DNA AND CELL BIOLOGY
Volume 30, Number 3, 2011

Pp. 137–147
DOI: 10.1089/dna.2010.1097

The Mouse Alpha-Albumin (Afamin) Promoter Is Differentially Regulated by Hepatocyte Nuclear Factor 1α and Hepatocyte Nuclear Factor 1β

Hua Liu,[1,2] Hui Ren,[1] and Brett T. Spear[1,3]

Alpha-albumin (*AFM*), a member of the albumin gene family that also includes *albumin*, *alpha-fetoprotein*, and *vitamin D-binding protein*, is expressed predominantly in the liver and activated at birth. Here, we identify two hepatocyte nuclear factor 1 (HNF1)-binding sites in the AFM promoter that are highly conserved in different mammals. These two sites bind HNF1α and HNF1β. The distal site (centered at −132, relative to AFM exon 1) is more important than proximal site (centered at −58), based on HNF1 binding and mutational analysis in transfected cells. Our data indicate that HNF1α is a more potent activator of AFM promoter than is HNF1β. However, HNF1β can act in a dominant manner to inhibit HNF1α-dependent transactivation of the AFM promoter when both proteins are expressed together. This suggests that the differential timing with which the albumin family genes are activated in the liver may be influenced by their responsiveness to HNF1α and HNF1β. Our comparison of HNF1-binding sites in the promoters in the albumin family of genes indicates that the primordial *albumin*-like gene contained two HNF1 sites; one of these sites was lost from the albumin promoter, but both sites still are present in other members of this gene family.

Introduction

ALPHA-ALBUMIN (*AFM*, also called *afamin*) is a member of the albumin gene family that also includes *albumin* (*Alb*), *alpha-fetoprotein* (*AFP*), *AFP-related gene* (*Arg*), and *vitamin D-binding protein* (*DBP*) (Gibbs *et al.*, 1998; Naidu *et al.*, 2010). These evolutionarily related genes arose from a series of duplications and encode serum transport proteins (Kioussis *et al.*, 1981; Gibbs and Dugaiczyk, 1987). In mice, these genes are found on chromosome 5 (Tilghman, 1985). *Alb*, *AFP*, *AFM*, and *Arg* are adjacent to each other and tandemly arranged in the same transcriptional orientation (5′ *Alb*–*AFP*–*AFM*–*Arg* 3′); *DBP* is less tightly linked and found roughly 1 Mb upstream of *Alb* and in the opposite transcriptional orientation (Naidu *et al.*, 2010). These genes are all expressed primarily in the liver but have different temporal patterns of expression. *AFP* and *Alb* are activated early in hepatogenesis and continue to be expressed at high levels in the fetal liver (Tilghman and Belayew, 1982). *Alb* expression persists at high levels in the adult liver, whereas *AFP* expression declines dramatically during the perinatal period and remains off in the normal adult liver. *DBP* is activated during midgestation, whereas *AFM* is activated at birth; expression of both genes continues in the adult liver (McLeod and Cooke, 1989; Belanger *et al.*, 1994). *AFP* is frequently activated in hepatocellular carcinomas, whereas *AFM*, whose activation parallels *AFP* repression at birth, is downregulated in liver cancer (Abelev, 1971; Wu *et al.*, 2000). *Arg* is activated at birth but is expressed at very low levels in mice; the *Arg* gene is intact in rodents but is a pseudogene in primates due to numerous mutations (Naidu *et al.*, 2010).

The transcriptional control of *Alb*, *AFP*, and *DBP* in the liver has been well studied. Many of the liver-enriched factors that have been identified, including hepatocyte nuclear factor 1 (HNF1), FoxA, HNF4, HNF6, and C/EBP, have been found to regulate members of this gene family (Gorski *et al.*, 1986; Chevrette *et al.*, 1987; Lichtsteiner *et al.*, 1987; Cereghini *et al.*, 1988; Feuerman *et al.*, 1989; Zhang *et al.*, 1991; Milos and Zaret, 1992; Bois-Joyeux and Danan, 1994; Thomassin *et al.*, 1996; Song *et al.*, 1998). These transcription factors are also expressed in tissues other than the liver, suggesting that the combined action of multiple factors is required for the liver-restricted expression of target genes, including members of the albumin family. Binding sites for HNF1 and C/EBP are found in the promoters of *Alb*, *AFP*, and *DBP*, suggesting an essential role for these factors in liver-specific control of this gene family (Courtois *et al.*, 1988; Feuerman *et al.*, 1989; Maire *et al.*, 1989; Thomassin *et al.*, 1992; Song *et al.*, 1998; Hiroki *et al.*, 2007).

[1]Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky, Lexington, Kentucky.
[2]Institute of Immunology, Shandong University School of Medicine, Jinan, China.
[3]Markey Cancer Center, University of Kentucky College of Medicine, Lexington, Kentucky.

HNF1 was initially identified by its interaction with an essential sequence for the liver-specific transcription of the β-fibrinogen (βFG), albumin, and α_1-antitrypsin promoters (Courtois *et al.*, 1987). Subsequent studies have identified HNF1-binding sites in numerous liver-specific genes (Schrem *et al.*, 2002). Two members of the HNF1 family, HNF1α and HNF1β, have been isolated and characterized (Baumhueter *et al.*, 1990; Mendel *et al.*, 1991). Both genes are expressed in the liver, kidney, and intestine, although at different levels (Blumenfeld *et al.*, 1991; Cereghini *et al.*, 1992). During embryonic development, HNF1β is induced upon the onset of hepatic differentiation, whereas HNF1α is activated later and continues to be expressed in terminally differentiated hepatocytes (Cereghini *et al.*, 1992). Inactivation of HNF1β in the developing liver leads to liver dysfunction and defects in the bile system (Coffinier *et al.*, 2002). HNF1α-deficient mice die during the perinatal period, due to hepatic dysfunction and phenylketonuria (Pontoglio *et al.*, 1996). These studies indicate an important role for HNF1 in hepatogenesis and normal hepatocyte function. Both HNF1 proteins have an amino-terminal dimerization domain, Pit, Oct, Unc (POU)-like homeodomain with DNA-binding activity, and carboxyl-terminal transcriptional activation domain, with the activation domains of these two proteins being more divergent than the DNA-binding domains (Mendel *et al.*, 1991).

In contrast to other members of the albumin gene family, the basis for liver-specific and developmental control of AFM remains unexplored. Here, we show that the human and mouse AFM promoters contain two highly conserved HNF1 sites. Analysis of the mouse AFM promoter by transient transfections and electrophoretic mobility shift assays (EMSAs) indicates that both sites bind HNF1 and are important for promoter activity. We also find that HNF1α is a more potent activator of AFM promoter than is HNF1β. Further, HNF1β can act in a dominant manner to inhibit HNF1α-dependent transactivation of the AFM promoter, a phenomenon that has also been observed with the DBP promoter (Song *et al.*, 1998). This raises the possibility that the differential timing with which the albumin family genes are activated in the liver may be influenced by their responsiveness to HNF1α and HNF1β.

