The murine SP-C promoter directs type II cell-specific expression in transgenic mice

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Glasser, Stephan W., Susan K. Eszterhas, Emily A. Detmer, Melissa D. Maxfield, and Thomas R. Korfhagen. The murine SP-C promoter directs type II cell-specific expression in transgenic mice. Am J Physiol Lung Cell Mol Physiol 288: L625–L632, 2005. First published December 3, 2004; doi:10.1152/ajplung.00250.2004.—Genomic DNA from the mouse pulmonary surfactant protein C (SP-C) gene was analyzed in transgenic mice to identify DNA essential for alveolar type II cell-specific expression. SP-C promoter constructs extending either 13 or 4.8 kb upstream of the transcription start site directed lung-specific expression of the bacterial chloramphenicol acetyl transferase (CAT) reporter gene. In situ hybridization analysis demonstrated alveolar cell-specific expression in the lungs of adult transgenic mice, and the pattern of 4.8 SP-C-CAT expression during development paralleled that of the endogenous SP-C gene. With the use of deletion constructs, lung-specific, low-level CAT activity was detected in tissue assays of SP-C-CAT transgenic mice retaining 318 bp of the promoter. In transient and stable cell transfection experiments, the 4.8-kb SP-C promoter was 90-fold more active as a stably integrated gene. These findings indicate that 1) the 4.8-kb SP-C promoter is sufficient to direct cell-specific and developmental expression, 2) an enhancer essential for lung-specific expression maps to the proximal 318-bp promoter, and 3) the activity of the 4.8-kb SP-C promoter construct is highly dependent on its chromatin environment.

PULMONARY SURFACTANT is a phospholipid and protein mixture that lines the gas-exchanging regions of the lung and prevents alveolar collapse during respiration (10). Pulmonary surfactant is synthesized and secreted in the alveolus by distinct cuboidal type II cells. The composition, alveolar concentration, and intracellular reservoir of pulmonary surfactant is precisely maintained in the normal lung. Alterations in surfactant composition or homeostasis can inhibit normal respiratory function. Neonatal respiratory distress is due to inadequate surfactant levels. Surfactant dysfunction can also result from microbial, particulate, or gaseous injury causing vascular leak that inhibits surfactant function. Surfactant inactivation contributes to the adult respiratory distress syndrome. A synthetic replacement surfactant consisting of surfactant phospholipids supplemented with a recombinant SP-C peptide as the only protein component restores pulmonary function in several animal models of surfactant-depleted acute lung injury (11). Addition of purified SP-C to synthetic phospholipid preparations lowers the surface activity to levels approximating whole native surfactant preparations (24).

The identification of naturally occurring SP gene mutations in humans and the development of gene knockout models in mice have provided insight into “nonsurfactant” functions. Correct temperospatial expression of the SP-C gene and subsequent processing of SP-C and its release from the type II cell is essential to normal pulmonary homeostasis. Mutations that alter normal intracellular pro-SP-C processing have been identified in cases of familial idiopathic interstitial pneumonitis (19, 23). Affected individuals within a family can present with highly variable onset and severity of clinical disease suggesting a complex etiology due to environmental influences or unknown modifier genes. SP-C-deficient mice generated by targeted gene inactivation develop a progressive interstitial lung disease consisting of cellular infiltrates and alveolar remodeling with fibrotic lesions in the most severely affected animals (8).

In the adult lung, SP-C mRNA expression is detected at high levels only in type II alveolar epithelial cells, whereas other SP genes are expressed in bronchiolar epithelial cells (SP-A and SP-B) and subsets of tracheal gland cells (SP-A and SP-D) as well as type II cells. Thus SP-C is the only gene expressed only in type II cells and has served as a marker of type II cell differentiation in the mammalian lung. In cell transfection experiments, the SP-C promoters are activated by an overlapping set of transcription factors that include thyroid transcription factor (TTF)-1, hepatocyte nuclear factors, GATA-6, and nuclear factor I (NFI). Analysis of SP gene promoters in transgenic mice has been used to correlate the arrangement of in vitro cis-active sites to actual in vivo regulation as well as to identify new cis-active regions, such as developmental control
and chromatin-dependent enhancers that may go undetected in transient cell experiments.

