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The Mouse Alpha-Fetoprotein Promoter is Repressed in HepG2 Hepatoma Cells by Hepatocyte Nuclear Factor-3 (FOXA)

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Abstract

The mouse alpha-fetoprotein gene is expressed at high levels in the fetal liver and is transcriptionally silenced at birth. The repression is governed, at least in part, by the 250 base pair (bp) AFP promoter. We show here that the AFP promoter is dramatically repressed by HNF3 in HepG2 hepatoma cells. This repression is governed by the region between –205 and –150. Furthermore, this fragment can confer HNF3-mediated repression on a heterologous promoter. The repression is abolished by a mutation that is centered at –165. EMSA analyses using *in vivo* and *in vitro* synthesized proteins indicate that HNF3 proteins do not bind DNA from the –205 to –150 region. We propose that HNF3 represses AFP promoter activity through indirect mechanisms that modulate the binding or activity of a liver-enriched factor that interacts with the –165 region of the AFP promoter.

INTRODUCTION

ALPHA-FETOPROTEIN (AFP), A MAJOR FETAL SERUM PROTEIN in mammals, is involved in ligand transport and maintaining physiologic osmolarity (reviewed in Deutsch, 1991). The AFP gene is transcribed at high levels in the yolk sac and fetal liver, and to a much lesser extent in the fetal gut and kidney (Tilghman, 1985). AFP transcription in the liver declines dramatically after birth, resulting in barely detectable AFP mRNA levels in the liver by 4 weeks of age (Tilghman and Belayew, 1982). Five *cis*-acting elements that regulate AFP expression, the promoter, repressor, and three upstream enhancers, have been identified and characterized using transgenic mouse and *in vitro* studies (Chen et al., 1997; Spear, 1999). The AFP promoter, localized within the first 250 bp upstream of the transcriptional start site, is active only in tissues where AFP is normally transcribed (Godbout et al., 1986). The repressor region, between –250 and –800, is required for complete postnatal silencing of AFP transcription (Vacher and Tilghman, 1990). Three distinct upstream enhancer regions, each roughly 300 bp in length, have also been identified (Godbout et al., 1988); transgenic studies indicated that all three are active in AFP-expressing tissues in both fetal and adult mice (Hammer et al., 1987; Ramesh et al., 1995).

Extensive analysis of the AFP promoter from mice, rats, and humans have shown that binding sites for several transcription factors exist within this region (Fig. 1A; reviewed in Chen et al., 1997). Overlapping binding sites for the liver-enriched transcription factors Hepatocyte Nuclear Factor 1 (HNF-1) and the ubiquitous factor Nuclear Factor 1 (NFI) exist in the –130 to –110 region (Feuerman et al., 1989; Zhang et al., 1991; Bernier et al., 1993; Bois-Joyeux and Danan, 1994). Constructs in which this HNF1/NFI binding site was mutated exhibited

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substantially less activity than wild-type promoter constructs when transiently transfected into AFP-expressing HepG2 human hepatoma cells (Feuerman et al., 1989; Bernier et al., 1993). Interestingly, a naturally occurring mutation in this region in the human AFP gene leads to incomplete postnatal AFP repression, resulting in continued AFP transcription in the adult liver (McVey et al., 1993). This single base substitution results in increased HNF1 binding, suggesting that the competition between HNF1 and NF1 binding could contribute to the developmental control of promoter activity. The region centered around -160 is also important for AFP promoter activity. Wen et al. (1993) identified a 12-bp inverted repeat located from -166 to -155 termed the promoter-linked coupling element (PCE), which was required for the stimulation of the rat AFP promoter by the upstream enhancers. Additional studies identified a factor, *nkx-2.8*, that bound this region (Apergis et al., 1998). This factor belongs to the nk-2 family of homeodomain-containing transcription factors. Separate studies showed that this 12 bp element from -166 to -155 also binds the factor Fetoprotein Transcription Factor (FTF; Bernier et al., 1993), an orphan nuclear receptor of the *Drosophila* fushi tarazu F1 (FTZ-F1) class of receptors (Galarneau et al., 1996). In addition to these well-defined elements, sequence analysis indicates the presence of a CAAT/enhancer binding protein (C/EBP) binding site at -75 of the AFP promoter (Zhang et al., 1991; Thomassin et al., 1992; Bois-Joyeux and Danan, 1994; Galarneau et al., 1996).

