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Hepatocyte Nuclear Factor 3 Activates Transcription of Thyroid Transcription Factor 1 in Respiratory Epithelial Cells

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Thyroid transcription factor 1 (TTF-1), hepatocyte nuclear factor 3α (HNF-3α), and HNF-3β regulate the transcription of genes expressed in the respiratory epithelium. To test whether members of the HNF-3/forkhead family influence TTF-1 gene expression, deletion constructs containing the 5′ region of the human TTF-1 gene were transfected into immortalized mouse lung epithelial (MLE) cells. DNase I protection and electrophoretic mobility shift assays identified elements in the 5′ region of the TTF-1 gene that bound MLE cell nuclear proteins consistent with the binding of HNF-3 to sites at positions −135 to −124 and −14 to −3. In MLE cells, TTF-1–luciferase reporter constructs were activated by cotransfection with HNF-3β, activated to a lesser extent by HNF-3α, but not activated by HFH-8. HNF-3α and HFH-8 inhibited the activation of TTF-1–luciferase by HNF-3β. Site-specific mutagenesis of each of the HNF-3 binding sites in the human TTF-1 gene inhibited the binding of MLE cell nuclear proteins and inhibited transactivation of the TTF-1–luciferase constructs after cotransfection with HNF-3β. Immunohistochemical staining demonstrated that both HNF-3β and TTF-1 were detected in bronchiolar and alveolar type II cells in the human lung. Modulation of TTF-1 gene expression by members of the HNF-3/forkhead family members may provide a mechanism by which distinct HNF-3/forkhead family members influence respiratory epithelial cell gene expression and cell differentiation.

Thyroid transcription factor 1 (TTF-1) is a 38-kDa member of the NKx-2 family of DNA-binding proteins expressed in endodermal tissues in the thyroid gland, lung, and in selected regions of the central nervous system (25, 29). TTF-1 binds to *cis*-active elements in genes expressed selectively in the thyroid gland (thyroperoxidase and thyroglobulin) and in the respiratory epithelium (Clara cell secretory protein [CCSP] and surfactant protein A [SP-A], SP-B, and SP-C), activating gene transcription and contributing to organ-specific expression of these genes (4, 5, 19, 20, 41). In some respiratory epithelial cells, TTF-1 is coexpressed with members of the hepatocyte nuclear factor 3 (HNF-3)/forkhead family of transcription factors that also influence the transcription of SP-B and CCSP. In the rat lung, TTF-1 is expressed in the developing lung buds (25). Thereafter, TTF-1 is expressed in respiratory epithelial cells in both the conducting and peripheral airways. Human TTF-1 is encoded by a 3.5-kb locus located on chromosome 14q13 (12, 17). The 5′ region of the TTF-1 gene activates transcription in a cell-type-restricted manner, and deletion analysis of the 5′ region of the TTF-1 gene identified sequences that may contribute to the control of TTF-1 gene expression (11, 17).

The spatial distribution of TTF-1 overlaps that of surfactant proteins, CCSP, and various members of the HNF-3/forkhead family of nuclear transcription proteins that are expressed in various cells in developing and mature foregut derivatives, including the lung (32). TTF-1 and, to a lesser extent, HNF-3α and HNF-3β modulate expression of SP-B and CCSP promoter constructs in human and mouse lung adenocarcinoma cells (3, 4, 7, 34). In the human SP-B gene, the HNF-3 binding site is located near TTF-1 binding sites. Both HNF-3α and HNF-3β stimulated SP-B gene expression, binding to an element located between −70 and −110 from the start of transcription of the SP-B gene (4). While HNF-3α modestly stimulated CCSP and SP-B transcription, HNF-3β inhibited the activity of CCSP gene constructs in vitro (4, 33, 34). Expression of HNF-3α and -β in the developing and mature lung and their ability to regulate surfactant protein gene expression support the concept that members of the HNF-3/forkhead family may regulate synthesis of surfactant proteins critical to postnatal adaptation to air breathing.

HNF-3α and -β are also expressed in endodermal deriva-

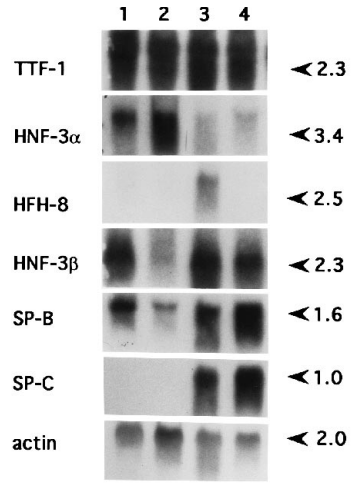

FIG. 1. Northern blot analysis of human and mouse cell lines and rat lung. Northern analysis was performed with 15 μg of total RNA from mouse cell lines MLE-15 passage 23 (lane 1), MLE-15 passage 45 (lane 2), and rat adult lung (lane 3) and freshly isolated rat type II cell RNA (lane 4). MLE-15 cells expressed TTF-1, HNF-3α, HNF-3β, and SP-B. The probe used for TTF-1 was a *Sac*II-*Sau*3A fragment of rat TTF-1 cDNA which does not contain any homeobox homology. The probes used for HNF-3α and HNF-3β contained the entire rat cDNAs. The probe used for β-actin was a 400-bp *Eco*RI fragment of human β-actin cDNA. Mouse SP-B and SP-C cDNA probes were used to detect the surfactant protein mRNAs. Sizes are indicated in kilobases.

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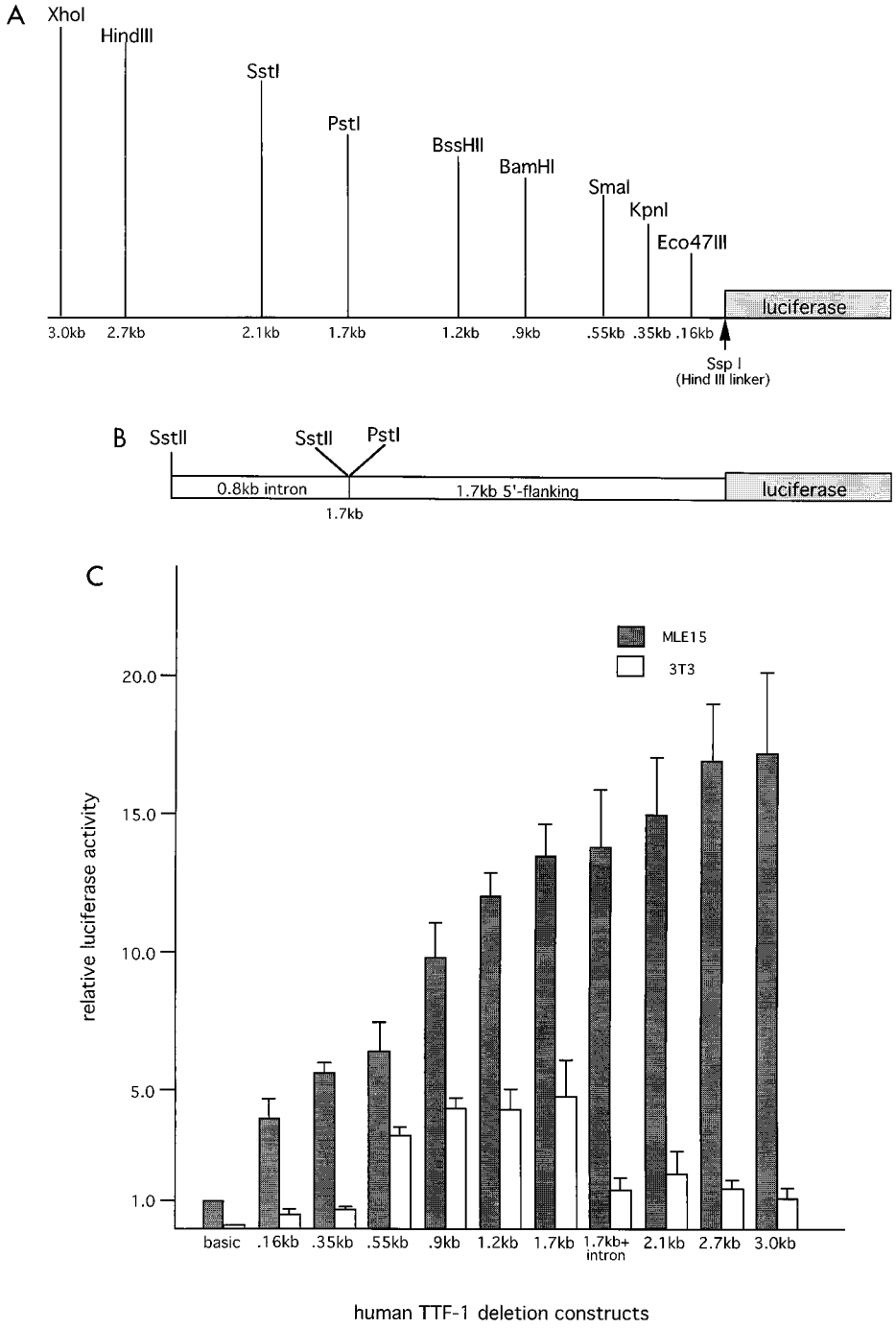

