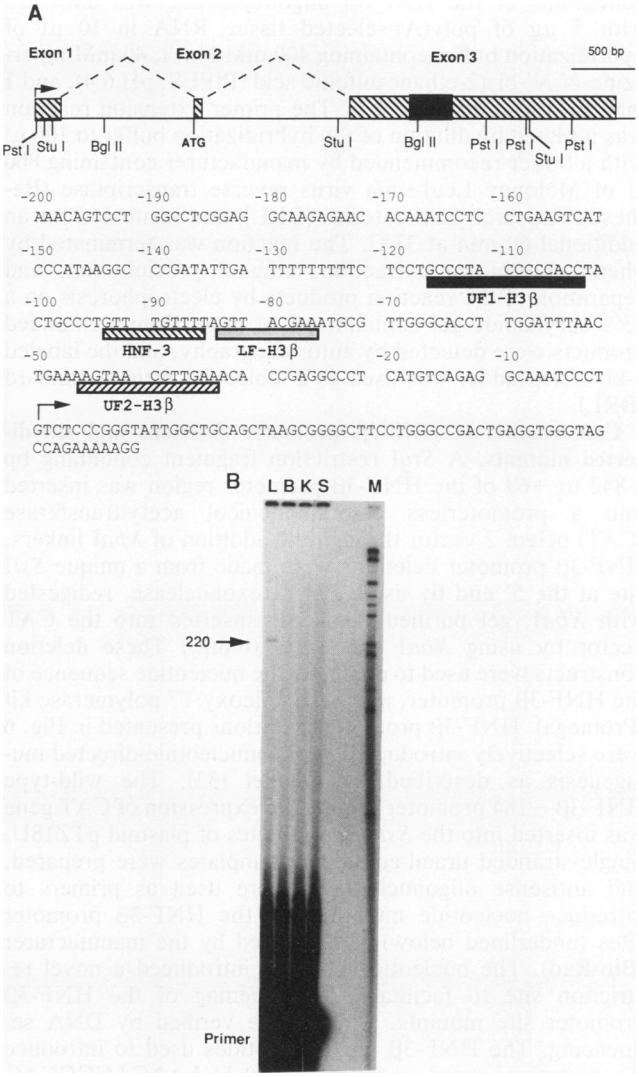


FIG. 1. HNF-3β genomic and promoter organization and localization of the transcriptional initiation site. (A) Schematic representation of HNF-3β genomic organization and positions of protein binding sites on the HNF-3β promoter sequence. Shown are the locations of the HNF-3β transcriptional initiation site (arrow), the three exons (striped boxes), and two introns (broken lines) of the gene as well as the positions of the initiating methionine (ATG), the DNA binding domain (black box), and various restriction endonuclease cleavage sites. Shown below is the DNA sequence of the HNF-3β promoter spanning nucleotides −200 to +69 5′ of the initiation site (indicated by arrow). Indicated on this sequence are the various protein binding sites that were identified in this study. These include the two ubiquitous binding factors UF1-H3β and UF2-H3β and the liver- and lung-specific factors LF-H3β and HNF-3. (B) Location of the HNF-3β transcriptional initiation site by primer extension. Poly(A)$^+$ RNA (5 μg) isolated from either liver (L), brain (B), kidney (K), or spleen (S) was hybridized with a radioactively labeled oligonucleotide (5 ng) made antisense to the HNF-3β cDNA sequence (nucleotides 214 to 189; 35) and elongated to the 5′ end of the mRNA by using reverse transcriptase. The primer-extended reaction products were fractionated on a denaturing 13% acrylamide gel along with labeled DNA molecular weight markers (M; 1-kb ladder; BRL) and subjected to autoradiography. Liver RNA yielded an expected 220-nucleotide product, indicating that HNF-3β cDNA was a full-length clone.

cleotide indicated above (nucleotides 214 to 189). Five nanograms of the HNF-3β oligonucleotide was annealed with 5 μg of poly(A)-selected tissue RNA in 10 μl of hybridization buffer containing 400 mM NaCl, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; pH 6.4), and 1 mM EDTA at 50°C for 2 h. The primer extension reaction was initiated by dilution of the hybridization buffer to 100 μl with a buffer recommended by manufacturer containing 800 U of Moloney Leukemia virus reverse transcriptase (Bethesda Research Laboratories [BRL]) and incubated for an additional 60 min at 37°C. The reaction was terminated by phenol-chloroform extraction, ethanol precipitation, and separation of the reaction products by electrophoresis on a 13% acrylamide gel containing 8 M urea. Primer-extended products were detected by autoradiography, and the labeled 1-kb DNA ladder was used as a molecular weight standard (BRL).

Construction of HNF-3β promoter deletions and site-directed mutants. A *Stu*I restriction fragment containing bp −842 to +69 of the HNF-3β promoter region was inserted into a promoterless chloramphenicol acetyltransferase (CAT) pGem-2 vector through the addition of *Xba*I linkers. HNF-3β promoter deletions were made from a unique *Sst*I site at the 5′ end by using Bal 31 exonuclease, redigested with *Xba*I, gel purified, and then inserted into the CAT vector by using *Xba*I and *Sma*I (blunt). These deletion constructs were used to complete the nucleotide sequence of the HNF-3β promoter, using the dideoxy/T7 polymerase kit (Promega). HNF-3β promoter mutations presented in Fig. 6 were selectively introduced by oligonucleotide-directed mutagenesis as described by Kunkel (32). The wild-type HNF-3β −184 promoter driving the expression of CAT gene was inserted into the *Sst*I-*Bam*HI sites of plasmid pTZ18U. Single-stranded uracil-containing templates were prepared, and antisense oligonucleotides were used as primers to introduce nucleotide mutations in the HNF-3β promoter sites (underlined below) as described by the manufacturer (Bio-Rad). The nucleotide changes introduced a novel restriction site to facilitate the screening of the HNF-3β promoter site mutants, which were verified by DNA sequencing. The HNF-3β oligonucleotides used to introduce the mutations were as follows: −90 5′-AAACAGGGCAG TAGGaattctTAGGGCAGGAGAAAA −125 (−105 to −110; UF1-H3β), −73 5′-CATTTCGTAACTAAAAgAtctAGGG CAGTAGGTGGG −108 (−89 to −93; HNF-3), −63 5′-GTGCCCAACGCATTTtcTagaTAAAACAAACAGGGC −98 (−78 to −83; LF-H3β), and −20 5′-GAGGGCCTCGGTG TTagtActTTACTTTTCAGT −55 (−35 to −40; UF2-H3β). The lowercase letters designate nucleotide changes introduced within the HNF-3β promoter binding sites.

