Analysis of genomic regions involved in regulation of the rabbit surfactant protein A gene in transgenic mice

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Abstract

Analysis of genomic regions involved in regulation of the rabbit surfactant protein A gene in transgenic mice. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21):L349–L361, 1999.—The gene encoding surfactant protein (SP) A, a developmentally regulated pulmonary surfactant-associated protein, is expressed in a lung-specific manner, primarily in pulmonary type II cells. SP-A gene transcription in the rabbit fetal lung is increased by cAMP. To delineate the genomic regions involved in regulation of SP-A gene expression, lines of transgenic mice carrying fusion genes composed of various amounts of 5′-flanking DNA from the rabbit SP-A gene linked to the human growth hormone structural gene were established. We found that as little as 378 bp of 5′-flanking DNA was sufficient to direct appropriate lung cell-selective and developmental regulation of transgene expression. The same region was also sufficient to mediate cAMP induction of transgene expression. Mutagenesis or deletion of either of two DNA elements, proximal binding element and a cAMP response element-like sequence, previously found to be crucial for cAMP induction of SP-A promoter activity in transfected type II cells, did not affect lung-selective or temporal regulation of expression of the transgene; however, overall levels of fusion gene expression were reduced compared with those of wild-type transgenes.

type II cell; lung; developmental regulation; hormonal regulation

The Synthesis of Pulmonary Surfactant, a developmentally regulated lipoprotein, is restricted to type II pneumonocytes of the lung alveoli where surfactant is stored as lamellated cytoplasmic inclusion bodies termed lamellar bodies. Surfactant synthesis in the fetal lung appears to be regulated by a number of hormones and factors, including glucocorticoids, prolactin, and hormones that increase cAMP formation, including catecholamines, acting through β2-adrenergic receptors (29, 33), prostaglandin E2, and vasactive intestinal peptide (V. Boggaram, M. E. Smith, and C. R. Mendelson, unpublished observations). In studies of fetal rabbit lung explants maintained in organ culture, we have found that glucocorticoids have both stimulatory and inhibitory effects on SP-A gene transcription. Treatment of lung explants from 21–24 day gestational age fetal rabbits with cortisol or dexamethasone (Dex; 10−7 M) caused an acute (6–24 h) inhibition of SP-A gene expression and reduced the magnitude of the stimulatory effect of cAMP. However, after 48–72 h of incubation, a stimulatory effect of glucocorticoids on SP-A gene transcription was observed, and there was found to be an additive stimulatory effect with dibutyryl cAMP (DBCAMP) (6).

In type II cell transfection studies, we observed that 378 bp of rabbit SP-A 5′-flanking DNA mediated cAMP induction of SP-A promoter activity (2). Elevated levels of basal expression were observed only in type II cells or
in type II-derived lung adenocarcinoma cell lines (2, 49). Furthermore, cAMP induction of SP-A promoter activity was evident only in primary cultures of type II cells and not in type II-derived lung adenocarcinoma cell lines (2, 49). These findings suggest that the cell lines either have lost the capacity for cAMP responsiveness or lack a transcription factor(s) that is an essential mediator of cAMP responsiveness. Our laboratory has also found that a cAMP response element (CRE)-like sequence [CRE for the SP-A promoter (CRESP-A); TGACCTC/TAG] present in the 5'-flanking regions of the rabbit (2, 31) and human (49) SP-A genes at –261 and –242 bp, respectively, is required for basal and cAMP induction of SP-A promoter activity. Michael et al. (31) have observed that the protein(s) that binds to this element is distinct from CRE binding protein (CREB)/activating transcription factor family members and is likely a member of the nuclear-receptor family.

A lung-specific DNase I-hypersensitive site at –100 bp from the SP-A gene transcription start site also has been identified (12). This hypersensitive site was detectable in chromatin isolated from lung tissues of 22- and 28-day gestational age fetal rabbits as well as from adults. Another DNase I-hypersensitive site at –1,300 bp was detected in the lung as well as in a number of other tissues of fetal and adult rabbits (12). Interestingly, these two hypersensitive sites are in close proximity to two elements that we previously found to be important in basal and cAMP-induced transcription of the rabbit SP-A gene; these are denoted as the proximal binding element (PBE) at –80 bp and the distal binding element (DBE) at –980 bp (13, 14). The DBE contains the core sequence CACGTG, which corresponds to an E-box motif to which members of the basic helix-loop-helix-leucine zipper family of gene regulatory proteins are known to bind. The PBE contains a related sequence, CTTCGTG. Our laboratory has recently found that the basic helix-loop-helix-leucine zipper family members upstream stimulatory factor-1 (14) and -2 (Gao and Mendelson, unpublished observations) bind to both of these elements. Our laboratory has also observed that cAMP induction of SP-A promoter activity is mediated by increased phosphorylation and binding of the homeodomain factor thyroid transcription factor-1 (26) and through the binding of Sp1 and related factors to a GT box (50) in the 5'-flanking regions of the baboon and human SP-A2 genes, respectively.

