Human SP-C gene sequences that confer lung epithelium-specific expression in transgenic mice

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Glasser, Stephan W., Michael S. Burhans, Susan K. Eszterhas, Michael D. Bruno, and Thomas R. Korfhagen. Human SP-C gene sequences that confer lung epithelium-specific expression in transgenic mice. Am J Physiol Lung Cell Mol Physiol 278: L933–L945, 2000.—We used transgenic mice to identify cis-active regions of the human pulmonary surfactant protein C (SP-C) gene that impart tissue- and cell-specific expression in vivo in the lung. Approximately 3.7 kb of genomic SP-C DNA upstream of the transcription start site was sufficient to direct chloramphenicol acetyltransferase (CAT) reporter gene expression specifically in bronchiolar and alveolar epithelial cells of the lung. To further define cis-active regulatory elements that mediate cell-specific expression, we tested deletions of the parental 3.7-kb human SP-C sequence in transgenic mice. Tissue CAT assays of mice generated with truncations or overlapping internal deletions of the 3.7-kb construct functionally map alveolar cell-specific regulatory elements to within –215 bp of the SP-C promoter. Analysis of SP-C promoter deletions demonstrate that sequences between –3.7 kb and –1.9 kb contain enhancer sequences that stimulate SP-C gene expression. In situ hybridization studies demonstrate that deletion of the –1,910- to –215-bp region abolishes the ectopic bronchiolar expression seen with the original 3.7-kb SP-C promoter construct. Comparison of sequences from –215 to –1 bp identified consensus binding sites for the homeodomain transcription factor thyroid transcription factor-1 (TTF-1). Cotransfection assays of the human 3.7-kb SP-C or –1,910- to –215-bp SP-C deletion construct with a TTF-1 expression plasmid demonstrates that TTF-1 activates the human SP-C gene. These results suggest that the TTF-1 cis-active sites are important in directing cell-specific expression of the SP-C gene in vivo.

thyroid transcription factor-1; chloramphenicol acetyltransferase

PULMONARY SURFACTANT is a protein and phospholipid complex that is essential for normal respiration and functions by reducing surface tension at the alveolar surface (6). Four surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) have been identified (16). Of these proteins, the two small hydrophobic proteins (SP-B and SP-C) are lung specific. They enhance the surface active properties of synthetic surfactant phospholipids in vitro and are a component of replacement surfactant preparations used to treat neonatal respiratory distress syndrome (15, 16, 30). SP-C gene transcription is initiated early in embryonic lung development with a temporal-spatial pattern of expression distinct from other surfactant protein genes (18, 31, 34). Murine SP-C mRNA is first detected on fetal day 11 (fetal day 20 is birth) and is maintained in epithelial cells at the distal migrating edge of the developing primitive airways (31). During prenatal development, SP-C mRNA expression is progressively extinguished in conducting airways (31). During prenatal development, SP-C mRNA expression is progressively extinguished in conducting airways along the proximal-to-distal axis of airway tubule growth. In the adult mouse lung, SP-C is expressed exclusively in mature type II cells, whereas SP-A and SP-B expression is detected in both bronchiolar epithelial and alveolar type II cells (7, 20). Thus SP-C is the only surfactant protein expressed exclusively in type II cells.

Several growth factors and humoral mediators have been identified that are required to sustain the type II phenotype and SP-C gene expression (24, 27). Such studies do not reveal the molecular basis for type II cell-specific SP-C transcription. The first insights into this complex process have come from limited in vitro gene activation studies of the SP-C promoter and candidate transcription factors. The murine SP-C gene is activated in vitro by coexpression of the homeodomain transcription factor thyroid transcription factor-1 (TTF-1) (3). Activation requires protein interactions with two adjacent cis-active sites, TTF-1 binding elements (TBE) of the murine SP-C promoter (17), implicating TTF-1 as a critical regulator of SP-C gene expression. Biochemical analysis of the murine SP-C promoter demonstrates that several other protein to DNA interactions occur near the TBE. The nature of these molecules and their role in specifying type II cell-specific transcription are unknown.

