An enhancer region determines hSP-B gene expression in bronchiolar and ATII epithelial cells in transgenic mice

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Yang, Li, Angela Naltner, Allison Kreiner, Dong Yan, Angelynn Cowen, Hong Du, and Cong Yan. An enhancer region determines hSP-B gene expression in bronchiolar and ATII epithelial cells in transgenic mice. Am J Physiol Lung Cell Mol Physiol 284: L481–L488, 2003; 10.1152/ajplung.00280.2002.—Regulation of the surfactant protein B gene (SP-B) is developmentally controlled and highly tissue specific. To elucidate the SP-B gene temporal/spatial expression pattern in lung development at the transcriptional level, a transgenic mouse model line carrying the human SP-B (hSP-B) 1.5-kb 5′-flanking regulatory region and the lacZ gene was established. Expression of hSP-B 1.5-kb lacZ gene started at the onset of lung formation [embryonic day 9 (E9)] and was restricted to epithelial cells throughout prenatal and postnatal lung development. In the adult lung, hSP-B 1.5-kb lacZ gene expression was restricted to bronchiolar and alveolar type II epithelial cells. In lung explant culturing studies, the hSP-B 1.5-kb lacZ gene was highly expressed in newly formed epithelial tubules during the respiratory branching process. In a second transgenic mouse line, an enhancer region, which binds to thyroid transcription factor-1, retinoic acid receptor, signal transducers and activators of transcription 3, and nuclear receptor coactivators (SRC-1, ACTR, TIF2, and CBP/p300), was deleted from the hSP-B 1.5-kb lacZ gene. The deletion abolished hSP-B lacZ gene expression in bronchiolar and epithelial cells and significantly reduced its expression level in alveolar type II epithelial cells in transgenic mice.

LUNG DEVELOPMENT CAN BE DIVIDED INTO PRENATAL AND POSTNATAL STAGES. Before birth, the primitive lung originates as a diverticulum from the foregut to form a small epithelial tube surrounded by mesenchymal cells. Through interaction with mesenchymal cells, lung epithelium undergoes multiplication of branches and subsequently forms airways and alveoli. After birth, maturation of the lung continues, entering the alveolarization stage. The alveolarization period starts from the late embryonic period to the neonatal period, accompanied with secretion of pulmonary surfactant primarily by nonciliated bronchial epithelial cells (Clara cells) and alveolar type II epithelial cells.

Among surfactant proteins, surfactant protein B (SP-B) is a 79-amino acid amphipathic peptide, produced by the proteolytic cleavage of SP-B proprotein in Clara cells and alveolar type II epithelial cells. The SP-B peptide is stored in lamellar bodies and secreted with phospholipids into the airway lumen. The most renowned function of SP-B is to facilitate the stability and rapid spreading of surfactant phospholipids during the respiratory cycle (16). Null mutations in the SP-B gene caused lethal respiratory distress in newborn infants and in SP-B-deficient mice produced by gene targeting (2, 9). Therefore, SP-B is essential for postnatal lung development, alveolar maturation, and postnatal respiratory adaptation in newborns.

Expression of the SP-B gene is highly tissue specific and developmentally controlled. In the adult lung, expression of SP-B is restricted to Clara cells and alveolar type II epithelial cells. The control of human SP-B (hSP-B) gene expression is determined by cis-acting DNA elements and trans-acting transcription factors. Through in vitro study, an important enhancer region has been located at −500 to −331 bp from the transcriptional starting site of the hSP-B gene in respiratory epithelial cells (19). Multiple transcription factors and coactivators have been identified to form an enhancosome on the enhancer, including thyroid transcription factor-1 (TTF-1), retinoic acid receptor (RAR), nuclear receptor coactivators (p160 coactivators and CBP/p300), and signal transducers and activators of transcription 3 (STAT3) (7, 8, 17, 18). These factors strongly stimulate hSP-B gene transcription in respiratory epithelial cells in a synergistic manner. TTF-1 is a homeodomain containing tissue-specific transcription factor of Nkx2 family members. In the lung, TTF-1 is present at the earliest stages of differentiation of epithelium and is later confined to respiratory epithelial cells (3, 8, 12, 21). In TTF-1−/− mice, lung branch-
ing morphogenesis is severely disrupted (11). RAR signaling is also required for lung organogenesis. RAR-α/− and RAR-β/− double knockout mice develop lung hypoplasia and aplasia (6). In addition, retinoic acid is a stimulatory reagent for alveolarization and realveolarization after lung injury (4, 5). STAT3 can be activated by a number of cytokines and growth factors, including interleukin (IL)-6 family cytokines and epidermal growth factor (20). IL-6 and IL-11 strongly stimulate SP-B gene expression in the mouse lung (18). Unlike in other STAT-deficient mice that are born alive, targeted disruption of the mouse STAT3 gene leads to early embryonic lethality (14), indicating that it is involved in normal embryonic development.