FIG. 2. Deletion analysis of human TTF-1–luciferase constructs. (A) 5′ region of the human TTF-1 gene and the restriction enzymes used for deletion mutant analysis. An *Ssp*I site ligated with a *Hin*dIII linker was used to make the 3′ ends of all deletion mutant constructs (17). (B) The fusion construct pGL2-intron-1.7kb consists of a 0.8-kb SstII-*Sst*II fragment within the intron and the *Pst*I-*Ssp*I 5′-flanking fragment. (C) Deletion mutagenesis of the 5′ region of the TTF-1 gene. Luciferase activity of the each deletion constructs was assessed in MLE-15 cells and 3T3 fibroblasts. Luciferase assays were performed after transfection of MLE-15 and 3T3 cells by using a promoterless plasmid with 5 pmol of either pGL2-basic or plasmids containing 0.16 to 3.0 kb of human TTF-1–luciferase. Cells were cotransfected with 2.5 pmol of pCMV-β-gal construct as described in Materials and Methods. Transcription was consistently more active in MLE-15 cells than in 3T3 fibroblast cells. Transcriptional activity diminished progressively with deletion of the 5′ region. Addition of the intron did not significantly alter the activity of the pGL2-1.7 kb TTF-1–luciferase construct in MLE-15 cells but contributed to the diminished activity of the construct in 3T3 cells. Results represent values relative to the activity of pRc/CMV from at least three separate experiments, each performed in triplicate. Values are means ± standard errors of three separate experiments.

tives in the developing and mature foregut prior to lung bud formation. The critical role of HNF-3β in dorsal-ventral patterning of the embryo was demonstrated in mice bearing a null mutation in the HNF-3β gene produced by gene targeting (1, 39). Homozygous HNF-3β$^{-/-}$ mice failed to form the notocord and floor plate in association with marked disruption of foregut morphogenesis. The early expression of HNF-3β in the foregut endoderm and the colocalization of TTF-1 and other

(-254)ggtaccccacaaaaaatgggggtgggggtgtccaagggaggggg
Kpn I
(-210) gaaatgctttgggtctcgtctctgcctctctctctctctttgagacct

(-160) aaaaatcctgacaagtgaaacttaaaggtgtttacctgtcatcagcatg
-135 -124
(-110) taagctaattatctcgggcaagatgtaggcttctattgtcttgttgcttt
BS2 -72 -61
(-60) agcgcttacgccccgcctctggtggctgcctaaaacctggcgccgggcta
Eco47III TATA like element -14
(-10) aaacaaacgcgaggcagcccccgagcctccactcaagccaattaaggagg
-3 +1 BS1
(+41) actcggtccactccgttacgtgtacatccaacaagatcggcgttaaggta

(+91) acaccagaat(+100)
Ssp I

FIG. 3. The 5′ genomic sequence of the human TTF-1 gene from the *Kpn*I (−254)-to-*Ssp*I (+100) site. Nucleotide sequencing was performed by standard dideoxy methods. The potential HNF-3 binding consensus sites (−135 to −124 and −14 to −3) are in boldface and underlined. BS1 and BS2 correspond to the Hox-B3 binding sites which were previously identified in the rat TTF-1 gene in assays using thyroid carcinoma cells (11). *Kpn*I-*Ssp*I and *Eco*47III-*Ssp*I fragments were used to produce constructs pGL2-0.35 kb and pGL2-0.16 kb, respectively (see Fig. 2A).

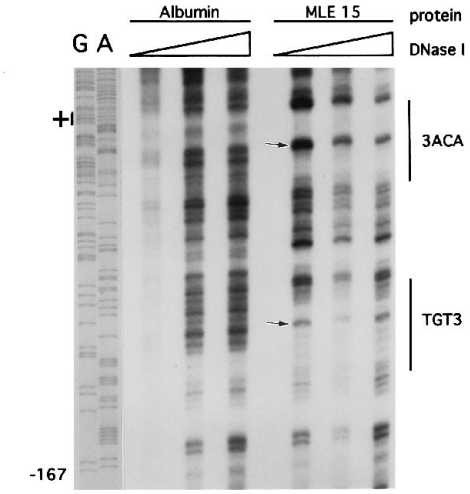

FIG. 5. DNase I footprinting analysis of the human TTF-1 promoter. Shown is an autoradiogram representing the in vitro DNase I digestion pattern of the 5′ region of the human TTF-1 gene from positions −167 to +1. DNase I digestion was performed for 1.5 min at room temperature in the presence of BSA (20 μg) or MLE-15 cell nuclear extract (30 μg). The triangle indicates increasing DNase I concentrations (0.05, 0.2, and 0.5 U of BSA per reaction; 1.25, 3, and 5 U of MLE-15 nuclear extract per reaction). Lanes G and A represent the G and A, respectively, from dideoxy sequencing. Bars at the right indicate the two regions protected from DNase I cleavage. Regions 1 (TGT3) and 2 (3ACA) include HNF-3 binding consensus sequences from positions −135 to −124 and −72 to −61, respectively. Both protected regions have DNase I-hypersensitive sites (arrows). Location (+1 to −167) is noted from the starting site (+1) at the top of the gel.

HNF-3/forkhead family members in some cells of the developing respiratory epithelium support the hypothesis that interactions between HNF-3/forkhead family members and TTF-1 may also contribute to cell differentiation and gene expression critical to lung development. An important role of TTF-1 in morphogenesis of the lung was recently demonstrated. Gene targeting of mouse TTF-1 caused severe pulmonary hypoplasia in vivo (22).

In this study, we demonstrate that (i) HNF-3β, HNF-3α, and HFH-8 differentially modulate expression of the human TTF-1 promoter in vitro and (ii) HNF-3β and TTF-1 are coexpressed in respiratory epithelial cells in human fetal lung. These findings provide support for a model by which HNF-3/forkhead family members may influence expression of TTF-1, which, in turn, may play a regulatory role in surfactant protein synthesis and lung cell differentiation.

HNF-3.TTF1("TGT3")	AGGTGTTTACCT
HNF-3.TTF1("3ACA")	GTTTGTTTTAGC
HNF-3.SPBf2	CAGTGTTTGTCT
HNF-3.TTR-S	GATTATTGACTT
HNF-3.TTR-W	TCATATTTGTGT
HNF-3.al-AT	AAATATTGACTT
HNF-3.AFP	CTTTATTGACTT
HNF-3W.H3B	CCCTGTTTGTTT
HNF-3.ALB	GAATGTTTGTTC
HNF-3.APOB	AAATACTGACTT
HNF-3.TAT	CAATATTTGCTC
HNF-3.TAT("HNF5")	ACTTGTTTGTTC
HNF-3.PEPCK	CATTATCGATCC
HNF-3.ALDB	AGGTGTTTATTC
HNF-3.CC10	GATTATTTGCTT
HNF-3.CC10	CACTATTTACTT
HNF-3.HNF1	CTCTGTTTACAT
CONSENSUS	VAWTRTTKRYTY

FIG. 4. Alignment of DNA binding sites recognized by HNF-3 protein. The HNF-3 protein binding sites are derived from the following endogenous promoters and enhancers: SP-B (SPBf2) (4), transthyretin (TTR), α1-antitrypsin (a1-AT), α-fetoprotein (AFP), HNF-3β, HNF-1, albumin (ALB), apolipoprotein B (APOB), tyrosine aminotransferase (TAT), phosphoenolpyruvate caboxykinase (PEPCK), aldolase B (ALDB), and CCSP (CC10) (28). TTF-1 (TGT3) and TTF-1 (3ACA) from this study match 9 and 8 of 12 nucleotides, respectively, of the consensus sequence. The competing oligonucleotides used in EMSA are represented by TTR, HNF-5, and SPBf2 (Fig. 6A) and correspond to HNF-3.TTR-S, HNF-3.TAT("HNF5"), and HNF-3.SPBf2. White letters indicate mismatches with the HNF-3 consensus from reference 28. Abbreviations for nucleotides: W = A or T; K = G or T; Y = C or T; R = G or A; V = A, C, or G.

