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Regulation of α-Fetoprotein Expression by Nkx2.8

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The α-fetoprotein (AFP) gene is an important model of developmental gene silencing and neoplastic gene reactivation. Nkx2.8 is a divergent homeodomain factor originally cloned through its binding to the promoter-coupling element (PCE), a regulatory region upstream of the AFP promoter that mediates stimulation by distant enhancers. Nkx2.8 is the only developmentally regulated factor that has been associated with AFP gene expression. Fetoprotein transcription factor, an orphan nuclear receptor, has also been shown to bind the PCE but is not developmentally regulated. The binding specificities of both families of transcription factor were determined, and overlapping sites for each were defined in the PCE. After modification of nuclear extract and gel shift analysis procedures, Nkx2.8 was identified in six AFP-positive cell lines. Transient-transfection analysis did not show transcriptional stimulation by Nkx2.8 or other active NK2 factors, which only interfered with gene expression. However, two sets of analysis demonstrated the relationship of Nkx2.8 to AFP expression: chromatin immunoprecipitation demonstrated that Nkx2.8 bound to the active AFP promoter, and antisense inhibition of Nkx2.8 mRNA translation selectively reduced expression of both the endogenous human AFP gene and transfected reporters containing the rat AFP promoter.

α-Fetoprotein (AFP) is the classical oncofetal tumor marker (1, 9). Its high expression in fetal liver is silenced shortly after birth but is reactivated in liver cancer. High-level expression of AFP characterizes numerous hepatocellular carcinoma cell lines, and examples from human, rat, and mouse were all used in this paper to recapitulate the fetal liver phenotype.

Studies from several laboratories have elaborated some of the very complex AFP gene transcription controls (10, 18, 22, 23, 30, 32, 42, 43, 46). In rodents, the gene is regulated by three strong upstream enhancers and a tissue-specific promoter (21, 42). The enhancers account for about 98% of total AFP expression and are active in adult liver—only the promoter undergoes developmental regulation. Studies have defined a critical region near the promoter, extending from −177 to −150 bp, that links the function of the promoter and the distant enhancers (41). This promoter-coupling element (PCE) is dispensable when the enhancers are placed near the promoter but is essential when they are at their normal distance. The PCE was used in a DNA-binding expression system to clone a novel transcription factor, Nkx2.8, from HepG2 cell and human fetal liver RNA (2).

The name Nkx2.8 is derived from a relationship to the *Drosophila* factor NK2, the prototype of a small group of factors with a characteristic but divergent homeodomain. Because the base-specific DNA contacts are conserved among the NK2 factors, all are expected to have nearly identical DNA binding and might regulate AFP in a receptive cell type (2, 17). Several other transcription factors have been shown to regulate the more proximal AFP promoter region, but Nkx2.8 is so far the only binding factor with appropriate developmental regulation. Its mRNA was demonstrated in fetal liver and AFP-positive hepatocytic cells but not in adult liver, AFP-negative hepatocellular lines, or nonhepatocytic cell lines.

Despite intriguing correlations, our previous studies did not demonstrate that Nkx2.8 actually regulates AFP. Nkx2.8 did function as a transcriptional activator in nonhepatocytic HeLa cells, but, when transfected into hepatocytic cell lines, an Nkx2.8 expression plasmid caused a moderate reduction of expression from AFP promoter reporter plasmids. Squelching might have caused this effect, but the negative result certainly did not confirm positive regulation. There was another significant problem: the Bélanger laboratory characterized the fetoprotein transcription factor (FTF, also known as LRH1 and CPF), an unrelated transcription factor that also bound the PCE (20). FTF is part of a small group of orphan nuclear receptors related to the *Drosophila* factor FtzF1. This family of factors has one other member in mammals, SF1, which is expressed in developing gonad and adrenal gland. They have identical DNA-binding domains (20), so either FTF or SF1 could regulate the AFP gene in an appropriate cell. FTF does not show developmental regulation like AFP. Expression persists in adult liver where FTF regulates the Cyp7A gene (33). Nevertheless, transfected FTF significantly increased the expression of AFP gene reporters in AFP-positive cells, in contrast to the weak negative effects of Nkx2.8.

The new studies were designed to resolve these apparent contradictions and establish the role of Nkx2.8 as a direct AFP gene regulator. We therefore characterized the distinct but overlapping binding specificities of the two classes of PCE-binding transcription factors, mapped binding sites for each within the PCE, characterized functional Nkx2.8 in a variety of AFP-positive cells, applied negative regulators to selectively inactivate each type of factor, and directly demonstrated the presence of Nkx2.8 on the active AFP promoter in vivo.

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MATERIALS AND METHODS

Cells and transfection. Human cell lines HepG2, Hep3B, and HuH7 (27, 31, 32), rat lines McA7777 (7777), McA8994 (8994), and H4-II-E (H4E) (39), and

mouse line Hepa1-6 (14) were derived from hepatocellular carcinomas. AML12, a nontransformed hepatocyte cell line, was derived from a transgenic mouse that overexpresses transforming growth factor alpha in the liver (44). HepG2, Hep3B, HuH7, McA7777, and McA8994 express high levels of AFP; Hepa1-6 and clone 2 express low levels; H4E and AML12 have a differentiated adult phenotype and are AFP negative. Human H441 lung carcinoma cells express high levels of TTF1 (25), and mouse αT3 cells express high levels of SF1 (4). All cells were propagated in standard medium (SM) (Williams E medium containing 5% fetal calf serum [FCS], penicillin-streptomycin, and glutamine), except AML12 cells, which were propagated in Dulbecco's modified Eagle medium-F12 plus 10% FCS plus 1% ITS (Sigma) plus 10^{-7} M dexamethasone plus 1% penicillin-streptomycin (44).

For transient-transfection assays, transfection in HuH7 and AML12 cells using the calcium phosphate method and analysis of chloramphenicol acetyltransferase (CAT) reporter genes were carried out as previously described (2, 42). For expression of Nkx2.8 in HeLa cells, a plasmid expressing the cDNA under control of the cytomegalovirus (CMV) promoter (2) was transfected using Lipofectamine (Life Technologies).

