MOLECULAR AND CELLULAR BIOLOGY, Dec. 2003, p. 9014–9024 Vol. 23, No. 24
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.24.9014–9024.2003

Nuclear Factor I/Thyroid Transcription Factor 1 Interactions Modulate Surfactant Protein C Transcription

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Received 19 May 2003/Returned for modification 21 July 2003/Accepted 18 September 2003

Surfactant protein C (SP-C; *Sftpc*) gene expression is restricted to pulmonary type II epithelial cells. The proximal SP-C promoter region contains critical binding sites for nuclear factor I (NFI) and thyroid transcription factor 1 (TTF-1; also called Nkx2.1). To test the hypothesis that NFI isoforms interact with TTF-1 to differentially regulate SP-C transcription, we performed transient transfection assays in JEG-3 cells, a choriocarcinoma cell line with negligible endogenous NFI or TTF-1 activity. Cotransfection of NFI family members with TTF-1 induced synergistic activation of the SP-C promoter that was further enhanced by p300. TTF-1 directly interacts with the conserved DNA binding and dimerization domain of all NFI family members in coimmunoprecipitation and mammalian two-hybrid experiments. To determine whether SP-C expression is regulated by NFI in vivo, a chimeric fusion protein containing the DNA binding and dimerization domain of NFI-A and the *Drosophila* engrailed transcriptional repression domain (NFIen) was conditionally expressed in mice under control of a doxycycline-inducible transgene. Induction of NFIen in a subset of type II cells inhibited SP-C gene expression without affecting expression of TTF-1 in doxycycline-treated double-transgenic mice. Taken together, these findings support the hypothesis that NFI family members interact with TTF-1 to regulate type II cell function.

Surfactant protein C (SP-C; mouse genomic designation, *Sftpc*) is a hydrophobic peptide that enhances the spreading and stability of surfactant phospholipids at the air-liquid interface during the respiratory cycle in the lung. Expression of the *Sftpc* gene is restricted to the distal respiratory epithelium during branching morphogenesis of the fetal lung and to alveolar type II (TII) epithelial cells in the postnatal lung. TII cell-specific regulatory elements were identified within −215 bp of the human SP-C promoter in transgenic mice (14). This region of the proximal promoter is highly homologous between humans and mice, containing conserved binding sites for nuclear factor I (NFI) (3) and thyroid transcription factor 1 (TTF-1; also termed T-EBP and Nkx2.1; mouse genomic designation, *Titf1*) (20). Mutagenesis of the NFI sites greatly diminished SP-C promoter activity in transient transfection experiments in mouse lung epithelial cells and blocked NFI-A-mediated activation of the SP-C promoter in HeLa cells (3). Whether NFI affects SP-C promoter activity in vivo is not known.

Isoforms of NFI arise from differential splicing of the gene products from the four NFI genes, *Nfia*, *Nfib*, *Nfic*, and *Nfix*. Although NFI is sometimes thought to be a ubiquitous transcription factor, these genes are expressed in specific overlapping patterns during fetal development and at lower levels in specific tissues in the adult (10). The relative expression levels of the four NFI genes vary with cell type, cell cycle, and differentiation status (16). Mice homozygous for deletion of *Nfib* die at birth from respiratory distress due to a block in lung maturation, suggesting that this NFI family member is required for late-fetal or perinatal lung development (18).

NFI family members are highly homologous in the amino-terminal DNA binding and dimerization domain but are divergent in the carboxyl-terminal transactivation-repression domain (1, 24; reviewed in reference 17). Further diversity of the transactivation domain is accomplished by alternative splicing that creates regions of variable proline richness. The significance of the proline-rich areas is not understood but they may form sites of protein-protein interaction. To test the hypothesis that changes in the repertoire of NFI family members directly modulate transcription of the SP-C gene, we measured promoter activation by selected isoforms of each of the NFI genes, alone and in combination with one another and TTF-1.

TTF-1 is a homeodomain-containing transcription factor that regulates morphogenesis and differential gene expression in the lung, thyroid, and ventral forebrain. Mice lacking TTF-1 protein do not undergo proper lung or thyroid differentiation and die at birth from respiratory distress (21). TTF-1 is expressed in the pulmonary epithelium during development and regulates the expression of the surfactant protein genes (reviewed in reference 42). TTF-1 interacts with retinoic acid receptor alpha (RARα) and TIF2 by mammalian two-hybrid analysis and synergistically interacts with RARα, SRC-1, TIF2, ACTR, CBP, and STAT3 to stimulate SP-B (31, 46, 44) and with CBP/p300 and SRC-1 to stimulate SP-A (47) promoter activity. TTF-1 was recently shown to directly interact with GATA-6 in the activation of SP-C transcription (28). This study was designed to test whether NFI family members interact with TTF-1 to regulate mouse SP-C gene transcription in vitro and in vivo. We now show that cotransfection of all NFI

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family members with TTF-1 causes synergistic activation of SP-C promoter activity, but to different extents. Mammalian two-hybrid and coimmunoprecipitation analysis demonstrated that TTF-1 interacts with all NFI family members by binding to the conserved DNA binding and dimerization domain. Additionally, doxycycline (Dox)-induced expression of a dominant-negative NFI-engrailed chimeric protein inhibited SP-C expression in double-transgenic mice, suggesting that SP-C is an NFI-regulated gene in vivo.

MATERIALS AND METHODS

Plasmid construction. The proximal murine SP-C promoter sequences from p0.32SP-C (20) were subcloned into the pGL3 vector (Promega), and p0.32SPC or pGL3SPCluc was used to assay SP-C promoter activity as indicated. SP-C promoter sequences containing point mutations were also subcloned into pGL3. pSP-C-NFI(−) contains mutations in all four NFI sites (3). In pSPC-TTF-1(−), both adjacent TTF-1 sites (underlined) were mutated (lowercase) in footprint C2 (−184 GGCCActGGCagTGGGG −168) by PCR-mediated site-directed mutagenesis.

To facilitate subcloning, a polylinker site was inserted into pBET, a chicken beta actin promoter-driven expression vector (19), replacing the *Bgl*II-to-*Sal*I sequence with a *Bgl*II-*Not*I-*Hin*dIII-*Sal*I polylinker to create pBETpl. Mouse NFI isoform cDNAs containing an amino-terminal hemagglutinin (HA) epitope were subcloned from pCHM-NFI-A1, -B2, -C2, and -X2 (11) as *Not*I-*Hin*dIII fragments into pBETpl and as *Hpa*I-*Hpa*I fragments into *Eco*RV-digested pPAC5Cpl, an expression vector containing the *Drosophila* beta actin promoter (23). To generate an HA-tagged carboxyl-terminal truncation mutant of NFI-A, pCHM-NFIA1 was digested with *Not*I and *Bst*XI and the 978-bp fragment encoding amino acids 1 to 308 was subcloned into pPAC5Cpl to create pPAC-HANFI-A-5′. The cytomegalovirus promoter-driven rat TTF-1 expression plasmid was previously described (5, 20).

For mammalian two-hybrid analysis, NFI and TTF-1 cDNAs were subcloned into the pM and pVP16 vectors (Clontech, Palo Alto, Calif.). These constructs direct the expression of NFI or TTF-1 fusion proteins containing an amino-terminal GAL4 DNA binding domain (dbd) from the pM vector or a VP16 transactivation domain (tad) from the pVP16 vector. The NFI constructs were designated either pM or pVP16 followed by NFI-A1, for the full-length coding sequence of mouse NFI-A1 from pBETNFIb1f (19); NFI-A-5′dbd for the DNA binding and dimerization domain of NFI-A (amino acids 1 to 308); and NFI-A1-3′tad for the NFI-A1 transactivation domain (amino acids 296 to 509). Construction of pMTTF-1 was previously described (31). The TTF-1 coding sequence was subcloned into pVP16 as an *Eco*RI fragment.

The dominant-negative NFI chimeric protein (NFIen) was constructed by fusing the amino-terminal HA-tagged DNA binding and dimerization domain (amino acids 1 to 296) of mouse NFI-A (11), via an eight-amino-acid linker, to the *Drosophila* engrailed transcriptional repression domain (amino acids 1 to 298) (8). This fusion protein was placed under control of the $(TetO)_7$CMV minimal promoter (15, 39), and the 3′ untranslated sequence and polyadenylation signal from the bovine growth hormone gene were added to generate a stable mRNA. All constructs were verified by sequencing. An expression vector containing the reverse tetracycline transactivator, pUHG17-1, was obtained from Hermann Bujard (University of Heidelberg).

Cell culture, transfection, and reporter gene assays. Human JEG-3 choriocarcinoma cells were maintained in minimum essential medium (MEM; Gibco) with 10% fetal bovine serum (FBS). JEG-3 cells, which do not express endogenous TTF-1 (data not shown) and have very low levels of endogenous NFI (7, 11), were used for the functional analysis of SP-C promoter reporter constructs by transient transfection. Cells were transfected by the calcium phosphate coprecipitation method, with modifications (3). Briefly, 6-well plates of JEG-3 cells at 50 to 60% confluence were transfected with 2 μg of SP-C luciferase plasmid, the indicated amounts of NFI or TTF-1 expression constructs, and 0.25 μg of pCMV-βgal per well in 2 ml of Dulbecco's modified Eagle's medium with 10% FBS. After 18 h, the precipitate was removed and the cells were fed MEM with 10% FBS. Two days after transfection, the cells were washed with phosphate-buffered saline, lysed in 150 μl of 1× reporter lysis buffer (Promega) per well, and frozen at −20°C. Luciferase and beta-galactosidase (β-Gal) assays were performed with 10 μl of the cleared lysates as described previously (3). Luciferase activity was normalized for β-Gal activity, and the relative activity of the p0.32SP-C promoter plus empty vector(s) was set to 1. All data for cotransfection reactions were reported as activities relative to the control. Transfections were performed in duplicate and the data were plotted as means ± standard errors (SE) for at least three independent experiments ($n = 6$) unless stated otherwise in figure legends.

