Developmental Biology **213,** 405–417 (1999)
Article ID dbio.1999.9380, available online at http://www.idealibrary.com on IDEAL®

HNF-3/Forkhead Homologue-4 Influences Lung Morphogenesis and Respiratory Epithelial Cell Differentiation *in Vivo*

Jay W. Tichelaar, Lorena Lim,* Robert H. Costa,* and Jeffrey A. Whitsett

*Division of Pulmonary Biology, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3039; and *Department of Biochemistry and Molecular Biology, University of Illinois-Chicago, 1819 West Polk Street, Chicago, Illinois 60612*

HNF-3/forkhead homologue 4 (HFH-4), a transcription factor of the winged helix/forkhead family, is expressed in various tissues including lung, brain, oviduct, testis, and embryonic kidney. In order to test whether the temporospatial expression of HFH-4 influences lung morphogenesis, HFH-4 was expressed in lungs of transgenic mice under control of the surfactant protein C (SP-C) promoter. The morphology of the lungs from SP-C/HFH-4 embryos (day 18 postconception) was distinctly abnormal, and the severity of the alterations correlated with the level of transgene expression as detected by *in situ* hybridization. At high levels of expression, HFH-4 altered epithelial cell differentiation and inhibited branching morphogenesis. Atypical cuboidal or columnar cells lined the lung periphery of SP-C/HFH-4 transgenic mice. The atypical epithelial cells seen in the SP-C/HFH-4 mice expressed thyroid transcription factor-1 and hepatocyte nuclear factor 3β (HNF-3β). However, surfactant proteins SP-B, SP-C, and Clara cell secretory protein, normally produced by nonciliated epithelial cells in lung parenchyma were lacking. β-Tubulin IV, a marker of ciliated cells, stained the atypical columnar cells produced by expression of high levels of the SP-C/HFH-4 transgene. Ectopic expression of HFH-4 in developing mouse lung altered epithelial cell differentiation and morphology, restricting the expression of markers typical of nonciliated cells of the distal lung parenchyma.

Key Words: **HFH-4; winged helix; lung morphogenesis; respiratory epithelial cell differentiation.**

INTRODUCTION

The respiratory tract is lined by numerous, distinct cell types, including squamous, goblet, ciliated, nonciliated secretory (Clara), basal, alveolar Type I, and alveolar Type II cells, that are derived from common progenitor cells of the foregut endoderm. The molecular events directing the complex pattern of respiratory epithelial cell differentiation during lung development are poorly understood at present. It is likely, however, that cell differentiation of the respiratory epithelium is influenced by complex cell–cell interactions and autocrine, paracrine, and humoral signals which influence the expression of transcription factors regulating gene expression in foregut tissues. Several families of transcription factors play critical roles in lung morphogenesis and gene expression, including the homeodomain protein thyroid transcription factor-1 (TTF-1) (Bohinski *et al.,* 1994; Kimura *et al.,* 1996), the winged-helix family member hepatocyte nuclear factor-3β (HNF-3β) (Ikeda *et al.,* 1996; Zhou *et al.,* 1997) and zinc finger transcription factors of the Gli family (Motoyama *et al.,* 1998). While these transcription factors are critical to commitment and differentiation of foregut endoderm, lung bud formation, and branching morphogenesis, their potential roles in respiratory epithelial cell differentiation later in development have not been elucidated.

HNF-3/forkhead homologue-4 (HFH-4) is a member of the winged-helix/forkhead family of transcription factors that is expressed in a number of tissues including lung, brain, oviduct, nasal and embryonic kidney epithelium, and the developing spermatids of testis (Clevidence *et al.,* 1994; Hackett *et al.,* 1995; Lim *et al.,* 1997; Murphy *et al.,* 1997; Pelletier *et al.,* 1998). In lung, expression of HFH-4 is restricted to the bronchial and bronchiolar epithelium from the midpseudoglandular stage of lung development and is maintained postnatally (Clevidence *et al.,* 1994; Hackett *et*

0012-1606/99 $30.00

al., 1995). In recent studies in the mouse lung, HFH-4 is expressed in ciliated cells of the respiratory tract, marking a subset of cells distinct from those expressing surfactant proteins (Chen *et al.,* 1998; Tichelaar *et al.,* 1999). Expression of HFH-4 is maintained in the lung rudiments of TTF-1 gene targeted mice (Tichelaar *et al.,* 1999), suggesting a role for HFH-4 in subsets of epithelial cells distinct from those dependent upon TTF-1. Thus, in the postnatal lung, cells expressing HFH-4 are distinct from those expressing TTF-1 and its targets, SP-B, SP-C, and CCSP.

To further clarify the role of HFH-4 in differentiation of respiratory epithelial cells *in vivo,* transgenic mice were generated in which HFH-4 was expressed ectopically under the control of the human SP-C promoter, targeting expression to a subset of progenitor cells involved in morphogenesis and differentiation of distal lung parenchyma (Wert *et al.,* 1993). Expression of HFH-4 in distal respiratory epithelial cells of transgenic mice dramatically altered the morphology of terminal airways, generating atypical cuboidal and columnar epithelial cells lining dilated lung saccules. Respiratory epithelial cells expressing the transgene lacked differentiated cell markers SP-B, SP-C, and CCSP and variably expressed β-tubulin IV, a marker of ciliated respiratory epithelial cells typical of more proximal regions of the lung epithelium.

MATERIALS AND METHODS

Production of SP-C/HFH-4 transgenic mice. A 2.4-kb (5′ *Xba*I–3′ *Sal*I) fragment of the murine HFH-4 cDNA (Clevidence *et al.,* 1994) was blunt-end ligated into the *Eco*RI site of an SP-C expression plasmid containing 3.7 kb of the human SP-C promoter, SV40 t-intron, and polyadenylation signal (Wert *et al.,* 1993). Plasmid DNA was propagated in *Escherichia coli* DH5α using standard methodology. The transgene was excised using *Nde*I and *Not*I, agarose gel purified, and dialyzed into 5 mM Tris–HCl, pH 7.4, 0.5 mM EDTA and microinjected into fertilized eggs of FVB/N mice. All mice were housed under pathogen-free conditions according to institutional and NIH guidelines. Embryos at 18 days of gestation were obtained from pregnant mothers. Transgenic mice were identified by PCR using transgene-specific primers (Fig. 1). Primers were as follows: upstream primer in human SP-C promoter, 5′-GACACATATAAGACCCTGGTCA-3′; and downstream primer in mHFH-4 cDNA, 5′-GTGTGGGTTGGTGGCATAGTC-3′. The presence of the transgene was subsequently confirmed by Southern blot using a probe specific for the SV40 t-intron–poly(A) sequences (Fig. 1).

Tissue preparation and histopathology. Mouse embryos were fixed in neutral buffered 4% paraformaldehyde for 16 h at 4°C. Embryos were dehydrated through a graded series of ethanols and embedded in paraffin. Five-micrometer transverse sections were loaded onto polylysine slides for immunohistochemistry or silanized slides for *in situ* hybridization. Paraffin sections were deparaffinized and rehydrated before staining with hematoxylin and eosin.

In situ hybridization. A plasmid containing the 478-bp SV40 t-intron–poly(A) fragment (Zhou *et al.,* 1996a) was used to generate anti-sense and sense riboprobes for the transgene. Anti-sense riboprobes were generated from linearized plasmid using T7 RNA polymerase, [^{35}S]UTP and the MaxiScript *in vitro* transcription kit (Ambion, Inc., Austin, TX). *In situ* hybridization was performed as described previously (Zhou *et al.,* 1996a). A plasmid containing the 758-bp mouse SP-C cDNA was used to generate anti-sense and sense riboprobes for endogenous SP-C. *In situ* hybridization conditions for SP-C were the same as those described previously (Wert *et al.,* 1993). Slides were autoradiographed with Kodak NTB2 emulsion for 3 weeks (SV40–t-intron–poly(A) transcript) or 6 days (SP-C transcript) and developed with Kodak D19 developer then counterstained with hematoxylin and eosin.

Antibodies and immunohistochemistry. Rabbit polyclonal antibody against a synthetic peptide spanning amino acid residues 1–101 of mouse HFH-4 fused to GST was generated as described previously (Lim *et al.,* 1997) and either affinity purified and used at a dilution of 1:1000 or Affi-Gel (Bio-Rad Laboratories, Hercules, CA) purified and used at a dilution of 1:2000. Rabbit polyclonal antibody generated against a synthetic peptide spanning amino acid residues 110–122 of rat TTF-1 was kindly provided by Dr. Roberto DiLauro (Guazzi *et al.,* 1990) and was used at a dilution of 1:8000 on fetal mouse lung or 1:1000 on adult mouse lung. Rabbit polyclonal antibody against HNF-3β was generated against amino acids 7–86 of rat HNF-3β and used at dilutions 1:2000 and 1:4000 (Jacob *et al.,* 1994). Rabbit antiserum against rat CCSP was a gift from Dr. Gurmukh Singh (Singh *et al.,* 1988) and was used at a dilution of 1:5000. Rabbit polyclonal antibodies to pro-SP-C (No. R68514) (Clark *et al.,* 1995), pro-SP-B (No. R55522) (Lin *et al.,* 1996) and mature SP-B (No. R28031) (Baatz *et al.,* 1990) were generated in our laboratory and used at dilutions of 1:2000, 1:2000, and 1:4000, respectively. Mouse monoclonal antibody to β-tubulin IV (Banerjee *et al.,* 1992) was obtained from BioGenex (San Ramon, CA) and used at a dilution of 1:40 and 1:80.