On the basis of in vitro characterization, we hypothesize that TTF-1, RAR/retinoid X receptor (RXR), nuclear receptor coactivators, and STAT3 on the enhancer (−500 to −375 bp) determine in vivo temporal/spatial expression of the hSP-B gene in the lung. Unlike in the in vitro systems, in vivo SP-B gene expression in lung development is largely dependent on multicellular interactions, especially mesenchymal-epithelial cell interactions. In this report, a transgenic mouse system containing the hSP-B 1.5-kb 5′-flanking regulatory region and the lacZ reporter gene was established to systematically study the hSP-B gene temporal/spatial expression pattern during lung development. In addition, a second transgenic mouse line containing enhancer deletion in the hSP-B 1.5-kb lacZ gene was also established to elucidate the functional role of the enhancer in hSP-B temporal/spatial expression.

MATERIALS AND METHODS

Antibody immunohistochemistry. The lungs from wild-type FVB/N adult mice were infused with a fixative solution (4% paraformaldehyde, 1× PBS) via the trachea, dissected out, and stored in fixative at 4°C for ~24 h. For postnatal lung day 1 and day 4, lungs were dissected out for fixation. For prenatal animals, embryos were dissected out for fixation. After being fixed and embedded in paraffin, embryos and lung tissue sections were cut 5 μm thick. The slides were baked at 60°C for 2 h and washed in a series of xylene and ethanol to remove paraffin from the tissues. Endogenous peroxidase activity was removed from tissues by incubating the tissue slides in methanol and hydrogen peroxide for 15 min. Tissue slides were incubated overnight at 4°C with a primary SP-B protein polyclonal antibody (1:1,000–1:2,000). The antibody was provided by the Morphology Core Facility of the Division of Pulmonary Biology, Cincinnati Children’s Hospital Medical Center (see Ref. 21). Preimmune serum was added in the negative control. The tissues were washed and treated with secondary conjugated antibodies 24 h later. The interactions were amplified with Vectastain Elite ABC kit to visualize the signals.

Construction of the wild-type and deletion mutant hSP-B 1.5-kb lacZ/pSV-β-galactosidase reporter gene vectors. The hSP-B 1.5-kb lacZ expression vector was made by subcloning the hSP-B 1.5-kb 5′-flanking regulatory region into the pSV-β-galactosidase (β-gal) vector (Promega) at the EcoRI/HindIII sites using a PCR strategy as previously described (19). The hSP-B 1.5-kb enhancer deletion mutation was made by removal of the −550 to −351-bp region using the double PCR strategy and subcloning into the pSV-β-gal vector at the EcoRI/HindIII sites as previously described (19).

H441 cell culture and transient transfection. H441 cells were cultured in RPMI supplemented with 10% fetal calf serum, glutamine, and penicillin/streptomycin. Cells were maintained and passaged weekly at 37°C in 5% CO2-air. H441 cells were seeded at a density of 2 × 105 cells/well in six-well plates. The hSP-B 1.5-kb lacZ reporter and control constructs (0.25 μg) were transfected into H441 cells by Fugene6 (Boehringer Mannheim). After 72 h, cells were fixed for immunohistochemical β-gal staining.

