Localization of Surfactant-associated Protein C (SP-C) mRNA in Fetal Rabbit Lung Tissue by *In Situ* Hybridization

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Surfactant is a lipoprotein substance that is synthesized and secreted by alveolar type II epithelial cells and acts to reduce surface tension at the air-alveolar interface. SP-C is a 5,000-D molecular weight, hydrophobic, surfactant-associated protein. In the present study, we used a ribonuclease protection assay to show that SP-C mRNA is induced in rabbit fetal lung tissue early in development, increases in relative concentration as development proceeds, and is present in maximal concentration at term (31 days of gestation). We also used the technique of *in situ* hybridization to localize SP-C mRNA in fetal, neonatal, and adult rabbit lung tissue. SP-C mRNA was present in all of the epithelial cells of the prealveolar region of day 19 gestational age rabbit fetal lung tissue, i.e., about 7 days before the appearance of differentiated alveolar type II cells in the fetal lung tissue. By day 27 of gestation, SP-C mRNA was restricted to epithelial cells with the morphologic characteristics of alveolar type II cells. SP-C mRNA was not detected in bronchiolar epithelium at any stage of lung development. The intensity of SP-C mRNA hybridization in the prealveolar and alveolar type II epithelial cells increased as a function of gestational age and was maximal at term. The pattern of SP-C mRNA localization in neonatal and adult rabbit lung tissue was consistent with the restriction of SP-C gene expression to differentiated alveolar type II cells. Our data are suggestive that SP-C may serve some as yet unknown function early in lung development because it is present in fetal lung prealveolar epithelial cells much earlier in gestation than are differentiated, surfactant-producing alveolar type II cells.

Pulmonary surfactant is a lipoprotein substance that acts to reduce surface tension at the air-alveolar interface (1). Surfactant is synthesized by the alveolar type II epithelial cell and is stored intracellularly in organelles called lamellar bodies. After being released from the apical portion of the alveolar type II cell by exocytosis, secreted lamellar bodies are transformed into tubular myelin, a structure that is thought to be the precursor of the monolayer of surfactant that lines the alveolus (1). Surfactant is composed of glycerophospholipids (~80% by weight), cholesterol (~10% by weight), and proteins (~10% by weight) (1). Although the surface tension-lowering properties of surfactant were originally attributed to the most abundant phospholipid present in surfactant, dipalmitoylphosphatidylcholine, it is now known that the surfactant-associated proteins are required to achieve the optimal functional properties of lung surfactant (2).

To date, four surfactant-associated proteins, SP-A, SP-B, SP-C, and SP-D, have been described (2). SP-A, a 35,000-D molecular weight sialoglycoprotein, is the most abundant and best characterized surfactant-associated protein (2, 3). SP-A, in the presence of Ca^{2+}, has been shown to mediate the structural transformation of lamellar body phospholipids into tubular myelin (4). SP-A has been shown to regulate the synthesis and secretion of surfactant phospholipids by purified alveolar type II cells (5, 6). SP-A may also influence alveolar macrophage function (7). SP-B and SP-C are low molecular weight, hydrophobic surfactant-associated proteins that have been shown to greatly increase the rate of spreading of surfactant phospholipids on an aqueous surface (8–10). SP-B is a 7,000-D molecular weight protein that intercalates with phospholipids in the surfactant monolayer (11, 12). SP-C, an ~5,000-D molecular weight protein, has an unusual, extremely hydrophobic region consisting of approximately 20 sequential valines (13, 14). The biophysical mechanism of action of SP-C is not clear; however SP-C has been shown to increase the rate of adsorption of surfactant phospholipids at an air-water interface (15). SP-D is a collagenous surfactant-associated protein of ~43,000 D that has recently been shown to be a calcium-dependent lectin (16).

The surfactant-associated proteins SP-A and SP-B have been immunolocalized within alveolar type II cells (2). Because of its hydrophobicity, SP-C is poorly antigenic; therefore, very few antibodies to this protein have been described. In one recent study, a polyclonal antisera that recognized

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Abbreviations: diethylpyrocarbonate, DEPC; phosphate-buffered saline, PBS; surfactant protein, SP; 3-aminopropyltrithoxysilane, TES.

both SP-B and SP-C was used to show that these proteins are present in alveolar type II cells and in noncilated, bronchiolar epithelial cells of human lung tissue (17). It is difficult to interpret the results of this study because the localized staining may reflect the presence of SP-B or SP-C or both of these antigens. SP-D has not been immunolocalized in lung tissue to date although it is known that SP-D is synthesized by alveolar type II cells (16).

The fetal lung alveolar type II cell differentiates late in gestation (18). The morphologic differentiation of the alveolar type II cell is characterized by the appearance of intracellular lamellar bodies. The induction of SP-A and SP-B protein is coincident with or slightly precedes the morphologic differentiation of alveolar type II cells in rat and rabbit fetal lung tissue (19, 20). SP-A protein and mRNA are also induced coincident with alveolar type II cell differentiation in human fetal lung tissue (21–23). In the human, SP-B and SP-C protein and their mRNA are present in fetal lung tissue many weeks before morphologic alveolar type II cell differentiation (24, 25). Likewise, SP-C protein and mRNA are induced in rat fetal lung tissue many days before morphologic alveolar type II cell differentiation occurs (20, 26).

Using *in situ* hybridization, SP-A mRNA has been localized within alveolar type II cells and nonciliated bronchiolar epithelial cells of adult and developing rabbit lung tissue and in adult human lung tissue (27, 28). SP-B mRNA has been detected within alveolar type II cells and small airway epithelial cells in human lung tissue (28). SP-C mRNA has not been localized in adult or fetal lung tissue of any species.

In the present study, we used the technique of *in situ* hybridization to localize SP-C mRNA in developing rabbit fetal lung tissue at several stages of differentiation as well as in neonatal and adult rabbit lung tissue. We used the rabbit fetus as a model because of its relatively long gestational period (31 days), which allows examination of many more stages of lung development than would be possible using the rat or mouse fetus as a model. Our results are suggestive that SP-C is expressed by cells that are precursors to both alveolar type I and type II cells. In addition, the SP-C protein must serve some physiologic function unrelated to pulmonary surfactant in undifferentiated fetal lung tissue as it is induced much earlier in gestation than are the other lipid and protein components of surfactant.

Materials and Methods

Animals

Pregnant New Zealand rabbits were obtained from a local rabbitry (Knapp Creek, Amana, IA) and were maintained in an approved animal care facility before use. Does were killed on days 19, 21, 24, 26, 27, 28, and 31 of gestation (term = 31 days) by the rapid injection of 500 mg pentobarbital into an ear vein. The fetuses were removed, lung tissue was dissected free, and the upper, middle, and lower lobes of the right lung were quickly frozen in liquid nitrogen. Fetal lung tissues from a minimum of three litters were examined for each gestational age. Lung tissues obtained from day 2 neonates and adult animals were also used for this study. Tissue samples were stored at −70° C before use.