Despite the fact that glucocorticoids stimulate rabbit SP-A transcription and have an additive stimulatory effect with cAMP (6), we previously observed that glucocorticoids antagonized the stimulatory effect of cAMP on the expression of SP-A:human growth hormone (hGH) fusion genes containing up to 1,754 bp of SP-A 5'-flanking DNA in transfected type II cells (2). An inhibitory effect of glucocorticoids was also observed when 650 bp of an SP-A downstream sequence (containing the first exon, intron, and second exon) were also included in the fusion gene constructs (2). To date, we have been unable to identify a DNA element that mediates a stimulatory effect of glucocorticoids on rabbit SP-A gene transcription.

To begin to define the regions within and surrounding the rabbit SP-A gene involved in developmental, lung cell-specific, and multifactorial regulation of expression, we have created transgenic mice carrying SP-A:hGH fusion genes composed of 47–4,000 bp of rabbit SP-A 5'-flanking DNA linked to the hGH structural gene as a reporter. We have observed that as little as 378 bp of SP-A 5'-flanking sequence is sufficient to mediate appropriate lung cell-selective, developmental, and hormonal regulation of SP-A promoter activity in the transgenic mice.

**EXPERIMENTAL PROCEDURES**

Construction of plasmids containing fusion genes. DNA purification, restriction endonuclease digestion, ligation, agarose gel electrophoresis, transformation, and maintenance of Escherichia coli were carried out according to methods of Sambrook et al. (37). The plasmid p0GH (40), which contains a promoterless hGH structural gene subcloned into pUC12, was used to construct the rabbit SP-A:hGH fusion genes. Schematic diagrams of the fusion genes used to create transgenic mice are shown in Fig. 1. To construct SP-A-378:hGH, a Hind III–Sau 3AI fragment of rabbit genomic DNA containing –47 bp of DNA flanking the 5'-end of the SP-A gene transcription initiation site (including the TATA box) and the first 20 bp of exon I (12) was fused to the first exon of the hGH structural gene in p0GH by ligation to a Bam HI site. SP-A-378:hGH was constructed by fusing an EcoRI I–Sau

![Fig. 1. Schematic diagram of rabbit surfactant protein (SP) A-human growth hormone (hGH) fusion genes. Solid bars, regions derived from rabbit SP-A gene (20 bp of 1st exon) and 5'-flanking DNA; hatched bars, DNA encoding hGH gene. Arrow, position of SP-A transcription initiation site and direction of transcription.](image-url)
Production and identification of transgenic mice. After digestion of recombinant plasmids with the appropriate restriction endonucleases to release the fusion genes from vector sequences, the fusion genes were isolated on agarose gels and purified as described by Short et al. (41). Fusion gene DNA was microinjected into the male pronucleus of fertilized F2 hybrid mouse eggs (obtained by mating C57B1/6× SJL hybrid adults), which then were cultured to the two-cell stage, reimplanted into pseudopregnant mice (10, 34), and allowed to develop to term. Transgenic progeny were identified, and the number of fusion gene copies per genome was determined by dot blot analysis of tail DNA, with hGH DNA as a probe. Copy number was determined by comparison of the hGH-specific signal present in the tail DNA with the signal of a known amount of hGH structural gene that was added to the tail DNA of a nontransgenic mouse.

Production of recombinant adenoviruses. Recombinant adenoviruses containing rabbit SP-A:hGH fusion genes were produced and characterized as described previously (2, 3).

Western analysis of SP-A proteins. Lung tissue explants from 15-day gestational age fetal transgenic and nontransgenic mice were maintained in organ culture in serum-free Waymouth MB752/1 medium (GIBCO BRL, Life Technologies, Grand Island, NY) in the presence of DBcAMP (1 mM), Dex (10−7 M), or both agents in combination for up to 5 days (42, 44). The explants were maintained in a humidified atmosphere of 95% air-5% CO2.

RESULTS

The 378-bp sequence flanking the 5′-end of the rabbit SP-A gene is sufficient to direct expression of the SP-A: hGH fusion genes in lung tissues of transgenic mice. Five SP-A:hGH fusion genes containing various amounts of SP-A 5′-flanking DNA (SP-A−47:hGH, SP-A−378:hGH, SP-A−991:hGH, SP-A−1765:hGH, and SP-A−4000:hGH) were constructed (Fig. 1) and introduced into the genomes of mice as described in EXPERIMENTAL PROCEDURES. The founder mice were bred to eliminate the effects of mosaicism; thus the analysis was conducted in established lines. Table 1 shows the copy numbers (as determined by Southern blot analysis described in EXPERIMENTAL PROCEDURES) and expression (as determined by Northern analysis of mRNA described in EXPERIMENTAL PROCEDURES) of the fusion genes in lung tissues of the established transgenic mouse lines. The lack of hGH expression in the lung tissues of 13 of the 13 lines of mice carrying the SP-A−47:hGH fusion gene and the presence of hGH expression in the lung tissues of 9 of 14 lines of mice carrying the SP-A−378:hGH fusion gene indicate that SP-A 5′-flanking sequences between −47 and −378 bp are required for expression in the lung. Expression in the lung of the SP-A:hGH transgenes containing 991 bp (expression in 15 of 15 lines) and −4,000 bp (expression in 2 of 6 lines) of the SP-A 5′-flanking sequence was also evident (Table 1). The relatively low proportion of mice expressing the 4,000-bp-containing fusion gene compared with that of the other constructs may be caused by the possible presence of silencer elements.
within the 4,000-bp 5′-flanking region that recruit transcriptional repressors and promote a "closed" chromatin structure. On the other hand, this merely could be due to the fact that, in comparison with the other transgenic constructs studied, a relatively small number of SP-A:4000:hGH lines were analyzed, and three-fourths of the nonexpressing founders carried only two copies of the transgene (Table 1). It should be noted that an absence of transgene expression can also be due to an in vivo rearrangement of the construct, and this possibility cannot be excluded.

