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Increased Expression of Thyroid Transcription Factor-1 (TTF-1) in Respiratory Epithelial Cells Inhibits Alveolarization and Causes Pulmonary Inflammation

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Thyroid transcription factor-1 (TTF-1), a member of the Nkx2 family of homeodomain-containing transcription factors, is expressed in the epithelium of the lung. TTF-1 is a critical regulator of transcription for the surfactant proteins (SP) A, B, and C and is essential for lung morphogenesis. Sites and levels of TTF-1 expression vary during lung morphogenesis and following injury. In order to determine the role of TTF-1 in lung formation, transgenic mice were generated in which TTF-1 was expressed in respiratory epithelial cells of wild-type and *Ttf1* null mutant (−/−) mice, using the lung-specific SP-C promoter. The *SP-C-Ttf1* transgene did not rescue the severe pulmonary hypoplasia characteristic of the *Ttf1* (−/−) mice. Increased expression of TTF-1, however, caused dose-dependent alterations in postnatal lung morphology of wild-type mice. Modest overexpression of TTF-1 caused type II cell hyperplasia and increased the cellular content of SP-B. In contrast, higher expression levels of TTF-1 disrupted alveolar septation, causing emphysema. In mice with the highest transgene expression, TTF-1 caused severe inflammation, pulmonary fibrosis, respiratory failure, and death, associated with eosinophil infiltration and increased expression of eotaxin and IL-6. Increased expression of TTF-1 altered alveolarization and caused chronic pulmonary inflammation, demonstrating that precise regulation of TTF-1 is critical for homeostasis in the postnatal lung.

Key Words: **mouse lung; morphogenesis; Nkx2.1; TITF-1; T/EBP; surfactant protein B; eosinophils; eotaxin; IL-6; emphysema.**

INTRODUCTION

Thyroid transcription factor-1 (TTF-1, TITF-1, T/EBP, Nkx2.1) is a 43-kDa member of the Nkx2 family of homeodomain-containing proteins and is selectively expressed in the forebrain, thyroid, and lung. TTF-1 is required for lung and thyroid morphogenesis (Kimura, 1996; Lazzaro, 1991; Minoo, 1999) and for the expression of genes selectively expressed in the respiratory epithelium of the lung (Whitsett, 1998). TTF-1 is expressed during early formation of the lung, the temporal–spatial regulation of TTF-1 production being precisely regulated throughout lung development (Morotti, 2000; Stahlman, 1996; Zhou, 1996a, 2001). TTF-1 expression is most abundant in epithelial cells of the developing pulmonary tubules, decreasing in the proximal conducting airways with advancing gestational age. Expression of TTF-1 is increased by transcription factors HNF-3β and GATA-6, and is induced by members of the fibroblast growth factor (FGF) family of polypeptides, including FGF-7 and FGF-10 (Clark, 2001; Ikeda, 1996; Shaw-White, 1999; Tichelaar, 2000). The levels of TTF-1 protein decrease markedly in late gestation and in the postnatal period of development (Morotti, 2000; Stahlman, 1996; Zhou, 2001). TTF-1 is expressed primarily in type II epithelial cells, less abundantly in subsets of nonciliated respiratory epithelial cells and basal cells in the conducting airways, and is not expressed in type I cells (Morotti, 2000;

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Stahlman, 1996; Zhou, 1996a, 2001). In the postnatal lung, TTF-1 is required for transcription of surfactant proteins (SP) A, B, and C, as well as the Clara cell secretory protein (CCSP) (Bohinski, 1994; Bruno, 1995; Kelly, 1996; Toonen, 1996; Zhang, 1997).

Lungs from *Ttf1* null mutant (−/−) mice consist of tracheal and bronchial tubules, with nearly complete loss of peripheral lung parenchyma, supporting the concept that TTF-1 plays an important role in lung morphogenesis (Kimura, 1996; Minoo, 1999). Since TTF-1 is also expressed in the lung after injury, TTF-1 may play a role in epithelial cell proliferation and differentiation during the repair process. While TTF-1 staining decreases in sites of acute epithelial injury, TTF-1 is markedly increased in regions of lung parenchyma undergoing regeneration and repair (Stahlman, 1996). Taken together, these findings support the concept that precise temporal–spatial regulation of TTF-1 may be important to the normal cytodifferentiation of the epithelium, which accompanies lung morphogenesis and repair following injury. In order to further discern the role of TTF-1 in pulmonary morphogenesis and function, a series of transgenic mice were generated in which the rat TTF-1 cDNA was overexpressed in respiratory epithelial cells. Increased copies of the *SP-C-Ttf1* transgene caused dose-dependent changes in alveolarization and inflammation of the lung.

METHODS

Generation of SP-C-Ttf1 Transgenic Mice

A 1.26-kb fragment containing the entire coding region of the rat TTF-1 cDNA (Guazzi, 1990) (the kind gift of Dr. Roberto DiLauro) was subcloned into a PUC-18 plasmid vector containing the human 3.7 SP-C promoter and SV40 t intron-poly(A) sequences (Fig. 1A). The transgene was propagated in *Escherichia coli* DH 5α cells, and the fragment was excised and purified by using the QIAEX DNA extraction kit (Qiagen Inc., Chatsworth, CA), as described by the manufacturer. After extensive dialysis, the linearized fragment was microinjected into the pronucleus of fertilized eggs from FVB/N mice. Transgenic mice were identified by PCR and Southern blot analyses. PCR products were designed to include TTF-1 cDNA and the SV40 t intron-poly(A) region (TPA) to ensure specificity for identification of the transgene. Southern blot analysis was performed, after restriction fragment digestion with *Eco*RI, to confirm the presence and size of the transgene using the TPA probe for hybridization. To estimate DNA copy number, Southern blot analysis was performed by using a 1.2-kb TTF-1 cDNA fragment as the probe. Quantitation was performed by phosphorimaging (Storm 860; Molecular Dynamics, Sunnyvale, CA). Phosphorimage units for the transgene were normalized to that for the endogenous *Ttf1* gene, and the average density ratios for each transgenic line were compared to estimate relative DNA copy number. Two permanent founder lines of *SP-C-Ttf1* transgenic mice, one with multiple copies of the transgene (Hi) and one with a single copy of the transgene (Lo), were established and maintained in the vivarium.

Animal Husbandry

All mice were maintained in a barrier facility, and animals were handled under IACCAC approved protocols. There was no serologic evidence of viral pathogens or bacterial infections in sentinal mice maintained with the colony. No serological evidence of viral infection or histological evidence of bacterial infection was detected in transgenic mice at necropsy. All genotypes were maintained in the FVB/N strain. DNA copy number and levels of *Ttf1* transgene expression were altered by crossing the separate Hi (+/−) and Lo (+/−) transgenic lines to produce Hi (+/−), Lo (+/−) and Hi (+/−), Lo (+/+) bitransgenic mice. The *Ttf1* (−/−) mice were originally generated in C57BL/6N (B6), 129S4/5vJae mice in the laboratory of Dr. Kimura at the National Institutes of Health (Kimura, 1996), maintained as heterozygotes (+/−), and bred to the *SP-C-Ttf1* transgenic line, Hi (+/−), to generate *Ttf1* (−/−), *SP-C-Ttf1* (+/−) mice.

mRNA Analysis

Northern blot analysis, using the [^{32}P]-labeled 0.47-kb TPA fragment from the transgene as a probe, was performed with mRNA isolated from the lungs of transgenic mice and wild-type littermate controls. A full-length TTF-1 cDNA probe was used to detect endogenous mouse TTF-1 mRNA. Mouse SP-C mRNA was detected by using a radiolabeled, full-length, 0.9-kb SP-C cDNA probe.