Generation of transgenic mice. The expression cassette containing the hSP-B 1.5-kb 5′-flanking regulatory region (or hSP-B 1.5-kb with deletion of the −550 to −351-bp region), the lacZ gene, and the simian virus 40 small T antigen poly(A) signals was dissected out, purified, and sent to the Transgenic Core Facility of the University of Cincinnati for microinjection. Founders were identified by PCR using a pair of primers spanning from the hSP-B promoter to the lacZ gene.

Immunohistochemistry of β-gal staining. For adult lungs and postnatal day 10 and day 15 lungs, whole lungs were prefixed with a fix solution (0.4 ml 25% glutaraldehyde, 1.0 ml 50 mM EGTA, 5.0 ml 1 M MgCl2, and 43.5 ml PBS) on ice for 4 h, followed by three washes (15 min each) in a wash buffer containing 1.0 ml 1 M MgCl2, 5.0 ml 2% sodium deoxycholate, 5.0 ml 2% Nonidet P-40, and 489 ml 100 mM sodium phosphate buffer, pH 7.3. Subsequently, lungs were stained with LacZ solution (48 ml wash buffer, 2 ml 25 mg/ml X-gal, 0.106 g potassium ferrocyanide, and 0.042 g potassium ferricyanide) overnight at room temperature. The next day, lungs were rinsed three times (10 min each) with PBS and fixed in a postfixative solution (3.2 ml 25% glutaraldehyde, 5 ml 16% paraformaldehyde, 4 ml 1 M sodium cacodylate, and 27.5 ml water) for 10 min. Lungs were dehydrated through a graded series of ethanol washes. After being fixed and embedded in paraffin, embryos and lung tissue sections were cut 5 μm thick. The slides were baked at 60°C for 2 h and washed in a series of xylene and ethanol to remove paraffin from the tissues. For prenatal lungs and postnatal lungs day 1 and day 4, cryostat sections of frozen embryos or lung tissues were used for β-gal staining. H441 cell β-gal staining was performed essentially the same.

Assay of β-gal activity. Various tissues were collected from both wild-type and transgenic FVB/N adult mice for homogenization in PBS. Approximately 1 ng of protein was used for β-gal assay as described previously (19). For cultured alveolar type II epithelial cells, cells were lysed in reporter lysis buffer for luciferase assay (Promega) and subsequently used for β-gal assay.

Lung explant culture and β-gal staining. Lung buds were dissected out from embryonic day 12 (E12) embryos of nontransgenic wild-type or hSP-B 1.5-kb lacZ gene transgenic mice. The buds were cultured on top of 1% low melting point agarose gel in DMEM/F-12 with supplementation of 10% fetal calf serum in a 30-mm dish. The next day, a final concentration of 0.2 mg/ml of X-gal was added to the culture medium and incubated for 2 more days. The cultured lung explants were changed with fresh medium each day.

Alveolar type II cell purification and culturing. The study was performed essentially the same as previously described (19). Two-mo-old mice were anesthetized by intraperitoneal injection. The abdominal cavity was opened, and mice were exsanguinated by severing the inferior vena cava and the left renal artery. The trachea was isolated and cannulated with a 20-gauge luer stub adapter. The diaphragm was cut, and the chest plate and thymus were removed. With the use of a
RESULTS

Temporal/spatial expression of endogenous SP-B in postnatal lungs. Cell type-specific expression of the endogenous SP-B was assessed using antibody against the SP-B protein by immunohistochemical staining. In the neonatal lungs, SP-B expression was detected in developing epithelial cells and later was restricted to the neonatal lungs, SP-B expression was detected in the SP-B protein by immunohistochemical staining. In endogenous SP-B was assessed using antibody against the SP-B protein. Original magnification, ×230. Br, bronchiole; Cap, capillary vessel.