Antisense phosphorothioate oligonucleotides were transfected into HuH7 using Oligofectamine (Life Technologies). Cells ($n = 10^5$)were plated in 3-cm microwells and were grown for 40 h in SM before transfection. At transfection this medium was removed and replaced with 800 μl of serum-free Williams E medium containing 1% glutamine (SFM). Oligonucleotide stocks were diluted to 100 nM; 1 to 10 μl of stock was mixed with Opti-Mem to a final volume of 185 μl. This was combined with a separate mix of 6 μl of Oligofectamine + 15 μl of Opti-Mem and was incubated for 20 min at room temperature. The Oligofectamine-oligonucleotide mixture was then overlaid on the 800 μl of medium to a final volume of 1 ml, with oligonucleotide concentrations of 100 to 1,000 nM. The cells were incubated for 4 h, and 500 μl of SFM supplemented with 1.5% dialyzed charcoal-treated FCS was added to give a final concentration of 0.5% FCS. Cells were incubated overnight. The medium was changed and collected for enzyme-linked immunosorbent assay (ELISA) after 24 h. Cells were then trypsinized and counted. Control plates received identical treatment including Oligofectamine but without oligonucleotide.

For combined transfection of reporter genes and oligonucleotide, cells were plated as above in 3-cm microwells in SM. After 40 h cells were first transfected with plasmid DNA using Lipofectamine-PLUS (Life Technologies). Plasmid DNA (1.25 μg) was combined with 6 μl of PLUS reagent diluted to 100 μl with SFM, combined with 4 μl of Lipofectamine mixed with 96 μl of SFM in a separate tube, and incubated for 20 min at room temperature. The mixture was then diluted with 800 μl of SFM to a final volume of 1 ml and overlaid on cells, which were then incubated for 3 h. The medium was removed, and oligonucleotide transfection was carried out as described above. Cell lysis for the CAT assay was carried out about 40 h after transfection.

Plasmids expressing the following proteins under control of the CMV promoter have been previously described: Nkx2.8 (2), SF1 (12), DAX1 (12), FTF-LRH1 (20), Nkx2.5 (8), and TTF1 (15). The reporter plasmids were pPCE4, which contained four copies of the AFP promoter region, from −166 to −153, upstream of the HIV promoter (2); pS2-CAT, containing three copies of the AFP promoter segment from −166 to −155, upstream of the TATA box in plasmid pE1b; pC5-E1bCAT, containing five copies of the TTF1 binding site in pE1bCAT (15); pAFP-246, the complete AFP promoter to −246 combined with the upstream enhancers (41); pAFP-166, the AFP promoter with a deletion to −166 combined with the enhancers (43); pAFP6000CAT, the intact AFP promoter and enhancer region (43); and pAFPE-Alb123CAT, the same enhancers fused to the albumin promoter (40).

Recombinant peptides and antibodies. A DNA segment encoding the full-length 240-amino-acid (aa) Nkx2.8 (bp 199 to 818) or an internal region encoding the homeobox and conserved peptide (bp 442 to 741, aa 82 to 181 in reference 2) (GenBank accession no. NM_014360) was PCR amplified using primers that added an *Nco*I site at the 5′ end and a *Hin*dIII site at the 3′ end. The segment was cloned into these sites in the histidine tag expression plasmid pProExHTb (Life Technologies). This was transfected into *Escherichia coli* strain BL21, and protein was purified using a nickel affinity resin according to the supplier's protocols (Life Technologies).

Polyclonal rabbit antisera were generated (ResGen, Huntsville, Ala.) against a peptide from the homeodomain (RRFRQQRRYLSAPE; aa 101 to 113; multiple antigen peptide resin conjugated) or the C terminus (YQHLASPALVSWNW; aa 226 to 239; keyhole limpet hemocyanin conjugated). The antibodies were then affinity purified using the immunizing peptide. Both antibodies demonstrated Nkx2.8 on a Western blot of the bacterial recombinant peptides. The C-terminal but not the homeodomain antibody demonstrated a supershift in electrophoretic mobility shift assays using bacterial recombinant Nkx2.8 or Nkx2.8 produced by cell-free translation (2), and this supershift was totally competed by the immunizing peptide. Antibodies against TTF1 (Lab Vision, Fremont, Calif.) and SF1 (Upstate, Lake Placid, N.Y.) were also used in supershift assays. Additional antibodies to hepatocyte nuclear factor 1α (HNF1α), HNF1β (Santa Cruz Biotechnology, Santa Cruz, Calif.), and the NF-Y A subunit (PharMingen, San Diego, Calif.) were used in chromatin immunoprecipitation analysis.

Nuclear extracts, gel shift, and supershift assays. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (NER and CER; Pierce). Cells (6×10^7) were washed twice with 10 ml of ice-cold phosphate-buffered saline to remove medium, incubated with 5 ml of detaching buffer (40 mM Tris, pH 7.6, 140 mM NaCl, and 1 mM EDTA) for 20 min, scraped from the plate, and sedimented at $800 \times g$. Cells were resuspended in 500 μl of CER I supplemented with 0.75 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg of aprotinin/μl, 2 μg of leupeptin/μl, and 2 μg of antipain/μl. Ice-cold CER II (27.5 μl) was added, and after mixing, the insoluble fraction containing nuclei was sedimented in a microcentrifuge at $16,000 \times g$ for 5 min at 4°C. The pellet was resuspended in 400 μl of NER supplemented with 2.0 mM PMSF, 2 μg of aprotinin/μl, 2 μg of leupeptin/μl, and 2 μg of antipain/μl; vortexed for 15 s; and incubated for 40 min with vortexing every 10 min. Following centrifugation at $16,000 \times g$ for 10 min, the nuclear extract supernatant was removed, glycerol was added to a final concentration of 20%, and the extract was stored at −80°C. Protein concentration was determined by the Bradford method (7). The composition of the Pierce extraction reagents is proprietary, but the final mixture with glycerol had a salt concentration of ~320 mM salt (Pierce, personal communication), a level which was too high for some gel shift interactions and for antibody binding to Nkx2.8. To allow dilution to lower salt, the extracts were concentrated to about 10 μg/μl, using Millipore Ultrafree-MC spin filtration cups at $2,500 \times g$. Some experiments also utilized standard nuclear extracts prepared as in previous studies (11, 16, 43).