Probes

A rabbit SP-C cDNA probe was isolated and sequenced in our laboratory using the reverse transcriptase–polymerase chain reaction (P. L. Durham *et al.*, manuscript submitted). The 0.5-kb SP-C cDNA probe was cloned into a Bluescript SK vector and digested with *Bss HII* before the synthesis of sense and antisense SP-C mRNA transcripts.

The distilled water used in the preparation of the probes and the solutions was treated before use with 0.01% diethylpyrocarbonate (DEPC) (Sigma Chemical Co., St. Louis, MO) to inactivate RNases (30). RNA transcripts were synthesized using [^{3}H]CTP (4.83×10^7 dpm/μg; New England Nuclear, Boston, MA) and [^{3}H]UTP (5.83×10^7 dpm/μg; New England Nuclear) as precursors. The labeling reaction was performed using 1 μg of the digested SP-C cDNA vector as a template, an RNA transcription kit (Gemini Riboprobe System II Buffers; Promega, Madison, WI) and the appropriate RNA polymerase in a 20-μl reaction volume. After completion of the labeling reaction, the mixture was incubated with RQ1 DNase (1 U; Promega) and yeast tRNA (20 μg) at 37° C for 10 min in order to degrade the template DNA. The newly synthesized RNA was then precipitated in sodium acetate (60 mM), ammonium acetate (500 mM), and ethanol (70%, vol/vol) for 1 h at −70° C. The precipitated RNA was centrifuged for 15 min at 4° C, the pellet washed with ethanol (70%, vol/vol), then dried using a Savant Speed Vac vacuum concentrator. The pellet was resuspended in DEPC-treated water and aliquots were taken for the determination of the percent incorporation of labeled percursors and for agarose gel electrophoresis (29).

All probes used for *in situ* hybridization were hydrolyzed to approximately 200-bp fragments as described by Cox and associates (30). The hydrolyzed probes were precipitated in sodium acetate (200 mM) and ethanol (70%, vol/vol) with yeast tRNA (5 μg) at −70° C for 1 h. The precipitated RNA was centrifuged at 4° C for 15 min, then dried and resuspended in 22 μl of DEPC-treated water. The length and integrity of the original RNA transcript as well as of the hydrolyzed probe were evaluated using 1.2% agarose, 5% formaldehyde gel electrophoresis (29). Probes were stored at −70° C until used. The final concentration of the radioactive probe in the hybridization buffer was 4.6×10^3 cpm/μl.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from fetal, neonatal, and adult rabbit lung tissue using the single-step method of Chomcyznski and Sacchi (31). The RNA was quantitated by determining the absorbance at 260 nm. For Northern blot analysis, 10 μg of adult rabbit lung total RNA was separated by electrophoresis on a 1.2% agarose, 5% formaldehyde gel (29). The RNA was transferred to a Nytran membrane (Schleucher & Schuell, Keene, NH) by the capillary blot method (29) and baked onto the membrane by heating at 80° C for 1 h. The RNA-containing membrane was hybridized with a ^{32}P-labeled rabbit SP-C cDNA probe radiolabeled to a high specific activity ($> 1 \times 10^9$ cpm/μg) using a random primer labeling kit (Boehringer Mannheim, Indianapolis, IN). The blot was air-dried, wrapped in plastic wrap, and exposed to X-ray film with an intensifier screen at −70° C.

Ribonuclease Protection Assay

Total RNA from rabbit fetal and adult lung tissues isolated as described above was also used in a ribonuclease protection assay (Ribonuclease Protection Assay [RPA] kit; Ambion,

Inc., Austin, TX). Five micrograms of total RNA from each sample was incubated with a ^{32}P-labeled antisense cRNA probe (5×10^5 cpm) in a solution-hybridization buffer for 16 h at 42° C. The nonhybridized single-stranded RNA was digested with a mixture of RNase A (0.05 U) and RNase T1 (10 U); the remaining hybridized RNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1, vol:vol) and then ethanol-precipitated (29). The hybridization products were separated on an 8 M urea, 5% acrylamide gel. The gel was wrapped in plastic wrap and exposed to X-ray film with an intensifier screen overnight at −70° C to visualize the protected, hybridized probe.

In Situ Hybridization

All glassware used for preparing solutions was treated with DEPC to inactivate RNases. Cleaned glassware was filled with distilled water and DEPC was added to a final concentration of 0.1%. The glassware was then incubated at 60° C overnight, the water poured out, and the treated glassware was autoclaved for 30 min. Glass slides were immersed in Chromerge solution (1%; Fisher Scientific, Pittsburgh, PA) overnight, rinsed for 2 h in distilled water, and coated with 3-aminopropyltrithoxysilane (TES) (2%; Aldrich, Milwaukee, WI) as described by Rentrop and colleagues (32). Coverslips (22 × 40 mm) were silanized by brief immersion in Sigmacote (Sigma), rinsed in ethanol (100%) and then DEPC- treated distilled water, and finally baked at 150° C for 2 h.

Five-micron-thick frozen sections were cut at −20° C and mounted on TES-treated glass slides. Routinely, sections from three different gestational ages, two sections per age, were mounted on each slide. Slides with adherent sections were either stored in an airtight slide box with dessicant at −20° C or were immediately fixed for 20 min at room temperature with freshly prepared paraformaldehyde (4%) in phosphate-buffered saline (PBS) (pH 7.4). After fixation, sections were rinsed 3 times in PBS and then dehydrated through an ethanol series. The sections were digested for 10 min at room temperature in 0.125 mg/ml Pronase E (Sigma), in Tris-HCl (50 mM, pH 7.5), EDTA (5 mM) buffer, and then rinsed for 30 s in PBS that contained glycine (2 mg/ml), followed by two 30-s rinses in PBS. To acetylate amino groups in the tissue and reduce the electrostatic nonspecific binding of the probe, the sections were dipped 10 times in triethanolamine buffer (0.1 M, pH 8.0), then incubated for 10 min in triethanolamine buffer that contained acetic anhydride (0.25%). Sections were then washed in 2× SSC (1× SSC = 150 mM sodium chloride, 15 mM sodium citrate [pH 7.0]), dehydrated through an ethanol series, and air-dried.

Thirty-five microliters of hybridization buffer (300 mM NaCl; 10 mM Tris-HCl [pH 8.0]; 1 mM EDTA; formamide [50%, vol/vol]; 1× Denhardt's solution; dextran sulfate [10%, vol/vol]; and yeast tRNA [0.28 mg/ml]) that contained the labeled probe (4.6×10^3 cpm/μl) was then applied to each slide, and a silanized coverslip was placed on top of the sections. To prevent drying, the coverslips were sealed around the edges with rubber cement, then placed in a humid chamber overnight at 42° C. The next morning, the rubber cement was removed using forceps, the sections were rinsed at room temperature in 4× SSC (four rinses, 5 min per rinse), and the coverslips were floated off. The slides were incubated for 30 min at 37° C in a Tris-HCl (10 mM, pH 8.0), EDTA (1 mM), NaCl (0.5 M) buffer that contained RNase A (20 μg/ml; Boehringer-Mannheim) and RNase T1 (3.0 U/ml; Boehringer-Mannheim). The sections were then rinsed for 30 min at 37° C in Tris-HCl (10 mM, pH 8.0), EDTA (2.5 mM), NaCl (0.5 M), followed by two rinses, 15 min per rinse, in 2× SSC and a 30-min rinse at 56° C in 0.1× SSC. Finally, the sections were rinsed in 0.1× SSC for 30 min at room temperature, dehydrated through an ethanol series, and air-dried.