Generation of the hSP-B 1.5-kb lacZ transgenic mouse line. To assess whether SP-B cell type-specific expression is controlled by the 5′-flanking sequence at the transcriptional level during lung development, an expression cassette containing the hSP-B 1.5-kb 5′-flanking regulatory region and the lacZ gene was constructed. To test lacZ gene expression, the hSP-B 1.5-kb lacZ gene construct was transfected into H441 cells. With the use of β-gal staining, expression of the lacZ gene was detected in H441 cells (data not shown). Next, the DNA fragment containing the hSP-B 1.5-kb lacZ gene expression cassette was microinjected into the mouse to establish founder lines. The positively genotyped lungs were inflated and dissected out for β-gal staining. As shown in Fig. 2, the adult lung from transgenic mice showed lacZ gene expression by β-gal staining (blue color). As control, the nontransgenic wild-type lung showed no β-gal activity.

Tissue and cell type-specific expression of the hSP-B 1.5-kb lacZ gene in transgenic animals. To examine tissue expression of the hSP-B 1.5-kb lacZ gene, various organs were collected from wild-type and trans-
genic animals for β-gal assay. Only the transgenic lungs and the intestines demonstrated β-gal activity (Fig. 3). To further identify cell type expression of the hSP-B 1.5-kb lacZ gene in each organ, adult tissue sections were prepared from various organs of the transgenic animals. Again, only lung and intestine tissue sections showed β-gal staining (Fig. 4). In the adult lung, β-gal staining was restricted to bronchiolar and alveolar type II epithelial cells, identical to endogenous SP-B expression, indicating that SP-B tissue and cell type-specific expression are controlled by this hSP-B 1.5-kb genetic sequence. There was no detectable β-gal staining in the nontransgenic lungs, except some weak endogenous staining in respiratory macrophages. In the intestines, β-gal staining was detected and restricted to goblet cells along the villi and Paneth cells. The hSP-B 1.5-kb lacZ gene expression in the intestines appears not to be affected by the position of DNA random insertion, because at least three transgenic founders showed intestinal expression of the hSP-B 1.5-kb lacZ gene. No specific staining was detectable in the nontransgenic intestines (data not shown).

**Temporal/spatial expression of the hSP-B 1.5-kb lacZ gene in the developing lungs.** To further assess the temporal/spatial expression pattern of the hSP-B gene in developing lungs, β-gal staining of hSP-B 1.5-kb...
lacZ gene expression was systematically analyzed during lung development. First, embryonic tissues from various developing stages were collected and stained for β-gal activity. The staining of β-gal activity was detected in respiratory epithelial cells as early as E9, the onset of lung formation when only one epithelial tubule was formed (Fig. 5). Throughout lung development, hSP-B 1.5-kb lacZ gene expression was continuously detected in and highly restricted to developing epithelial tubules.

Next, lung tissues from various postnatal days of transgenic animals were collected for β-gal staining when the alveolar maturation process was accelerated. Throughout the entire neonatal stage, hSP-B 1.5-kb lacZ gene expression was highly expressed in differentiated epithelial cells (Fig. 6). At postnatal day 15, peripheral alveolar structure was formed, and β-gal activity of hSP-B 1.5-kb lacZ gene expression was restricted to newly formed bronchiolar and alveolar type II epithelial cells. Therefore, the hSP-B 1.5-kb fragment recapitulated specificity of endogenous SP-B expression in the lung. In nontransgenic animals, no β-gal staining activity was observed in both prenatal and postnatal developing lungs (data not shown).

Expression of the hSP-B 1.5-kb lacZ gene in branching morphogenesis of cultured lung explants. To study whether expression of the hSP-B 1.5-kb lacZ gene is associated with respiratory branching morphogenesis, lung buds from E12 day embryos were dissected out and cultured in vitro in the presence of X-gal. After 2 days of incubation, β-gal activity was highly integrated into the newly branched epithelial tubules (Fig. 7). There was faint staining in the lung explants isolated from nontransgenic animals in the cultured conditions.