Drosophila melanogaster Schneider line 2 (SL2) cells were maintained in Schneider's *Drosophila* medium (Gibco) with 10% FBS at 27°C in ambient air. SL2 cells were transiently transfected by the calcium phosphate coprecipitation method in 100-ml tissue culture dishes, with 20 μg of a *Drosophila* expression vector, pPAC5cpl, per dish driving the expression of the indicated HA-tagged mouse NFI family member or rat TTF-1. Two days after transfection, cells were washed and collected for preparation of nuclear extracts.

Coimmunoprecipitation and Western blot analysis. *Drosophila* SL2 cell nuclear extracts containing individual HA-tagged NFI family members or TTF-1 were prepared by a miniextract procedure essentially as previously described (6). The indicated nuclear extracts were incubated with polyclonal anti-HA antibody and protein A/G-agarose beads in IP buffer (20 mM TRIS [pH 7.6], 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg of pepstatin A per ml, 10 μg of leupeptin per ml, 30 μg of bacitracin per ml, 1 mM *o*-phenanthroline) with rotation at 4°C overnight. The beads were washed extensively with IP buffer without Triton X-100 and were boiled in sodium dodecyl sulfate (SDS) loading dye. The coimmunoprecipitated proteins were separated by electrophoresis under reducing conditions on SDS–10 to 20% polyacrylamide gradient gels (Novex, San Diego, Calif.) in Tris-glycine buffer and were then transferred to nylon membranes. Western blots were blocked with 5% nonfat dry milk in TBST (10 mM Tris [pH 8], 150 mM NaCl, 0.1% Tween 20) and incubated with anti-TTF-1 monoclonal antibody (a kind gift from J. A. Whitsett) followed by horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Calbiochem, La Jolla, Calif.). Western blots were developed with the enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, Ill.) and exposed to X-OMAT film (Kodak). Western blots were stripped and the procedure was repeated with a mouse monoclonal anti-HA antibody (12CA5; Roche Molecular Biochemicals) to detect the HA-tagged NFI isoforms.

Mammalian two-hybrid analysis. The reporter construct pG5LUC, containing five GAL4 DNA binding sites and the adenovirus E1b minimal promoter, was previously described (31). For two-hybrid analysis, JEG-3 cells were cotransfected with the indicated combinations of GAL4 DNA binding domain and VP16 transactivation domain chimeric plasmids, 2 μg of pG5LUC reporter construct, and 1 μg of pRSVβgal per well. Data are presented as relative luciferase activities, with the activity of pG5LUC cotransfected with the indicated pM chimeric plasmid and pVP16 empty vector set to 1. Since TTF-1 has been previously reported to self-associate (2), cotransfection of pMTTF-1 with pVP16TTF-1 was used as a positive control.

EMSA. Annealed oligonucleotides were gel purified by use of 4% Biogel and the Mermaid kit (Bio 101, Vista, Calif.) for use as electrophoretic mobility shift assay (EMSA) probes. Purified probes were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. EMSAs were performed as previously described, with slight modifications (3). Briefly, nuclear extracts from transiently transfected cells expressing similar amounts of HA-tagged NFI family members were incubated in EMSA binding buffer [20 mM Tris (pH 7.6), 50 mM KCl, 2 mM $MgCl_2$, 40 ng of poly(dI-dC) (Boehringer Mannheim) per μl, 10% glycerol, 1 mM dithiothreitol, 0.1 mM fresh phenylmethylsulfonyl fluoride] for 5 to 10 min at room temperature. End-labeled probe was added (100,000 cpm) and the mixture was incubated for an additional 10 min at room temperature. Bound and free probes were separated by nondenaturing polyacrylamide gel electrophoresis using 5% acrylamide-bisacrylamide (29:1)–2.5% glycerol gels in 0.5× Tris-borate-EDTA buffer.

Generation of double-transgenic mice. Homozygous CCSP-rtTA activator transgenic mice (39), containing the reverse tetracycline transactivator under the control of the 2.3-kb rat Clara cell secretory protein (CCSP) promoter, were kindly provided by Jeffrey Whitsett (Cincinnati Children's Hospital, Cincinnati, Ohio). Animals were housed under pathogen-free conditions according to federal and institutional guidelines. The $(TetO)_7$CMV promoter-driven dominant-negative NFI-engrailed (NFIen) construct was microinjected into F/VBN mouse oocytes by the transgenic core at CCHMC, and NFIen transgene-positive mice were bred with CCSP-rtTA transgenic activator mice. Double-transgenic mice were identified by PCR using primers specific for each transgene. Primers used for the $(TetO)_7$CMV-NFIen transgene were a 5′ primer for the CMV minimal promoter (5′-GCCATCCACGCTGTTTTG) and a 3′ primer for the *Drosophila* engrailed sequence (5′-CCAGTTGCTGTTGGTGCAAC). Primers used to detect CCSP-rtTA were a 5′ primer for the rat CCSP promoter sequence (5′-ACTGCCCATTGCCCAAACAC) and a 3′ primer in the reverse tetracycline transactivator sequence (5′-AAAATCTTGCCAGCTTTCCCC).

Double- and single-transgenic mice at 6 weeks of age were fed Dox-containing mouse chow for 3 or 8 days as previously described (34) to induce expression of the NFIen transgene in a subset of TII cells and Clara cells. Total lung RNA (10

μg/lane) was separated in 1% agarose–formaldehyde gels and Northern blot analysis was performed as described previously (4), using cDNA probes for mouse SP-C, TTF-1, ribosomal protein L32, and the *Drosophila* engrailed domain to detect NFIen transgene expression.

ISH and IHC. Lungs were inflation fixed with 4% paraformaldehyde at 25-cm water pressure and were processed for paraffin embedding. Serial 5-μm sections were prepared for in situ hybridization (ISH) and immunohistochemistry (IHC). ISH was performed by using [α-^{35}S]UTP-labeled riboprobes for sense and antisense mouse SP-C cDNA under high-stringency conditions essentially as previously described (41). Slides were dipped in Kodak NTB2 emulsion, exposed for 3 to 4 days, and developed with Kodak D19 developer. Slides were imaged by dark field illumination to detect the ISH signal and by phase-contrast microscopy to detect the underlying structures.

To detect NFIen transgene expression, antigen retrieval was performed by heating of slides in the microwave oven in citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with hydrogen peroxide, and slides were incubated with polyclonal anti-HA antiserum (1:200) (HAprobe1; Santa Cruz). For immunodetection, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G and visualized with the Vectastain Elite kit. The diaminobenzamidine reaction products were enhanced with nickel cobalt and the slides were counterstained with nuclear fast red essentially as previously described (48).

Statistical analysis. Relative luciferase activity from transient transfections was analyzed by the two-tailed *t* test statistic with Statview 4.5 software (Abacus Concepts, Berkeley, Calif.). Significance was accepted when $P < 0.05$.

RESULTS

NFI family members differentially activate the SP-C promoter. We previously identified four NFI binding sites in the 0.32-kb mouse SP-C promoter (3). To determine the role of NFI family members in the transcriptional regulation of the SP-C gene, representative isoforms of each of the four NFI genes were tested in cotransfection assays with a minimal mouse SP-C promoter-luciferase expression vector. Transient transfections were performed in JEG-3 cells, which express very little endogenous NFI (11) and no TTF-1 (data not shown). Cotransfection with all NFI family members activated SP-C transcription in a dose-dependent manner, but to different extents (Fig. 1). NFI-B2 and NFI-A1 induced SP-C promoter activity 50- to 65-fold, whereas expression of equivalent amounts of NFI-C2 induced 10-fold less promoter activity. Cotransfection of NFI-C2 in combination with the other NFI family members resulted in intermediate promoter activation, consistent with the formation of heterodimers between isoforms. NFI-C2 expression inhibited transactivation by all other isoforms tested (Fig. 1).

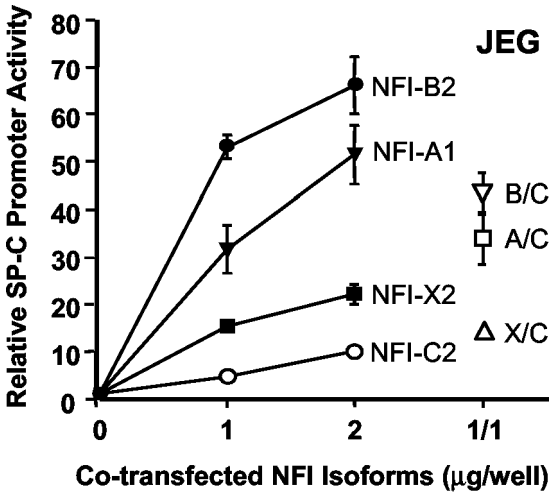

FIG. 1. Coexpression of NFI isoforms in JEG cells modulates SP-C promoter activity. JEG-3 cells were transiently transfected in 6-well plates with 2 μg of p0.32SPCluc, the indicated amounts of expression plasmids for NFI family members, and 0.5 μg of pCMV-βgal/well. Relative luciferase activity was determined by correcting for β-Gal activity and then setting the activity of p0.32SPCluc cotransfected with empty vectors to 1. Data are shown as means ± SE ($n = 9$; three independent experiments). All cotransfections with other NFI family members were significantly different from that with NFI-C alone ($P < 0.01$).