Histology and Immunohistochemistry

Tissue sections were prepared from fetal (E18–E18.5), neonatal (newborn, postnatal days 7 and 14), juvenile (postnatal days 21 and 28), and adult lungs fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). To obtain fetal tissue, dams were anesthetized with a 4:1:1 mixture of ketamine, acepromazine, and xylazine and then exsanguinated by severing the inferior vena cava and descending aorta. The fetuses were removed by hysterotomy and bisected below the diaphragm, which was stripped away from the rib cage, before immersion in fixative overnight. Postnatal animals were anesthetized, weighed, and then exsanguinated as described above. The trachea was cannulated, and the lungs were collapsed by piercing the diaphragm. The lungs were inflation-fixed at 25 cm of water pressure for 1 min. Tissue was immersion-fixed overnight, washed in PBS, dehydrated through a series of alcohols and xylenes, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin, Masson's trichrome stain, and orcein in order to analyze histopathological changes in the tissue. Immunohistochemistry for eosinophil major basic protein (Matthews, 1998), TTF-1, SP-B, SP-C preproprotein (proSP-C), and proliferating cell nuclear antigen (PCNA) (clone PC10, Zymed Laboratories Inc., South San Francisco, CA) was performed as described previously (Zhou, 1996a), using biotinylated primary or secondary antibodies and avidin-biotin-peroxidase (Vector Elite ABC kit; Vector Laboratories Inc., Burlingame, CA) or strepavidin (Zymed Laboratories) detection systems. The enzymatic reaction was enhanced with nickel cobalt, and the sections were 2counterstained with nuclear fast red. Elimination of the primary antibody from the reaction served as a negative control for nonspecific binding of the secondary antibody, as well as for other kit components. Mouse intestine and esophagus were used as positive controls for the PCNA reaction; normal adult mouse lung served as a negative control for this cellular proliferation assay.

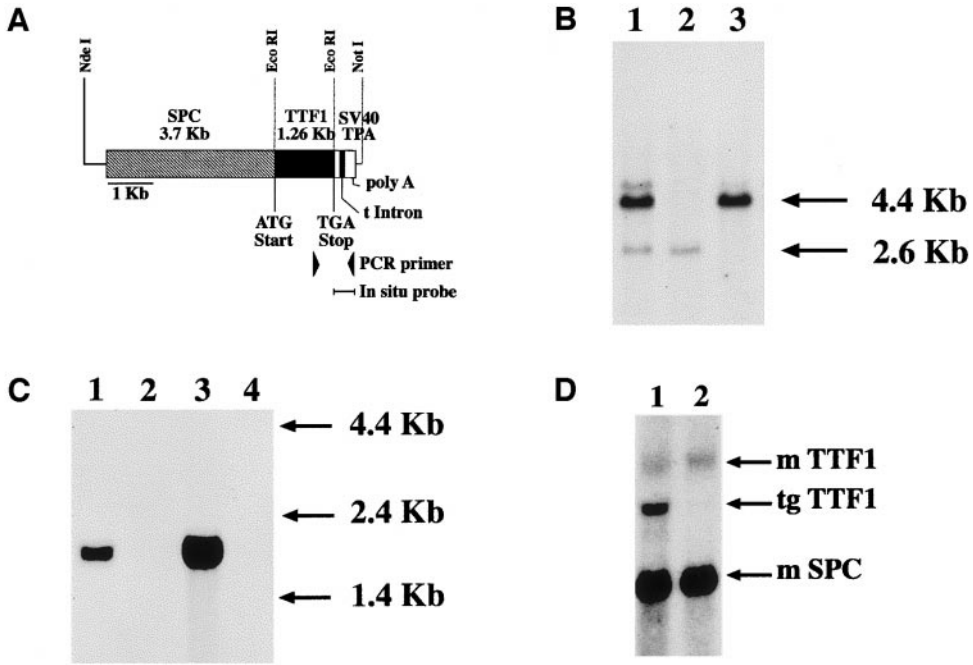


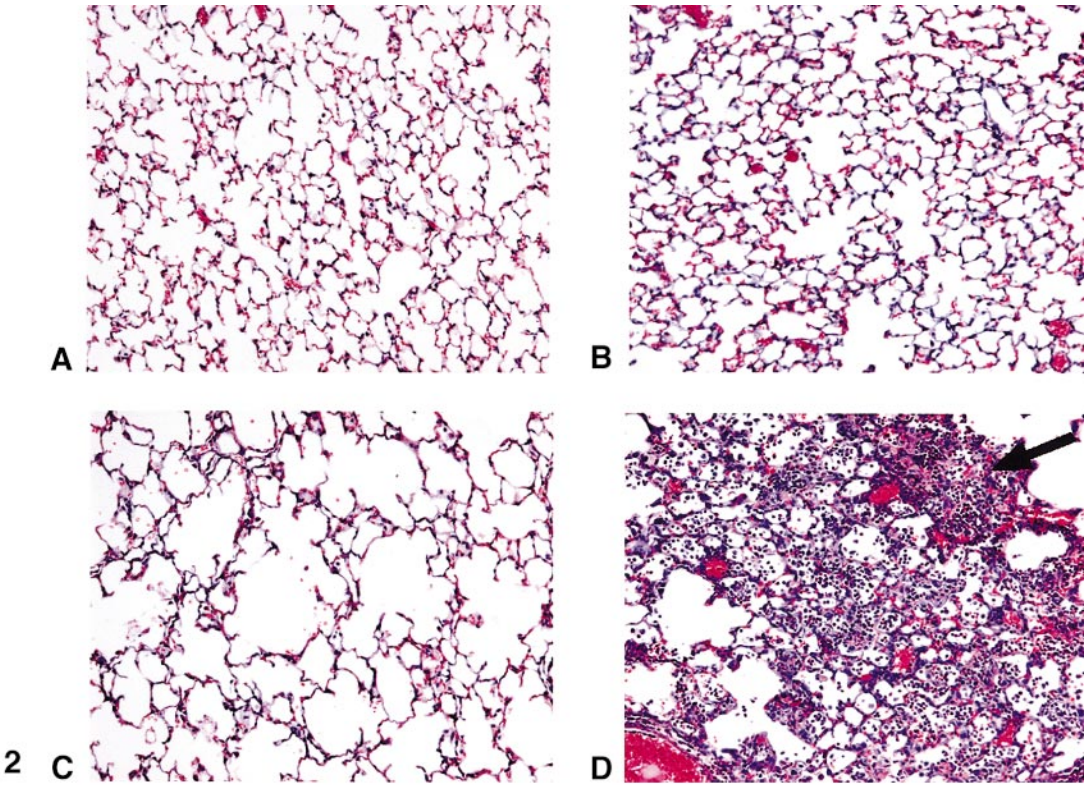
FIG. 1. *SP-C-Ttf1* transgenic construct, genomic integration, and mRNA expression in the lung. (A) Construction of the human 3.7 *SP-C-Ttf1* transgene. A 1.26-kb fragment of the coding region of rat TTF-1 cDNA was inserted into an *Eco*RI site in the multiple cloning site of a PUC-18 plasmid vector, between the human 3.7 SP-C promoter and the TPA. The TTF-1 start and stop codons and the SV40 t intron and polyadenylation signal are indicated. Arrowheads indicate the sites of primers used for the PCR genotyping. Bar represents the 0.47-kbp TPA fragment used to generate probes for DNA, mRNA, and *in situ* hybridization analysis of transgene expression. (B) Southern blot analysis of transgene integration and germ line transmission. Tail DNA (3 μg) was digested with *Eco*RI and hybridized with a [^{32}P]-labeled TPA probe. The 4.4-kb band represents transgenic DNA that integrated into the Hi (+/−) copy founder line as concatamers. The 2.6-kb band represents integration of a single copy of the transgene into the Lo (+/−) copy founder line. Lane 1, cross between Hi (+/−) and Lo (+/−) copy founder lines to produce Hi (+/−), Lo (+/−) bitransgenic mouse; Lane 2, Lo (+/−) copy founder line; Lane 3, Hi (+/−) copy founder line. (C) Northern blot analysis of transgene expression in the lung. Northern blot analysis of total lung mRNA using a [^{32}P]-labeled TPA probe was performed to confirm transgene expression in the lung. Higher levels of transgenic mRNA were observed in the Hi (+/−) copy founder line (lane 3) compared with the Lo (+/−) copy founder line (lane 1). Lane 1, Lo (+/−) copy founder line; Lanes 2 and 4, nontransgenic control; Lane 3, Hi (+/−) copy founder line. (D) Northern blot analysis of TTF-1 and SP-C expression in the lung. Northern blot analysis of total lung mRNA using a [^{32}P]-labeled TTF-1 probe was performed to compare the expression of endogenous and transgenic TTF-1 mRNA in the Hi (+/−) copy founder line. Transgenic TTF-1 mRNA expression was greater than that observed for endogenous TTF-1 in transgenic mice (lane 1). Endogenous levels of TTF-1 and SP-C mRNA in transgenic mice were comparable to those observed in nontransgenic controls (lane 2). Lane 1, Hi (+/−) copy founder line; Lane 2, nontransgenic control.

In Situ Hybridization

Expression of *SP-C-Ttf1* transgene mRNA was assessed by *in situ* hybridization using [^{35}S]-labeled TPA riboprobes, as previously described (Wert, 1993; Zhou, 1996b). Tissue sections were hybridized overnight at 42°C, washed, treated with 20 μg/ml of RNAse for 10 min at room temperature, dehydrated, and coated with Kodak NTB2 emulsion. Slides were autoradiographed for 2–6 weeks before being developed with Kodak D19 developer. Results were photographed with darkfield illumination.

