Localization of Cystic Fibrosis Transmembrane Conductance Regulator mRNA in Human Fetal Lung Tissue by In Situ Hybridization

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Abstract

The fetal pulmonary epithelium secretes fluid. Cl transport is presumed to provide the driving force for net fluid secretion, although the cellular mechanisms have not been well identified in the fetus. The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP- and nucleoside triphosphate–regulated Cl channel; mutations in CFTR cause cystic fibrosis. We hypothesized that if CFTR is involved in fetal lung fluid transport, the fetal pulmonary epithelium should express CFTR mRNA. We used the technique of in situ hybridization with ^{3}H-anti-sense and, as a control, ^{3}H-sense CFTR cRNA probes to localize CFTR mRNA in human fetal lung tissue and cultured lung explants and determine when in gestation it is expressed. Epithelial cells of both first and second trimester lung tissues expressed CFTR mRNA. A decreasing gradient of CFTR mRNA expression was present from the proximal to the distal pulmonary epithelium. Cultured second trimester lung tissue explants expressed more CFTR mRNA than the uncultured starting tissue, suggesting CFTR gene expression increased during the five days in culture. Furthermore, alveolar type II cells in cultured explants expressed CFTR mRNA, suggesting that these cells are Cl-secretory and may be involved in lung fluid transport. These data confirm that CFTR mRNA is expressed in the human fetal pulmonary epithelium, consistent with the Cl-secretory properties of the fetal lung. (*J. Clin. Invest.* 1992. 90:619–625.) Key words: lung fluid • chloride secretion • alveolar type II cell

Introduction

Secretion of fluid by the fetal pulmonary epithelium is thought to play a role in normal lung development (1–3). Studies in fetal lambs have shown that lung fluid transport is coupled to chloride (Cl) secretion and is hormonally regulated, although the cellular mechanisms for fluid secretion are not well identified (2, 3). There is also evidence that regulated fluid transport is linked to Cl secretion in human fetal lung. In cultured first trimester human fetal lung tissue explants, the transepithelial potential of the pulmonary epithelium hyperpolarized in response to a membrane permeant analogue of cAMP (CPT-cAMP), and this effect was inhibited by bumetanide (4). Likewise, cAMP analogues stimulated an increase in lung fluid secretion in first and second trimester lung tissue explants as assessed by an increase in explant luminal area (4, 5).

The cystic fibrosis gene product, cystic fibrosis transmembrane conductance regulator (CFTR),[1] is a membrane-associated protein present in many epithelial tissues that transport fluid (6–8). Recent studies have shown that CFTR can function as a Cl channel activated by protein kinase A phosphorylation and nucleoside triphosphates (9, 10); mutations in CFTR cause cystic fibrosis (CF) (11, 12). Some complications of CF, including meconium ileus and pancreatic duct obstruction, occur before birth (13–15), suggesting the defective gene product is expressed in epithelia of these tissues prenatally. Therefore, the developmental regulation of CFTR gene expression in the human may bear directly on the timing of the disease onset. Expression of CFTR mRNA in human fetal lung, pancreas, cultured pancreatic duct epithelial cells, male genital ducts, and intestine has been reported (16). We have previously identified CFTR mRNA in human fetal lung by polymerase chain reaction amplification of reverse-transcribed total RNA using specific primers (17). In addition, CFTR protein was detected in homogenates of human fetal lung tissue explants by immunoprecipitation with a monoclonal antibody directed against CFTR (17). In contrast to normal fetal lung tissue explants, cultured tissue from a fetus homozygous for the most common CFTR mutation, ΔF508, failed to show a significant change in either transepithelial potential or fluid secretion in response to CPT-cAMP, suggesting that there is a physiologic defect in cAMP-mediated fluid secretion in the CF lung prenatally (17). Together, these findings confirmed that CFTR is expressed prenatally in human lung tissue and suggested that it may be involved in fetal lung fluid transport, but the cellular localization of CFTR was not identified.

We were interested in further understanding the developmental expression of the CF gene in human fetal lung. To learn how CFTR may affect fluid transport in the developing lung, it is important to know when the gene is expressed during development, and where it is expressed. To this end, we localized CFTR mRNA in human fetal lung tissues and in cultured human fetal lung explants using in situ hybridization.