Gel shift assays were performed by combining approximately 10 μg of nuclear extract protein in 1 μl with 5 μl of binding buffer (30 mM HEPES, pH 8.3, 25% glycerol, 0.3 mM EDTA, pH 8.0, 1.0 mM PMSF, 6.0 mM dithiothreitol, 1.0 μg of aprotinin/ml, 1.0 μg of leupeptin/ml, and 1.0 μg of antipain/ml) and 1 μl of poly(dIdC) (5 mg/ml). The mixture was incubated for 5 min at room temperature, and then 2 ng of labeled oligonucleotide (~100,000 dpm/ng), 200 ng of double-stranded oligonucleotide DEIV, and 0 to 200 ng of unlabeled double-stranded competitor oligonucleotide were added, together dissolved in 3 μl of a solution of 10 mM Tris, pH 8.0, and 1 mM EDTA. Oligonucleotide DEIV (top strand, CTTCAGATGGCAAACATACTTAA) was included as a nonspecific competitor. It is from a region upstream of the Alb gene and has been observed to eliminate most nonspecific bands from gel shifts (40). The final mixture, with a salt concentration of ~100 mM, was incubated for 20 min at room temperature and was loaded on an electrophoretic gel or further incubated with antibody. For supershift assays, 1 μl of affinity purified antibody was added and the mixture was incubated for 3 h at 4°C. Gel shifts were resolved by electrophoresis in a 6% polyacrylamide gel including 0.16% glycerol in low-ionic-strength buffer (0.5× Tris-borate-EDTA), run at 4°C.

AFP and albumin protein determinations. ELISA quantification of human albumin and AFP was carried out using commercial ELISA kits (Alpha Diagnostic International, San Antonio, Tex.) according to the supplier's protocols.

Chromatin immunoprecipitation. The method of Boyd and Farnham (5, 6) was followed as adapted by Forsberg et al. (19), with the following modifications: before fixation, HuH7 cells were trypsinized, washed, and suspended in complete tissue culture medium to a final concentration of 2×10^7 cells per 50 ml. Fixed chromatin was sheared in a Branson 450 Sonifier at 4°C for a total of 6 min at a power setting of 70% and duty cycle of 25%, conditions determined to shear the chromatin-encased DNA to an average size of 400 to 800 bp. One microgram of each antibody was used, and after the preclearing step, the antibody-bound supernatant was transferred to a tube containing 30 μl of protein A-agarose (Nkx2.8 H and C, NF-YA) or protein G-agarose (HNF1α, HNF1β) and was rotated for 1.5 h at 4°C. The agarose-antibody-protein complex was sedimented at $8,000 \times g$ for 2 min and was washed twice (5 min each at 4°C) with low-salt buffer (0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, and 130 mM NaCl), once (3 min at 4°C) with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, and 230 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris pH 8.1), and three times (5 min each) with 10 mM Tris, pH 8.0, and 1 mM EDTA. The DNA-protein-antibody complexes were eluted from the beads by vortexing in 150 μl of 0.1 M $NaHCO_3$–1% SDS, which was pooled with a second extraction. Following DNA isolation, standard PCR conditions were used for 30 cycles with an annealing temperature of 65°C. The AFP promoter was detected with primers CATTTTCAACCTAAGGAAATACCAT AAAGTAAC and ACCTATTCCATATTCATTTCTATGCAGTGTTC to give

a 240-bp product; the albumin promoter was detected with primers ATTGAC AAGGTCTTGTGGAGAAAACAG and AAGAGAAAAGCTAGGACAAAC GGAGG to give a 209-bp product.

RESULTS

Characterization of NK2 and FtzF1 family sites near the AFP promoter. Previous gel shift analysis indicated that Nkx2.8 synthesized in a cell-free translation system bound the same rat-AFP promoter site and migrated to the same position as FTF (2, 20). The binding specificities were resolved with preparations that gave unambiguous gel shift patterns for each family of factors: bacterial recombinant Nkx2.8, NK2 family factor TTF1 in H441 cell extract (25), and FtzF1 family factor SF1 in αT3 cell extract (4). From the literature and sequence databases, we analyzed 18 sites for binding and competition. Eleven sites bound both families, and two were discriminating: an NK2-specific site in the proximal rat thyroglobulin promoter (13) and an FtzF1-specific site in an enhancer downstream of the *H19* gene (45) (GenBank accession no. MUSH19ENII). Two family-specific consensus sequences were derived: NK2, YMCTYSA; and FtzF1, WGNACCTTG ANY.

Gel shift assays were then used to map NK2 and FtzF1 sites in the PCE (Fig. 1). These demonstrated dual binding at the previously characterized site at −164 and a strong new NK2-specific site at −176. The new site was unexpected, because it was not conserved in the human AFP promoter, which matched the rat PCE to −166 (38) (GenBank accession no. AC008076). No other NK2 or FtzF1 sites were detected in either the AFP promoter or enhancers.

Demonstration of native Nkx2.8 binding. Despite the demonstration of Nkx2.8 mRNA and the use of strong specific binding sites, native Nkx2.8 binding could not be detected in gel shifts using standard nuclear extract preparations (2). Alternative procedures were investigated until we found that extracts obtained with a commercial kit (Pierce) demonstrated clear gel shifts (Fig. 2). For supershift assays, further modifications were required to stabilize weak binding and to reduce the salt concentration enough to allow antibody binding. Subsequently, specific binding, competition, and supershifts confirmed the identity of Nkx2.8.

Nkx2.8 was demonstrated in HeLa cells transfected with an Nkx2.8 expression plasmid and in six cell lines that we used as models for AFP expression (Table 1). In all expressing cell lines, the Nkx2.8 band was the only significant band that showed appropriate competition (Fig. 2B and C), strongly suggesting the absence of other NK2 family factors. Notably, the Nkx2.8 band shift was completely absent from the AFP-negative adult phenotype cell line H4E. Resolution of binding at the actual PCE, however, was inconclusive, because this 27-bp DNA segment strongly bound a very complex mixture of factors (Fig. 2C) that were not fully resolved with either competition or supershift assays.

Functional studies in HuH7 cells. We chose HuH7 cells for reporter gene transfection experiments because these cells expressed balanced levels of Nkx2.8 and FTF. One set of comparisons suggested the importance of Nkx2.8: partial deletion of the stronger distal NK2 site caused a significant reduction of gene expression (Fig. 3A). This deletion removed the stronger NK2 site but left intact the weaker NK2-FtzF1 site and included the entire region that is conserved with the human promoter. The shorter promoter was still significantly stimulated by enhancers, though further deletion to −153 abolished enhancer stimulation (41). Overexpression of NK2 factors did not stimulate expression. Instead, three different NK2 factors all reduced expression of both reporters, an effect previously described in HepG2 cells (2). In contrast, FtzF1 factors never inhibited and frequently stimulated expression. The direct participation by FTF was demonstrated by using DAX1, a negative transcriptional regulator known to specifically bind SF1 (12). We first verified SF1-mediated repression by DAX1 and demonstrated a similar interaction with FTF. This analysis was carried out in AML12, a receptive cell line that had an adult phenotype and expressed little FTF and no Nkx2.8 (Fig. 3B). DAX1 completely blocked stimulation by either SF1 or FTF, reducing expression below the basal level. In HuH7 cells, DAX1 strongly inhibited AFP reporter gene expression (Fig. 3A), demonstrating the importance of endogenous FTF in regulating this promoter.