Autoradiography was performed using NTB-2 liquid emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with distilled water at 42° C. Slides were coated with emulsion, air-dried for 2 h, and packed into light-tight slide boxes containing dessicant. Slides were exposed for 1 to 4 wk, but to facilitate comparison most of the data presented in this study were obtained from slides that were exposed for 2 wk. To examine the possibility of a weak hybridization signal, some slides were exposed for as long as 4 wk. Slides were developed for 3 min in D19 developer (Kodak), rinsed for 30 s in distilled water, and fixed in a 1:1 dilution of Rapid Fixer (Kodak) for 3 min. After rinsing for 5 min in running water, the slides were stained briefly in hematoxylin (Surgipath, Grayslake, IL), dehydrated through an ethanol series, cleared with xylene, and mounted using Coverbond (Scientific Products, Chicago, IL). The slides were examined by two independent investigators.

Results

Probe Specificity

The 0.5-kb rabbit SP-C cDNA probe recognized a 0.9-kb mRNA species in adult rabbit lung tissue by Northern blot hybridization (Figure 1A). The rabbit SP-C cDNA probe, cloned into a Bluescript vector, was used to generate ^{32}P-labeled antisense SP-C cRNA probes, which were utilized in a ribonuclease protection assay (Figure 1B). SP-C mRNA was detected in rabbit fetal lung tissue at day 19 of gestation (the earliest time point studied) and increased in relative concentration at later stages of lung development. The highest concentration of SP-C mRNA in rabbit fetal lung tissue was detected at day 31 of gestation (term). The levels of SP-C mRNA were slightly lower in neonatal and adult lung tissue when compared with those present at day 31 of gestation.

In Situ Hybridization of SP-C mRNA in Fetal Rabbit Lung Tissue

Day 19 of gestation. SP-C mRNA was easily detectable in day 19 rabbit fetal lung tissue using a ^{3}H-labeled antisense cRNA probe (Figure 2). SP-C mRNA was present in epithelial cells lining the distal portions of the prealveolar ducts (Figures 2A and 2B). SP-C mRNA was evenly distributed in all the epithelial cells of the distal portion of the tubules and was concentrated in the apical portion of the epithelial cells (Figures 2C and 2E). All the distal portions of the prealveolar tubules that contained SP-C mRNA were rounded. No SP-C mRNA was detected in presumptive small airways, which were identified by their architecture and on the basis of the shape and staining characteristics of their epithelial cells (Figures 2A, 2B, and 2E). An abrupt demarcation was found between the presumptive small airway epithelium, which was negative for SP-C mRNA, and the distal, rounded portion of the prealveolar tubules, which contained

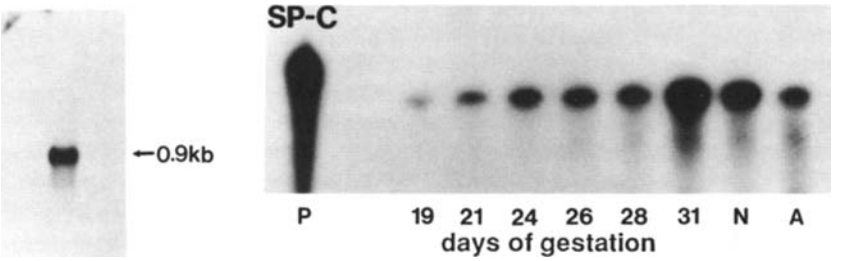

Figure 1. Northern blot analysis and ribonuclease protection assay for rabbit surfactant protein (SP)-C mRNA. *Left panel:* Northern blot analysis for SP-C mRNA in adult rabbit lung tissue. Ten micrograms of total RNA isolated from adult rabbit lung tissue was separated, then hybridized with a ^{32}P-labeled rabbit SP-C cDNA probe. A single 0.9-kb SP-C mRNA species was detected (*arrow*). *Right panel:* Ribonuclease protection assay for SP-C mRNA in fetal, neonatal, and adult rabbit lung tissue. A ^{32}P-labeled 550-bp antisense SP-C cRNA that included 50 bp of the vector sequence was synthesized and run in lane P for comparison. The SP-C mRNA present in the fetal, neonatal, and adult lung samples protected an approximately 500-bp fragment of the labeled probe. SP-C mRNA was present in fetal lung tissue obtained from 19 to 31 days of gestation and in neonatal (*lane N*) and adult rabbit lung tissue (*lane A*).

SP-C mRNA (Figures 2B and 2E). No hybridization of the day 19 rabbit fetal lung tissue with a ^{3}H-labeled sense cRNA probe was detected (Figure 2D).

Day 21 of gestation. On day 21 of gestation, SP-C mRNA was present in the epithelium of the rounded, distal portion of the prealveolar tubules and in the apical portion of the epithelial cells, similar to the pattern observed at day 19 of gestation (Figures 3A and 3B). SP-C mRNA was not present in bronchioles (Figures 3C and 3D). In addition, in profiles in which the rounded distal prealveolar ducts were seen to