As a complement to in vitro studies, transgenic mouse models have been used to detect cis-active DNA that is required to drive the correct temporal and cell-specific pattern of gene expression. The transgenic analysis of promoter function has often identified relevant cis-active regions that are not identified in vitro. We have generated transgenic mice with segments of human SP-C DNA to identify cis-active sequences that direct lung-specific transcription in vivo. Analysis of transgenic mice demonstrated that 3.7 kb of DNA upstream from the transcription start site were sufficient to cause reporter gene expression in lung epithelial cells of the developing mouse (9, 10). That study identified the first genomic sequence that could be used...
to selectively drive lung-specific expression in vivo. The 3.7-kb SP-C promoter has been used subsequently to express a variety of biologically active molecules in vivo to alter lung development and to model lung diseases. Examples include 3.7-kb SP-C-driven transforming growth factor-α expression, which has produced a model of progressive pulmonary fibrosis (21). The 3.7-kb SP-C-promoted expression of the SV40 T antigen has generated a model of bronchoalveolar lung cancer (32). Further characterization of multiple 3.7-kb SP-C-driven chloramphenicol acetyltransferase (CAT) transgenic lines would be useful then to define the range of cell specificity that can be obtained when using this promoter to develop new transgenic models. The high levels of expression from 3.7-kb SP-C-CAT mice indicated that analysis of 3.7-kb SP-C deletions in vivo could be successful in mapping regions essential for type II-specific expression. In the present study, we have identified transcriptional control elements of the SP-C gene that activate and sustain transgene expression in subpopulations of distal respiratory epithelial cells.

MATERIALS AND METHODS

Generation of SP-C-CAT constructs and transgenic mice. A 3.7-kb fragment of human SP-C genomic DNA containing basal promoter elements and additional 5′ sequences (extending from +21 of exon 1 to −3668) was subcloned into the unique Hind III restriction site of the expression plasmid pSV0-CAT. This plasmid and the SP-C genomic dones have been described previously (9, 10). For this study, the −1225- and −590-bp regions of the promoter were cloned into pSV0-CAT with Hind III linkers and oriented by Xba I and Ava I restriction digests. Internal deletion constructs were generated by restriction digest of the original 3.7-kb SP-C-CAT plasmid at paired restriction sites unique to the genomic SP-C sequence, followed by plasmid self-ligation with T4 DNA ligase. Deletion clones were mapped relative to restriction sites 5′ and 3′ of the deleted SP-C DNA. Nde I-BamH I double-digested fragments were purified by CsCl gradient ultracentrifugation, and transgenic mice were generated at the University of Cincinnati transgenic core facility in FVB/N mice. Founder (F₀) transgenic mice were identified by genomic Southern blot of tail-tip DNA using the bacterial CAT gene as probe, as previously described (10). F₀ mice were bred to wild-type FVB/N mice to establish heterozygotes, which were bred to generate mice homozygous for the SP-C-CAT transgene. For in vitro expression analyses, the 3.7-kb SP-C genomic DNA was either subcloned into M13 vectors for conventional dideoxynucleotide sequencing or directly sequenced in the plasmid vectors using a model 377 Applied Biosystems automated sequencer at the University of Cincinnati DNA-sequencing core facility. Human and murine SP-C sequences were compared using the alignment and DNA-sequencing core facility. Human and murine SP-C were sequenced in the plasmid vectors using a model 377 Applied Biosystems automated sequencer at the University of Cincinnati DNA-sequencing core facility. Human and murine SP-C DNA sequences were compared using the alignment and matrix programs of MacVector 6.5 (Symantec).

