Developmental and Hormonal Regulation of Surfactant Protein C (SP-C) Gene Expression in Fetal Lung

ROLE OF TRANSCRIPTION AND mRNA STABILITY*

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Pulmonary surfactant protein C (SP-C) gene expression is developmentally and hormonally regulated in fetal lung. In the present study, we investigated the role of transcriptional and posttranscriptional mechanisms in the developmental, cAMP, and dexamethasone induction of SP-C mRNA. We found that developmental induction of SP-C mRNA was not coincident with induction of SP-C gene transcription. SP-C mRNA levels reached ~90% of levels in adult lung on day 24 of gestation, whereas SP-C gene transcription was only ~4% of level in adult lung and did not increase until day 28 of gestation (term in rabbit = 31 days). Treatment of fetal lung tissues in vitro with dibutyryl cyclic AMP (Bt,cAMP) and dexamethasone increased SP-C mRNA accumulation by different mechanisms. Increase in SP-C mRNA accumulation by Bt,cAMP was the result of increased SP-C gene transcription, whereas increased SP-C mRNA accumulation by dexamethasone was due to stabilization of RNA. In control tissues the SP-C mRNA half-life (t½) was 11.2 h, and after dexamethasone treatment it increased to 30 h. These data show that both transcriptional and mRNA stabilization mechanisms regulate induction of SP-C gene expression during fetal lung development and by cAMP and dexamethasone in fetal lung in vitro.

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# The abbreviations used are: SP, surfactant protein; Bt,cAMP, dibutyryl cyclic AMP; MOPS, 3-(N-morpholino)propanesulfonic acid; kb, kilobase(s).

Organ Culture of Fetal Lung Tissues—Lung tissues of 21-day gestational age fetal rabbits were maintained in organ culture in serum-free Waymouth’s medium MB 752/1 (Life Technologies, Inc.) according to Snyder et al. (12). Lung explants were maintained in culture for up to 3 days in either control medium or medium containing Bt,cAMP (1 mm) or dexamethasone (10⁻⁵ M) or a combination of the two agents. Dexamethasone was dissolved in ethanol before addition to the culture medium. The concentration of ethanol in culture medium was 0.01%; at this concentration ethanol had no effect on SP-C mRNA levels. Actinomycin D (actinomycin D-mannitol, water-soluble, Sigma) (5 μg/ml) was added in experiments designed to measure the rate of SP-C mRNA degradation. Culture medium was changed every 24 h.

Isolation of RNA and Northern Blot Analysis—Total RNA from fetal lung tissues was extracted with TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer’s instructions. RNA (5–10 μg) was electrophoresed in agarose gels (1.2%) containing 20 mM MOPS and 1.1% formaldehyde (13). RNAs were transferred to Hybond N⁺ (Amersham Corp.) membrane by capillary action using 30 mM sodium hydroxide. Membranes were hybridized to 32P-labeled rabbit SP-C cDNA (0.9 kb) and human β actin cDNA (~1.7 kb) at 60 °C, and final washes were done in 1 x SSC at 60 °C. Membranes were also hybridized to a 3P-labeled antisense oligonucleotide (30-mer) of ribosomal 18 S RNA to assess the integrity of RNA preparations, hybridization of equivalent amounts of RNA, and quantitative transfer of RNA. Autoradiographic signals were quantified by scanning densitometry (Milli-
pore BioImage Analyzer). Densities of SP-C and actin mRNA signals were normalized to the density of 18S rRNA signal to correct for the amount of RNA loaded on the gels.