NFI family member interaction with NFI half-sites from the SP-C promoter. The above results suggest that NFI-C2 is a weak activator and NFI-B2 is a strong activator of SP-C promoter activity. To determine whether NFI family members have different relative binding affinities for the SP-C promoter NFI half-sites (3) (diagrammed in Fig. 2B), nuclear extracts were prepared from JEG-3 cells transiently transfected with individual HA-tagged NFI family members. All family members were expressed at similar levels, as detected by anti-HA Western blot analysis (Fig. 2A). NFI family members range in apparent molecular mass from ~47 kDa for NFI-B2 to ~56 kDa for NFI-A1. Consistent with previously published data (16), all NFI family members interacted with the palindromic NFI binding site with similar affinities (Fig. 2B, compare lanes 1, 4, 7, 10, and 13). However, NFI family members bound NFI half-site probes from the C1 and C3 footprinted regions of the SP-C promoter with variable lesser affinities (Fig. 2B, lanes 2, 5, 8, 11, and 14 [probe C1] and lanes 3, 6, 9, 12, and 15 [probe C3]). NFI-C2 had the strongest relative binding to the NFI half-site probes in titration experiments (data not shown), suggesting that binding affinity does not correlate precisely with transcriptional activation (see Discussion).

Cooperativity between TTF-1 and NFI family members in regulation of SP-C gene expression. We and others previously showed that TTF-1, a homeodomain transcription factor expressed in the pulmonary epithelium, is required for SP-C transcription (20, 27). To determine whether NFI family members influence TTF-1-mediated SP-C promoter activation, JEG-3 cells were cotransfected with individual NFI family members with or without TTF-1. As shown in Fig. 3, all NFI family members increased SP-C promoter transactivation by TTF-1. NFI-C2 was least effective, primarily due to its modest effect on SP-C promoter activity alone. Cotransfection of NFI-B2 or -A1 with TTF-1 induced SP-C promoter activity to high levels, but the cooperative effects of cotransfection of all NFI family members with TTF-1 were similar. The synergistic activation of SP-C promoter activity by TTF-1 and all NFI family members suggests that all family members interact with TTF-1.

All NFI family members interact with TTF-1. Direct protein-protein interactions between TTF-1 and NFI isoforms were tested in vitro by coimmunoprecipitation of mixtures of nuclear extracts from transiently transfected SL2 cells expressing HA-tagged mouse NFI family members and rat TTF-1. Mixtures were incubated with anti-HA antiserum and protein G-Sepharose beads, and the immunoprecipitated proteins were detected by Western blotting using monoclonal antibodies against TTF-1 and HA. In initial studies, TTF-1 was specifically coimmunoprecipitated with both NFI-A1 and NFI-C2 (Fig. 4A).

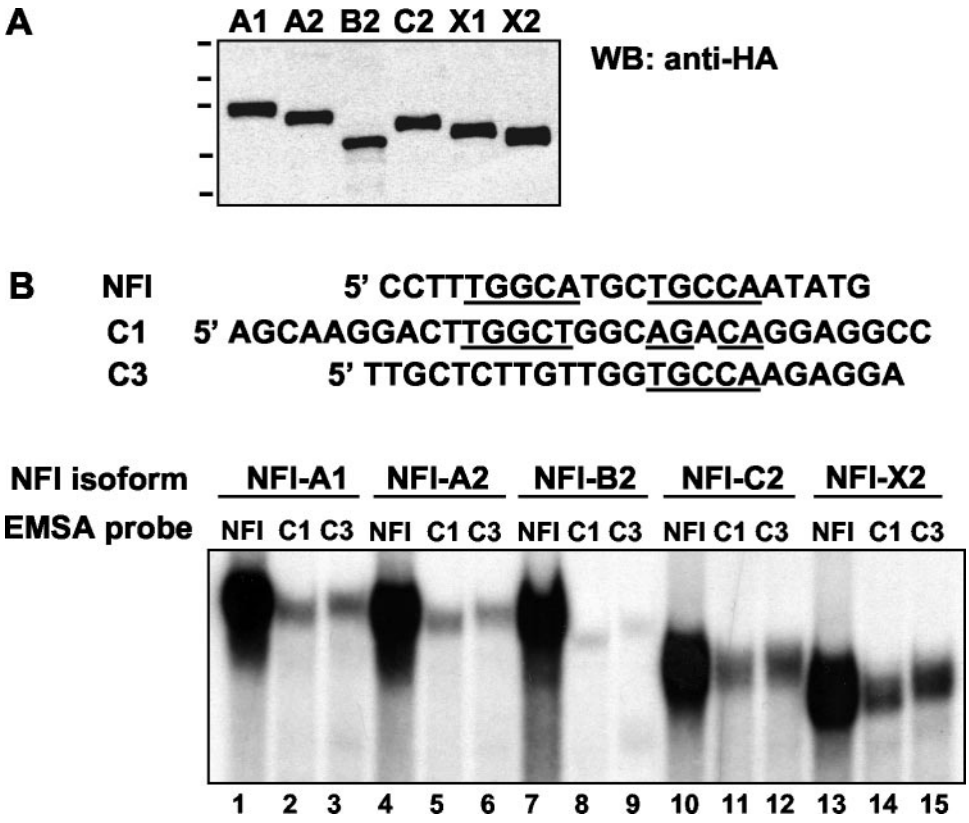

FIG. 2. Homodimers of all NFI isoforms bind similarly to a palindromic NFI site probe (NFI) but with varying weaker affinities to C1 and C3 NFI half-site probes from the SP-C promoter. (A) Western blot analysis of nuclear extracts from JEG cells transiently transfected with HA-tagged NFI family members detected by anti-HA monoclonal antibody. (B) EMSA analysis of selected nuclear extracts from panel A. Extracts were incubated with the indicated ^{32}P-labeled double-stranded oligonucleotides and electrophoresed on nondenaturing gels. The sequence of each EMSA probe is shown, and the NFI consensus binding sites are underlined.

Since the amino-terminal DNA binding and dimerization domain is highly conserved among all NFI family members, we hypothesized that TTF-1 may interact with this domain. To test this hypothesis, we subcloned representative full-length HA-tagged NFI family members or the region of the HA-tagged NFI-A cDNA encoding amino acids 1 through 330 into the *Drosophila* expression vector pPAC5cpl and used nuclear extracts from SL2 cells transfected with each construct for coimmunoprecipitation. TTF-1 was coimmunoprecipitated with all NFI isoforms tested and with the truncation mutant containing only the amino-terminal DNA binding and dimerization domain of NFI-A, suggesting that TTF-1 interacts with this conserved amino-terminal region (Fig. 4B).

NFI–TTF-1 protein-protein interactions were confirmed by use of the mammalian two-hybrid system. Fusion protein expression vectors were constructed containing the putative interaction domains fused to the GAL4 DNA binding domain (dbd) or the VP16 transactivation domain (tad). Protein-protein interactions between fusion partners induce promoter activity from a cotransfected luciferase expression vector containing multiple copies of the GAL4 DNA binding site adjacent to a minimal promoter (diagrammed in Fig. 5). Relative luciferase activity was compared to control transfections with the respective GALdbd fusion protein and VP16tad alone. The full-length TTF-1 and NFI-A1 coding sequences were fused to the GAL4dbd and VP16tad domains and the NFIA-dbd and -tad domains were fused to VP16tad. Since TTF-1 has been shown to self-associate in vitro (2, 38), we used cotransfection with GAL4TTF-1 and VP16TTF-1 as a positive control for protein-protein interactions (Fig. 5). NFI-A–TTF-1 cotransfection reactions induced luciferase activity to levels similar to those of the positive controls for both fusion partner combinations tested. Consistent with the coimmunoprecipitation assays, the conserved NFIA-dbd showed binding activity to TTF-1, whereas the NFIA-tad did not.