The 378-bp 5′-flanking sequence of the rabbit SP-A gene is sufficient to direct lung-selective expression of SP-A:hGH fusion genes in transgenic mice. To determine which genomic regions are required for lung-specific expression of the rabbit SP-A gene, expression of the transgenes in lungs and other tissues was analyzed. Total RNA isolated from various tissues of adult transgenic mice carrying the SP-A:378:hGH, SP-A:4000:hGH, and SP-A:991:hGH transgenes that were found to be expressed in lung was analyzed for hGH mRNA by Northern blotting. Equivalent amounts of RNA from the lungs and other tissues to be analyzed from each mouse line were electrophoresed on the same gel and probed for hGH transcripts on the same Northern blot. The levels of hybridizable hGH mRNA in various tissues were determined by scanning densitometry of the autoradiograms (computing densitometer model 300A and ImageQuant software version 3.3, Molecular Dynamics, Sunnyvale, CA) and corrected for loading and transfer of RNA by comparison to levels of hybridizable 18S RNA present in each lane of the gels. For each transgenic line, the corrected value of hGH mRNA expressed in the lung was given an arbitrary number of 100, and the levels of hGH mRNA in the other tissues (muscle, fat, intestine, brain, thymus, spleen, mammary tissue, kidney, liver, and heart) are presented as a percentage of expression in the lung. The results of these analyses are presented in Table 2.

In the two lines of mice that expressed the SP-A:4000:hGH fusion gene in the lung, expression was undetectable in all other tissues examined. Expression of the SP-A:991:hGH transgene was detected in the lung tissues of all 15 lines of transgenic mice established. However, in two of the five randomly chosen lines studied for lung-specific expression, substantial levels of expression were detected in certain other tissues. In two lines, expression of the transgene in heart tissue was ~40–50% of that detected in lung tissue. In one of these lines, transgene expression also was detected in the kidney, mammary gland, and spleen at levels between 2 and 7% of those in lung and at <1% of those in the other tissues studied. In the three other lines of mice carrying the SP-A:991:hGH transgene, expression in the brain, thymus, spleen, mammary gland, kidney, and liver was either undetectable or ≤1% of that in lung. In two of four of the transgenic lines studied carrying the SP-A:378:hGH transgene, substantial levels of hGH expression were detected in the spleen and thymus; in one of these lines, a considerable level of expression was detected in the heart as well. Relatively low levels of transgene expression were also detected in fat, small intestine, kidney, and liver tissues of each of these lines. These findings suggest that although SP-A 5′-flanking sequences between −378 and −47 bp are sufficient to mediate transgene expression in a lung-
selective manner, sequences upstream of −991 bp appear to be required for lung-specific expression. In a previous study with transfected type II cells, Alcorn et al. (2) observed that basal and DBcAMP-stimulated levels of expression of SP-A:hGH fusion genes containing 378 bp of SP-A 5'-flanking DNA were considerably lower than those of fusion genes containing 991 bp of the 5'-flanking sequence. In the present study, equivalent amounts of lung RNA from a number of transgenic lines containing the different fusion gene constructs were analyzed on the same Northern blot. We were surprised to find that the corrected values for hGH mRNA in transgenic lines carrying the SP-A 2378:hGH and SP-A 2991:hGH fusion genes were roughly equivalent (data not shown).

The 378-bp 5'-flanking sequence of the rabbit SP-A gene is sufficient for appropriate lung cell-selective expression of SP-A:hGH fusion genes in transgenic mice. To analyze cellular localization of transgene expression, immunocytochemical and in situ hybridization analyses were performed as described in EXPERIMENTAL PROCEDURES. Adjacent sections of lung tissue from an adult transgenic mouse carrying the SP-A 2400:hGH fusion gene (line 3-3) were immunostained for SP-A and hGH. As can be seen in Fig. 2, the same alveolar epithelial cells that contained hGH immunoreactivity (B) also immunostained for SP-A (A); however, other cells that were immunoreactive for SP-A did not contain detectable hGH protein. The decreased number of cells immunopositive for hGH compared with SP-A may be due to different secretory pathways of the two proteins. SP-A is primarily associated with lamellar bodies in type II cells (16), whereas hGH may be constitutively secreted (40). During sectioning of the tissue, there is also a potential for removing a portion of the cell in which one of the proteins is localized while the other protein remains.