Immunohistochemical staining for HFH-4, TTF-1, and HNF-3β was carried out as follows: 5-μm paraffin sections were deparaffinized and slides were heated to 90°C in 0.1 M citric acid, 0.1 M sodium citrate (pH 6.0) for 20 min. Sections were then treated with 3% hydrogen peroxide in methanol for 15 min, blocked with 2% normal goat serum for 2 h, and incubated at 4°C overnight with primary antibody and developed using biotinylated goat anti-rabbit secondary antibody and a Vector Elite ABC kit (Vector Laboratories, Burlingame, CA). Antigen localization was enhanced with Ni-DAB, followed by incubation with Tris-Cobalt and counterstaining with Nuclear Fast Red. Staining for β-tubulin IV was carried out similarly, but slides were blocked in 5% normal horse serum for 4 h and the secondary antibody used was biotinylated horse anti-mouse IgG. Staining for CCSP, mature SP-B, pro-SP-B, and pro-SP-C was performed identically to above but without high temperature/sodium citrate antigen retrieval. All immunohistochemical reactions were carried out in parallel with reactions lacking primary antibody to ensure the specificity of the observed staining.

RESULTS

Production of SP-C/HFH-4 Transgenic Mice

To investigate the role of HFH-4 *in vivo,* transgenic mice were generated using the construct diagrammed in Fig. 1G. The transgene consists of a 3.7-kb promoter fragment from the human SP-C promoter ligated to the murine HFH-4 cDNA and SV40 t-intron and polyadenylation signal. Because of the potential neonatal lethality of lung-specific

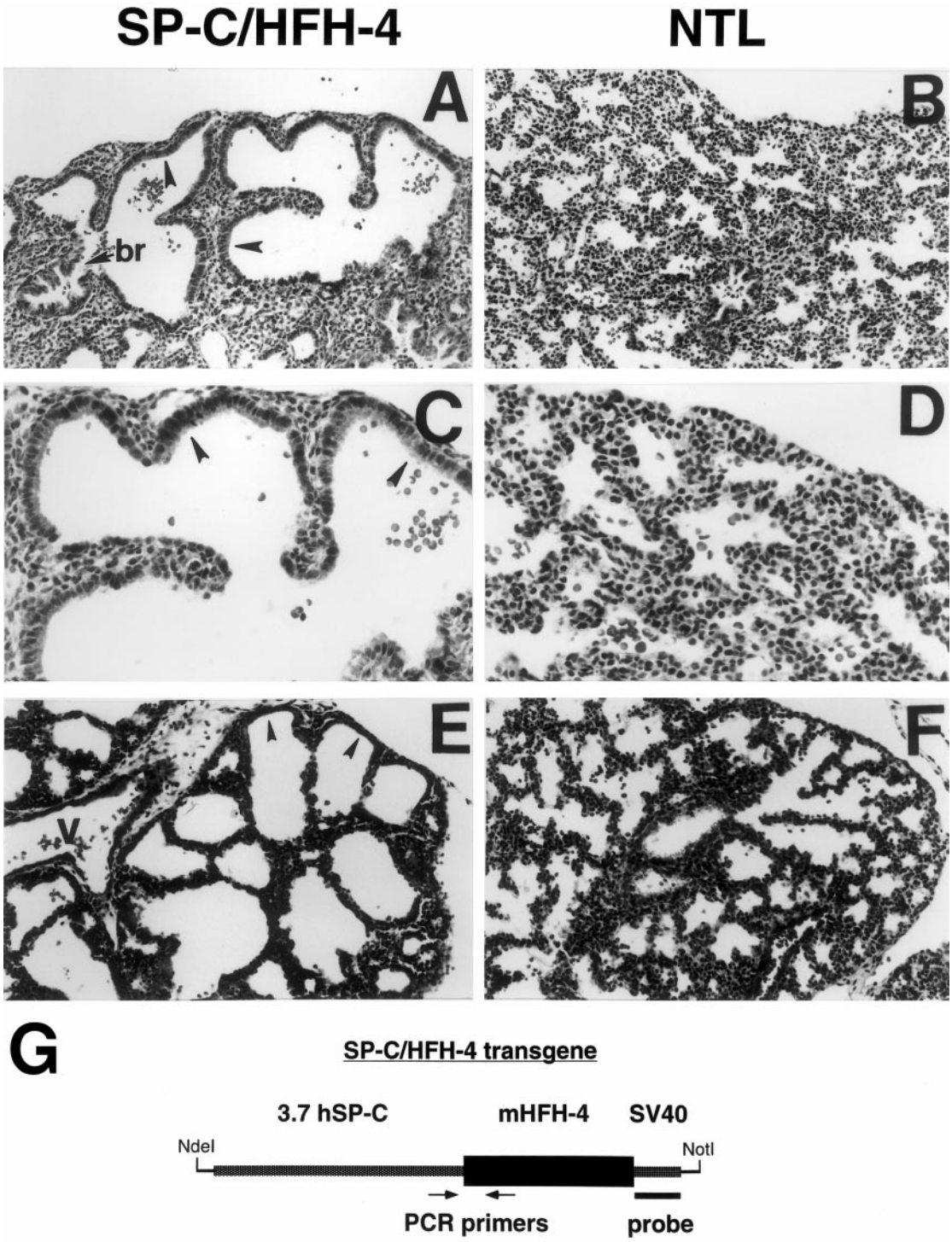

FIG. 1. Lung histology of SP-C/HFH-4 transgenic mice at day 18 pc. Lung sections from SP-C/HFH-4 transgenic mice (A, C, E) and nontransgenic littermates (NTL; B, D, F) at day 18 pc were examined by hematoxylin and eosin staining. Lungs from SP-C/HFH-4 mice contained dilated saccules lined by columnar and cuboidal epithelium (arrowheads). Two founders, F3-1 (A, C) and F32 (E), are shown. (G) The SP-C/HFH-4 transgene construct, consisting of the 3.7-kb human SP-C promoter, the 2.4-kb murine HFH-4 cDNA and SV40 t-antigen intron, and early polyadenylation signal. The arrows indicate the location of PCR primers used for genotype analysis and the bar below the construct indicates the fragment used as a probe for *in situ* hybridization. br, bronchiole; v, vessel.

TABLE 1
Genotype of Potential SP-C/HFH-4 Founders

	Total no. of pups examined	Transgenic pups	Pups/lines with altered lung histology
Day 18 pc pups	74	13	4
Weanling pups	33	7	0

Note. Screening for SP-C/HFH-4 founders was done at day 18 pc or at weaning by PCR and Southern blot. Of 13 transgenic pups identified at day 18 pc, 4 had altered lung morphology and respiratory epithelial cell differentiation. Transgenic lines were established from 5 of the 7 founder pups identified at weaning. All established lines of SP-C/HFH-4 mice had absent or low levels of transgene expression (see Fig. 7).

expression of winged-helix transcription factors (Zhou *et al.,* 1997), pregnant mice and fetuses were sacrificed at day 18 postconception (pc). The presence of the transgene was assessed by PCR and Southern blot (data not shown). Of 74 potential founder mice screened at day 18 pc, 13 carried the SP-C/HFH-4 transgene (Table 1), consistent with previous experience in our laboratory with transgenes that are nonlethal *in utero.*

Lung Morphology in SP-C/HFH-4-Expressing Mice

Histological inspection of the SP-C/HFH-4 fetuses revealed no abnormalities except in the lungs. Lung morphology of SP-C/HFH-4 transgenic mice varied widely, from unaffected to severely disrupted. Four of the 13 transgenic founders examined at day 18 pc (founders F1, F32, F1-1, and F3-1) had abnormal lung morphology as assessed by light microscopy (Fig. 1), and the extent of abnormalities seen in the respiratory tract varied among the affected founders. The disruption of lung morphology correlates with epithelial cells that ectopically express high levels of HFH-4 protein (see below). In the most severely affected mouse (F3-1), columnar epithelium, usually characteristic of the proximal conducting airway, was noted throughout the alveolar region of the lung (Figs. 1A and 1C). Similar morphologic alterations were observed in lungs from founder mice F1, F32, and F1-1, but the extent of abnormalities in epithelial cell morphology was less widespread, consisting of abnormally distributed columnar and cuboidal epithelial cells (Fig. 1E).

Atypical cuboidal and columnar cells lined the terminal saccules of lungs of the transgenic mice. In the founder F3-1, columnar epithelium lined dilated terminal saccules in all lobes of the lung. Cuboidal and squamous epithelial cells, Type II and Type I cells, respectively, normally line the airspaces in these regions of the lung. The abnormal epithelial cells in the SP-C/HFH-4 mice contained large oval nuclei (Fig. 1C). The abnormalities in lung morphology were similar, but less extensive in founders F1 and F32, being detected in some lobes but absent in others. In F1 and F32 founders, the abnormal tubules were lined by primarily low columnar epithelium compared to the tall columnar cells characteristic of the F3-1 founder. In founder F1-1, abnormal epithelium consisted of scattered rows of uniform, cuboidal epithelium without interspersed squamating epithelial cells. In all affected founders, the regions of lung parenchyma with abnormal morphology had enlarged acinar buds, consistent with abnormalities in branching morphogenesis.