Cooperative interactions of NFI–TTF-1–p300 drive SP-C promoter activity. TTF-1 has been shown to interact with other transcription factors, including RAR, and transactivating factors, including CBP/p300 to activate SP-B transcription. Since CBP/p300 can act as a scaffolding protein to bring together transcription factors and basal transcriptional machinery to promote transcriptional activity and may be limiting in tissue culture cells, NFI and TTF-1 were cotransfected in the presence or absence of an expression vector for p300. Cotransfection with p300 had no effect on basal SP-C promoter activity or transactivation by NFI-A1 alone and had a minimal effect on TTF-1 transactivation. However, cotransfection with p300 enhanced the synergistic interaction of NFI and TTF-1 on SP-C promoter activity (Fig. 6, lower panel). Mutation of all NFI binding sites in footprinted regions C1, C3, C4, and C5 in

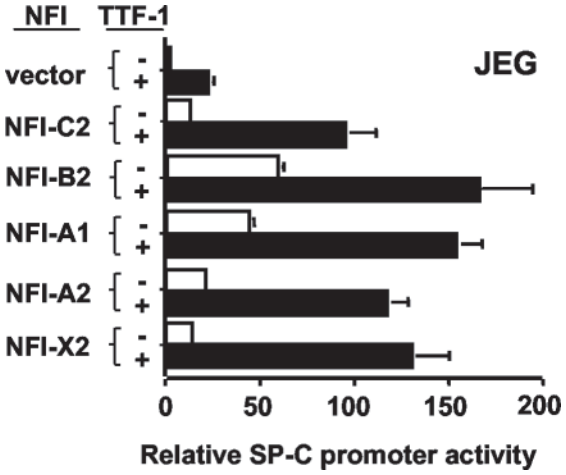

FIG. 3. Synergistic SP-C promoter activation by TTF-1 and NFI family members. JEG cells were cotransfected with 1.5 μg of p0.32SPCluc per well, 1 μg of the indicated HA-tagged NFI isoform per well, and 0.5 μg of either pCMVTTF-1 (+) or pCDNA3 empty vector (−) per well. Relative SP-C promoter activity was calculated as described for Fig. 1. Data are plotted as means ± SE (n = 4 to 6).

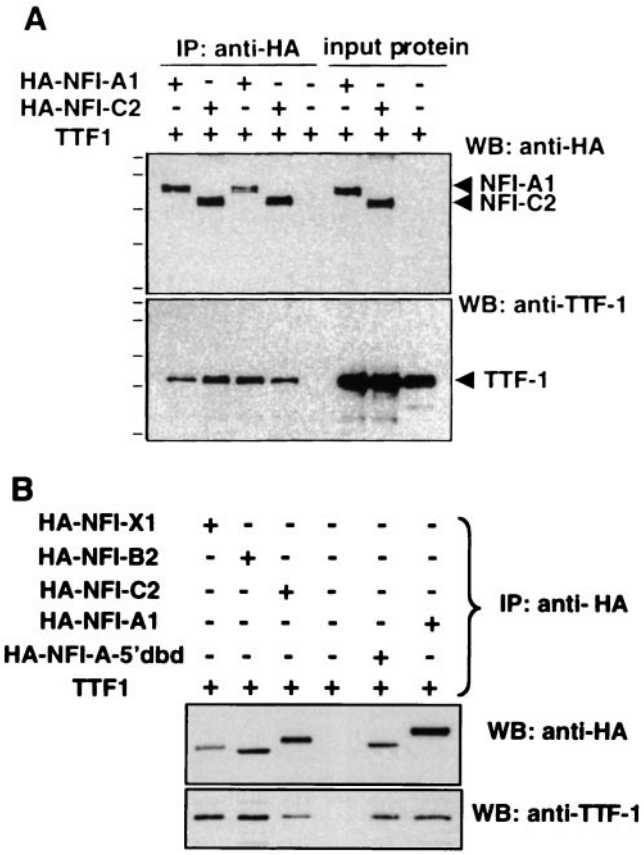

FIG. 4. Coimmunoprecipitation of NFI–TTF-1 complexes. (A) Nuclear extracts of SL2 cells transfected with HA-tagged NFI-A1 or NFI-C2 were incubated as indicated with extracts of SL2 cells transfected with TTF-1, and complexes were immunoprecipitated (IP) with polyclonal anti-HA antiserum and protein G-agarose. Immunoprecipitated proteins or equivalent amounts of input protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to Hybond membranes, and sequentially probed with monoclonal antibodies against HA and TTF-1. (B) Nuclear extracts of SL2 cells transfected with the indicated NFI isoforms or a truncation mutant of NFI-A containing the DNA binding and dimerization domain (dbd) was incubated with TTF-1, and complexes were collected and visualized as for panel A.

pSPC-NFI(−) (Fig. 6, middle panel) or of the TTF-1 binding sites in footprinted region C2 in pSPC-TTF-1(−) (Fig. 6, upper panel) interfered with the cooperative transactivation of SP-C promoter activity by NFI, TTF-1, and p300. These data suggest that direct DNA binding of both transcription factors is required for cooperative interactions with p300 to maximally activate transcription of the SP-C promoter.

NFIen inhibits SP-C promoter activity in vitro. To determine whether the SP-C gene is an NFI-regulated gene in vivo, we constructed a dominant-negative NFI fusion protein under the control of an inducible promoter. This chimeric gene was made by fusing the HA-tagged N-terminal DNA binding and dimerization domain of NFI-A (residues 1 to 296) to the well-characterized repression domain of the *Drosophila* engrailed protein (residues 1 to 298) via an eight-amino-acid linker (diagrammed in Fig. 7A). Expression was made inducible by Dox by using a minimal CMV promoter linked to multiple tetracycline operator sites. This promoter is active only in the presence of Dox-activated rtTA and has been used for the Dox-regulated expression of transgenes in both tissue culture cells and transgenic mice (15, 22, 39).

To test the efficacy of this dominant-negative NFI construct, p$(TetO)_7$CMV-HA-*nfi-a-engrailed* was transfected, along with a vector expressing rtTA and vectors driving constitutive expression of wild-type NFI proteins, into JEG-3 cells. As shown in Fig. 1, the SP-C promoter had little activity in JEG cells in the absence of coexpressed NFI proteins and Dox-induced expression of NFIen had no effect (Fig. 7B, vector). In the absence of Dox, NFI-A1 and NFI-B2 strongly activated the SP-C promoter while NFI-C2 and NFI-X2 activated the promoter only weakly (Fig. 7B, white bars). Dox treatment repressed the NFI-activated expression of the SP-C promoter three- to fourfold (Fig. 7B, gray bars). No repression was seen in the presence of the *Drosophila* engrailed domain alone or in the absence of NFIen (C. J. Bachurski and G.-H. Yang, unpublished data). These data indicate that the dominant-negative NFIen chimeric protein can repress transcriptional activation by all four NFI family members.

The SP-C gene is an NFI-regulated gene in vivo. To examine the functional consequences of NFIen expression in the pulmonary epithelium, we generated double-transgenic mice using the lung epithelial cell-specific Dox-inducible system (34) (Fig. 8A). Single-transgenic "operator" mice were generated that contained the $(TetO)_7$-CMV HA-*Nfi-a-engrailed* transgene. These single-transgenic mice do not express NFIen and have no detectable phenotype. When crossed with transgenic activator mice expressing rtTA protein in the pulmonary epithelium, double-transgenic progeny express NFIen in a tissue-specific, Dox-regulated manner. We used CCSP-rtTA activator mice from J. A. Whitsett (Cincinnati Children's Hospital) in which the rat CCSP (mouse genome designation *Scgb1a1*) promoter drives rtTA expression in a subset of bronchiolar Clara cells and alveolar TII cells. Double-transgenic mice harboring

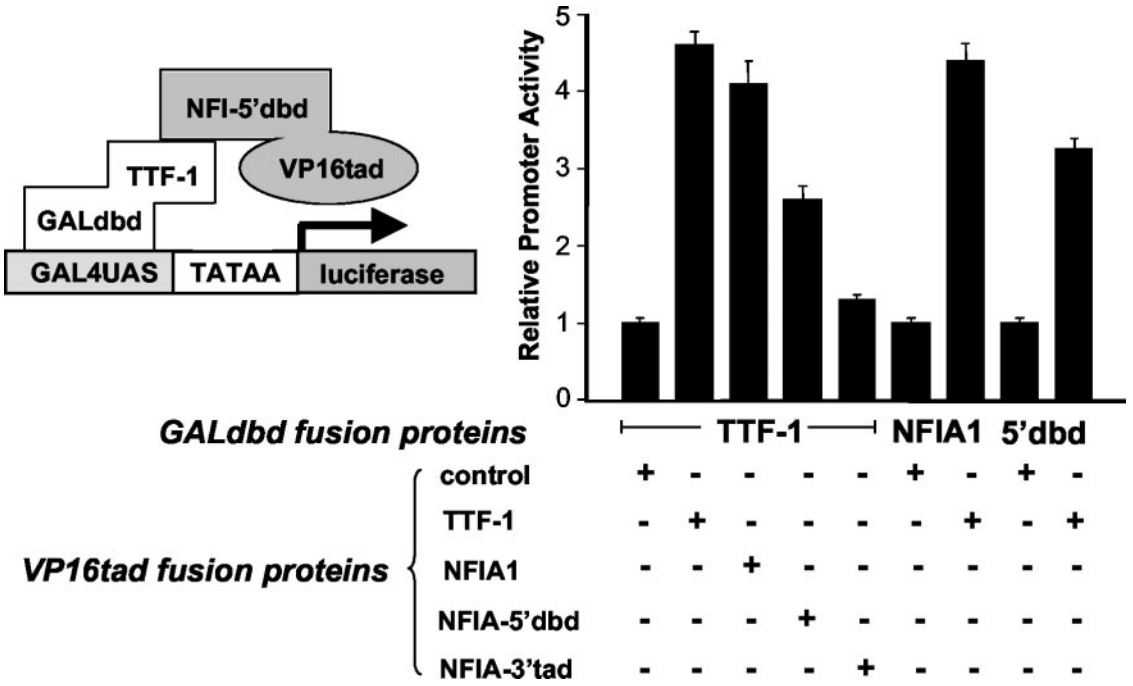

FIG. 5. NFI and TTF-1 fusion proteins interact via the conserved 5′ DNA binding and dimerization domain (dbd) of NFI. JEG cells were cotransfected with the indicated combinations of vectors expressing fusion proteins of the GAL dbd and the VP16 transactivation domain (tad), a luciferase reporter plasmid driven by the GAL4 upstream activator sequences, and pCMVβgal as an internal control. Relative luciferase activity was calculated as described for Fig. 1 except that luciferase activity stimulated by each GALdbd fusion protein (full-length TTF-1, NFI-A1, or NFI-A5′dbd) cotransfected with the VP16tad vector pM (control) was set to 1. Data are plotted as means ± SE ($n = 6$).