Cell lines, DNA transfection, and transient expression assays. Human hepatoma (HepG2) cells (29) were maintained as monolayer cultures and grown in Ham's F12 medium supplemented with 7% heat-inactivated fetal calf serum, 0.5× minimal essential medium amino acids, 100 U of penicillin-streptomycin (GIBCO Laboratories) per ml, and 0.5 U of insulin per ml. During transfection experiments, HepG2 and HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 7% heat-inactivated fetal calf serum, 1× nonessential amino acids, and 0.5 U of insulin per ml. HNF-3β promoter–CAT constructs (40 μg) and the cytomegalovirus (CMV) immediate-early promoter–β-galactosidase construct (0.5 μg; gift from T. A. Van Dyke, University of Pittsburgh) were both transfected into HepG2 or HeLa cells (100-mm dish) by a modification of the calcium phosphate coprecipitation method as described by Chen and Okayama (6). Cells were incubated for 16 to 20 h with calcium phosphate-precipitated DNAs, followed by two rinses with phosphate-buffered saline and addition of fresh medium. Cellular protein extracts were made 24 to 36 h later by three cycles of freeze-thawing, cellular debris was removed by centrifugation, and protein concentration was determined by the Bio-Rad protein assay. After normalization for equal amounts of protein, cellular extracts were used to measure CAT enzyme activity as described by Gorman et al. (21), and β-galactosidase enzyme activity was determined as described by Miller (46). CAT activity was quantified by densitometer scans of autoradiographs as described previously (8) and normalized to the β-galactosidase enzyme activity, which served to monitor variations in transfection efficiency. HNF-3β expression levels are presented as a ratio of mutant to wild-type levels in which wild-type CAT activity was set at 1.0.

In vitro translation, gel mobility shift assay, and methylation interference. Double-stranded oligonucleotides were 5′ end labeled with [γ-^{32}P]ATP (ICN) and T4 polynucleotide kinase (BRL). Preparation of nuclear protein extracts from rat tissues, gel shift assays, and methylation interference were performed as described previously (9, 11). For methylation interference experiments, uniquely labeled double-stranded DNA was prepared by annealing 5′-end-labeled and unlabeled oligonucleotides together. Treatment of liver nuclear extracts with 5% deoxycholate was performed for 30 min on ice, followed by neutralization with an equal amount of Nonidet P-40 detergent before use for gel shift. T7 RNA polymerase was used to synthesize HNF-3β RNA from a linearized cDNA template (pGem-1), and this RNA was used to program the synthesis of HNF-3β protein, using rabbit reticulocyte lysate extracts as described by the manufacturer (Promega). We used 5 μl of the 50-μl in vitro translation reaction during protein complex formation in the gel shift assay. Competition experiments were performed by including a 150-fold molar excess of the indicated unlabeled oligonucleotide in the binding reaction: HNF-3 site, TTR −111 to −85 (9); HNF-1 site, rat β-fibrinogen gene −90 to −61 (12); HNF-4 site, human apolipoprotein C-III gene −91 to −66 (50, 53); AP-1 site (38) α_1-antitrypsin −279 to −272 (oligonucleotide B; 23), C/EBP site (36); TTR enhancer site 2 (10); retinoic acid-responsive element, retinoic acid receptor β promoter −59 to −32 (55); and Sp1 site (15).

RESULTS

Isolation and characterization of HNF-3β genomic clones. The transcription factor HNF-3 plays a critical role in expression of several hepatocyte-specific genes through interaction with other liver and basal transcription factors. Since the basis for tissue-specific distribution of HNF-3 is at the level of transcription (35, 60), we wanted to isolate one of the HNF-3 promoter regions and dissect its regulation by transfecting deletion mutants of the promoter region into hepatoma cells. Analysis of HNF-3 promoter regions will allow us to identify the transcription factors which are important for establishment of hepatocyte differentiation. We chose to limit our initial analysis to the HNF-3β clone, since it is the most abundantly expressed family member and appeared to be a full-length cDNA insert.

To isolate the HNF-3β promoter region, we screened a rat genomic λ Dash II library (Sprague-Dawley testis; Stratagene) with a radioactively labeled *Eco*RI-*Pvu*II restriction fragment containing the 5′ portion of the HNF-3β cDNA (nucleotides 1 to 470; 35). This screen yielded 10 strongly

hybridizing genomic clones which were further characterized by probing Southern blots of the λ clones with a variety of labeled oligonucleotides spanning the length of the cDNA. This analysis revealed that the HNF-3β gene was 4 kb in length and contained only a few introns. The position of intron-exon boundaries was further mapped by using appropriate sense and antisense HNF-3β oligonucleotides for PCR with the λ clones. PCR products generated from genomic clone amplification that were larger in molecular weight than predicted from the cDNA provided the approximate length of the intron. We determined the sequence of the intron-exon boundaries by sequencing these PCR products as well as other portions of the exons contained in the HNF-3β genomic clones. The results of these analyses yielded the HNF-3β genomic structure diagramed in Fig. 1A, which illustrates the locations of three exons and two introns of approximately 1 kb in length. The first exon is noncoding and terminates at nucleotide 188 of the HNF-3β cDNA (35), while the second exon contains the initiation codon and ends at amino acid codon 23. The HNF-3β third exon contains the majority of the coding region and the 3′ untranslated portion of the cDNA. Both the sequence of this third exon and various genomic Southern blots show that the HNF-3β gene is distinct from other members of this family (35). It may be a general structural feature of the HNF-3 gene family that the majority of the coding region is contained in one exon, as determined by preliminary PCR analysis of the HNF-3 α genomic structure (data not shown).