HNF3 (FOXA) was originally identified as a liver-enriched factor that regulated the rat transthyretin gene (Costa et al., 1989). Additional analysis revealed three HNF3 isoforms, HNF3 α , HNF3 β , and HNF3 γ , that are encoded by distinct genes (Lai et al., 1990, 1991). The HNF3 family of factors regulate numerous liver genes, and are among the earliest factors to be expression during hepatogenesis (Kaestner, 2000). Although HNF3 proteins are most often associated with activation, there are several examples whereby HNF3 acts to repress transcription (Gregori et al., 1994; Rouet et al., 1995). The HNF3 proteins are related to the *Drosophila* forkhead protein, which is important for foregut and hindgut development (Weigel and Jackle, 1990). Members of the HNF3/forkhead family of proteins are defined by a novel "winged-helix" DNA binding motif (Clark et al., 1993). Studies with the albumin enhancer suggest that HNF3 proteins may regulate transcription, at least in part, by positioning nucleosomes (Shim et al., 1998).

AFP is among the earliest genes that are induced during hepatogenesis and, like many liver genes, is regulated by HNF3. An HNF3 site is found in the 5' end of enhancer III, and mutational studies have demonstrated that this site is important for full enhancer activity (Group et al., 1994; Thomassin et al., 1996). AFP enhancers I and II do not contain consensus HNF3 binding sites and do not appear to be regulated by HNF3 (K. Li and B. Spear, unpublished observation). The mutually exclusive binding of HNF3 and p53 at the -850 region appears to govern AFP repressor activity (Lee et al., 1999). We previously showed by transient transfection in Hela cells and *in vitro* transcription assays that a 1-kb fragment containing the AFP promoter and repressor is regulated by HNF3 (Crowe et al., 1999). To further define this regulation, we tested whether the 250-bp promoter could be the target of this control. We show here that the 250-bp promoter is activated by HNF3 in Hela cells. However, we found that HNF3 represses AFP promoter activity in HepG2 cells. This repression acts through the -205 to -150 region, and mutation of the *nkx2.8*/FTF site at -165 alleviates this repression. EMSA analysis indicates that HNF3 does not bind this region. Based on these data, we propose that HNF3 downregulates AFP promoter activity by modulating the binding or transactivating potential of other liver-enriched transcription factors.

METHODS AND MATERIALS

Plasmids and DNA fragments

The $\Delta 44$ -*lacZ* and (HNF3)₁₂- $\Delta 44$ -*lacZ* expression vectors were described previously (Spear et al., 1995). The 250-*lacZ* and 150-*lacZ* expression vectors were generated by replacing the promoter of $\Delta 44$ -*lacZ* with fragments of the AFP promoter from -250 to +23 or -150 to +23, respectively. Two mutated versions of the 250-*lacZ* vector, in which the region centered at -165 was changed from CTTTGTCC to ACTGCAGA or the region centered at -75 was changed from TGTTCGCT to ACTGCAGA, were generated by PCR-based mutagenesis as described (Fig. 1B; Zaret et al., 1990) to produce 250^{m165}-*lacZ* and 250^{m75}-*lacZ*, respectively. The AFP promoter "A" fragment, extending from -205 to -137, was generated by PCR amplification. A mutated version of the "A" fragment, with the region centered at -165 mutated as described above, was generated by PCR amplification as described (Zaret et al., 1990). The AFP promoter "B" fragment, extending from -250 to -192, was generated by PCR amplification. The A-150-*lacZ* expression vector was generated by PCR amplifying the AFP promoter from -205 to -23; this amplified product was used to replace the promoter of $\Delta 44$ -*lacZ*. The "B" fragment was linked to 150-*lacZ* in its normal orientation to generate B-150-*lacZ*. Wild-type and mutated versions of the AFP promoter A and B fragments were fused in their normal orientations 5' of the promoter of $\Delta 44$ -*lacZ* to produce A- $\Delta 44$ -*lacZ*, A^{m165}165- $\Delta 44$ -*lacZ*, and B- $\Delta 44$ -*lacZ*. All fragments generated by PCR were confirmed by DNA sequencing. The pCAT plasmid was obtained from Promega (Madison, WI). The CMV-based rat HNF3 α and HNF3 β eukaryotic expression vectors were provided to us by Dr. Robert Costa (University of Illinois at Chicago; Qian and Costa, 1995; Qian et al., 1995; Rausa et al., 1997). The entire cDNA insert containing HNF3 α was removed as an *EcoRI* fragment from the CMV-HNF3 α expression vector to generate the CMV empty vector (E.V.). A truncated version of the rat HNF3 β cDNA that encodes the DNA-binding domain (DBD; amino acids 121-272) was produced by PCR amplification and inserted into the CMV expression vector; this truncated insert was confirmed by DNA sequencing. Full-length cDNAs for rat HNF3 β and mouse HNF6 in pBluescript were provided by Dr. Robert Costa and used for *in vitro* protein synthesis.