In transgenic mice generated with human SP-C genomic DNA, 3.7 kb of the human SP-C promoter directed lung-specific expression of a diphtheria toxin A or chloramphenicol acetyl transferase (CAT) reporter gene in transgenic mice (9, 14). Subsequent experiments demonstrated that this region of DNA directed developmental transgene expression similar to the endogenous gene and that expression was sustained in bronchiolar and alveolar type II cells of the adult lung (26). Further dissection of the elements critical for type II cell-specific transcription have proven complicated by the bronchoalveolar pattern of expression. To discern what elements are essential for accurate temporal spatial expression in the lung, we have analyzed segments of the mouse SP-C gene promoter in transgenic mice.

MATERIALS AND METHODS

SP-C-CAT constructs and transgenic mice. A 4.8-kb fragment of murine SP-C genomic DNA was isolated from a 129/J library and subcloned into the promoterless pBLCAT 6 expression plasmid. The –318 to –230-bp deletion constructs were generated by restriction digestion of the 4.8-kb fragment. The 13-kb SP-C-CAT construct was generated by ligation of an 8.2-kb SalI-BamHI genomic fragment to a unique 5’ BamHI site in the proximal 4.8-kb SP-C promoter fragment (12). Restriction digestion at unique sites in the flanking multicloning sites was used to liberate the transgene constructs. Transgene constructs were purified from plasmid vector DNA by electrophoresis through low-melt agarose gels. DNA was recovered and purified for microinjection using Magic PCR columns (Promega, Madison, WI). DNA was resuspended in 5 mM Tris microinjection solution using Magic PCR columns (Promega, Madison, WI). DNA was recovered and purified for microinjection using Magic PCR columns (Promega, Madison, WI). DNA was resuspended in 5 mM Tris microinjection solution using Magic PCR columns (Promega, Madison, WI).

Materials and methods were removed and replaced with selection media (HITES, 4% neo, 0.125 pmol of pGL-2, or 0.18 pmol of SP-C-luciferase (luc) DNA isolated from tail clips of control and candidate SP-C-CAT transgenic mice. Five micrograms of DNA were digested with the restriction enzyme EcoRI and separated on 0.8% agarose gel for Southern blot analysis. A 550-bp CAT fragment was labeled with α-dCTP by nick translation and used as the hybridization probe. Blots were hybridized overnight and washed at a final stringency of 0.5× SSC at 65°C and subjected to autoradiography. Films were scanned, and relative densitometer values of the SP-C-CAT diagnostic bands were compared.

RESULTS

Transgenic mouse lines were generated with two large fragments of genomic DNA from the mouse SP-C gene directing CAT reporter gene expression. One construct incorporated 4.8 kb of DNA upstream of the basal promoter. By sequence comparison, the mouse 4.8-kb DNA fragment was equivalent to a 3.7-kb human SP-C promoter fragment used in previous transgenic studies. Analysis of the 4.8-kb SP-C promoter in vivo could potentially distinguish expression differences due to species-specific variation between mouse and human sequences. A second construct was made with 13 kb upstream of the promoter to functionally identify any distal enhancer sequence that would contribute to type II cell specificity or that

In situ hybridization. Lungs of adult mice were inflation fixed at 20-cm pressure with freshly prepared 4% paraformaldehyde. Animals taken for developmental time points were fixed by immersion after the thoracic cavity was opened. Tissue was embedded in paraffin, and 4-μm sections were cut. 35S-labeled riboprobes for bacterial CAT and murine SP-C were synthesized from pGEM plasmids. Probe synthesis, hybridization, and wash conditions have been previously described (26). Specificity of CAT hybridization was established by comparison of antisense CAT probe hybridization to nontransgenic lung and hybridization signal from CAT and SP-C sense strand probes. Equivalent total counts of CAT or SP-C riboprobes were used in individual hybridizations.