**Isolation of Nuclei and Transcription Run-on Assay—**Methods for isolation of nuclei, transcription run-on assay, and hybridization of RNA have been described previously (14). RNA from nuclei was extracted with TRI reagent by modification of the method for extraction of RNA from cells/tissue. Nuclei (20 x 10^6 in 200 µl) were lysed by adding 1.4 ml of TRI reagent to the reaction mixture, vigorously vortexed for 30–60 s, and stored at room temperature for 10 min. Chloroform (0.28 ml) was added to the lysed nuclei, and the samples were shaken vigorously for 15 s and stored at room temperature for 5 min. Samples were centrifuged for 15 min at 12,000 x g at 4°C. The upper aqueous phase was mixed with 100 µg of yeast tRNA (Escherichia coli tRNA served as a carrier), and total RNA was precipitated with an equal volume of isopropanol for 10 min at room temperature. RNA was collected by centrifugation in 200 µl of hybridization buffer, and radioactivity of RNA was determined by liquid scintillation spectrometry. The final RNA preparations were checked for presence of unincorporated [3H]UTP.

This method for isolation of RNA does not involve many steps, and the entire procedure can be completed in a relatively short time. It is especially convenient for extraction of RNAs from a large number of samples. RNA extracted by our method produced hybridization results similar to those RNAs obtained by digestions with DNase I, proteinase K, and multiple phenol and chloroform extractions. A similar method for isolation of RNA from labeled nuclei was described recently (15).

Equal amounts of radioactive RNAs were hybridized to 10 µg of linearized plasmid DNA containing rabbit SP-C cDNA or human β actin cDNA that had been bound to nitrocellulose membranes. In some experiments RNAs were also hybridized to membranes containing antisense SP-C cDNA (9.5 kb) or antisense SP-C gene fragments in M13. Linearized plasmid DNAs were denatured by treatment with 0.3 N NaOH for 30 min at 37°C and neutralized with 1 N NH₄OAc. M13 DNA and denatured plasmid DNAs were applied to nitrocellulose membranes using a dot blot apparatus, and the DNAs were fixed to the membrane by baking at 80°C for 2 h under vacuum. Hybridizations were carried out in a final volume of 200 µl at 45°C for 5 days. Washed membranes were subjected to autoradiography using an intensifying screen, and signals were quantified by scanning densitometry (Millipore BioImage Analyzer). After autoradiography membranes were counted in a liquid scintillation counter to determine the amount of bound radioactivity. Usually we detected no degradation of RNA during hybridization. Transcription rates determined by either quantification of autoradiographic signals or by quantification of bound radioactivity were similar. Signal/radioactivity bound to linearized pUC DNA/M13 DNA (background) was subtracted from radioactivity obtained with plasmids containing SP-C/actin cDNAs or M13 containing antisense SP-C cDNA/antisense SP-C gene fragments.

**Accumulation of Half-life of SP-C and Actin mRNAs—**Half-lives of SP-C and actin mRNAs were determined by measuring their rate of degradation after inhibition of RNA synthesis with actinomycin D (5 µg/ml). Actinomycin D at this concentration inhibited total RNA synthesis by ~80% after 3 h of incubation. Tissues were incubated in the presence or absence of dexamethasone (10⁻⁷ M) for 24 h, and incubation was continued for an additional 24 h in the presence or absence of dexamethasone in medium containing actinomycin D (5 µg/ml). Tissues were collected at 0, 6, 9, 12, 18, and 24 h after addition of actinomycin D, and SP-C and actin mRNA levels were determined by Northern blot analysis. Half-life of RNA was calculated from the equation >80% after 3 h of incubation. Tissues were incubated in the presence or absence of dexamethasone (10⁻⁷ M) for 24 h, and incubation was continued for an additional 24 h in the presence or absence of dexamethasone in medium containing actinomycin D (5 µg/ml). Tissues were collected at 0, 6, 9, 12, 18, and 24 h after addition of actinomycin D, and SP-C and actin mRNA levels were determined by Northern blot analysis. Half-life of RNA was calculated from the equation \( t_{1/2} = \frac{ln2k}{k} \) (degradation rate constant \( k = -0.3305 \) (slope) (16)).