RESULTS

Tissue-specific expression of the transgene was assessed in adult transgenic mice generated with the human 3.7-kb SP-C-CAT construct. Transgenic lines were subsequently generated with deletions of the 3.7-kb human SP-C DNA to identify regions of DNA that determine lung-specific expression. The deletion constructs tested and number of transgenic mouse lines expressing each construct are summarized in Fig. 1.
The 3.7-kb SP-C-CAT transgene expression does not alter levels of endogenous SP-C expression. To determine whether the level of transgene expression altered the overall level of endogenous SP-C expression by competing for essential transcription factors, we compared CAT mRNA and SP-C mRNA levels in lungs from the various founder lines. Total lung RNA was prepared from lungs of nontransgenic mice and four 3.7-kb SP-C-CAT founder lines and used for Northern blot analysis. Transgene mRNA expression was detected using a 550-bp portion of the bacterial CAT gene as probe (Fig. 2A). CAT mRNA was not detected in the nontransgenic control (Fig. 2A, lane 1), whereas high levels of CAT mRNA were detected in the lungs from transgenic lines 5.5, 5.7 and 3.8.8. Relatively low levels of CAT mRNA were detected from line 3.8.1 (Fig. 2A, lane 4). To determine the level of endogenous SP-C expression, the blot was stripped of CAT probe and rehybridized with an SP-C cDNA probe (Fig. 2B). Similar levels of SP-C expression were detected in lungs from all transgenic lines and were similar to the nontransgenic wild-type mice, indicating that transgene expression did not alter the level of endogenous SP-C mRNA. Differences in SP-C signal levels reflected slight differences in loading as determined by methylene blue staining of the membrane before hybridization analysis.

Lung-specific expression of SP-C-CAT transgenes. To determine sequences required to direct lung-specific gene expression in vivo, we generated transgenic mice with progressively smaller deletions of the 3.7-kb sequence directing the CAT reporter gene. Constructs were generated that were either 5’ directional, eliminating segments of DNA distal to the transcription start site, or overlapping internal deletions of proximal SP-C DNA (Fig. 1).

Transgenic lines were established that retained either −1,225 or −590 bp of promoter proximal genomic SP-C DNA. CAT activity was detected in 4 of the 10 independent lines, two −1,225-bp lines and two −590-bp lines and was lung specific. CAT activity was not detected in other examined tissues. Representative tissue CAT activity of a −590-bp SP-C-CAT transgenic line is shown (Fig. 3). The level of lung CAT activity from the −1,225-bp transgenic lines was nearly identical to the −590-bp SP-C-CAT transgenic lines and is not shown. The levels of lung-specific CAT activity in lung tissue from transgenic mice with the −1,225- and −590-bp SP-C, promoters were much less than the activity detected in the 3.7-kb SP-C-CAT lines (Fig. 3). Therefore, loss of enhancer elements located in the deleted distal sequence may account for the diminished level of in vivo expression. Nevertheless, lung-selective CAT expression from the two independent −590-bp SP-C-CAT transgenic lines demonstrated that cis-active determinants of lung-specific transcription were located within this small region of the promoter.

To more precisely identify sequences conferring lung-specific expression, we generated transgenic mice with small internal deletions that would overlap into the −590-bp region retaining only 365 bp (−860 to −365) or 215 bp (−1,910 to −215) of the promoter proximal DNA. CAT activity was detected in lung tissue from two independent founder lines bearing each construct (Fig. 4). The 3′ boundaries of these constructs functionally map cis-active elements that mediate lung-specific expression to within 215 bp of the human SP-C promoter.

Cell-specific expression of SP-C-CAT transgenes. The cellular sites of SP-C-CAT transgene expression were localized by in situ hybridization with a CAT mRNA-specific probe and compared with the pattern of endog-
ous murine SP-C mRNA expression. Three independent lines of the 3.7-kb SP-C-CAT construct and the four internal deletion transgenic lines were evaluated. CAT mRNA was detected along the distal bronchiolar epithelium and focally throughout the lung parenchyma of the 3.7-kb SP-C-CAT lines. The focal CAT expression in the parenchyma is a pattern consistent with the distribution of type II cells (Fig. 5). A uniform pattern of focal sites of endogenous SP-C mRNA expression was detected throughout the lung parenchyma, consistent with the distribution of alveolar type II cells (Fig. 6). To determine if all SP-C mRNA-expressing cells also expressed the CAT transgene mRNA, the number of alveolar cellular sites of CAT expression and endogenous SP-C expression were determined. In 3.7-kb SP-C-CAT transgenic lines 5.5 and 5.7, 36 and 56% of alveolar cells expressing SP-C also expressed the CAT transgene. CAT expression in line 3.8.8 most closely approximated the endogenous pattern of SP-C expression, with 72% of SP-C-expressing cells also expressing the transgene (compare Fig. 5B and Fig. 6B). The number of sites of SP-C expression in nontransgenic mice and transgenic mice varied by ±12%, indicating that endogenous SP-C expression is unaffected by expression of SP-C-CAT transgene constructs in vivo.