Relative copy number estimation. Genomic DNA was isolated from tail clips of control and candidate SP-C-CAT transgenic mice. Five micrograms of DNA were digested with the restriction enzyme EcoRI and separated on 0.8% agarose gel for Southern blot analysis. A 550-bp CAT fragment was labeled with α-dCTP by nick translation and used as the hybridization probe. Blots were hybridized overnight and washed at a final stringency of 0.5× SSC at 65°C and subjected to autoradiography. Films were scanned, and relative densitometer values of the SP-C-CAT diagnostic bands were compared.

Fig. 1. Organ-specific expression of the mouse 13- and 4.8-kb surfactant protein C-chloramphenicol acetyl transferase (SP-C-CAT) transgenes. CAT enzyme assays were performed as described in MATERIALS AND METHODS with tissue extracts prepared from organs of the 4.8 SP-C-CAT transgene-positive mouse line 4.4 (A), age-matched nontransgenic FVB/N mice (B), and 13-kb SP-C-CAT C line (C). Lane 1, lung; lane 2, heart; lane 3, liver; lane 4, kidney; lane 5, spleen; lane 6, muscle; lane 7, salivary gland; lane 8, no extract, negative control (C only). Significant CAT enzyme activity is detected only in lung extract of transgene-positive lung from 4.8 SP-C-CAT and 13 SP-C-CAT mice (arrows). Nearly identical lung-specific expression was obtained with tissue extracts of a second 4.8 SP-C-CAT transgenic line 6.1 (not shown). Presented data are representative of four 4.8 SP-C-CAT mice and eight 13 SP-C-CAT mice.
would silence bronchiolar expression seen with the human 3.7-kb SP-C promoter.

**Tissue-specific expression of 4.8-kb SP-C-CAT transgenes.** Three 4.8-kb SP-C-CAT transgene founder mice were identified by genomic Southern blot. Two of the 4.8-kb SP-C-CAT founders were bred to establish lines 4.4 and 6.1. Tissue extracts were prepared from organs of adult mice homozygous for the transgene from both lines and assayed for CAT enzyme activity. High levels of CAT activity were detected only in lung extracts of both 4.4 SP-C-CAT and 6.1 SP-C-CAT mice and not in other tissues. Lung and other organ extracts were uniformly negative from nontransgenic control mice (Fig. 1). The murine 4.8-kb promoter segment targeted lung-specific gene expression.

**Cell-specific expression of 4.8-kb SP-C-CAT transgenes.** The cellular sites of 4.8-kb SP-C-CAT expression from lines 4.4 and 6.1 in the lung were determined by in situ hybridization with CAT riboprobes. Sections of adult lung and *ed* 13 and *ed* 16 lung from both 4.4 and 6.1 mice were compared with the pattern of expression for the endogenous SP-C gene. Strong focal alveolar CAT expression was detected in sections of adult lung from both lines (Fig. 2). Bronchiolar expression was absent, whereas CAT expression was restricted to alveolar cells and overlapped expression of SP-C. These findings from two independent founder lines indicate that the 4.8-kb region is sufficient to restrict expression to the adult type II cells.

**Development expression of 4.8-kb SP-C-CAT transgenes.** Tissue CAT assays and in situ analysis were used to assess whether developmental expression was encoded by the 4.8-kb SP-C-CAT transgene. Extremely low levels of lung-specific CAT activity was detected in extracts from *ed* 13 embryos. Significantly higher levels of transgene CAT activity was detected in lung extracts from *ed* 16 embryos (Fig. 3). Low-level 4.8-kb SP-C-CAT expression was localized by in situ hybridization over the epithelial cells at the distal boundary of the developing tubules in *ed* 13 lungs (Fig. 4). CAT or SP-C expression was not detected over large proximal airways or mesenchymal tissue. At *ed* 16, the relative level of CAT signal was increased relative to *ed* 13 and was confined to the numerous small distal airway epithelia, consistent with apparent increased branching (Fig. 5). Whereas the developmental pattern of 4.8-kb SP-C-CAT transgene expression was similar to the pattern of SP-C expression, in situ signals of 4.8-kb...
SP-C-CAT expression were modest in comparison with the intensity of endogenous SP-C signal.