Protein Measurements

Mice were anesthetized and exsanguinated as described above. The trachea was cannulated, and the lungs were lavaged three times with 1-ml aliquots of sterile PBS. Bronchoalveolar lavage fluid (BALF) for each animal was pooled, and the volumes were measured. The cells were removed by centrifugation and the samples were stored at −20°C. SP-B content was measured by enzyme-linked immunosorbent assay (ELISA), using bovine SP-B as the standard, and was expressed as ng/mg of protein (Pryhuber, 1991). Total protein in the pooled samples was determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Eotaxin content was quantitated as previously described, using mouse eotaxin as the quantitation standard (Mishra, 2001), and expressed as pg/ml of BALF. Tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and macrophage inflammatory protein-2 (MIP-2) levels were quantitated by using murine sandwich ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's directions, and expressed as mg or pg per ml of BALF. All plates were read on a microplate reader at



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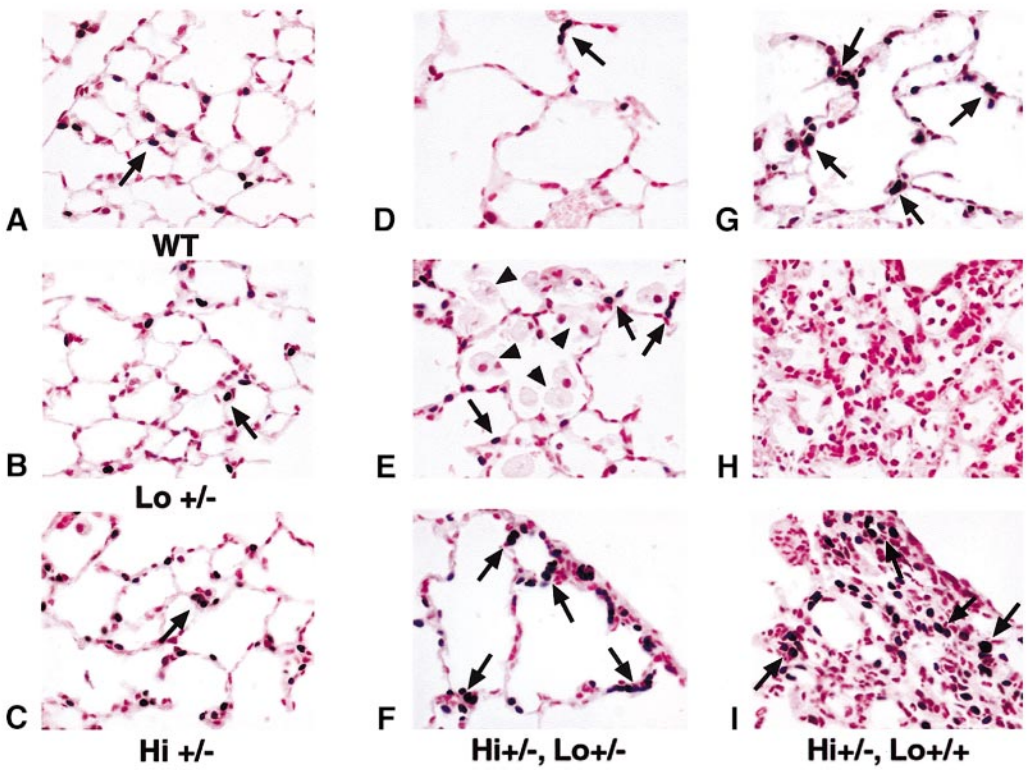

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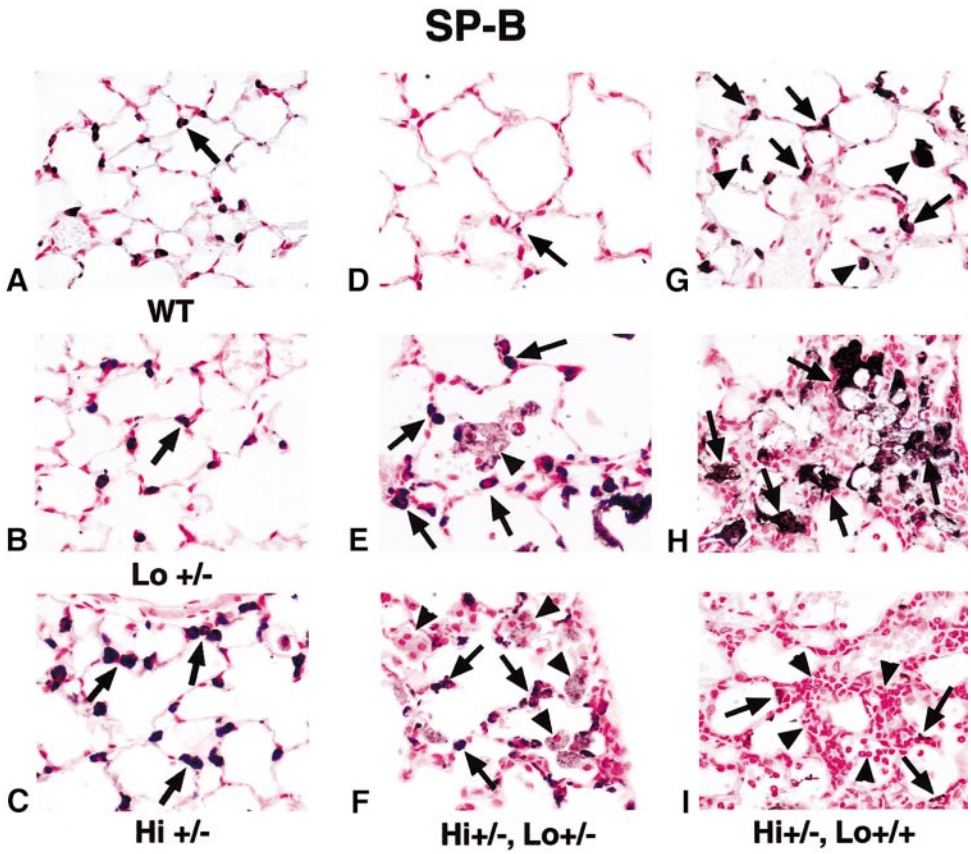


FIG. 4. Immunohistochemistry for SP-B. SP-B was detected by immunostaining with a rabbit polyclonal antibody to the mature, hydrophobic SP-B peptide as described in Methods. SP-B was detected as a black precipitation product in the cytoplasm of alveolar type II cells (arrows) throughout the lungs of both wild-type (WT) littermate controls (A) and Lo (+/−) transgenic mice (B). SP-B-positive cells were distributed in a ratio of one to two cells per alveoli, similar to that for TTF-1-positive cells in these genotypes. Increased cytoplasmic staining for SP-B was detected in clusters of alveolar type II cells (arrows) found in the Hi (+/−) transgenic mice (C). Enlarged alveoli with decreased staining for SP-B (arrow) were found in the Hi (+/−), Lo (+/−) bitransgenic mice (D), although increased staining for SP-B could still be found in alveolar type II cells (arrows) adjacent to focal areas of macrophage infiltration (arrowheads) (E and F). Increased staining for SP-B was detected in alveolar type II cells (arrows) of the Hi (+/−), Lo (+/+) bitransgenic mice, as well as in alveolar macrophages (arrowheads) (G). Increased staining for SP-B was detected in secreted, proteinaceous material (arrows) found in focal areas of fibrosis, while decreased staining for SP-B (arrows) was found in regions of acute eosinophilic inflammation (arrowheads) (I). Figures are representative of $n > 4$ animals per genotype. Original magnification for all panels, 440×.

FIG. 2. Histopathology of transgenic and bitransgenic mouse lung. Both heterozygous Lo (+/−) and homozygous Lo (+/+) transgenic mice had normal appearing lungs with no evidence of inflammation or alveolar remodeling (data not shown). Heterozygous Hi (+/−) mice (A) also had normal-appearing lungs compared with their nontransgenic littermate controls (B). Enlarged, emphysematous alveoli were found throughout the lung in the heterozygous Hi (+/−), Lo (+/−) bitransgenic mice (C). Eosinophilic infiltrates with focal areas of fibrosis (arrow) were found throughout the lungs of bitransgenic mice heterozygous for the Hi (+/−) copy transgene and homozygous for the Lo (+/+) copy transgene (D). Perivascular infiltrates were also found in the latter genotype (not shown). Figures are representative of $n > 4$ individual animals per genotype. Original magnification for all panels, 110×.