In a similar manner, the expression patterns of the internal deletion constructs were determined by in situ hybridization. The −860- to −365-bp SP-C-CAT transgene was detected in bronchiolar and alveolar cells similar to the pattern of CAT expression produced by the parental 3.7-kb SP-C-CAT transgene (Fig. 7). The pattern of expression found in the −1,910- to −215-bp SP-C-CAT mice lacked the bronchiolar expression but maintained focal alveolar-specific expression (Fig. 8). As with the other transgenes, the number of sites of alveolar expression were reduced compared with the endogenous SP-C gene. The loss of bronchiolar expression in the −1,910- to −215-bp line, but not in −860- to −365-bp lines, suggests that deletion of the more distal

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**Fig. 2.** Northern blot analysis of SP-C and SP-C-CAT expression in lungs of adult transgenic mice. Total RNA was isolated from lung homogenates of 6-mo-old mice. Five micrograms were loaded per lane and blots were probed with CAT-specific (A) or SP-C-specific probes (B). CAT expression is not present in nontransgenic mouse lung (lane 1). Low-level CAT expression is detected for line 3.8.1 (lane 4). High-level CAT mRNA expression is detected in lines 5.5, 5.7 and 3.8.8 (lanes 2, 3, and 5, respectively). Bottom band correlates with correct unit length CAT gene expression, whereas top band is most likely read through tandem copies of 3.7-kb SP-C-CAT transgenes. SP-C levels of transgenic lines are similar to SP-C mRNA levels of nontransgenic mouse lines (B). Variation reflects minor differences in RNA loading as assessed by methylene blue staining of blots after electrophoresis and transfer.

**Fig. 3.** Expression of truncated human SP-C-CAT transgenes is restricted to lungs of transgenic mice. Representative CAT assays of tissue extracts from nontransgenic and −590-bp SP-C-CAT transgenic mice are shown. CAT activity was not detected in nonlung organs of transgene-negative (A) or transgene-positive (B, left lanes) mice. Low-level lung CAT expression was detected in −590-bp SP-C-CAT lung extracts (+) but not in extracts prepared from nontransgene lung (−) (B, right lanes). CAT activity from lung extract of 3.7-kb SP-C-CAT transgenic mouse is shown at far right for comparison of relative expression levels. The other −590-bp SP-C-CAT and 2−1,225-bp SP-C-CAT lines expressed at levels equal to or less than −590-bp SP-C-CAT line (data not shown).
DNA (from 286 to 21,960 bp) removed sequences that mediated the incorrect bronchiolar expression. Collectively, the in vivo CAT assays, which map lung specificity to 2215 bp, and the in situ data, which show sustained alveolar expression, suggest that key cis-active elements that guide type II cell-specific expression are located within 2215 bp of the human SP-C promoter.