We next sought to determine the location of the HNF-3β transcription initiation site within the genomic clone and therefore performed a primer extension assay with an antisense oligonucleotide spanning the initiating methionine on RNA isolated from various tissues. Five nanograms of this end-labeled oligonucleotide was hybridized with 5 μg of poly(A)$^+$ tissue RNA, elongated to the 5′ end of the mRNA with reverse transcriptase, and then subjected to denaturing gel electrophoresis and autoradiography (Fig. 1B). The primer-extended product of 220 bp was detected only in liver RNA, and the size of this product indicated that the isolated HNF-3β cDNA was a full-length clone. The primer extension analysis allowed us to precisely map the start of transcription and thereby locate and sequence the 5′ flanking region within the HNF-3β λ clone (Fig. 1A; bp −200 to +69). Schematically shown on the HNF-3 β promoter sequence is a summary of the protein binding sites defined in this study. It includes two distinct sites for ubiquitous binding factors (UF1-H3β and UF2-H3β), a binding site for HNF-3 itself, and a site for a novel factor found only in liver and lung nuclear extracts (LF-H3β). In addition, the HNF-3β promoter region lacks a discernible TATA box sequence, though we were able to map a specific transcriptional initiation site (Fig. 1B). This result is reminiscent of the terminal deoxynucleotidyltransferase promoter, which possesses an element termed "initiator" that can direct specific initiation without any TATA sequences (54).

The HNF-3 β promoter requires −134 for maximal activity in hepatoma cells. To identify the minimal HNF-3β promoter region, we attached various amounts of 5′ flanking region to the CAT gene, using a *StuI* site in the first exon (bp +69). These constructs were transfected into HepG2 hepatoma cells (29) by calcium phosphate coprecipitation, using the CMV–β-galactosidase gene as an internal control for transfection efficiency. Cellular protein extracts were made from transfected HepG2 cells 36 h later and then assayed for CAT activity after normalization for protein concentration (21). Only DNA sequences contained within the −134-bp HNF-3β promoter were required for maximal expression of the CAT enzyme (Fig. 2). Deletion mutants that proceeded further toward the transcriptional initiation site had a deleterious effect on transcriptional activity of the HNF-3β promoter (Fig. 2, mutants −97 and −67). None of the HNF-3β promoter constructs were active when transfected into HeLa (human epithelial) cells, which is consistent with the cell-specific distribution of HNF-3 mRNA (data not shown). This directed us to sequences between −134 to −67 as targets for nuclear binding proteins important in HNF-3β transcription.

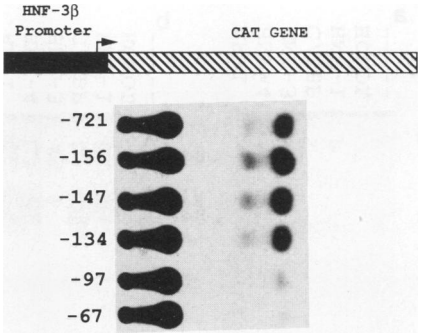


FIG. 2. Evidence that HNF-3β promoter requires −134 nucleotides for expression in HepG2 (human hepatoma) cells. Different amounts of the indicated HNF-3β promote region (black box) located 5′ to the transcriptional initiation site (indicated by the arrow) were joined with the CAT gene (striped box) at +69 bp of the HNF-3β gene and transfected into HepG2 cells by the calcium coprecipitation method (6). Protein extracts were prepared from transfected cells 36 h later, and then equal amounts of protein extract were assayed for CAT activity (21). The acetylated chloramphenicol products generated from CAT enzyme activity migrated faster than chloramphenicol on ascending thin-layer chromatography, which was then visualized by autoradiography as shown below. The CMV-driven β-galactosidase gene was included as an internal control and showed nearly identical enzyme activity.

The HNF-3β promoter region (−134 to −24) binds five protein complexes in liver extracts, one of which is the HNF-3 protein. To investigate nuclear proteins binding to these HNF-3β promoter sequences, we synthesized two oligonucleotides, −128 to −98 and −97 to −67, to use in gel mobility shift assays (18, 19). These labeled, double-stranded oligonucleotides were incubated with liver nuclear extract and then subjected to electrophoresis on a native acrylamide gel, and protein-DNA complexes were visualized by autoradiography (Fig. 3a and b). The −128 to −98 oligonucleotide bound two protein complexes (labeled B-1 and B-2) that were competed for by inclusion of homologous DNA but not by oligonucleotides containing several other transcription factor binding sites (Fig. 3A). Because the −128 to −98 sequence was rich in G residues, we performed competitions with DNA fragments from the simian virus 40 enhancer region and an oligonucleotide containing the Sp1 binding site (15), but we were unable to disrupt complex formation (data not shown). The −97 to −67 oligonucleotide also produced two different protein complexes [designated (proximal) pB-1 and pB-2] with liver nuclear extracts which were not competed for by heterologous oligonucleotides (Fig. 3b). Interestingly, the faster-migrating complex was effectively competed for by the HNF-3 binding site oligonucleotide (Fig. 3b, pB-2 protein complex in lane HNF-3), while the slower complex remained intact. In liver nuclear extracts, the HNF-3 complex is composed of three proteins that recog-