CCSP-rtTA and $(TetO)_7$CMV-NFIen were fed Dox for 3 or 8 days to induce NFIen expression in the pulmonary epithelium. NFIen expression was not detected in untreated double-transgenic mice by Northern blot analysis of 10 μg of total lung RNA (Fig. 8B). Dox treatment induced expression of NFIen at both time points tested and decreased SP-C mRNA by approximately 55%. TTF-1 mRNA expression was not affected by Dox treatment.

To confirm the cellular sites of NFIen transgene expression and its effect on SP-C gene expression, NFIen was detected by IHC and SP-C mRNA was detected by ISH of serial sections of lungs from double-transgenic mice fed Dox for 8 days. HA-tagged NFIen expression was detected by anti-HA IHC. Consistent with the Northern blot analysis, NFIen was not detected in untreated double-transgenic mice (Fig. 9A). NFIen was detected in the nuclei of a subset of bronchiolar (data not shown) and alveolar epithelial cells of double-transgenic mice fed Dox for 8 days (Fig. 9E). Transgene expression was variable: in some areas only a few TII cells were positive, whereas in other areas most TII cells and many bronchiolar epithelial cells were positive. The Dox-induced NFIen expression pattern was consistent with that of other transgenes activated by the CCSP-rtTA–Dox system (34, 39).

TTF-1 is specifically expressed in pulmonary TII cells and is expressed at lower levels in Clara cells in the adult mouse lung (reviewed in reference 42). TTF-1 IHC was performed on serial sections as a marker for TII cells and to verify that TTF-1 expression was not affected by NFIen. TTF-1-expressing TII cells were readily detected in both treatment groups (Fig. 9B and F). ISH analysis of serial sections showed the normal pattern of TII cell expression of SP-C mRNA in control double-transgenic mice. Mice fed Dox had decreased expression of SP-C mRNA in regions of lung parenchyma expressing NFIen (Fig. 9, compare panels A and C to panels E and G). Phase-contrast images (Fig. 9D and H) reveal the underlying structure of the same regions as those shown in Fig. 9C and G). Taken together, these data show that NFIen inhibits SP-C expression both in vitro and in vivo.

DISCUSSION

These results provide evidence that NFI directly interacts with TTF-1 and that these interactions mediate SP-C gene transcription. Using a dominant-negative approach, we have shown that the SP-C gene is an NFI-regulated gene in vivo. The inducible NFIen double-transgenic mouse system will be a useful model to define the role of NFI in lung development and regulation of genes required for lung epithelial cell differentiation.

All NFI family members are expressed in the lung (10). In the developing mouse lung, NFI family members are expressed in both epithelium and mesenchyme (10; C. Bachurski, unpublished observations), whereas TTF-1 is restricted to the epithelium (48). We previously showed by transient transfection analysis that NFI and TTF-1 independently regulate the minimal SP-C promoter (3, 20). This study suggests that all NFI family members can directly interact with the homeodomain transcription factor TTF-1 in the pulmonary epithelium to regulate SP-C promoter activity. Our data support the hypothesis that TTF-1 interactions with different NFI family members

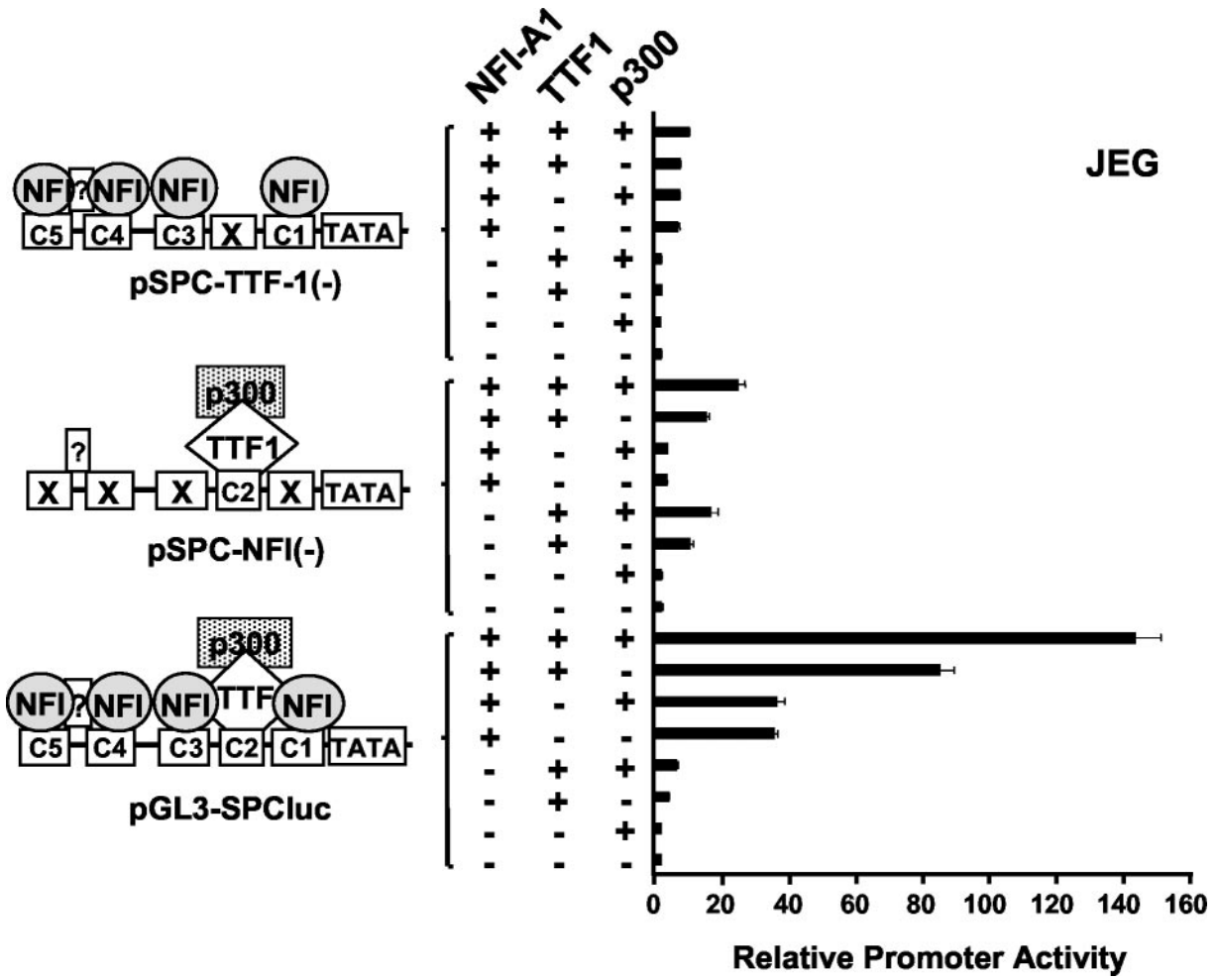

FIG. 6. Cotransfection of NFI and TTF-1 with the coactivator protein p300 caused synergistic activation of the 0.32 SP-C promoter. JEG cells were cotransfected with 1.5 μg of the SP-C luciferase construct indicated at the left and combinations of expression vectors for NFI-A1 (1 μg), TTF-1 (0.5 μg), and p300 (0.5 μg), with pCMVβgal (0.5 μg) to control for transfection efficiency. The relative activity of each luciferase construct cotransfected with the relevant empty vector was set to 1 and data were plotted as means ± SE (n = 9). Schematic representations of the transcription factor interactions with each construct are shown at the left, and mutant sites are indicated by an "X." All NFI sites are mutated in pSPC-NFI(−) and both TTF-1 sites are mutated in the C2 footprinted region of pSPC-TTF-1(−).

modulate SP-C promoter activity. TTF-1 interaction with NFI-A1 or -B2 induced SP-C promoter activity to high levels, whereas NFI-C2 was least effective (Fig. 2).

NFI-B2, the strongest transactivator of SP-C transcription, had the lowest apparent binding affinity and consistently produced a slower mobility complex in EMSA, even though it is the smallest protein as assessed by SDS gel electrophoresis. The discrepancy between apparent protein size and relative mobility in EMSA could be caused either by intrinsic surface charge differences between isoforms or by covalent modifications such as phosphorylation or glycosylation. NFI family members are both glycosylated and phosphorylated (reviewed in reference 17), but the sites of these modifications have not been mapped. Domain swap experiments are being considered to map the region(s) in NFI that is involved in differential SP-C promoter activation.