Sequence comparison of murine and human SP-C promoters. The pattern of bronchiolar expression obtained with the 3.7-kb human SP-C promoter suggested that there may be sequence differences between the promoters of the human and murine genes. We have sequenced the human 3.7-kb DNA and DNA upstream from the murine SP-C promoter to determine the extent of sequence similarity between both SP-C promoter regions. Sequence homology alignment of the two SP-C sequences demonstrated that there is considerable sequence divergence with portions of the human 3.7-kb fragment common to both species (Fig. 9). Segments of the human promoter sequences are arranged as three blocks of conserved DNA dispersed over ~4.8 kb of murine genomic DNA (Fig. 9A). The highest degree of homology was found in the promoter proximal sequences (81%). The three regions of sequence conservation account for ~2.8 kb of DNA common to the two SP-C promoters. The position and size of the human- or murine-specific sequence differences are diagrammed as insertions relative to the 2.8 kb of sequence common to both human and mouse SP-C DNA (Fig. 9B). In the human 3.7-kb SP-C sequence, four small regions were identified that were absent in the murine SP-C sequence. One of these inserts (~250 bp in Fig. 9B) has identity with the human Alu family of repetitive elements. The largest of the human inserts was a sequence of ~550 bp in length. Both the distal Alu and proximal 550-bp inserts begin with 32-bp direct repeats. These unique direct repeats suggest sites for specific insertional or recombination events. The largest region of nonhomology between human and murine sequences can be accounted for by three adjacent but distinct insertions in the murine DNA relative to the human sequence in the promoter proximal region (~110 bp, 1,120 bp, and 650 bp, Fig. 9B).

Activation of human SP-C promoter requires transcription factor TTF-1. TTF-1 is expressed in airway epithelial cells and stimulates the murine surfactant protein gene promoters (3, 4, 17). The consensus site for TTF binding to DNA has been identified as the conserved nucleotide motif CAAG. Two TTF-1 elements essential for in vitro activation of the murine SP-C gene lie within the ~215-bp region, which is highly conserved between the human and mouse SP-C genes (17). To determine whether the human gene is also regulated by TTF-1, we cotransfected the human 3.7-kb SP-C-CAT construct into HeLa cells with a TTF-1-expressing plasmid. Constitutive expression of TTF-1 with the 3.7-kb SP-C-CAT construct stimulated a sevenfold increase in CAT expression (Fig. 10). The increased stimulation of the human SP-C promoter by TTF-1 was similar to the increased induction reported for the murine SP-C promoter (17). However, the relative level of human 3.7-kb SP-C-CAT expression in these transient transfection assays was significantly less than expression levels obtained with the murine 4.8-kb SP-C-CAT construct. TTF-1 did not transactivate the empty CAT reporter plasmid, pBLCAT6 (Fig. 10A) nor the plasmid pBLCAT5 where the CAT reporter gene is driven by the herpes thymidine kinase gene promoter (data not shown). The ~1,910- to ~215-bp human SP-C construct was also transactivated by TTF-1 in vitro (Fig. 10B, ΔAva SP-C). This construct retained the two proximal CAAG elements that were shown by site-specific mutation to mediate the TTF-1 response of the
murine SP-C promoter (17). Thus the human SP-C sequences that mediate the TTF-1 response in vitro map to the region necessary for in vivo SP-C transgene expression.

DISCUSSION

We have analyzed the in vivo expression of SP-C-CAT transgenic constructs to identify the cis-active regulatory regions that mediate lung cell-specific expression of SP-C. We previously determined that 3.7 kb of human DNA extending upstream from the SP-C gene transcription start site directed lung-specific expression of reporter genes in transgenic mice (11). In the current study, we have determined that an essential cis-active region for in vivo SP-C expression in alveolar epithelial cells maps to within 215 bp of the basal promoter. In transgenic lines generated with extensive deletions of distal 5' SP-C sequence, the level of lung-specific expression was dramatically reduced in comparison to expression levels obtained with the parental

Fig. 5. The 3.7-kb SP-C-CAT transgene is expressed in bronchiolar and alveolar epithelial cells of adult lung. Lungs of transgenic 3.7-kb SP-C-CAT (line 3.8.8) mice were prepared at 7 mo of age for cellular localization of transgene CAT mRNA. Dark-field images are presented in A, B, D, and F. Bright-field images corresponding to D and F are presented in C and E, respectively. Sense CAT probe did not hybridize to lung tissue (A). Intense hybridization of antisense probe over alveolar and bronchiolar epithelia was detected in transgenic lungs (B, D, and F). Arrows, bronchiolar epithelium. Presented data are representative of 3.8.8 line. Three other lines showed significantly less alveolar expression (see RESULTS). Magnification: A and B, ×43; C and D, ×215; E and F: ×860.
3.7-kb SP-C promoter fragment. These results indicate that a significant enhancer region(s) lies upstream from the tissue-specific sequences.