MATERIALS AND METHODS

Cell culture, RNA extraction, and Northern (RNA) analysis. MLE-15 cells and other cell lines were maintained as described previously (16, 40). NIH 3T3 cells were grown in Dulbecco's modified Eagle medium containing 10% heat-inactivated bovine serum. Primary rat type II cells were isolated after elastase digestion and differential adherence on immunoglobulin G-coated plates. Total RNA was isolated by an adaptation of the method of Chirgwin et al. (6). Tissue was homogenized in 4 M guanidine thiocyanate–0.5% *N*-lauroylsarcosine–25 mM sodium citrate–0.1 M β-mercaptoethanol. Cultured cells were lysed directly on the plate in the same buffer. Phase Lock gels (5 Prime-3 Prime, Inc., Boulder, Colo.) were used to prepare RNA. RNA concentration was determined by A_{260}. Total RNA (15 μg) was electrophoresed through a 0.8% agarose–7% formaldehyde gel, transferred to Nytran (Schleicher & Schuell), and bound to the filter by UV cross-linking. Filters were hybridized overnight at 42°C in 50% formaldehyde with sodium phosphate-EDTA solution. Probes were radiolabeled by the random primer method. Filters were washed to a final stringency of 0.2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–0.1% sodium dodecyl sulfate at 60°C and exposed to Kodak XAR-2 film.

Deletion mutants. pGL2, a luciferase reporter vector, was purchased from Promega. Nine 5′ fragments of the human TTF-1 gene (*Xho*I-*Ssp*I, *Hin*dIII-*Ssp*I, *Sst*I-*Ssp*I, *Pst*I-*Ssp*I, *Bss*HII-*Ssp*I, *Bam*HI-*Ssp*I, *Sma*I-*Ssp*I, *Kpn*I-*Ssp*I, and *Eco*47III-*Ssp*I) were subcloned into the multiple cloning site of the pGL2 vector to generate pGL2-3.0 kb, pGL2-2.7 kb, pGL2-2.1 kb pGL2-1.7 kb, pGL2-1.2 kb, pGL2-0.9 kb, pGL2-0.55 kb, pGL2-0.35 kb, and pGL2-0.16 kb (see Fig. 2A). The 0.8-kb *Sst*II-*Sst*II fragment was fused to the 1.7-kb *Pst*I-*Ssp*I 5′ fragment to generate pGL2-intron-1.7kb (see Fig. 2B).

Expression constructs were transfected in 10-cm^2 dishes treated with DNA precipitates containing 6 μg of promoter-luciferase fusion plasmid, 6 μg of pCMV-β-gal, and either the empty expression vector pRc/CMV (Invitrogen) or an expression vector containing the entire rat HNF-3α, HNF-3β, HFH-8, or TTF-1 open reading frame (pCMV-HNF-3α, pCMV-HNF-3β, pCMV-HFH-8, or pCMV-TTF-1) (7, 12, 23, 24). pCMV plasmid concentrations were held constant in cotransfection experiments. HNF-3α, HNF-3β, and HFH-8 expression vectors were kindly provided by Robert Costa.

Luciferase assays. Cell transfection was performed by the calcium phosphate coprecipitation method except that glycerol shock was not used (31). Cells were

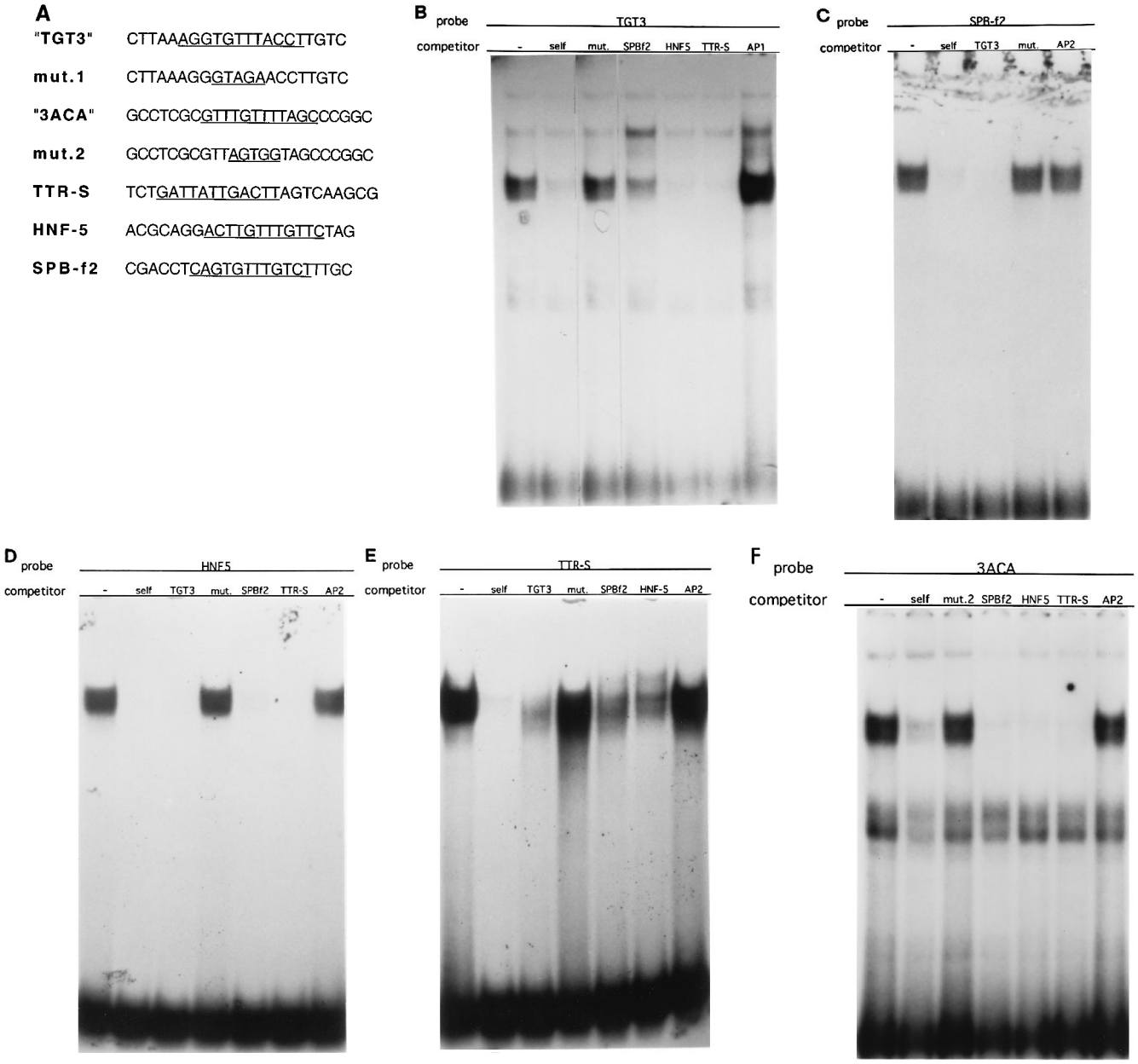

FIG. 6. EMSA with wild-type and mutant HNF binding sites. (A) Double-stranded oligonucleotides used in EMSA. The oligonucleotides containing mutated region 1 (TGT3) and region 2 (3ACA) are designated mut.1 and mut.2, respectively. Oligonucleotides from each HNF-3 motif were used as unlabeled competitors in EMSA. MLE-15 nuclear extracts (5 μg) were incubated with a 100-fold excess of unlabeled probe, demonstrating efficient cross-competition between the motifs. HNF-3 DNA recognition sites from SPBf2 (4), HNF-5 (30), or the strongest HNF-3 site from the TTR gene promoter, TTR-S (8), were also efficient competitors. Mutant motifs (mut.1 and mut.2), which do not bind HNF-3, did not compete (B and F). Complementary experiments in which SPBf2, HNF-5, and TTR-S were labeled showed that oligonucleotide competitor TGT3 (100-fold excess over the labeled probe) inhibited their binding to MLE-15 nuclear extracts (C, D, and E).

incubated for approximately 20 to 22 h, washed once with Hanks' balanced salt solution (Gibco BRL), and returned to culture in the original medium for additional 24 h for MLE-15 cells and 48 h for 3T3 cells. Cells were harvested with Reporter Lysis Buffer (Promega). The lysates were prepared, and aliquots were assayed for β-galactosidase activity and for luciferase activity with a luminometer (Analytical Luminescence Laboratory, San Diego, Calif.). To correct for variations in transfection efficiency, activity was normalized to β-galactosidase activity (27).