Materials and Methods

Molecular biology/synthesis of HNF1 expression vectors

All oligonucleotides were purchased from Integrated DNA Technologies. The mouse AFM promoter fragments were cloned by polymerase chain reaction (PCR) amplification of mouse genomic DNA. For promoter fragments with various 5′ endpoints, amplicons were generated using the following series of forward primers (−320F: GGATCCA GGCCCCAGAAACTTAACTTAATG; −234F: GGATCCGG AGGATTATTCTTACCCTGTG; −145F: GGATCCCCTAGT TAATAATTACCTAGA; −124F: GGATCCAGAAATTTGCA CCAGGACCGAA) and a common reverse primer (+14R: AAGCTTTAAAGGAGCAATGTGACTGGGG; transcription start site = +1). Fragments were cloned into pGEM-T Easy (Promega), sequenced, excised with *Bam*HI and *Hin*dIII, and re-cloned into the promoterless luciferase vector pGL3-Basic (Promega) that had been linearized with *Bgl*II and *Hin*dIII. Using the full-length (−320) promoter as a template, mutations were introduced into site 1, site 2, or both sites 1 and 2, by the megaprimer method (Sarkar and Sommer, 1990). Mutated constructs were confirmed by DNA sequencing. The pGL3-Enhancer (Promega) was used as a positive control for transfections. Full-length expression vectors for mouse HNF1α and HNF1β were generated by PCR amplification of mouse liver cDNA. The 5′ oligonucleotide (GCCACCATGGTTTCTAAGCTGAGC and GCCACCAT GGTGTCCAAGCTCACGT for HNF1α and HNF1β, respectively) contained a Kozak consensus, whereas the 3′ oligonucleotide (GGATCCCTGGGAAGAGGAGGC and GGAT CCCCAGGCTTGCAGTGGACA for HNF1α and HNF1β, respectively) was flanked by a *Bam*HI site. Amplicons were cloned into pGEM-T Easy, sequenced, excised using *Eco*RI and *Bam*HI, and cloned into the pcDNA3.1 myc-His expression vector (Invitrogen), which provides C-terminal Myc and His epitope tags.

Tissue culture cells/transfections/mRNA analysis

Human hepatoma cell line HepG2 was maintained in Dulbecco's modified Eagle's medium-F12 (1:1) supplemented with 10% fetal bovine serum and 10 μg/mL of bovine pancreatic insulin. Human hepatoma Hep3B cells and human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, penicillin/streptomycin, and glutamine. Insulin was obtained from Sigma Chemical Corp.; all other reagents were from Life Sciences. Cells were incubated at 37°C in the presence of 5% CO_2.

All transfections were carried out by the calcium phosphate method as described using a total of 15 or 7.5 μg of DNA (for 10 or 6 cm dishes, respectively) (Long and Spear, 2004). Cells were transfected with the AFM-luciferase constructs alone or with expression vectors for HNF1α, HNF1β, or empty vector control; the Renilla luciferase expression vector pRL-CMV was included to normalize for variations in transfection efficiency. Six hours after transfection, plates were washed with phosphate-buffered saline (PBS) and a fresh medium was added. Forty-eight hours after the addition of DNA, cells were washed three times in PBS, scraped from plates into 1.5 mL of PBS, and transferred to 1.5 mL microcentrifuge tubes. Cells were pelleted by centrifugation and were stored at −80°C or used immediately for mRNA production, the dual-luciferase assay, or the preparation of nuclear extracts as described below.

Dual luciferase assays and RNA analysis

Cell pellets from transient transfections were resuspended in 400 μL of lysis buffer (Promega) and lysed for 15 min at room temperature and stored at −80°C. Dual-luciferase reporter assays were performed in triplicate following the Promega protocol using 20 μL of cell extracts and 100 μL of Luciferase Assay Reagent II Reagent and 100 μL Stop&Glo™ Reagent with the Luminoskan TL plus (Labsystems). Firefly luciferase activity was normalized against the Renilla reniformis luciferase activity. Transfection data shown in this study were obtained from at least three independent experiments.

For RNA analysis, RNA was prepared from cell pellets using Trizol as described (Long and Spear, 2004). RNA was converted to cDNA using the iScript cDNA synthesis kit

(Bio-Rad). For AFM, the forward primer was from exon 4 (TTTCCCTACCCTGGATC) and the reverse primer was from exon 7 (GATGCACTGCACAACATCCCC); the resulting amplicon from cDNA should be 420 bp. For β-actin, the forward primer was from exon 3 (ATTGGCAATGAG CGGTTCCG) and the reverse primer was from exon 5 (TGATCCACATCTGCTGGAAGG); the resulting amplicon from cDNA should be 323 bp. PCR was carried out using a Perkin-Elmer GeneAmp 9700 thermal cycler; products were resolved by polyacrylamide gel electrophoresis and bands were observed by ethidium bromide staining.

EMSA and Western analysis

EMSAs were carried out using nuclear extracts prepared from Hep3B or transient transfected HEK293 cells as described (Li *et al.*, 2000). Protein concentrations were determined using the BCA assay kit (Pierce Biochemicals). Oligonucleotides were annealed and used as radiolabeled probes (end-labeled with γ^{32}P-ATP using T4 polynucleotide kinase) or unlabeled cold competitors. Reactions used 5 μg of nuclear extract and 0.25 μg of poly dI:dC. Reaction mixtures were incubated on ice for 15 min in the presence or absence of nonradioactive competitors or super-shift antibodies, followed by 30 min incubation at room temperature after adding radiolabeled probe (~20,000 cpm). Reaction mixtures were resolved on nondenaturing 8% polyacrylamide gels in 1×TBE (2.2 mM Tris, 2.2 mM boric acid, and 0.5 mM ethylenediaminetetraacetic acid) running buffer. Gels were dried and subjected to autoradiography and analyzed using Phosphorimaging analysis. Antibodies for supershift experiments were obtained from Santa Cruz Biotechnology (anti-HNF1α: C19; anti-HNF1β: C20; anti NFI: H300). The following double-stranded oligonucleotides were used as probes for EMSAs: site 1 (CTCAGTTAATAATTACCTAG), site 2 (TT AACTAAGTTACTTTTTAACAAATGTT), site 1 mutant (CT CAGTAC TCGAGAACCTAG), site 2 mutant (TTAAC TAAGCCTCGAGCGGACAAATGTT), rat βFG HNF1 site (ACCAAACTGTCAAATATTAACTAAAGGGAG), and unrelated (ATACAAGTGACCCCTGCTCT). Western analysis was carried out using standard procedures, using antibodies against the myc epitope. Bands were observed using the ECL Western Kit (Pierce Biochemicals).