Transfections

The human HeLa cell line was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), Penicillin/Streptomycin (Pen/Strep), and glutamine. The human hepatoma HepG2 cell line was maintained in DMEM/F12 (1:1) medium with 10% FBS, PenStrep, glutamine, and 10 μ g/ml insulin. Insulin was obtained from Sigma Chemical Corporation (St. Louis, MO), all other reagents were from Life Sciences (Gaithersburg, MD). Transient transfections were carried out by the calcium phosphate method as described for HepG2 cells (Spear and Tilghman, 1990) or using Lipofectamine (Life Technologies) according to manufacturer's directions for HeLa cells. For HeLa cotransfections, cells were plated at 1×10^6 cells/10 cm dish. The following day, cells were transfected with a total of 20 μ g of DNA consisting of 12 μ g of the *lacZ* reporter construct, 3 μ g of the HNF3 expression vector, and 5 μ g of pCAT. For HepG2 cotransfections, cells were plated at 0.5×10^6 cells/6 cm dish. The following day, cells were transfected with 6 μ g of the *lacZ* reporter construct, 1.5 μ g of the HNF3 expression vector, and 1 μ g of pCAT. For HeLa synthesized HNF3 proteins, cells were plated at 1×10^6 cells/10 cm dish and transfected with 15 $\times$ g of HNF3 vectors. Forty-eight hours after the addition of DNA, cells were washed three times in $1 \times$ PBS and scraped from plates into 1.5 ml of PBS and transferred to 1.5 ml microcentrifuge tubes. Cells were pelleted by centrifugation and used for β gal/CAT assays or the preparation of nuclear extracts as described below.

βgal/CAT assays

Cell pellets from transient transfections were resuspended in 100 μl of 0.25 M Tris, pH 7.4, and thoroughly vortexed. Cells were lysed by three cycles of freeze thawing using dry ice, followed by centrifugation at $14,000 \times g$ for 5 min. Protein concentrations were determined by the BCA assay kit (Pierce Biochemicals, Rockford, IL). The β-galactosidase activity was measured using the colorimetric substrate chlorophenolred-β-D-galactopyranoside (CPRG; Boehringer Mannheim, Indianapolis, IN) as described (Spear et al., 1995). Cell extracts (10–50 μl) were added to microfuge tubes containing 200 μl of buffer A (100 mM NaPO₄, pH 7.2, 10 mM KCl, 10 mM MgCl₂, and 10 mM 2-mercaptoethanol [2-ME]). Thirty microliters of CPRG solution (15 μg CPRG/ml Buffer A lacking 2-ME) was added to the tubes and reactions were incubated at 37°C. Reactions mixtures were stopped by the addition of 2× sample volumes of Buffer A lacking 2-ME. Absorbance was measured at 570 nm using a control reaction without cell extracts as a blank. The β-gal activity was normalized to CAT activity to control for variations in transfection efficiency. Chloramphenicol acetyltransferase (CAT) assays were performed as described (Sambrook et al., 1989). Briefly, cell extracts were incubated at 65°C for 10 min to inactivate deacetylases. Particulate material was removed by centrifugation at $14,000 \times g$ for 2 min at 4°C. Cell extracts (10–50 μl) were mixed with 10 μl of the CA solution (10 μl of ³H-chloramphenicol [1 mCi/ml, NEN, Boston, MA], 10 μl of nonradioactive chloramphenicol [20 mM, Sigma, St. Louis, MO], and 380 μl of 0.25 M Tris-HCl, pH 8.0), 10 μl of *n*-butyryl Coenzyme A (5 mg/ml, Sigma), and 0.25 mM Tris-HCl (pH 8.0) buffer to bring the total volume to 125 μl. The *n*-butyryl Coenzyme A was added last as it initiated the reaction. Reaction mixtures were incubated at 37°C and 300 μl of xylene (Baxter, Deerfield, IL) were added to stop the reactions. Reactions were mixed and the xylene was removed. The xylene was back-extracted once with 100 μl of 0.25 M Tris-HCl buffer. The resulting xylene layer was transferred to a scintillation vial containing 3 ml of scintillation fluid (Bio-Safe NA, RPI, Mount Prospect, IL) and the cps were determined using a scintillation counter.

Nuclear extracts/in vitro synthesized proteins

Nuclear extracts were prepared from Hela or HepG2 cells (Li et al., 2000). Monolayers of untransfected or transfected cells were harvested by scraping as described above and resuspended in buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After a 10-min incubation on ice, nuclei were collected by centrifugation at $14,000 \times g$ for 2 min. The nuclear pellet was resuspended in buffer containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% (v:v) glycerol, 0.5 mM DTT, and 0.2 mM PMSF. After a 20-min incubation on ice, samples were centrifuged at $14,000 \times g$ for 2 min. The supernatant was removed and stored in aliquots at –80°C. Protein concentrations were determined using the BCA assay kit (Pierce Biochemicals). The TNT-coupled reticulocyte lysate system (Promega Biotech) was used for the production of *in vitro* synthesized HNF3 and HNF6 proteins according to the manufacturer's instructions. Proteins were aliquoted and stored at –80°C until use.