**RESULTS**

**Developmental Regulation of SP-C Gene Transcription and SP-C mRNA Levels—**We found previously that SP-C mRNA accumulation is induced during fetal lung development (10). To determine if the induction of SP-C mRNA levels is regulated at the transcriptional level, we analyzed SP-C gene transcription and SP-C mRNA levels in fetal lung tissues (19–30-day gestational ages) and adult rabbit lung (Fig. 1A). We found a marked lag between the temporal induction of SP-C gene transcription and elevation of SP-C mRNA levels. The increases in SP-C mRNA accumulation were not associated with similar increases in SP-C gene transcription. SP-C mRNA levels in 24-day fetal lung was ~90% of the level in adult lung, whereas SP-C gene transcription level was only ~4% of the level in adult lung (Fig. 1B). An increase in SP-C gene transcription was not observed until day 28 of gestation, and after day 28 of gestation it increased to reach peak levels in adult lung tissue. Total RNA transcription in nuclei of different gestational age fetal lungs did not change significantly, but a decrease of ~50% was consistently found in nuclei from adult lungs. SP-C gene transcription rates were similar or identical whether measured with double-stranded SP-C cDNA or single-stranded antisense SP-C cDNA probes.

**cAMP and Glucocorticoid Regulation of SP-C Gene Transcription and SP-C mRNA Levels—**In previous studies we found that cyclic AMP analogs and dexamethasone increases the SP-C mRNA accumulation in 21-day fetal lung tissues in vitro in a time-dependent manner. Induction by cAMP analogs occurred consistently after only 48 h of incubation, whereas induction by dexamethasone was found after 24 h of incubation. In tissues treated with a combination of β,γcAMP and dexamethasone, the effects on SP-C mRNA levels were less than additive after 24 h of incubation, whereas after 48–72 h of incubation they were additive. To determine if the changes in SP-C mRNA accumulation are due to changes in SP-C gene transcription, we analyzed SP-C gene transcription and SP-C mRNA levels in tissues treated with β,γcAMP or dexamethasone or a combination of the two agents (Fig. 2). Total RNA transcription rate was unaffected by treatment with hormonal agents.
Regulation of Surfactant Protein C Gene Expression

FIG. 2. Effects of Bt,cAMP (B) and dexamethasone (D) on SP-C gene transcription and SP-C mRNA accumulation in fetal rabbit lung in vitro. A, 21-day gestational age fetal rabbit lung tissues were maintained in the absence (C) or presence of Bt,cAMP (1 mM) or dexamethasone (10^{-6} M) or a combination of the two agents for 24–72 h. Nuclei were isolated from fetal lung tissues in culture and SP-C gene transcription rates were determined by run-on assay as described under "Materials and Methods." 

b) 

We also observed that SP-C gene transcription and SP-C mRNA accumulation occurred at different times during culture of fetal lung tissues. SP-C mRNA accumulation increased during culture of fetal lung tissues.

DISCUSSION

The temporal induction of SP-C mRNA during fetal lung development is remarkably different from that of SP-B and SP-A mRNAs. In rat (18), rabbit (7, 10), and human fetal lungs (19, 20), SP-C mRNA is detected prior to the appearance of morphologically identifiable alveolar type II epithelial cells. Differentiated alveolar type II cells are first observed in rabbit
lung tissue at approximately day 26 of gestation (21). In rabbit
fetal lung, SP-C mRNA is detected at day 19 of gestation, the
earliest time point examined (7, 10, 22). SP-C mRNA levels
increase as a function of gestation, and in neonatal and adult
lung tissues its level decreases significantly compared to levels
found in lung tissues of 28–31-day gestational age fetal rabbits.

We found a marked discrepancy between the temporal induc-
tion of SP-C gene transcription and SP-C mRNA accumulation.
The lag between SP-C gene transcription and SP-C mRNA
accumulation suggested that significant stabilization of SP-C
mRNA can account for the accumulation of SP-C mRNA during
prenatal lung development. In adult lung, however, transcrip-
tion appears to regulate SP-C mRNA accumulation. Because
of experimental difficulties involved, we did not attempt to
measure SP-C mRNA turnover in vivo during fetal lung
development.