Results

Candidate HNF1-binding sites are present in the mouse AFM promoter region

The *AFM* gene is activated in the liver at birth and continues to be expressed at high levels in the adult liver (Belanger *et al.*, 1994). The basis for this hepatic regulation has not been investigated. To identify potential liver-enriched factors that govern AFM expression, we aligned the region upstream of AFM exon 1 from several different species. These studies indicate that roughly 300 bp region upstream from exon 1 shows considerable conservation, but this conservation falls off dramatically further upstream (data not shown). Several segments of considerable conservation are found between the mouse and human AFM promoter regions (Fig. 1). A search for transcription factor binding sites identified two candidate sites for HNF1 that are found in both the mouse and human promoters. The site centered at −132 (site 1) and −58 (site 2) of the mouse promoter shows 11/12 and 10/12 match, respectively, to the consensus HNF1-binding site (Fig. 1; corresponding sites in the human promoter show 10/12 and 9/12 matches, respectively). This analysis also identified a conserved C/EBP consensus site from −84 to −75 of the mouse AFM promoter (−82 to −73 of the human AFM promoter).

To investigate the transcriptional activity of the AFM promoter, a 336 bp region was amplified from mouse DNA (from −320 to +16 within exon 1), sequenced, and cloned into the pGL3-basic luciferase expression vector to generate AFM(320)-Luc. Activity of this reporter construct was analyzed by transient transfections into the human hepatoma cell lines HepG2 and Hep3B and HEK293 cells; a Renilla

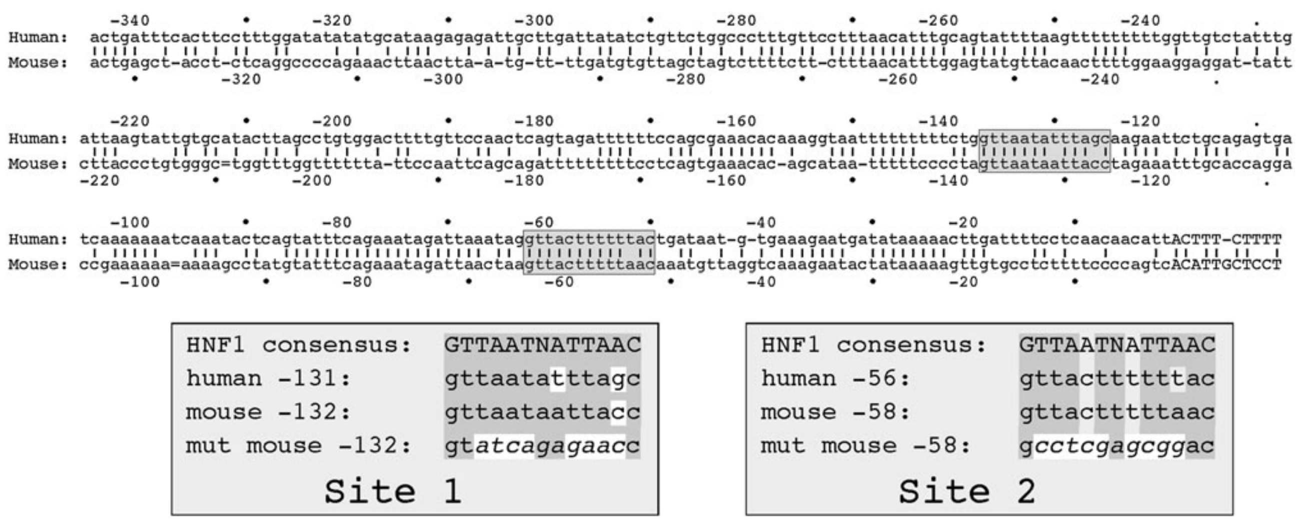

FIG. 1. Alignment of the human and mouse alpha-albumin (AFM) promoters. Regions shown are from −345 (human) and −334 (mouse) relative the to the beginning of exon 1 (exonic sequences in upper case). The two putative hepatocyte nuclear factor 1 (HNF1)-binding sites centered at −130 (site 1) and −55 (site 2) are highlighted in gray. A comparison of these two sites with the HNF1 consensus sequence are shown at the bottom.