FIG. 3. Immunohistochemistry for TTF-1. TTF-1 was detected by immunostaining with a rabbit polyclonal antibody generated to rat TTF-1, as described in Methods. TTF-1 was detected as a black precipitation product in the nuclei of alveolar type II cells (arrows) in both wild-type (WT) littermate controls (A) and Lo (+/−) transgenic mice (B). TTF-1 immunopositive cells were distributed throughout the lung at a ratio of one to two cells per alveoli in both WT and Lo (+/−) transgenic mice. In Hi (+/−) transgenic mice, TTF-1 staining was detected in clusters of two to four type II cells per alveoli (arrow), indicating a mild hyperplasia of the alveolar cells expressing this transgene (C). Enlarged alveoli with decreased TTF-1 staining of the remaining alveolar type II cells were detected in the lungs of bitransgenic Hi (+/−), Lo (+/−) bitransgenic mice (D), although clusters of two to three TTF-1-positive cells were found occasionally (arrow). Increased staining for TTF-1 was detected in the nuclei of type II cells (arrow) adjacent to focal areas of macrophage accumulation (arrowhead) (E) and at the pleural surface (F). In bitransgenic Hi (+/−), Lo (+/+) bitransgenic mice, clusters of TTF-1-positive type II cells (arrows) were detected in focal areas of emphysema (G) and fibrosis (H), but not in regions of acute eosinophilic inflammation (I). Figures are representative of $n > 4$ animals per genotype. Original magnification for all panels, 440×.

495 nm, and the results were determined with the use of computer-assisted analysis.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical significance was determined by using the Student's *t* test for comparison of SP-B concentrations in BALF, the Mann–Whitney test for comparison of eotaxin levels in BALF (due to unequal variances in the comparison groups), and analysis of variance followed by the Student–Newman–Keuls multiple comparison test for analysis of the total number of cells recovered in BALF from bitransgenic and control mice.

RESULTS

Two separate *SP-C-Ttf1* founder lines with high (Hi) and low (Lo) levels of transgene expression were generated and maintained in the FVB/N strain. The transgene were maintained in *SP-C-Ttf1* heterozygous (+/−) mice. Southern blot analysis was used to determine germ line transmission (Fig. 1B) and to demonstrate increased copy number of the transgene in each line. Phosphorimaging was used to estimate copy numbers, which were approximately three copies in the Hi (+/−) and four copies in the Hi (+/−),Lo (+/−) bitransgenic lines when compared to the single copy Lo (+/−) transgenic line (n = 3–4 animals per genotype; data not shown). Northern blot analysis using the TPA probe was performed to compare the expression of transgene mRNA in the lungs of both Hi (+/−) and Lo (+/−) copy founder lines (Fig. 1C). Transgenic mRNA expression was greater in the Hi (+/−) copy line compared with the Lo (+/−) copy line. Northern blot analysis, using the rat TTF-1 probe, demonstrated that expression of the *Ttf1* transgene mRNA species appeared to be higher than that of the endogenous TTF-1 mRNA in the Hi (+/−) copy line (Fig. 1D). Endogenous levels of SP-C mRNA in transgenic mice were comparable to those observed in nontransgenic controls (Fig. 1D). While lung histology in the Lo (+/−), Lo (+/+), and Hi (+/−) transgenic mice was relatively unaltered, dramatic histologic changes were evident in both Hi (+/−), Lo (+/−) and Hi (+/−), Lo (+/+) bitransgenic mice (Fig. 2). The severity of the abnormalities varied with transgene dosage. Bitransgenic Hi (+/−), Lo (+/+) mice failed to thrive and did not live beyond 3–4 weeks of age. Animals with the Hi (+/+) genotype were almost never encountered in the colony. Lung histology of a single Hi (+/+) transgenic mouse available for analysis demonstrated severe emphysema and pulmonary fibrosis (data not shown).

Histology and Immunohistochemistry for TTF-1 and SP-B

While no clear abnormalities were seen by histologic assessment of lungs from adult Lo (+/−), Lo (+/+), or Hi (+/−) transgenic mice when compared with wild-type littermates, abnormalities in lung morphology were readily observed in the bitransgenic mice (Fig. 2). Increased numbers of type II epithelial cells were observed in the Hi (+/−) transgenic mice, often appearing as abnormal clusters of type II cells whose nuclei stained intensely for TTF-1 (Fig. 3C). Lung histology was more severely disrupted in both the Hi (+/−), Lo (+/−) and Hi (+/−), Lo (+/+) bitransgenic mice. Lungs of Hi (+/−), Lo (+/−) bitransgenic mice were clearly abnormal, consisting of emphysematous lesions with poor alveolar septation (Fig. 2C), as well as a paucity of type II cells with decreased TTF-1 staining (Fig. 3D). Robust staining for TTF-1 was detected, however, in alveolar type II cells located adjacent to focal regions of increased macrophage infiltration and in prefibrotic lesions at the periphery of the lung (Figs. 3E and 3F). Even more dramatic abnormalities in lung morphology were noted in the Hi (+/−), Lo (+/+) bitransgenic mice. These lungs were markedly emphysematous and infiltrated with macrophages and eosinophils (Fig. 2D). Interstitial thickening and fibrosis were observed in the lung periphery. Regions of hyperplastic type II cells staining intensely for TTF-1 were noted in both emphysematous and fibrotic lesions (Figs. 3G and 3I). Absent and/or decreased TTF-1 staining was observed, however, in regions of severe inflammation (Fig. 3H).

Differences in the staining patterns for SP-B expression correlated with that for TTF-1 in all genotypes. Robust cellular expression of SP-B was found by immunohistochemistry in alveolar type II cells of wild-type, Lo (+/−), and Hi (+/−) transgenic mouse lungs (Figs. 4A–4C). Increased cellular staining for SP-B was detected in the clusters of hyperplastic type II cells found in the Hi (+/−) transgenic mice (Fig. 4C). Cellular staining for SP-B was absent or decreased in emphysematous regions of bitransgenic Hi (+/−), Lo (+/−) mouse lungs (Fig. 4D), similar to that observed for TTF-1 staining, while SP-B staining was maintained or increased in alveolar type II cells adjacent to foci of alveolar macrophage accumulation and in prefibrotic lesions found at the periphery of the lung (Figs. 4E and 4F). Staining for SP-B was decreased or absent in regions of severe eosinophil infiltration in the Hi (+/−), Lo (+/+) bitransgenic mice (Fig. 4I), but was maintained or increased in alveolar type II cells located in noninflamed regions of the lung (Fig. 4G), correlating with TTF-1 expression in these foci. SP-B-positive alveolar macrophages and secreted proteinaceous material were found throughout the lungs of the Hi (+/−), Lo (+/+) bitransgenic mice (Figs. 4G and 4H), suggesting increased synthesis and secretion of SP-B. This was confirmed by quantitative analysis of the SP-B content of BALF from the Hi (+/−), Lo (+/+) bitransgenic mice. SP-B concentration in the BALF of postnatal day-21, Hi (+/−), Lo (+/+) bitransgenic mice was elevated significantly compared with age-matched, nontransgenic controls: 95.4 ± 17.9 ng/mg protein (n = 4) vs. 43.3 ± 9.85 ng/mg of protein (n = 5), respectively (P = 0.025).

Pulmonary Inflammation and Cytokines

BALF from postnatal day-21 and -28 Hi (+/−), Lo (+/+) bitransgenic mice contained increased numbers of eosinophils, large foamy macrophages, and neutrophils, consistent with the histopathologic findings (Fig. 5). Differential cell counts revealed a significant increase in eosinophils (70%), with a modest increase in neutrophils (5%), when compared with that for nontransgenic littermates, in which 98% of the cells were macrophages and <2% were neutrophils (Jain-Vora, 1997). The total number of inflammatory cells was markedly increased from $4.8 \pm 0.4 \times 10^4$ cells in the nontransgenic controls to $3.4 \pm 0.5 \times 10^5$ cells in the Hi (+/−), Lo (+/+) bitransgenic mice, ($P = 0.03$; $n = 3$ for each genotype). Inflammation was associated with increased production of eotaxin, an eosinophil-selective chemoattractant normally produced by pulmonary epithelial cells (Rothenberg, 1999). Eotaxin levels in the BALF from the Hi (+/−), Lo (+/−) bitransgenic mice were 3840 ± 2460 pg/ml ($n = 9$) vs. 194 ± 32 pg/ml ($n = 5$) for the nontransgenic control mice ($P = 0.001$). Similarly, IL-6 was markedly increased in the BALF of the Hi (+/−), Lo (+/+) bitransgenic mice to 430.5 ± 77 pg/ml ($n = 5$), being undetectable in the controls ($n = 8$). Extremely low levels of IL-1β (5.5 ± 2 pg/ml) and MIP-2 (3.15 ± 1.5 mg/ml) were observed in the BALF of the Hi (+/−), Lo (+/+) bitransgenic mice ($n = 5$), while neither cytokine was detectable in the controls ($n = 8$). TNF-α was not detected in the BALF from either genotype.