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1. *Abbreviations used in this paper:* CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; CPT-cAMP, membrane permeant analogue of cAMP.

Methods

Fetal lung samples. Human fetal lung tissue was obtained from first and second trimester abortuses as described previously (4). The study was approved by the Institutional Review Board of the University of Iowa. Both fresh lung tissue and cultured lung tissue explants were examined. The lung tissues were snap frozen in liquid nitrogen and stored at −70°C until used.

Explant culture. Second trimester human fetal lung tissue was rinsed several times with sterile culture medium, Waymouth's MB 752/1, that contained 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone. Major airways and blood vessels were dissected from the lung and the peripheral rim of parenchymal tissue (~ 5 mm in thickness) was dissected from the specimens. Distal lung tissue pieces were then minced with a sterile razor blade into 1–2-mm explants. Five tissue pieces were distributed evenly on a 1-cm^2 piece of lens paper supported by a stainless steel grid in a 35-mm plastic petri dish. The dish contained 1 ml of serum-free Waymouth's MB 752/1 medium. Explants were incubated for 5 d in an incubator at 37°C, in an atmosphere of 5% CO_2 in air. Tissue culture medium was changed daily. Explants were harvested after 5 d in culture, quick frozen in liquid nitrogen, and stored at −70°C for in situ hybridization.

In situ hybridization. In situ hybridization for the localization of CFTR mRNA was performed using methods described previously (18). The CFTR cDNA probe 10-1, which encodes exons 1 to 6 of CFTR mRNA (7), was obtained from the American Type Culture Collection, Rockville, MD. The Bluescript SK vector containing the CFTR cDNA insert was purified, then linearized with ClaII and NotI and used to synthesize the sense and anti-sense CFTR RNA transcripts, respectively. RNA transcripts were synthesized using [^{3}H]CTP (4.82 × 10^7 dpm/μg) and [^{3}H]UTP (5.83 × 10^7 dpm/μg, both from New England Nuclear, Boston, MA). RNA synthesis was performed using an RNA transcription kit (Gemini Riboprobe System II; Promega Corp., Madison, WI), 1 μg of digested plasmid DNA, and the appropriate RNA polymerase. The radiolabeled cRNA probes were subsequently hydrolyzed to ~ 200-bp fragments as described by Cox et al. (19).

6-μm thick frozen sections of the fetal lung tissue were prepared, then mounted on *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES)-coated glass slides. Sections were fixed for 20 min in freshly prepared paraformaldehyde (4%, wt/vol, pH 7.4), rinsed three times in PBS, pH 7.4, and dehydrated through a graded ethanol series. The sections were then digested in Pronase E (0.125 mg/ml; Sigma Chemical Co., St. Louis, MO) in Tris-HCl (50 mM, pH 7.5), EDTA (5 mM) buffer. The slides were rinsed in PBS, pH 7.4, followed by a brief immersion in triethanolamine buffer (TEA, 0.1 M, pH 8.0), incubated for 10 min in TEA buffer that contained acetic acid (0.25%, vol/vol) and rinsed in 2× standard saline citrate (SSC) before being dehydrated through an ethanol series.

The sections were hybridized overnight at 50°C in 35 μl hybridization buffer (NaCl, 300 mM; Tris-HCl, 10 mM, pH 8.0; EDTA, 1 mM; formamide, 50% vol/vol; 1× Denhardt's solution; dextran sulfate, 10%, wt/vol; and yeast tRNA, 0.28 mg/ml) which contained the appropriate ^{3}H-CFTR cRNA probe. The hybridization buffer contained 4.6 × 10^3 cpm/μl. After hybridization, the slides were rinsed in 4× SSC, then incubated for 30 min at 37°C in Tris-HCl (10 mM, pH 8.0), EDTA (1 mM), NaCl (0.5 M) buffer that contained RNase A (20.0 μg/ml) and RNase T1 (3.0 U/ml). After dehydration, the slides were coated with NTB-2 liquid emulsion (Kodak, Rochester, NY) diluted 1:1 with distilled water, sealed in a black slide box containing desiccant, and exposed for up to 16 wk at 4°C. The slides were immersed in D-19 developer (Kodak) for 3 min, rinsed in distilled water, immersed in Rapid Fixer (Kodak) diluted 1:1 with distilled water for 3 min, air dried, and rinsed in tap water for 5 min. The slides were then briefly stained in hematoxylin, dehydrated, mounted, and examined by two investigators. Samples from two first trimester lungs, five second trimester lungs, and five cultured explants were examined in this study.