The NFI proteins have a modular structure with a highly conserved amino-terminal DNA binding and dimerization domain followed by a central region and a more divergent C-terminal transactivation-repression domain. NFI family members form stable hetero- and homodimers through interactions between the conserved amino-terminal 250 to 300 amino acids (25). This region is sufficient to drive specific protein-DNA interactions of fusion proteins. We took advantage of the heterodimerization ability of the NFI DNA binding domain to create a dominant-negative NFI-engrailed domain chimeric gene. Previously, Schuur et al. reported the creation of a dominant-negative NFI truncation mutant that partially inhibited the activity of some NFI family members (36). To improve the transcriptional repression of NFI-regulated promoters, we fused the transcriptional repressor domain from the *Drosophila* engrailed gene to the NFIA-dbd. This strategy was previously used to create a dominant-negative GATA-6 transcriptional repressor expressed in the pulmonary epithelium (28, 45). These studies also showed that expression of the engrailed domain alone had no effect on lung development or epithelial maturation.

Dox-induced expression of NFIen inhibited SP-C promoter

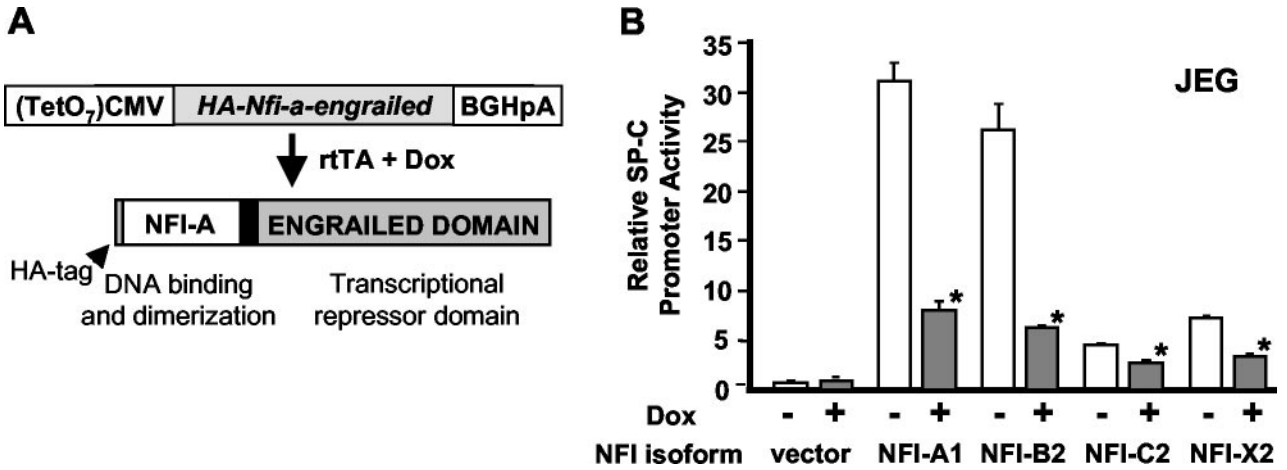

FIG. 7. Dox-induced expression of the dominant-negative NFIen chimeric protein inhibited transactivation of the SP-C promoter by coexpressed NFI family members. (A) Schematic representation of the Dox-inducible chimeric dominant-negative NFI-A–engrailed (NFIen) expression vector. The HA-tagged chimeric protein consists of the DNA binding and dimerization domain of mouse NFI-A and the transcriptional repressor domain of *Drosophila* engrailed. Cotransfection with the rtTA and treatment with 1 μg of Dox per ml induces NFIen expression. (B) JEG cells were cotransfected with p0.32SPCluc, pUHG expressing rtTA, pCMVβgal, and expression vectors for the indicated NFI family members and were incubated for 24 h in the absence (white bars) or presence (gray bars) of Dox. ∗, $P < 0.01$ versus no Dox (means ± SE; $n = 4$).

activity in transient transfection analysis and SP-C gene expression in TII cells in the adult mouse lung. Whether specific NFI family members regulate SP-C promoter activity in vivo is unknown. Three of the four NFI genes, *Nfia* (12), *Nfib* (18), and *Nfic* (37), have been targeted in mice. *Nfia* and *Nfic* gene-targeted mice have no known lung defects, whereas lung development fails to progress through the canilicular phase in *Nfib* gene-targeted mice, causing respiratory failure and perinatal death (18). Whether the pulmonary defects are caused by epithelial or mesenchymal deficiencies remains to be determined.

TTF-1 regulates lung morphogenesis and SP gene expression in TII cells of the adult lung (reviewed in reference 42). Protein-protein interactions with TTF-1 regulate lung epithe-

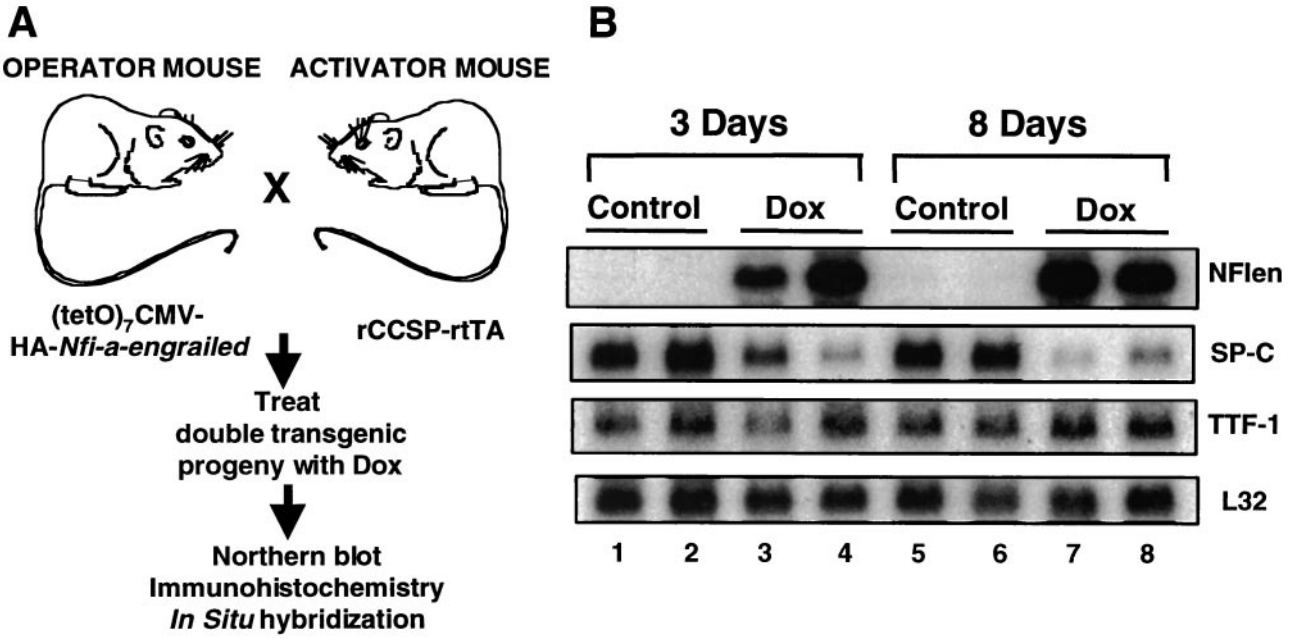

FIG. 8. Generation of double-transgenic mice. (A) Diagram of breeding scheme. Activator mice are transgenic for rtTA under control of the rat CCSP promoter, which targets expression to a subset of bronchiolar Clara cells and alveolar TII cells. Operator mice harbor the HA-Nfi-a-engrailed transgene (NFIen) under the control of the minimal CMV promoter, with seven copies of the Tet operator sequence, which is only active in the presence of rtTA bound to Dox. (B) Dox-induced expression of NFIen in double-transgenic mice inhibited SP-C mRNA but not TTF-1 mRNA. Northern blots of total lung RNA (10 μg/lane) were prepared from adult double-transgenic littermates that were untreated (lanes 1, 2, 5, and 6) or fed Dox-treated food for 3 days (lanes 3 and 4) or 8 days (lanes 7 and 8). Blots were sequentially hybridized with ^{32}P-labeled probes for the NFIen transgene, SP-C, TTF-1, and ribosomal protein L32 mRNA as a control for loading.