EMSAs

Dephosphorylated, gel-purified DNA fragments were end-labeled with ³²P-ATP using T4 polynucleotide kinase as described (Li et al., 1996). Oligonucleotides corresponding to –111 to –85 of the rat transthyretin promoter were chemically synthesized, annealed, and used as the HNF-3 site probe (Costa et al., 1989). The AFP promoter “A” fragments from –205 to –137 (wild-type and –165 mutation) were amplified by PCR, dephosphorylated, and gel purified prior to labeling. EMSAs were carried out using 10 μg of extract and 2 μg of polydI:dC as described (Li et al., 1996). Reaction mixtures were resolved on nondenaturing 8%

polyacrylamide gels in 1 × TBE (2.2 mM Tris, 2.2 mM boric acid, 0.5 mM EDTA) running buffer. Gels were dried and subjected to autoradiography using Kodak X-OMAT AR film.

RESULTS

HNF3 α and HNF3 β repress AFP promoter activity in HepG2 hepatoma cells

We showed previously that HNF3 could transactivate an AFP(1.0)–*lacZ* reporter gene in Hela cells (Crowe et al., 1999). Additional studies found that the 250-bp AFP promoter was also activated by HNF3 in Hela cells (M.-C. Huang and B.T. Spear, unpublished observations). AFP is not normally expressed in Hela cells, but is synthesized in several hepatoma lines including HepG2 human hepatoma cells, which have been used extensively to study AFP regulation. To investigate further HNF3 control of the AFP promoter, transient cotransfections were performed in HepG2 cells. A construct with the 250-bp AFP promoter fused to *lacZ* (250–*lacZ*) was transiently transfected into HepG2 cells along with a CMV expression vector that contained no insert (empty vector) or cDNA insert encoding HNF3 β (Fig. 1C). In addition, pCAT was included to control for variations in transfection efficiency. Forty-eight hours after the addition of DNA, cell lysates were prepared and *lacZ* and CAT activities were determined. In contrast to the activation that was seen in Hela cells, the 250 bp AFP promoter was repressed nearly fivefold by HNF3 β in HepG2 cells (Fig. 1C). This repression was specific to the AFP promoter because the (HNF3)₁₂– Δ 44–*lacZ* vector was transactivated nearly 26-fold by HNF3 α in HepG2 cells (see Fig. 3). To further localize the region that is required for this repression, additional AFP–*lacZ* constructs were analyzed in HepG2 cells (Fig. 1C). A promoter extending from –150 was linked to *lacZ* to obtain 150–*lacZ*; this was, in turn, fused to regions of the AFP promoter from –205 to –150 (A-150–*lacZ*) or from –250 to –192 (B-150–*lacZ*). When cotransfections were performed with the empty vector, 150–*lacZ* exhibited roughly 10% of the activity of 250–*lacZ*, whereas the β -gal levels in cells transfected with A-150–*lacZ* were nearly identical to cells transfected with 250–*lacZ*. This indicates that the –205 to –150 region contributes substantially to promoter activity. The β -gal levels in cells transfected with B-150–*lacZ* were roughly the same as cells transfected with 150–*lacZ*, suggesting that the region between –250 and –192 does not contribute to promoter activity in HepG2 cells. Similar results were observed with these AFP–*lacZ* constructs were transfected in the absence of the empty vector (data not shown). When cotransfections were performed with the HNF3 β expression vector, A-150–*lacZ* was repressed similarly to the 250–*lacZ* (4.3-fold versus 4.2-fold reduction, respectively). In contrast, the AFP(150)–*lacZ* and B-150–*lacZ* constructs were not repressed, but were instead activated slightly by HNF3 β . This indicates that HNF3 represses AFP promoter activity through the –200 to –150 region.

DNA binding by HNF3 proteins is mediated by the internal winged-helix domain. Previous studies had shown that this domain could act in a dominant negative manner to repress numerous liver genes when stably transfected in hepatoma cells (Vallet et al., 1995). A 152-amino acid form of HNF3 β that contains the internal DNA binding domain (HNF3 β -DBD) was generated by PCR. The ability of this truncated HNF3 β -DBD to repress 250–*lacZ* was tested in HepG2 cells (Fig. 2A). HNF3 β -DBD was almost as effective as HNF3 α and full-length HNF3 β at repressing AFP promoter activity in HepG2 cells. We also tested whether lower amounts of the HNF3 expression vectors could still repress AFP promoter activity. HNF3 α , HNF3 β , and HNF3 β -DBD were all still potent repressors when titrated to 0.1 μ g, which is 30-fold lower than the levels used in Figure 1C.