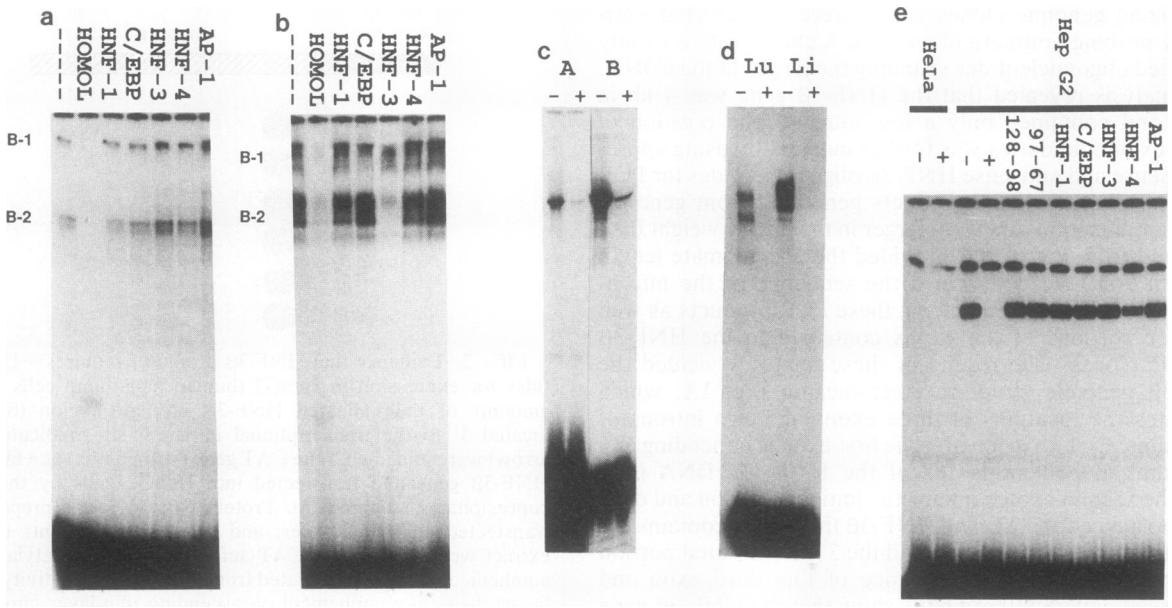


FIG. 3. Gel shift assay showing that the HNF-3β promoter (−128 to −24) contains five liver protein complexes. (a and b) Two oligonucleotides (−128 to −98 [a] and −97 to −67 [b]) bind four liver protein complexes, one of which competes with the HNF-3 site. The indicated labeled oligonucleotides were incubated with liver nuclear extracts to allow for protein complex formation, and the protein-DNA complexes (B-1 and B-2) were separated from unbound DNA by electrophoresis on a low-ionic-strength 9% acrylamide gel and visualized by autoradiography. We will designate the −97 to −67 protein complexes as (proximal) pB-1 and pB-2. These binding reactions were carried out in the absence (----) or in the presence of a 150-fold molar excess of an unlabeled oligonucleotide that includes either itself (homol) or other indicated transcription factor binding sites (see Materials and Methods). (c and d) In vitro-translated HNF-3β protein binds to −97 to −67. The HNF-3β protein, synthesized by in vitro transcription and translation of the HNF-3β cDNA, was used to form a specific protein complex with HNF-3β −97 to −67 oligonucleotide by the gel shift assay (c; probe A, − and + competition). The TTR HNF-3 binding site oligonucleotide is included as a control for HNF-3 β complex formation (probe B; −111 to −85). A gel shift assay with lung (Lu) nuclear extracts reveals binding to TTR HNF-3 site (d; − and + competition lanes); liver extract (Li) is presented for comparison. (e) A HepG2 nuclear protein binds to the HNF-3β −51 to −24 sequences. A gel shift assay between HepG2 nuclear extracts and −51 to −24 oligonucleotide was carried out in the absence (−) or presence (+) of homologous competitor as well as other oligonucleotides containing the indicated transcription factor binding sites. HeLa nuclear extracts lack this binding activity.

nize the same binding sequence and result in multiple bands (Fig. 3d). Nuclear extracts prepared from the lung, which is a tissue that expresses abundant amounts of HNF-3α and -β mRNA, also demonstrate protein complex formation with the HNF-3 oligonucleotide. These results suggest that two distinct proteins are binding to the −97 to −67 oligonucleotide, and one of these protein complexes (pB-2) is the result of HNF-3 binding.

To verify that HNF-3 protein could bind to this promoter site, we used the HNF-3β cDNA to produce the encoded polypeptide by in vitro transcription/translation for the gel shift assay. The in vitro-translated HNF-3 β protein was able to form a protein complex with the −97 to −67 oligonucleotide, which was effectively competed for by homologous DNA (Fig. 3c, probe A). This translation product produced an identical complex with the TTR HNF-3 binding site oligonucleotide (Fig. 3c, probe B). Therefore, HNF-3β will bind to its own promoter region, and this binding site may be involved in autoactivation of HNF-3β transcription (see below).

To determine whether any other binding proteins were interacting with sequences more proximal to the start site, we performed a gel shift assay with a DNA fragment containing sequences between bp −70 and +5 of the HNF-3β promoter. HepG2 extracts produced a single protein complex which required two G residues at positions −39 and −40, as determined by methylation interference (data not shown). To further characterize this protein interaction, a third double-stranded oligonucleotide was synthesized to the −51 to −24 region, and this sequence formed a specific protein-DNA complex with HepG2 but not with HeLa extracts (Fig. 3e). This protein complex was not competed for by several oligonucleotides containing different transcription factor binding sites. Competition with the HNF-4 site (150-fold molar excess), which binds a protein that is a member of the steroid hormone superfamily, resulted in a 50% reduction in complex formation. However, the in vitro-translated HNF-4 protein (gift from F. Sladec and J. E. Darnell, Jr.) did not form a complex with the −51 to −24 oligonucleotide, and the HNF-4 complex migrated at a significantly different position on the gel (data not shown). This result demonstrates that a protein with recognition properties different from those of HNF-4 was binding to these sequences. In addition, the nucleotides between −51 to −24 did not resemble other steroid hormone receptor target sequences, nor were we able to compete the −51 to −24 band with an oligonucleotide containing a retinoic acid-responsive element (data not shown; 55). Thus, the HNF-3 β promoter appears to contain a binding site for HNF-3 and to associate with at least four other promoter-specific factors in liver nuclear extract.

Methylation interference delineates four different DNA binding sites. To define the nucleotides important for recognition by these proteins, we used methylation interference. The HNF-3β oligonucleotides were uniquely labeled at the 5′ end; G residues were partially methylated by dimethyl