HFH-4 Transgene Expression in Regions of Lung with Altered Morphology

The expression of the transgene-specific mRNA was determined by *in situ* hybridization and tissue autoradiography using a probe for the SV40 t-intron, which splices into the 3′ region of the chimeric HFH-4/t-intron mRNA (Zhou *et al.,* 1996a). The HFH-4 transgenic mRNA was detected in the abnormal respiratory epithelium of all affected mice (Figs. 2A and 2C) but was not detected with a sense probe (data not shown) or in nontransgenic littermates (Fig. 2B). Transgene-specific mRNA was detected in the bronchioles and peripheral saccules, consistent with previous studies with the human 3.7-kb SP-C promoter in the mouse lung (Wert *et al.,* 1993).

The distribution of HFH-4 protein was assessed by immunohistochemistry. In human, rat and mouse, HFH-4 is normally restricted to the epithelium of the conducting airways (Clevidence *et al.,* 1994; Hackett *et al.,* 1996; Pelletier *et al.,* 1998), being expressed in subsets of basalar and ciliated cells (Tichelaar *et al.,* 1999). In the lungs of SP-C/HFH-4 transgenic mice, intense staining for HFH-4 protein was detected in the atypical cells in the alveolar regions of the lung (Figs. 3A and 3C). HFH-4 staining was also detected in epithelial cells of the conducting airways at levels comparable to those found in nontransgenic littermates (Fig. 3B). Analysis of the lungs of transgenic founder mice lacking altered morphology demonstrated that few cells expressed HFH-4 in the lung periphery and the normal pattern of HFH-4 staining in conducting airways was maintained (data not shown).

HFH-4 Expression Alters TTF-1 and HNF-3β

TTF-1 and HNF-3ß are transcription factors that play a critical role in lung morphogenesis (Kimura *et al.,* 1996; Zhou *et al.,* 1997) and epithelial specific gene expression in the lung (Bohinski *et al.,* 1994; Bruno *et al.,* 1995; Ikeda *et al.,* 1996). TTF-1 is expressed in most cells of the conducting airways and in Type II and pre-Type II alveolar cells in the fetal lung (Stahlman *et al.,* 1996; Zhou *et al.,* 1996b). TTF-1 and HNF-3β decrease with advancing gestational age and are most intense in the lung periphery (Stahlman *et al.,* 1998; Zhou *et al.,* 1996b). In the adult lung, TTF-1 and HNF-3β are expressed primarily in alveolar Type II cells.

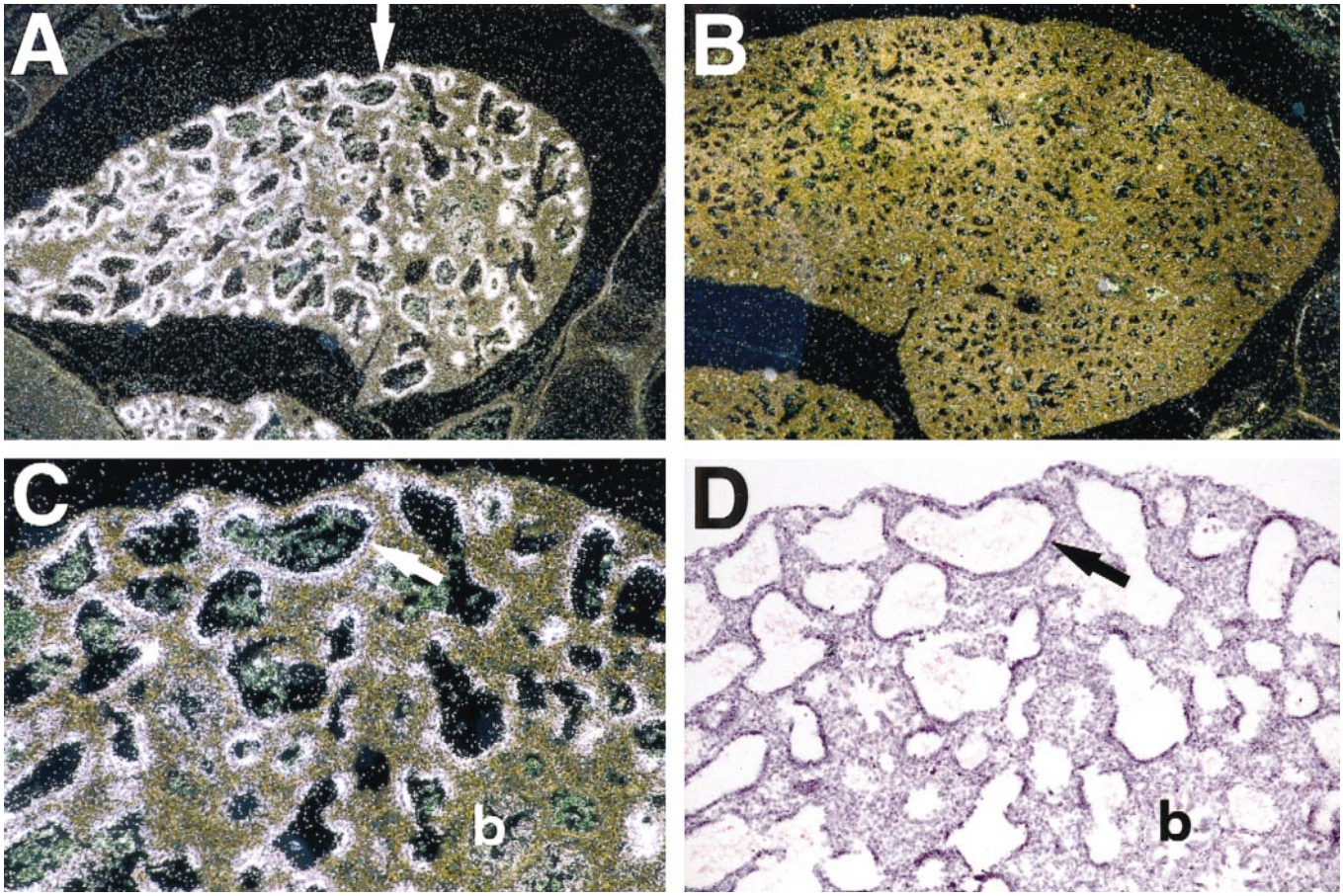

FIG. 2. Localization of transgene mRNA in fetal lungs. *In situ* hybridization detected transgene specific mRNA in the abnormal epithelial cells lining the dilated saccules of transgenic mouse lung at day 18 pc (arrow; A, C, D) and at lower levels in distal bronchiolar epithelium (b, bronchiole; C, D). No signal was detected in the lungs of nontransgenic littermates (B).

Intense nuclear staining for TTF-1 was observed in the affected epithelium of SP-C/HFH-4 mice at sites consistent with the expression of the HFH-4 transgene. TTF-1 was detected in nearly all cells lining the dilated saccules of SP-C/HFH-4 transgenic mice (Fig. 4A). The pattern and intensity of TTF-1 staining seen in the SP-C/HFH-4 mice was distinct from that in normal littermates where TTF-1 staining was observed primarily in Type II epithelial cells (Fig. 4B). HNF-3β was detected in a similar pattern in abnormal epithelial cells characteristic of the transgenic mice (Figs. 4C and 4D). Thus, ectopic expression of HFH-4 altered the typical distribution of TTF-1 and HNF-3β in the fetal mouse lung.

HFH-4 Alters Respiratory Epithelial Cell Gene Expression

To address cell differentiation in the SP-C/HFH-4 transformed epithelium, immunohistochemical localization of several lung epithelial cell proteins was assessed. At day 18 pc, expression of pro-SP-C is normally restricted to cuboidal Type II epithelial cells in the lung periphery, while pro-SP-B is detected in alveolar Type II cells and nonciliated columnar cells (Clara cells) lining conducting airways (Khoor *et al.*, 1994; Stahlman *et al.*, 1992; Zhou *et al.*, 1996b). Neither SP-B nor pro-SP-B was detected in the atypical cells in the lung periphery of SP-C/HFH-4 mice (data not shown). Weak staining for pro-SP-C was observed in scattered cells lining the dilated lung saccules in founder F3-1 coinciding with breaks in the abnormal columnar epithelium. In addition, small buds positive for pro-SP-C are present in the lung parenchyma of SP-C/HFH-4 transgenic mice (Fig. 5E, arrowhead). Pro-SP-C staining was not detected in the abnormal epithelium of other affected founder animals (data not shown). The intensity and distribution of staining for the surfactant proteins in unaffected areas of the lungs from transgenic mice was similar to that seen in nontransgenic littermates.

