Transcriptional Regulation of the Gene Encoding the Major Surfactant Protein (SP-A) in Rabbit Fetal Lung

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The expression of the major protein of rabbit pulmonary surfactant (SP-A), a glycoprotein of Mr = 29,000–36,000, is regulated during development and by hormones. In the present study, utilizing a cDNA insert complementary to mRNA coding for SP-A and nuclear transcription elongation assays, we have investigated the developmental and hormonal regulation of transcription of the SP-A gene in rabbit fetal lung tissue. The relative rates of transcription of SP-A mRNA increased as a function of the gestational age of the fetus. The rate of transcription reached a maximum level in lung tissues of 28-day gestational age fetuses and declined slightly in those of neonatal rabbits. The relative rate of transcription of SP-A mRNA increased in rabbit fetal lung explants maintained in organ culture in control medium as a function of incubation time. Dibutyryl cyclic AMP (Bt2cAMP) treatment of fetal lung explants increased the rate of transcription of SP-A mRNA over that of control tissues by several-fold; after 12 h of incubation in the presence of Bt2cAMP, there was >4-fold increase in the rate of transcription of SP-A mRNA as compared to control lung explants. In contrast, glucocorticoids had a rapid effect to decrease the rate of SP-A mRNA transcription. The rapid effect of glucocorticoids to inhibit the transcription of SP-A mRNA was transient; in fetal lung explants incubated in the presence of dexamethasone for >24 h, there was an increase in the rate of transcription of SP-A mRNA over that of control explants. Cycloheximide caused an inhibition of both basal as well as Bt2cAMP-stimulated rates of transcription of SP-A mRNA in the rabbit fetal lung tissue in vitro. This finding is suggestive of a role of labile protein factor(s) in mediating transcription of the SP-A gene as well as its induction by Bt2cAMP. The magnitude of changes in the relative rates of transcription of SP-A mRNA during development of rabbit fetal lung in vitro as well as those effected by hormones in vitro were similar to changes in the steady-state levels of SP-A mRNA, suggestive that the regulation of the levels of SP-A mRNA in fetal rabbit lung tissue both in vivo and in vitro occurs primarily at the transcriptional level.

The major pulmonary surfactant-associated protein (SP-A), a 29–36-kDa glycoprotein synthesized by type II pneumocytes, has been reported to bind to surfactant glycerophospholipids and to enhance the rate of adsorption of dipalmitoylphosphatidylcholine to an air-liquid interface (1). Also, it has been suggested that SP-A and calcium may function in the structural organization of tubular myelin (2, 3), a lattice-like structure that may serve as an intermediate between the secreted lamellar body and the mono-molecular phospholipid film at the alveolar air-liquid interface. Through these actions, it is believed that SP-A facilitates the reduction of surface tension at the alveolar air-liquid interface and thereby promotes normal respiration. Inadequate production of surfactant at birth results in respiratory distress syndrome of the newborn. SP-A may also facilitate the recycling of surfactant glycerophospholipids and, thus, may serve as a feedback regulator of surfactant glycerophospholipid synthesis and secretion (4, 5).

Using polyclonal antibodies, as well as a cDNA complementary to mRNA encoding rabbit SP-A, we have previously observed that the levels of SP-A and its mRNA are induced in rabbit fetal lung tissue during development and also that Bt2cAMP and glucocorticoids increase the accumulation of SP-A and its mRNA in rabbit fetal lung in vitro (6, 7). Also, we have reported that the induction of SP-A mRNA by Bt2cAMP is dependent upon on-going protein synthesis (7). Cyclic AMP analogues (8, 9) and β-adrenergic agonists (8) also increase the accumulation of SP-A and its mRNA in human fetal lung in vitro. The presence of β-adrenergic receptors on alveolar epithelial cells (10) and the action of catecholamines to stimulate adenylate cyclase activity of fetal lung tissue (11) suggest a key role for cyclic AMP as a mediator of regulation of SP-A gene expression in vivo. Glucocorticoids have been reported to have both inhibitory (9) and stimulatory (12) effects on the accumulation of SP-A and its mRNA in human fetal lung in vitro. We recently have observed that glucocorticoids have a biphasic effect on the levels of SP-A and its mRNA in human fetal lung in culture; at concentrations of 10⁻¹⁰ and 10⁻⁸ M, dexamethasone increased the levels of SP-A and SP-A mRNA over those of control tissues, whereas at concentrations >10⁻⁸ M, the steroid was found to be markedly inhibitory (13).