Cellular Proliferation

Because type II cell hyperplasia, emphysema, and fibrosis were noted in the histologic studies, immunohistochemistry for PCNA was used to determine whether proliferative activity was increased in the pulmonary epithelium. In general, PCNA staining was not detected in the alveolar epithelium of wild-type, Lo (+/−), or Hi (+/−) transgenic mice (data not shown). In the lung periphery, foci of PCNA-positive alveolar type II cells admixed with PCNA-positive cells with condensed nuclei were detected in the Hi (+/−), Lo (+/−) bitransgenic mice (Figs. 6A and 6B). Large, emphysematous alveoli with no PCNA staining were found in the centrolobular regions of these lungs (Fig. 6F). This pattern of PCNA expression suggests that increased cellular proliferation followed by rapid cell death might account for the loss of alveolar epithelium in these mice, which results in emphysema. On the other hand, robust nuclear staining for PCNA was detected in a variety of cells found throughout the lungs of the Hi (+/−), Lo (+/+) bitransgenic mice, i.e., in alveolar type II cells, in inflammatory cells, and in presumptive fibroblasts located in focal regions of fibrosis (Figs. 6D–6F). This reactive, hyperplastic pattern of PCNA staining in alveolar type II cells correlates with that for TTF-1 and SP-B staining shown previously in these cells. This suggests that high levels of TTF-1 may be involved in reparative and/or proliferative responses to acute and/or chronic inflammation in the lung.

Development of TTF-1 Induced Lesions

In order to assess whether TTF-1 altered lung morphogenesis during fetal development or caused alterations in postnatal alveologenesis, lung histology was compared in fetal mice at E18.5 and in postnatal mice at day 14. At E18.5, lung morphology of all genotypes was normal, and no difference in TTF-1, SP-B, or PCNA staining were detected (data not shown). Abnormalities in alveolar structure were detected in the Hi (+/−) transgenic mice and in both the Hi (+/−), Lo (+/−) and Hi (+/−), Lo (+/+) bitransgenic mice at postnatal day 14, indicating that overexpression of TTF-1 perturbed alveolarization (Fig. 7). Alveologenesis appeared to be delayed in the 14-day-old Hi (+/−) transgenic mice (Fig. 7B) compared with the adult Hi (+/−) transgenic mouse lung (Fig. 2A), while enlarged, emphysematous alveoli and inflammation were detected in both the Hi (+/−), Lo (+/−) and Hi (+/−), Lo (+/+) bitransgenic mice at 14 days of age (Figs. 7C and 7D).

In Situ Hybridization for the TTF-1 Transgene

In order to detect exogenous transgene expression in lungs of the *SP-C-Ttf1* transgenic mice, a unique sequence from the 3′ region of the *SP-C-Ttf1* transgene, containing the TPA, was utilized as an *in situ* hybridization probe. Transgene mRNA was detected in most type II epithelial cells in lungs of both Lo (+/−) and Hi (+/−) transgenic mice (Figs. 8B and 8C), consistent with the known cellular expression of the 3.7 human SP-C promoter element used in the construct (Wert, 1993). Transgene expression was more abundant in the Hi (+/−) transgenic mice than in the Lo (+/−) transgenic mice, consistent with the Northern blot analysis, immunohistochemistry for TTF-1 and SP-B, and with the increased copy number of transgenic DNA observed in the Hi vs. Lo transgenic lines. Expression of the transgene was also readily detected in both the Hi (+/−), Lo (+/−) and Hi (+/−), Lo (+/+) bitransgenic mice (Figs. 8D and 8E), although the transgene mRNA was not uniformly distributed. Transgene mRNA was detected in areas of lung hyperplasia and fibrosis, but was decreased in areas of the lung with severe inflammation and emphysema, consistent with immunohistochemical localization of TTF-1 protein.

SP-C-Ttf1 Fails to Rescue Pulmonary Hyperplasia in Ttf1 (−/−) Mice

In order to assess whether the expression of TTF-1 by the SP-C promoter might rescue the *Ttf1* (−/−) mice during fetal development, the Hi (+/−) transgenic mice were bred to heterozygous *Ttf1* (+/−) mice to generate *Ttf1* (−/−), *SP-C-Ttf1* (Hi +/−) transgenic mice. The lung phenotype of these mice was similar to that of the *Ttf1* (−/−) mice, demonstrating a lack of correction of the severe pulmonary hypoplasia found in these animals by insertion of the *SP-C-Ttf1* transgene (data not shown). This result is consistent with the requirement of TTF-1 expression for activity of the SP-C promoter and, therefore, is consistent with the

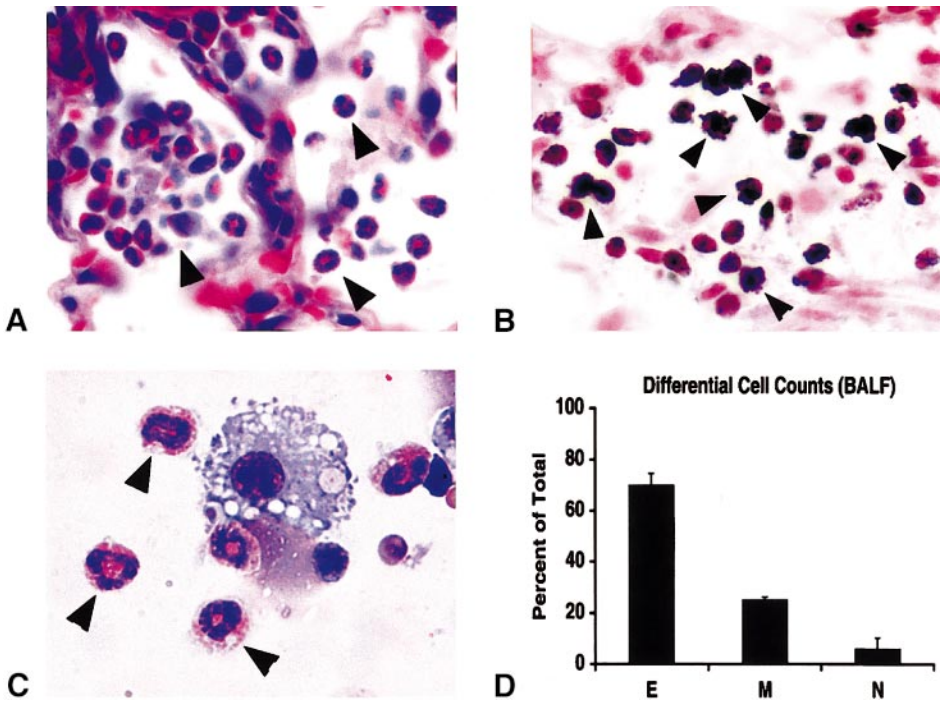


FIG. 5. Inflammation in the Hi (+/−), Lo (+/+) bitransgenic mice. Inflammatory infiltrates in the Hi (+/−), Lo (+/+) bitransgenic mice were composed primarily of eosinophils (arrowheads; A), which were immunopositive for eosinophil major basic protein (arrowheads; B). Cytospins of inflammatory cells recovered by bronchoalveolar lavage were composed primarily of eosinophils (arrowheads) and large foamy macrophages (C). Very few neutrophils were found in either cytospins or tissue sections. Differential cell counts of inflammatory cells recovered by bronchoalveolar lavage revealed that 70% of the cells were eosinophils (E), 25% were macrophages (M), and 5% were neutrophils (N) (D). Original magnification of (A) and (B), 800×; (C), 1600×.