Transgene expression overlaps but is not identical to endogenous SP-C expression. The 3.7-kb SP-C-CAT transgene expression was detected in both bronchiolar and alveolar epithelial cells of each 3.7-kb SP-C-CAT founder line, whereas native SP-C gene transcription was detected only in the surfactant-producing type II cell. To determine whether position effects or cis-active sequences of the human SP-C transgene controlled bronchiolar expression, levels and cellular sites of transgene expression were compared. Eight independent SP-C-CAT lines were generated by independent microinjection and subsequent transgene integration events. Four of these transgenic lines were bred to homozygosity and subject to further analysis. Additionally, the 3.7-kb SP-C-CAT transgenic lines resulted...
from low and high copy number transgene integrations, yet all the lines sustained bronchiolar CAT expression with no alteration of endogenous SP-C expression. Therefore, it is unlikely that the bronchiolar transgene expression is due to position effects or copy number. The possibility that multiple copies of transgene SP-C cis-active sequences sequester an essential SP-C transcriptional repressor is unlikely to account for the observed bronchiolar expression because the endogenous SP-C expression is not activated in bronchiolar epithelial cells by the loss of such a hypothetical repressor. A plausible interpretation is suggested by sequence homology between the human segment of SP-C DNA and the murine equivalent of upstream SP-C cis-acting sequences. Homology between the 3.7 kb of human DNA is not sequence identical but is arranged as three large regions of homology distributed over 4.8 kb of murine genomic DNA (Fig. 9A). Near identity between the human and murine DNA is preserved only in the promoter proximal 320 bp. When the two SP-C sequences are compared, multiple distinct inserts are identified that are specific to either the human or murine gene. Such species differences in sequence may partially disrupt precise binding of transcription factors to their intended cognate site or may displace the alignment of bound factors necessary to silence airway epithelial cell expression of the human transgene and sustain only alveolar type II-specific expression. Moreover, the type II cell only expression of the −1,910- to −215-bp deletion construct suggests that alveolar cell specificity resides within the conserved proximal region. When transgenic mice were generated with 2.4 kb of rat Clara cell secretory protein (CCSP) promoter, the 2.4-kb CCSP-CAT pattern of expression was similar to but not identical to the pattern of murine CCSP expression. The 2.4-kb CCSP-CAT mRNA was detected in epithelial cells along the trachea, bronchi, and large bronchioles. The 2.4-kb CCSP-CAT was diminished in distal bronchioles where murine CCSP expression was still maintained (29). Such species differences may
A second difference between the sites and levels of activity of the 3.7-kb SP-C-CAT transgene and endogenous SP-C mRNA was a reduced number of alveolar cellular sites of transgene expression. Each 3.7-kb SP-C-CAT and 3.7-kb SP-C-CAT deletion transgenic line had significantly less transgene-expressing alveolar cells than endogenous SP-C-expressing cells. Transgene expression levels were high enough that loss of hybridization signal could not account for reduced numbers of transgene-expressing cells. Variation in the number of target cells, which express a transgene, has been widely reported for tissue-specific genes analyzed in transgenic mice. Extensive transgene analysis of β-globin gene regulation represents the most complete studies to date, which define complex elements that determine uniform cell appropriate expression in vivo and minimize insertion site effects. Minimal β-globin transgenes demonstrated cell type- and developmental stage-specific expression but with a dramatic variation in the percentage of cells expressing the transgene between different lines made with the same construct (12). Transgene-expressing cells ranged from 0.3 to 88% in different lines, whereas little variation was seen in the relative levels of expression per cell. The variation in number of sites of globin transgene expression is similar to the variation in alveolar sites of human SP-C transgene expression described in this study. Further analysis of the globin locus control regulatory sites in transgenic mice defined multiple elements that cooperate to open chromatin structure and mediate uniform transgene transcription (5). To date, such equivalent locus control regions of the surfactant protein genes have not been identified. The β-globin transgene studies provide the conceptual basis for future experiments to characterize elements from the SP-C locus that mediate uniform alveolar cell expression. Other studies have indicated heterogeneity in type II cells. Wikenheiser and co-workers (33) noted...
that a subpopulation of type II cells maintained SP-B expression in the lungs of mice exposed to high levels of oxygen. In a separate study, Piedboeuf et al. (25) determined that in mice a subset of SP-C-positive type II cells expressed intercellular adhesion molecule-1 in response to oxygen exposure. Therefore, levels of gene expression can vary among type II cells in vivo. The present study suggests that additional cis-active sequences in the transgenes may be required to reduce the variation in number of type II cells expressing a SP-C transgene.