The temporal induction of SP-C gene transcription and
mRNA during fetal rabbit lung development is quite different
from the temporal induction of SP-A gene transcription and
mRNA. SP-A gene transcription increases as early as day 24 of
gestation and continues to increase to reach highest levels in
28-day lung (14). SP-A gene transcription in neonatal lung is
decreased compared to levels in 28-day lung. The temporal
induction of SP-A gene transcription coincides with temporal
induction of SP-A mRNA (14), suggesting that our observed
differences between temporal induction of SP-C gene transcrip-
tion and SP-C mRNA accumulation represent a specific phe-
nomenon, not a pleiotropic effect.

Snyder and co-workers (7) used in situ hybridization analysis
to detect SP-C mRNA in epithelial cells of prealveolar region of
19-day rabbit fetal lung. They found that the SP-C mRNA con-
tent increased as a function of gestational age, and by day 27 of
gestation and thereafter expression of SP-C mRNA was re-
stricted to epithelial cells with morphologic characteristics of
alveolar type II cells (7). Taken together, the data of Snyder
and co-workers and our finding that SP-C gene transcription
does not increase until day 28 of gestation suggest that an increase
in SP-C gene transcription is linked to differentiation of alve-
olar type II cells.

The number of identifiable type II cells increases as a pro-
portion of total epithelial cells in lung after day 26 of gestation
in the rabbit (21, 23). This makes accurate assessment of in-
creases in SP-C gene transcription rate during development
difficult. SP-C mRNA content of prealveolar and alveolar ep-
ithelial cells increase during fetal rabbit lung development to
reach highest levels at term (7). This suggests that an increase
in SP-C mRNA content of type II epithelial cells during devel-
opment must be the result of increases in SP-C gene transcrip-
tion/SP-C mRNA stability. These data suggest that increases in
SP-C gene transcription rate during development must be due
both to an increase in the number of type II epithelial cells and
to enhanced transcription/stability of SP-C mRNA.

The physiological significance of high levels of SP-C mRNA
in embryonic lung is unclear. A similar pattern of accumulation
was also noted for SP-A and SP-B mRNAs (22). The transition
from an aqueous to a gaseous environment that accompanies
tal lung development and enhance surfactant synthesis (24).
Glucocorticoids increase SP-C mRNA accumulation in human
fetal lung in vitro (19, 20) and in rat lung both in vitro (25) and
in vivo (26, 27). The effects of maternal administration of glu-
cocorticoids on SP-C mRNA levels in fetal lung are not clear.
Maternal administration of glucocorticoids to pregnant rabbits
on day 26 of gestation resulted in a decrease in SP-C mRNA
level in lungs of fetal rabbits delivered on day 27 (28). A recent
study showed that glucocorticoids administered to pregnant
rabbits on days 25 and 26 of gestation increased SP-C mRNA
levels 2-fold compared to un.injected control animals (29). In
the same study injection of saline to pregnant animals increased
SP-C mRNA levels in fetal lung tissues 2-fold, leading the au-
tors to suggest that maternal stress related factors alone can
increase SP-C mRNA levels. Glucocorticoid regulation of SP-C
mRNA levels in vivo might depend on the developmental stage
of the fetus when the hormone was administered maternally.

We found that cAMP and dexamethasone regulate SP-C
mRNA levels in fetal lung in vitro by distinct mechanisms; the
effects of cAMP are mediated at the transcriptional level, whereas
the effects of dexamethasone are mediated solely at the
posttranscriptional level. We also found that after 48 h of
incubation dexamethasone inhibited both basal and Bt,cAMP-
dependent increases in SP-C gene transcription. This effect
may explain why we did not observe a substantial increase in
SP-C mRNA levels in tissues treated with a combination of
Bt,cAMP and dexamethasone for 48–72 h, even though dexam-
ethasone increased the half-life of SP-C mRNA after 54 h and
Bt,cAMP increased SP-C gene transcription after 48 h. Mec-
hanisms by which dexamethasone inhibited SP-C gene transcrip-
tion are not known. Glucocorticoids were found to negatively
regulate human glycoprotein a-subunit gene expression by in-
terfering with a cAMP responsive element (30). Whether dexa-
methasone inhibits CAMP-induced SP-C gene transcription by
a similar mechanism remains to be investigated.