In situ hybridization results were quantitated in second trimester tissue and cultured explants by counting the number of grains per cell. Two observers counted 60–100 cells/anatomic region in a sample of tissue containing bronchial, bronchiolar, and distal lung tissue. Results from the grain counts were pooled for statistical analysis by analysis of variance or unpaired *t* test.

Results

In situ hybridization of CFTR mRNA in first trimester lung tissue. CFTR mRNA hybridization was clearly visualized in first trimester lung tissue with the ^{3}H-anti-sense CFTR cRNA probe (Fig. 1). The anti-sense CFTR riboprobe hybridized to mRNA in the epithelial cells of the developing airways, while no hybridization was seen in the mesenchymal tissue. Epithelial cells of bronchioles and prealveolar tubules all expressed CFTR mRNA (Fig. 1, *A* and *B*). While CFTR mRNA was present throughout the pulmonary epithelium, there was a gradient of intensity of CFTR mRNA hybridization. The anti-sense CFTR cRNA probe hybridized more intensely to the epithelial cells of the bronchioles and less intensely to the epithelium of the prealveolar tubules. CFTR mRNA hybridization in the fetal lung was of low intensity, requiring 12–14 wk exposure for visualization. No hybridization was seen with the control ^{3}H-sense CFTR cRNA probe (Fig. 1, *C* and *D*).

In situ hybridization of CFTR mRNA in second trimester lung tissue. Human fetal lung tissues of ~ 20 wk gestational age were also studied with the ^{3}H-anti-sense cRNA probe (Figs. 2 and 3). Similar to the first trimester tissue, hybridization was evident throughout the pulmonary epithelium. The ^{3}H-anti-sense CFTR cRNA probe hybridized most strongly to mRNA in the epithelium of the large, cartilage-containing bronchi (Fig. 2, *A* and *B*). Again, there was a gradation of CFTR mRNA expression, with the large conducting airway epithelium expressing message at a greater level than the small bronchioles or epithelial cells of prealveolar tubules (Fig. 2, *C* and *D*). This gradation of CFTR mRNA expression was quantitated by counting the number of grains per cell. Grain counts in the sample shown in Fig. 2 revealed that bronchial epithelial cells contained 16.0±0.95 grains/cell, bronchiolar cells 13.3±0.7 grains/cell, and epithelial cells of the prealveolar tubules contained 9.6±0.6 grains/cell (mean±SEM, $P < 0.05$ by analysis of variance). There was a patchy distribution of mRNA expression in the bronchial epithelial cells of the second trimester lung (Fig. 3, *A* and *B*). Not all epithelial cells showed hybridization to the anti-sense cRNA probe, suggesting there may be cell to cell variation in the expression of CFTR mRNA.

The intensity of hybridization in the epithelia of prealveolar tubules was the least intense, but was nonetheless clearly evident (Fig. 2, *E* and *F*). While there are no alveolar type II cells present at this stage of lung development, this finding suggests that type II cell precursors express CFTR. When second trimester tissue was incubated with the ^{3}H-sense CFTR riboprobe, no hybridization was observed (Fig. 2, *G* and *H*).

In situ hybridization of CFTR mRNA in cultured second trimester lung explants. We also looked for CFTR mRNA in cultured second trimester lung tissue explants. The intensity of CFTR mRNA hybridization in the cultured lung tissue explants was markedly greater than in the starting tissue (Fig. 3). The epithelial cells in the cultured explants expressed relatively abundant CFTR message (Fig. 3, *C* and *D*). This appeared to represent an increase in CFTR mRNA expression in the cultured explants since the ^{3}H-anti-sense cRNA probe showed