By in situ hybridization analyses of lung tissues of transgenic mice containing the SP-A 2991:hGH (line 1699) and SP-A 2378:hGH (line 62-9) fusion genes with an hGH antisense cRNA probe, it is apparent that hGH mRNA transcripts are present both in alveolar epithelial cells and in the cells of the bronchiolar epithelium (Fig. 3). In situ hybridization analysis of lung tissue of a nontransgenic adult mouse lung with an antisense hGH cRNA probe is shown in Fig. 3, A and B. As can be

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Values are levels of hGH expression relative to expression in lung in each line. CRE, cAMP response element; PBE, proximal binding element; ND, not detected. Only tissues from mice that expressed transgenes in lung were analyzed. hGH expression was determined by scanning densitometry of autoradiographs of Northern analysis of RNA isolated from tissues.
seen, no hybridization of the hGH cRNA was detectable. By contrast, hGH mRNA transcripts were readily detectable in the lung tissues of transgenic mice carrying the SP-A-378:hGH (Fig. 3, C and D) and SP-A-993:hGH (Fig. 3, E and F) fusion genes. The finding that silver grains were localized to cells in the “corners” of alveoli (Fig. 3, C and E, arrowheads) is suggestive of transgene expression in type II cells. Expression of the transgene in bronchiolar epithelial cells (Fig. 3, C and E, arrows) of transgenic mice carrying either the SP-A-378:hGH (Fig. 3C) or SP-A-993:hGH (Fig. 3E) fusion genes was also apparent. These patterns of expression are identical to in situ analyses previously performed by others for both mouse (23) and rabbit (4, 47) SP-A mRNA transcripts. No hybridization signal was detected in lung sections from the transgenic mice when a sense hGH probe was utilized (data not shown). These findings suggest that the fusion genes are expressed in type II cells and in bronchiolar epithelial cells and that 378 bp of the sequence flanking the 5′-end of the rabbit SP-A gene is sufficient for appropriate lung cell-selective expression in transgenic mice.

The 378-bp 5′-flanking sequence from the rabbit SP-A gene is sufficient to direct appropriate developmental regulation of SP-A:hGH fusion gene expression in transgenic mouse lung tissue. To begin to analyze SP-A 5′-flanking sequences that mediate appropriate temporal regulation of SP-A promoter activity, the developmental regulation of SP-A-4000:hGH (line 3-3) and SP-A-378:hGH (line 62-9) fusion genes was analyzed in lung tissues of fetal, neonatal, and adult transgenic mice. Figure 4A shows Northern blots of RNA isolated from lung tissues of 16- to 19-day gestational age transgenic fetuses carrying the SP-A-4000:hGH fusion gene and their nontransgenic littermates. The blots were probed either with hGH cDNA to analyze transgene expression or with an SP-A cDNA to analyze endogenous SP-A expression. As can be seen, the expression of the SP-A-4000:hGH transgene was detectable as early as day 17 and increased through day 19. Transgene expression was developmentally regulated in concert with the endogenous SP-A gene (Fig. 4A). In transgenic mice carrying SP-A-378:hGH fusion genes, transgene expression was detectable as early as day 17 (day 16 tissues were not analyzed in this group) and appeared to reach maximal levels by day 19 (Fig. 4B). These findings suggest that the genomic regions responsible for appropriate developmental regulation of the rabbit SP-A gene lie within the 378-bp region upstream of the SP-A transcription initiation site.

Transgene expression in fetal mouse lung tissue in organ culture is regulated by cAMP and glucocorticoids. In previous type II cell transfection studies, Alcorn et al. (2) observed that cAMP induced expression of SP-A:hGH fusion genes containing –378 to –1,765 bp of the rabbit SP-A 5′-flanking DNA, whereas Dex, which had little effect on basal expression, antagonized the stimulatory effects of cAMP. In a study using fetal rabbit lung explants, Boggaram and Mendelson (6) have found that glucocorticoids have stimulatory effects on SP-A gene transcription after 24–72 h of incubation; however, acutely, glucocorticoids inhibit SP-A gene transcription and antagonize the stimulatory effects of DBCAMP. In the present study, the expression of SP-A-4000:hGH (line 3-3) and SP-A-378:hGH (line 62-9) fusion genes was analyzed in transgenic fetal mouse lung explants incubated for up to 5 days in the absence and presence of DBCAMP and Dex. Transgene expression was assessed by the accumulation of hGH in the culture medium over each 24-h period. As can be seen in Fig. 5, expression of the SP-A-4000:hGH fusion gene in 16-day fetal mouse lungs in organ culture increased as a function of time in culture. Treatment of fetal lung explants with DBCAMP (1 mM) resulted in a three- to sixfold increase in fusion gene expression. The inductive effect of DBCAMP on transgene expression was of a similar magnitude to the stimulatory effect of cAMP on...
the expression of the SP-A gene in rabbit fetal lung explants (6). On the other hand, Dex (10⁻⁷ M) caused a slight decrease in hGH expression compared with that in control tissues and antagonized the stimulatory effect of DBcAMP on fusion gene expression, reducing the levels of expression two- to fourfold.

Similar findings of the effects of DBcAMP and Dex on SP-A promoter activity were obtained with fetal lung explants from a transgenic mouse line carrying the SP-A₂₃₇₈:hGH fusion gene. Figure 6A shows the effects of DBcAMP and of DBcAMP and Dex in combination on the expression of SP-A₂₄₀₀:hGH and SP-A₂₃₇₈:hGH fusion genes in lung explants of 16-day gestational age fetal transgenic mice after 5 days of organ culture. Shown are the levels of hGH that accumulated in the culture medium over a 24-h period between days 4 and 5 of culture. As can be seen, DBcAMP stimulated expression of both fusion genes approximately threefold compared with fusion gene expression in lung explants maintained in control medium, whereas Dex antagonized the stimulatory effect of DBcAMP. The effect of Dex to antagonize cAMP induction of SP-A promoter activity is similar to that observed in type II cells transiently transfected with SP-A:hGH fusion gene constructs (2, 3). These findings suggest that sequences responsible for cAMP stimulation and glucocorticoid inhibition of rabbit SP-A gene promoter activity lie within the 378-bp SP-A 5′-flanking region.