We found previously that adenosine 3',5'-cyclic monophos-
phorothioate (Sp-diastereomer) (0.1–0.25 mM), an analog of
cyclic AMP, increases SP-C mRNA accumulation in fetal rabbit
lung in vitro by a magnitude similar to that of Bt,cAMP (1 mM)
(10). This suggested that the effects of Bt,cAMP to increase
SP-C mRNA accumulation are not due to butyric acid, a by-
product of metabolism of Bt,cAMP. Butyric acid modulates glo-
in gene expression in erythroid cells (31, 32). Recently, butyric
acid was found to modulate surfactant protein gene expression
in fetal rat lung in vitro with significant inhibitory effects on
SP-A and SP-B mRNA accumulation (33). The effects of butyric
acid on SP-C mRNA levels were complex; butyric acid inhibited
SP-C mRNA accumulation below control levels after 6 h of
incubation but increased to control levels after 24 h of incuba-
tion (33). Both transcription and mRNA stability were found to
mediate butyric acid-induced changes in SP-C mRNA accumu-
lation. The authors have suggested that elevated levels of buty-
ic acid analogs as found in diabetic mothers and fetuses
might lead to increased incidence of newborn respiratory dis-
press syndrome. It remains to be determined whether butyric
acid has similar effects on surfactant protein gene expression in
fetal lungs of other species. Incubation of fetal rabbit lung
explants with butyric acid for 5 days did not inhibit levels of
immunoreactive SP-A (34).

Glucocorticoids and cAMP influence expression of a number
genes in different cell types of fetal lung (35). This might con-
found our analysis of the effects of these agents on SP-C gene
transcription rate in fetal lung explants. In fetal rabbit lung in
vitro SP-A (14), SP-B,5 and SP-C (10) genes are regulated in-
dependently by both cAMP and glucocorticoids. By in situ hy-
bridization dexamethasone was found to increase SP-B mRNA
content of both alveolar and bronchiolar epithelial cells of fetal
rat lung explants (36). This suggests that dexamethasone-de-
pendent increase in SP-B mRNA levels in fetal rat lung ex-
plants must be due to increased accumulation of SP-B mRNA
in alveolar and bronchiolar epithelial cells of fetal lung. By de-

5 R. K. Margana and V. Boggaram, submitted for publication.
tion mapping and transfection analysis a region of SP-A gene, -378 to +20, was shown to mediate cAMP induction of SP-A chloramphenicol acetyltransferase fusion gene expression in purified alveolar type II epithelial cells (37). This suggests that the inductive effects of cAMP on SP-A gene transcription in fetal rabbit lung in vitro are indeed due to activation of SP-A gene transcription in type II cells. These data suggest that glucocorticoids and cAMP regulate surfactant protein gene expression by altering their expression by specific cell types of lung and that changes in surfactant protein gene expression are not the result of pleiotropic effects of hormones.

We investigated the effects of dexamethasone on SP-C mRNA stability since stabilization of RNA plays a major role in post-transcriptional regulation of gene expression (17). We found that dexamethasone increased the half-life of SP-C mRNA by >2.5-fold. The action of dexamethasone that results in increased SP-C mRNA levels in rabbit fetal lung in vitro is different from that found in rat (25) and human fetal lung (38) tissues. In both rat and human fetal lung tissues in vitro, glucocorticoids increase SP-C mRNA levels by increasing gene transcription. However, mRNA stabilization and its effect on SP-C mRNA levels in rat and human lung in vitro cannot be ruled out, because the time course of the effects of glucocorticoid on SP-C gene transcription and SP-C mRNA accumulation were not investigated. The lack of effect of dexamethasone on SP-C gene transcription in rabbit fetal lung might be species-specific. SP-A gene expression appears to be regulated differently by glucocorticoids in rabbit (14) and human (39) fetal lung in vitro. In rabbit fetal lung in vitro, glucocorticoids regulate SP-A gene expression predominantly at the transcriptional level (14), whereas in human fetal lung in vitro, they exert dose-dependent effects on transcription and mRNA stability (40).