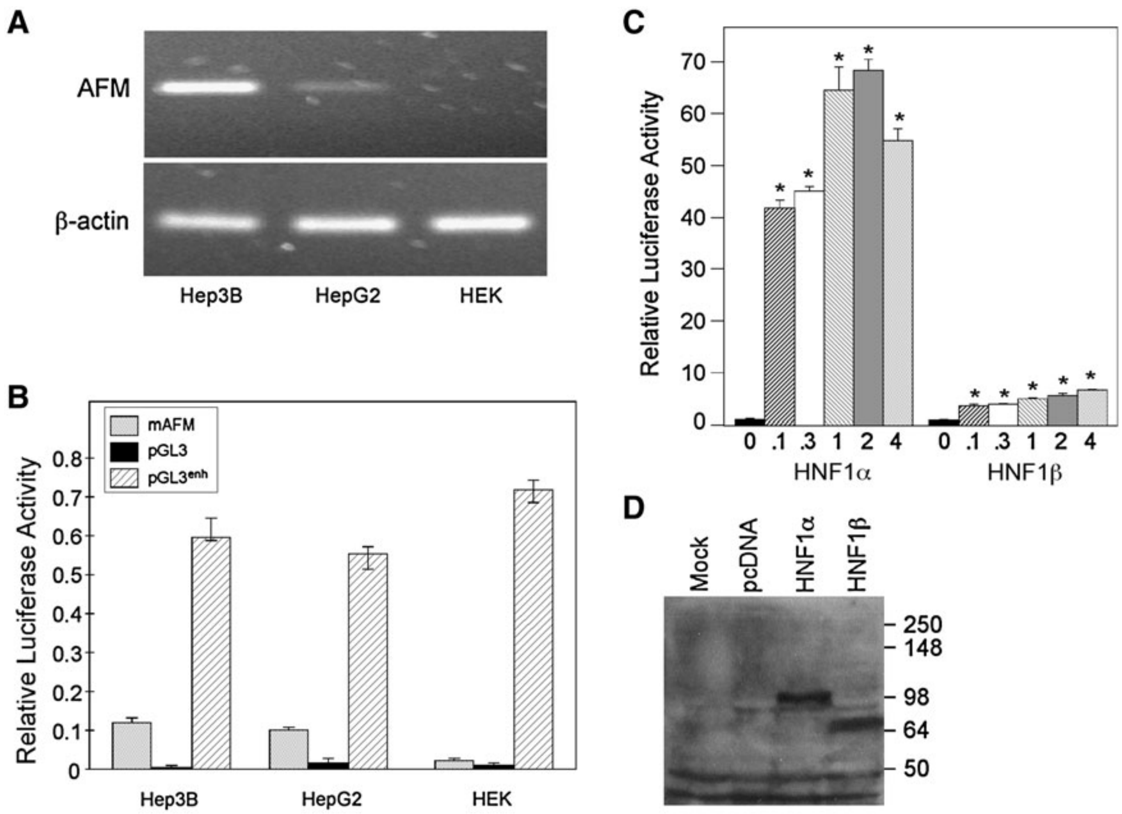

FIG. 2. AFM promoter activity in different cell lines and responsiveness to HNF1α and HNF1β. **(A)** Expression of endogenous AFM gene in Hep3B, HepG2, and nonliver human embryonic kidney 293 (HEK293) cells determined by RT-polymerase chain reaction analysis of total RNA. The β-actin levels were used as a control. **(B)** Expression of luciferase reporter genes in Hep3B, HepG2, and HEK293 cells. Cells were transiently transfected with AFM(320)-Luc (AFM promoter from −320 to +16 fused to luciferase), the promoterless pGL3 luciferase vector, and pGL3enh (SV40 promoter/enhancer linked to luciferase). Luciferase levels were normalized to the cotransfected Renilla luciferase. **(C)** Transient cotransfections of AFM(320)-Luc and HNF1 expression vectors in HEK293 cells. Amounts of transfected HNF1α and HNF1β vectors are shown on the X-axis (μg). Luciferase levels were normalized to the cotransfected Renilla luciferase. Asterisks indicate statistically significant increase in expression compared to no HNF1 expression vector ($p < 0.01$). **(D)** Western analysis of HNF1 proteins in transfected HEK293 cells. Lysates from untransfected cells (Mock) or cells transfected with the pcDNA3.1 empty vector (pcDNA), HNF1α, or HNF1β expression vectors were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with antiserum against the Myc epitope.

luciferase construct was cotransfected to normalize for variations in transfection efficiency. To monitor expression of endogenous AFM expression in these cells, RNA was isolated and AFM mRNA levels were analyzed by reverse transcriptase–PCR. These data indicate that AFM is expressed in Hep3B and HepG2 cells at moderate and low levels, respectively (Fig. 2A). In contrast, AFM mRNA levels were not detected in HEK293 cells (Fig. 2A). In transient assays, the AFM(320)-Luc was active in both hepatoma cell lines (Fig. 2B). This activity was considerably higher than the promoterless pGL3 vector but lower than the pGL3-positive control, which contains the strong SV40 promoter/enhancer. In contrast, the 320 bp AFM promoter had little, if any, activity in HEK293 cells (Fig. 2B). Similar studies indicated that a 330 bp human AFM promoter fragment was also active in Hep3B and HepG2 cells, but not in HEK293 cells (data not shown).

To test whether the mouse AFM promoter was regulated by HNF1, cotransfections were performed in HNF1-deficient HEK293 cells. Full-length cDNAs for both HNF1α and HNF1β were amplified from mouse adult liver RNA, cloned into the pGEMT-Easy vector, confirmed by DNA sequencing, and then subcloned into the pcDNA3.1-Myc/HIS expression vector. Cotransfections were performed with AFM(320)-Luc and increasing amounts of HNF1 vectors (Fig. 2C). HNF1α was a potent activator of the AFM promoter even at the lowest concentration tested. HNF1β could also transactivate the AFM promoter, but to a much lesser extent than HNF1α. Even at the highest concentration, transactivation by HNF1β was only ∼10% that of HNF1α. This dramatic difference in the transactivation by the two HNF1 isoforms was not due to different levels of HNF1 proteins, since Western analysis indicated that similar levels of HNF1α and HNF1β were present in extracts of transfected HEK293 cells (Fig. 2D). Taken together, these data confirm that the mouse AFM promoter is transactivated by both HNF1 isoforms.

HNF1α/β binds to two HNF1 sites in the mouse AFM promoter

To determine whether either of the computer-predicted HNF1 sites could bind HNF1α or HNF1β, EMSAs were performed. The well-characterized HNF1-binding site from