To compare the effects of cAMP and Dex on transgene expression with those on expression of the endogenous SP-A gene in the same lung tissue samples, lung explants isolated from 16-day gestational age transgenic mice carrying the SP-A₂₄₀₀:hGH fusion gene were maintained in organ culture for 5 days in the absence and presence of DBcAMP, Dex, or DBcAMP plus Dex at various concentrations. Figure 6B shows a Northern blot of RNA isolated from the transgenic fetal lung explants that was probed for hGH and SP-A mRNA transcripts. As can be seen, although DBcAMP increased transgene expression, there was no detectable effect of cAMP on expression of the endogenous mouse SP-A gene. Although Dex (10⁻⁷ M) inhibited transgene expression and had a dose-dependent effect to antagonize the stimulatory effect of cAMP, Dex caused a dose-dependent increase in the levels of endogenous mouse SP-A mRNA. These findings indicate that species-specific differences in the effects of cAMP and glucocorticoids on SP-A transcription are due to species differences in cis-acting elements rather than to differences in trans-acting factors.
Mutation of the PBE and CRESP-A elements within the 5′-flanking sequence of the rabbit SP-A gene has no effect on tissue-specific or developmental regulation of SP-A:hGH transgene expression. Previously, our laboratory (2, 13, 31) observed that an E box and a putative nuclear-receptor binding site, termed PBE and CRESP-A, respectively, are required for basal and cAMP-induced expression of rabbit SP-A:hGH fusion genes transfected into rat, human, and rabbit type II cells. To investigate the regulatory influences of these elements on the levels of expression in the lung as well as on lung-specific and developmental regulation of transgene expression, transgenic mice were created carrying SP-A:hGH fusion genes in which one of these elements, PBE or CRESP-A, was mutated (Fig. 1). The CRESP-A sequence TGACCTCA was mutated either to TGAC-GACA (CRESP-A2) or to the sequence TTCTAGAA (CRE-S). These mutations were previously found to markedly reduce cAMP induction of rabbit SP-A:hGH fusion gene expression in transfected type II cells (2, 31). The PBE (CCTCGTGA) was mutated to ACTCTAGA, a sequence found to markedly reduce basal and prevent cAMP induction of SP-A promoter activity in transfected type II cells (13). The CREP-A– and PBE– mutations were created in the SP-A-375:hGH fusion gene (SP-A-375CREP-A:hGH and SP-A-375PBE:hGH, respectively), whereas, the CRE-S mutation was created in the SP-A-991:hGH fusion gene (SP-A-991CRE-S:hGH). These mutated fusion genes were then introduced into transgenic mice; levels of expression of the SP-A-375:hGH transgenes with and without the mutations were determined in five to nine independently derived lines of mice by Northern analysis. The blots were reprobed for 18S rRNA to correct for differences in loading and transfer. As can be seen in Fig. 7, top, when transgene expression levels were analyzed without regard to transgene copy number in each of the lines, the PBE mutation was found to have little effect on expression of the SP-A-375:hGH fusion gene compared with mice carrying the wild-type SP-A-375:hGH fusion gene. By contrast, the CRE mutations were found to decrease expression by ~50%. As can be seen in Fig. 7, bottom, when transgene copy number was taken into account, the PBE– mutation was found to cause a 50% reduction in expression of the SP-A-375:hGH fusion gene, whereas the inhibitory effect of the CRE mutation was more pronounced, resulting in a 65% reduction compared with wild-type levels. These results indicate that these elements do act as enhancers of rabbit SP-A gene expression. Surprisingly, neither of the CRE– nor PBE– mutations had an apparent effect on lung-specific expression (Table 2). In fact,}

![Fig. 4. Developmental expression of SP-A:hGH transgenes in fetal mouse lung. A: total RNA (30 mg) isolated from lung tissues of 16- to 19-day fetal transgenic mice carrying SP-A-4000:hGH transgene (line 3-3) and their nontransgenic littersmates was analyzed for SP-A and hGH mRNA transcripts by Northern blotting. B: total RNA (30 mg) isolated from lung tissues of 15-, 17, and 19-day fetal, 1-day neonate (N), and adult (Ad) transgenic mice carrying SP-A-375:hGH transgene (line 62-9) was analyzed for hGH mRNA by Northern blotting.

![Fig. 5. Effects of dibutyryl cAMP (DBcAMP) and dexamethasone (Dex) on expression of SP-A-4000:hGH transgene in fetal mouse lung in organ culture. Lung tissues from 16-day fetal transgenic mice carrying SP-A-4000:hGH transgene (line 3-3) were placed in organ culture and incubated for up to 5 days in serum-free medium in presence of DBcAMP (1 mM), Dex (10−7 M), or the agents in combination. Medium was collected every 24 h and replaced with fresh medium. Amount of hGH that accumulated in culture medium during each 24-h period was analyzed by radioimmunoassay. Values are means ± SE from 6 offspring of a single pregnant transgenic female.](http://ajplung.physiology.org//)
in the case of the CRE-S and PBE- mutations in the context of the SP-A<sub>-996</sub>:hGH and SP-A<sub>-378</sub>:hGH fusion genes, respectively, there was generally greater lung specificity of expression compared with the corresponding wild-type fusion gene constructs. Furthermore, in transgenic mice carrying the SP-A<sub>-996</sub>:hGH fusion gene containing the CRE-S mutation, we observed that fusion gene expression appeared to be developmentally regulated in a manner similar to that of the SP-A<sub>-4000</sub>:hGH and SP-A<sub>-378</sub>:hGH fusion genes and to the endogenous mouse SP-A gene (data not shown).