Lung- and cell-specific expression of 13-kb SP-C-CAT transgenes. Four transgenic lines were established with the 13-kb SP-C-CAT construct. CAT reporter gene activity was lung specific (Fig. 1C). Transgene expression was detected in a focal pattern throughout the parenchyma. No aberrant bronchiolar expression was detected (Fig. 2, E and F). The number of alveolar sites of transgene expression varied between 13 SP-C-CAT lines similar to the findings obtained from the 4.8-kb SP-C-CAT lines. The extended 13 kb of SP-C DNA, therefore, do not appear to harbor enhancers that confer uniform expression among all type II cells. Whereas the cis-active elements in the SP-C sequences confer lung- and type II cell-specific expression, other chromatin-dependent elements associated with the SP-C locus may be required to elicit uniform transcription in all type II cells.

Specificity of truncated 0.32 SP-C-CAT transgene expression. To map essential cis-active regulatory regions, eight founder transgenic lines were established from constructs consisting of 318 or 230 bp of 5'-DNA. Tissue CAT activity was not detected from the 230-bp SP-C-CAT lines. Very weak CAT activity was detected in lung extracts from the 318-bp SP-C-CAT line 1.3 (Fig. 6). This finding localizes an essential cis-active determinant of SP-C transcription to the proximal 318 bp of the SP-C promoter. The relative transgene copy number for 4.8 SP-C-CAT and 318 SP-C-CAT lines was compared. Both 4.8 SP-C-CAT lines 4.4 and 6.1, expressed at high levels, were extremely low copy number (line 4.4, 1 copy) or low copy number (line 6.4, 3 copies). The only 318 SP-C-CAT line to express had 30 copies relative to 4.8 SP-C-CAT line 4.4. Therefore, the high-level 4.8 SP-C-CAT expression did not reflect an increased transgene copy number.

Increased 4.8-kb promoter activity is due to chromatin. Whereas the 4.8-kb promoter was far more active in transgenic mice than the 318-bp promoter, the 4.8-kb promoter construct was only slightly more active than the 318-bp promoter in transient cell transfection experiments (12). To determine whether the increased activity of 4.8-kb SP-C promoter activity in vivo could be explained by a chromatin-dependent mechanism, 4.8-kb SP-C-luc and 318-bp SP-C-luc gene expression was compared when stably integrated in cells. Stable transduced MLE-15 cells were generated by cotransfection with a pGKneo plasmid and selection of neomycin (G418)-resistant colonies. Equal molar amounts of 318-luc or 4.8-luc DNA were used in transfections. A similar number of G418-resistant colonies were obtained in each of seven independent transfections, indicating that efficiency of integration was nearly identical with both 318-SP-C-luc and 4.8-SP-C-luc constructs. In each experiment, between 200 and 500 G418-resistant colonies were harvested per plate so that measured reporter activity reflected numerous independent integrations. Luciferase assays were normalized to total cell protein. Luciferase reporter activity was 90-fold higher in extracts from 4.8-kb SP-C-luc-transfected colonies than in the 318-bp SP-C-luc cell extracts. In contrast, the 4.8-kb SP-C-luc luciferase activity was only seven- to eightfold higher than 318-bp SP-C-luc activity in transient transfection experiments (Fig. 7). This finding indicates that incorporation of the 4.8-kb SP-C DNA into chromatin structure stimulates enhancer activity by more than an order of magnitude. Virtually no luciferase activity was detected in cells transfected with the promoterless luciferase plasmid, indicating that potential stimulation of luciferase activity due to integration of test DNA adjacent to active but unrelated cellular promoters was minimal.