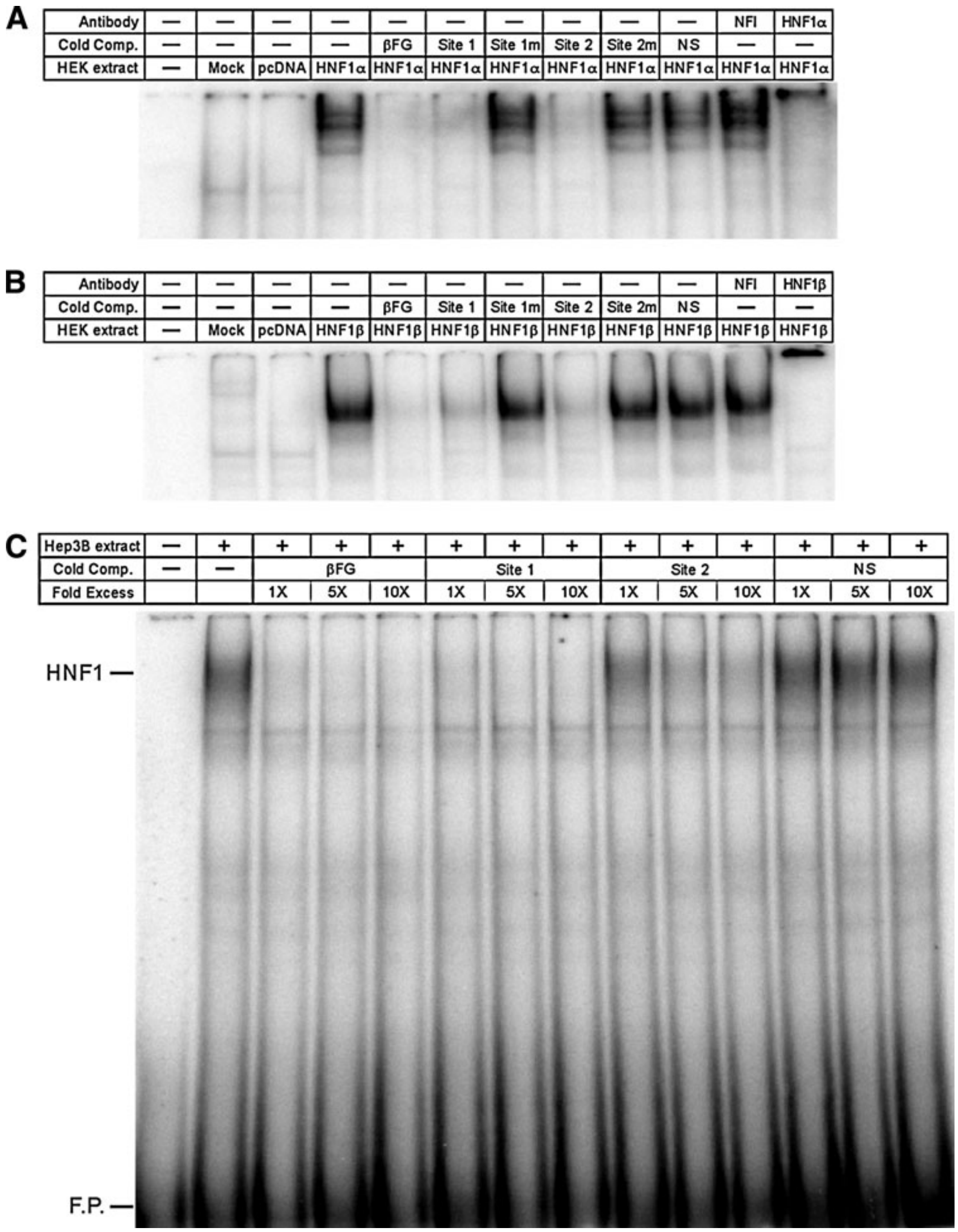

FIG. 3. Electrophoretic mobility shift assay (EMSA) analysis using the β-fibrinogen (βFG) HNF1 site as a radiolabeled probe. **(A)** EMSA performed with the βFG probe and extracts from HEK293 cells that were mock transfected or transfected with the pcDNA empty vector or HNF1α expression vector. EMSAs were performed with no competitor or with a 40-fold excess of cold competitor as listed, including wild-type and mutant versions of AFM promoter site 1 and site 2. Supershift experiments were performed with antibodies against nuclear factor I (NFI) and HNF1α. NS, nonspecific oligonucleotide. **(B)** EMSAs were performed as in **(A)** except HNF1β was transfected instead of HNF1α, and an anti-HNF1β was used in supershift assays. **(C)** EMSAs using the radiolabeled βFG probe and extracts from Hep3B cells, using no competitor or the cold competitors shown at 1-, 5-, or 10-fold excess.

the rat βFG promoter was first used as a radiolabeled probe and fragments containing the predicted HNF1 sites in the AFM promoter were used as cold competitors. Since HNF1 proteins are not expressed in HEK293 cells, nuclear extracts were prepared from these cells that were transiently transfected with HNF1 expression vectors. Complexes between HNF1α and the βFG probe were readily detected; the presence of HNF1α in these complexes was confirmed by supershift assays (Fig. 3, top panel). In competition experiments with a 40-fold excess of unlabeled competitor, the βFG oligonucleotide effectively competed for binding. The oligonucleotides corresponding to AFM site 1 and site 2 (centered at −132 and −58, respectively) could also compete for binding. In contrast, mutated versions of site 1 or site 2, as well as a nonspecific competitor, did not compete. Similar results were obtained with extracts from HEK293 cells that were transfected with the HNF1β expression vector (Fig. 3, middle panel).

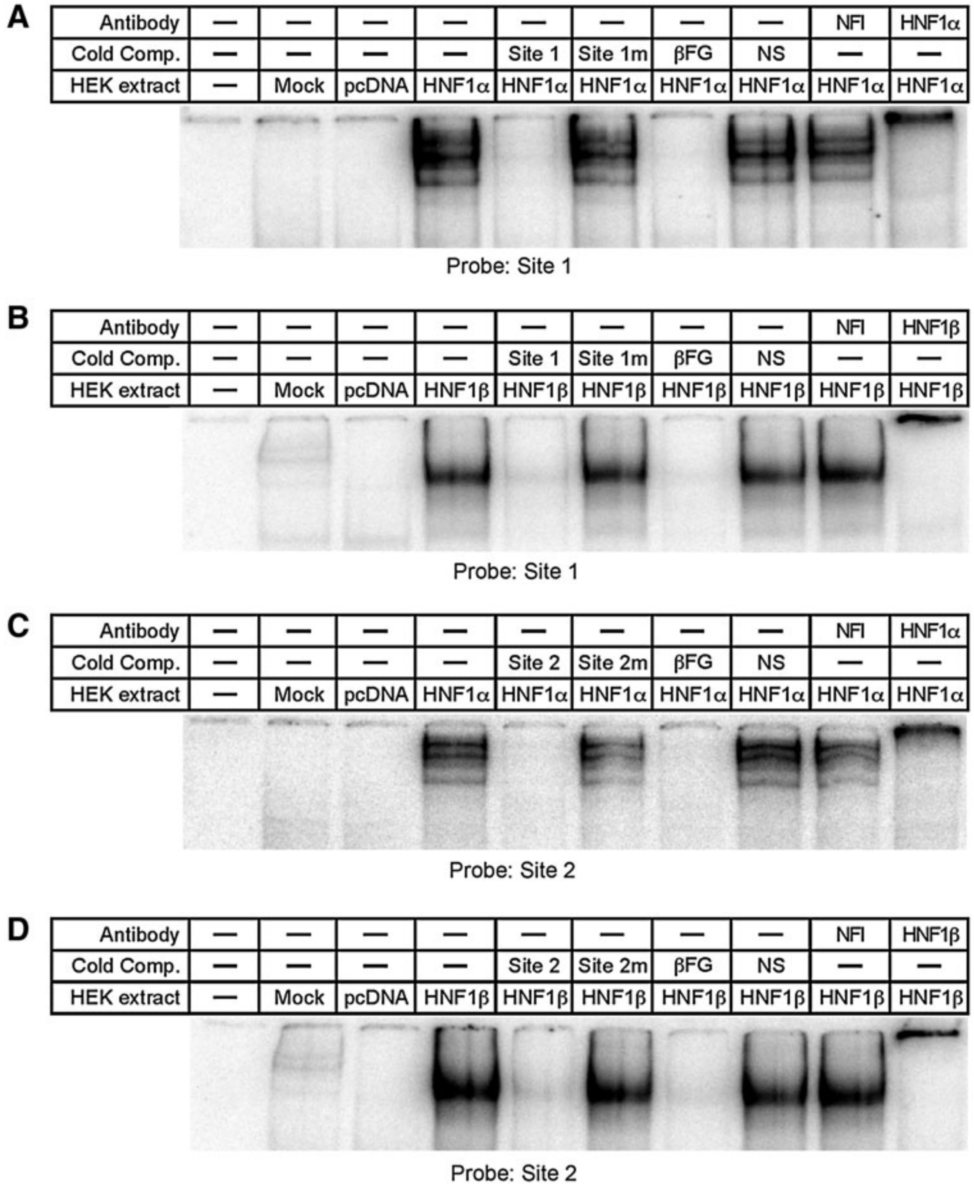

FIG. 4. EMSA analysis of HNF1 binding to AFM promoter site 1 and site 2. Nuclear extracts were prepared from HEK293 cells that were untransfected, or transfected with empty vector (pcDNA), HNF1α **(A, C)**, or HNF1β **(B, D)**. Extracts were incubated with radiolabeled probes corresponding the site 1 **(A, B)** or site 2 **(C, D)** of the AFM promoter. EMSAs were performed with no competitor or with a 40-fold excess of cold competitors as listed, including wild-type and mutant versions of site 1 and site 2. Supershift experiments were performed with antibodies against NFI, HNF1, or HNF1β as shown.