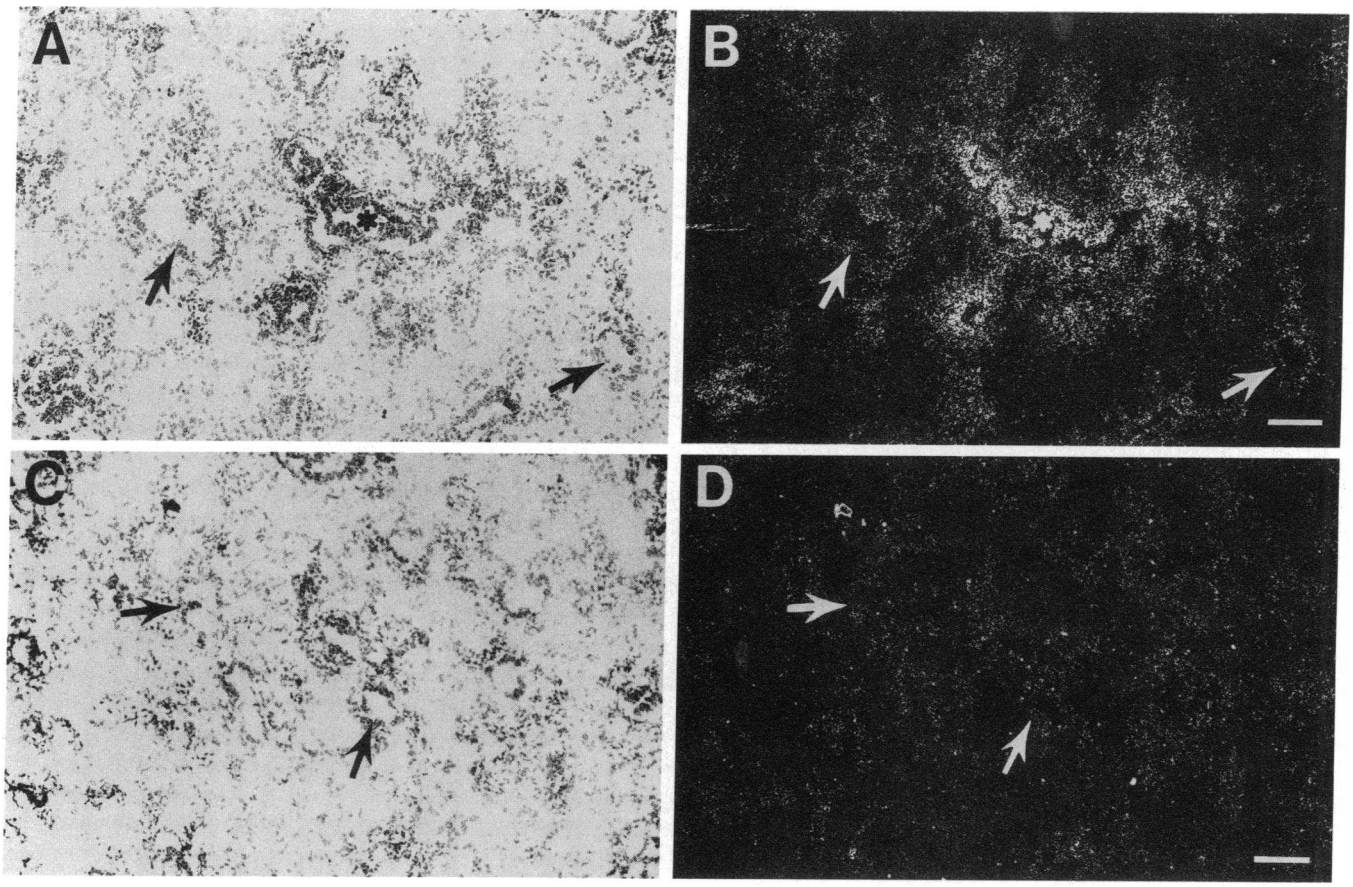


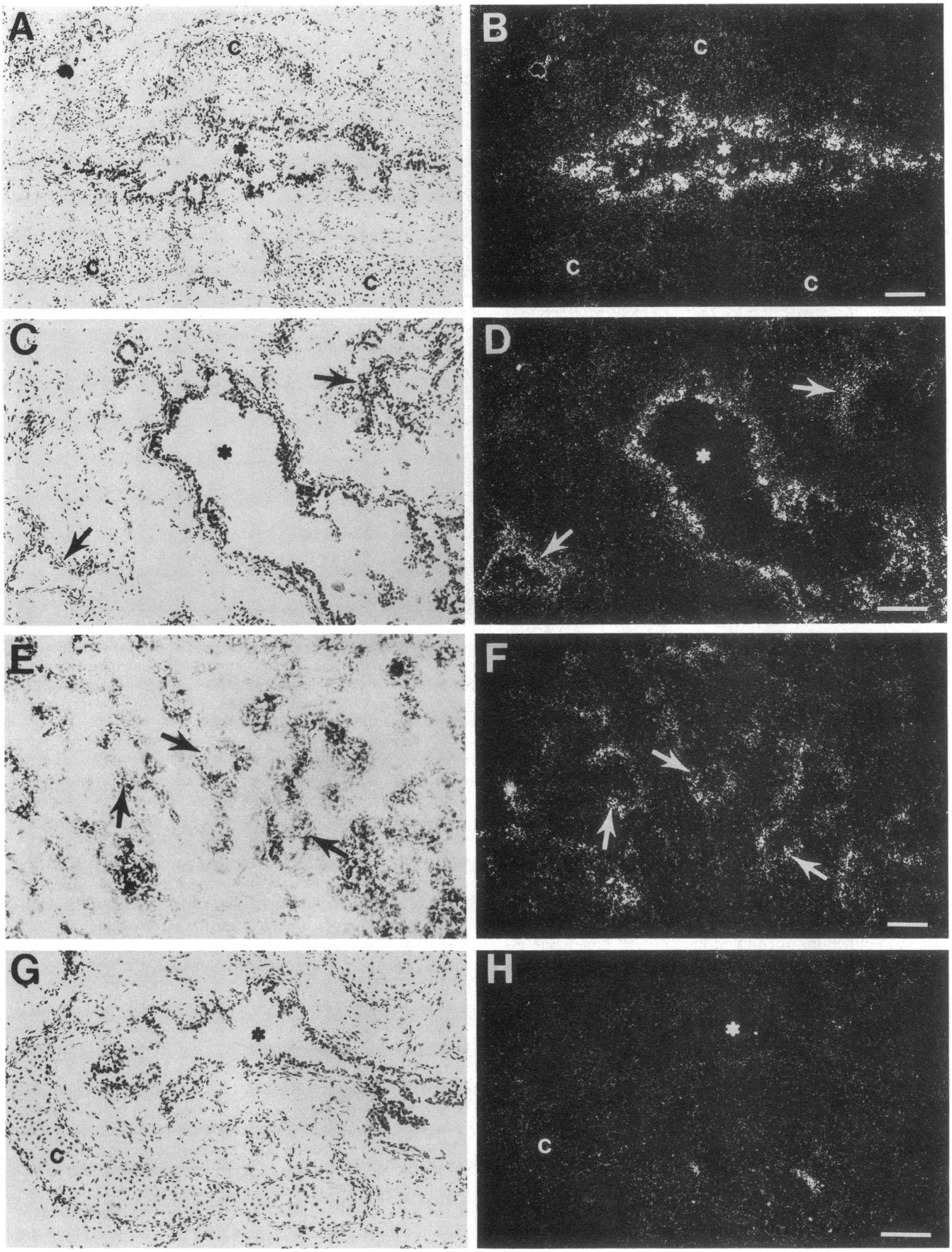
Figure 1. Localization of CFTR mRNA in first trimester human fetal lung (11.5-wk gestation). In situ hybridization with sense and anti-sense ^{3}H-CFTR cRNA probes was performed as described in Methods. Exposure time was 16 wk. (*A* and *B*) Light- and dark-field photomicrographs of lung tissue demonstrating hybridization of the anti-sense ^{3}H-CFTR cRNA probe to the epithelium of bronchioles and prealveolar tubules. (*C* and *D*) Light- and dark-field photomicrographs showing no significant hybridization of the control sense ^{3}H-CFTR cRNA probe. Arrows denote epithelium of prealveolar tubules. The asterisk denotes the lumen of a bronchiole. Bar = 100 μm.

relatively intense hybridization after an exposure time as short as 4 wk. In contrast, 20-wk gestation lung tissue that had not been in explant culture required a 13-wk exposure time (Fig. 2, *E* and *F*). Grain counts in the sample shown in Fig. 2, *E* and *F*, and Fig. 3, *C* and *D*, revealed that the epithelial cells of the prealveolar tubules in the uncultured tissue contained 9.8±0.6 grains/cell while the cultured explant epithelial cells contained 24.0±1.2 grains/cell (mean±SEM, $P < 0.001$ by unpaired t test). The epithelium in cultured human fetal lung tissue explants has previously been shown to be composed almost entirely of lamellar body–containing, alveolar type II cells (20). Based on this observation, we conclude that the fetal alveolar type II cell expressed CFTR mRNA. There was no hybridization of the ^{3}H-sense CFTR cRNA probe to the explant tissue (data not shown).