Localization of a regulatory region that is essential for lung epithelial cell transcription. An in vivo deletion analysis of the 3.7-kb promoter region was used to map sequences that mediate lung-specific gene transcription. CAT activity was detected only in the lungs of transgenic mice when the SP-C promoter was reduced to −1,225 or −590 bp in length (Fig. 3). In a separate study, lung-specific expression of a single transgenic line generated with 1277 bp of human SP-C promoter DNA was reported. Expression was localized to type II cells, but bronchiolar expression was not evaluated (8). This report is consistent with our transgenic analysis. The deletion SP-C-CAT transgenic lines were similar in high copy number to the 3.7-kb lines, yet the lung CAT expression was at the lower limits of detection. These results suggested that the distal sequences eliminated from the constructs included an enhancer(s) and that essential lung-specific elements were located in the −590-bp region. The presumptive enhancer may be associated with the two distal segments of conserved homology eliminated in the deletion constructs (Fig. 9).

Because of the very low level lung-specific CAT activity detected in the −590-bp SP-C-CAT lines, it was
unlikely that we would be able to detect expression from shorter deletions that might further reduce the CAT reporter signal. We therefore generated two sets of internal deletions that removed regions of DNA closer to the transcription start site than −590 bp but with intact distal sequences. By including distal regions of the original construct, we hoped to maintain transgene expression levels that were high enough to detect. The 3' boundary of the constructs retained either 365 or 215 bp of DNA adjacent to the basal TATAAA box of the SP-C gene. CAT activity was present only in lung extracts from transgenic animals generated with these transgenes. The results of this analysis precisely map a cis-active region for lung-specific expression to the 215 bp directly adjacent to the human SP-C promoter.

Deletion analysis of the rabbit SP-A gene promoter in transgenic mice demonstrated that a region between −378 and −47 bp is critical for bronchiolar and alveolar cell expression of SP-A-hGH (human growth hormone) transgenes. Low-level SP-A-hGH expression was also detected in heart, thymus, and spleen of some −991-bp SP-A-hGH or −378-bp SP-A-hGH transgenic lines. Extended −4,000-bp SP-A promoter constructs were expressed exclusively in the lung, indicating that distal sequences may be required to silence nonlung SP-A expression (1). In a separate study, regions necessary for cell-specific expression of the murine CCSP gene were identified in transgenic mice. A −166-bp region was shown to confer bronchiolar-specific expression, whereas a second region between −803 and −166 bp was required for high-level CCSP expression (26). Consistent with our findings, both of these studies indicate that multiple cis-active regions contribute to pulmonary cell-specific expression.