Sequence comparisons indicate that site 1 is more similar to the HNF1 consensus motif than is site 2, suggesting that this site might be more effective at binding HNF1 isoforms. To test this, EMSAs were also performed with extracts from Hep3B cells. These cells contain HNF1α and HNF1β at moderate and low levels, respectively (data not shown). Here, increasing amounts of cold competitors (1X, 5X, and 10X) were used to compete for binding to the βFG radiolabeled probe (Fig. 3, lower panel). Both the βFG and site 1 oligonucleotides could effectively compete for binding at a onefold molar excess. In contrast, site 2 was a less effective competitor, but could still compete for binding at higher concentrations. Phosphorimage analysis indicated that equimolar amounts (1X competitor) of the βFG, site 1, and site 2 reduced the radioactive complex by 100%, 95%, and 60%, respectively.

To confirm HNF1 binding to site 1 and site 2, EMSAs were performed using these as radiolabeled probes with extracts from transfected HEK293 cells (Fig. 4). Both sites could effectively bind both HNF1α and HNF1β. For both sites, the βFG and self-fragments could effectively compete for binding, whereas the mutated self-fragment and a nonspecific competitor could not. Supershift EMSAs with anti-HNF1α and anti-HNFβ confirmed the presence of these proteins in the shifted complexes. Taken together, these data confirm the ability of site 1 and site 2 to bind HNF1α/β, and suggest that site 1 binds with a higher avidity than site 2.

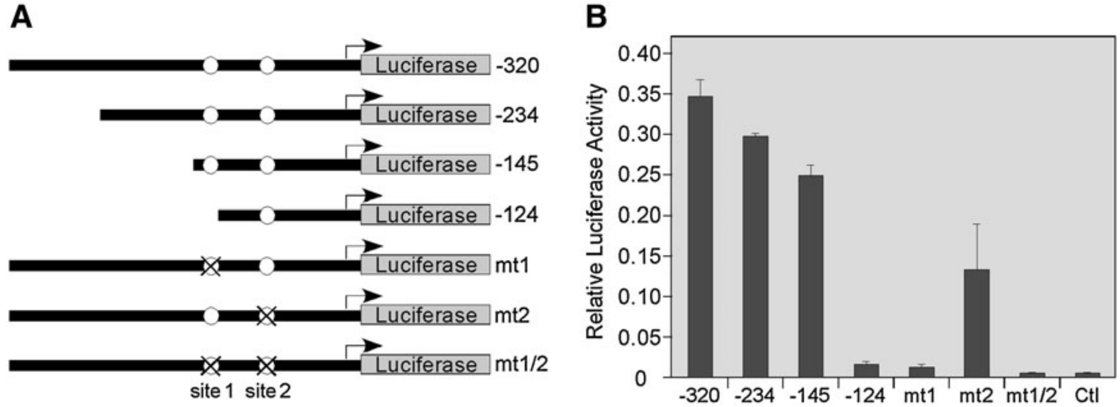

FIG. 5. Analysis of site 1 and site 2 activity by transient transfections in Hep3B cells. **(A)** A series of 5′ deletions of the AFM promoter were generated and fused to the luciferase reporter gene. In addition, derivatives of the full-length AFM promoter (−320) were generated in which site 1 and site 2 were mutated, individually (mt1 and mt2, respectively, or together, mt1/2). **(B)** Analysis of AFM promoter fragments (shown in **A**) by transient transfection in Hep3B cells. Cells were harvested 48 h after transfection and firefly luciferase levels were normalized to Renilla luciferase. Open circles indicate wild-type HNF1 sites; cross-hatched circles indicate mutated HNF1 sites.

Functional analysis of HNF1 sites in the mouse AFM promoter

To determine the functional significance of the two HNF1 sites, a series of luciferase constructs were generated. These include a series of 5′ truncations ending at −320 (full-length), −234, −145, and −124 and a full-length promoter (−320) in which site 1, site 2, or both site 1 and site 2 were altered by site-directed mutagenesis (Fig. 5A). These constructs were transiently transfected into Hep3B cells; luciferase levels were normalized to the cotransfected Renilla luciferase (Fig. 5B). The full-length −320 promoter had the highest activity, whereas a slight reduction in promoter activity was seen with the −234 and −145 truncations, both of which still contain the two HNF1 sites. In contrast, the −124 truncation resulted in a promoter with roughly 10% the activity of the full-length 320 bp promoter. In fact, this truncation had the same level of activity as the full-length promoter with a site 1 mutation (mt1). Taken together, these constructs demonstrate the critical role of site 1 for AFM promoter activity in Hep3B cells. The site 2 mutation (mt2) resulted in a ∼60% reduction in promoter activity, demonstrating that this site also contributes to promoter activity. The activity of the double mutant (mt1/2), in which both sites 1 and 2 are mutated, was essentially the same as the promoterless control, demonstrating the importance of both HNF1 site for AFM promoter activity.