In situ hybridization was used to examine the effects of ectopic HFH-4 expression on SP-C mRNA levels. No hy-

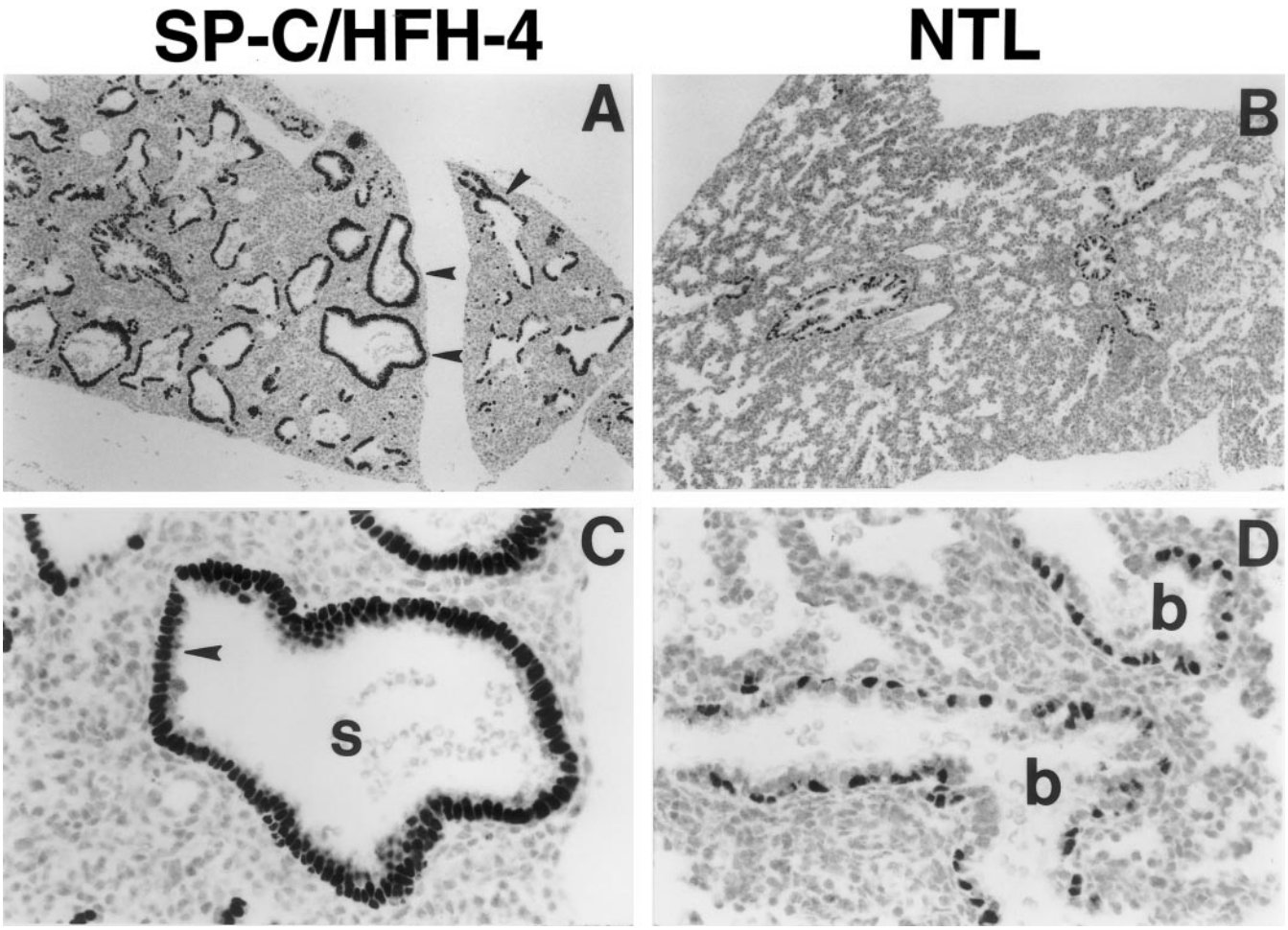

FIG. 3. Immunohistochemical staining for HFH-4. HFH-4 staining was apparent in the abnormal epithelium in the lung periphery of SP-C/HFH-4 transgenic mice at day 18 pc (arrowheads; A, C) but was restricted to conducting airway epithelium in nontransgenic littermates (NTL; B, D). b, bronchiole; s, dilated saccule.

bridization signal for SP-C mRNA was seen in the abnormal columnar epithelium of SP-C/HFH-4 transgenic mice (Figs. 6A and 6B) that are making high levels of transgene-specific mRNA (see Fig. 2A). High levels of SP-C mRNA were present in acinar buds in the lung parenchyma of SP-C/HFH-4 transgenic mice and in nontransgenic littermates (Figs. 6C and 6D), agreeing with the immunohistochemical localization. Hybridization with a sense SP-C probe did not detect any signal (data not shown).

Clara cell secretory protein (CCSP) is normally restricted to nonciliated columnar epithelial cells in the conducting airways (Singh *et al.*, 1988). Previous studies demonstrated that HFH-4 activated the CCSP promoter in transient transfection assays (Lim *et al.*, 1997), supporting the concept that CCSP may be a downstream target of HFH-4. However, CCSP staining was not detectable in the atypical respiratory epithelium in SP-C/HFH-4 transgenic mice, even in the founders expressing the highest levels of HFH-4 (F3-1, Fig. 5G). In addition, in founder mice wherein the transgene was expressed in the bronchiolar epithelium, CCSP staining was reduced (compare Figs. 5G and 5H), suggesting that HFH-4 restricted CCSP expression or negatively influenced differentiation of CCSP-expressing cells.

β-Tubulin IV staining was detected in ciliated cells in the airway epithelium where it was co-localized with HFH-4 (Tichelaar *et al.*, 1999). β-Tubulin IV was readily detectable in the atypical epithelium of F3-1 (Fig. 5C) but was not detected in the peripheral regions of lungs from nontransgenic littermates (Fig. 5D). β-Tubulin staining in the F3-1 founder was noted on the apical surface of some abnormal cells, consistent with staining of cilia, while in some cell staining appeared to be cytoplasmic. Areas of atypical epithelium that were positive for β-tubulin IV were also positive for HFH-4 (Figs. 5A and 5C, asterisks). In all other affected founders, β-tubulin IV was not detectable in the abnormal epithelial cells and β-tubulin IV staining was

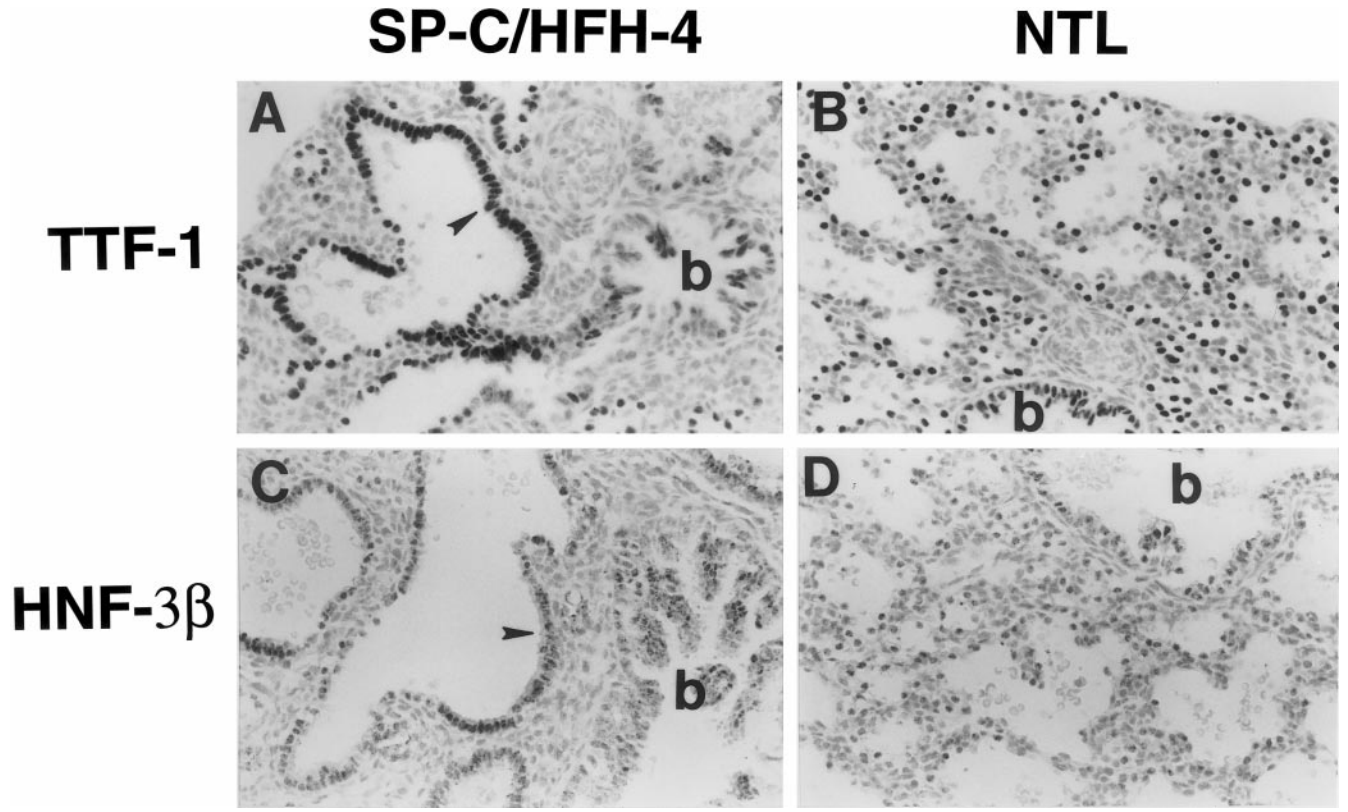

FIG. 4. Immunohistochemical staining for TTF-1 and HNF-3*β*. Staining for TTF-1 (A, B) and HNF-3*β* (C, D) was examined in lung sections from SP-C/HFH-4 transgenic mice (A, C) and nontransgenic littermates (NTL; B, D) at day 18 pc. Uniform staining for TTF-1 and HNF-3*β* was observed in the abnormal epithelium in the lung periphery of transgenic mice (arrowheads), while in nontransgenic littermates staining was restricted to Type II alveolar epithelium in the lung periphery. Bronchiolar staining was detected in both transgenic and nontransgenic littermates. b, bronchiole.

decreased in the bronchiolar epithelium of transgenic animals compared to nontransgenic littermates (Figs. 5C and 5D). Platelet/endothelial cell adhesion molecule (PECAM) staining, a marker for endothelial cells, was readily detectable surrounding the altered epithelium of SP-C/HFH-4 transgenic mice, indicating that vessel formation proceeded in the dysmorphic areas in lungs of SP-C/HFH-4 mice (data not shown).