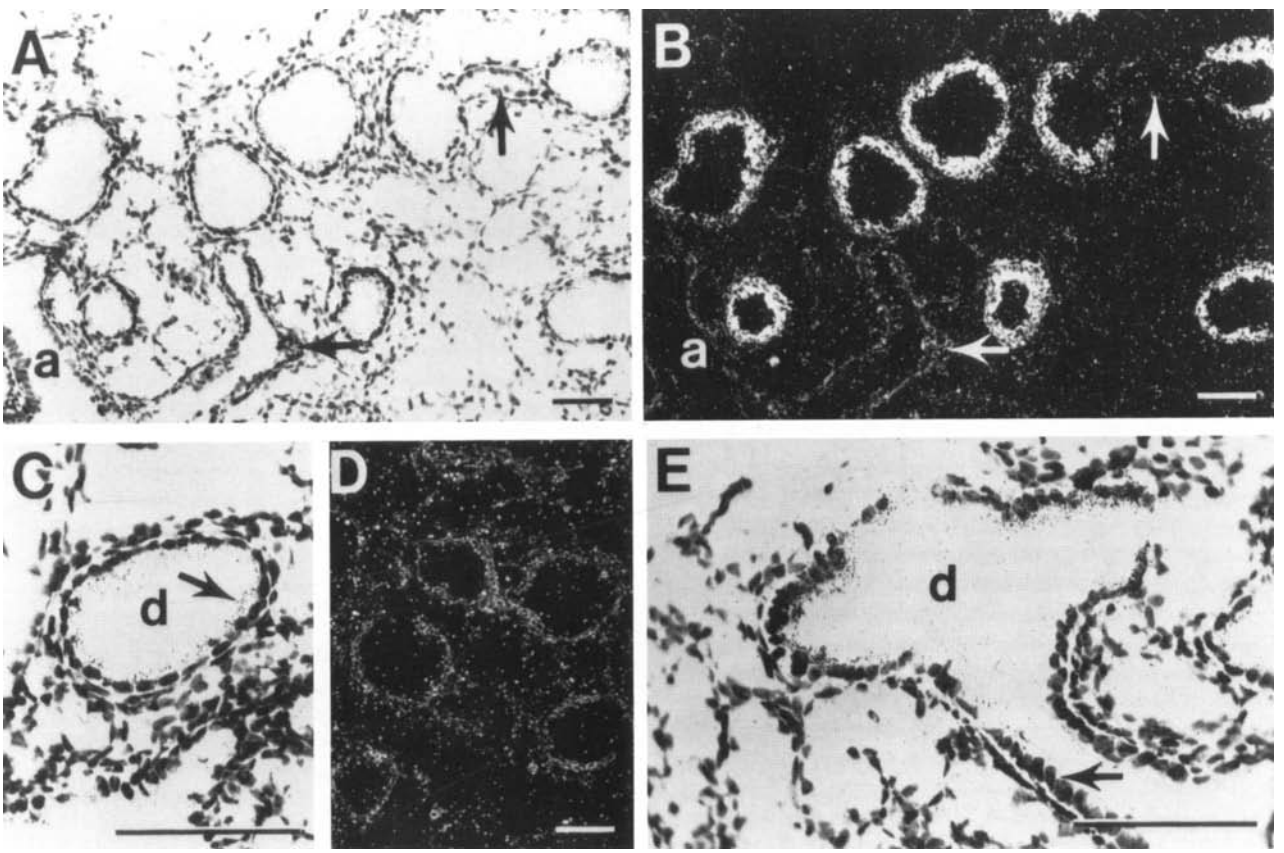

Figure 2. In situ hybridization of SP-C mRNA in day 19 gestational age rabbit fetal lung tissue. Bar = 50 μm. (A) Bright-field photomicrograph of day 19 gestational age rabbit fetal lung tissue hybridized with a [^{3}H]-antisense SP-C cRNA probe. The lung tissue at this stage of development was characterized by many epithelium-lined ducts. Straight portions of the ducts, i.e., presumptive small airways (lumen labeled "a"), are indicated by the arrows. (B) Dark-field photomicrograph of the area depicted in panel A. The labels correspond to the labels in panel A. (C) Higher magnification photomicrograph of the distal portion of a prealveolar duct (lumen labeled "d") in day 19 gestational age rabbit fetal lung tissue that has been hybridized with the [^{3}H]-antisense SP-C cRNA probe. The arrow points to autoradiographic grains concentrated in the apical portion of the epithelial cells. (D) Dark-field photomicrograph of day 19 gestational age rabbit fetal lung tissue hybridized with a [^{3}H]-sense SP-C cRNA probe. No hybridization was detected. (E) Higher magnification photomicrograph of the straight portion of a duct, a presumptive small airway, leading to the distal prealveolar region (lumen labeled "d"). The arrow indicates the region in the straight portion of the duct with little SP-C mRNA hybridization.

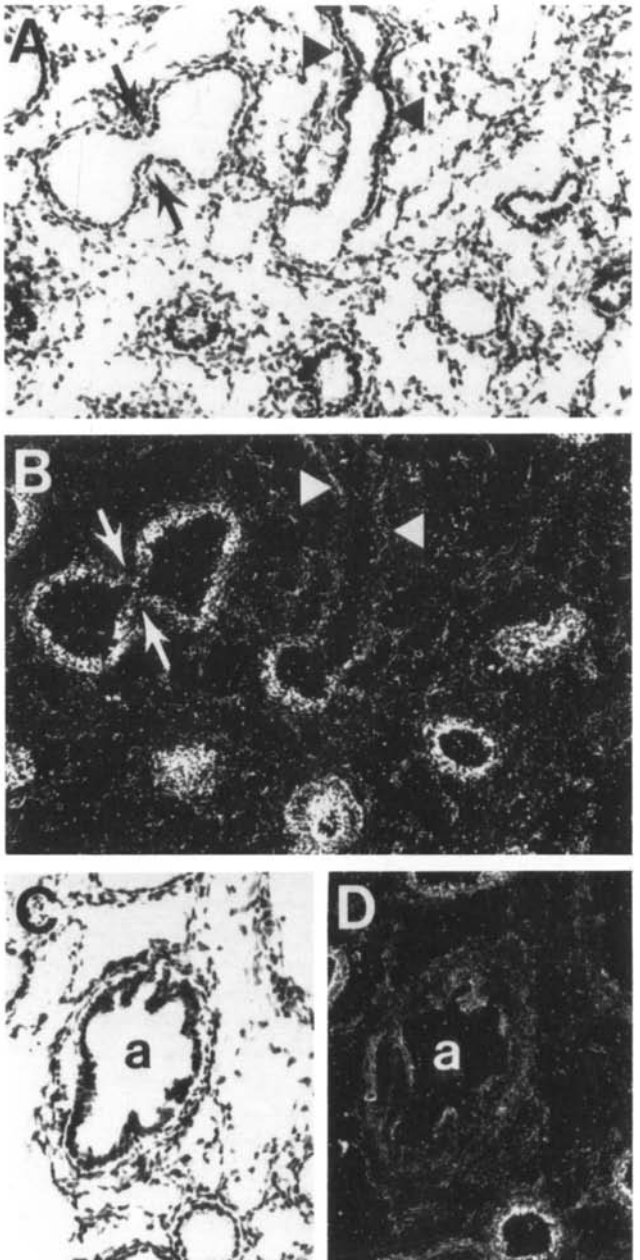

Figure 3. In situ hybridization of SP-C mRNA in day 21 gestational age rabbit fetal lung tissue. Bar = 50 μm. (A) Bright-field photomicrograph of day 21 gestational age rabbit fetal lung tissue hybridized with a [^{3}H]-antisense SP-C cRNA probe. The constricted portion between two distal, prealveolar duct regions is indicated by the solid arrows. A straight, presumptive small airway leading to a distal prealveolar region is indicated by the arrowheads. (B) Dark-field photomicrograph of the same area depicted in panel A. The labels correspond to those in panel A. There is little hybridization in the straight, presumptive small airway indicated by the arrowheads. (C) Bright-field photomicrograph of day 21 gestational age rabbit fetal lung tissue. The lumen in a small airway is indicated by an "a". (D) Dark-field photomicrograph of the area depicted in panel C. The labels correspond to those in panel C. No hybridization with the [^{3}H]-antisense SP-C cRNA probe was detected in the airway epithelium.

be branching, little SP-C mRNA was present in the constricted portion of the ducts (Figure 3A and 3B). The mesenchyme beneath the constricted areas was condensed and more abundant than in the rounded portions of the tubules (Figures 3A and 3B). The diameters of the SP-C mRNA-containing, distal portions of the prealveolar tubules on day 21 of gestation were generally smaller than observed in rabbit fetal lung tissue at day 19 of gestation.