Transcription from human SP-C promoter is stimulated in vitro by TTF-1. TTF-1 is a member of the dispersed family of Nkx homeodomain transcription factors and is expressed in developing thyroid, brain, and lung (22). Targeted mutation of the TTF-1 gene disrupted development of the distal lung, demonstrating that TTF-1 is a critical determinant of lung maturation (19). The onset of TTF-1 expression precedes SP-C expression and is maintained along the entire airway epithelium during development (22, 37). These observations implicated TTF-1 as a potential regulator of surfactant protein gene expression. Cotransfection of TTF-1 expression plasmids with test promoter constructs demonstrated that TTF-1 activates expression of murine SP-A, human and murine SP-B, murine SP-C, and rat CCSP promoters (3, 4, 17, 36). TTF-1 transactivation of the murine SP-C promoter was mediated by binding to two adjacent sites at −186 to −163 bp of the murine SP-C promoter (17). These two TTF-1 responsive sites are conserved between the murine and human SP-C promoters and are located within the −215-bp human SP-C region shown in the current study to be essential for lung-specific transgene expression. We demonstrate that the human 3.7-kb SP-C-CAT and −1,910- to −215-bp deletion SP-C-CAT constructs are transactivated by coexpression of TTF-1. These results indicate that TTF regulation of SP-C is conserved across species and that the deletion clone, which mapped alveolar-specific expression at −215 bp in vivo, also harbors the TTF-1 responsive region of the human SP-C promoter.
Each TTF-1 responsive gene has been shown to have a unique arrangement and number of TTF-1 binding sites with distinct TTF-1 binding affinities. In addition to distinct configurations of TTF-1 sites, the DNA binding activity of TTF-1 has been shown to be dependent on phosphorylation state and responsive to redox regulation (2, 35). Analysis of the TTF-1 gene identifies multiple promoter and transcription initiation sites that might produce alternate isoforms of TTF-1 as well (13, 23). Collectively these reports suggest that numerous subtle modifications of TTF-1 may alter selective expression of a gene in type II cells relative to Clara or other airway epithelial cells.

It is unlikely that TTF-1 alone is sufficient to dictate the precise expression of SP-C and other surfactant protein genes in differentiated airway and alveolar cells because each surfactant protein gene is expressed in a unique cellular and developmental pattern. The transcription factors hepatocyte nuclear factor (HNF)-3 and forkhead homolog (HFH) family members have been shown to be expressed in the bronchial epithelium where the SP-A, SP-B, and CCSP genes are expressed (37). HNF-3β stimulates human SP-B and human TTF-1 gene expression, whereas HNF-3α suppresses HNF-3β stimulation of the TTF-1 gene (3, 14). The transcription factor GATA-6 is expressed along the developing airway epithelium and transactivates the TTF-1 promoter in transient transfection experiments (28). These experiments are the first evidence for complex networks regulating gene expression in developing lung cells. Footprint analysis of the active promoter regions of SP-A, SP-B, and SP-C indicate that numerous other protein-DNA interactions have occurred (4, 3, 17). The combination of this current biochemical data with HNF-TTF-1 transactivation data suggests that combinatorial interactions of TTF-1 and other transcription factors orchestrate the abundance and distribution of SP-A, SP-B, SP-C, and CCSP in a cell-specific manner along the respiratory epithelium.

The present study demonstrates that the ∼590-bp segment of human SP-C DNA is sufficient to initiate and sustain lung-specific transgene expression. Transgenic mice with internal deletions of the SP-C DNA map cis-active elements, which mediate transcription in alveolar cells to ∼215 bp and a second distal segment of DNA that sustains a high level of SP-C expression in vivo. An extensive deletion analysis of the 4.8-kb murine SP-C promoter in vitro did not identify any distal enhancer activity, suggesting that elevated in vivo expression requiring the distal human SP-C region is chromatin dependent (17). The 3.7-kb human SP-C promoter has been used to generate transgenic mice expressing growth factors, cytokines, and other reporters in vivo. The present findings indicate that in such models the extent of type II cell expression may vary for each founder line and should be considered in the interpretation of phenotypes. The human SP-C promoter is also transactivated by TTF-1 at cis-active sites in close proximity to the SP-C promoter, supporting the concept that TTF-1 is critical in regulating cell-specific SP-C gene expression.

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REFERENCES