SP-C/HFH-4 Transgenic Mice That Survive Postnatally Express Low Levels of the Transgene

The variable severity of the lung phenotype in SP-C/HFH-4 mice led us to investigate the potential effects of the transgene in adult animals. At weaning, the transgene was detected in 7 of 33 pups as determined by PCR (Table 1). These transgenic animals were indistinguishable from nontransgenic littermates and histology of their lungs was unaltered. Transgene-specific mRNA was low or undetectable in all established lines as determined by Northern blot and RT-PCR (data not shown). Transgene-specific *in situ* hybridization and HFH-4 immunohistochemistry were used to further characterize the two lines (F33 and F46) that had the highest levels of transgene expression as determined by RT-PCR. Respiratory epithelial cells expressing the transgene were observed infrequently in adult and fetal mice as determined by *in situ* hybridization (Figs. 7A and 7B). Immunohistochemistry confirmed that HFH-4 staining cells in the alveolar epithelium of transgenic mice were rare (Figs. 7C and 7D). In no fetal offspring from SP-C/HFH-4 lines F33 and F46 was the level and distribution of the transgene as great as that seen in fetal founders F1, F32, F1-1, or F3-1 collected at day 18 pc (compare Fig. 7 with Figs. 2 and 3). The distribution and intensity of staining for SP-B, SP-C, CCSP, *β*-tubulin IV, TTF-1, and HNF-3*β* in established SP-C/HFH-4 lines F33 and F46 were not altered at any time point examined (day 13.5 pc to adult, data not shown). The absence of altered lung morphology in surviving SP-C/HFH-4 mice suggests that founders expressing high levels of the transgene may not survive postnatally. However, the extent of the loss of transgenic pups is unclear since the dams often cannibalize abnormal offspring.

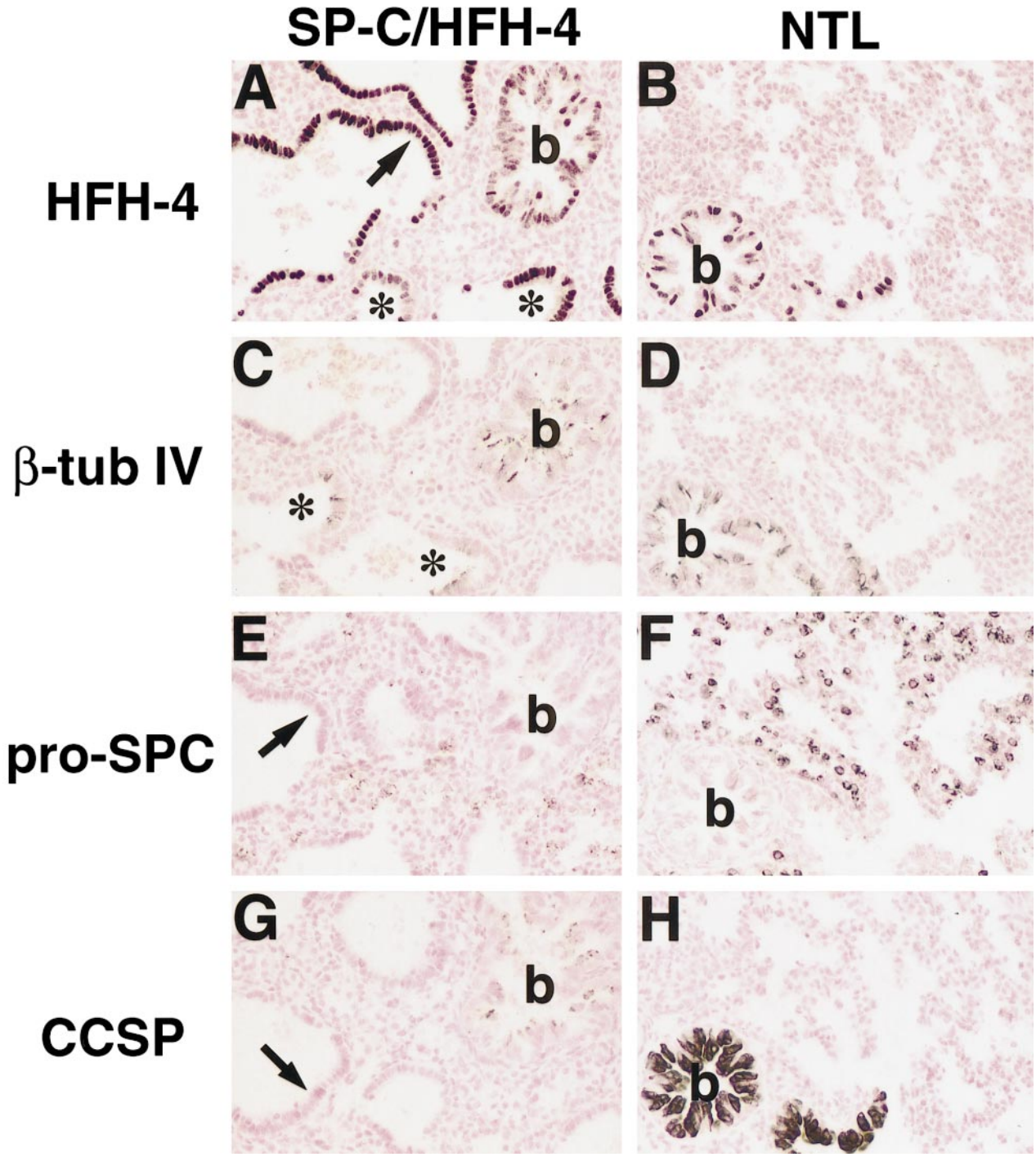

FIG. 5. Immunohistochemical staining for HFH-4, β-tubulin IV, pro-SP-C, and CCSP. Staining for HFH-4 was detected throughout the atypical columnar epithelium at day 18 pc (arrow, A). Staining for β-tubulin IV was detected in some abnormal respiratory epithelial cells at day 18 pc (asterisks, C) corresponding to sites of HFH-4 staining (asterisks, A). β-Tubulin IV staining was maintained in bronchiolar epithelium (C, D). Staining for pro-SP-C was undetectable in most of the atypical respiratory epithelial cells found in SP-C/HFH-4 transgenic mice (E, arrow) but was present in small acinar buds in the lung periphery. Staining for pro-SP-C was restricted to alveolar Type II cells in nontransgenic littermates (F). Staining for CCSP was absent from abnormal respiratory epithelium in transgenic mice at day 18 pc (G, arrow). Bronchiolar staining for CCSP was reduced in transgenic mice (G) compared to nontransgenic littermates (H). b, bronchiole.