**DISCUSSION**

In the present study, transgenic mice were used to define genomic regions upstream of the rabbit SP-A gene that mediate lung type II cell-specific, developmental, and hormonal regulation of SP-A gene expression. We found that SP-A:hGH fusion genes containing 4,000

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**Fig. 6. Effects of DBcAMP and Dex on expression of SP-A<sub>-378</sub>:hGH and SP-A<sub>-4000</sub>:hGH transgenes and endogenous mouse SP-A gene in fetal mouse lung in organ culture.** Lung tissues from 16-day fetal transgenic mice carrying SP-A<sub>-378</sub>:hGH (line 3-3) and SP-A<sub>-4000</sub>:hGH (line 62-9) transgenes were placed in organ culture and incubated in serum-free medium in absence and presence of DBcAMP (1 mM), Dex (10<sup>-7</sup> M), or medium containing DBcAMP and Dex at concentrations of 10<sup>-10</sup> to 10<sup>-7</sup> M. **A**: fetal lung explants were incubated for 5 days in absence and presence of DBcAMP or DBcAMP + Dex. Shown are levels of hGH that accumulated in medium during a 24-h period between days 4 and 5 of culture. **B**: fetal lung explants were incubated for 5 days in the absence (C) and presence of 10<sup>-7</sup> M Dex (Dx), DBcAMP (Bt), or Bt + Dx at 10<sup>-10</sup> to 10<sup>-7</sup> M. Total RNA was isolated and analyzed by Northern blotting with <sup>32</sup>P-labeled hGH cDNA and 18S rRNA oligonucleotide probes. hGH mRNA transcripts and 18S rRNA were visualized by autoradiography.

**Fig. 7. Expression of SP-A<sub>-378</sub>:hGH transgenes containing mutations in PBE (PBE<sup>-</sup>) or CRE (CRE<sup>-</sup>) in lung tissues of adult transgenic mice.** RNA isolated from lung tissues of lines of adult transgenic mice carrying SP-A<sub>-378</sub>:hGH (378WT), SP-A<sub>-378PBE<sup>-</sup></sub>:hGH (378PBE<sup>-</sup>), or SP-A<sub>-378CRE<sup>-</sup></sub>:hGH (378CRE<sup>-</sup>) transgenes were subjected to Northern analysis with <sup>32</sup>P-labeled hGH cDNA and 18S rRNA oligonucleotide probes. hGH mRNA transcripts and 18S rRNA were visualized by autoradiography. Relative levels of hGH mRNA from lung tissues in the various lines were determined by scanning densitometry and expressed relative to 18S rRNA alone (top) or relative to 18S rRNA and gene copy number (bottom). Values are means ± SE from 5–9 independently derived lines of mice; n, no. of mice.
bp of the sequence flanking the 5′-end of the rabbit SP-A gene were expressed in transgenic mice in a lung-specific manner and were developmentally regulated in concert with the endogenous mouse SP-A gene. To begin to define the minimum genomic region required for lung cell-specific and appropriate developmental regulation of SP-A promoter activity, transgenic mice were also created carrying SP-A-991:hGH, SP-A-378:hGH, and SP-A-473:hGH fusion genes. In three of the five lines of transgenic mice carrying SP-A-991:hGH that were analyzed for tissue-specific expression, fusion gene expression was essentially lung specific. However, in the two other lines examined, considerable levels of transgene expression were also detected in the heart. In two of five lines of transgenic mice carrying SP-A-378:hGH fusion genes, expression was essentially lung specific, whereas in three other lines, there were also substantial levels of transgene expression detected in the heart, thymus, and spleen. Expression in the lung was always greater than in the other tissues. The variability in ectopic expression from one line of mice to the other is likely due to positional effects of transgene integration (34).

Expression of the SP-A gene is essentially lung specific (7); however, low levels of SP-A mRNA have been reported in epithelium of the small intestine (36) and the thymus and prostate (27). In the present study, there was no evidence for transgene expression in the small intestine; however, when ectopic expression of SP-A:hGH transgenes containing ≤991 bp of SP-A 5′-flanking DNA was observed, the highest levels were found in the heart, thymus, and spleen. The finding that in mice carrying SP-A-991:hGH transgenes with a scramble mutation in CRESP-A (SP-A-991CRES:hGH) there was little or no ectopic expression in the heart suggests that transcription factors in the heart may bind to this element and activate the SP-A promoter in the absence of constraints imposed by upstream sequences. Similarly, the finding that expression of SP-A-378:hGH transgenes with a mutation in the PBE (SP-A-378PBE-hGH) was essentially lung specific suggests that transcription factors in the heart, spleen, and thymus may activate the SP-A promoter by binding to this element in the absence of constraints imposed by upstream sequences.