In the present study, we have utilized a cDNA complementary to the mRNA encoding rabbit SP-A and nuclear transcription elongation assays to investigate the molecular mechanisms that mediate the regulation of SP-A mRNA levels in rabbit fetal lung tissue during development, as well as the effects of Bt2cAMP and glucocorticoids on SP-A mRNA levels in rabbit fetal lung tissue in organ culture. To date, no
information is available on the developmental and hormonal regulation of the SP-A gene at the transcriptional level.

**EXPERIMENTAL PROCEDURES**

**Organ Culture of Rabbit Fetal Lung Tissue—**Lung tissues of fetal rabbits of 21-days gestational age were maintained in organ culture in serum-free Waymouth's MB752/1 medium (GIBCO) as described previously (14). Lung explants were maintained in organ culture for up to 3 days in control medium or in medium containing Bt(c)AMP (1 mM), cortisol (10^{-7} M), or dexamethasone (10^{-7} M). Prior to the addition of hormones or Bt(c)AMP, lung explants were maintained in culture in control medium for 1 day. Methods for isolation of RNA and Northern analysis have been published previously (7).

**Isolation of Nuclei and Transcription Elongation Assay—**Methods for isolation of nuclei, transcription elongation assay, and isolation of ^32P-labeled RNA were essentially as described by Sasaki et al. (15). All procedures for isolation of nuclei were performed on ice at 4°C. Fetal lung explants, after various treatments, were homogenized in 10 volumes of 0.25 M sucrose, 10 mM Hepes, pH 8.0, 0.5 mM MgCl2, 0.1% (v/v) Triton X-100 using a glass/dounce homogenizer and 10 hand-driven strokes. The homogenate was filtered through six layers of gauze, and a crude nuclear pellet was isolated by centrifugation at 600 × g for 5 min. The crude nuclei were washed twice in homogenization buffer and finally centrifuged through 1.3 M sucrose in homogenization buffer at 10,000 × g for 10 min (1 volume of nuclei to 3 volumes of 1.3 M sucrose). The nuclei were suspended in 50 mM Hepes, pH 7.5, 10 mM MgCl2, and 0.4 mM each of ATP, CTP, GTP, and 300 μCi of (α-32P)UTP (3000 Ci/mmole) and 2 mM dithiothreitol. Typically, nuclei from 1.0 g of tissue were suspended in 1.0 ml of glycerol-containing buffer. The suspended nuclei were divided into 100-pl aliquots, immediately frozen in liquid nitrogen, and stored at −70°C for future use. Nuclei were quantitated using a hemacytometer.

