Antibodies directed against the major apoprotein of rabbit lung surfactant, a 29-36-kDa glycoprotein, were used to study changes in the levels of translatable surfactant apoprotein mRNA in rabbit lung tissue during development, as well as the effects of cortisol and cyclic AMP analogues on the levels of surfactant apoprotein and its mRNA in fetal rabbit lung tissue in organ culture. The major surfactant apoprotein and its mRNA were undetectable in lung tissues of 21-day gestational age fetal rabbits. Translatable mRNAs specific for the major surfactant apoprotein was first detectable in lung tissues of 26-day fetuses, increased 25-fold on day 28, reached peak levels at day 31, and declined after birth. Incubation of 21-day fetal rabbit lung explants with cortisol in serum-free medium resulted in an increase in the specific content of the 29-36-kDa apoprotein. Cyclic AMP analogues and forskolin, an activator of adenylate cyclase, increased surfactant apoprotein accumulation were associated with comparable changes in the levels of translatable surfactant apoprotein mRNA. When fetal lung explants were incubated with cortisol and dibutyryl cyclic AMP in combination, the major surfactant apoprotein (1) increased to levels greater than that of explants treated with either cortisol or dibutyryl cyclic AMP alone. These effects of dibutyryl cyclic AMP and cortisol on surfactant apoprotein accumulation were associated with comparable changes in the levels of translatable surfactant apoprotein mRNA. Thus, we have shown for the first time that the induction of pulmonary surfactant apoprotein synthesis during differentiation in vitro and in vivo is associated with an increase in the level of translatable mRNA and that cortisol and cyclic AMP increase both the accumulation of the major surfactant apoprotein and the corresponding mRNA in fetal rabbit lung tissue in vitro.

The synthesis and secretion of pulmonary surfactant is initiated in fetal lung tissue during the latter stages of gestation. Surfactant is a lipoprotein that is synthesized in the type II pneumocyte where it is stored as lamellar bodies. After birth, the secreted surfactant lines the alveolus and reduces surface tension at the alveolar-air interface. Approximately 80-85% by weight of surfactant is glycerophospholipid; the major glycerophospholipid of surfactant is dipalmitoyl phosphatidylcholine. Approximately 5-10% of surfactant is protein (1).

The functions of the apoproteins of surfactant remain uncertain. The proteins may be enzymes involved in glycerophospholipid synthesis and/or degradation; phosphatidate phosphohydrolase (2), phospholipase A₂ (3), and lyssolecithin acyltransferase (4) activities, as well as the activities of a number of lysosomal enzymes (3), have been found to be associated with purified lamellar body fractions. The proteins may also serve a structural role in the packaging of surfactant glycerophospholipids within the type II cell and/or in the formation of tubular myelin within the alveolar lumen (5). It has also been suggested that the proteins may alter the physicochemical properties of surfactant by enhancing the rate of diffusion of surfactant glycerophospholipids over the alveolar surface (6). By use of two-dimensional PAGE, we have identified at least 8 proteins that are associated with surfactant purified from lavage of adult rabbit lungs; four of these are glycoproteins with acidic isoelectric points (pI ≤ 5.6) (7). The major surfactant-associated protein of rabbit lung lavage is a 29-36 kDa sialic acid-containing glycoprotein (7). The apoproteins of human (8-10) and rat (11, 12) surfactant also are sialoglycoproteins with molecular weights similar to those of rabbit lung. The association of a sialoglycoprotein with an organelle comprised almost entirely of phospholipid is suggestive of its amphipathic nature and, therefore, its possible role in surfactant structure and function. Recently, the amino acid sequences of the human (13) and canine (14) surfactant apoproteins have been determined by analysis of the nucleotide sequences of genomic and complementary DNA clones, respectively. It was found that the protein is comprised of both collagen-like and globular domains. The collagen-like sequences are in the amino-terminal third of the molecule, while the carboxyl-terminal half of the protein is enriched in aromatic amino acids.