Evidence for the role of upstream sequences in conferring binding specificity to CRESP-A was obtained in studies with type II cells transfected with SP-A-378:hGH and SP-A-991:hGH fusion genes with and without mutations in CRESP-A. When CRESP-A (TGACCTCA) was mutated to the canonical palindromic CRE (CREpal) sequence (TGACCTCA), known to bind the transcription factor CREB, in the context of the SP-A-378:hGH fusion gene, basal and cAMP-induced expression were increased compared with that of fusion genes containing the wild-type sequence (2). In contrast, in the context of the SP-A-991:hGH fusion gene, mutagenesis of CRESP-A to CREpal resulted in a marked decrease in basal and cAMP-induced expression (31). These findings suggest that in the absence of upstream sequences, mutagenesis of CRESP-A to a sequence that binds CREB can increase expression and cAMP responsiveness, whereas in the context of the 991-bp 5′-flanking sequence, CREB homodimers may be prevented from binding to CREpal because of restraints imposed by other trans-acting factors bound to upstream response elements. We have obtained evidence that CRESP-A does not bind CREB; rather, a member of the nuclear-receptor family appears to bind to this site (31).

By in situ hybridization, the SP-A-378:hGH fusion gene was found to be expressed in alveolar epithelial cells that have characteristics of type II cells in terms of their localization at the corners of the alveoli. Expression also was evident in bronchiolar epithelial cells. These patterns of expression correspond to those of the endogenous SP-A gene (4, 23, 47). The finding that the SP-A-378:hGH fusion gene was developmentally regulated in association with the endogenous mouse SP-A gene suggests that the genetic elements required for accurate developmental timing of expression are also contained within the 378-bp region. Based on these findings, we suggest that the regulatory elements required for lung cell-selective and developmental regulation of SP-A gene expression are localized within the 378-bp 5′-flanking region and that sequences upstream of this region are required for preventing activation of the SP-A promoter in other tissues.

Transgenic technology has been used to begin to define sequences that mediate lung cell-specific and developmental timing of expression of SP-C and the Clara cell secretory protein/Clara cell 10-kDa protein (CCSP/CC10) genes. In studies of the rat CCSP/CC10 gene, it was found that 2.25 kb of the 5′-flanking sequence directed lung- and Clara cell-specific expression of an hGH reporter gene (17); however, transgene expression was detected as early as embryonic day 12.5, at least 4 days before the time that expression of the endogenous CCSP/CC10 gene is initiated (18). In subsequent studies, it was found that although 166 bp of the mouse CCSP/CC10 5′-flanking region were sufficient to mediate Clara cell-specific expression, enhancer sequences between −166 and −803 bp were required to achieve maximal levels of transgene expression (35).

In transgenic mouse studies to define the genomic elements required for lung cell-specific and developmental timing of expression of the human SP-C gene, it was found that 3.7 kb of the SP-C 5′-flanking region linked to bacterial chloramphenicol acetyltransferase mediated high levels of chloramphenicol acetyltransferase expression in cells of the alveolar and bronchiolar epithelia (15, 46). Interestingly, although SP-C expression is restricted to type II cells in the mouse lung, proSP-C in the human lung is detectable both in alveolar and in distal bronchiolar cells (22). These findings suggest the presence of sequences within the 5′-flanking region of the human SP-C gene that mediate bronchiolar as well as type II-specific expression.

The effects of cAMP and Dex on SP-A gene expression differ among species (29). Whereas expression of rabbit (7), human (33), and baboon (39) SP-A genes is mark-
edly stimulated by cAMP, expression of rat (32) and mouse (11) SP-A genes is relatively unaffected by cAMP treatment. In the present study, we observed that DBcAMP treatment of lung explants from 15- to 16-day gestational age fetal transgenic mice increased expression of rabbit SP-A -4000:hGH and SP-A -378:hGH integrative transgenes in a manner similar to its effects on transcription of the endogenous rabbit SP-A gene in fetal rabbit lung explants (7) and on expression of rabbit SP-A:hGH fusion genes in transfected type II cells (2, 3, 31). Because the endogenous mouse SP-A gene is not responsive to cAMP, the present findings suggest that mouse type II cells contain the cellular proteins and transcription factors required to mediate cAMP induction of rabbit SP-A promoter activity. Therefore, it is likely that species-specific differences in cAMP responsiveness are due to genetic differences in enhancer elements that confer cAMP responsiveness to the promoter rather than to species-specific differences in trans-acting factors. In contrast to the stimulatory effects of cAMP on transgene expression, Dex treatment of the transgenic fetal mouse lung explants inhibited fusion gene expression and antagonized the stimulatory effects of DBcAMP. The inhibitory effects of glucocorticoids on cAMP induction of SP-A:hGH fusion gene expression are similar to those observed previously with type II cells transfected with rabbit SP-A: hGH fusion genes (2). However, these findings are in contrast to the stimulatory effects of glucocorticoids when added alone and in combination with cAMP on the transcriptional activity of the endogenous SP-A gene in fetal rabbit lung explants (7). In the present study, we observed that Dex caused a dose-dependent increase in the levels of endogenous mouse SP-A mRNA in the transgenic fetal lung explants; this was coordinate with a dose-dependent decrease in hGH mRNA levels.