Plasmid constructions and site-directed mutagenesis. The 5′ region of the human TTF-1 gene was isolated by using restriction enzymes and linker primers as previously reported (17). pGL2-0.35 kb was used as the template, and site-directed mutagenesis was performed by the PCR mutagenesis method (15). The final product was digested with *Kpn*I (5′ side) and *Hin*dIII (3′ side) and subcloned into the respective sites of pGL2-basic vector (Promega), changing TGTTT (−132 to −128) to GTAGA and AAACA (−10 to −6) to CCACT, respectively. Mutated human TTF-1–luciferase fusion plasmids were designated pGL2-0.35kb,mut.1 and pGL2-0.35kb,mut.2, respectively. The double mutant, pGL2-0.35kb,mut.12, in which both TGTTT and AAACA sites were mutated, was made by using the same method. Identities were confirmed by sequencing of double-stranded templates.

Preparation of nuclear extracts. MLE-15 nuclear extracts were prepared by using a miniextract procedure (35). All procedures for nuclear extraction were performed on ice and with ice-cold reagents. Confluent monolayers from four 10-cm^2 dishes were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS), harvested by scraping into 1 ml of PBS, and pelleted in a 1.5-ml microcentrifuge tube at 3,000 rpm for 5 min. The cell pellet was washed once in 1 ml of PBS, and pelleted as described above. The pellet was resuspended in 1 packed cell volume of fresh buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-

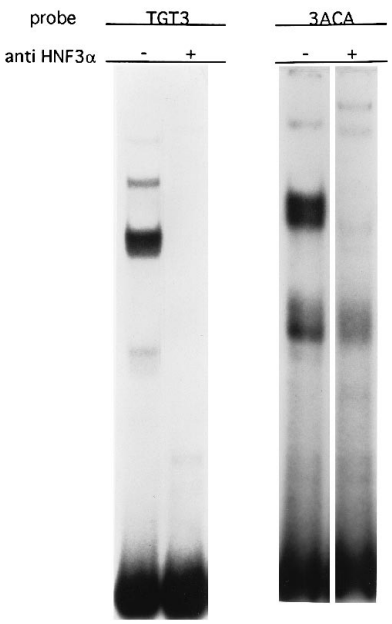

FIG. 7. Antibody interference analysis of the HNF-3 sites. HNF-3α antibody (1 μl) was added to EMSA reaction mixtures after labeled oligonucleotide TGT3 or 3ACA (10 fmol) was incubated with MLE-15 nuclear extracts (5 μg) (18). Anti-HNF-3α inhibited formation of the complexes at both HNF-3 sites with MLE-15 nuclear extracts.

ethanesulfonic acid [HEPES; pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% [vol/vol] Nonidet P-40, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]), and the cells were lysed during a 5-min incubation with occasional gentle vortexing. A nuclear pellet was obtained by centrifugation at 3,000 rpm for 5 min, and the supernatant was used as the cytoplasmic extract. The nuclear pellet was resuspended in 1 packed nuclear volume of buffer (20 mM HEPES [pH 7.9], 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% [vol/vol] glycerol, 1 mM DTT, 0.5 mM PMSF), and nuclei were extracted during a 10-min incubation with occasional gentle vortexing. Extracted nuclei were pelleted in a microcentrifuge at 14,000 rpm for 10 min. The supernatant was recovered and typically contained 5 to 9 μg of extracted nuclear protein per μl. Nuclear extracts were stored at −80°C without loss of activity for at least 6 months.

DNase I footprinting. For DNase I footprinting, 5′-end-labeled probe was generated by PCR, using the pGL2-0.35 kb construct as the template and vector-specific GL1 and GL2 primers from Promega. The DNase I protection assay was performed in a 50-μl reaction as described by Kelly et al. (20). The DNA binding reaction was carried out on ice in a buffer containing 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, 50 μM EDTA, 75 mM KCl, 12% glycerol, 0.5 mM DTT, and 0.2 mM PMSF. Nuclear extracts (30 μg) or bovine serum albumin (BSA; 20 μg; Boehringer Mannheim) was first incubated with 2 μg of poly(dI-dC) (Boehringer Mannheim) for 30 min. Then 20,000 dpm (approximately 0.3 ng) of probe was added, and incubation continued for 1 h. Probes were then subjected to DNase I (RQ DNase; Promega) digestion for 1.5 min at room temperature, using various concentration of DNase I (0.05, 0.2, and 0.5 U of naked DNA per reaction; 1.25, 3, and 5 U of MLE-15 nuclear extract per reaction). The DNA fragments were separated on 6% polyacrylamide–7 M urea sequencing gels, using the dideoxy sequencing reaction product (Sequenase; U.S. Biochemical) as a size marker.

Synthetic oligonucleotides. Synthetic oligonucleotides were annealed at 10 mM in 100 μl of restriction enzyme buffer M (10 mM Tris [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl; Boehringer Mannheim) by placing the mixture in a preheated 95°C water bath, which was then slowly cooled to room temperature. A_{260} was determined, and dilutions of this mixture were made in 10 mM Tris (pH 8.0)–1 mM EDTA and used directly in electrophoretic mobility shift assay (EMSA) as unlabeled competitor DNA. Probes were purified by using a 4% Bio-Gel and MERmaid kit as specified by the manufacturer (Bio 101). A_{260} was determined, and 1.5 to 2.0 pmol of the annealed and gel-purified oligonucleotide was end labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. End-labeled probes were purified from unincorporated [γ-^{32}P]ATP by using a Pharmacia Nick column and used at an activity of approximately 25,000 dpm μl^{-1}.

Oligonucleotides used in EMSA are listed in Fig. 6A. TGT3 and 3ACA are from the 5′ region of the human TTF-1 positions −140 to −120 and −19 to +5, respectively. mut.1 and mut.2 are mutants of TGT3 and 3ACA, respectively. TTR-S, HNF-5, and SPBf2 are oligonucleotides containing known HNF-3 binding sites and are derived from the mouse transthyretin gene promoter (8), from the rat tyrosine aminotransferase gene promoter (29), and from the human SP-B gene promoter (4), respectively.

EMSA. The procedure for EMSA was adapted from methods described by Hennighausen and Lubon (14). Briefly, nuclear extract (5 μg) and unlabeled oligonucleotide competitor DNA (100-fold excess over the labeled probe) were preincubated in buffer [12 mM HEPES (pH 7.9), 4 mM Tris-Cl (pH 7.9), 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 50 ng of poly(dI-dC) per ml, 0.2 mM PMSF] for 10 min on ice. Probe (100,000 dpm) was added, and the mixture was incubated for an additional 20 min on ice. For antibody interference assays, 1 μl of antibody was added after the addition of probe, and the mixture was incubated for an additional 20 min on ice. Rabbit anti-HNF-3α polyclonal antiserum was kindly provided by R. H. Costa (18). Bound and free probe were resolved by nondenaturing polyacrylamide gel electrophoresis. Five percent gels (acrylamide/bisacrylamide, 29:1; 0.5× TBE [44.5 mM Tris, 44.5 mM borate, 1 mM EDTA {pH 8.3}, 2.5% {vol/vol} glycerol]; 1.5 mm thick) were run in 0.5× TBE running buffer at constant current (30 mA) for approximately 80 min. Gels were blotted to Whatman 3MM paper, dried under vacuum, and exposed to X-ray film for 1 to 2 h at −80°C with an intensifying screen.