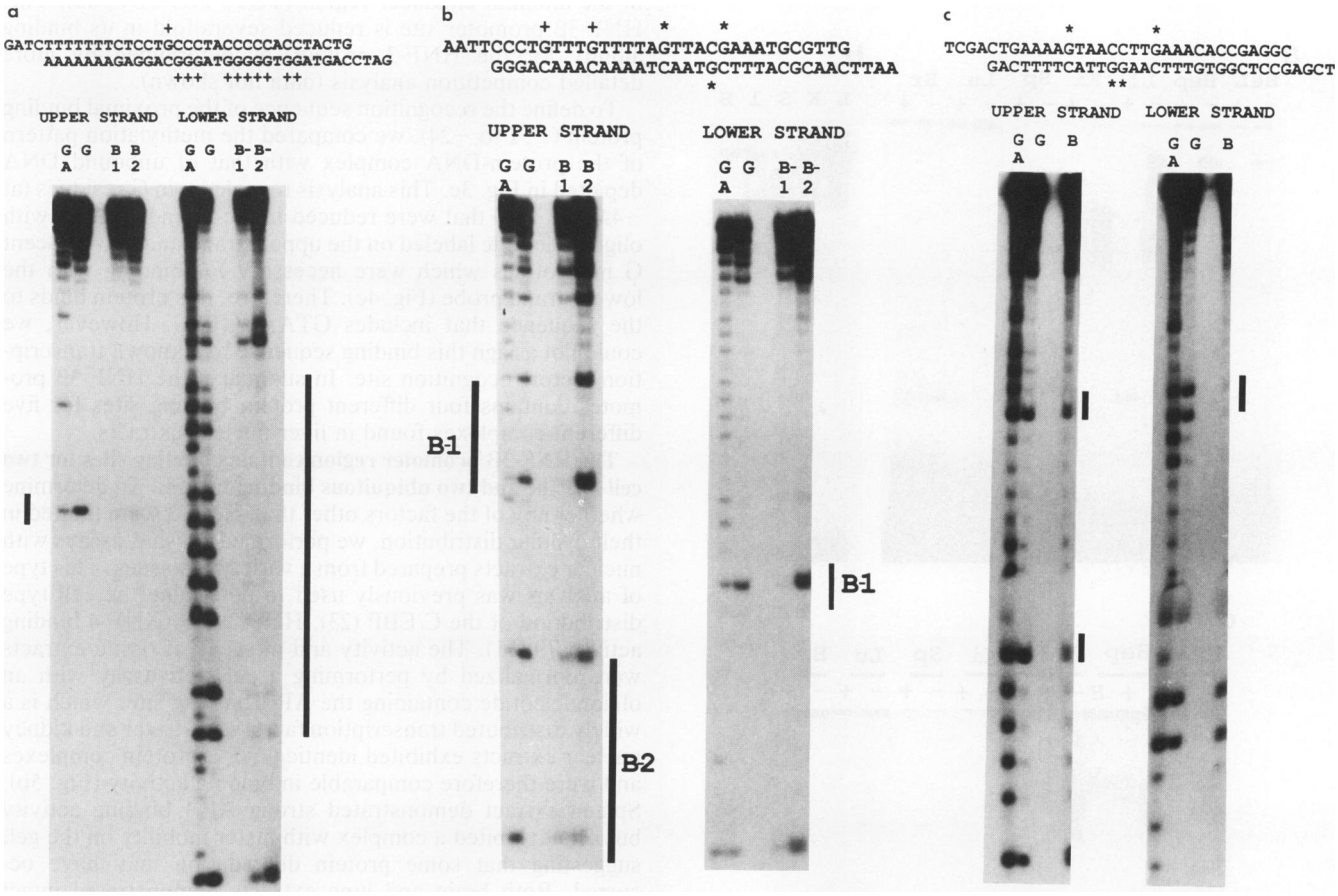
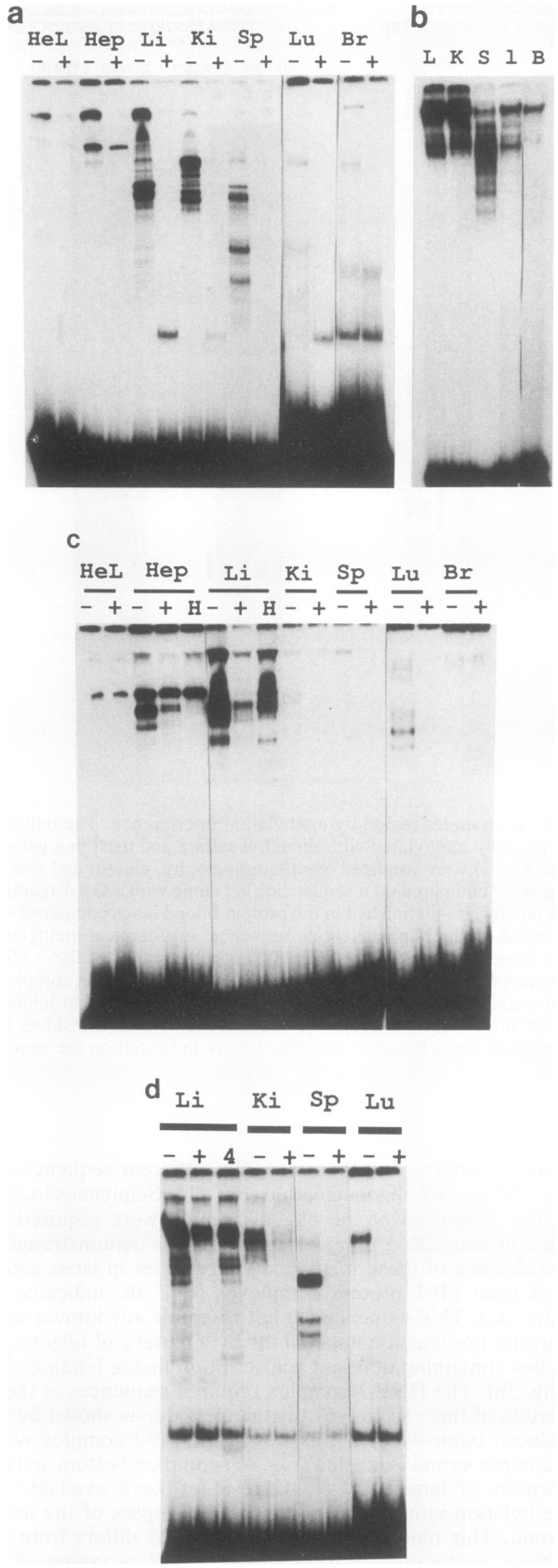


FIG. 4. Identification of four binding sites within the −128 to −24 HNF-3β promoter region by methylation interference. The indicated oligonucleotides were uniquely labeled at the 5′ end, and G residues were partially methylated with dimethyl sulfate and used in a gel shift assay with liver nuclear extract. Protein complexes and unbound DNA (see Fig. 3) were localized by autoradiography, eluted, and cleaved at methylated G residues with piperidine. The protein-bound and free DNAs were compared on a sequencing gel along with a G+A reaction, as shown above the lanes. Methylated G residues that interfere with protein binding are diminished in the protein-bound lanes compared with unbound DNA. These G residues are highlighted by vertical bars and indicated on the oligonucleotide sequence. (a) Identical methylation interference patterns were produced by the B-1 and B-2 protein complexes (lanes B-1 and B-2; see Fig. 3a) on both strands of the −128 to −98 oligonucleotide compared with unbound DNA (lane G; indicated on sequence by +). (b) The pB-1 and pB-2 (HNF-3) protein complexes elicited different methylation interference patterns on the −97 to −67 oligonucleotide (see Fig. 3b). *, Methylated G residues that inhibited pB-1 complex formation (lane B-1; LF-H3β); +, residues important for pB-2 complex (lanes B-2; HNF-3 site). (c) Methylated G residues that are involved in complex formation with the −51 to −24 oligonucleotide (compare lanes B and G; see Fig. 3e) are indicated on the sequene by asterisks.

sulfate and then used in a gel shift assay with liver nuclear extract. The protein complexes (B-1 and B-2) and unbound DNA (Fig. 3a and b) were localized by autoradiography and eluted, and methylated G residues were cleaved with piperidine for comparison on a sequencing gel. Methylated G residues that interfered with binding are absent in the protein-bound bands, as shown in Fig. 4 and summarized on the oligonucleotide sequences above the gels. The two protein complexes (B-1 and B-2) obtained with −128 to −98 sequences produced identical methylation interference patterns on both DNA strands, and the recognition required participation of a large number of G residues on the lower strand (Fig. 4a).