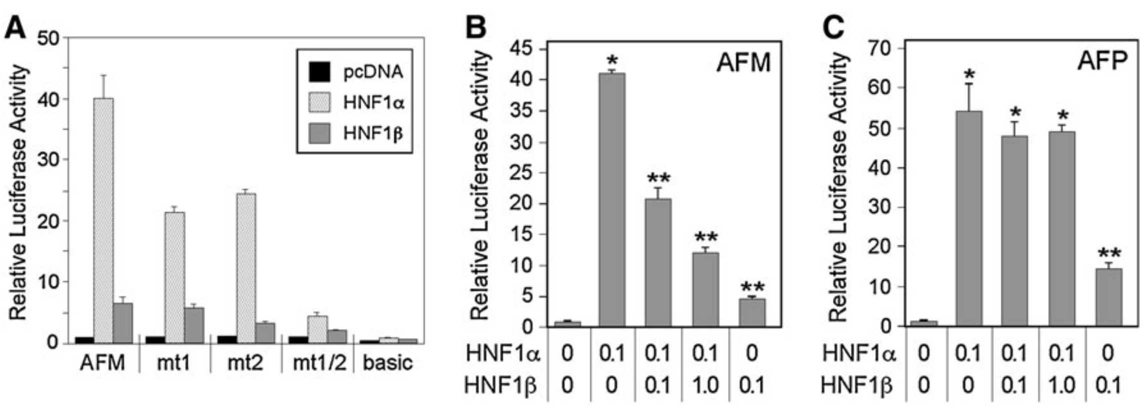

FIG. 6. Responsiveness of AFM and alpha-fetoprotein (AFP) promoter fragments to HNF1α and HNF1β. Cotransfections with luciferase plasmids and HNF1 expression vectors were performed in HEK293 cells. The pRL-CMV Renilla luciferase was included to control for variations in transfection efficiency. After 48 h, cells were harvested and firefly luciferase levels were normalized to the Renilla luciferase. **(A)** The AFM(320)-Luc plasmid, wild-type (AFM) or with mutations in site 1 (mt1), site 2 (mt2), or both sites (mt1/2), or the promoterless pGL3basic luciferase vector (basic), were cotransfected with empty vector (pcDNA) or expression vectors for HNF1α or HNF1β. **(B)** The AFM(320)-Luc plasmid was cotransfected with expression vectors for HNF1α or HNF1β, at the concentrations shown. **(C)** The AFP(250)-Luc plasmid was cotransfected with expression vectors for HNF1α or HNF1β, at the concentrations shown. In **(B)** and **(C)**, *a statistically significant increase in expression compared to no HNF1 expression vector ($p < 0.01$) and **a statistically significant increase in expression compared to no HNF1 expression vector and statistically significant decrease in expression compared to 0.1 μg of HNF1α alone ($p < 0.01$).

Table 1. Hepatocyte Nuclear Factor 1-Binding Sites in Albumin Family Gene Promoters

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*
HNF1 consensus	**G**	**T**	**T**	**A**	**A**	**T**	**n**	**A**	**T**	**T**	**A**	**A**	**C**
Human Alb (−63 to −51)	G	T	T	A	A	T	a	A	T	C	T	A	C
Mouse Alb (−64 to −52)	G	T	T	A	A	T	g	A	T	C	T	A	C
Dog Alb (−63 to −51)	G	T	T	A	A	T	a	A	T	C	T	A	C
Horse Alb (−63 to −51)	G	T	T	A	A	T	a	A	T	C	T	A	T
Human AFP (−59 to −47)	G	T	T	A	C	T	a	G	T	T	A	A	C
Mouse AFP (−62 to −50)	G	T	T	A	C	T	a	G	T	T	A	A	C
Dog AFP (−59 to −47)	A	T	T	A	C	T	a	A	T	T	A	A	C
Horse AFP (−59 to −47)	A	T	T	A	C	T	a	A	T	T	A	A	C
Human AFM (−62 to −50)	G	T	T	A	C	T	t	T	T	T	T	A	C
Mouse AFM (−64 to −52)	G	T	T	A	C	T	t	T	T	T	A	A	C
Dog AFM (−62 to −50)	G	T	T	A	C	T	t	T	T	T	T	A	C
Horse AFM (−64 to −52)	G	T	T	A	C	T	t	T	T	T	T	A	C
Human DBP (−88 to −76)	A	T	T	A	A	T	a	A	T	T	G	A	T
Mouse DBP (−64 to −52)	A	T	T	A	A	T	a	A	T	T	G	A	T
Dog DBP (−87 to −75)	A	T	T	A	A	T	a	A	T	T	G	A	T
Horse DBP (−88 to −76)	A	T	T	A	A	T	a	A	T	T	G	A	T
Human AFP (−130 to −118)	G	T	T	A	A	T	t	A	T	T	G	G	C
Mouse AFP (−128 to −116)	G	T	T	A	A	T	t	A	T	T	G	G	C
Dog AFP (−134 to −122)	G	T	T	A	A	T	t	A	T	T	G	A	C
Horse AFP (−134 to −122)	G	T	T	A	A	T	t	A	T	T	G	G	C
Human AFM (−137 to −125)	G	T	T	A	A	T	a	T	T	T	A	G	C
Mouse AFM (−138 to −126)	G	T	T	A	A	T	a	A	T	T	A	C	C
Dog AFM (−140 to −128)	G	T	T	A	A	T	a	A	T	T	A	G	C
Horse AFM (−142 to −130)	G	T	T	A	A	T	a	A	T	T	A	G	C
Human DBP (−233 to −221)	G	T	T	A	A	T	g	A	T	T	A	A	A
Mouse DBP (−185 to −173)	G	T	T	A	A	T	g	A	T	T	A	A	A
Dog DBP (−195 to −183)	G	T	T	A	A	T	g	A	T	T	A	A	T
Horse DBP (211 to −199)	G	T	T	A	A	T	g	A	T	T	A	A	A

HNF1 consensus site is on top (in bold). Nucleotide matches to the consensus are highlighted in gray. Location of the sites is relative to the start of exon 1 (+1); start sites of dog and horse genes are not known but estimated based on comparison to human genes.

Alb, albumin; AFM, alpha-albumin; AFP, alpha-fetoprotein; DBP, D-binding protein; HNF1, hepatocyte nuclear factor 1.

HNF1β functionally competes with HNF1α on the AFM promoter but not the AFP promoter

Our earlier studies (Fig. 2C) indicated that HNF1α is a more potent activator of the AFM promoter than HNF1β. Our data also suggested that site 1 binds HNF1 proteins more effectively than site 2 (Fig. 3C). To explore further the control of the AFM promoter by the two HNF1 isoforms, we tested the ability of the HNF1α and HNF1β to activate mutant AFM promoters in HEK293 cell cotransfections. As seen previously (Fig. 2), the wild-type AFM promoter was activated more effectively by HNF1α than HNF1β (roughly 40-fold and 6-fold, respectively, compared to pcDNA control plasmid) (Fig. 6A). Mutations in site 1 or site 2 reduced HNF1α-dependent transactivation by roughly 50%, demonstrating the importance of both sites in AFM promoter activation by HNF1α. Mutations of either site also reduced the transactivation by HNF1β, although this reduction was not as great as that seen with HNF1α. Also, the mt1 had less of an effect on HNF1β responsiveness than did the mt2, suggesting that site 2 may be more important than site 1 for HNF1β regulation. The double mutant (mt1/2) was less responsive to HNF1α or HNF1β than the individual mutants, but was still slightly responsive to both HNF1 isoforms; this may be due to weak HNF1-like sites elsewhere in the AFM promoter.

Due to the differential ability of HNF1α and HNF1β to transactivate the AFM promoter, we cotransfected HNF1α and HNF1β together to determine whether one isoform could act in a transdominant manner over the other. Since the evolutionarily related *AFP* gene also contains two HNF1 sites (centered at −122 and −56, relative to the transcription start site), a 250 bp AFP promoter fragment fused to luciferase [AFP(250)-Luc] was also included in this analysis. As seen previously, HNF1α was a more potent transactivator of the wild-type AFM promoter than was HNF1β (Fig. 6B). When a constant amount of HNF1α was cotransfected with increasing amounts of HNF1β, we saw a dose-dependent decrease in luciferase activity, suggesting that HNF1β can act in a dominant manner to inhibit the action of HNF1α on the AFM promoter. The AFP promoter was also activated to a greater extent by HNF1α than by HNF1β (Fig. 6C). However, in contrast to the AFM promoter, HNF1β could not functionally compete with HNF1α on the AFP promoter when both factors were transfected together.