Day 24 of gestation. SP-C mRNA was more abundant in rabbit fetal lung tissue on day 24 of gestation than in the previous stages of lung development examined in our study. The SP-C mRNA was present in the distal tubules of the fetal lung, most of which much were smaller in diameter than on day 21 of gestation (Figures 4A and 4B). Bronchiolar airway epithelial cells did not express SP-C mRNA on day 24 of gestation (data not shown). In addition, epithelial cells in profiles that may be small distal airways were also negative (Figures 4A and 4B). As shown in Figure 4C, not all of the epithelial cells in the distal tubules expressed SP-C mRNA. Some cells in the distal tubules contained much more SP-C mRNA than did others (Figure 4C). In addition, at day 24 of gestation, the SP-C mRNA was present throughout the entire epithelial cell as opposed to the apical localization observed in the previous gestational ages examined (Figure 4C).

Days 26 and 27 of gestation. The pattern of SP-C mRNA localization on day 26 of gestation was very similar to that observed on day 24 of gestation (Figures 5A and 5B). The epithelial cells in the straight, presumptive airways leading to the distal, rounded prealveolar tubules did not contain SP-C mRNA (Figures 5A and 5B). The only major difference in the SP-C mRNA localization pattern at day 26 of gestation when compared with earlier time points was the occasional appearance of isolated SP-C mRNA-containing cells in the distal, rounded tubules (Figure 5C). The epithelial cells in the most distal, rounded ends of the tubules contained the greatest concentration of SP-C mRNA (Figure 5C). On day 27 of gestation, the pattern of SP-C mRNA-containing epithelial cells was quite different from that observed on day 26 of gestation. Only discrete, isolated cells with a localization pattern similar to that of differentiated alveolar type II cells contained the SP-C mRNA (Figures 5D and 5E).

Days 28 and 31 of gestation. In the day 28 gestational age rabbit fetal lung tissue, it was possible to identify terminal and respiratory bronchioles that led to alveolar ducts and alveolar sacs (Figure 6A). The epithelium in the terminal and respiratory portion of the bronchiolar airways did not contain SP-C mRNA (Figure 6B). However, clusters of cells that contained high levels of SP-C mRNA were present in the alveolar duct region. Within the epithelium of each alveolar sac and alveolus were many discrete cells that contained SP-C mRNA (Figure 6B). The numerical density of the SP-C mRNA positive cells in the alveolar regions was higher in the day 28 gestational age fetal lung tissue than observed at day 27 of gestation. The visceral pleural epithelium did not contain significant amounts of SP-C mRNA (Figure 6C). The pattern of SP-C mRNA localization observed on day 31 of gestation (term) was essentially identical to that observed on day 28 of gestation (Figures 6D and 6E).

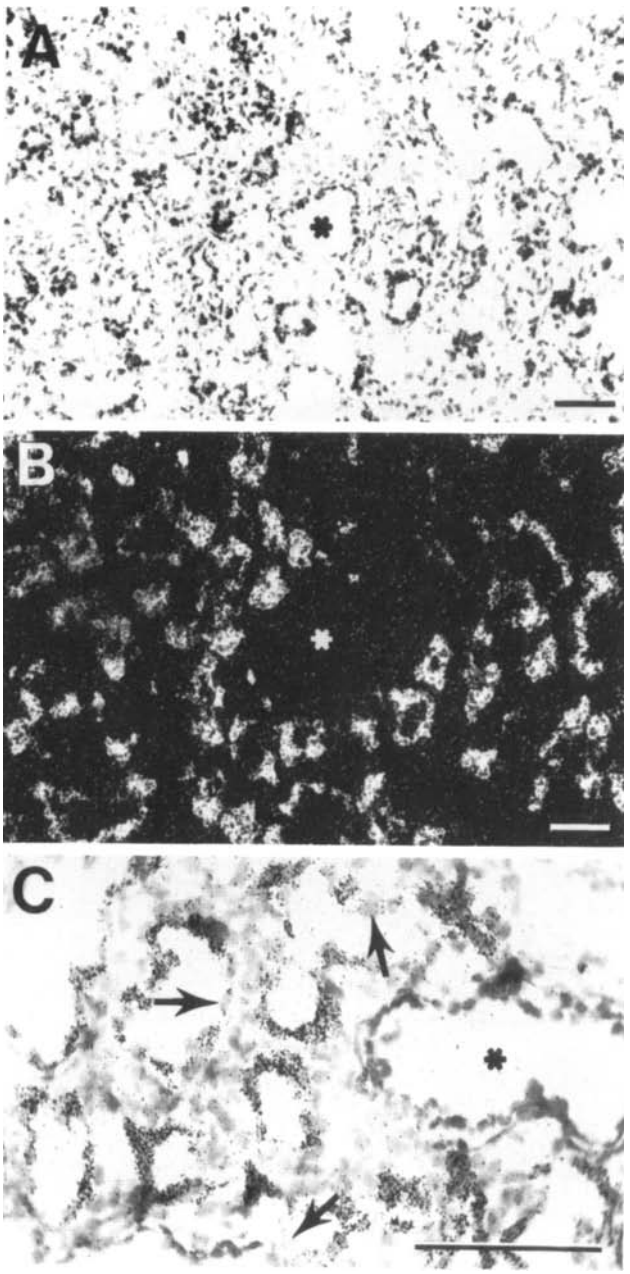

Figure 4. In situ hybridization of SP-C mRNA in day 24 gestational age rabbit fetal lung tissue. Bar = 50 μm. (A) Bright-field photomicrograph of day 24 gestational age rabbit fetal lung tissue hybridized with a [^{3}H]-antisense SP-C cRNA probe. The lumen in a cross-section of a presumptive small airway is indicated by the asterisk. (B) Dark-field photomicrograph of the same area depicted in panel A. The label corresponds to that in panel A. No SP-C mRNA was detected in the presumptive small airway epithelium. (C) Higher magnification photomicrograph of day 24 gestational age rabbit fetal lung tissue that has been hybridized with a [^{3}H]-antisense SP-C cRNA probe. Some cells in the distal, prealveolar regions did not contain SP-C mRNA (*arrows*). The lumen in a cross-section of a small airway is indicated by an asterisk. No SP-C mRNA was detected in the epithelium that lined the small airway.