The enhancer −550 to −351 bp determines hSP-B gene expression in bronchiolar epithelial cells. As discussed earlier, a series of in vitro studies identified an important enhancer region on the hSP-B gene, which
binds to multiple trans-acting factors that form an enhanceosome. To identify the functional role of this enhancer in hSP-B temporal/spatial expression in the lung, a lacZ transgenic mouse line containing an hSP-B 1.5-kb deletion mutation that lacks the enhancer region was made (Fig. 8A). The transcriptional activity of the deletion construct had been tested in H441 cells that showed much lower β-gal activity compared with the wild-type hSP-B 1.5-kb lacZ construct (data not shown). Analysis of this transgenic mouse line revealed that there was no lacZ gene expression in bronchiolar epithelial cells (Fig. 8B). Therefore, the enhancer is essential for hSP-B gene expression in bronchiolar epithelial cells. Although β-gal activity could be detected in alveolar type II epithelial cells, the expression level was dramatically reduced (Fig. 8B). To quantitatively compare expression levels between the wild-type hSP-B 1.5-kb lacZ gene and the enhancer deletion mutant mice, alveolar type II epithelial cells were purified from both transgenic lines and cultured in vitro for β-gal assay. The β-gal activity comparison indicated that removal of the enhancer region resulted in ~70% β-gal activity reduction in cultured alveolar type II epithelial cells (Fig. 8C).

**DISCUSSION**

In contrast to other surfactant protein (SP-A, SP-C, and SP-D)-deficient mice that are born alive, targeted disruption of the mouse SP-B gene leads to lethality of newborn mice (2), suggesting that SP-B is essential for postnatal lung function. It appears that SP-B gene activation starts at the onset of lung development, since expression of the hSP-B 1.5-kb lacZ reporter gene could be detected at E9 of embryonic lung development (Fig. 5), a stage with only one epithelial tubule present. After fetal lung development, the hSP-B 1.5-kb lacZ gene was continuously expressed in branching epithelial tubules in animals (Figs. 5 and 6) and in in vitro lung explants (Fig. 7), suggesting transcription of the hSP-B gene was activated throughout respiratory epithelial differentiation and proliferation during lung development. This process recapitulates endogenous mouse SP-B expression and agrees with the observation made by others using the chloramphenicol acetyltransferase as a reporter gene (13).

The observation made in this report suggests that the 1.5-kb 5′-flanking regulatory region of the hSP-B gene contains sufficient positive cis-acting genetic sequences required for tissue and cell type-specific expression in the developing and mature mouse lungs. The observation suggests that common genetic sequences and mechanisms control both human and mouse SP-B gene regulation during lung development. One of the common sequences shared by human and mouse is the enhancer region located in −500 to −331 bp of the hSP-B 5′-flanking regulatory region (19). The enhancer region binds to multiple transcription factors (including TTF-1, RAR/RXR, STAT3) and nuclear receptor coactivators (CBP, SRC-1, ACTR, TIF2) with intrinsic histone acetyltransferase activity that form an enhanceosome (7, 8). These factors mediate retinoic acid and IL-6 family cytokine stimulation of the hSP-B gene in respiratory epithelial cells (7, 8, 17, 18). Among them, TTF-1 is a tissue-specific transcription factor and plays a central role in enhanceosome complex formation (3, 7, 8, 19, 21). Importantly and interestingly, the temporal/spatial expression pattern of TTF-1 (8, 21) matches very well with the hSP-B 1.5-kb lacZ gene expression pattern in both developing and mature lungs (Figs. 4–6). It has been shown that TTF-1 is a strong transcription activator for SP-B gene expression and lung branching morphogenesis in respiratory epithelial cells (1, 11, 15, 19). TTF-1 can interact with RAR, nuclear receptor coactivators, and STAT3 to regulate hSP-B gene expression in mediating retinoic acid signaling and IL-6 family cytokine signaling (7, 8, 17, 18). These factors interact with each other on the enhancer as identified by mammalian two-hybrid assay, glutathione S-transferase pull down assay, chromatim immunoprecipitation assay, and confocal microscope colocalization assay (7, 8, 17, 18). These factors plus other unidentified factors on the enhancer exercise a combinatorial effect to control temporal/spatial expression of the SP-B gene in the lung. In this report, after deletion of the enhancer region, the hSP-B 1.5-kb lacZ gene was no longer expressed in bronchiolar epithelial cells of the transgenic mouse. Therefore, the enhancer region and associated trans-acting factors are essential for hSP-B gene expression in bronchiolar epithelial cells. The study indicates that cell type-specific expression of the hSP-B gene in bronchiolar and alveolar type II epithelial cells is controlled by different sets of genetic elements and transcription factors. It remains to be determined which region(s) within the hSP-B 1.5-kb 5′-flanking regulatory region controls alveolar type II epithelial cell expression, although deletion of the enhancer substantially reduced lacZ reporter gene expression in these cells (Fig. 8C).