Discussion

Using in situ hybridization we have localized CFTR mRNA to the epithelium of human fetal lung tissues. CFTR mRNA was present in a concentration gradient, with the level of expression diminishing from bronchi to bronchioles to the prealveolar tubules. Not all epithelial cells within a given region expressed CFTR mRNA at the same intensity, suggesting there may be differences in CFTR mRNA expression between neighboring cells as well as regional differences in expression. Hybridization to the lung mesenchymal tissue was not observed in this study. This contrasts with a recent report of CFTR mRNA localization in adult rat lung in which the investigators reported a broad hybridization signal in the lung parenchyma beneath the epithelial cells (21). Crystal and colleagues have also reported that cultured nonepithelial cells express CFTR mRNA, although the levels of expression may be several-fold lower than that seen in epithelial cells (22). In situ hybridization suggests that CFTR mRNA expression is confined to the epithelial cells of the human fetal lung.

CFTR mRNA was expressed in the epithelium of the distal lung as well as the conducting airway epithelium. A surprising finding was the increased intensity of CFTR mRNA expression in the epithelial cells of cultured 20-wk gestation lung tissue explants. In explants cultured for 5 d, CFTR mRNA hybridization grain counts were ~ 2.5 fold greater in comparison to 20-wk gestation tissue that was not cultured, suggesting that CFTR mRNA expression increased during the time in explant culture. It has previously been demonstrated in cultured human fetal lung explants that gene expression of surfactant-associated proteins SP-A, SP-B, and SP-C increases with time in explant culture (23, 24). Human fetal lung tissue explants are known to undergo spontaneous differentiation when maintained in culture in serum-free media and the majority of epi-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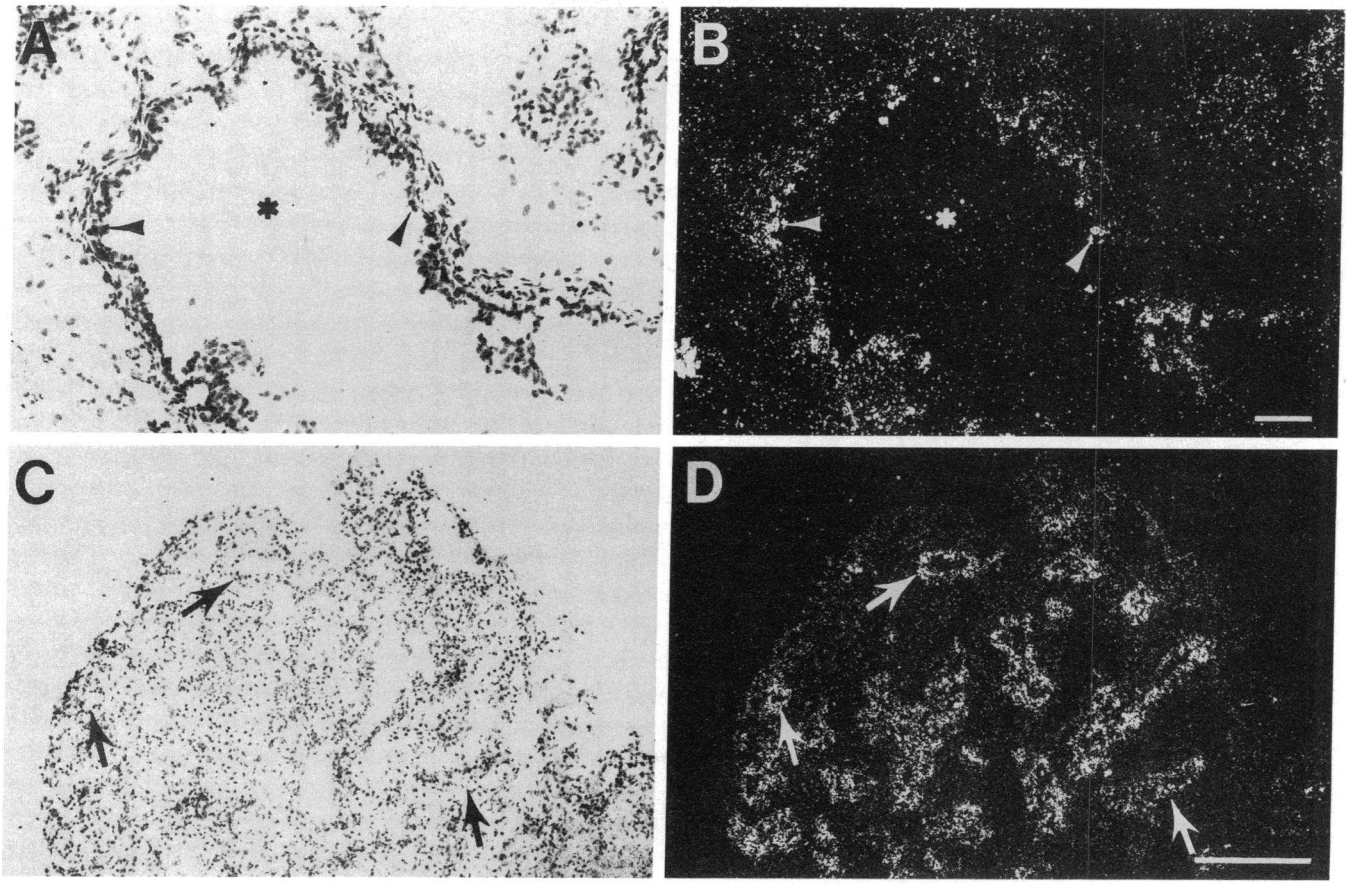