Transcription elongation assays were performed in a total volume of 200 μl that contained 20 mM Hepes, pH 8.0, 5.0 mM MgCl2, 90 mM NaCl, 0.5 mM MnCl2, 16% (v/v) glycerol, 0.04 mM EDTA, 2 mM dithiothreitol, 0.4 mM each of ATP, CTP, GTP, and 300 μCi of (α-32P)UTP (3000 Ci/mmole) and 200 μl of nuclei. The reaction was carried out for 20 min at 25°C. The reaction was terminated by digestion with 100 units of DNase I ( Worthington) for 20 min at 37°C followed by digestion with proteinase K (100 μg/ml) in the presence of 15 mM EDTA, 0.5% SDS, and 30 μg of yeast tRNA for 30 min at 37°C. Samples were extracted twice with an equal volume of phenol/chloroform (1:1, v/v), and the RNA was precipitated with ammonium acetate (2.5 M) and 2 volumes of ethanol. The RNA precipitate was subjected to digests with DNase I, and proteinase K again, phenol/chloroform extracted, and precipitated with ammonium acetate and ethanol. The final RNA precipitate was dissolved in 100 μl of 1 mM Tris-HCl, pH 7.5, 0.5 mM EDTA and 0.1% SDS, and radioactivity was determined. Equivalent amounts of radioactive RNA were then hybridized to nitrocellulose filters either without or with 5.0 ml of wash buffer containing 0.1% SDS and 100 μg of salmon sperm DNA and added to vials containing two nitrocellulose filters; one contained immobilized cDNA for rabbit SP-A (1.0 μg of pUC18 DNA containing SP-A cDNA), the other contained nonspecific DNA (5.7 μg of pUC18 DNA) or else no added DNA. Plasmid DNAs were previously immobilized to nitrocellulose according to the method described by Diamond and Goodman (16). Hybridization was performed at 45°C for 4 days in a shaking water bath. The nitrocellulose filters had been prehybridized for 12 h under identical conditions.

Following hybridization, the filters were washed four times in 5 ml of wash buffer (0.3 M NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 7.5) containing 0.1% SDS at 45°C for 30 min each time. The filters were then washed once in 5.0 ml of wash buffer at 45°C for 30 min and incubated in 1.0 ml of wash buffer containing 10.0 μg of RNA “A” and 1.0 μg of RNA “T” at 37°C for 30 min. They were then incubated in 1.0 ml of wash buffer containing 0.1% SDS and 100 μg of proteinase K for 20 min at 37°C. The filters were washed twice with 5.0 ml of wash buffer containing 0.1% SDS at 45°C for 30 min each time. After the washes, the filters were subjected to autoradiography using intensifying screens. Relative rates of transcription were assessed by scanning densitometry of the autoradiograms. In all cases, the background (signal obtained after hybridization of radiolabeled RNA to filters containing pUC18 DNA or to blank filters) was subtracted from the signal obtained using the corresponding filters containing the rabbit SP-A cDNA plasmid. In some cases, following autoradiography the bound RNA was released by treating filters with 0.25 ml of 0.4 M NaOH for 30 min at room temperature, neutralized with 0.1 ml of 1 M acetic acid, and the radioactivity was determined by liquid scintillation spectrometry. When the results obtained by scintillation spectrometry were compared to those obtained by scanning densitometry, it was found that relative changes in SP-A gene transcription in control and treated samples were similar.

**RESULTS**

**Transcription of SP-A mRNA during Development—**We have previously observed that SP-A mRNA is undetectable in rabbit fetal lung tissue until day 26 of gestation, the levels of SP-A mRNA reach a maximum on day 31 of gestation and decline slightly in the neonate (7). To determine whether the changes in the levels of SP-A mRNA during development are regulated at the transcriptional level, the rate of transcription of SP-A mRNA was determined in nuclei isolated from lung tissues of fetal rabbits of 21–28 days gestational age and from neonates. It can be seen (Fig. 1) that the transcription rate of SP-A mRNA is detectably increased in nuclei from 24-day rabbit fetal lung tissue (2.0-fold increase as compared to that of nuclei from 21-day fetal lung tissue) and that the rate is increased further as a function of gestational age of the fetus, reaching a maximum level in lung nuclei from 28-day fetal rabbits (11.5-fold increase as compared to that of nuclei from 21-day fetal lung tissue) and declined slightly after birth (8.8-fold increase as compared to that of nuclei from 21-day fetal lung tissue). It should be noted that the overall transcription rate was similar in nuclei obtained from lung tissues at different developmental stages. A similar pattern was observed when the levels of SP-A mRNA were analyzed by Northern blot analysis of total RNA isolated from the same tissues (data not shown).