The negative effect of NK2 factors on AFP might have represented transcriptional squelching. Cotransfection with the full-length AFP promoter was therefore evaluated over a range of concentrations, but there was no stimulation at any concentration (Fig. 3C). All of the factors in the series were also evaluated with simple reporter plasmids (Fig. 3D). With these reporters, SF1 was a strong activator at all concentrations; FTF was a strong activator at higher concentrations; TTF1 was a weak activator at the highest concentration; and Nkx2.8 and Nkx2.5 showed no activation at any concentration. These factors all have been shown to stimulate expression in other cell types, so it appears that transcriptional activation by each NK2 factor requires a highly specific promoter and cell context. Even so, one of these factors, TTF1 could activate in HepG2 cells but instead moderately inhibited the AFP promoter, presumably by interfering with the factors already bound there. Taken together, these transfection experiments showed a role for FTF as an activator of AFP expression. They did not confirm Nkx2.8 regulation but were consistent with transcriptional interference through overexpression of an activator.

Antisense and chromatin immunoprecipitation studies. Due to the limitations of the transfection and gel shift experiments, two other approaches were used to demonstrate a role for Nkx2.8 in the regulation of AFP expression in HuH7 cells: antisense inhibition of mRNA translation (Fig. 4) and chromatin immunoprecipitation (Fig. 5). Two antisense oligonucleotides were compared with a sense-strand control oligonucleotide, to discriminate nonspecific and toxic effects of the transfection procedure. Cells were dually transfected, first with plasmid DNA and 3 h later with oligonucleotide. Two strongly expressed rat-gene plasmids were compared. These contained the same AFP enhancers in combination with either the albumin or AFP promoter (40, 43). In these two reporters, the enhancers contributed about 85 or 98% of the total gene expression, respectively. The first transfection reduced the efficiency of the second, probably leading to an underestimation of the magnitude of the antisense effect, but the differences were clear. Both antisense oligonucleotides selectively inhibited the AFP promoter by up to 50% and had no effect on the

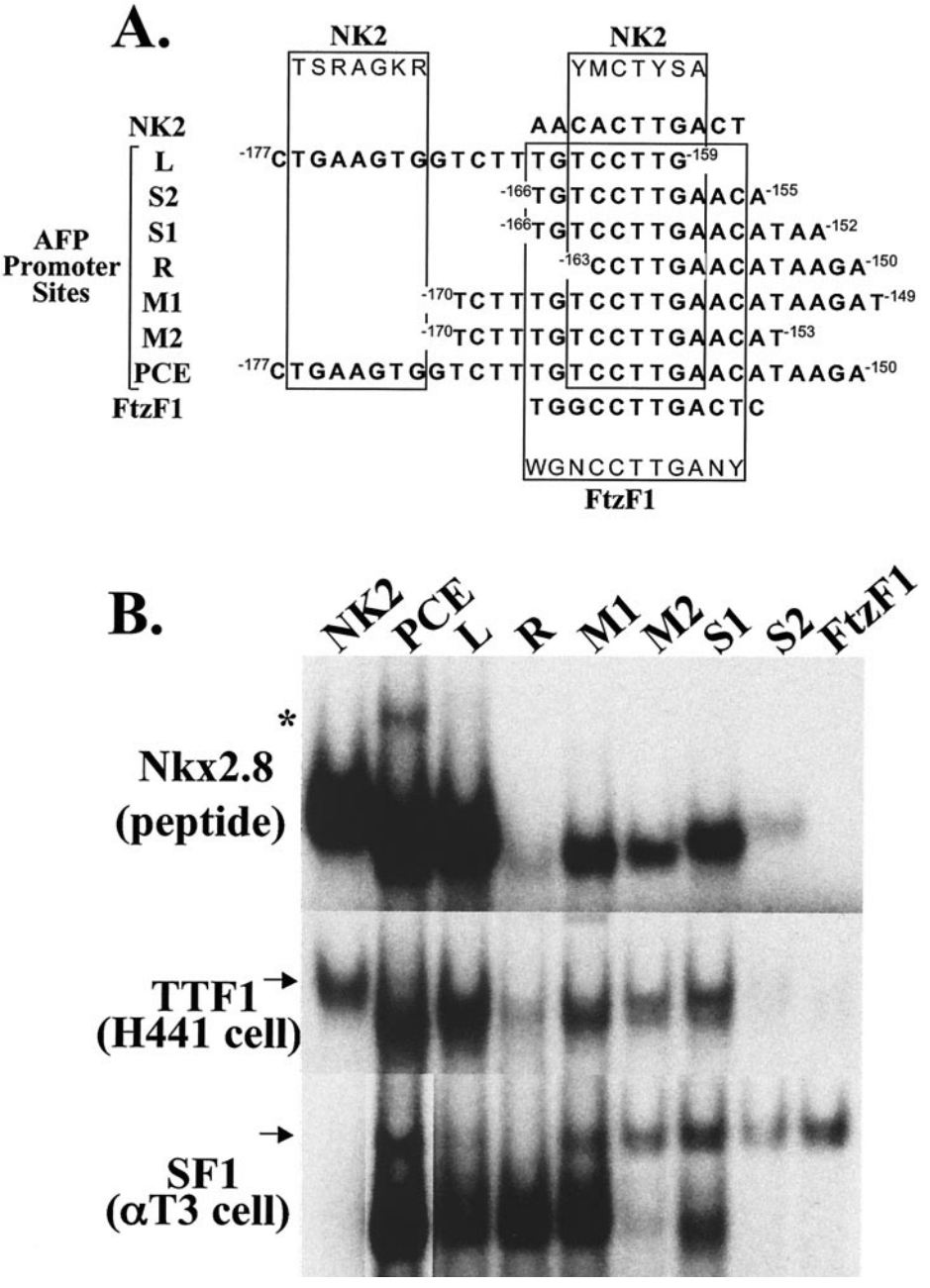

FIG. 1. Mapping of binding sites in the PCE region of the AFP promoter. (A) Oligonucleotides L, S2, S1, R, M2, M1 and PCE, spanning the AFP promoter region from −177 to −150, are aligned with NK2 family- specific and FtzF1 family-specific oligonucleotides and with derived consensus binding sites for these families. The NK2 consensus is shown in both its direct and inverted forms. (B) Gel shifts were carried out using a recombinant peptide consisting of the Nkx2.8 homeobox and conserved region (aa 82 to 181), H441 cell, or αT3 cell extracts. One hundred and fifty nanograms of recombinant peptide or ~6 μg of cell extract protein was utilized. The arrows indicate the positions of TTF1- and SF1-specific bands, and the asterisk shows the position of an upper band caused by binding of a second Nkx2.8 peptide. A strong αT3 cell band shift at a lower position was also apparent, but it was not further characterized since it did not correspond to an activity present in hepatocytic cell lines.