Immunohistochemistry. Surgical specimens of human neonatal lung were obtained from the Division of Pathology, Children's Hospital Medical Center, Cincinnati, Ohio. Affinity-purified rabbit polyclonal antiserum to amino acids 7 to 86 of rat HNF-3β was provided by Robert H. Costa (18) and used at dilutions of 1:500 to 1:1,000. A Vectastain ABC Peroxidase Elite Rabbit IgG kit (Vector Laboratories Inc.) was used for detection of the antigen-antibody complexes (38). An antigen retrieval method, using microwave irradiation, was required for immunolocalization of the antigen (10, 26). Four- to 6-μm-thick sections were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol to water. Sections selected for antigen retrieval were immersed in sodium citrate buffer (pH 6.0) and heated in a microwave for 15 min at 90°C. Endogenous peroxidase was quenched for 15 min at room temperature with 3% H_2O_2 in methanol. Sections were blocked with 2% normal goat serum in PBS plus 0.2% Triton for 2 h at room temperature before incubation overnight at 4°C with the primary antibody at dilutions of 1:500 and 1:1,000. The sections were washed six times (5 min each) in PBS plus 0.2% Triton and then incubated for 30 min at room temperature with biotinylated goat anti-rabbit antibody diluted 1:200 in the blocking solution. Sections were incubated for 30 min at room temperature with the avidin-biotin-peroxidase complex, incubated for 4 min with Ni-diaminobenzidine in 0.1 M acetate buffer, and then incubated for 4 min at room temperature with Tris-cobalt. Finally, the sections were counterstained with nuclear fast red and photographed with a Nikon Microphot FXA photomicroscope, using differential interference contrast optics. Omission of the primary antibody was used to check for endogenous biotin and peroxidase activity as well as for nonspecific binding of the secondary antibody.

RESULTS

Northern blot analysis of RNA from simian virus 40 large-T-antigen-immortalized mouse lung epithelial (MLE) cells (16, 40) and other pulmonary and nonpulmonary cell lines was used to identify SP-B, TTF-1, HNF-3α, HNF-3β, and HFH-8 mRNAs. Coexpression of SP-B, TTF-1, HNF-3α, and HNF-3β mRNAs was observed in MLE-15 cells (Fig. 1). NCI-H441 cells, a human pulmonary adenocarcinoma cell line used widely for analysis of surfactant protein synthesis, also expressed TTF-1, HNF-3α, and SP-B mRNAs but not HNF-3β mRNA (data not shown). SP-B, HNF-3α, HNF-3β, and TTF-1 mRNAs were detected in rat type II cells and in several lung epithelial cell lines (MLE-15, MLE-12, and H441 [data not shown]). HFH-8 mRNA was not detected in primary cultures of rat type II cells or in the lung epithelial cell lines tested but was readily detected in whole lung tissue. MLE-15 cells expressed SP-B, TTF-1, HNF-3α, and HNF-3β mRNAs. Because MLE cells were readily transfected, these cells were chosen for further study of TTF-1 gene expression.

As previously reported, constructs containing the 5′ region of the human TTF-1 gene were transcriptionally active, expressing luciferase after transfection into MLE-15 cells (17). Transcriptional activity of TTF-1–luciferase constructs was consistently greater in MLE-15 cells than in 3T3 fibroblast cells. Transcriptional activity of this region of the TTF-1 gene was demonstrated in deletion mutants generated at convenient restriction enzyme sites, deleting increasing segments of the 5′ region of the TTF-1 gene (Fig. 2A). Intronic sequence (0.8 kb) was also added to the construct containing the 1.7-kb flanking

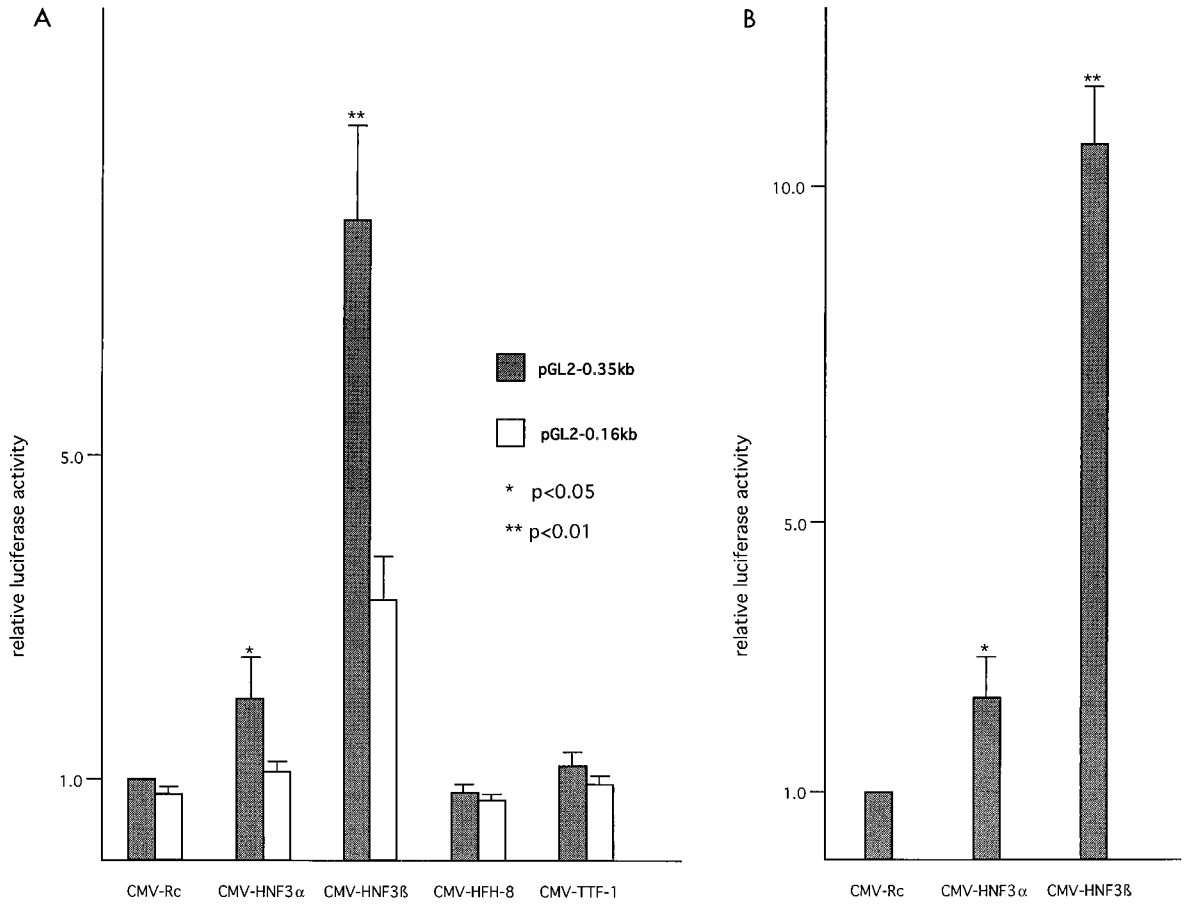

FIG. 8. Transactivation of TTF-1–luciferase by cotransfection with HNF family members. Transactivation of pGL2-0.35 kb and pGL2-0.16 kb (6 μg of each) by pCMV-HNF-3α, pCMV-HNF-3β, and pCMV-HFH-8 (10 μg of each) was assessed in MLE-15 cells (A). Transactivation of the pGL2-0.35 kb (6 μg) construct by pCMV-HNF-3α and β (10 μg of each) was tested in 3T3 cells (Fig. 8B). Basal activity in 3T3 cells was much lower than in MLE-15 cells (see Fig. 2C). Luciferase activity is expressed relative to the activity of pRc/CMV. Values are means ± standard errors of three separate experiments. * and **, $P < 0.05$ and $P < 0.01$ versus pRc/CMV added control (unpaired t test).

region to test whether it contributed to transcriptional activity (Fig. 2B). Transfection studies with the TTF-1–luciferase constructs in MLE-15 cells demonstrated that transcriptional activity diminished progressively with deletion of the 5′ region (Fig. 2C). Addition of intronic sequences did not significantly alter the activity of the pGL2-1.7 kb TTF-1–luciferase construct in MLE-15 cells (Fig. 2B) but contributed to the diminished activity of the construct in 3T3 cells (Fig. 2C).