Fig. 3. CAT activity in tissue extracts during mouse embryonic development. CAT assays were performed using extracts prepared from 4.8 SP-C-CAT transgenic mice at embryonic day 13 (A) and embryonic day 16 (B) and nontransgenic mice (C). Arrows indicate specific acetylated chloramphenicol reaction products. CAT activity was barely detected in day 13 lung and was abundant in day 16 lung extracts. Organ extracts in A and C: lane 1, liver; lane 2, heart; lane 3, intestine; lane 4, lung. Organ extracts in B: lane 1, liver; lane 2, heart; lane 3, intestine; lane 4, kidney; lane 5, stomach; lane 6, lung. Presented data are representative of 4 mice from 4.8-kb SP-C-CAT transgenic line.
DISCUSSION

SP-C is distinguished from other SP by being expressed only in alveolar type II cells and serves as a specific type II cell marker during lung maturation or injury response. SP-C gene expression has also been analyzed in vitro in cell transcription assays and in vivo transgenic models to ascertain mechanisms regulating SP-C gene transcription. The mouse SP-C gene promoter was analyzed in transgenic mice with the goal of identifying cis-active regions that support alveolar type II cell expression. Previous transgenic studies using the human SP-C promoter demonstrated lung-specific expression but were complicated by the finding that human 3.7-kb SP-C transgenes did not recapitulate the expression pattern of the endogenous SP-C gene. The human SP-C transgene was expressed in alveolar type II cells and robustly in bronchiolar cells (7, 26). Analysis of the mouse SP-C promoter constructs in transgenic mice identified promoter regions that direct cell-appropriate expression, a proximal region essential for in vivo promoter activity, and a distal chromatin-dependent regulatory domain that functions as an enhancer.

Tissue CAT assays and CAT in situ hybridization experiments demonstrated that 4.8 kb of mouse SP-C promoter and 5’-flanking DNA targeted lung and alveolar cell-specific expression in adult mice. In an independent study, the mouse 4.8-kb SP-C promoter was used to restore SP-B expression in lungs of SP-B−/− mice. SP-B mRNA and protein expression was type II cell specific for two of the three lines and was detected only in a subset of the alveolar type II cells (17). The murine 4.8-kb promoter segment is the sequence equivalent to the 3.7 kb human promoter wherein three regions of homology are conserved between the two species (7). The bronchiolar expression obtained from the human promoter likely results from altered assembly of murine transcription complexes on human binding sites or from displacement of complexes that silence SP-C expression in bronchiolar cells. The number of alveolar cells expressing 4.8 SP-C-CAT in the transgenic lines was reduced relative to sites of endogenous SP-C expression, recapitulating results obtained with the human SP-C promoter. This consistent finding suggested either that additional enhancers with type II cell activity were missing or that uniform type II cell expression is context dependent, requiring sequences and higher-ordered chromatin unique to the SP-C locus. Such modifiers have been documented and include matrix attachment regions that tether chromatin loops at highly transcribed

Fig. 4. Expression of 4.8 SP-C-CAT transgene at embryonic day 13. Dark-field images of sense (A) and antisense (B) SP-C hybridization. Sense (C) and antisense (D) images of CAT hybridization. Magnification, ×4. Weak CAT and strong SP-C signals are detected by antisense probes in peripheral tubules. E and F are high magnification. Arrowheads indicate central tubule that is negative for CAT expression, and small arrows indicate peripheral airways positive for CAT expression. Magnification, ×20.
gene loci, locus control regions that modify gene expression over distance and/or insulator elements that limit active gene transcription and selective alteration of histone acetylation (6). Transgenic mice were generated with extended 13 kb of SP-C promoter and upstream DNA to test whether extended sequences harbored such type II cell normalizing elements. Each of four separate 13-kb SP-C-CAT founder lines supported alveolar (type II) cell-specific expression. Surprisingly, the number of CAT-positive alveolar cells varied between each founder line, with one line having 79% of cells expressing the CAT gene compared with the number of cells expressing SP-C. Expression from the other three 13-kb SP-C-CAT lines had diminished number of sites of expression similar to the 4.8 SP-C-CAT lines. The current analysis with the extended promoter does not rule out intron or 3′/H11032 enhancer(s) that would confer uniform type II cell expression. The structural features of the SP-C locus limit the possibility of 3′ regulatory elements wherein the SP-C gene terminates adjacent to the promoter of a new gene. The sequence immediately 3′ of SP-C exon 6 is G-C rich (82%) and serves as the promoter of the bone morphogenetic protein (BMP)-1 gene, with the BMP-1 transcription start site only 700 bp downstream of the SP-C gene (22). The BMP-1 gene encodes an essential developmental procollagenase C gene. The BMP-1 exon 1 is followed by extended CA dinucleotide repeats that are highly polymorphic. The structural features of the unusual sequence immediately 3′ of the SP-C gene suggest that 3′ cis-active regulation of the SP-C gene is unlikely.