The mechanisms whereby glucocorticoids enhance transcriptional activity of the endogenous SP-A gene have not been defined. From the findings of the present study and from those with transfected type II cells (2), it is likely that the stimulatory effects of glucocorticoids on expression of the rabbit SP-A gene are mediated by response elements that lie >4,000 bp upstream of the transcription initiation site, within the structural gene, or in the 3'-flanking region. In cell transfection studies, Alcorn et al. (2) also observed that sequences within the first exon and intron of the rabbit SP-A gene are mediated by glucocorticoid induction of SP-A promoter activity. The stimulatory effects of glucocorticoids may be mediated by glucocorticoid-receptor binding to a glucocorticoid response element(s) or by induction or activation of another transcription factor. If the latter is the case, such a transcription factor must also act through an element(s) that lies outside the confines of the SP-A promoter constructs used in these studies. Because the observed effects of glucocorticoids on expression of SP-A:hGH transgenes are inhibitory, it is possible that in the absence of a functional glucocorticoid response element, the glucocorticoid receptor represses cAMP induction of SP-A promoter activity by an inhibitory interaction with a cAMP-responsive transcription factor or by competition for binding to an essential coactivator. Glucocorticoids have been reported to inhibit transcription factor activator protein-1 activation by direct interaction of the glucocorticoid receptor with c-Jun (38, 48) and by competition for binding to limiting amounts of the coactivator CBP/p300 (21).

In previous studies using transfected type II cells, Alcorn et al. (2) and Gao and colleagues (13, 14) observed that basal and cAMP induction of rabbit SP-A promoter activity requires the cooperative interaction of transcription factors bound to two E boxes, termed DBE (−980 bp) and PBE (−80 bp), and to a CRE-like element (CRE SP-A; −261 bp). SP-A -976:hGH and SP-A -378:hGH fusion genes, which lack the DBE, manifested a marked reduction in basal and cAMP-stimulated expression compared with that of SP-A -991:hGH fusion genes that contain this sequence; however, an inductive effect of cAMP was still observed. Mutagenesis of the CRE SP-A within the SP-A -991:hGH fusion gene had similar effects to reduce overall levels of basal and cAMP-induced expression; again, an ~10-fold inductive effect of cAMP on SP-A promoter activity was still evident (2, 31). On the other hand, mutagenesis of the PBE caused a marked decrease in basal expression and loss of cAMP-inducible expression (13). In contrast to our findings using transfected type II cells, we were surprised to find that SP-A -378:hGH fusion genes, which lack the DBE, were expressed in lung tissues of transgenic mice at levels roughly equivalent to those of SP-A:hGH fusion genes containing 991 bp of the SP-A 5'-flanking sequence. Furthermore, an SP-A -378PBE::hGH fusion gene (which lacks both DBE and PBE) and SP-A -378CRE::hGH and SP-A -991CRE-S::hGH fusion genes (containing mutations in the CRE SP-A) were also expressed in the lung and in a lung-specific manner, albeit at lower levels than the wild-type constructs.

These findings suggest a redundancy of response elements within the SP-A 5'-flanking region that have the capacity to mediate high levels of SP-A promoter activity in the context of multiple inserted copies of a transgene in tandem arrays. Similar results were obtained in studies of the pancreatic elastase I gene in which it was found, with transfected pancreatic acinar cells, that each of three genomic domains, which bind different nuclear proteins, were required for enhanced levels of expression in an acinar cell-specific manner (24). By contrast, in transgenic mice, it was found that any two of the three domains were sufficient to direct pancreas-specific expression (45). A similar mechanism may apply to the rabbit SP-A gene; redundancy of dissimilar elements allows for appropriate expression of SP-A promoter activity in transgenic mice when single elements are eliminated.

In type II cell transfection studies, our laboratory has found that basal and cAMP-induced expression of SP-A promoter activity are mediated by the cooperative interaction of ubiquitously expressed [e.g., upstream stimulatory factor-1 (13, 14), Sp1 (50)] and tissue-selective [e.g., thyroid transcription factor-1 (26)] tran-
scription factors as well as unidentified members of the nuclear-receptor family (2, 31, 49). We suggest that increased ectopic expression of SP-A:hGH fusion genes containing \( \leq 991 \) bp of SP-A 5′-flanking DNA compared with SP-A \(-4000\):hGH transgenes may be due to the presence of binding sites for ubiquitously expressed transcription factors within the 991-bp SP-A 5′-flanking region and to the absence of putative inhibitory elements that block expression in other tissues. Random integration of the transgenes into chromosomal regions adjacent to other enhancer/promoter elements may also cause their inappropriate expression; however, this would not explain why the shorter SP-A:hGH transgenes manifest a higher degree of ectopic expression than the SP-A \(-4000\):hGH transgene.

In summary, the results of this study indicate that a 378-bp genomic sequence upstream of the transcription initiation site of the rabbit SP-A gene directs expression of the SP-A promoter in lung type II and bronchial epithelial cells. This genomic region is also sufficient for appropriate developmental regulation of SP-A promoter activity in transgenic mice. Sequences between \(-991\) and \(-4,000\) bp appear to prevent ectopic expression of the transgene, which was found to occur predominantly in the heart, thymus, and spleen. In subsequent studies, we will attempt to more fully define the regulatory elements within this 378-bp region that are responsible for the temporal and spatial regulation of SP-A gene expression. In this manner, a better understanding of the molecular mechanisms involved in developmental and tissue-specific regulation of the genes expressed in pulmonary epithelium can be achieved.

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