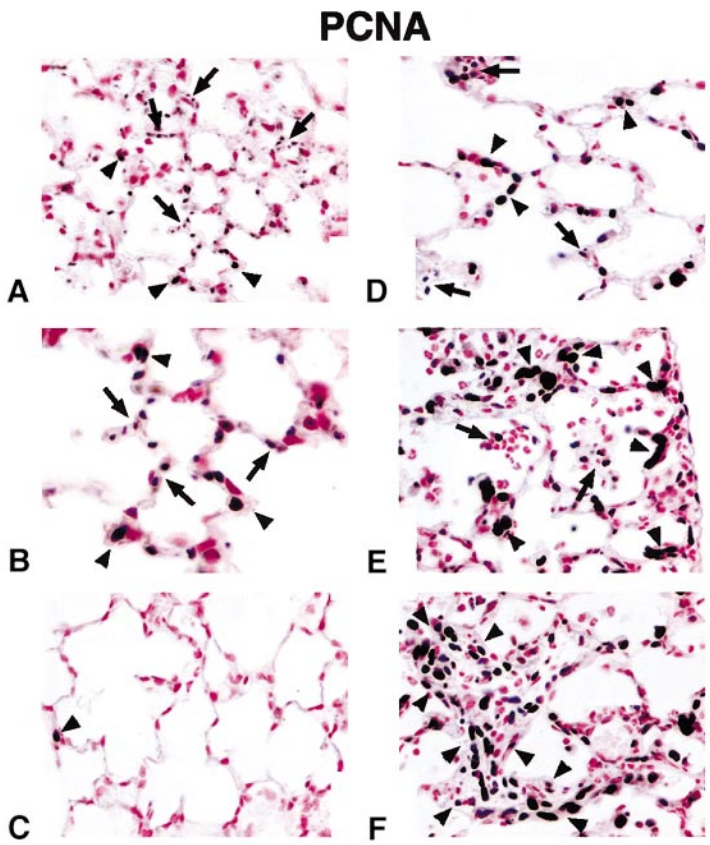
proposed role of TTF-1 as an upstream regulator of SP-C gene expression.

DISCUSSION

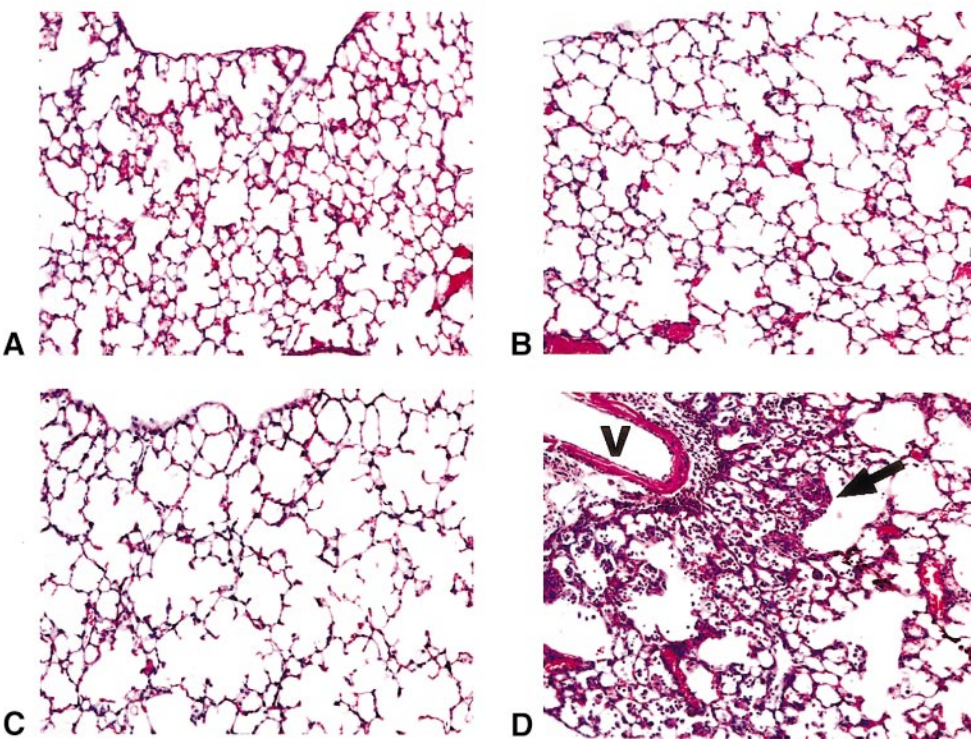
Increased expression of TTF-1 in alveolar type II cells caused dose-dependent alterations in alveolar morphology, epithelial cell hyperplasia, emphysema, and pulmonary inflammation. Although the *SP-C-Ttf1* transgene failed to rescue the severe pulmonary hypoplasia typical of the *Ttf1* (−/−) mice, increased expression of TTF-1 did not alter prenatal lung formation but caused abnormalities in alveolarization in the postnatal period. The present study demonstrates that precise temporal–spatial expression of TTF-1 is required for normal

FIG. 6. Immunohistochemistry for cellular proliferation using PCNA antibody. PCNA was detected by immunostaining with a biotinylated mouse monoclonal antibody as described in Methods. PCNA staining was detected as a black precipitation product that was localized in the cell nucleus. No PCNA staining was detected in the alveolar epithelium of wild-type (WT) littermate controls or in Lo (+/−) or Hi (+/−) transgenic mice (data not shown). In the bitransgenic Hi (+/−), Lo (+/−) mice, focal areas of PCNA-positive type II cells (arrowheads) and PCNA-positive, alveolar epithelial cells with condensed nuclei (arrows) were found in the periphery of the lung (A). These cells are illustrated at a higher magnification in (B). Only rarely were there any PCNA-positive cells (arrowhead) detected in the more emphysematous, centrolobular regions of the lungs from these mice (C). In contrast, PCNA-positive cells were detected throughout the lungs of the Hi (+/−), Lo (+/+) bitransgenic mice (D, E), including PCNA-positive alveolar epithelial cells (arrowheads), PCNA-positive inflammatory cells (arrows), and presumptive fibroblasts with elongated PCNA-positive nuclei, which were found in multiple focal areas of fibrosis (arrowheads, F). Figures are representative of $n > 4$ animals per genotype. Original magnification of (B), 880×; for all other panels, the original magnification, 440×.

FIG. 7. Developmental progression of the histopathological findings. Alveolar septation was nearly complete at 14 days after birth in nontransgenic littermate controls (A). Alveologenesis was delayed in the Hi (+/−) transgenic mice (B) and disrupted in the Hi (+/−), Lo (+/−) bitransgenic mice (C). Acute eosinophilic inflammation with focal areas of fibrosis (arrow) was observed in the Hi (+/−), Lo (+/+) bitransgenic mice at this time point. Figures are representative of two to three animals per genotype; v, pulmonary vein. Original magnification of all panels, 110×.