SP-C gene transcription and SP-C mRNA accumulation increased during explant culture in the absence of either serum or hormones. Several other reports indicate increases in mRNAs for rabbit SP-A (14), SP-C (10), and human SP-B (19) during explant culture of fetal lung tissues in serum-free defined medium. Increases in SP-A mRNA in rabbit fetal lung in vitro appeared to be coincident with increase in gene transcription (14). Increased expression of surfactant protein mRNAs during explant culture is probably due to an increased number of type II epithelial cells as a result of differentiation of fetal tissues (12). The factors and mechanisms that mediate spontaneous differentiation of fetal lung tissues in vitro are not well understood. Prostaglandins acting through cAMP may promote differentiation of type II cells and increase SP-A gene expression in human fetal lung tissues in vitro (41).

In addition to regulating gene expression at the transcriptional level, hormonal and developmental signals also modulate gene expression at the post-transcriptional level by altering mRNA stability (42). Changes in mRNA stability occur during maturation of frog and mouse oocytes (43, 44). Both frog and mouse oocytes store a major fraction of maternal mRNA in a stable and translationally inactive form. During development oocyte-specific mRNAs are recruited for translation and later undergo degradation at an increased rate. Developmental changes in mRNA stability also play important roles in regulating gene expression in terminally differentiated cells. During red cell development, accumulation of globin is attributed to stabilization of globin mRNA and selective destabilization of non-globin mRNAs (45, 46). In cell lines that reflect different stages of B cell development, stabilization of immunoglobulin mRNA mediates accumulation of this mRNA in differentiated B cells (47, 48). Our investigation has revealed that changes in both mRNA stability and transcription regulate accumulation of SP-C mRNA during development and differentiation of fetal lung.

Glucocorticoids regulate gene expression at posttranscriptional level by exerting both positive and negative effects on mRNA stability. Glucocorticoids increase stability of growth hormone (49), fibroactin (50), and phosphoenolpyruvate carboxykinase (51) mRNAs and decrease the stability of interleukin-1β (52), interferon β (53), and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (54) mRNAs. In human fetal lung in vitro, they stabilize SP-B mRNA (38) and destabilize SP-A mRNA (40).

Molecular mechanisms underlying regulation of mRNA stability by hormonal and developmental signals are not well understood. 3' Untranslated regions of mRNAs are required for regulation of mRNA stability, and alterations in RNA stability are mediated by the interaction of discrete protein factors (turnover factors) with specific domains and sequence elements (turnover elements) within mRNAs (42). A region located in the 3' untranslated region of phosphoenolpyruvate carboxykinase mRNA has been shown to confer glucocorticoid stabilization upon a heterologous mRNA when stably transfected into a rat hepatoma cell line (51). A glucocorticoid-inducible protein factor was suggested to interact with the 3' untranslated region. SP-C mRNAs contain a 3' untranslated region of 240–271 nucleotides. Sequence elements within SP-C mRNA and development- and glucocorticoid-specific putative protein factors that interact with RNA sequence elements to regulate RNA stability remain to be investigated.

Our investigation provides new information on the regulation of SP-C gene expression during fetal lung development and by glucocorticoid and cAMP in fetal lung in vitro. Results of our experiments reveal that, whereas post-transcriptional (mRNA stability) mechanisms play a major role in SP-C gene regulation in prenatal lung development, transcriptional mechanisms regulate its expression in adult lung. In fetal lung in vitro glucocorticoids and cAMP, agents that have profound effects on lung maturation and surfactant synthesis, regulate SP-C gene expression by diverse mechanisms. The effects of cAMP are exerted at the transcriptional level, whereas the effects of glucocorticoids occur at the level of mRNA stabilization.

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Regulation of Surfactant Protein C Gene Expression

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