Nucleotide sequence analysis of the 5′ region of the human TTF-1 gene identified three possible consensus sequences (−135 to −124, −72 to −61, and −14 to −3) consistent with HNF-3 protein binding sites as shown in Fig. 3. The HNF-3 DNA recognition sequence has been shown by Overdier et al. (28) to consist of 12 nucleotides. The regions from −135 to −124, −72 to −61, and −14 to −3 match 9, 9, and 8 of 12 nucleotides, respectively (Fig. 4). DNase I footprinting with MLE-15 cells identified protected sites flanking a DNase I-hypersensitive site in region 1 (TGT3) and region 2 (3ACA), corresponding to positions −135 to −124 and −14 to −3, respectively (Fig. 5). In contrast, no DNase I footprint or EMSA was detected in the region from −72 to −61.

EMSAs were performed with nuclear extracts from MLE-15 cells, using competitor oligonucleotides consistent with known HNF-3 binding sites (Fig. 6A). Both region 1 (probe TGT3) and region 2 (probe 3ACA) from the TTF-1 gene promoter were shifted by MLE-15 nuclear extracts. Binding was competed for by self and by oligonucleotides containing known HNF-3 binding sites (Fig. 6A) from the mouse transthyretin promoter (TTR-S) (8), rat tyrosine aminotransferase gene promoter (HNF-5) (30), and human SP-B gene promoter (SPBf2) (4) (Fig. 6B and F). Mutant oligonucleotides did not compete for binding to both TGT3 and 3ACA sites in the TTF-1 gene promoter (Fig. 6B and F). Antisera generated against HNF-3α (18) blocked the formation of the DNA-protein complexes with both sites (Fig. 7). In complementary experiments in which known HNF-3 binding sites from SPBf2 (4), HNF-5 (30), and TTR-S (8) were labeled, oligonucleotide probes containing the region 1 (TGT3) and region 2 (3ACA) from the TTF-1 gene promoter inhibited their binding to MLE-15 cell nuclear extracts (Fig. 6C, D, and E and data not shown). In contrast, mutant TTF-1 and AP-2 probes failed to compete in the EMSA analysis, providing further support for the conclusion that HNF-3/forkhead family members bind to the TTF-1 gene, at least in part, by binding to region 1 and region 2.

To directly assess the effect of HNF-3/forkhead family members on TTF-1 gene transcription, TTF-1–luciferase constructs and pCMV-HNF-3α, pCMV-HNF-3β, or pCMV-HFH-8 were cotransfected into MLE-15 cells (7, 12, 23, 24). Transcriptional activity of a TTF-1–luciferase construct consisting of 0.35 kb of the 5′ region of the TTF-1 gene was stimulated by pCMV-

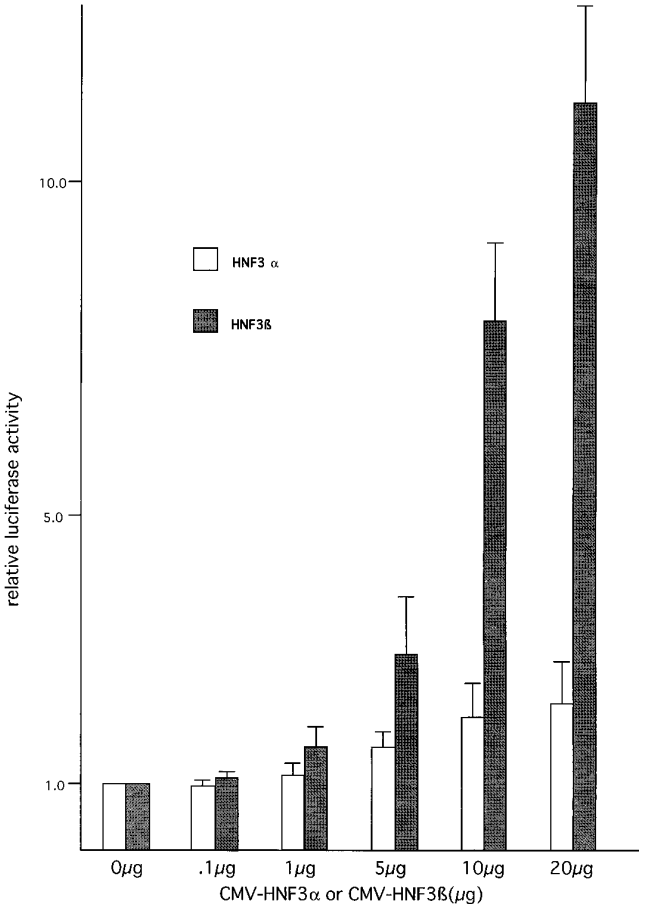

FIG. 9. Dose response of the activation of the pGL2-0.35 kb reporter construct by cotransfection with HNF-3α and HNF-3β. Increasing concentrations of pCMV-HNF-3α or pCMV-HNF-3β (0 to 20 μg) were added to pGL2-0.35 kb (6 μg) and transfected into MLE-15 cells. Cells were cotransfected with pCMV-β-gal (5 μg) to assess transfection efficiency. HNF-3β protein transactivated the TTF-1 luciferase construct in a dose-dependent manner. Luciferase activity is expressed in relative to that of the control after correction for β-galactosidase activity. Values are means ± standard errors of three separate experiments.

HNF-3β and to a lesser extent by pCMV-HNF-3α. In contrast, cotransfection with an HFH-8 or TTF-1 expression vector did not alter TTF-1–luciferase activity (Fig. 8A). The effects of HNF-3β and HNF-3α on the pGL2-0.35 kb construct were dose dependent. HNF-3β consistently activated gene transcription to a greater extent than HNF-3α (Fig. 9). To test the potential cell selectivity of the effects of HNF-3α and β, cotransfection experiments were also performed with 3T3 cells, using the pGL2-0.35 kb construct. The TTF-1–luciferase construct was much less active in 3T3 cells than in MLE-15 cells and was stimulated by HNF-3β (Fig. 8B). Site-specific mutagenesis of region 1 (TGT3) and region 2 (3ACA) in the 0.35-kb TTF-1–luciferase construct inhibited the effects of pCMV-HNF-3β in MLE-15 cells; however, the mutation of both sites did not completely block the effects of HNF-3β (Fig. 10).

HNF-3α and HFH-8 were cotransfected with HNF-3β to test whether HNF-3α or HFH-8 could compete with HNF-3β for activation. Dose-dependent inhibition of the stimulatory effects of HNF-3β on TTF-1–luciferase activity were observed after cotransfection with HNF-3α or HFH-8 (Fig. 11).

Immunohistochemistry. HNF-3β was detected by immunohistochemistry in the nuclei of epithelial cells located in both bronchiolar and alveolar epithelia of the postnatal human lung (Fig. 12A). In the alveolar epithelium, HNF-3β was restricted to type II pneumocytes (Fig. 12C). In the bronchiolar epithelium, HNF-3β was detected in nuclei of both ciliated and nonciliated epithelial cells (Fig. 12B). Staining was also observed in the apical cytoplasm of cells in the conducting airway in human lung. Such staining was not observed in epithelial cells of developing mouse lung, wherein staining was observed only in nuclei of respiratory epithelial cells in the airways of type II epithelial cells (43). The significance of the nonnuclear staining is unclear. Immunostaining of nuclei of type II pneumocytes and cells located in the alveolar-bronchiolar portals was more intense than that detected in cells of the bronchiolar epithelium. The distribution of cells expressing HNF-3β in the human neonatal lung overlaps that reported for TTF-1 expression in the human fetal, neonatal, and adult lung (2, 17, 37). However, immunolocalization of HNF-3β in both ciliated and nonciliated cells of the bronchiolar epithelium diverges from that reported for TTF-1, which is restricted to nonciliated cells of the bronchiolar epithelium in the neonatal lung. Similar results were obtained for fetal mouse lung tissue (data not shown).