Discussion

The *albumin*, *AFP*, and *DBP* genes have been studied extensively and proven to be valuable models to advance our understanding of liver-restricted transcriptional control. The fourth member of this family, *AFM*, was first identified in 1994, but the basis for liver-specific regulation of this gene had not been investigated. Here, we show that the two highly conserved HNF1 sites in the mouse AFM promoter can bind HNF1α and HNF1β and that both sites are required for AFM promoter activity. In EMSA cold-competition assays, the upstream site (site 1) is a more potent competitor for HNF1 binding than is the downstream site (site 2). Further, mutating site 1 had a more dramatic effect on promoter activity than did the mutating site 2 when transfections were performed in Hep3B cells. In contrast, mutating site 1 and site 2 had similar effects on HNF1 responsiveness when AFM-luciferase reporter genes were cotransfected with HNF1 expression vectors in HEK293 cells, but in these experiments the HNF1 levels were likely to be substantially higher than those found in Hep3B cells. Taken together, these data indicate that site 1 is more important than site 2 for promoter activity.

The albumin gene family arose from a series of duplication events. The first event gave rise to *DBP* and a precursor to the other members. A second event generated *albumin* and the *AFP-AFM* precursor; a final duplication resulted in *AFP* and *AFM*. A comparison on the HNF1 sites in the promoters of the albumin gene family is consistent with the evolutionary history of these genes (Table 1). *AFM*, *AFP*, and *DBP* contain two HNF1 sites, whereas *Alb* contains a single site. This suggests that the primordial albumin-like gene contained two HNF1 sites; both sites have remained in *DBP*, *AFP*, and *AFM*, whereas the upstream site has been lost from *Alb* and did so after the divergence of *Alb* and the *AFP-AFM* precursor. The location of the downstream HNF1 site has remained relatively fixed, relative to exon 1, particularly for the three most related members (*Alb*, *AFP*, and *AFM*). The upstream HNF1 site is roughly 130 bp upstream of exon 1 in the AFP and AFM promoters, but further from exon 1 (180–225 bp) in the less related DBP promoter. The downstream HNF1 site has a noncanonical "C" residue in the fifth position of both *AFP* and *AFM*; this site is a consensus "A" in all other HNF1 sites. The 12th nucleotide of the upstream HNF1 site is a "G" in most of the *AFM* and *AFP* genes. Taken together, this would suggest that these two changes occurred in the *AFP*/*AFM* precursor, but before the duplication that gave rise to AFP and AFM. Overall, each of the HNF1 sites is quite conserved in the different species analyzed here (human, mouse, dog, and horse), which diverged roughly 90–100 million years ago (Murphy *et al.*, 2004). Recently, we identified a new member of the Albumin gene family, which we have called *Arg* (Naidu *et al.*, 2010). *Arg* is no longer functional in primates due to a number of mutations, but the gene is intact in mice, rat, horse, and dog. However, Arg is expressed at very low levels in mice, suggesting that the functional importance of *Arg* is less than other members of the albumin gene family even in species where the gene is still intact. Interestingly, there are no HNF1 sites in the promoter of the weakly expressed *Arg* gene, indicative of the importance of the HNF1 in the hepatic expression of albumin family of genes in the liver.

Whereas the *AFM* and *AFP* genes are closely related, expression of these two genes in the liver is quite different. *AFP* is activated very early in liver development and *AFM* is activated during the perinatal period. While the basis for this difference in timing of activation is not known, the AFP and AFM promoters exhibit different responses to HNF1α and HNF1β. With both promoters, HNF1α is a more potent activator than HNF1β, a phenomenon that has been seen with other HNF1-target genes (Wu *et al.*, 1994; Song *et al.*, 1998; Erickson *et al.*, 2000). When co-transfected together, the HNF1β isoform can act in a transdominant manner over HNF1α on the AFM promoter, whereas HNF1α is transdominant over HNF1β on the AFP promoter. The different responses of these two promoters could be due to the ability of HNF1β to bind sites in the AFM promoter with a higher affinity than to sites in the AFP promoter and thus compete more effectively with HNF1α for binding; future studies will be needed to address this possibility. However, HNF1β is activated earlier than HNF1α during liver development, and HNF1α is the predominant isoform in the adult liver. The relatively higher levels of HNF1β in the fetal liver may keep AFM repressed before birth, at which time HNF1α levels increase. In contrast, AFP is activated earlier during hepatogenesis even though HNF1α levels, relative to HNF1β, are lower at this time. In this regard, it is interesting that this transdominant inhibition of HNF1α by HNF1β is also seen with the *DBP* gene, which is also activated later in liver development (Song *et al.*, 1998). Taken together, these data suggest that the relative response to HNF1α and HNF1β could help determine the timing of activation of albumin family genes in the developing liver.

While our studies have characterized the several *cis*-acting sites in the AFM promoter, it is possible that other factors are also involved in AFM regulation. While a conserved consensus C/EBP site was found in the AFM promoter, C/EBPα did not activate AFM(320)-luc in HEK293 cell cotransfections (H.L., data not shown). It is reasonable to believe that other general and liver-specific factors will regulate the AFM promoter. Even if this is the case, HNF1 appears to be an essential factor since mutation of both HNF1 sites resulted in a promoter with little, if any, activity. It is also not known whether other elements, including enhancers, control AFM expression. We have performed a genomic comparison of the region upstream of the AFM gene from several mammals, and have not found any conserved regions. Since enhancers tend to be conserved between species, this might suggest that there are no enhancers in this region. We recently deleted the AFP enhancer region by homologous recombination in embryonic stem cells (Jin *et al.*, 2009). Whereas AFP expression was dramatically reduced in these mice, AFM activation occurred normally, leading us to conclude that the AFP enhancers do not influence AFM expression. It is possible that the albumin enhancer could contribute to AFM activation later in liver development. Additional studies will be needed to characterize further the *trans*-acting factors and *cis*-acting sites that govern AFM expression during liver development.

Acknowledgments

The authors thank members of the Spear lab and Martha Peterson for helpful discussions and review of the article. This work is supported by Public Health Service Grant DK-074816.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Brett T. Spear, Ph.D.
Department of Microbiology, Immunology,
and Molecular Genetics
University of Kentucky College of Medicine
Lexington, KY 40536

E-mail: bspear@uky.edu

Received for publication June 10, 2010; received in revised form August 24, 2010; accepted August 24, 2010.