In Situ Hybridization of SP-C mRNA in Neonatal and Adult Lung Tissue

The pattern of SP-C mRNA localization in the day 2 neonate lung tissue was very similar to that observed in the day 31 fetal lung tissue (Figure 7A). In rabbit adult lung tissue, the degree of SP-C mRNA hybridization per reactive cell appeared to be less than that observed in the neonate and day 31 gestational age fetal lung tissue (Figures 7B and 7C). The cells that contained SP-C mRNA in the neonatal and adult lung tissue had an anatomical localization consistent with their identification as alveolar type II epithelial cells. Airway epithelial cells in the adult lung tissue did not contain SP-C mRNA (Figures 7B and 7C). When adult lung tissue was hybridized with the ^{3}H-labeled sense SP-C cRNA transcript, no hybridization was detected (Figure 7D).

Discussion

We detected significant amounts of SP-C mRNA in rabbit fetal lung tissue at day 19 of gestation, the earliest time point examined in our study. Differentiated alveolar type II cells are first observed in rabbit fetal lung tissue at approximately 26 days of gestation (19). Whitsett and colleagues (24) and Liley and co-workers (25) detected SP-C mRNA in human fetal lung tissue at 13 wk of gestation, i.e., many weeks before the commencement of alveolar type II cell differentiation, which begins at approximately 24 wk of gestation in the human. Schellhause and associates (26) detected SP-C mRNA in rat fetal lung tissue on day 17 of gestation, the earliest time point examined in their study; differentiated alveolar type II cells are first detected on day 19 of gestation in the rat (term = 21 days). Together, the results of studies in the human, rat, and rabbit species are suggestive that the expression of SP-C mRNA is not temporally linked to the morphologic differentiation of alveolar type II cells, i.e., to the appearance of intracellular lamellar bodies.

The results of the ribonuclease protection assay showed that the SP-C mRNA content of rabbit fetal lung tissue increases gradually from day 19 of gestation to term with a slight decrease in relative concentration apparent in neonatal and adult rabbit lung tissue. Our results using *in situ* hybridization are also suggestive that the levels of SP-C mRNA present in rabbit fetal lung tissue increase with advancing gestational age. Also, the intensity of SP-C mRNA hybridization within alveolar type II cells of adult rabbit lung tissue was less than detected in alveolar type II cells in day 31 rabbit fetal lung tissue. Whitsett and colleagues (24) and Liley and co-workers (25) have detected a gradual increase in SP-C mRNA levels in human fetal lung tissue over the period of 16 to 24 wk of gestation. However, the levels of SP-C mRNA present in human fetal lung tissue at 24 wk of gestation were not as great as the SP-C mRNA levels present in adult human lung tissue. Schellhause and associates (26) found that SP-C mRNA levels increased in rat fetal lung tissue with increasing gestational age and reached a maximum in adult lung tissue. Thus, the developmental pattern of SP-C mRNA induction in the rabbit differs from that observed in the human and rat species.

We detected SP-C mRNA in every epithelial cell of the rounded, distal portions of the prealveolar ducts of rabbit fe-