Extremely low level but lung-specific CAT activity was detected when the mouse 4.8-kb SP-C promoter was deleted to 318 bp. This finding indicates that significant enhancer activity is located in the deleted distal region. The nature of the enhancer is unclear, but initial cell transfection experiments suggest that it is responsive to higher-ordered DNA structure. When previously tested in transient cell transfection, only modestly elevated 4.8 SP-C-CAT expression levels were seen compared with 318 SP-C-CAT activity (12). By selecting for stable chromosome integration, the relative expression of the 4.8 SP-C to the 318-bp SP-C promoter was 90-fold greater than differences detected in transient assays. This finding is consistent with strong 4.8 SP-C vs. 318 SP-C expression in transgenic mice and indicates chromatin association is necessary for activity of an enhancer upstream of 318 SP-C sequence. The 318 SP-C-CAT transgenes were present at >10-
An enhancer was mapped to the rabbit SP-A promoter studies provide initial insights into elements required for bronchoalveolar expression, identification of cis-elements may be affected by species sequence variation as seen with the SP-C promoters.

The essential 318-bp SP-C sequence conferring lung-specific activity identified in this study spans a region where multiple factors interact to influence SP-C transcription in vitro. The homeobox transcription factor TTF-1 (also termed Nkx2.1) trans-activates the 318-bp SP-C promoter (12). With the use of mutagenesis assays, NFI binding sites were identified and shown to be required for SP-C promoter activity (3). Co-transfection experiments demonstrated that NFI and TTF-1 synergistically activate 318 SP-C promoter activity (4). TTF-1 also directly interacts with the transcription factor TAZ to enhance 318 SP-C promoter activity (20) and interacts cooperatively with GATA-6 to increase SP-C expression (18). In addition, TTF-1/GATA-6 interactions enhance SP-A activity (5). Deletion mapping of the human SP-C promoter in transgenic mice showed that the equivalent TTF-1-responsive region was required for lung-specific expression in vivo, similar to the current findings (7). Thus the combined in vitro promoter activation studies and functional in vivo mapping implicate a critical role for the TTF-1-responsive region in controlling the level and cell-specific expression of SP-C.

The current study demonstrates that 4.8 kb of promoter sequence activates and sustains alveolar cell-specific expression in the lungs of transgenic mice. A proximal 318-bp region containing known cis-active NFI, TTF-1, and GATA-6 binding sites is critical for lung-specific expression. High-level expression from the 4.8-kb promoter is mediated by an element that directs vigorous expression as an integrated transgene. Two recent reports have assessed SP-B promoter studies provide initial insights into elements required for bronchoalveolar expression, identification of cis-elements may be affected by species sequence variation as seen with the SP-C promoters.

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is dependent on a chromatin environment. The pattern of CAT expression directed by 4.8 SP-C promoter was alveolar type II cell specific. The aberrant bronchiolar component of expression obtained with the human 3.7 SP-C promoter in transgenic mice is due to species/sequence differences. The 3.7 SP-C promoter has been used successfully to express a variety of bioactive reporter molecules in lungs of transgenic mice. The 4.8-kb murine SP-C promoter may have further utility in directing expression restricted to just type II cells in vivo and thus provides a useful tool for developing transgenic models.

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