Figure 3. Localization of CFTR mRNA in second trimester human fetal lung and cultured lung tissue explants. (*A* and *B*) Higher magnification view of airway epithelium of 20-wk gestation human fetal lung tissue, light- and dark-field views, after hybridization with the anti-sense ^{3}H-CFTR cRNA probe. The hybridization signal is localized over the epithelial cells and not all cells express the signal to the same degree. An asterisk denotes lumen of prealveolar tubule. Arrowheads indicate cells with more intense hybridization. (*C* and *D*) Light- and dark-field photomicrographs of cultured 20-wk gestation human fetal lung tissue explants in situ hybridization with anti-sense ^{3}H-CFTR cRNA probe. A strong hybridization signal is seen over the epithelial cells in the explants which consist almost entirely of alveolar type II cells. This level of hybridization is more intense than that seen in uncultured 20-wk gestation tissue (Fig. 2, *E* and *F*). Exposure time was 16 wk. Arrows denote epithelium of the prealveolar tubules which is composed primarily of alveolar type II cells. Bars = 100 μm.

thelial cells in the explants develop lamellar bodies with a phospholipid composition of the surfactant produced by the human fetal lung at 36 wk of gestation (20). These findings are consistent with CFTR mRNA expression in alveolar type II cell precursors and the more differentiated, lamellar body–containing alveolar type II epithelial cell.

The presence of CFTR mRNA in human fetal lung alveolar epithelial cells raises questions regarding the role these cells play in fetal lung fluid transport. In the fetal lung, it has been hypothesized that the distal lung units, including the developing alveolar epithelium, are the most likely origin of the majority of the secreted lung fluid (2, 3). The ion transport properties of fetal and adult alveolar epithelium has been investigated in cultured cells. Monolayers of 18-d gestation fetal rat distal pulmonary cells demonstrated a bumetanide-sensitive short-circuit current, consistent with Cl secretion by developing fetal alveolar cells (25). In another report, monolayers of later gestation fetal rat alveolar epithelial cells exhibited an amiloride-sensitive, bumetanide-insensitive short-circuit current, suggesting sodium absorption; these cells may be involved in neonatal pulmonary fluid clearance (26). Alveolar type II cells cultured from adult rat lungs are primarily sodium absorptive, and may be important for alveolar fluid reabsorption (27). Alveolar type II epithelial cells also express the enzyme sodium-potassium-ATPase, consistent with the notion that they are involved in electrolyte transport (28). CFTR mRNA expres-