albumin promoter (Fig. 4B). The antisense therefore acted only on the AFP promoter, not the enhancers. Gel shift analysis (Fig. 4C) verified a selective strong reduction of Nkx2.8 binding activity. FTF was unaffected. Further experiments measured secretion of endogenous human albumin and AFP following oligonucleotide transfection (Fig. 5). In this case, antisense inhibited AFP by up to 80%, while albumin was unaffected.

Chromatin immunoprecipitation demonstrated a selective association between Nkx2.8 and the AFP promoter (Fig. 6). This analysis was controlled by detection of the albumin promoter. We compared two Nkx2.8-specific antibodies to an antibody directed against NF-Y, a factor known to bind the albumin but not the AFP promoter, and to HNF1α and HNF1β, factors known to bind both promoters. Although the Nkx2.8 detections were somewhat weaker than the others, both

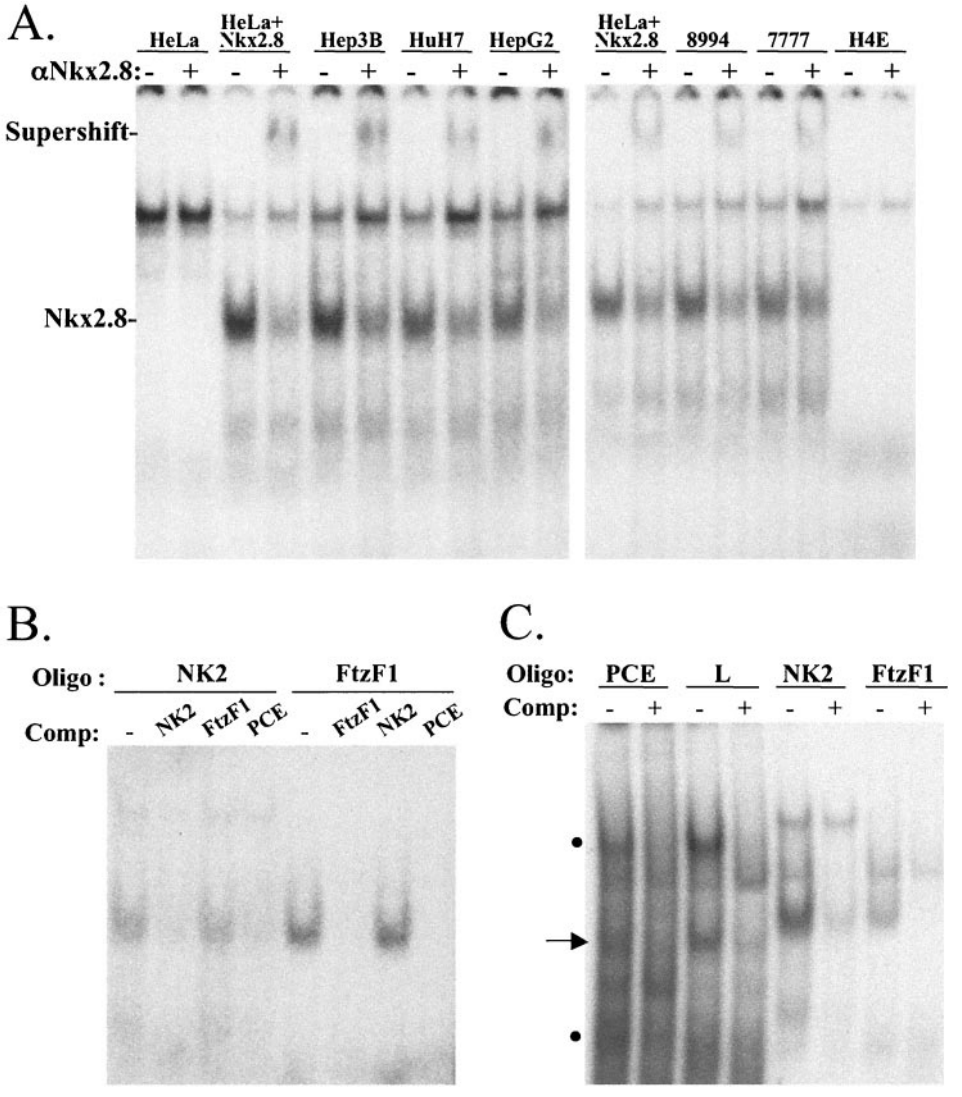

FIG. 2. Binding of native cell extracts. (A) Demonstration of Nkx2.8 in nuclear extracts. Nuclear extracts, gel shift, and supershift assays were carried out as described in Materials and Methods, utilizing the NK2-specific binding oligonucleotide AACACTTGACT. Supershifts utilized an antibody to the Nkx2.8 C-terminal. (B) Binding by 8994 cell nuclear extract. Binding of two labeled oligonucleotides (Oligo) is shown with a series of specific competitions (Comp). The oligonucleotides were described in the Fig. 1 legend. (C) Binding by Hep3B cell nuclear extract. Gel shifts were obtained with nuclear extract (~10 μg of protein) prepared as described in Materials and Methods. For the PCE gel shift pattern, a band with the approximate mobility of Nkx2.8 and FTF binding is marked with an arrow, and two regions that show a level of self-competition indicating specific binding are marked with filled circles.

antibodies detected Nkx2.8 on the AFP but not the albumin promoter. The control studies showed the appropriate specificities: NF-Y was detected on the albumin promoter but not the AFP promoter, and both HNF1 isoforms were detected on both promoters.