Glucocorticoids are considered to serve an important regulatory role in the synthesis of surfactant glyceroophospholipids by fetal lung tissue (cf. Ref. 15). We have found that cortisol increases by 3-6-fold the rate of synthesis of lamellar body phosphatidylincholine in fetal rabbit lung tissue explants (16). Cortisol also promotes the synthesis of "mature" surfactant, i.e. lamellar bodies enriched in phosphatidylglycerol and depleted of phosphatidylinositol (16). The binding of glucocorticoid receptors in fetal lung type II cells (17) is suggestive of a direct action of these steroids in type II cell function.
Cyclic AMP analogues (18–20) and phoshodiesterase in-hibitors (18, 21–23) also have been reported to increase sur-factant glycerophospholipid synthesis in fetal lung tissue. Adeynylate cyclase activity is detectable in fetal rabbit lung (24) and has appeared to be increasingly responsive to epinephrine with advancing gestational age. β-Adrenergic re-ceptors have been identified as early as day 22 of gestation (25); an increase in receptor concentration was observed in fetal rabbit lung tissue with increasing gestational age (25, 26). In studies using fetal rat lung in organ culture, Gross and Wilson (27) found that dexamethasone and cyclic AMP act synergistically to increase phosphatidylcholine synthesis.

In consideration of the potential importance of surfactant-associated proteins in the biogenesis and structural organi-zation of the lamellar body within the type II cell, as well as the possible role of these proteins in surfactant function, it was an objective of the present study to utilize antibodies specific for the major apoprotein of rabbit lung surfactant to investigate developmental changes in the levels of translatable mRNA specific for this protein and the effect of glucocorti-coids and analogues of cyclic AMP on the synthesis of this protein in fetal rabbit lung tissue maintained in vitro.

MATERIALS AND METHODS

Antibody Preparation—Antibodies directed against the major apoprotein of rabbit lung surfactant, a 29–36-kDa glycoprotein (G), were raised in a goat after immunization with the protein excised from unstained two-dimensional polyacrylamide gels according to previously described methods (28). By use of immunoblot analysis we found that an IgG fraction prepared from the serum of the immunized goat contained antibodies against the major surfactant apoprotein as well as against the other glycoproteins of rabbit surfactant (55–70, 26). In studies using fetal rat lung in organ culture, Gross and Jackson (37) using a commercially available rabbit reticulocyte lysate translation system (New England Nuclear) with [3H]methionine as the radiolabeled amino acid. The incorporation of radioactivity into protein was determined by assay of trichloroacetic acid-precipitable radioactivity. An aliquot of total translation products was subjected to SDS-PAGE and autoradiography. The major surfactant apoprotein was immunoscreened from equivalent amounts of trichloroacetic acid-precipitable radioactivity using the anti-rabbit surfactant apoprotein and Staphylococcus aureus cell membranes (Pansorbin, Behring Diagnostics) as a source of Protein A (38). The immunosolubilates were analyzed by SDS-PAGE and autoradiography. Changes in the relative amounts of immunoreactive surfactant apoprotein on autoradiograms of immunoblots or immunosolubilates were assessed by scanning of the autoradiograms using a Transidyne scanning densitometer.

RESULTS

Effect of Cortisol on the Specific Content of Immunoreactive 29–36-kDa Surfactant Apoprotein in Fetal Rabbit Lung Explants—The specific content of the major surfactant apoprotein in fetal lung explants maintained in organ culture for up to 5 days in the absence or presence of cortisol (10−7 M) was assessed by immunoblot analysis. The results of a typical experiment are shown in Fig. 1. Immunoreactive surfactant apoprotein was undetectable in homogenates of 21-day fetal lung tissue before culture (data not shown) or in explants incubated for 1 day in medium with or without cortisol. After 3 days of incubation, however, surfactant apoprotein was detectable in homogenates of control tissue and was increased about 5-fold in cortisol-treated explants. After 5 days of incubation, a further increase in the specific content of the apoprotein was observed in control tissues; in cortisol-treated explants the apoprotein was increased about 2-fold as compared to control. The effect of cortisol on the incorporation of [3H]choline into phosphatidylcholine of tissue homogenates was similar to that previously reported (16). No stimulatory effect of cortisol was detected on day 1; however, an increase in phosphatidylcholine synthesis was observed in cortisol-treated explants as compared to controls after 3 and 5 days of incubation.