**Effect of Bt(c)AMP and Dexamethasone on the Rate of Transcription of SP-A mRNA**—In previous studies, we found that incubation of rabbit fetal lung explants in the presence of Bt(c)AMP (1 mM) or cortisol (10^{-7} M) resulted in increased accumulation of SP-A and the levels of translatable SP-A mRNA (6). In other studies, we found that Bt(c)AMP (1 mM) treatment of rabbit fetal lung explants resulted in an increased accumulation of cytoplasmic and nuclear RNAs encoding SP-A as rapidly as 2 h after its addition to the culture medium (7). In the present study, we compared the effects of Bt(c)AMP and cortisol on the levels of SP-A mRNA in rabbit fetal lung explants as a function of incubation time (Fig. 2). As we have found previously (7), levels of the two species of SP-A mRNA of 2.0 and 3.0 kilobases in size were increased in control explants as a function of incubation time. Bt(c)AMP caused a rapid induction of SP-A mRNA in fetal lung explants; a stimulatory effect was observed as early as 6 h after its addition to the culture medium. In explants incubated with cortisol for 48 and 72 h, SP-A mRNA levels were greater than

![FIG. 1. Changes in the transcriptional activity of the SP-A gene during development of rabbit fetal lung. Nuclei were isolated from tissues of fetuses of different gestational ages and from neonates (Neo) and the transcription of SP-A mRNA was analyzed. ^32P-Labeled RNA (32 × 10^6 cpm) was hybridized to nitrocellulose filters containing cDNA specific for SP-A and an autoradiogram was obtained.](image-url)
those of control tissues. We were surprised to find, however, that after 6 and 24 h of incubation in the presence of cortisol, the levels of SP-A mRNA were reduced as compared to those of control explants. This rapid effect of glucocorticoids to inhibit accumulation of SP-A mRNA also was observed in two other experiments.

To determine whether the effects of cyclic AMP analogues and glucocorticoids to alter the levels of SP-A mRNA are the result of changes in the rate of transcription of the SP-A gene, nuclei were isolated from 21-day rabbit fetal lung explants after 12 h of incubation in the absence or presence of BtZcAMP (1 mM) or the synthetic glucocorticoid, dexamethasone (10^{-7} M), added alone or in combination, and nuclear transcription elongation assays were performed. Also, cytoplasmic RNA from the same tissues was isolated and subjected to Northern blot analysis. Incubation of lung explants in the presence of BtZcAMP (1 mM) for 12 h caused a 4.7-fold increase in the rate of transcription of SP-A mRNA over that of tissues incubated in control medium (Fig. 3). In contrast, when lung explants were incubated with dexamethasone (10^{-7} M) for 12 h, there was a 50% decrease in the rate of transcription of SP-A mRNA as compared to that of untreated tissues. When explants were incubated with dexamethasone (10^{-7} M) and BtZcAMP (1 mM) in combination, the rate of transcription of SP-A mRNA was reduced to a level that was ~50% of that observed in nuclei incubated with BtZcAMP alone (2.1-fold greater than that of control explants). Northern blot analysis (Fig. 3) of cytoplasmic RNAs isolated from the same tissues was indicative of similar effects of BtZcAMP and dexamethasone on the levels of SP-A mRNA (hybridizable SP-A mRNA relative to control: BtZcAMP-treated, 5.0-fold; dexamethasone-treated, 0.4-fold; BtZcAMP plus dexamethasone, 3.5-fold). Treatment of fetal lung explants with BtZcAMP or with dexamethasone had no effect either on the overall transcriptional activity of the nuclei or on the amount of total RNA isolated per gram wet weight of tissue.