We already demonstrated that two distinct factors were binding to the −97 to −67 sequence and that the pB-2 protein–DNA complex was due to interaction with the HNF-3β protein (Fig. 3). Consistent with these results, the two protein complexes exhibited different methylation interference patterns and thus recognize different sequences on the −97 to −67 oligonucleotide (Fig. 4b). Sequences located at the 3′ portion of the oligonucleotide were required for pB-1 protein–DNA complex formation, as demonstrated by the absence of these methylated G residues in lanes generated from pB-1 protein complexes (Fig. 4b, indicated by asterisks). This sequence did not resemble any known binding site, nor was it competed for by a battery of oligonucleotides containing different transcription factor binding sites (Fig. 3b). The HNF-3 complex required sequences at the 5′ portion of the −97 to −67 oligonucleotide, as shown by the reduced intensity of G residues in the pB-2 complex when the upper strand is labeled (Fig. 4b; compare bottom and top portions of lane B-2). No G residues were available for methylation interference in the pB-2 complex of the lower strand. This binding site (TGTTTGTTTT) differs from the HNF-3 recognition sequence in the TTR promoter (TAT TGACTTAG) but resembles the HNF-3 binding site located



in the albumin enhancer region (TGTTTGTTCT; 42). This HNF-3β promoter site is reduced severalfold in its binding affinity for the HNF-3 protein, as determined by more detailed competition analysis (data not shown).

To define the recognition sequence of the proximal binding protein (−51 to −24), we compared the methylation pattern of the protein-DNA complex with that of unbound DNA depicted in Fig. 3e. This analysis revealed two G residues (at −44 and −36) that were reduced in the bound fraction with oligonucleotide labeled on the upper strand and two adjacent G nucleotides which were necessary for binding with the lower-strand probe (Fig. 4c). Therefore, this protein binds to the sequence that includes GTAACCTTG. However, we could not assign this binding sequence to a known transcription factor recognition site. In summary, the HNF-3β promoter contains four different protein binding sites for five different complexes found in liver nuclear extracts.

The HNF-3β promoter region contains binding sites for two cell-specific and two ubiquitous binding factors. To determine whether any of the factors other than HNF-3 were limited in their cellular distribution, we performed gel shift assays with nuclear extracts prepared from a variety of tissues. This type of analysis was previously used to determine the cell type distribution of the C/EBP (23), HNF-3, and HNF-4 binding activity (9, 11). The activity and integrity of tissue extracts were normalized by performing a gel shift assay with an oligonucleotide containing the AP-1 binding site, which is a widely distributed transcription factor (38). Liver and kidney nuclear extracts exhibited identical AP-1 protein complexes and were therefore comparable in binding activity (Fig. 5b). Spleen extract demonstrated strong AP-1 binding activity but also exhibited a complex with faster mobility on the gel, suggesting that some protein degradation may have occurred. Both brain and lung extracts demonstrated intact protein complexes but exhibited fivefold-lower AP-1 binding activity. Therefore, we compensated for this reduced activity by displaying longer autoradiograph exposure for these tissues in our comparison.

Each of the tissue and cell nuclear extracts that were tested bound the −128 to −98 oligonucleotide in a gel shift assay (Fig. 5a). One specific protein-DNA complex that was similar to the migration of B-1 complex in liver extracts was exhibited by HepG2 and HeLa nuclear extracts, (compare

FIG. 5. Gel shift assay with various tissue extracts and HNF-3β promoter oligonucleotides, revealing two widely distributed binding proteins and two cell-specific factors. Labeled HNF-3β oligonucleotides were incubated with nuclear extracts prepared from HeLa (HeL), HepG2 (Hep), Liver (Li), kidney (Ki), spleen (Sp), lung (Lu), or brain (Br) cells and analyzed for protein complex formation by the gel shift assay. The binding reaction was carried out in either the absence (−) or (+) of homologous competitor DNA. The −128 to −98 oligonucleotide formed protein complexes with all of the indicated nuclear extracts (a); the AP-1 positive control (38) (b) served to normalize the various tissue extracts. Longer exposures for lung and brain nuclear extracts are shown in panels a, c and d, since they possessed reduced AP-1 binding activity. (c) The −97 to −67 oligonucleotide binds two different lung- and liver-specific proteins: HNF-3 (pB-2) and a second complex designated LF-H3β (pB-1). The −97 to −67 sequence binds two complexes in HepG2, liver, and lung nuclear extracts that are not apparent in the other indicated extracts. The slower-migrating LF-H3β complex is distinct from HNF-3, since it is not competed for by the HNF-3 oligonucleotide (lane H). (d) The −51 to −24 sequence binds a protein that is widely distributed in tissue nuclear extracts and is not competed by HNF-4 (lane 4).

Fig. 3a and 5a), but these cell extracts did not contain the B-2 complex. Kidney, lung, and brain extracts formed protein complexes with −128 to −98 that differ in migration from those in liver nuclear extracts (Fig. 5a and b). Spleen extracts displayed several complexes but showed slight degradation of the AP-1 band. We were unable to disrupt these complexes with 5% deoxycholate treatment, suggesting that the slow-mobility bands may not be due to protein-protein interaction. These altered complexes could be due to a different or modified binding protein than the liver −128 to −98 binding protein (Fig. 5). Since several extracts possessed binding activity with the −128 to −98 sequence, we will designate these different protein-DNA complexes ubiquitous binding factors 1–HNF-3β (UF1-H3β). We are in the process of investigating the nature of these different protein-DNA complexes by UV cross-linking experiments.