6 **Hi +/-, Lo+/-** **Hi+/-, Lo+/+**



7

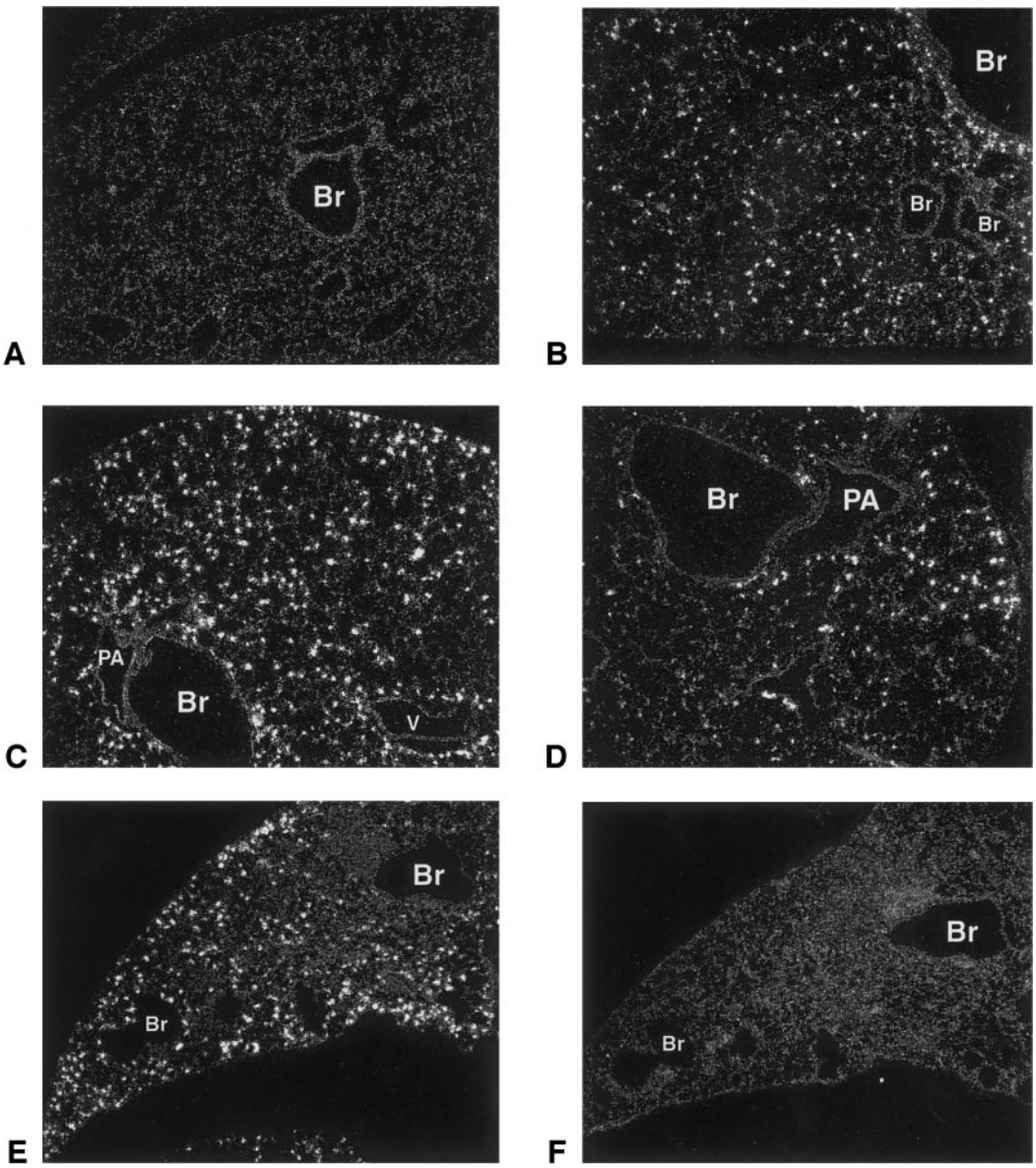


FIG. 8. *In situ* hybridization analysis for transgene expression in the lung. Distribution of the *SP-C-Ttf1* transgene was analyzed by *in situ* hybridization using [^{35}S] labeled TPA sense and anti-sense riboprobes as described in Methods. Results were photographed with dark field illumination. No hybridization signals were detected for transgene expression in nontransgenic littermate controls (A). Uniform signals for transgene mRNA expression were detected throughout the alveolar regions of mice expressing the Lo (+/−) copy transgene (B). In mice with the Hi (+/−) copy transgene, there was an increase in the number of alveolar cells expressing the transgene, as well as an increase in signal strength (C). A mixture of scattered foci of cells exhibiting either weak, intense, or no hybridization signals for transgene expression was found in the Hi (+/−), Lo (+/−) bitransgenic mice (D). Intense hybridization signals for transgene mRNA were found in fibrotic regions of the lungs of Hi (+/−), Lo (+/+) bitransgenic mice, while regions of acute inflammation lacked transgene expression (E). No hybridization signals were detected when tissue from the latter genotype was hybridized with the radiolabeled TPA sense control probe (F). Br, bronchiole; PA, pulmonary artery. Figures are representative of $n > 4$ animals per genotype. Original magnification for all panels, 44×.

postnatal lung maturation. A surprising link between the expression of TTF-1, a nuclear transcription factor expressed only in epithelial cells, and recruitment of inflammatory cells was observed in mice bearing the highest copy number of the transgene, supporting unexpected relationships between transcriptional control of respiratory epithelial cell proliferation, differentiation, and function (via TTF-1) and inflammation and alveolar remodeling.

Failure of the SP-C-Ttf1 Transgene to Rescue the Ttf1 (−/−) Hypoplastic Lungs

The *SP-C-Ttf1* transgene failed to rescue the severe pulmonary hypoplasia typical of the *Ttf1* (−/−) mice. This finding perhaps is not surprising, since *in vitro* studies demonstrated that TTF-1 itself is required for transcriptional activity of the SP-C promoter (Kelly, 1996), while *in vivo* studies demonstrated that SP-C is not detected in *Ttf1* (−/−) mice (Tichelaar *et al.*, 1999). The human SP-C promoter is active as early as day 9.5–10 of gestation, while the endogenous mouse SP-C mRNA is readily detectable as early as day 10–11 of gestation (Glasser, 1991; Wert, 1993). TTF-1 protein is detected at post-conception 9–9.5 in the lung buds at sites consistent with those of SP-C expression (Zhou, 1996a). The failure of the transgene to rescue the hypoplastic lungs of the *Ttf1* (−/−) mice suggests that either TTF-1 is required for the activation of the *SP-C-Ttf1* transgene or the commitment of cells to lineages expressing SP-C has not occurred in the embryonic lung in the absence of TTF-1. This result supports the concept that TTF-1 is an upstream regulator required for commitment and proliferation of cells forming the alveolar parenchyma, as well as for the expression of the surfactant proteins.

TTF-1 Regulation of SP-B Expression

TTF-1 has been shown to be an important transcriptional regulator of the SP-B gene (Bohinski, 1994). In this study, increased cellular staining and secretion of SP-B was correlated with TTF-1 expression. Increased SP-B staining was observed in Hi (+/−) transgenic mice in the absence of inflammation, as well as in bitransgenic mice with emphysema and/or severe eosinophilic inflammation. Increased and/or decreased cellular staining patterns for TTF-1 and SP-B were associated in all genotypes, as well as within various histopathological lesions, suggesting that a very tight linkage exists between the expression of these two genes.

Alveolar Abnormalities in SP-C-Ttf1 Transgenic Mice

Gene dose-dependent abnormalities in lung morphology were consistently observed in the alveolar regions of *SP-C-Ttf1* transgenic mice. In spite of the fact that the *SP-C-Ttf1* transgene is active during lung development (data not shown), no abnormalities in branching morphogenesis or lung structure were observed in the transgenic mice at E18.5, regardless of gene dosage. Since levels of TTF-1 are normally high in the developing lung (Morotti, 2000; Stahlman, 1996; Zhou, 1996a, 2001), expression of the *SP-C-Ttf1* transgene in cells expressing high levels of endogenous TTF-1 did not appear to perturb lung morphogenesis. However, sustained high concentrations of TTF-1 later in postnatal development, when endogenous levels of TTF-1 decrease, perturbed alveolarization in the *SP-C-Ttf1* bitransgenic mice.

Effects of TTF-1 on lung morphogenesis were clearly evident during the postnatal period of alveolarization, a process that is highly active between postnatal days 5 and 14 in the mouse (Amy, 1977). In the normal lung, the levels of TTF-1 decrease dramatically in the postnatal period (Morotti, 2000; Stahlman, 1996; Zhou, 1996a, 2001), ultimately being detected in type II epithelial cells but not in type I epithelial cells, the latter comprising an increasing proportion of the lung parenchyma in the postnatal period of development. Disruption of alveolarization suggests that precise temporal–spatial regulation of TTF-1 is required for maturation and maintenance of alveolar structures.

Increased expression of TTF-1 caused type II cell hyperplasia in the Hi (+/−) transgenic mice, indicated by clusters of SP-B-positive type II epithelial cells at alveolar sites normally containing single type II cells. These findings support the concept that TTF-1 influences type II cell proliferation and differentiation or, alternatively, inhibits the differentiation of type II to type I epithelial cells. Similar type II cell hyperplasia was previously observed in transgenic mice expressing the SV40 large T gene driven by the SP-C promoter (Wikenheiser, 1998). In that model, type II cell hyperplasia was associated with emphysema and altered alveolarization prior to tumor formation. The present findings support the concept that decreased TTF-1 activity may be critical for type II-to-type I cell differentiation, which accompanies alveolarization. Thus, persistently increased expression of TTF-1 in the postnatal lung may disrupt normal epithelial cell proliferation and differentiation, leading to cell loss and emphysema. This hypothesis is supported by the findings in the Hi (+/−), Lo (+/−) bitransgenic mice, where foci of PCNA staining cells with condensed nuclei were found in peripheral regions of the lung undergoing remodeling and loss of epithelial cells. Whether the remodeling process represents activation of apoptotic or abnormal proliferative pathways is unclear at present.

Severe inflammation and fibrosis were observed in lungs of Hi (+/−), Lo (+/+) bitransgenic mice in the absence of infection or pathogens. Cell proliferation was associated with high levels of TTF-1 expression in fibrotic regions of the lung, but was generally decreased in areas associated with inflammation and acute alveolar injury. This histopathology is similar to that of lungs undergoing repair in response to injury or following exposure to proliferative stimuli. For example, increased expression of fibroblast growth factors FGF-7 and FGF-10 caused type II cell hyperplasia that was associated with increased staining for TTF-1, as well as for the surfactant proteins (Clark, 2001; Tichelaar, 2000). Increased TTF-1 staining was also observed in regenerating regions of human lung following infection, neonatal respiratory distress syndrome, and bronchopulmonary dysplasia (Stahlman, 1996). Thus, increased expression of TTF-1 may play a role in repair of the lung following injury.