DISCUSSION

Problem of demonstrating Nkx2.8 activity. Nkx2.8 is an intriguing developmental factor that was first cloned from HepG2 cells through its binding of the PCE. However, it was a rare clone in cDNA libraries, and the Nkx2.8 mRNA and peptide were subsequently difficult to detect (2). This difficulty posed a significant experimental problem. Did high-sensitivity cloning isolate a factor that was expressed at a low level inadequate to regulate target genes in HepG2? Alternatively, at least three significant technical problems could have blocked detection of Nkx2.8. First, its mRNA had an exceptionally high G+C content that interfered with specific hybridization. Second, Nkx2.8 DNA-binding activity in cells was obscured by the easily detected SF1 or FTF, which had similar binding specificity and nearly identical gel shift mobility. Third, even when SF1 or FTF binding was eliminated, transcription factor isolation and gel shift conditions were not optimum for Nkx2.8.

To resolve the binding specificities of the two factor families, we examined known sites that had already been reported and then predicted new sites in genes that were likely to be common regulatory targets for factors that regulate AFP. The *H19*

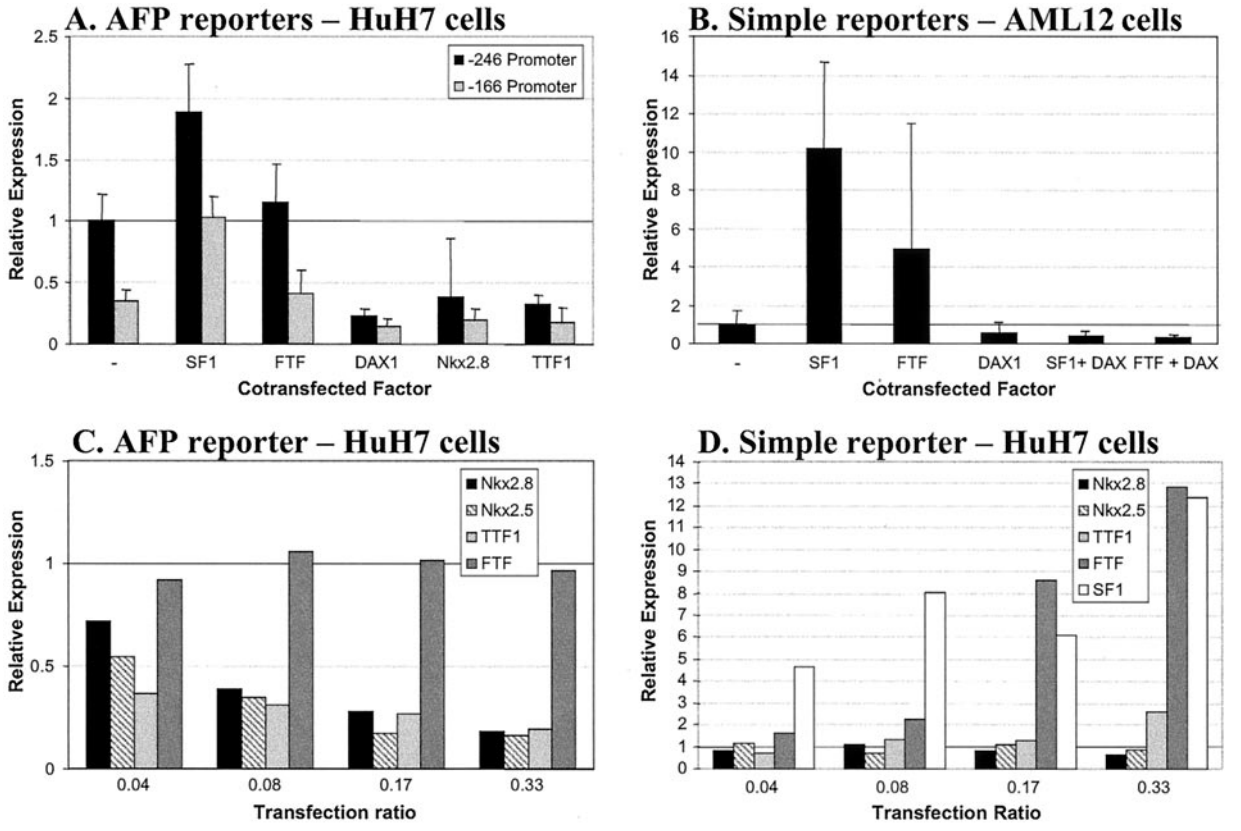

FIG. 3. Transfection experiments. (A) Comparison of an intact AFP promoter and an AFP promoter with a partial deletion. CAT gene reporter plasmids consisted of the AFP enhancers combined with the intact (pAFP-246) AFP promoter or the AFP promoter (pAFP-166) with a partial deletion. These were expressed alone or cotransfected with CMV promoter plasmids expressing SF1, FTF, DAX1, Nkx2.8, or TTF1, all at an expression-to-reporter ratio of 0.33 to 1.0. Plots show the mean and standard deviation of four determinations. (B) DAX1 inhibition of FtzF1 factors. Reporter plasmid pPCE4 was transfected into AML12 cells alone or in combination with expression plasmids at a ratio of 0.33 to 1.0. Plots show the mean and standard deviation of four determinations. The reporter had detectable basal expression, probably due to a low level of endogenous FTF expression. (C) Effects of factor concentration on AFP promoter expression. pAFP-246 was cotransfected with various amounts of expression plasmid. The plots show the average of two determinations normalized to the expression of the reporter plasmid alone (1.0). (D) Detection of transcriptional activation. Reporter plasmids pS2-CAT (with FTF and SF1) and pC5-E1bCAT (with Nkx2.8, Nkx2.5, and TTF1) were cotransfected with various amounts of expression plasmid. The plots show the average of two determinations normalized to the expression of the reporter plasmid alone (1.0).

gene, for example, was first characterized through its coregulation with AFP in fetal liver (34, 35). Moreover, like the case for AFP, distant enhancers regulate *H19* expression (29, 45). Among the new sites that we detected, one in the *H19* enhancer strongly bound FTF or SF1 but not NK2 factors. Similarly, a previously described strong NK2 site from the thyroglobulin gene showed no binding of FtzF1 factors (13). Studies using native TTF1, recombinant forms of Nkx2.8, and native SF1 or FTF all clearly demonstrated the binding specificities. However, detection of native Nkx2.8, even with the NK2-specific oligonucleotide, was weak and difficult to reproduce until nuclear protein isolation and the gel shift conditions were modified. Once modified, however, the assays demonstrated that Nkx2.8 binding activity was comparable to and sometimes stronger than that of FTF in hepatocytic cell lines. Notably, this analysis did not demonstrate NK2 family factors with mobility different from Nkx2.8, which thus appeared to be the only significant NK2 factor expressed in hepatocytic cells. Six AFP-positive cell lines were positive for Nkx2.8: 4 showed comparable levels of FTF; HepG2 had a much higher level of SF1; and Hepa1-6 had Nkx2.8 but not FtzF1 factor expression.