The effect of cortisol on surfactant apoprotein accumulation was dose dependent. Lung explants from 21-day fetal rabbits were incubated in the absence or presence of cortisol (10−12–10−8 M) for 3 days and analyzed for surfactant appro-
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**Fig. 1.** Changes in the specific content of immunoreactive surfactant apoprotein in 21-day fetal rabbit lung tissue maintained in organ culture for 1, 3, or 5 days in the absence or presence of cortisol (10^{-7} M). Proteins (30 μg) of 600 x g supernatant fractions of homogenates of the fetal lung explants were separated by SDS-PAGE and analyzed for the major surfactant apoprotein by immunoblotting as described under "Materials and Methods." This is an autoradiogram of the immunoblot.

**Fig. 2.** Effect of cortisol in concentrations of 10^{-10}-10^{-5} M on the specific content of the major surfactant apoprotein in fetal rabbit lung explants. Lung explants from 21-day fetal rabbits were maintained in organ culture for 3 days in the absence or presence of cortisol (10^{-10}-10^{-5} M). Proteins (30 μg) of 600 x g supernatant fractions of homogenates of the fetal lung explants were analyzed for surfactant apoprotein content by immunoblotting. This is an autoradiogram of the immunoblot.

**Fig. 3.** In vitro translation of RNA isolated from lung tissues of 19-31-day gestational age fetal, 1-day neonatal (N), and adult (Ad) rabbits; immunolocalization of surfactant apoprotein. Equal amounts of RNA from each age group were translated in the presence of [35S]methionine using a rabbit reticulocyte lysate system. Surfactant apoprotein was immunolocalized from equal amounts of trichloroacetic acid-precipitable radioactivity and analyzed by SDS-PAGE and autoradiography as described under "Materials and Methods." This is an autoradiogram from such an experiment.

**Fig. 4.** Immunolocalization of radiolabeled surfactant apoprotein in the absence or presence of an excess of nonradiolabeled 29-36-kDa surfactant apoprotein or total surfactant apoproteins. Surfactant apoprotein was immunolocalized from in vitro translation assays using RNA isolated from 21-day fetal rabbit lung tissue explants after 6 days of organ culture in control medium. The immunolocalizations were conducted in the absence (lane A) or in the presence of nonradiolabeled 29-36-kDa surfactant apoprotein (lane B) or total delipidated surfactant proteins (lane C) and analyzed by SDS-PAGE and autoradiography.

Changes in the Levels of Translatable mRNA Specific for the Major Surfactant Apoprotein during Rabbit Lung Development—Equal amounts of RNA from lung tissues of 19-31-day gestational age fetal, 1-day neonatal, and adult rabbits were translated in vitro using a rabbit reticulocyte lysate system with [35S]methionine as the radiolabeled amino acid. The incorporation of [35S]methionine into trichloroacetic acid-precipitable radioactivity per μg of RNA did not change significantly as a function of gestational age. The major surfactant apoprotein was immunolocalized and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3, a faint radiolabeled band of 31 kDa was first detectable in the immunolocalize of the translation assay using RNA from 26-day fetal rabbit lung. A 25-fold increase in the concentration of translatable mRNA specific for this protein was observed in 28-day fetal lung tissue, and peak levels (40-fold greater than the levels in 26-day fetal rabbits) were reached at day 31. In lung tissues of day 1 neonates and adult rabbits the level of translatable surfactant apoprotein mRNA was reduced as compared to that observed in the 31-day gestational age fetal lung tissue.