The rate of transcription of SP-A mRNA also was determined in nuclei isolated from 21-day rabbit fetal lung explants incubated in the absence or presence of either BtZcAMP (1 mM) or dexamethasone (10^{-7} M) for 24–72 h. It was observed that the rate of SP-A mRNA transcription increased in control explants as a function of incubation time (Fig. 4). After 24 h of incubation, the inhibitory effect of dexamethasone (10^{-7} M) on SP-A mRNA transcription was no longer apparent, whereas in fetal lung explants incubated for 24 h in the presence of BtZcAMP (1 mM), a 4.8-fold increase in the rate of transcription of SP-A mRNA as compared to control tissues was observed. After 48 h of incubation, dexamethasone (10^{-7} M) clearly increased the rate of transcription of SP-A mRNA; a 1.5-fold increase in the rate of transcription was observed compared to that of untreated tissues. BtZcAMP (1 mM) caused a 3-fold increase in the rate of SP-A mRNA transcription over that of control explants at this time point. By 72 h of incubation, however, the stimulatory effects of BtZcAMP (1 mM) and dexamethasone (10^{-7} M) on SP-A mRNA transcription as compared to control explants were diminished, presumably because of the increased rate of transcription of SP-A mRNA in control tissues.

![Image](image_url)
A RNA-We previously observed that treatment of rabbit transcription were analyzed. The 32P-RNA (33A (prSP-A), cDNA specific for chicken actin (pcAct), or nonspecific of transcription of Bt2cAMP fetal lung explants with cycloheximide for 6 h inhibited the accumulation of SP-A mRNA in control as well as in hybridized to nitrocellulose filters containing cDNA specific for SP- increase the levels of SP-A mRNA occurs at the transcriptional presence of cycloheximide (2 pg/ml) for 6 h. Also, the rate of transcription of actin mRNA was evaluated in the same set of nuclei (Fig. 5). Treatment of fetal lung explants with cycloheximide for 6 h resulted in a 30% decrease in the rate of transcription of actin mRNA. These results taken together indicate that the inhibitory effects of cycloheximide on the fetal lung tissue and the induction of SP-A gene transcription are not understood.

In the present study, we have investigated the transcriptional regulation of expression of the gene encoding the major surfactant-associated protein, SP-A, in rabbit fetal lung tissue during development, as well as the effects of Bt2cAMP and glucocorticoids on SP-A gene transcription in 21-day gestational age fetal rabbit lung tissue in vitro. We observed that Bt2cAMP caused a detectable increase in the rate of transcription of SP-A mRNA in nuclei obtained from the rabbit fetal lung explants within 12 h of its addition to the culture medium. These relatively rapid changes in the rates of transcription of SP-A mRNA in nuclei obtained from the rabbit fetal lung explants were associated with comparable changes in steady-state levels of SP-A mRNA. Cycloheximide treatment, however, did not have any significant inhibitory effect on the overall rate of transcription of actin mRNA. These results taken together indicate that the effects of cycloheximide to inhibit SP-A mRNA transcription are relatively specific and are not due to a general toxic effect of cycloheximide on the fetal lung tissue. In previous studies, we have found that the inhibitory effects of cycloheximide on SP-A mRNA accumulation are reversible (7). Also, the present findings support the concept that a rapidly turning over protein factor(s) is required for transcription of the SP-A gene and its induction by Bt2cAMP.

Cyclic AMP has been found to increase the transcription of a number of eucaryotic genes including phosphoenolpyruvate carboxykinase (15, 17, 18), vasoactive intestinal peptide (19), somatostatin (20), prolactin (21, 22), plasminogen activator (23), tyrosine hydroxylase (24), and a number of steroidogenic genes of cytochrome P-450 (25). In the cases of phosphoenolpyruvate carboxykinase (15) and plasminogen activator (23), the induction of gene transcription has been shown to be independent of new protein synthesis. In the case of cyclic AMP regulation of cytochrome P-450, (25), cytochrome P-450(m) (26), and aromatae cytochrome P-450 (27), the induction of mRNA levels were found to be blocked by cycloheximide, indicating that a labile protein factor(s) is required for the transcriptional activation of these genes. The mechanisms by which cyclic AMP regulates eucaryotic gene expression are not clear. Putative cyclic AMP regulatory elements have been identified in the 5'-flanking regions of a number of genes including somatostatin (20), vasoactive intestinal peptide (19), tyrosine hydroxylase (24), and phos-
phosphoenolpyruvate carboxykinase (28). Recently, a nuclear protein of $M_r = 43,000$ was isolated that binds selectively to the cyclic AMP response element of the somatostatin gene (29). This protein is phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase in vitro, and its phosphorylation state is increased in vivo in response to forskolin treatment. In eucaryotes, cyclic AMP, after binding to cyclic AMP-dependent protein kinase, induces the phosphorylation of cellular proteins, some of which might directly interact with target genes to modulate their expression. Recently, it has been demonstrated that in rat liver, increases in intracellular cyclic AMP levels cause increased association of the catalytic and regulatory subunits of cyclic AMP-dependent protein kinase with transcriptionally active chromatin (30). In mutant PC12 cells, which lack cyclic AMP-dependent protein kinase type II activity, cyclic AMP fails to regulate somatostatin gene expression (20).