HNF3-mediated repression of the AFP promoter is abolished by a mutation at –165

HNF3-mediated repression of AFP promoter activity is governed by the 55-bp region between –205 and –150, but sequence analysis failed to identify potential HNF3 sites in this region. However, this region contains overlapping binding sites for two factors, *nkx-2.8* and FTF, that

are important regulators of AFP promoter activity (Fig. 1A). To explore the potential role of this motif in HNF3-mediated repression, this site centered at -165 was mutated (Fig. 1B). The mutation was confirmed by DNA sequencing and the mutated AFP promoter was fused to *lacZ* to generate $250^{\text{m165}}\text{-lacZ}$. When cotransfected with the empty vector, $250^{\text{m165}}\text{-lacZ}$ had roughly 10% of the activity of 250-lacZ (Fig. 2B), consistent with a role for the *nkx-2.8/FTF* site in promoter function. Whereas the wild-type promoter was repressed when HNF3 α or HNF3 β was cotransfected, the mutated promoter was resistant to repression and was, in fact, slightly activated. This modest activation is similar to what was seen previously with 150-lacZ and $\text{B-}150\text{-lacZ}$. This demonstrates the importance of the -165 site for normal promoter activity and HNF3-mediated repression.

The AFP promoter constructs that were subjected to repression by HNF3 were much more active in HepG2 cells than those that were not repressed. This raised the possibility that the less active promoters were simply too weak to be repressed by HNF3. To test this, a putative C/EBP binding site at -75 was mutated to generate $250^{\text{m75}}\text{-lacZ}$ (Fig. 1B). This mutation should diminish promoter activity, but should not influence HNF3-mediated repression through the AFP promoter A fragment. Both $250^{\text{m165}}\text{-lacZ}$ and $250^{\text{m75}}\text{-lacZ}$ had equally low activity when transfected into HepG2 cells alone (data not shown) or with the empty vector (Fig. 2B). Despite its low activity, $250^{\text{m75}}\text{-lacZ}$ could still be repressed by HNF3 β and HNF3 α (Fig. 2B).

If the AFP promoter region between -205 and -150 is responsible for HNF3-mediated repression, this fragment should confer repression when fused to a heterologous promoter. We previously used a minimal promoter ($\Delta 44$) from the human liver/bone/kidney alkaline phosphatase gene (Spear et al., 1995) because this promoter is highly responsive to linked elements (Kiledjian and Kadesch, 1990). The AFP A fragment, the A fragment with the -165 mutation, and the AFP B fragment were fused to $\Delta 44\text{-lacZ}$. The (HNF3) $_{12}\text{-}\Delta 44\text{-lacZ}$ vector was used as a positive control. Cotransfections were performed in HepG2 cells, and changes in *lacZ* levels with the HNF3 α expression vector versus empty vector was determined (Fig. 3). Most of the $\Delta 44\text{-lacZ}$ -based reporter constructs had little activity in the presence of the empty vector, consistent with studies showing low basal activity of the $\Delta 44$ promoter. The one exception was $\text{A-}\Delta 44\text{-lacZ}$, which was nearly as active as 250-lacZ . This confirms the robust activity of the A fragment in HepG2 cells. Activities of $\Delta 44\text{-lacZ}$ and $\text{B-}\Delta 44\text{-lacZ}$ reporter constructs were increased roughly three- to fourfold by the HNF3 α . This demonstrated that $\Delta 44\text{-lacZ}$ was somewhat responsive to HNF3 α , and that the B fragment did not influence this response. The positive control (HNF3) $_{12}\text{-}\Delta 44\text{-lacZ}$ vector was activated 26-fold when HNF3 α was cotransfected, consistent with previous studies by us and others (Pani et al., 1992; Spear et al., 1995). In contrast, $\text{A-}\Delta 44\text{-lacZ}$ was repressed fourfold by HNF3 α ; this repression is similar to the 3.7-fold HNF3-mediated inhibition of 250-lacZ . The $\text{A}^{\text{m165}}\text{-}\Delta 44\text{-lacZ}$ vector was activated roughly fourfold, similarly to $\Delta 44\text{-lacZ}$, further demonstrating that this mutation abolished HNF3-mediated repression.