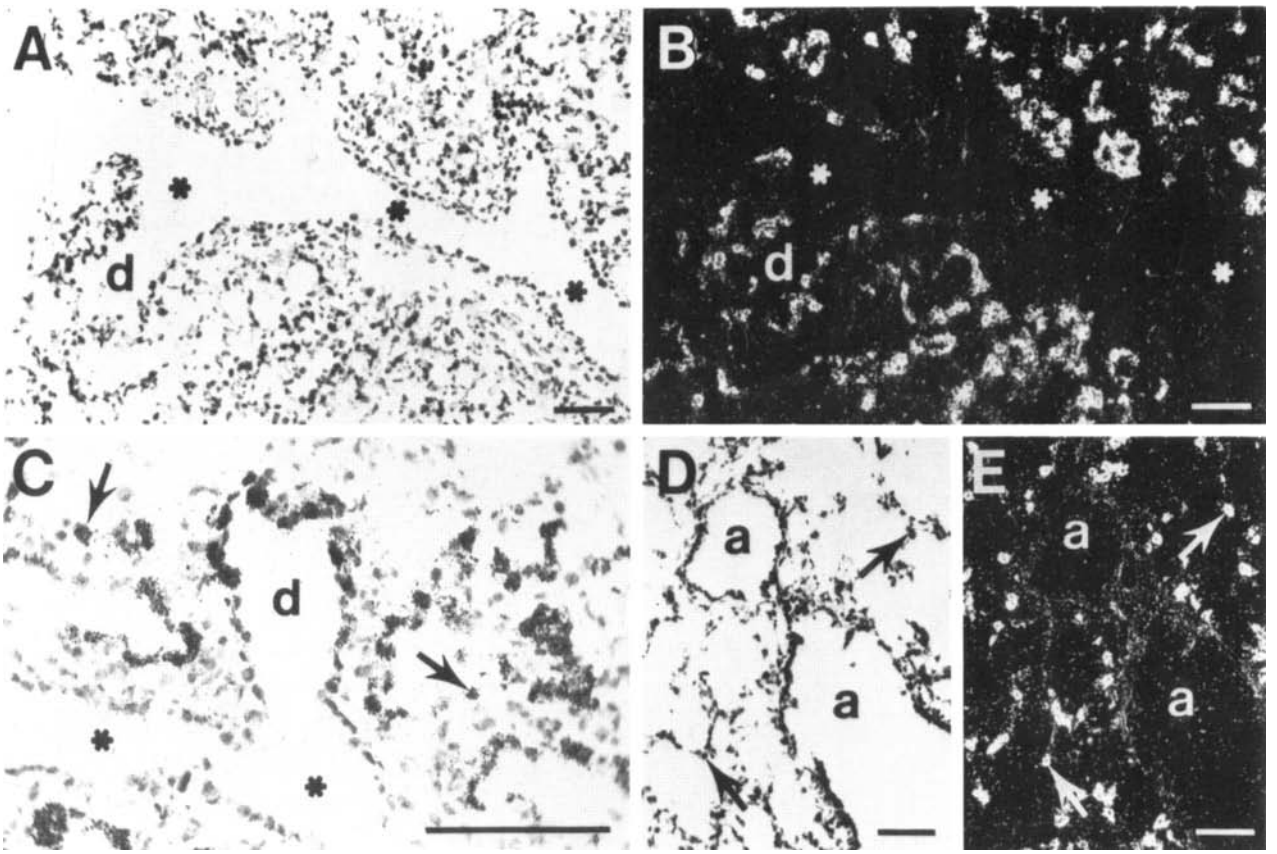

Figure 5. *In situ* hybridization of SP-C mRNA in day 26 and 27 gestational age rabbit fetal lung tissue. Bar = 50 μm. (A) Bright-field photomicrograph of day 26 gestational age rabbit fetal lung hybridized with a [^{3}H]-antisense SP-C cRNA probe. The straight portions of the branching ducts are indicated by the asterisks. The lumen of a distal prealveolar region is indicated with a "d". (B) Dark-field photomicrograph of the area depicted in panel A. The labels correspond to those in panel A. Very little SP-C mRNA hybridization was detected in the epithelium lining the straight portions of the branching ducts. (C) Higher magnification bright-field photomicrograph of day 26 gestational age rabbit fetal lung tissue hybridized with a [^{3}H]-antisense SP-C cRNA probe. The lumen of a distal, prealveolar region is indicated by a "d", while the lumen of the straight, presumptive airway regions is indicated by asterisks. Isolated epithelial cells that contain SP-C mRNA are indicated by the arrows in the prealveolar regions of the tissue. (D) Bright-field photomicrograph of day 27 gestational age rabbit fetal lung tissue hybridized with a [^{3}H]-antisense SP-C cRNA probe. The lumen of an airway is indicated by an "a" and representative individual epithelial cells with a localization pattern consistent with alveolar type II epithelial cell morphology are indicated by the arrows. (E) Dark-field photomicrograph of the same area depicted in panel D. The labels correspond to those in panel D. No SP-C mRNA hybridization was detected in the airway epithelial cells; however, SP-C mRNA was detected in cells with a type II cell morphology (*arrows*).

tal lung tissue at days 19 and 21 of gestation. Starting on day 24 of gestation, a few cells in the distal, rounded portion of the prealveolar ducts contained smaller amounts of SP-C mRNA than the majority of epithelial cells in this location. This trend was also apparent on day 26 of gestation, and by day 27 of gestation only a fraction of the epithelial cells contained SP-C mRNA. Thereafter in development, only cells with anatomical characteristics of alveolar type II cells contained SP-C mRNA. Thus, our data are suggestive that the precursor to alveolar type I and type II epithelial cells present early in lung development contains SP-C mRNA and, as alveolar type I cells differentiate starting on day 24 of gestation, they no longer express SP-C mRNA. In contrast, alveolar type II cells continue to express SP-C mRNA throughout the remainder of development.

We noted a striking asymmetric localization of SP-C mRNA in the epithelial cells of the distal lung prealveolar region on days 19 and 21 of gestation. The SP-C mRNA was present predominantly in the apical portion of the epithelial cells. At this stage of development, the epithelial cells in the distal lung tubules are tall columnar with abundant, glycogen-containing cytoplasm in both the apical and basal portions of the cell (33). We have never noted an asymmetric distribution of the rough endoplasmic reticulum at days 19 and 21 of gestation in these epithelial cells (C. Wohlford-Lenane and J. Snyder, unpublished observations). Therefore, we hypothesize that SP-C mRNA may be localized in the apical portion of the epithelial cell because SP-C protein is preferentially synthesized and secreted from the apical part of the cell. Starting on day 24 of gestation, when the fetal lung prealveolar region epithelial cells have attained a more cuboidal shape, SP-C mRNA was detected throughout the cell

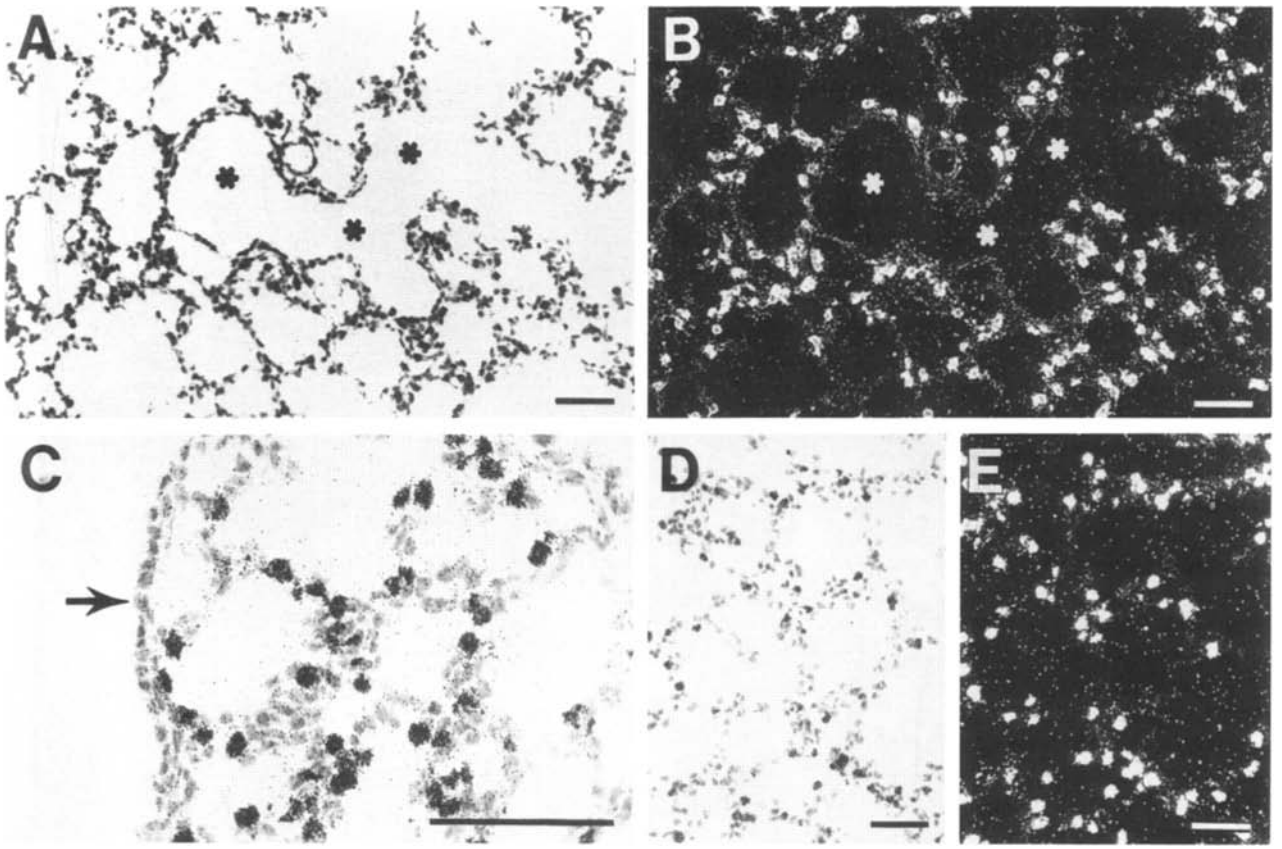

Figure 6. In situ hybridization of days 28 and 31 gestational age rabbit fetal lung tissue hybridized with a [^{3}H]-antisense SP-C cRNA probe. Bar = 50 μm. (A) Bright-field photomicrograph of day 28 rabbit fetal lung tissue hybridized with a [^{3}H]-antisense SP-C cRNA probe. The lumina of terminal and respiratory bronchioles are indicated by asterisks. (B) Dark-field photomicrograph of the same area depicted in panel A. Labels correspond to those in panel A. No SP-C mRNA hybridization was detected in the airway epithelium; SP-C mRNA was only detected in alveolar type II cells. (C) Higher magnification of day 28 gestational age rabbit fetal lung tissue hybridized with a [^{3}H]-antisense SP-C cRNA probe. The visceral pleura, which was negative, is indicated by an arrow. (D) Bright-field photomicrograph of day 31 gestational age rabbit fetal lung tissue hybridized with a [^{3}H]-antisense SP-C cRNA probe. (E) Dark-field photomicrograph of the same area depicted in panel D. The alveolar type II cells contained SP-C mRNA.