To provide evidence that the 31-kDa protein was the primary translation product of the major surfactant apoprotein, immunolocalizations of translation assays were conducted in the presence of either the 29-36-kDa surfactant apoprotein eluted from unstained two-dimensional polyacrylamide gels or delipidated total surfactant proteins (Fig. 4). In the absence of added protein the immunolocalize consisted of a protein band of 31 kDa (lane A). When immunolocalizations were conducted in the presence of the 29-36-kDa surfactant apoprotein (lane B) or total surfactant apoprotein (lane C), no radiolabeled band was observed.
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FIG. 5. In vitro translation of RNA isolated from 21-day fetal rabbit lung tissue before culture and after 1–3 days of organ culture in the absence or presence of cortisol (10^{-7} M); immunoprecipitation of the major surfactant apoprotein. RNA was isolated from the fetal lung tissue and translated in the presence of ^{35}S)methionine in a reticulocyte lysate system. The major surfactant apoprotein was immunoprecipitated from the translation mixtures and analyzed by SDS-PAGE and autoradiography as described under "Materials and Methods." This is an autoradiogram of a representative experiment.

FIG. 6. Effect of cortisol and Bt$_2$cAMP on the accumulation of immunoreactive surfactant apoprotein in fetal rabbit lung explants. Lung explants from 21-day rabbits were incubated for 3 days in control medium (C) or in medium containing cortisol (F, 10^{-7} M), Bt$_2$cAMP (Bt$_2$, 1 mM), or Bt$_2$cAMP + cortisol. The amount of immunoreactive surfactant apoprotein was analyzed by immunoblotting. This is an autoradiogram of an immunoblot from a typical experiment.

Effect of Cortisol on the Translatability of mRNA Isolated from Fetal Rabbit Lung Explants—RNA was isolated from 21-day fetal rabbit lung tissue before culture and after incubation for 1–3 days in the absence or presence of cortisol. The RNA was translated in vitro, and newly translated surfactant apoprotein was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The autoradiogram from such an experiment is shown in Fig. 5. No translatable RNA specific for the major surfactant apoprotein was detectable in the 21-day fetal lung tissue before culture. After 1 day of organ culture, a radiolabeled band of ~31 kDa was detectable in the immunoprecipitate from in vitro translations using RNA from control tissue; a further increase in the amount of translatable surfactant apoprotein mRNA was observed in control explants after 2 and 3 days of organ culture. In cortisol-treated explants, the level of translatable surfactant apoprotein mRNA was increased ~2.5-fold as compared to controls on day 1 and ~1.6- and 1.5-fold over that of controls on days 2 and 3, respectively. In this experiment, the induction of surfactant apoprotein mRNA by cortisol on days 2 and 3 was relatively modest. Although there appears to be a discrepancy between the -fold induction by cortisol of the level of translatable mRNA (Fig. 5) and immunoreactive surfactant apoprotein (Fig. 1), it should be noted that these data were obtained from two separate experiments. In three other experiments wherein the levels of immunoreactive apoprotein and mRNA were determined using the same tissue samples, it was found that the -fold induction by cortisol of mRNA and apoprotein were similar (3.9 ± 0.7-fold induction of apoprotein and 3.6 ± 1.9-fold induction of mRNA).

Effect of Cyclic AMP Analogues on the Levels of Surfactant Apoprotein and Its mRNA in Fetal Lung in Vitro—To evaluate...
the effects of cyclic AMP on surfactant apoprotein synthesis, fetal lung explants were incubated for 3 days in control medium or in medium that contained Bt2cAMP (1 mM), cortisol (10^{-7} M), or Bt2cAMP plus cortisol; the specific content of surfactant apoprotein was assessed by immunoblot analysis, and surfactant apoprotein mRNA levels were assayed by immunoprecipitation of surfactant apoprotein from in vitro translation of RNA isolated from control and treated lung explants. In explants incubated for 3 days in the presence of Bt2cAMP there was a 5-fold increase in the accumulation of surfactant apoprotein as compared to control (Fig. 6). We consistently observed that the stimulatory effect of Bt2cAMP was greater than that of cortisol at this time point. When explants were incubated with cortisol plus Bt2cAMP, the level of immunoreactive surfactant apoprotein was greater than that of explants incubated with either stimulatory factor alone and was increased ~10-fold as compared to control (Fig. 6).