Consistent with the cell-specific activity of the HNF-3β promoter, the −97 to −67 DNA-protein complexes (pB-1 and pB-2) that are apparent with either liver or HepG2 nuclear extracts were observed only with lung extracts (Fig. 5c). Nuclear extracts from HeLa cells, kidney, spleen, and brain, which produced protein complexes with both the −128 to −98 and AP-1 oligonucleotides, were not able to form any specific complex with the −97 to −67 sequences (compare Fig. 5a and b with Fig. 5c). Liver and HepG2 extracts produce multiple HNF-3 complexes with the −97 to −67 sequence which are the result of sequence recognition by the three different HNF-3 family members (Fig. 5c; compare lanes − and H). As demonstrated in Fig. 3, the pB-1 complex is distinct from HNF-3 complex since it is not competed for by the HNF-3 oligonucleotide (Fig. 5c; compare lanes −, +, and H). In liver, lung, and HepG2 extracts, the pB-1 complex migrates more slowly than HNF-3 bands, and in HepG2 extracts, the pB-1 complex migrates at a position above the nonspecific band (Fig. 5c, lanes H). This pB-1 complex will be designated liver and lung factor–HNF-3β (LF-H3β). The cell-specific distribution pattern of LF-H3β suggests that it may participate in the restricted transcription pattern of HNF-3β.

Even though HeLa nuclear extracts lack binding activity to the −51 to −24 bp oligonucleotide (Fig. 3e), this sequence produced a protein complex with a variety of tissue extracts (Fig. 5d) that migrated to a similar position in the gel shift assay. Spleen possessed a faster-migrating protein complex due to slight degradation of this extract. The wide distribution of this factor suggests that it may participate in basal gene transcription, and thus we will designate it UF2-H3β. In summary, this functional analysis has revealed two widely distributed binding factors and two cell-specific factors, LF-H3β and HNF-3.

Site-directed mutagenesis reveals that all of the protein binding sites contribute to HNF-3β promoter activity. Nucleotide changes were made in each of the four protein binding sites to assess their contribution to HNF-3β promoter activity. These HNF-3β mutant constructs were transfected into HepG2 cells, analyzed for CAT activity, and normalized to the CMV–β-galactosidase internal control. The mutant expression data are presented as a ratio of wild-type levels in Fig. 6. Mutations in each of the protein binding sites resulted in reduced HNF-3β promoter activity, illustrating that HNF-3β expression is due to a combinatorial effect of these transcription factors. Disruption of the UF1-H3β site (−110 to −105) showed the most dramatic drop in the promoter activity (Fig. 6), which is consistent with expression levels produced by removal of this binding site (Fig. 2). However, this factor alone is not sufficient for HNF-3β promoter activity, since HeLa cells, which possess this binding activity, are inactive for HNF-3β expression. The binding site for the other widely distributed UF2-H3β also contributed to promoter activity but to a lesser extent than UF1-H3β (Fig. 6).

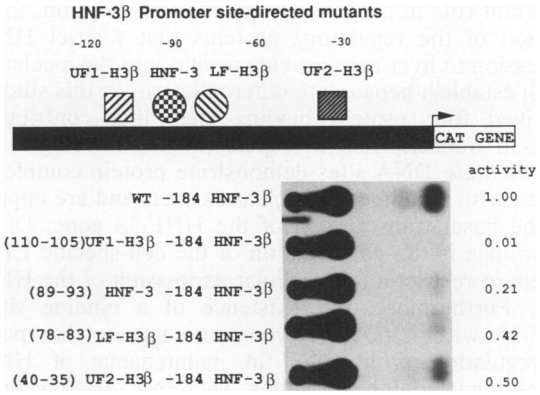


FIG. 6. Contribution of each site to HNF-3 β promoter activity as revealed by site-directed mutagenesis of protein binding sites. Shown is a schematic drawing of the HNF-3β promoter and locations of protein binding sites identified in this study. a single-stranded −184 HNF-3β promoter–CAT construct was used for oligonucleotide site-directed mutagenesis to introduce nucleotide changes in each of these protein binding sites (see Materials and Methods). Each construct was transfected into HepG2 cells along with a CMV–β-galactosidase internal control, and then protein extracts from transfected cells were analyzed for CAT enzyme activity. The results of this experiment are shown, with the indicated HNF-3β site-directed mutation and the wild-type included as a control. The amount of CAT activity was normalized to the internal control and tabulated as the ratio of mutant to wild-type expression levels.

Substantial reductions in HNF-3β-driven CAT expression were also observed with mutations in the cell-specific binding sites HNF-3 and LF-H3β (Fig. 6). Therefore, HNF-3β promoter activity requires both these sites for maximal expression. In support of the thesis that three factors are required for expression, we were unable to activate the HNF-3β promoter in HeLa cells by cotransfection with a HNF-3β expression construct (data not shown). This implies that the restricted transcription pattern of HNF-3β could be due to the absence of both HNF-3 and LF-H3β proteins in other tissues (Fig. 5c). In addition, the site-directed mutations also illustrate that the HNF-3 site is important for maximal activity but that the disruption of this site is not sufficient to completely inactivate HNF-3β transcription. This finding allows us to propose a model describing the cell-specific transcriptional induction of the HNF-3β gene by LF-H3β and subsequent autoactivation through the HNF-3 site within its promoter region.

DISCUSSION

The transcription factor HNF-3 plays an important role in the coordinate expression of several hepatocyte-specific genes, since it binds to the DNA regulatory regions of the TTR, α_1-antitrypsin, α-fetoprotein (9, 20), albumin (25, 42), phosphoenolpyruvate carboxykinase (27), and apolipoprotein B (4) genes. Hepatocyte-specific transcription requires multiple binding proteins that are present in a limited number of other cell types and are intimately involved in the maintenance of liver-specific expression. Since HNF-3 plays an

important role in hepatocyte-specific transcription, the elucidation of the regulatory proteins that restrict HNF-3β expression to liver may provide insight into the mechanisms which establish hepatocyte differentiation. In this study, we identified four protein binding sites that contribute to HNF-3β transcription in HepG2 (human hepatoma) cells. Two of these DNA sites demonstrate protein complex formation with a number of nuclear extracts and are important for the basal transcription of the HNF-3β gene. Of most importance is the participation of the cell-specific LF-H3β protein in restricting the cellular expression of the HNF-3β gene. Furthermore, the existence of a binding site for HNF-3β within its own promoter suggests that positive autoregulation is involved in maintenance of HNF-3β expression in adult hepatocytes. Therefore, establishment of HNF-3β transcription may involve two different types of mechanisms: transcriptional induction by a cell-specific factor (LF-H3β) and autoactivation by its own gene product.