DISCUSSION

In the rat embryo, TTF-1 mRNA is present in embryonic and postnatal thyroid and lung and in some parts of central nervous system (25). Postnatally, TTF-1 is expressed in respiratory epithelial cells, the pattern of its expression changing with advancing lung development (17). During lung development, TTF-1 protein is increasingly restricted to subsets of respiratory cells in the distal conducting airway and in alveolar type II cells, consistent with its role in the regulation of surfactant protein and CCSP gene expression (4, 5, 17, 20, 33). The present study demonstrates binding and activation of TTF-1 gene transcription by HNF-3α and HNF-3β but not by HFH-8. HNF-3α and HNF-3β mRNAs, but not HFH-8 mRNA, were coexpressed in several respiratory epithelial cell lines, in primary cultures of rat type II cells, and in the epithelial cells of the lung in vivo (this report and reference 13). The control of TTF-1 expression in the respiratory epithelium may therefore provide important temporal and spatial signals involved in both lung morphogenesis and gene expression.

The 5′ region of the human TTF-1 gene has been highly conserved, and both HNF-3 binding sites, consisting of 12 nucleotides, are completely conserved among human, rat, and mouse genes (9, 11, 21). However, the transcriptional mechanisms controlling the TTF-1 gene expression are relatively poorly understood. Transcriptional activity of this region of the TTF-1 gene locus has been demonstrated. Guazzi et al. identified binding of nuclear proteins to the Hox-B3 consensus element in the 5′-flanking region of the rat TTF-1 gene and demonstrated that cotransfection with Hox-B3 activated TTF-1 gene promoter constructs in vitro (11). This Hox-B3 site has been highly conserved among human, rat, and mouse genes. However, Hox-B3 is not expressed in the NCI-H441 cells or MLE cells (unpublished observations) and appears to be expressed in the developing mesenchyme rather than within the epithelial cells of the lung (36).

This study demonstrates that HNF-3β is expressed in the respiratory epithelium of the human lung, consistent with its potential role in lung development and function. Likewise, HNF-3α mRNA was detected in pulmonary epithelial cells (7). HNF-3α and HNF-3β are more widely distributed than TTF-1, being expressed in the central nervous system and foregut

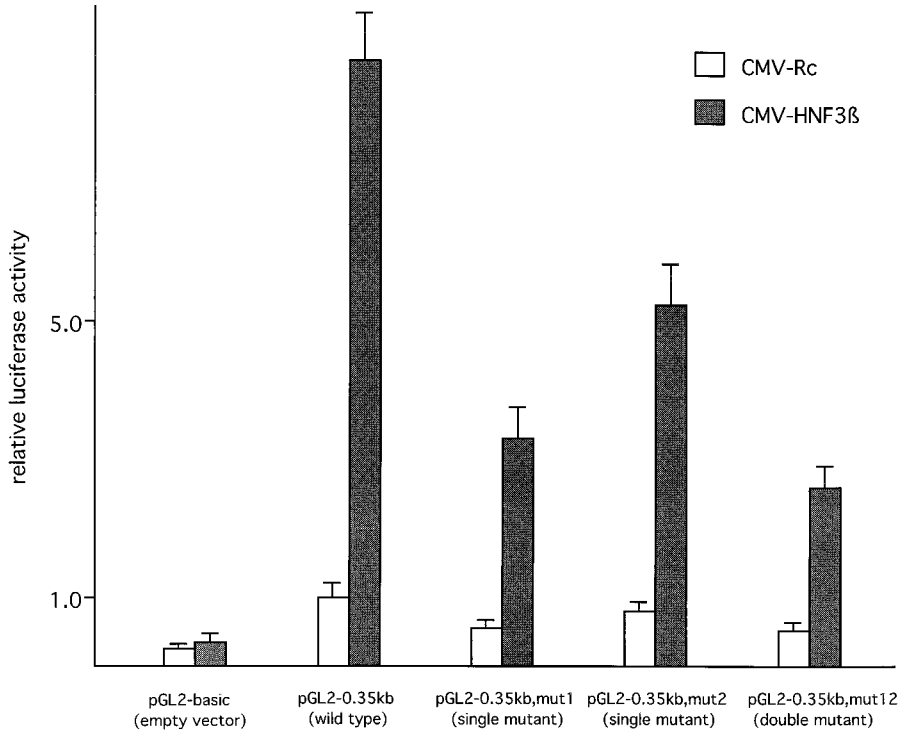

FIG. 10. Site-directed mutation and cotransfection with pCMV-HNF-3β. pRc/CMV or pCMV-HNF-3β DNA (10 μg) was cotransfected with the 6 μg of mutant (pGL2-0.35 kb,mut.1, pGL2-0.35 kb,mut.2, or pGL2-0.35 kb,mut.12) or wild-type (pGL2-0.35 kb) TTF-1–luciferase DNA. Activities of the mutated constructs were less than that of the wild-type TTF-1 construct. Transactivation of each of the mutant constructs by HNF-3β was significantly reduced. Luciferase activity is expressed relative to that of the control, pRc/CMV. Values are means ± standard errors of three separate experiments; $P < 0.01$ by analysis of variance.

endoderm prior to lung bud formation (32). HNF-3α and HNF-3β bind to the same consensus sites, influencing transcription of many genes in the liver, lung, and other tissues (Fig. 4). In the present study, site-specific mutagenesis, EMSA, and transactivation studies with the human TTF-1 gene promoter demonstrated that HNF-3β, and to a lesser extent HNF-3α but not HFH-8, activated TTF-1 gene transcription, providing a distinct manner by which HNF-3/forkhead family

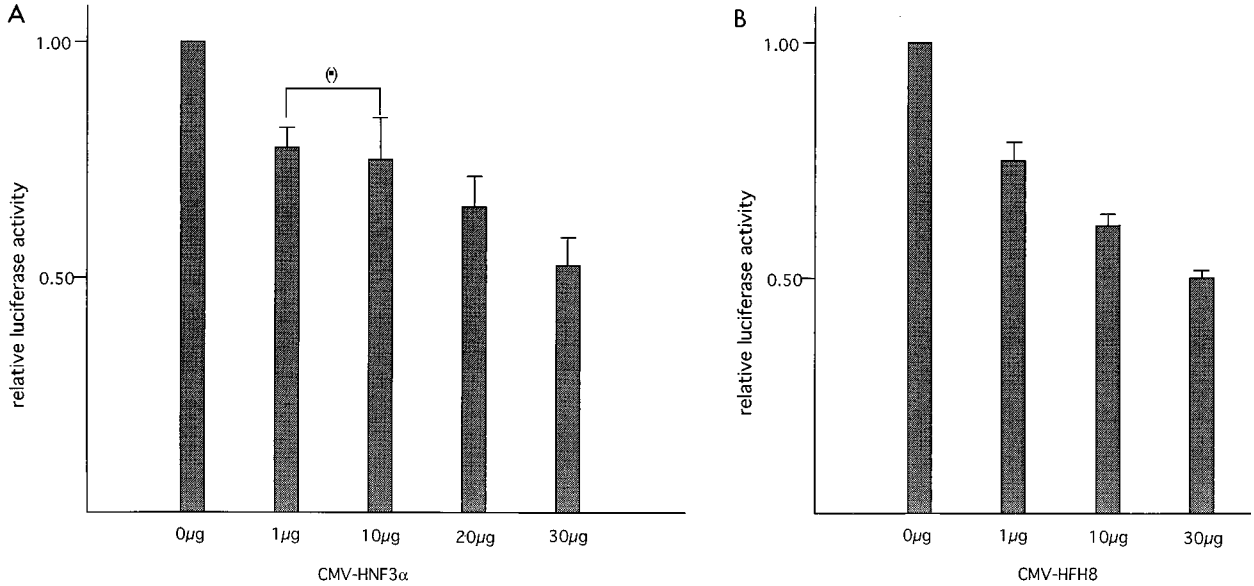

FIG. 11. Inhibitory effects of HNF-3α and HFH-8 on HNF-3β activation of TTF-1 transcription. Increasing concentrations (0 to 30 μg) of pCMV-HNF-3α (A) or pCMV-HFH-8 (B) were added to 6 μg of pGL2-0.35 kb, 10 μg of pCMV-HNF-3β, and 5 μg of pCMV-β-gal. HNF-3β-induced luciferase activity was inhibited by HFH-8 and HNF-3α in a dose-response manner. Values are means ± standard errors of three separate experiments; $P < 0.01$ by analysis of variance except where indicated by (■).