In addition to the lung, expression of the hSP-B 1.5-kb lacZ gene was also observed in adult intestinal epithelial cells (goblet and Paneth cells) of transgenic mice (Fig. 4). Weak β-gal expression has also been observed in trachea (goblet cells) and thyroid (data not shown). Other researchers have also made the same observation using the chloramphenicol acetyltransferase reporter gene (13). This raises a very interesting...
issue. It suggests that some negative sequences beyond the hSP-B 1.5-kb 5′-flanking regulatory region are important contributors for tissue-specific expression of the SP-B gene. The sequences appear to suppress hSP-B gene transcription in the intestine, trachea, and thyroid. In addition, detection of hSP-B 1.5-kb lacZ gene expression in the intestine and thyroid implies that these organs originate from the same progenitor cells as the lung. Similar to the lung, intestine development starts at a rather late embryonic stage. Both organs have an internal interface to separate the body from the outside environment, and both organs are repeatedly exposed to external microorganisms and pathogens. In addition to serving as barriers, both organs actively participate in host defense through cytokine- and chemokine-mediated inflammatory responses. The enhancer deletion mutant of the hSP-B 1.5-kb lacZ gene transgenic mice still retained low

Fig. 8. Deletion of the hSP-B enhancer (~550 to ~351 bp) abolished hSP-B 1.5-kb lacZ gene expression in bronchiolar epithelial cells. A: illustration of the hSP-B 1.5-kb lacZ gene construct and the enhancer deletion mutant hSP-B 1.5-kb lacZ gene construct. B: lung sections from hSP-B 1.5-kb lacZ gene transgenic mice or enhancer deletion mutant mice were prepared and stained for β-gal activity. Original magnification, ×230. Arrows point to representative alveolar type II epithelial cells. Arrowheads point to bronchiolar epithelial cells. C: alveolar type II epithelial cells were purified from nontransgenic mice, hSP-B 1.5-kb lacZ gene transgenic mice, or the enhancer deletion mutant transgenic mice. Cells were cultured in in vitro condition for 3 days. Expression levels of the β-gal activity from these animals were determined. Values are means ± SD, n = 3. TTF-1, thyroid transcription factor-1; RAR, retinoic acid receptor; RXR, retinoid X receptor; STAT3, signal transducers and activators of transcription 3.
β-gal activity in the intestine (data not shown), suggesting that the enhancer is required for lung-specific activity in bronchiolar epithelial cells.

In summary, generation of the hSP-B 1.5-kb lacZ gene and enhancer deletion mutant transgenic mice reveals the temporal/spatial expression pattern of hSP-B transcription in vivo, suggesting that both positive and negative DNA regulatory sequences contribute to hSP-B gene tissue and cell type-specific expression. The studies demonstrate that the enhancer region (~550 to ~351 bp) and its associated trans-acting factors have at least two functions in vivo: 1) to determine bronchiolar epithelial cell-specific expression and 2) to elevate SP-B gene basal level expression in alveolar type II epithelial cells. The studies have identified a correlation between SP-B transcription and lung epithelial cell differentiation, proliferation, and alveolar maturation. Our findings support the concept that SP-B is critical for postnatal lung development and the pulmonary surfactant structure. These transgenic mouse lines will provide useful tools for characterization of cis-acting elements and transcription factors that control SP-B transcription in vivo. They can serve as markers for studying respiratory epithelial cell differentiation, proliferation, and regeneration during prenatal and postnatal lung development.

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