In the present studies, epithelial cell hyperplasia, increased cytokine production, and inflammation were asso-

ciated with increased expression of TTF-1. Similar findings were observed following exposure of the adult lung to members of the FGF family of polypeptides (Clark, 2001; Tichelaar, 2000). FGF-induced hyperplasia was accompanied by macrophage infiltration, increased expression of TTF-1, surfactant proteins, and proinflammatory cytokines and chemokines. Since a number of cytokines, including IL-6, IL-1β, MIP-2, and TNF-α, cause inflammation and remodeling when expressed in the lung (DiCosmo, 1994; Kuhn, 2000; Miyazaki, 1995; Sueoka, 1998), their induction by TTF-1 may contribute to the morphological abnormalities observed in the *SP-C-Ttf1* bitransgenic mice. In the present study, inflammatory cell infiltrates in the Hi (+/−), Lo (+/+) bitransgenic mice consisted primarily of eosinophils and foamy macrophages. Markedly increased concentrations of eotaxin and IL-6 were also observed in the BALF fluid from these mice. While IL-6 is synthesized in inflammatory cells, fibroblasts, and endothelial cells, eotaxin is expressed primarily in the epithelium of the lung (Cook, 1998; Lilly, 1997). Although it is unclear at present whether changes in eotaxin or IL-6 contributed to the emphysema and fibrosis observed in the *SP-C-Ttf1* mice, the present studies support the concept that TTF-1 may influence gene transcription, directly and/or indirectly, to enhance the expression of selected chemokines and/or cytokines, which mediate inflammatory responses in the lung. Thus, TTF-1 appears to regulate type II cell proliferation, differentiation, and function in complex ways that may influence the expression of proinflammatory or signaling molecules involved in the process of host defense and repair of the lung.

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REFERENCES

Amy, R. W., Bowes, D., Burri, P. H., Haines, J., and Thurlbeck, W. M. (1977). Postnatal growth of the mouse lung. *J. Anat.* **124,** 131–151.

Bohinski, R. J., *et al.* (1994). Lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3 indicating common mechanisms for organ-specific gene expression along the foregut axis. *Mol. Cell. Biol.* **14,** 5671–5681.

Bruno, M. D., *et al.* (1995). Lung cell-specific expression of the murine surfactant protein A (SP-A) gene is mediated by interaction between the SP-A promoter and thyroid transcription factor-1. *J. Biol. Chem.* **270,** 6531–6536.

Clark, J. C., *et al.* (2001). FGF-10 disrupts lung morphogenesis and causes pulmonary adenomas *in vivo*. *Am. J. Physiol.* **280,** L705–L715.

Cook, E. B., *et al.* (1998). Epithelial cells are a major cellular source of the chemokine eotaxin in the guinea pig lung. *Allergy Asthma Proc.* **19,** 15–22.

DiCosmo, B. F, *et al.* (1994). Airway epithelial cell expression of interleukin-6 in transgenic mice: Uncoupling of airway inflammation and bronchial hyperreactivity. *J. Clin. Invest.* **94,** 2028–2035.

Glasser, S. W., *et al.* (1991). Genetic element from human surfactant protein SP-C gene confers bronchiolar-alveolar cell specificity in transgenic mice. *Am. J. Physiol.* **261,** L349–L356.

Guazzi, S., *et al.* (1990). Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. *EMBO J.* **9,** 3631–3639.

Ikeda, K., *et al.* (1996). Hepatocyte nuclear factor 3 activates transcription of thyroid transcription factor 1 in respiratory epithelial cells. *Mol. Cell. Biol.* **16,** 3626–3636.

Jain-Vora, S., *et al.* (1997). Interleukin-4 alters epithelial cell differentiation and surfactant homeostasis in the postnatal mouse lung. *Am. J. Respir. Cell Mol. Biol.* **17,** 541–551.

Kelly, S. E., *et al.* (1996). Transcription of the lung-specific surfactant protein C gene is mediated by thyroid transcription factor-1. *J. Biol. Chem.* **271,** 6881–6888.

Kimura, S., *et al.* (1996). The T/ebp null mouse: Thyroid specific enhancer-binding protein is essential for the oganogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* **10,** 60–69.

Kuhn, C. III, *et al.* (2000). Airway hyperresponsiveness and airway obstruction in transgenic mice. Morphologic correlates in mice expressing interleukin (IL)-11 and IL-6 in the lung. *Am. J. Respir. Cell Mol. Biol.* **22,** 289–295.

Lazzaro, D., *et al.* (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **113,** 1093–1104.

Lilly, C. M., *et al.* (1997). Expression of eotaxin by human lung epithelilal cells: Induction by cytokines and inhibition by glucocorticoid. *J. Clin. Invest.* **99,** 1767–1773.

Lowry, O. H., *et al.* (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Matthews, A. N., *et al.* (1998). Eotaxin is required for the baseline level of tissue eosinophils. *Proc. Natl. Acad. Sci. USA* **95,** 6273–6278.

Minoo, P., *et al.* (1999). Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(−/−) mouse embryos. *Dev. Biol.* **209,** 60–71.

Mishra, A., *et al.* (2001). IL-5 mediated allergic airway inflammation inhibits the human surfactant protein C promoter in transgenic mice. *J. Biol. Chem.* **276,** 8453–8459.

Miyazaki, Y., *et al.* (1995). Expression of a tumor necrosis factor-α transgene in murine lung causes lymphocytic and fibrosing alveolitis. A mouse model of progressive pulmonary fibrosis. *J. Clin. Invest.* **96,** 250–259.

Morotti, R. A., *et al.* (2000). Expression of thyroid transcription factor-1 in congenital cystic adenomatoid malformation of the lung. *Pediatr. Dev. Pathol.* **3,** 455–461.

Pryhuber, G. S., *et al.* (1991). Ontogeny of surfactant proteins A and B in human amniotic fluid as indices of fetal lung maturity. *Pediatr. Res.* **30,** 597–605.

Rothenberg, M. E. (1999). Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. *Am. J. Respir. Cell Mol. Biol.* **21,** 291–295.

Shaw-White, J. R., *et al.* (1999). GATA-6 activates transcription of thyroid transcription factor-1. *J. Biol. Chem.* **274,** 2658–2664.

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

Sueoka, N., *et al.* (1998). Molecular pathogenesis of interstitial pneumoitis with TNF-alpha transgenic mice. *Cytokine* **10,** 124–131.

Tichelaar, J. W., *et al.* (1999). HNF-3/forkhead homolog-4 (HFH-4) is expressed in ciliated cells in the developing mouse lung. *J. Histochem. Cytochem.* **47,** 823–831.

Tichelaar, J. W., *et al.* (2000). Conditional expression of fibroblast growth factor-7 in the developing and mature lung. *J. Biol. Chem.* **275,** 11858–11864.

Toonen, R. F. G., *et al.* (1996). The lung enriched transcription factor TTF-1 and the ubiquitously expressed proteins Sp1 and Sp3 interact with elements located in the minimal promoter of the rat Clara cell secretory protein gene. *Biochem. J.* **316,** 467–473.

Wert, S. E., *et al.* (1993). Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. *Dev. Biol.* **156,** 426–443.

Whitsett, J. A, and Glasser, S. W. (1998). Regulation of surfactant protein gene transcription. *Biochim. Biophys. Acta* **1408,** 303–311.

Wikenheiser, K. A., *et al.* (1998). SV40 early region genes alter alveolarization and surfactant protein mRNA expression in transgenic mice. *Transgenics* **2,** 311–326.

Zhang, L., *et al.* (1997). Regulating Clara cell secretory protein gene transcription by thyroid transcription factor-1. *Biochim. Biophys. Acta* **1350,** 359–367.

Zhou, H., *et al.* (2001). Expression of thyroid transcription factor-1, sufactant proteins, type I cell-associated antigen, and Clara cell secretory protein in pulmonary hypoplasia. *Pediatr. Dev. Pathol.* **4,** 364–371.

Zhou, L., *et al.* (1996a). 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.

Zhou, L., *et al.* (1996b). Arrested lung morphogenesis in transgenic mice bearing a SP-C-TGFβ1 chimeric gene. *Dev. Biol.* **175,** 227–238.

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