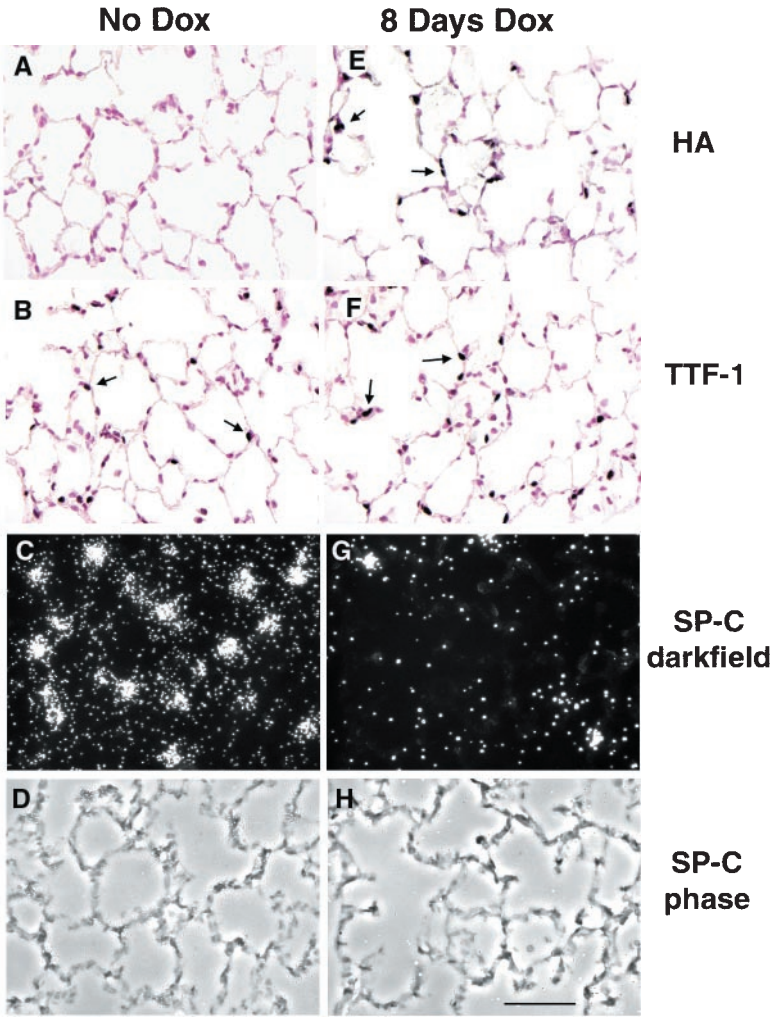

FIG. 9. Dox-induced expression of NFIen inhibited SP-C expression but not TTF-1 expression in transgene-positive regions of lung parenchyma. Adult double-transgenic mice were fed normal (A to D) or Dox-treated (E to H) food for 8 days. Anti-HA IHC was used to detect HA-tagged NFIen transgene expression. NFIen was not detected in double-transgenic mice that were fed normal mouse chow (A) but was readily detected in a subset of TII cells in double-transgenic mice that were fed Dox for 8 days (E). TTF-1 was detected in the nuclei of TII cells in both transgene-positive and transgene-negative areas (B and F). SP-C mRNA was detected by ISH on serial sections of the same regions used for IHC, viewed by dark-field (C and G) and phase-contrast (D and H) optics. Arrows indicate representative IHC-positive TII cells. Bar = 50 μm.

lial, thyroid follicular, and brain-specific gene expression. Calreticulin, a Ca^{2+} binding chaperone, binds to the homeodomain of TTF-1 and stimulates transcriptional activity (35). In the lung, TTF-1 interacts with GATA-6 to activate SP-C (28) and Wnt7b transcription (40). RAR/RXR, CBP, SRC-1, and BR22 have been shown to interact with TTF-1 to regulate SP-B transcription (43, 46). TTF-1 also interacts with two coactivator proteins, CBP and SRC-1, to activate SP-A transcription (47). We now add NFI to the list of transcription factors that interact with TTF-1 to activate lung epithelial cell-specific gene expression.

In thyroid follicular cells, TTF-1 interacts with TTF-2 and Pax8, both of which are tissue-restricted transcription factors, to activate thyroglobulin and thyroid peroxidase expression (13, 29, 33). NFI also directly interacts with TTF-2 (a forkhead domain transcription factor; also termed Foxe1) to modulate hormone-induced expression of thyroid peroxidase in thyroid cells (32). In the FRTL-5 rat thyroid follicular cell line, NFI was shown to regulate constitutive expression of TTF-1 (30). Inhibition of NFI binding to the TTF-1 promoter was suggested to be a mechanism by which thyroglobulin mediates inhibition of thyroid-specific gene expression. However, Dox-induced expression of dominant-negative NFIen in the pulmonary epithelium had no effect on TTF-1 expression in TII cells (Fig. 8B and 9F). These apparently contradictory findings suggest that either TTF-1 expression is regulated differently in the lung and thyroid or TTF-1 regulation in FRTL-5 cells is distinct from that in lung cells. Conditional expression of NFIen could be used to clarify the role of NFI in other target systems, including the thyroid, mammary gland, brain, and liver, where NFI family members have been implicated in gene regulation (reviewed in reference 17).

Our data also suggest that the coactivator CBP/p300 can interact with the NFI–TTF-1 transcription complex to further

enhance SP-C transcription. p300 synergistically enhanced the cooperative NFI–TTF-1 transactivation of SP-C promoter activity by all NFI family members (Fig. 6). NFI-C interacts with the CREB binding domain of CBP in the two-hybrid assay (26). Whether all NFI family members interact directly with CBP or p300 is unknown. Leahy et al. showed that different NFI family members have opposite effects on transcription of the PEPCK gene. They proposed a model in which CBP coordinates diverse signals to regulate gene transcription by acting as a scaffold for bringing together specific transcription factors with the basal transcriptional machinery in response to insulin, steroid hormones, and cyclic AMP (cAMP) (26). Treatment of fetal human lung explants with cAMP induces interaction of CBP with TTF-1, acetylation of TTF-1, and increased SP-A transcription (47). In the present experiments, p300 had no effect on cotransfected NFI or TTF-1 alone, and synergistic activation only occurred in the presence of both transcription factors and when both NFI and TTF-1 binding sites were intact in the SP-C promoter sequence. CBP/p300 may act as a scaffold protein to bring together the cotransfected factors with the basal transcription complex or may act to open chromatin and allow access of transcription factors to their binding sites through its histone acetyltransferase activity (reviewed in reference 9). The present experiments do not distinguish between the two possible roles for p300 in SP-C promoter activation by NFI and TTF-1.

Our studies indicate that TTF-1 and NFI family members cooperate to regulate the transcription of SP-C in vitro. In addition, the ability of the dominant-negative NFIen protein to repress SP-C expression both in vitro and in vivo indicates that NFI family members likely play an important role in SP-C expression in pulmonary TII cells. Finally, the strong enhancement of TTF-1 and NFI-dependent SP-C reporter expression by p300 suggests that p300 or related coactivators may play essential roles in SP-C expression. Together with recent findings that lung development is delayed in *Nfib*-deficient mice (18), these data strongly support the hypothesis that NFI family members have multiple important roles in lung development and SP-C gene expression.

ACKNOWLEDGMENTS

We thank J. A. Whitsett for providing the *Drosophila* engrailed transcriptional repression domain, monoclonal TTF-1 antibody, and the CCSP-rtTA activator mice and for helpful discussions and suggestions. We also thank H. Bujard for the rtTA expression plasmid, C. Yan for providing some of the plasmids used in the mammalian two-hybrid analysis, and T. Inoue for the pBETNFIb1f untagged mouse NFI-A expression plasmid. Susan Wert and the Molecular Morphology Core helped with ISH and Kathryn Foss provided expert technical assistance.

This work was supported by NIH grants HL60907 (C.J.B.), HD34908, and DK58401 (R.M.G.) and the Charlotte R. Schmidlapp Women Scholars Award (C.J.B.).