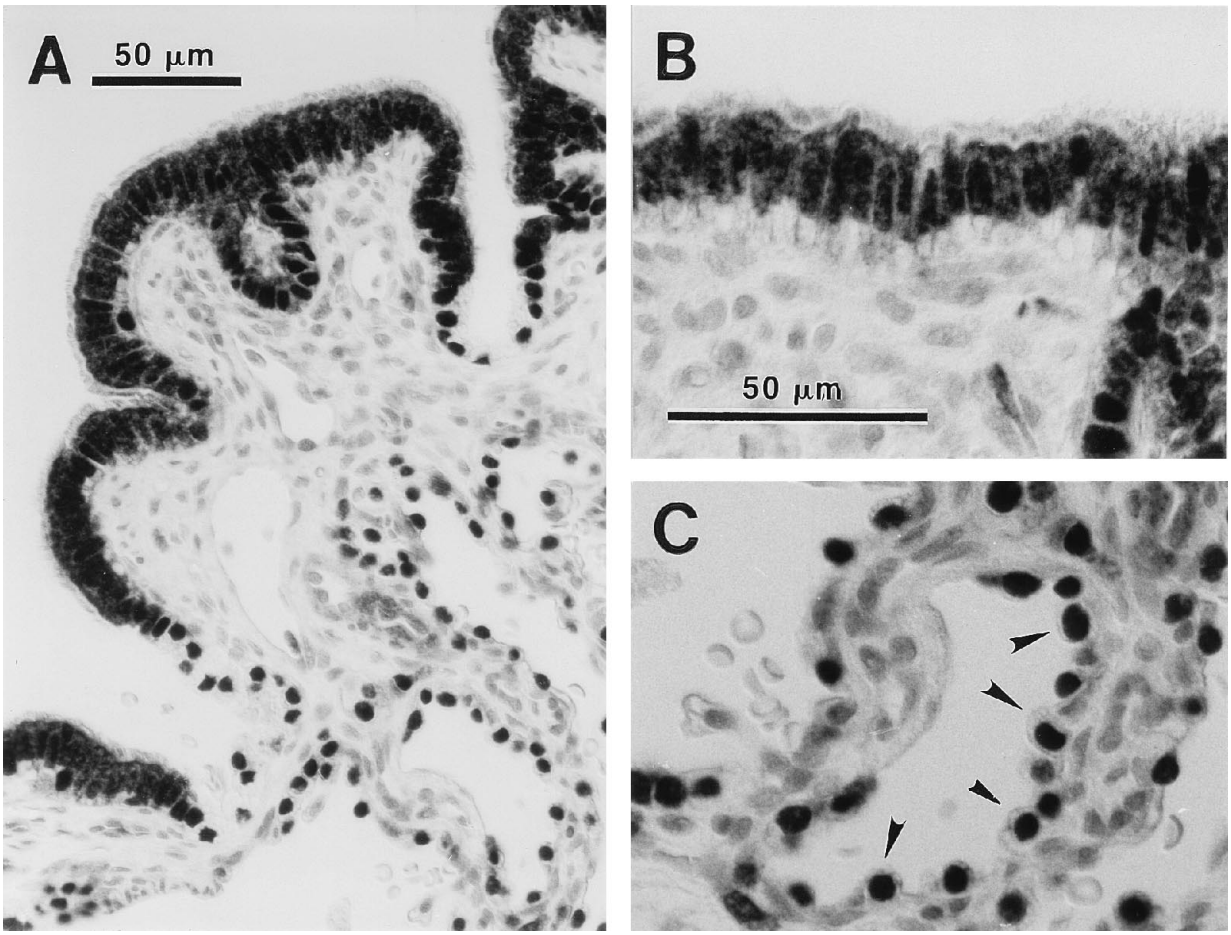

FIG. 12. Immunohistochemical localization of HNF-3β in human neonatal lung. (A) HNF-3β was localized to the nuclei of epithelial cells located in both bronchiolar and alveolar epithelia. (B) In the bronchiolar epithelium, HNF-3β was detected in the nuclei of both ciliated and nonciliated epithelial cells. (C) In the alveolar epithelium, HNF-3β was restricted to nuclei of type II pneumocytes (arrowheads). The bar in panel B applies also to panel C.

members may influence both TTF-1 and surfactant protein gene expression.

The patterns of expression of HNF-3α and -β in the lung are consistent with their potential role in the regulation of TTF-1, surfactant protein, and CCSP gene transcription and, in combination, may provide a mechanism by which surfactant protein or CCSP expression is regulated in distinct subsets of the respiratory epithelial cells. The finding that HNF-3α and HFH-8 inhibited HNF-3β activation of TTF-1 gene transcription provides yet another mechanism influencing gene expression in the respiratory epithelium. Variations in the cellular sites and levels of TTF-1 or HNF-3/forkhead family members and their distinct interactions with transcriptional elements that are unique to their target genes may regulate gene expression in subsets of respiratory epithelial cells.

Site-specific mutagenesis of the HNF-3 binding sites, region 1 and region 2, in the TTF-1 gene decreased but did not completely inhibit HNF-3β-dependent TTF-1 transcription. EMSA showed that site-specific mutations in these elements completely blocked their binding to nuclear extracts from MLE cells. Similar mutations in HNF-3 binding sites in the SP-B gene inhibited activation of the SP-B gene promoter (SPBf2 region) by HNF-3 (4). The residual effects of HNF-3β on the TTF-1 element containing mutations in both HNF-3 sites may represent activity mediated by yet unrecognized HNF-3/forkhead family binding sites in the TTF-1 construct or may be mediated by indirect effects of HNF-3β. The finding that HNF-3α and HFH-8 inhibited HNF-3β-dependent expression provides a mechanism by which the local concentrations or activities of these or other HNF-3/forkhead family members may alter TTF-1 synthesis, thereby regulating expression of downstream TTF-1 targets in the respiratory epithelium.

Axial patterning of the foregut endoderm occurs on postconception days 5 to 6 in the embryonic mouse. Differentiation of progenitor cells of endodermal origin ultimately form the foregut organs that express HNF-3α and -β early in embryogenesis. The primary role of the HNF-3/forkhead family members in formation of endodermal derivatives is supported by the temporal-spatial distribution of the HNF-3α and -β in the early embryo (32). Genetic ablation of HNF-3β by gene targeting resulted in the loss of foregut endoderm formation and disrupted anterior-posterior organization of the mouse embryo, supporting a critical role for HNF-3β in the endoderm. Subsequent foregut endodermal cell differentiation is associated with expression of organ-selective transcription factors that are temporally and spatially restricted along the foregut axis. In embryonic thyroid development, PAX-8 and TTF-1 genes are coexpressed in the early thyroid epithelium and play an important role in both thyroid gland differentiation and thyroid-specific gene expression (42). In contrast, TTF-1, HNF-3α, and HNF-3β are coexpressed in the embryonic lung bud, perhaps

providing signals by which foregut endodermal derivatives are distinguished to form distinct organs derived from the foregut. These same transcription factors are cooperatively involved in the regulation of tissue-specific genes later in development. For example, PAX-8 and TTF-1 regulate thyroperoxidase and thyroglobulin mRNAs in thyroid epithelial cells. TTF-1 or HNF-3/forkhead family members also regulate the transcription of SP-A, SP-B, and SP-C as well as CCSP. These latter genes are each expressed in overlapping subsets of respiratory epithelial cells in the developing and mature lung. The present finding that the HNF-3/forkhead family members activate the transcription of the TTF-1 gene promoter in lung epithelial cells provides support for the concept that HNF-3/forkhead family members may play a role in gene expression in the respiratory epithelium by regulating TTF-1 transcription.

In summary, HNF-3β, HNF-3α, and TTF-1, but not HFH-8, are coexpressed in immortalized respiratory epithelial cells and in the respiratory epithelial cells of the human lung. HNF-3β and HNF-3α, but not HFH-8, activated the transcription of the TTF-1 gene in lung epithelial cells in vitro. HNF-3/forkhead family members may therefore influence transcription both of TTF-1 and surfactant protein genes, the latter being critical for the formation of surfactant required for postnatal adaptation to air breathing.

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