TABLE 1. Relative levels of AFP regulatory factors in hepatocytic cell lines

Cell line	Species	AFP level	Level of factor[a]		
			Nkx2.8	SF1	FTF−LRH1
HuH7	Human	+++	++	−	++
Hep3B	Human	++++	+++	−	++
HepG2	Human	++++	++	++++	−
Clone 2	Human	+	−	−	++
8994	Rat	++	++	−	+++
7777	Rat	+++	+++	−	++
H4E	Rat	−	−	−	+
Hepal-6	Mouse	+	++	−	−

[a] FTF and SF1 were discriminated using conventional gel shift and supershift assays. Relative factor abundance was then determined by comparing modified gel shifts of oligonucleotides NK2 and FtzF1, as done for Fig. 2B and C.

The expression of SF1 in HepG2 cells was distinctive. This factor is usually associated with developing and mature gonadal and endocrine tissues and was not detected in fetal or

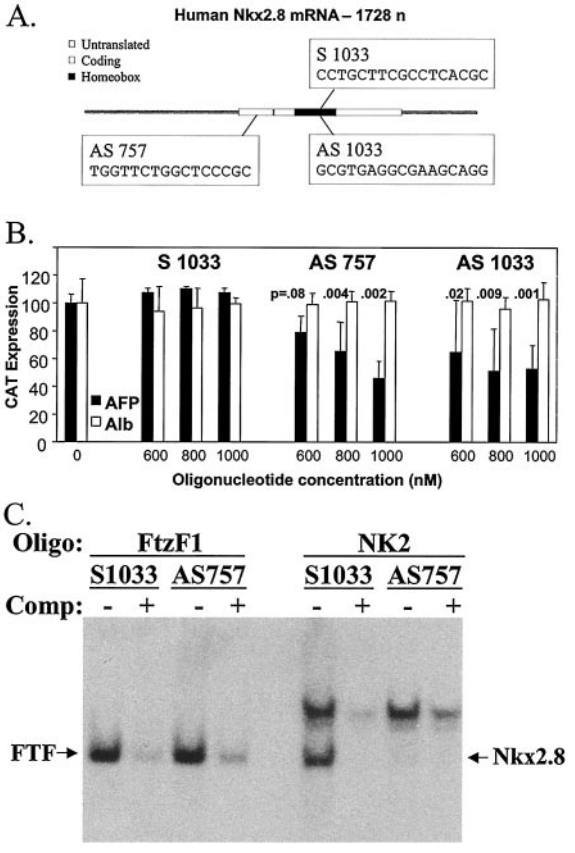

FIG. 4. Antisense oligonucleotides and cotransfection with reporter plasmids. (A) Oligonucleotides. The positions of phosphorothioate antisense (AS) and a control sense (S) strand oligonucleotide are aligned with a map of the Nkx2.8 mRNA. (B) Dual transfection. Plasmid DNA (pAFP6000CAT or pAFPE-Alb123CAT) was transfected into HuH7 cells using Lipofectamine PLUS; 3 h later, the cells were washed and oligonucleotides were transfected using Oligofectamine. Cells were collected for CAT assay after an additional 40 h. The plots show the mean and standard deviation of six determinations from three separate platings, normalized to the expression of plasmids transfected alone (100%). The pAFP6000CAT AS and S control oligonucleotide results were compared using Student's *t* test. (C) Gel shift analysis. Nuclear extract was prepared from cells treated with either the AS 757 or the control S 1033 oligonucleotides, and comparative gel shifts were carried out as done for Fig. 2B and C. To discriminate the specific bands, alternate lanes show competition at ×50 magnification with the same oligonucleotide.

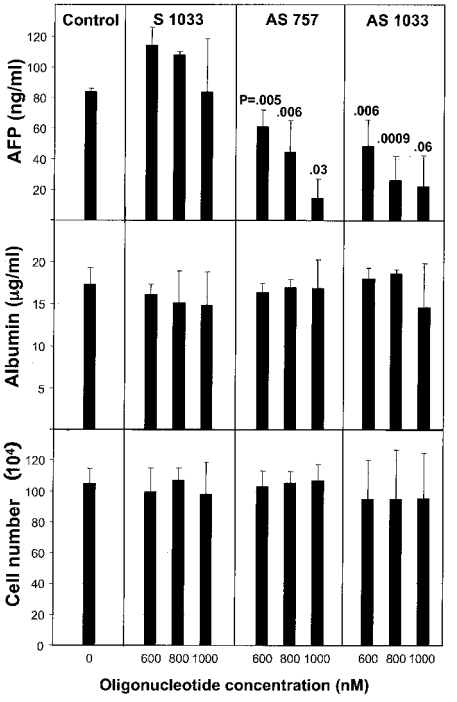

FIG. 5. Antisense effect on secreted AFP. HuH7 cells plated in 3-cm microwells were transfected with oligonucleotides and were incubated overnight. The medium (1 ml) was then changed and collected after 24 h. Human albumin and AFP were quantified using commercial ELISA kits. The plots show the mean and standard deviation of six determinations from three separate platings normalized to the expression of plasmids transfected alone (100%). AFP AS and S results were compared at each concentration using Student's *t* test.

adult liver (3, 24, 28). HepG2 is derived from a hepatoblastoma, and it will be of considerable interest to evaluate whether the abnormal activation of SF1 is an oncogenic mechanism in such tumors.