Model for cell-specific transcription of HNF-3β and autoregulation. The analysis of the HNF-3 β promoter in HepG2 cells led to identification of proteins that initiate the cell-specific transcription pattern of this regulatory gene. Site-directed mutations with each of the four binding sites have illustrated that all of the sites contribute to HNF-3β promoter activity (Fig. 6). One of the promoter sequences is recognized by a novel cell-specific factor, LF-H3β, which exhibited binding activity only in liver and lung nuclear extracts (Fig. 5). A second site is occupied by the HNF-3 protein and thus provides an autoregulatory mechanism for transcriptional control. This HNF-3 site is divergent from the HNF-3 consensus, and its lower binding affinity may provide a less dramatic response to HNF-3 recognition. The other two sites (UF1-H3β and UF2-H3β) bind proteins (in various nuclear extracts) that vary in their gel shift mobilities and are necessary components for expression in HepG2 cells. In addition, we have shown that lung nuclear extracts possess the requisite factors and may utilize these proteins to control HNF-3β expression in the lung.

With regard to the cell-specific transcription pattern of the HNF-3β gene, the LF-H3β protein plays a critical role in eliciting hepatocyte expression. We propose that HNF-3β transcription in liver involves promoter recognition by the cell-specific LF-H3β protein and two ubiquitous binding proteins, UF1-H3β and UF2-H3β. The presence of these three factors produces expression levels exhibited by the HNF-3 site-directed promoter mutant (Fig. 6). Tissues other than liver and lung possess only the two ubiquitous binding factors, which are insufficient to elicit HNF-3β transcription. Therefore, the LF-H3β protein is an important component for promoting the cell-specific expression of the HNF-3β gene. Subsequent to this initial activation by the LF-H3β protein, a feedback loop is involved in which the HNF-3 protein binds to a site within the HNF-3β promoter and activates its own expression. Together with the LF-H3β protein, this positive feedback loop also serves as a mechanism to sustain HNF-3β expression in adult hepatocytes. This model is consistent with the data presented herein and provides a working hypothesis to explain cell-specific transcription of the HNF-3β gene.

The fact that none of the other known liver factors (HNF-1/LF-B1, C/EBP, or HNF-4) bind to the HNF-3β promoter suggests that HNF-3 is at an equivalent position within the hierarchy of factors involved in the developmental cascade. Furthermore, conservation of the HNF-3 gene in evolution as shown by the homology with the *Drosophila* homeotic gene fork head, suggests the importance of the HNF-3 regulatory protein during hepatocyte differentiation. Although our experiments have defined proteins necessary for the transcription of HNF-3β, they do not evaluate whether these regulatory elements are sufficient to initiate HNF-3β transcription at the appropriate period during embryonic development. Transgenic experiments in *C. elegans* using a *C. elegans* MyoD promoter–β-galactosidase construct demonstrated expression in early blastula, yet negative regulation subsequently restricted expression to body wall muscle cell lineage (31). Transgenic mice harboring wild-type and mutant HNF-3β promoters driving the expression of the β-galactosidase gene will be used to determine the requirement for developmental expression. In addition, since all of the HNF-3 family members (α and γ) can recognize the HNF-3 site, it is possible that a cross-regulating mechanism exists in the control of HNF-3 expression. The isolation of promoters for the other HNF-3 genes and the generation of HNF-3 knockout mutants will allow for further study of this issue.

Regulation of HNF-3β in comparison with regulation of other cell-specific transcription factors. A positive autoactivation loop is involved in the establishment and maintenance of HNF-3β expression in hepatocytes. Autoregulation may also be used by the *Drosophila* fork head (HNF-3 homolog), since the 5′ flanking region contains several putative recognition sites for fork head protein binding (58, 59). Similar autoregulatory schemes were first discovered in promoter or enhancer regions of the *Drosophila* homeotic genes fushi tarazu (26), deformed (2), and Ultrabithorax (30). In mammals, ectopic expression of muscle-specific MyoD1 in other cell types stimulates the transcription of endogenous MyoD1 and results in differentiation toward the myogenic pathway (56). Detailed functional analysis of the promoters for pituitary-specific Pit-1/GHF-1 (7, 44) and erythrocyte-specific GATA-1 (57) revealed autoregulatory sequences and sites for widely distributed factors. However, this initial characterization did not demonstrate the binding of cell-specific activators or repressors. Recent in vitro transcription analysis of the GHF-1 promoter revealed that sequences flanking the TATA box interact with a cell-type-specific factor and may explain pituitary-specific expression of GHF-1 (43). Indeed, requirement for the cell-specific LF-H3β factor in HNF-3β promoter activity provides the molecular basis for restricted expression of the HNF-3β gene. Moreover, the LF-H3β protein may also play a more extensive role in hepatocyte differentiation, since it controls the expression of a transcription factor that participates in regulation of liver-specific genes.

A general feature of tissue-specific promoters is the involvement of multiple cell-specific factors in their restricted activity (28). The existence of two cell-specific binding sites for HNF-3β promoter activity (HNF-3 and LF-H3β) parallels the architecture of other tissue-specific promoters. Because HNF-3 expression is required to elicit the transcription of liver genes, the HNF-3β promoter factors may be reserved for regulatory genes that are expressed early in liver differentiation. This hypothesis is supported by experiments which correlate the differentiation of simian virus 40-infected hepatocytes (H2.35) with an increase in both HNF-3α mRNA levels and HNF-3α binding activity (14). Further analysis of the regulation of the HNF-3 family members will determine whether HNF-3α and -γ promoter activity depends on the LF-H3β, UF1-H3β, and UF2-H3β proteins. Moreover, the visceral yolk sac represents an extra embryonic tissue that expresses many liver genes and their transcription factors, including HNF-3 (11, 45). Treatment of

F9 cells with retinoic acid provides an opportunity to investigate the events involved in the differentiation of the visceral endoderm cell type (33). The analysis presented herein will provide the framework for study of mechanisms involved in induction of HNF-3 activity during F9 differentiation. Finally, transgenic analysis using HNF-3β promoter constructs will enable us to determine whether these factors are sufficient for the correct developmental expression in liver, lung, and visceral yolk sac.

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