RNA was isolated from the same set of tissues as those used for immunoblot analysis, and the amount of translatable surfactant apoprotein mRNA was assessed by in vitro translation-immunoprecipitation. We found that the effects of cortisol and Bt2cAMP on surfactant apoprotein accumulation were associated with comparable increases in the levels of translatable surfactant apoprotein mRNA (Fig. 7). When the immunoprecipitated proteins from translation assays programmed with RNA from control and treated lung explants were analyzed on two-dimensional gels, no differences in the patterns of the immunoprecipitated proteins were observed (data not shown).

In other studies, we found that surfactant apoprotein accumulation was also increased by 8-bromo-cyclic AMP and by forskolin, an activator of adenylate cyclase, but not by butyrate (Fig. 8).

**DISCUSSION**

In the present study, we have demonstrated for the first time that cortisol and cyclic AMP increase the rate of accumulation of the major surfactant apoprotein and its mRNA in fetal rabbit lung tissue. The induction of surfactant apoprotein by cortisol in the present study followed a similar time course as that observed for the cortisol-induced increase in the synthesis of phosphatidylcholine in the cultured fetal lung explants. The stimulatory effect of cortisol on phosphatidylcholine synthesis and on the specific content of the 29–36-kDa apoprotein in 21-day fetal rabbit lung tissue explants was first detectable on the 3rd day of organ culture, although a stimulatory effect of cortisol on the level of translatable surfactant apoprotein mRNA was detectable as early as the first day of incubation. Treatment of explants with cortisol and Bt2cAMP in combination resulted in an induction of surfactant apoprotein accumulation that was greater than that observed with either factor alone. The stimulatory effects of cortisol and Bt2cAMP on surfactant apoprotein accumulation were associated with comparable increases in the levels of translatable surfactant apoprotein mRNA. A synergistic effect of dexamethasone and Bt2cAMP on phosphatidylcholine synthesis by fetal rat lung explants has been reported (27).

The cortisol-induced increase in surfactant apoprotein accumulation in the fetal rabbit lung tissue in vitro is associated with an increase in the number of identifiable type II cells. In preliminary studies, we found no identifiable type II cells in the 21-day fetal rabbit lung explants after 3 days of organ culture in control medium. After 3 days of culture in the presence of cortisol, however, 64% of the ductular epithelial cells contained lamellar bodies and could, therefore, be identified as type II cells. After 5 days of incubation in control medium, 47% of the epithelial cells were now identifiable as type II cells, while in cortisol-treated tissues the percentage of type II cells was 66%. Since the presence of lamellar bodies is the most appropriate marker for the type II cells, it is difficult to distinguish an effect of cortisol on type II cell differentiation from one on the induction of surfactant synthesis.

The results of the in vitro translation studies of the present investigation are indicative that the primary translation product of rabbit surfactant apoprotein mRNA is a protein of ~31 kDa. Whitsett et al. (12) reported recently that the primary translation product of the major surfactant apoprotein of rat lung has an apparent Mr = 26,000. These authors suggested that the 32- and 36-kDa forms of the rat surfactant apoprotein arise from the 26-kDa primary translation product by the addition of N-linked oligosaccharide side chains (12). Floros et al. (39) suggested that two different mRNAs may encode the major surfactant apoprotein of human lung tissue, since the primary translation products consisted of a doublet of 29–31 kDa.

In the present study, we found a marked increase in the level of translatable mRNA specific for the major surfactant apoprotein in lung tissues of 28-day fetal rabbits; the level was increased further in lung tissues of 31-day fetuses but declined in lung tissues of day 1 neonates and was reduced further in adults. These changes in the levels of surfactant apoprotein mRNA are associated with similar alterations in the levels of immunoreactive surfactant apoprotein.2 The marked increase in surfactant apoprotein mRNA in rabbit lung tissue at day 28 of gestation is coincident with the time that increased numbers of type II cells and augmented surfactant phosphatidylcholine synthesis are observed (40). The reduced levels of surfactant apoprotein and its mRNA in neonatal and adult lung tissues may reflect a decreased requirement for de novo synthesis of surfactant apoprotein and glycerophospholipids after birth, perhaps due to increased surfactant recycling by the type II cells (41).