HNF3 α or HNF3 β proteins do not bind the -205 to -150 region of the AFP promoter in vitro

Computer analysis did not reveal any obvious HNF3 binding sites in the region between -205 and -150 . However, the repressive effects of HNF3 on this fragment prompted us to use electrophoretic mobility shift assays (EMSAs) to investigate the possibility that HNF3 binding sites might exist within this region. Nuclear extracts were prepared from Hela cells that were transfected with the CMV expression vector that contained no insert, HNF3 α , or HNF3 β or from untransfected HepG2 cells. These extracts were incubated with radiolabeled DNA fragments containing the rat transthyretin promoter HNF3 site (-86 to -111), the wild-type AFP "A" fragment, or the "A" fragment with the -165 mutation (A*; Fig. 4). The HNF3 probe did not bind any proteins in extracts from Hela cells transfected with the empty vector (Fig. 4,

lane 4). A predominant band was detected when this probe was incubated with extracts from Hela cells transfected with HNF3 α or HNF3 β (Fig. 4, lanes 7 and 10). These two bands had roughly the same mobility, although the HNF3 α band had a slightly slower mobility when gels were run further (data not shown), consistent with previous studies (Lai et al., 1990). A faint band corresponding to HNF3 α/β was also present in HepG2 cell extracts (Fig. 4, lane 13). The Hela extracts contained three faint complexes that bound to the AFP “A” fragment (Fig. 4, lanes 5, 8, and 11). These complexes were also present in extracts from untransfected Hela cells (data not shown). Interestingly, the intensity of the complex with intermediate mobility (asterisk) increased when cells were transfected with HNF3 α or HNF3 β . These three complexes were also present in HepG2 extracts, as was another complex of higher mobility that was not present in Hela extracts (Fig. 4, lane 14). Although extracts from transfected Hela cells and HepG2 cells contained HNF3 proteins, HNF3 binding to the AFP “A” fragment could not be detected with these extracts. The mutated “A*” fragment did not bind any proteins in Hela or HepG2 cell extracts, indicating that the site at -165 is critical for all the protein binding to this fragment (Fig. 4, lanes 6, 9, 12, and 15).

To explore further the possibility of HNF3 binding to the AFP “A” fragment, EMSAs were performed with *in vitro* synthesized proteins. When the HNF3, wild-type “A” and mutated “A” fragments were incubated with lysates that were programmed with BlueScript alone (B/S), no complexes were seen (Fig. 5, lanes 4–6). Extracts programmed with the HNF3 β vector contained a complex that bound the HNF3 probe (Fig. 5, lane 7). However, the HNF3 β extract did not bind the wild-type or mutant “A” fragments (Fig. 5, lanes 8 and 9). Previous studies have shown that some HNF3 sites, including that from the transthyretin promoter, can also be recognized by the CUT-Homeodomain protein HNF6 (Lemaigre et al., 1996; Samadani and Costa, 1996). Therefore, we tested whether this factor might bind the AFP “A” fragment. Extracts programmed with the HNF6 vector did bind the transthyretin HNF3 site, as expected, but showed no binding to the AFP promoter fragments (Fig. 5, lanes 10–12).

Because a truncated form of HNF3 β containing only the DNA binding domain was a potent inhibitor of AFP promoter activity in HepG2 cells, it was somewhat surprising that the EMSA data indicated that HNF3 did not bind to the AFP promoter “A” fragment. The truncated form is substantially smaller than the full-length HNF3 β protein, so we tested whether we could detect HNF3 β -DBD binding to the AFP “A” fragment in EMSAs. Nuclear extracts were prepared from Hela cells transfected with the empty vector, HNF3 α , HNF3 β , or HNF3 β -DBD, and used in EMSAs with the HNF3 probe and AFP “A” fragment (Fig. 6). Consistent with Figure 4, Hela-synthesized HNF3 α and HNF3 β could bind the HNF3 probe (Fig. 6, lanes 5 and 7). HNF3 β -DBD could also bind effectively to this probe; the HNF3 probe/HNF3 β -DBD complex had a high mobility that could be readily distinguished from other bands in this EMSA gel (Fig. 6, lane 9). However, none of these HNF3 molecules bound to the AFP “A” fragment (Fig. 6, lanes 6, 8, and 10). As was seen in Figure 4, the “A” probe bound three complexes in Hela cells and the intensity of the middle band was enhanced by the presence of HNF3 proteins including HNF3 β -DBD (asterisk in Fig. 6).

DISCUSSION

We previously showed that HNF3 α and HNF3 β can activate AFP-linked reporter genes in Hela cells through the 1-kb promoter/repressor region. We extended these studies using transient transfections to show that this induction in Hela cells is mediated, at least in part, by the 250-bp AFP promoter. In contrast to the transactivation that was seen in Hela cells, we have shown here that HNF3 acted as a potent repressor of AFP promoter activity when transient transfections were performed in AFP-expressing HepG2 hepatoma cells. A truncated form of HNF3 β that contained the winged-helix DNA binding domain, but lacked the N-terminal and C-terminal activation domains, could still repress AFP promoter activity. Repression was

governed by the region between -205 and -137 of the AFP promoter, and was abolished by a mutation centered at -165. EMSA analysis indicated that HNF3 could not bind to a fragment spanning the -205 to -137 region. This argues that repression does not require the direct binding of HNF3 to this region.