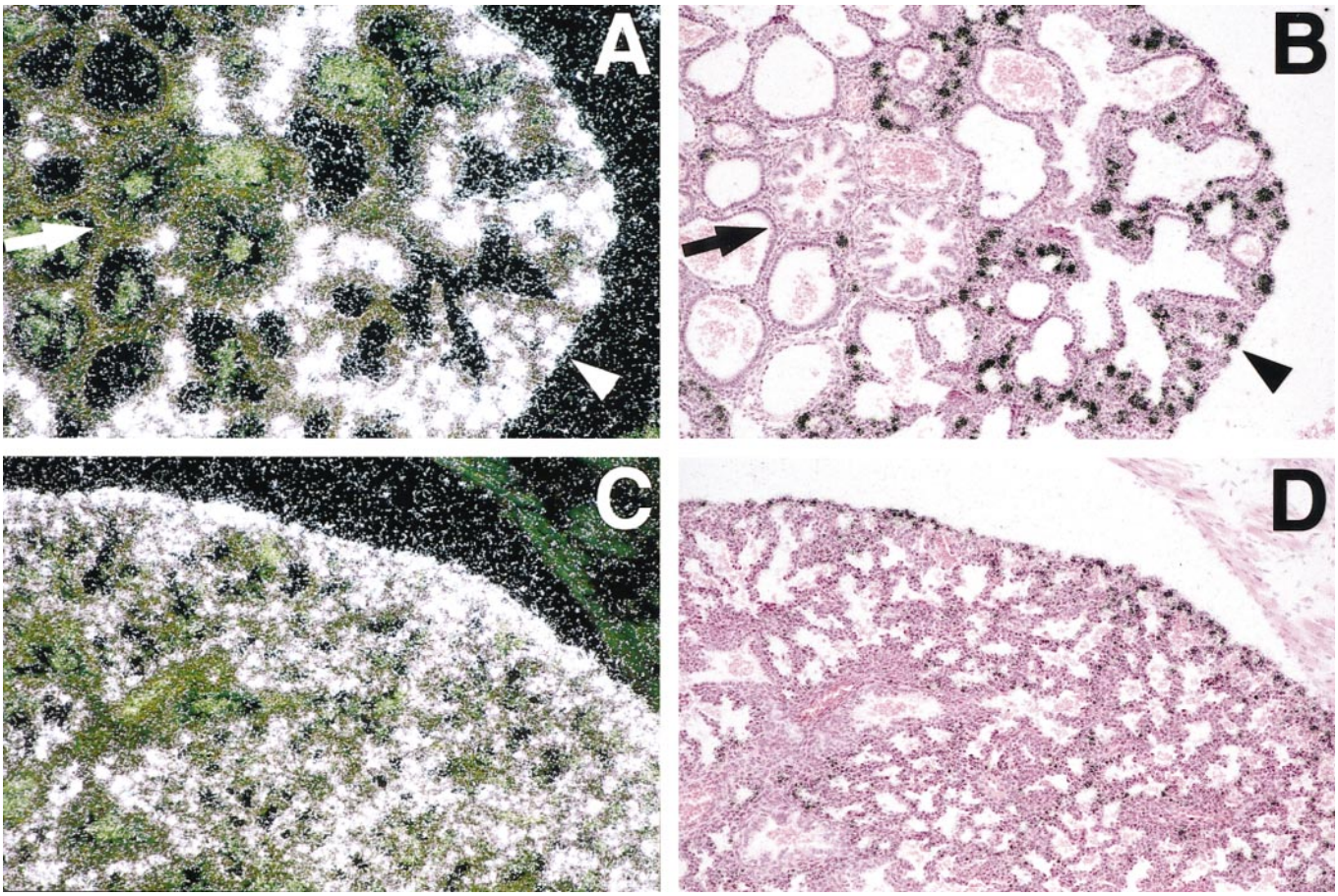

FIG. 6. Localization of SP-C mRNA in fetal lungs. SP-C mRNA was not detected in the atypical columnar epithelium of SP-C/HFH-4 transgenic mice at day 18 pc (arrows, A, B), but was present in scattered acinar buds in the lung parenchyma (arrowheads, A, B). SP-C mRNA in nontransgenic littermates was restricted to alveolar Type II cells (C, D).

DISCUSSION

Lung morphogenesis and respiratory epithelial-specific gene expression was assessed at day 18 pc in transgenic mice bearing a chimeric SP-C/HFH-4 transgene. *In situ* hybridization and immunohistochemistry was used to demonstrate high levels of HFH-4 mRNA in the lungs of some of the transgenic mice. Lungs of affected transgenic mice contained dilated saccules in the peripheral airspace associated with decreased or absent expression of markers typical of alveolar Type II or Clara cells. At high levels of expression, HFH-4 was associated with the ectopic appearance of β-tubulin IV and ciliated cells in the lung periphery. The present findings support the concept that HFH-4 influences respiratory epithelial cell differentiation, enhancing columnar and ciliated cell differentiation and restricting the expression of genes normally expressed in Clara and Type II cell lineages.

Early in lung development the branching respiratory tree is lined with a relatively undifferentiated, columnar epithelium (Adamson, 1997). TTF-1 and HNF-3β, transcription factors critical in regulating respiratory epithelial cell differentiation and gene expression (Ikeda *et al.*, 1996; Kimura *et al.*, 1996; Zhou *et al.*, 1997), are present throughout the respiratory epithelium, being detected in the lung bud as early as day 10 pc in the mouse (Zhou *et al.*, 1996b). TTF-1 and HNF-3β are expressed in subsets of respiratory epithelial cells in both conducting and peripheral airways, their abundance generally decreasing with age and becoming increasingly restricted to the lung periphery (Zhou *et al.*, 1996b). Nonciliated respiratory epithelial cells (Clara cell) and alveolar Type II epithelial cells express both HNF-3β and TTF-1 at sites coinciding with that of CCSP, SP-B and SP-C. Surfactant proteins SP-B and SP-C mRNAs are present in the respiratory epithelium by day 11 pc (Zhou *et al.*, 1996b). As branching morphogenesis proceeds, SP-B and SP-C mRNAs are present at higher concentrations in bronchiolar and alveolar regions in the lung periphery. While HNF-3β and TTF-1 are critical to formation of foregut endoderm and lung, respectively, their presence or absence

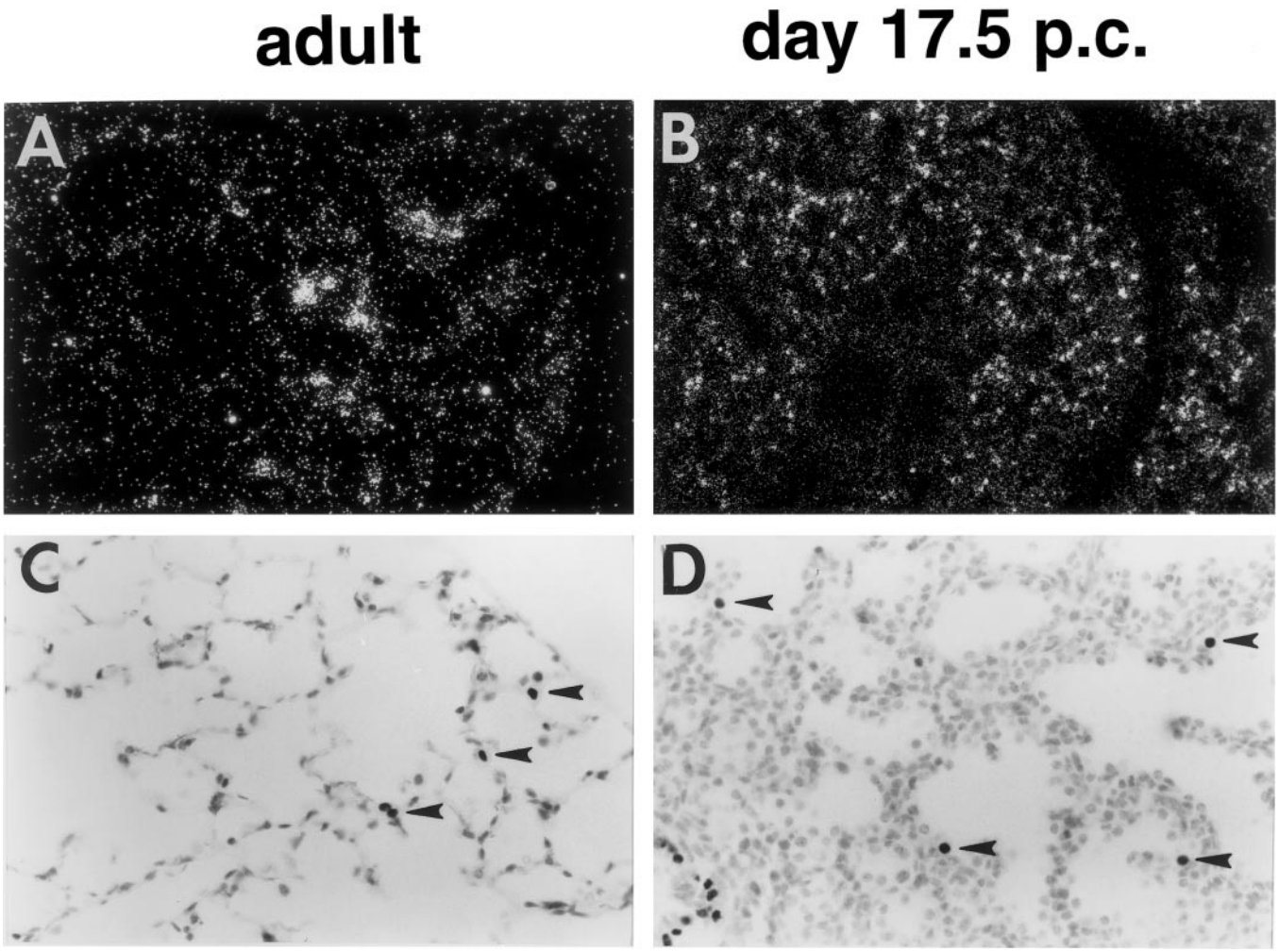

FIG. 7. Low levels of transgene expression in established lines of SP-C/HFH-4 mice. Levels of transgene expression in SP-C/HFH-4 line F46 were determined by *in situ* hybridization (A, B) and immunohistochemistry (C, D) in adult and fetal lung (day 17.5 pc). Transgene mRNA and HFH-4 protein was detected in a few scattered cells in the lung periphery (arrowheads). Similar results were observed in other established lines (data not shown). pc, postconception.

is not sufficient to explain the marked heterogeneity of cell types present in the mature respiratory epithelium. The timing and pattern of HFH-4 expression led us to investigate whether this molecule plays an important role in determining respiratory epithelial cell differentiation. The atypical respiratory epithelial cells seen in all affected SP-C/HFH-4 transgenic mice express high levels of TTF-1 and HNF-3β protein but not pro-SP-B or pro-SP-C, findings similar to that seen in the conducting airways during the early pseudoglandular period of lung development as the heterogeneity of cell types begins to expand in the developing respiratory epithelium. The finding that HFH-4 restricted expression of genes normally seen in Clara and Type II cell lineages is consistent with the hypothesis that HFH-4 may promote columnar or ciliated cell differentiation rather than Clara, Type II, or Type I cell differentiation.