Figure 2. Localization of CFTR mRNA in 20-wk gestation human fetal lung tissue. In situ hybridization with sense and anti-sense ^{3}H-CFTR cRNA probes was performed as described in Methods. (*A* and *B*) Light- and dark-field photomicrographs of a bronchus showing anti-sense ^{3}H-CFTR cRNA hybridization to the epithelial cells. (*C* and *D*) Light- and dark-field views of a bronchiole and prealveolar tubules showing anti-sense ^{3}H-CFTR cRNA hybridization to the bronchiolar epithelium and, in smaller amounts, to the epithelium of the prealveolar tubules. (*E* and *F*) Light- and dark-field views of distal lung parenchyma showing a lower level of hybridization with the anti-sense ^{3}H-CFTR cRNA probe to the epithelial cells of the prealveolar tubules. (*G* and *H*) Light- and dark-field views of the area depicted in panels *A* and *B* showing no hybridization of the sense ^{3}H-CFTR cRNA probe to the tissue. The sections were exposed for 13 wk. The asterisk indicates the lumen of a bronchus; C signifies cartilage in the wall of a bronchus; an arrow denotes the epithelium in a prealveolar tubule. Bar = 100 μm.

sion in human fetal alveolar cells suggests that the alveolar epithelium is Cl-secretory. Since CFTR mRNA is expressed in lamellar body–containing human fetal alveolar type II cells, it is possible that the alveolar type II cell is involved in the secretion and maintenance of the alveolar subphase postnatally. This 1-μm thick fluid layer is important for the function of surfactant and must be secreted, and presumably reabsorbed, to maintain an optimal functional thickness (29, 30).

The function of CFTR in human fetal lung tissue is unclear. Patch clamp analysis has demonstrated that CFTR has ion transport characteristics of a Cl channel which is activated by protein kinase A and nucleoside triphosphates (9, 10). Since the CFTR gene is expressed in the fetal pulmonary epithelium, it may be involved in a cAMP-dependent, Cl-secretory process that generates fetal lung fluid transport. The finding of higher levels of expression in the bronchial epithelium when compared to the distal lung suggests that CFTR-mediated Cl transport may be more abundant in the proximal lung. We do not yet know if CFTR mRNA expression correlates directly with CFTR protein expression or Cl secretion in fetal lung. In one recent study, steady-state levels of CFTR mRNA were unchanged despite progressive loss of cAMP-induced Cl secretion in cultures of the human intestinal cell line CaCo-2 (31), suggesting that mRNA expression does not directly correlate with Cl secretion in this cell line. On the other hand, there is evidence that cAMP stimulates Cl secretion and fluid transport in human fetal lung explants and in developing fetal rat alveolar epithelial cells, suggesting CFTR is involved in this secretory process (4, 32).

Since CFTR mRNA is expressed in the fetal pulmonary epithelium, it is possible that mutations in CFTR could lead to lung disease prenatally. Studies of tissues from fetuses and infants with CF have documented anatomical abnormalities in the pancreas (33), intestine (15), and vas deferens (34), but the results of examination of the lungs have been controversial. Morphological studies have described anatomically normal lungs in neonates and infants with CF and, in general, the earliest anatomic abnormalities observed in the lung consist of mucus obstruction of bronchioles followed by inflammatory changes in the bronchiolar epithelium and mucous gland hyperplasia, usually appearing after the neonatal period (35, 36). One report described the deposition of periodic acid Schiff–positive granules in tracheal submucosal glands along with dilatation of these glands in CF-affected fetuses, although the significance of this finding is unknown (14). We recently reported that the transepithelial potential in cultured lung explants from a human fetus homozygous for the ΔF508 CFTR mutation failed to hyperpolarize in response to CPT-cAMP, the response seen in normal tissues of the same gestation (17). Furthermore, the CF explants did not show evidence of fluid secretion in response to CPT-cAMP, another response observed in normal tissues. In spite of the lack of responsiveness to cAMP, the CF fetal lung tissue had a normal morphology (17). Since the CF fetal lung expresses CFTR and there is no conclusive evidence of abnormal lung development in CF, it is unlikely that functional CFTR is required for normal lung development. Alternatively, it is possible that the importance of fluid secretion in normal lung development has been overemphasized. The localization of CFTR mRNA in the human fetal pulmonary epithelium suggests that CFTR represents one route for Cl secretion in the fetal lung. Other, non–cAMP-dependent pathways may also exist, allowing for the CF fetal lung to compensate for defective cAMP-mediated fluid transport.

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