(33). It is possible that the change in shape of the prealveolar region epithelial cells is correlated with the change in the intracellular localization of SP-C mRNA.

SP-C mRNA was not present in structures we identified as presumptive small airways, i.e., in the straight ducts leading to the distal, rounded, prealveolar regions. Even in tissue sections that were exposed for many weeks, no SP-C mRNA hybridization was detected in identifiable bronchioles or the presumptive small airways. We conclude that SP-C mRNA is only expressed in the prealveolar and alveolar regions of fetal, neonatal, and adult lung tissue.

de Mello and colleagues (17) reported that SP-B and SP-C are immunolocalized in alveolar type II cells and in some columnar epithelial cells of small airways. The antiserum used in the study of de Mello and colleagues (17) was directed against both SP-B and SP-C. Therefore, it was not possible to ascribe a specific localization for SP-C protein in this study. Whitsett and associates (24), using an antiserum directed against the surfactant proteolipids, SP-B and SP-C, described the localization of these proteins in ductal epithelial cells of human fetal lung explants. The exact localization of SP-C protein in the study of Whitsett and associates (24) also cannot be determined because of the mixed specificity of the antiserum. Interestingly, however, the staining pattern described in that study is suggestive of a predominantly membranous localization of the surfactant proteolipids in the tall columnar epithelial cells of the explants. It has been difficult to generate antibodies directed specifically against SP-C, presumably because of its extreme hydrophobicity. Oetomo and co-workers (34) recently described a monoclonal antibody directed against porcine SP-C. They reported that the monoclonal antibody directed against porcine SP-C only stained clumped material along the surface of the alveolus, presumably surfactant, and did not stain cells in the porcine lung tissue. We have no direct evidence that the SP-C mRNA we detected in our study was translated into SP-C protein. However, Whitsett and associates (24) have shown that SP-C protein and mRNA are detectable simultaneously in human fetal lung tissue.

Whitsett and associates (35) have constructed a 3.6-kb chimera of the human SP-C promoter and 5′ flanking region joined to the diphtheria toxin A gene and have used the chi-

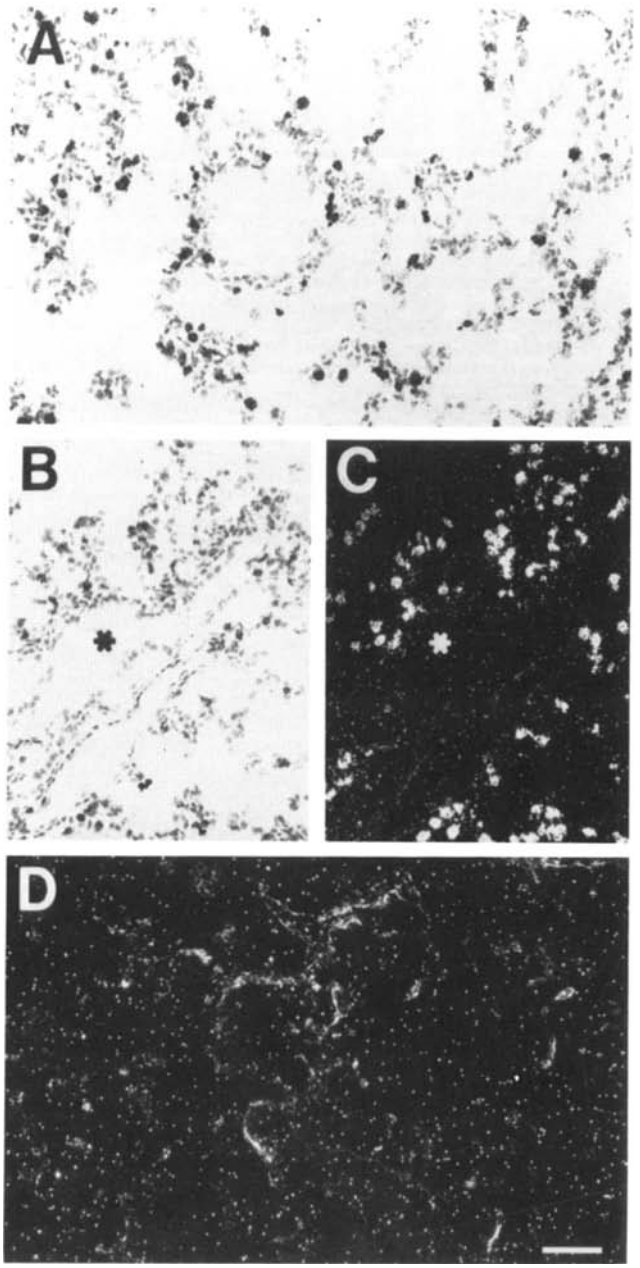

Figure 7. In situ hybridization of SP-C mRNA in rabbit day 2 neonatal and adult lung tissue. Bar = 50 μm. (A) Bright-field photomicrograph of day 2 neonatal rabbit lung tissue that was hybridized with a [^{3}H]-antisense SP-C cRNA probe. Strong SP-C mRNA hybridization was detected in alveolar type II cells. (B) Bright-field photomicrograph of adult rabbit lung tissue that was hybridized with a [^{3}H]-antisense SP-C cRNA probe. The lumen of a small airway is indicated by the asterisk. (C) Dark-field photomicrograph of the same area depicted in panel B. The label corresponds to that in panel B. The epithelial cells of the small airway did not contain SP-C mRNA. (D) Dark-field photomicrograph of adult rabbit lung tissue hybridized with a [^{3}H]-sense SP-C cRNA probe. No specific hybridization was detected.

meric gene to produce transgenic mice. They reported that the lungs of the transgenic fetal mice were characterized by extensive necrosis of the distal respiratory epithelium. The more proximal portions of the tracheobronchial tree were unaffected. These results are consistent with our findings concerning the localization of SP-C mRNA in the distal portions of the ductal system in fetal lung tissue.

SP-C is thought to be involved in facilitating the surface tension–lowering properties of surfactant phospholipids (8–10, 15). To date, no other functions have been proposed for SP-C. Our data are suggestive that SP-C must play another role in lung development because we can detect SP-C mRNA at least 7 days before the appearance of lamellar bodies within differentiated alveolar type II cells in rabbit fetal lung tissue. The role of SP-C in the epithelium of the prealveolar ducts of the fetal lung early in gestation remains to be determined.

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