When lung tissue explants from 21-day fetal rabbits were placed in organ culture in medium without hormones, the level of translatable surfactant apoprotein mRNA was detectable after 1 day of organ culture and was increased with increasing time of incubation. The level of translatable surfactant apoprotein mRNA was increased further by cortisol and by Bt2cAMP treatment. When explants were incubated with cortisol and Bt2cAMP in combination, the levels of translatable RNA were greater than those observed with either factor alone. In preliminary studies, using a cloned cDNA specific for rabbit surfactant apoprotein we have found that cortisol and Bt2cAMP cause an induction in the levels of hybridizable surfactant apoprotein mRNA of similar magnitude to that observed using in vitro translation.3 These findings are suggestive that the changes in mRNA translatability are correlated with changes in surfactant apoprotein mRNA content. The finding that the -fold induction of surfac-tant apoprotein and its mRNA by cortisol is more marked at early than at late time points of incubation (Figs. 1 and 5) is due largely to the induction of surfactant apoprotein mRNA to relatively high levels in control explants by day 5 of incubation. The factors that cause the induction of surfactant apoprotein mRNA in fetal lung explants maintained in control medium are unknown. Previously, we reported that lung tissue explants of 19- (29) and 21-day (16) gestational age fetal rabbits differentiate when maintained in organ culture in serum-free medium. After several days of culture the alveolar

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3. V. Boggaram and C. R. Mendelson, unpublished observations.
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epithelium of the explants is comprised entirely of type II pneumonocytes that contain numerous lamellar bodies. These changes in morphology are accompanied by an increase in the synthesis of phosphatidylcholine. Addition of cortisol to the medium causes a further increase in the synthesis of lamellar body glycerophospholipids (16).

The findings of other investigators that cyclic AMP analogues and inhibitors of phosphodiesterase stimulate surfactant phosphatidylcholine synthesis in fetal lung tissue in vivo (21, 22) and in vitro (18-20, 23, 27) and that β-adrenergic receptors are present in fetal lung tissue and increase with advancing gestational age (25, 26) are suggestive that catecholamines may also serve a role in the regulation of surfactant synthesis by fetal lung tissue.

The results of the present study are indicative that concomitant with type II cell differentiation and increased surfactant glycerophospholipid synthesis in vitro there is a marked increase in the levels of translatable mRNA specific for the major surfactant apoprotein and an induction of synthesis of this protein in the fetal lung tissue. Cortisol may increase the levels of translatable surfactant apoprotein mRNA by binding to specific receptors, which in turn may exert a direct stimulatory effect on the rate of surfactant apoprotein gene transcription. The identification of a putative glucocorticoid receptor-binding region in the 5’-flanking region of the human surfactant apoprotein gene (13), together with our observations of the effects of cortisol on surfactant apoprotein mRNA, are suggestive that the glucocorticoid-receptor complex may directly regulate surfactant apoprotein gene transcription. On the other hand, glucocorticoid effects on RNA processing have also been reported (42). Cyclic AMP is known to exert its effects on cellular metabolism in higher eucaryotes by activation of cyclic AMP-dependent protein kinases with resulting phosphorylation of a number of cellular proteins resulting in their altered activity. It has recently become apparent, however, that cyclic AMP increases the rate of transcription of a number of genes, including phosphoenolpyruvate carboxykinase (43-45) and prolactin (46, 47). The mechanism(s) by which cyclic AMP alters gene transcription is unknown. Studies are in progress to define the molecular mechanisms that mediate the increase in surfactant apoprotein synthesis during fetal development, as well as those that mediate the action of glucocorticoids and cyclic AMP on this process.

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