REFERENCES

1. **Apt, D., Y. Liu, and H. U. Bernard.** 1994. Cloning and functional analysis of spliced isoforms of human nuclear factor I-X: interference with transcriptional activation by NFI/CTF in a cell-type specific manner. Nucleic Acids Res. **22:**3825–3833.
2. **Arnone, M. I., M. Zannini, and R. Di Lauro.** 1995. The DNA binding activity and the dimerization ability of the thyroid transcription factor I are redox regulated. J. Biol. Chem. **270:**12048–12055.
3. **Bachurski, C. J., S. E. Kelly, S. W. Glasser, and T. A. Currier.** 1997. Nuclear factor I family members regulate the transcription of surfactant protein-C. J. Biol. Chem. **272:**32759–32766.
4. **Bachurski, C. J., G. S. Pryhuber, S. W. Glasser, S. E. Kelly, and J. A. Whitsett.** 1995. Tumor necrosis factor-alpha inhibits surfactant protein C gene transcription. J. Biol. Chem. **270:**19402–19407.
5. **Bohinski, R. J., R. Di Lauro, and J. A. Whitsett.** 1994. The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. Mol. Cell. Biol. **14:**5671–5681.
6. **Bohinski, R. J., J. A. Huffman, J. A. Whitsett, and D. L. Lattier.** 1993. *Cis*-active elements controlling lung cell-specific expression of human pulmonary surfactant protein B gene. J. Biol. Chem. **268:**11160–11166.
7. **Bruggemeier, U., L. Rogge, E. L. Winnacker, and M. Beato.** 1990. Nuclear factor I acts as a transcription factor on the MMTV promoter but competes with steroid hormone receptors for DNA binding. EMBO J. **9:**2233–2239.
8. **Bruno, M. D., T. R. Korfhagen, C. Liu, E. E. Morrisey, and J. A. Whitsett.** 2000. GATA-6 activates transcription of surfactant protein A. J. Biol. Chem. **275:**1043–1049.
9. **Chan, H. M., and N. B. La Thangue.** 2001. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J. Cell Sci. **114:**2363–2373.
10. **Chaudhry, A. Z., G. E. Lyons, and R. M. Gronostajski.** 1997. Expression patterns of the four nuclear factor I genes during mouse embryogenesis indicate a potential role in development. Dev. Dyn. **208:**313–325.
11. **Chaudhry, A. Z., A. D. Vitullo, and R. M. Gronostajski.** 1998. Nuclear factor I (NFI) isoforms differentially activate simple versus complex NFI-responsive promoters. J. Biol. Chem. **273:**18538–18546.
12. **das Neves, L., C. S. Duchala, F. Godinho, M. A. Haxhiu, C. Colmenares, W. B. Macklin, C. E. Campbell, K. G. Butz, and R. M. Gronostajski.** 1999. Disruption of the murine nuclear factor I-A gene (Nfia) results in perinatal lethality, hydrocephalus, and agenesis of the corpus callosum. Proc. Natl. Acad. Sci. USA **96:**11946–11951.
13. **Di Palma, T., R. Nitsch, A. Mascia, L. Nitsch, R. Di Lauro, and M. Zannini.** 2003. The paired domain-containing factor Pax8 and the homeodomain-containing factor TTF-1 directly interact and synergistically activate transcription. J. Biol. Chem. **278:**3395–3402.
14. **Glasser, S. W., M. S. Burhans, S. K. Eszterhas, M. D. Bruno, and T. R. Korfhagen.** 2000. Human SP-C gene sequences that confer lung epithelium-specific expression in transgenic mice. Am. J. Physiol. Lung Cell Mol. Physiol. **278:**L933–L945.
15. **Gossen, M., and H. Bujard.** 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA **89:**5547–5551.
16. **Goyal, N., J. Knox, and R. M. Gronostajski.** 1990. Analysis of multiple forms of nuclear factor I in human and murine cell lines. Mol. Cell. Biol. **10:**1041–1048.
17. **Gronostajski, R. M.** 2000. Roles of the NFI/CTF gene family in transcription and development. Gene **249:**31–45.
18. **Grunder, A., T. T. Ebel, M. Mallo, G. Schwarzkopf, T. Shimizu, A. E. Sippel, and H. Schrewe.** 2002. Nuclear factor I-B (Nfib) deficient mice have severe lung hypoplasia. Mech. Dev. **112:**69–77.
19. **Inoue, T., T. Tamura, T. Furuichi, and K. Mikoshiba.** 1990. Isolation of complementary DNAs encoding a cerebellum-enriched nuclear factor I family that activates transcription from the mouse myelin basic protein promoter. J. Biol. Chem. **265:**19065–19070.
20. **Kelly, S. E., C. J. Bachurski, M. S. Burhans, and S. W. Glasser.** 1996. Transcription of the lung-specific surfactant protein C gene is mediated by thyroid transcription factor 1. J. Biol. Chem. **271:**6881–6888.
21. **Kimura, S., Y. Hara, T. Pineau, P. Fernandez-Salguero, C. H. Fox, J. M. Ward, and F. J. Gonzalez.** 1996. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev. **10:**60–69.
22. **Kistner, A., M. Gossen, F. Zimmermann, J. Jerecic, C. Ullmer, H. Lubbert, and H. Bujard.** 1996. Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. Proc. Natl. Acad. Sci. USA **93:**10933–10938.
23. **Krasnow, M. A., E. E. Saffman, K. Kornfeld, and D. S. Hogness.** 1989. Transcriptional activation and repression by Ultrabithorax proteins in cultured Drosophila cells. Cell **57:**1031–1043.
24. **Kruse, U., and A. E. Sippel.** 1994. The genes for transcription factor nuclear factor I give rise to corresponding splice variants between vertebrate species. J. Mol. Biol. **238:**860–865.
25. **Kruse, U., and A. E. Sippel.** 1994. Transcription factor nuclear factor I proteins form stable homo- and heterodimers. FEBS Lett. **348:**46–50.
26. **Leahy, P., D. R. Crawford, G. Grossman, R. M. Gronostajski, and R. W. Hanson.** 1999. CREB binding protein coordinates the function of multiple transcription factors including nuclear factor I to regulate phosphoenolpyruvate carboxykinase (GTP) gene transcription. J. Biol. Chem. **274:**8813–8822.
27. **Li, C., J. Cai, Q. Pan, and P. Minoo.** 2000. Two functionally distinct forms of NKX2.1 protein are expressed in the pulmonary epithelium. Biochem. Biophys. Res. Commun. **270:**462–468.
28. **Liu, C., S. W. Glasser, H. Wan, and J. A. Whitsett.** 2002. GATA-6 and thyroid transcription factor-1 directly interact and regulate surfactant protein-C gene expression. J. Biol. Chem. **277:**4519–4525.

29. **Miccadei, S., R. De Leo, E. Zammarchi, P. G. Natali, and D. Civitareale.** 2002. The synergistic activity of thyroid transcription factor 1 and Pax 8 relies on the promoter/enhancer interplay. Mol. Endocrinol. **16:**837–846.
30. **Nakazato, M., H. K. Chung, L. Ulianich, A. Grassadonia, K. Suzuki, and L. D. Kohn.** 2000. Thyroglobulin repression of thyroid transcription factor 1 (TTF-1) gene expression is mediated by decreased DNA binding of nuclear factor I proteins which control constitutive TTF-1 expression. Mol. Cell. Biol. **20:**8499–8512.
31. **Naltner, A., M. Ghaffari, J. A. Whitsett, and C. Yan.** 2000. Retinoic acid stimulation of the human surfactant protein B promoter is thyroid transcription factor 1 site-dependent. J. Biol. Chem. **275:**56–62.
32. **Ortiz, L., P. Aza-Blanc, M. Zannini, A. C. Cato, and P. Santisteban.** 1999. The interaction between the forkhead thyroid transcription factor TTF-2 and the constitutive factor CTF/NF-1 is required for efficient hormonal regulation of the thyroperoxidase gene transcription. J. Biol. Chem. **274:**15213–15221.
33. **Ortiz, L., M. Zannini, R. Di Lauro, and P. Santisteban.** 1997. Transcriptional control of the forkhead thyroid transcription factor TTF-2 by thyrotropin, insulin, and insulin-like growth factor I. J. Biol. Chem. **272:**23334–23339.
34. **Perl, A. K., J. W. Tichelaar, and J. A. Whitsett.** 2002. Conditional gene expression in the respiratory epithelium of the mouse. Transgenic Res. **11:**21–29.
35. **Perrone, L., G. Tell, and R. Di Lauro.** 1999. Calreticulin enhances the transcriptional activity of thyroid transcription factor-1 by binding to its homeodomain. J. Biol. Chem. **274:**4640–4645.
36. **Schuur, E. R., U. Kruse, J. S. Iacovoni, and P. K. Vogt.** 1995. Nuclear factor I interferes with transformation induced by nuclear oncogenes. Cell Growth Differ. **6:**219–227.
37. **Steele-Perkins, G., K. G. Butz, G. E. Lyons, M. Zeichner-David, H. J. Kim, M. I. Cho, and R. M. Gronostajski.** 2003. Essential role for NFI-C/CTF transcription-replication factor in tooth root development. Mol. Cell. Biol. **23:**1075–1084.
38. **Tell, G., L. Perrone, D. Fabbro, L. Pellizzari, C. Pucillo, M. De Felice, R. Acquaviva, S. Formisano, and G. Damante.** 1998. Structural and functional properties of the N transcriptional activation domain of thyroid transcription factor-1: similarities with the acidic activation domains. Biochem. J. **329:**395–403.
39. **Tichelaar, J. W., W. Lu, and J. A. Whitsett.** 2000. Conditional expression of fibroblast growth factor-7 in the developing and mature lung. J. Biol. Chem. **275:**11858–11864.
40. **Weidenfeld, J., W. Shu, L. Zhang, S. E. Millar, and E. E. Morrisey.** 2002. The WNT7b promoter is regulated by TTF-1, GATA6, and Foxa2 in lung epithelium. J. Biol. Chem. **277:**21061–21070.
41. **Wert, S. E., S. W. Glasser, T. R. Korfhagen, and J. A. Whitsett.** 1993. Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. Dev. Biol. **156:**426–443.
42. **Whitsett, J. A., and S. W. Glasser.** 1998. Regulation of surfactant protein gene transcription. Biochim. Biophys. Acta **1408:**303–311.
43. **Yan, C., A. Naltner, J. Conkright, and M. Ghaffari.** 2001. Protein-protein interaction of retinoic acid receptor alpha and thyroid transcription factor-1 in respiratory epithelial cells. J. Biol. Chem. **276:**21686–21691.
44. **Yan, C., A. Naltner, M. Martin, M. Naltner, J. M. Fangman, and O. Gurel.** 2002. Transcriptional stimulation of the surfactant protein B gene by STAT3 in respiratory epithelial cells. J. Biol. Chem. **277:**10967–10972.
45. **Yang, H., M. M. Lu, L. Zhang, J. A. Whitsett, and E. E. Morrisey.** 2002. GATA6 regulates differentiation of distal lung epithelium. Development **129:**2233–2246.
46. **Yang, Y. S., M. C. Yang, B. Wang, and J. C. Weissler.** 2001. BR22, a novel protein, interacts with thyroid transcription factor-1 and activates the human surfactant protein B promoter. Am. J. Respir. Cell. Mol. Biol. **24:**30–37.
47. **Yi, M., G. X. Tong, B. Murry, and C. R. Mendelson.** 2002. Role of CBP/p300 and SRC-1 in transcriptional regulation of the pulmonary surfactant protein-A (SP-A) gene by thyroid transcription factor-1 (TTF-1). J. Biol. Chem. **277:**2997–3005.
48. **Zhou, L., L. Lim, R. H. Costa, and J. A. Whitsett.** 1996. Thyroid transcription factor-1, hepatocyte nuclear factor-3beta, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. J. Histochem. Cytochem. **44:**1183–1193.