Glucocorticoids have been reported to act both as positive (31) and negative (32, 33) regulators of gene expression. The rapid but transient action of glucocorticoids to inhibit the transcription of SP-A mRNA observed in the present study may be mediated by an interaction of the glucocorticoid receptor complex with the regulatory element of the SP-A gene. The glucocorticoid-receptor complex has been reported to have a direct effect to inhibit the transcription of the proopiomelanocortin gene by interacting with a regulatory element that is located upstream of the start site of transcription (34). The mechanisms whereby glucocorticoids increase the transcription of SP-A mRNA after longer periods of incubation are not clear. The results of recent studies on the hormonal regulation of prolactin gene expression have revealed that positive and negative transcriptional effects of estrogens and glucocorticoids, respectively, are mediated by distinct functional domains of the steroid receptors (35). Of particular interest are the findings of studies of the regulation of expression of the human glycoprotein hormone $\alpha$-subunit gene, which are suggestive that the glucocorticoid receptor complex may negatively regulate $\alpha$-subunit expression by interference with the binding of specific transcription factors to cyclic AMP regulatory elements within the 5'-flanking region of the gene (36). The mechanisms whereby glucocorticoids exert both positive and negative regulatory effects on SP-A gene expression remain to be determined. The identification of putative glucocorticoid regulatory DNA-sequence elements in both human (37) and the rabbit SP-A genes is suggestive that these actions may be mediated by the interaction of the glucocorticoid receptor with distinct regulatory regions of the SP-A gene. Alternatively, glucocorticoids may increase SP-A gene transcription through their action to elevate intracellular levels of cyclic AMP. Glucocorticoids have been reported to cause inhibition of phoshoesterase in cultured HTC hepatoma cells (38) and in rat fetal lung tissue (39) and to increase the levels of Gs, the stimulatory guanine nucleotide binding protein of adenylate cyclase, in ROS17/2.8 cells (40).

In the present study, we found that the transcriptional activity of the SP-A gene is increased in rabbit fetal lung tissue during development. Appreciable rates of transcription were observed in nuclei from 24-day rabbit fetal lung. The transcriptional activity of the SP-A gene increased with gestational age and reached a maximum level in lung tissues of 28-day fetal rabbits. The transcription rate of the SP-A gene was slightly decreased in the lungs of neonates as compared to lungs of 23-day fetal rabbits. Again, the developmental changes in the relative transcription rate of this gene were similar to the changes in steady-state levels of SP-A mRNA (6, 7). The factors that regulate the expression of SP-A mRNA during rabbit fetal lung development in vitro are not known. The findings of this and our previous investigations (6, 7) are suggestive that cyclic AMP may serve an important role in the transcriptional activation of the SP-A gene in developing fetal lung and that glucocorticoids may have both stimulatory and inhibitory effects that are dependent upon the stage of type II cell differentiation. The results of our studies are further suggestive that changes in the levels of SP-A mRNA in rabbit fetal lung tissue during development in vivo or after treatment with cyclic AMP or glucocorticoids in vitro are regulated primarily at the transcriptional level.

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