The ability of HNF3 to transactivate or repress the AFP promoter in HeLa or HepG2 cells, respectively, argues that HNF3-mediated control is modulated by factors that are differentially expressed in these two cell lines. In HeLa cells, the AFP promoter has little, if any, activity. Forced expression of HNF3 in these cells is able to induce AFP promoter activity. This data is consistent with previous *in vitro* chromatin-based transcription assays (Crowe et al., 1999). In contrast to HeLa cells, the AFP promoter has robust activity in HepG2 cells. This activity is due to the presence of multiple liver-enriched factors, including FTF and *nkx-2.8*, both of which bind to a site centered at -165 of the AFP promoter. This site is required for full promoter activity (this paper and Wen and Locker, 1994; Galarneau et al., 1996; Apergis et al., 1998), and is also required for HNF3-mediated repression, but does not bind HNF3. Thus, the most likely targets of HNF3-mediated repression would therefore be FTF or *nkx-2.8*.

HNF3 has been shown to negatively regulate the adolase P promoter and the α -1-microglobulin/bikunin enhancer (Gregori et al., 1994; Rouet et al., 1995). These two examples involve direct binding of HNF3 to DNA, and are thus likely to be different than repression of the AFP promoter in HepG2 cells. Several explanations could account for HNF3-mediated repression of the AFP promoter via indirect mechanisms. HNF3 may simply inhibit promoter activity by nonspecific squelching whereby HNF3 overexpression titrates other general transcription factors (Natesan et al., 1997). However, $\Delta 44$ -*lacZ*, when fused to the transthyretin promoter HNF3 binding sites, was activated by HNF3 in HepG2 cells; this would argue against a nonspecific squelching mechanism. A second possibility is that HNF3 could regulate the transcription of other transcription factors that control AFP promoter activity. HNF3 is known to regulate several known liver-enriched transcription factors including HNF1 α , HNF1 β , and HNF4 α (Costa, 1994; Kaestner, 2000); HNF1 regulates the AFP promoter through a site centered at -120 (Feuerman et al., 1989; McVey et al., 1993), but there is no evidence that these factors bind sites within the AFP promoter "A" fragment. In addition, it seems unlikely that this type of indirect regulation would be detected in a transient assay.

A third possibility, which we favor, is that HNF3 represses AFP promoter activity by modulating the binding or activity of other transcriptional regulators that bind to the "A" fragment. Recent studies have demonstrated that transcription factors can bind to other factors and influence their transactivation potential. For example, the glucocorticoid receptor (GR) can control transcription of some genes by a "tethering" mechanism that allows GR to bind other transcription factors and regulate their transactivating potential (Yamamoto et al., 1998). GR can repress NF- κ B-mediated activation of the ICAM-1 and IL-8 promoters by such a mechanism; GR tethers to the RelA/p65 component, which interferes with NF- κ B-dependent phosphorylation of the RNA Polymerase II C-terminal domain (Nissen and Yamamoto, 2000). Alternatively, HNF3 binding elsewhere in the AFP control region could influence nucleosome positioning and therefore modulate the accessibility of factors to sites within the "A" fragment. Elegant studies by Zaret and colleagues have shown that HNF3 binding to the albumin enhancer can alter nucleosome positioning (Shim et al., 1998). By this mechanism, HNF3 could alter chromatin in such a way to reduce the binding of FTF or *nkx-2.8* to their target sites at -165 of the AFP promoter, leading to a reduction in AFP promoter activity. In this regard, it is interesting that 150-*lacZ* and $\Delta 44$ -*lacZ* were slightly transactivated by HNF3, suggesting the presence of HNF3 binding sites in these plasmids. A prediction of the nucleosome positioning model would be that an expression vector containing the AFP promoter "A" fragment, but lacking any other HNF3 binding sites, would not be repressed by HNF3 in HepG2 cells.

The AFP gene is dramatically repressed within the first several weeks after birth. The basis for this repression is not well understood, and specific *trans*-acting factors that govern this repression have not been identified. Transgenic mouse studies have shown that repression is mediated, at least in part, by the promoter (Peyton et al., 2000). In addition, a mutation in the overlapping HNF1/NF1 site at -120 in the AFP promoter is associated with the hereditary persistence of AFP in humans (McVey et al., 1993). Our studies raise the possibility that HNF3 may also be involved in postnatal AFP repression, possibly by blocking the activity or binding of other liver-enriched factors to the AFP promoter. A more thorough understanding of the factors that control AFP promoter activity, and the analysis of these factors during liver development, will be needed to further understand this control.