HFH-4 mRNA is first detected in the normal mouse lung at day 14.5 pc in the bronchial and bronchiolar epithelium (Hackett *et al.,* 1995), preceding the appearance of either ciliated or nonciliated secretory (Clara) columnar cells that line the conducting airways. In the normal fetal lung HFH-4 is expressed in subsets of cells that express TTF-1 and HNF-3β (Zhou *et al.,* 1996b; Tichelaar *et al.,* 1999). β-Tubulin IV, a marker of ciliated cells (Fanucchi *et al.,* 1997; Renthal *et al.*, 1993), colocalized with HFH-4 but not with CCSP, a marker of nonciliated secretory cells (Singh *et al.,* 1988). Thus, HFH-4 is normally found in ciliated cells that do not express CCSP, SP-B, or SP-C. In the SP-C/HFH-4 founder F3-1, expression of β-tubulin IV was induced in the atypical epithelium in association with high levels of HFH-4 mRNA and protein. Colocalization of HFH-4 with β-tubulin IV in the normal lung and the presence of

β-tubulin IV in the atypical epithelium in the SP-C/HFH-4 mouse supports the concept that HFH-4 may influence commitment of progenitor cells toward columnar and ciliated cell phenotypes. Increased levels of HFH-4 changed the normally cuboidal distal respiratory epithelium toward columnar or ciliated cell phenotypes. The present findings are consistent with recent observations in mice with a targeted disruption of the HFH-4 locus (Chen *et al.*, 1998). HFH-4-deficient mice lacked ciliated cells in all tissues and exhibited varying degrees of situs inversus and hydrocephalus. Thus, HFH-4 plays a critical role in the formation of ciliated epithelium in various tissues.

While ectopic expression of HFH-4 resulted in decreased staining for the respiratory epithelial cell markers SP-B and pro-SP-C, it was not known if this inhibition was occurring at a transcriptional or posttranscriptional level. No SP-C mRNA was detected in the atypical respiratory epithelium of SP-C/HFH-4 transgenic mice by *in situ* hybridization. However, strong signal for SP-C mRNA was maintained in the lung parenchyma of SP-C/HFH-4 transgenic mice in regions distinct from the abnormal epithelium. The differential response of the endogenous SP-C promoter and the 3.7-kb human SP-C promoter to elevated levels of HFH-4 is consistent with previous observations indicating nonidentical regulation of these promoters in the mouse lung (Wert *et al.*, 1993). The absence of SP-C mRNA in the atypical respiratory epithelial cells of SP-C/HFH-4 transgenic mice suggests that the inhibitory effect of HFH-4 on SP-C levels is at the transcriptional level.

Consistent with previous studies using the 3.7-kb human SP-C promoter (Wert *et al.*, 1993), transgene expression was detected by *in situ* hybridization in the distal bronchiolar and alveolar epithelium. While the bronchiolar epithelium in SP-C/HFH-4 mice was indistinguishable from nontransgenic littermates as assessed by light microscopy, immunohistochemical analysis revealed altered patterns of gene expression of bronchial epithelial cell markers in the absence of morphological alterations. Previous *in vitro* studies reported that HFH-4 activated a CCSP promoter in transient transfection assays (Lim *et al.*, 1997). However, the present *in vivo* findings demonstrated that HFH-4 inhibited CCSP expression in cells expressing the transgene in the bronchiolar epithelium, as CCSP staining in the bronchiolar epithelium was markedly reduced. Interestingly, β-tubulin IV staining was also reduced in the bronchiolar epithelium of all affected SP-C/HFH-4 founders. While the mechanism underlying this observation is unclear, it is possible that the transdifferentiation of nonciliated bronchiolar cells altered gene expression in adjacent ciliated cells indirectly, perhaps via cell–cell or paracrine mechanisms. The present *in vivo* findings support the concept that HFH-4 restricts cells from the Clara cell lineage, directly or indirectly, and inhibits expression of CCSP, a marker of non-ciliated bronchiolar epithelial cells.

The presence of dilated saccules in the lungs of the affected SP-C/HFH-4 transgenic mice is likely related to defects in branching morphogenesis, a complex process involving inductive interactions between the epithelium and surrounding mesenchyme (Post, 1997). The aberrant differentiation of respiratory epithelial cells in SP-C/HFH-4 transgenic mice may in turn influence responses to inductive signals from the surrounding mesenchyme, in turn disrupting branching morphogenesis. However, the presence of developing acinar buds positive for SP-C mRNA and proprotein in the atypical peripheral regions of lungs from SP-C/HFH-4 founder F3-1 (Figs. 4A and 5E) suggests that the epithelium continues to respond to signals from the mesenchyme.

In established SP-C/HFH-4 lines, lung morphology was unaffected and few cells expressed the SP-C/HFH-4 transgene. Low levels of transgene expression in adult lung may indicate that ectopic expression of HFH-4 in the alveolar epithelium inhibited activity of the 3.7-kb human SP-C promoter earlier in development. This is consistent with the absence of pro-SP-C protein and SP-C mRNA in the abnormal epithelial cells of affected SP-C/HFH-4 transgenic mice on day 18 pc. Thus, high levels of HFH-4 expression may downregulate the SP-C promoter and extinguish transgene expression. Evidence to support this was observed in founder F32, in which certain regions of abnormal epithelium had decreased signal for transgene-specific mRNA, while expression was abundant in other areas of abnormal epithelium. Alternatively, atypical cells expressing the transgene may have lacked appropriate differentiated features necessary for their survival and are simply lost during postnatal morphogenesis and differentiation.

The lack of abnormal lung morphology in established SP-C/HFH-4 lines suggests that high levels of expression of the transgene may have caused neonatal death. The relative rarity of affected animals among fetal founders (4 of 13) and the relationship between transgene expression levels and the severity of the phenotype suggest that relatively high levels of HFH-4 expression were required to alter lung morphogenesis. Neonatal lethality of affected founders could result from inefficient gas exchange due to the presence of the cuboidal and columnar epithelium lining the alveolar compartment and/or an overall reduction in surface area due to the disrupted branching morphogenesis in the lungs of transgenic mice. Lungs of affected founders also contained reduced or absent mature SP-B. Decreased SP-B may limit postnatal survival since gene targeting of SP-B in mice caused respiratory failure in the immediate postnatal period (Clark *et al.*, 1995).

Findings in the SP-C/HFH-4 transgenic mice are similar but distinct from those seen after expression of HNF-3β with the SP-C promoter (Zhou *et al.*, 1997), although both models are consistent with the concept that precise temporal and spatial expression of winged-helix family members is critical to lung morphogenesis and cell differentiation. Atypical columnar epithelium was observed in the lungs of both SP-C/HFH-4 and SP-C/HNF-3β mice, but lung morphology was distinct in the two models. Lungs of SP-C/HNF-3β mice contain convoluted tubules lined by atypical columnar epithelium and have no apparent sacculation,

while lungs of SP-C/HFH-4 mice contain large dilated saccules lined by atypical columnar and ciliated epithelium. Ciliated cells were not observed in the atypical cells seen in SP-C/HNF-3β transgenic mice and disruption of lung morphogenesis was more pronounced in that model. Marked disruption of vasculogenesis was evident in the lungs of SP-C/HNF-3β mice as assessed by electron microscopy, in association with decreased vascular endothelial growth factor mRNA. In contrast, vasculogenesis in the lungs of SP-C/HFH-4 mice was normal as determined by staining for the endothelial cell marker PECAM.

In both SP-C/HFH-4 and SP-C/HNF-3β transgenic mice, expression of differentiated cell markers (SP-B, SP-C, and CCSP) was lacking, demonstrating that altering the levels of winged-helix family members disrupts respiratory epithelial cell gene expression patterns. Interestingly, winged-helix proteins share common structural motifs with linker histones (Clark *et al.*, 1993) and are capable of displacing linker histones from chromatin and positioning nucleosomes (Cirillo *et al.*, 1998; Shim *et al.*, 1998). Thus, altered levels of winged-helix proteins in the developing respiratory epithelium of SP-C/HFH-4 and SP-C/HNF-3β mice may alter gene expression patterns via the ability of the winged-helix domain to affect chromatin structure. The finding that HNF-3β disrupted lung morphogenesis more severely than HFH-4 is perhaps consistent with a primary role of HNF-3β in the regulation of foregut endodermal differentiation. Studies investigating the role of HNF-3β in liver specification and differentiation have placed HNF-3β at the top of a hierarchy of transcription factors required for hepatic cell differentiation (Duncan *et al.*, 1998). Since HFH-4 in the lung is expressed later in development and in a more restricted subset of cells than is HNF-3β, the alterations in lung structure produced by ectopically expressing HFH-4 may be less profound.

Ectopic expression of HFH-4 restricted the normal differentiation of Clara cell and Type II cell lineages in the lung periphery. High levels of HFH-4 expression induced characteristics of ciliated or columnar cells in regions of the lung normally lined by cuboidal and squamous cells. The present findings support the concept that precise levels and combinations of winged-helix transcription factors are required to correctly regulate gene expression and respiratory epithelial cell differentiation during lung development.

ACKNOWLEDGMENTS

The authors thank Dr. Susan Wert and the Molecular Morphology Core at Children's Hospital Research Foundation and Dr. John Neumann and the transgenic mouse core facility at the University of Cincinnati for their excellent help and suggestions. This work was supported by NIH grants HL 56387 (J.A.W. and J.W.T.) and HL 41496 (J.A.W. and J.W.T.) and a grant from the Council for Tobacco Research (R.H.C. and L.L.).