Complex binding at the PCE. The specific binding assays led to resolution of two regulatory sites in the PCE, one that bound both NK2 and FtzF1 factors and one that bound only NK2, but comparison among deletions and the human and rat promoters indicated that NK2 binding at both sites was important. In simple mixing experiments (data not shown), we were unable to demonstrate simultaneous binding of both types of factor, although transfection studies suggest that gene expression reflects a combination of activities. More significantly, the binding of the PCE by native cell extracts did not simply represent the two activities. The total binding was much stronger, and much of the binding could be self competed but not competed by NK2- or FtzF1-specific oligonucleotides. The PCE has been reported to include a glucocorticoid response element (23), but several observations indicated that the glucocorticoid receptor was not a significant part of the pattern we observed: glucocorticoid receptor binding was weak in HuH7, Hep3B, and HepG2 cells, and specific competition with a glucocorticoid response element oligonucleotide did not alter the binding patterns (data not shown). Shorter binding sites clearly showed appropriate binding of Nkx2.8, FTF, and SF1 but did not produce the more complex binding of the longer oligonucleotides, which probably resulted from cooperative recruitment through factors bound at different parts of the site. These complex patterns may represent architectural factors as well as ternary complexes that include Nkx2.8 and FTF. A full characterization of PCE-binding complexes will be of considerable interest because of the importance of this site in mediating enhancer-promoter interactions and developmental regulation.

Transient-transfection assays and AFP regulation. Transient-transfection assays were used to analyze the effects of Nkx2.8 on AFP gene expression, but responsive cells with a fetal liver phenotype already contained the factor. Reconstitution of AFP expression in other cell types was unsuccessful. The AFP promoter remained inactive in cells with an adult hepatocyte phenotype even when transcription factors were

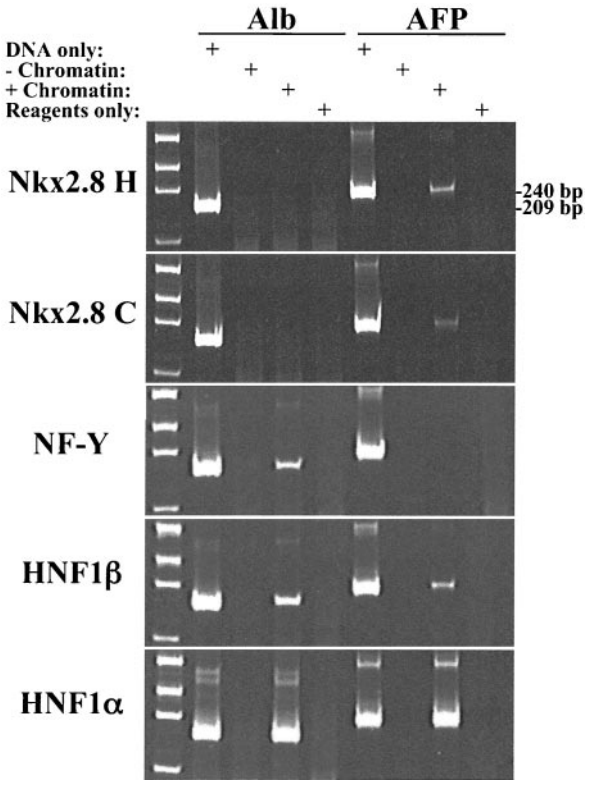

FIG. 6. Chromatin immunoprecipitation. Sheared formalin-fixed chromatin was prepared from HuH7 cells and precipitated with antibodies directed against the homeodomain and C-terminal regions of Nkx2.8, the NF-Y A subunit, HNF1α, or HNF1β. Each comparison represents a single precipitation followed by PCR detection of the albumin (Alb) (209 bp) and AFP (240 bp) promoters. A marker lane at left shows bands of 476, 341, 258, and 141 bp.

supplemented. Further, Nkx2.8 did not activate from simple site reporters, although the related factors Nkx2.5 and TTF1 did activate in adult-phenotype hepatocytic cells (2). Nonhepatocytic HeLa was the only cell type where clear Nkx2.8 activation could be detected, in this case from simple PCE site reporters. However, the AFP promoter and enhancers could not be activated in these nonhepatic cells. Thus, Nkx2.8 effects on AFP expression could only be studied in cells where AFP and Nkx2.8 were already expressed, and in these cells, additional Nkx2.8 only interfered with gene expression.

In contrast to Nkx2.8, SF1 stimulated most reporters in all of the cell types that were evaluated. SF1 was generally stronger than FTF, which also activated in most settings, and even in cells where added FTF did not further stimulate gene expression, there was no inhibition by high levels. We employed only one strategy, cotransfection with DAX1, to demonstrate that endogenous of FTF was necessary for high-level AFP expression, because the role of FTF has been further demonstrated by studies from the Bélanger laboratory (20, 36, 37). DAX1 acts like a dominant negative for SF1. It is an orphan member of the nuclear receptor family that lacks its own DNA-binding domain but heterodimerizes to SF1 and then recruits corepressors (12). Our experiments first showed the same effect on FTF. Cotransfection of DAX1 was then used to significantly inhibit expression directed by the AFP promoter, demonstrating that FTF, like Nkx2.8, was bound to the active AFP promoter in HuH7 cells.

Deletion of the more distal NK2 site was evaluated, but mutation of the dual-binding proximal site was more problematic. Elimination of one of the two characterized binding specificities would be likely to change the strength of the other and might also alter binding of other components to the PCE. These mutations could not, moreover, be directly evaluated in transfection experiments with NK2 factors. Therefore, alternate experimental strategies were chosen. The experiments demonstrated the presence of Nkx2.8 on the human AFP promoter in vivo and its major contribution to AFP gene expression through the rat or human promoter. We could not restrict this function to a single site in the rat promoter, nor could we demonstrate that FTF and Nkx2.8 occupied the proximal site at the same time.

Model of enhancer-promoter interactions. The PCE is required for the interaction of the AFP promoter with distant enhancers (41). To model the albumin-AFP locus, the enhancers were combined with both promoters on a single plasmid, and surprisingly, they stimulated both promoters at maximal levels. This noncompetitive enhancer sharing implied that each promoter interacts with enhancers through different factors and cofactors (26, 40). We hypothesize that Nkx2.8 and FTF (or SF1) combine their activities to mediate AFP enhancer-promoter interactions. This is indicated by preliminary experiments in which FTF or SF1 stimulated PCE-containing promoters similarly in the presence or absence of enhancers, while NK2 factors activated only when promoter and enhancer were combined. In these enhancer-promoter combinations, the two activities were additive (Y. Kajiyama and J. Locker, unpublished data). The albumin promoter can interact with the same enhancers, and in this case the interaction is through a proximal region that binds entirely different factors: HNF1, NF-Y, and additional architectural transcription factors (40). The detailed resolution of the full complement of factors that bind these two promoter regions, their cooperative interactions, and the cofactors that they recruit will be necessary to define how the distinct enhancer-promoter interactions of albumin and AFP are mediated.

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