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FIG. 1.

(A) Region of the AFP promoter used in this study. The AFP promoter is defined as a 250-bp fragment upstream of the AFP transcription start site. Binding sites for *nkx2.8*/FTF, NF-1, HNF1, and C/EPB and the endpoints of the “A” and “B” fragments are shown. (B) AFP promoter mutations used in this study. Sequence of the AFP promoter regions centered at –165 and –75 and the corresponding mutations, designated by lower case letters, that were used to alter these regions. (C) HNF3 represses AFP promoter activity in HepG2 cells. Cells were transfected with vectors containing *lacZ* fused to the entire 250-bp AFP promoter, 150-bp promoter, or 150-bp promoter fused to the AFP “A” or “B” fragments, pCAT, and either empty vector (e.v) or HNF3 β expression vectors as designated. The β -galactosidase activity normalized to CAT is shown. Error bars represent standard deviation.

FIG. 2.

(A) HNF3 α , HNF3 β , and 3 β -DBD are equally capable of repressing the AFP promoter in HepG2 cells. Cells were transfected with 250-*lacZ*, RSV-CAT, and HNF3 expression vectors at the designated concentrations. The β -galactosidase activity normalized to CAT is shown.

(B) Mutations at -165 and -75 reduce AFP promoter activity and the -165 mutation relieves HNF3-mediated repression in HepG2 cells. Cells were transfected with *lacZ* fused to the wild-type 250-bp AFP promoter or 250-bp promoter with mutations centered at -165 or -75 (250-*lacZ*, 250^{m165}-*lacZ*, and 250^{m75}-*lacZ*, respectively), pCAT, and HNF3 expression vectors as shown. The β -galactosidase activity normalized to CAT is shown. Error bars represent standard deviation.

FIG. 3.

The AFP promoter “A” fragment confers HNF3 α -mediated repression to a heterologous promoter. A minimal promoter of the human liver/bone/kidney alkaline phosphatase gene ($\Delta 44$), was fused to *lacZ* to generate $\Delta 44$ -*lacZ*. Fragments from the AFP promoter or tandem copies of the rat transthyretin promoter HNF3 binding site were inserted upstream of $\Delta 44$ -*lacZ*. These *lacZ* reporter constructs were transfected with pCAT and the HNF3 α expression vector or empty vector as shown. The β -galactosidase activity normalized to CAT is shown. Error bars represent standard deviation.

FIG. 4.

In vivo synthesized HNF3 α or HNF3 β do not bind the AFP promoter “A” fragment. EMSAs were performed with radiolabeled probes corresponding to the rat transthyretin promoter HNF3 binding site (HNF3), the AFP promoter “A” fragment extending from –205 to –137 (A), or the AFP promoter “A” fragment containing the mutation centered at –165 as shown in Figure 1B (A*). Probes were incubated with no extract (free probe, F.P.; lanes 1–3), or extracts from HeLa cells that were transfected with the empty vector (E.V.; lanes 4–6), HNF3 α expression vector (lanes 7–9), HNF3 β expression vector (lanes 10–12), or extracts from untransfected HepG2 cells (lanes 13–15). The asterisk to the right of the figure designates a band that is found in HeLa cells and HepG2 cells; the intensity of this band is increased with extracts from cells transfected with HNF3 α or HNF3 β compared to cells transfected with the empty vector.

FIG. 5.

In vitro synthesized HNF3 α or HNF6 fail to bind the AFP promoter “A” fragment. EMSAs were performed with radiolabeled probes corresponding to the rat transthyretin promoter HNF3 binding site (**HNF3**) or the wild-type or mutated AFP promoter “A” fragment (**A** and **A***, respectively). EMSAs were performed with no proteins (free probe, F.P.; lanes 1–3) or with proteins generated from TnT reticulocyte lysates that had been programmed with BlueScript (B/S; lanes 4–6), or vectors for HNF3 β (lanes 7–9) or HNF6 (lanes 10–12).

FIG. 6.

In vivo synthesized HNF3 β -DBD truncated protein binds the transthyretin promoter HNF3 site but does not bind the AFP promoter “A” fragment. EMSAs were performed with radiolabeled probes corresponding to the rat transthyretin promoter HNF3 binding site (**HNF3**) or the AFP promoter “A” fragment extending from –205 to –137 (**A**). Probes were incubated with no extract (free probe, F.P.; lanes 1–2), or extracts from Hela cells that were transfected with the empty vector (E.V.; lanes 3–4), HNF3 α vector (lanes 5–6), HNF3 β expression vector (lanes 7–8), 3 β -DBD vector (lanes 9–10) or extracts from untransfected HepG2 cells (lanes 11–12). The asterisk corresponds to the band described in the legend to Figure 4.