REFERENCES

Adamson, I. Y. R. (1997). Development of lung structure. *In* "The Lung: Scientific Foundations" (R. G. Crystal, J. B. West, E. R. Weibel, and P. J. Barnes, Eds.), Vol. 1, pp. 993–1001. Lippincot-Raven, Philadelphia.

Baatz, J. E., Elledge, B., and Whitsett, J. A. (1990). Surfactant protein SP-B induces ordering at the surface of model membrane bilayers. *Biochemistry* **29,** 6714–6720.

Bannerjee, A., Roach, M. C., Trcka, P., and Luduena, R. F. (1992). Preparation of a monoclonal antibody specific for the class IV isotype of β-tubulin: Purification and assembly of $\alpha\beta_{II}$, $\alpha\beta_{III}$ and $\alpha\beta_{IV}$ tubulin dimers from bovine brain. *J. Biol. Chem.* **267,** 5625–5630.

Bohinski, R. J., Di Lauro, R., and Whitsett, J. A. (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.

Bruno, M. D., Bohinski, R. J., Huelsman, K. M., Whitsett, J. A., and Korfhagen, T. R. (1995). Lung cell-specific expression of the murine surfactant protein A (SP-A) gene is mediated by interactions between the SP-A promoter and thyroid transcription factor-1 *J. Biol. Chem.* **270,** 6531–6536. [published erratum appears in J. Biol. Chem. **270**(27), 16482, 1995].

Chen, J., Knowles, H. J., Hebert, J. L., and Hackett, B. P. (1998). Mutation of the mouse hepatocyte nuclear factor/forkhead homologue 4 gene results in an absence of cilia and random left–right asymmetry. *J. Clin. Invest.* **102,** 1077–1082.

Cirillo, L. A., McPherson, C. E., Brossard, P., Stevens, K., Cherian, S., Shim, E. Y., Clark, K. L., Burley, S. K., and Zaret, K. S. (1998). Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. *EMBO J.* **17,** 244–254.

Clark, J. C., Wert, S. E., Bachurski, C. J., Stahlman, M. T., Stripp, B. R., Weaver, T. E., and Whitsett, J. A. (1995). Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc. Natl. Acad. Sci. USA* **92,** 7794–7798.

Clark, K. L., Halay, E. D., Lai, E., and Burley, S. K. (1993). Co-crystal structure of the HNF-3/forkhead DNA-recognition motif resembles histone H5. *Nature* **364,** 412–420.

Clevidence, D. E., Overdier, D. G., Peterson, R. S., Porcella, A., Ye, H., Paulson, K. E., and Costa, R. H. (1994). Members of the HNF-3/forkhead family of transcription factors exhibit distinct cellular expression patterns in lung and regulate the surfactant protein B promoter. *Dev. Biol.* **166,** 195–209.

Duncan, S. A., Navas, M. A., Dufort, D., Rossant, J., and Stoffel, M. (1998). Regulation of a transcription factor network required for differentiation and metabolism. *Science* **281,** 692–695.

Fanucchi, M. V., Murphy, M. E., Buckpitt, A. R., Philpot, R. M., and Plopper, C. G. (1997). Pulmonary Cytochrome P450 monooxygenase and Clara cell differentiation in mice. *Am. J. Respir. Cell. Mol. Biol.* **17,** 302–314.

Guazzi, S., Price, M., DeFelice, M., Damante, G., Mattei, M.-G., and DiLauro, R. (1990). Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. *EMBO J.* **9,** 3631–3639.

Hackett, B. P., Bingle, C. D., and Gitlin, J. D. (1996). Mechanisms of gene expression and cell fate determination in the developing pulmonary epithelium. *Annu. Rev. Physiol.* **58,** 51–71.

Hackett, B. P., Brody, S. L., Liang, M., Zeitz, I. D., Bruns, L. A., and Gitlin, J. D. (1995). Primary structure of hepatocyte nuclear

factor/forkhead homologue 4 and characterization of gene expression in the developing respiratory and reproductive epithelium. *Proc. Natl. Acad. Sci. USA* **92,** 4249–4253.

Ikeda, K., Shaw-White, J. R., Wert, S. E., and Whitsett, J. A. (1996). Hepatocyte nuclear factor 3 activates transcription of thyroid transcription factor 1 in respiratory epithelial cells. *Mol. Cell. Biol.* **16,** 3626–3636.

Jacob, A., Budhiraja, S., Qian, X., Clevidence, D., Costa, R. H., and Reichel, R. R. (1994). Retinoid acid-mediated activation of HNF-3a during ES stem cell differentiation. *Nucleic Acids Res.* **22,** 2126–2133.

Khoor, A., Stahlman, M. T., Gray, M. E., and Whitsett, J. A. (1994). Temporal–spatial distribution of SP-B and SP-C proteins and mRNAs in developing respiratory epithelium of human lung. *J. Histochem. Cytochem.* **42,** 1187–1199.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M., and Gonzalez, F. J. (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.

Lim, L., Zhou, H., and Costa, R. H. (1997). The Winged helix transcription factor HFH-4 is expressed during choroid plexus epithelial development in mouse embryo. *Proc. Natl. Acad. Sci. USA* **94,** 3094–3099.

Lin, S., Akinbi, H. T., Breslin, J. S., and Weaver, T. E. (1996). Structural requirements for targeting of surfactant protein B (SP-B) to secretory granules in vitro and in vivo. *J. Biol. Chem.* **271,** 19689–19695.

Motoyama, J., Liu, J., Mo, R., Ding, Q., Post, M., and Hui, C.-c. (1998). Essential function of Gli2 and Gli3 in the formation of lung, trachea and esophagus. *Nature Gen.* **20,** 1–4.

Murphy, D. B., Seeman, S., Wiese, S., Kirschner, R., Grzeschik, K. H., and Thies, U. (1997). The human hepatocyte nuclear factor 3/forkhead gene FKHL13: Genomic structure and pattern of expression. *Genomics* **40,** 462–469.

Pelletier, G. J., Brody, S. L., Liapis, H., White, R. A., and Hackett, B. P. (1998). A human forkhead/winged-helix transcription factor expressed in developing pulmonary and renal epithelium. *Am. J. Physiol. (Lung Cell Mol. Physiol.)* **274,** L351–L359.

Post, M. (1997). Tissue interactions. *In* "The Lung: Scientific Foundations" (R. G. Crystal, J. B. West, E. R. Weibel, and P. J. Barnes, Eds.), Vol. 1, pp. 1003–1011. Lippincot–Raven, Philadelphia, PA.

Renthal, R., Schneider, B. G., Miller, M. M., and Luduena, R. F. (1993). β_{IV} is the major β-tubulin isotype in bovine cilia. *Cell. Motil. Cytoskel.* **25,** 19–29.

Shim, E. Y., Woodcock, C., and Zaret, K. S. (1998). Nucleosome positioning by the winged helix transcription factor HNF3. *Genes Dev.* **12,** 5–10.

Singh, G., Singh, J., Katyal, S. J., Brown, W. E., MacPherson, J. A., and Squeglia, N. (1988). Identification, cellular localization, isolation and characterization of human Clara cell-specific 10 KD protein. *J. Histochem. Cytochem.* **36,** 73–80.

Stahlman, M. T., Gray, M. E., and Whitsett, J. A. (1992). The ontogeny and distribution of surfactant protein B in human fetuses and newborns. *J. Histochem. Cytochem.* **40,** 1471–1480.

Stahlman, M. T., Gray, M. E., and Whitsett, J. A. (1996). Expression of thyroid transcription factor-1(TTF-1) in fetal and neonatal human lung. *J. Histochem. Cytochem.* **44,** 673–678.

Stahlman, M. T., Gray, M. E., and Whitsett, J. A. (1998). Temporal–spatial distribution of hepatocyte nuclear factor-3beta in developing human lung and other foregut derivatives. *J. Histochem. Cytochem.* **46,** 955–962.

Tichelaar, J. W., Wert, S. E., Costa, R. H., Kimura, S., and Whitsett, J. A. (1999). HNF-3/forkhead homologue-4 (HFH-4) is expressed in ciliated epithelial cells in the developing mouse lung. *J. Histochem. Cytochem.* **47,** 823.

Wert, S., Glasser, S., Korfhagen, T., and Whitsett, J. (1993). Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev. Biol.* **156,** 426–443.

Zhou, L., Dey, C. R., Wert, S. E., and Whitsett, J. A. (1996a). Arrested lung morphogenesis in transgenic mice bearing an SP-C-TGF-β1 chimeric gene. *Dev. Biol.* **175,** 227–238.

Zhou, L., Dey, C. R., Wert, S. E., Yan, C., Costa, R. H., and Whitsett, J. A. (1997). Hepatocyte nuclear factor-3β limits cellular diversity in the developing respiratory epithelium and alters lung morphogenesis in vivo. *Dev. Dyn.* **210,** 305–314.

Zhou, L., Lim, L., Costa, R. H., and Whitsett, J. A. (1996b). Thyroid transcription factor-1, Hepatocyte nuclear factor-3β, surfactant protein B, C and Clara cell secretory protein in developing mouse lung. *J. Histochem. Cytochem.* **44,** 1183–1193.

Received for publication December 22, 1998
Revised June 7, 1999
Accepted June 10, 1999