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Modulation of Tropoelastin Production and Elastin Messenger Ribonucleic Acid Activity in Developing Sheep Lung†

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ABSTRACT: During fetal development of the sheep lung, elastin content continually increases. For examination of the processes controlling this elastin accumulation, an explant culture system was characterized with respect to changes in tropoelastin production in sheep lung during fetal and early postnatal development. Relative tropoelastin production in cultured lung explants, quantitated by immunoprecipitation, was about 0.3% of total [^{14}C]valine incorporation during the period from 55 to 104 days after conception. This percentage began to increase by 112 days after conception, reached a maximum value of about 1.0% by 135 days after conception, and then declined to 0.5% soon after birth. The absolute rate of tropoelastin production paralleled these changes in relative tropoelastin production. For evaluation of the processes controlling tropoelastin production in the developing sheep lung, total cellular RNA prepared from 68-day-old fetal, 107-day-old fetal, and 147-day-old fetal lung was translated in a rabbit reticulocyte lysate system. Elastin mRNA activity, expressed as the amount of elastin precursor translated per microgram of DNA, increased about 3-fold during fetal lung development, and elastin precursor synthesis, expressed as a proportion of total translational activity, increased in parallel. It appears, therefore, that elastin production in developing fetal lung is modulated, at least in part, by the amount of available translatable elastin mRNA present in the tissue.

During fetal life, the lung undergoes marked morphological changes to become a stable gas-exchange system at birth. Beyond the very early embryonic stages of morphogenesis, three prenatal stages of lung development are traditionally recognized: the glandular, canalicular, and alveolar or saccular stages (Emery, 1969; Meyrick & Reid, 1977). Morphological development continues after birth and through infancy (Loosli & Potter, 1959; Boyden & Tompsett, 1965).

Connective tissue elements appear to play a major role in lung development (Emery, 1969). One of these elements, elastin, is the component of elastic fibers which possesses the elastic properties critical to the mechanical behavior of the lung as a gas-exchanging organ. Morphologic studies of fetal lung of a variety of species have indicated marked age-related changes in the amount and distribution of elastic fibers (Loosli & Potter, 1959; Jones & Barson, 1971; Collet & Des Biens, 1974) that are thought to be essential to the proper architectural development of lung.

In this context, the present study was designed to evaluate how the lung regulates elastin production during fetal life and the early perinatal period. Three aspects of lung elastin were evaluated in the developing sheep: (1) the amount of elastin present, (2) the production of tropoelastin[1] by lung explants, and (3) the role of elastin mRNA levels in regulating elastin synthesis during lung development. To accomplish these objectives, we developed in vitro systems for quantitating the amount of tropoelastin production by lung and corresponding levels of lung elastin mRNA activity.

Materials and Methods

Materials. All reagents were of highest commercial grade available. Culture media were prepared by the NIH Media Unit. Killed *Staphylococcus aureus* cells ("Pansorbin") were from Calbiochem (La Jolla, CA), L-[2,3,4-^{3}H]valine (11.1 Ci/mmol) was from New England Nuclear (Boston, MA), and L-[^{14}C]valine (250 mCi/mmol) was from Schwarz/Mann (Orangeburg, NY).

Solutions. The "extraction" solution used to extract labeled proteins from lung explants was a mixture of 5% deoxycholate, 5% Triton X-100, 70 mM *N*-ethylmaleimide, 175 μg/mL toluenesulfonylphenylalanyl chloromethyl ketone, 175 μg/mL

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[1] The term "tropoelastin" is used to refer to the soluble elastin precursor present in tissue extracts and culture media of lung explants. The term "elastin precursor" designates the primary translation product of elastin mRNA.

tosyllysyl chloromethyl ketone, 35 μg/mL phenylmethanesulfonyl fluoride, 70 mM ε-aminocaproic acid, 14 mM ethylenediaminetetraacetic acid tetrasodium salt (EDTA),[2] and 100 μg/mL β-aminopropionitrile (Eichner & Rosenbloom, 1979). The "RIA" buffer (the buffer for radioimmune assay) consisted of 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 7.5, 0.05% Nonidet P-40, 0.01% sodium dodecyl sulfate ($NaDodSO_4$), 0.05% deoxycholate, 0.02% NaN_3, 1 mg/mL bovine serum albumin, 40 mM ε-aminocaproic acid, 5 mM benzamidine hydrochloride, 20 mM sodium iodoacetate, and 2 mM L-valine.

Tissue Source. Fresh lungs were obtained from lambs between 55 days of gestation and 14 days after birth (Veterinary Resources Branch, National Heart, Lung, and Blood Institute). Their age was based upon the conception date given by the supplier; the average full term age of newborn lambs used was 147 days after conception. Fetal lambs were delivered by caesarean section and newborn lambs were delivered spontaneously. Three stages of sheep lung development were confirmed by histologic evaluation as follows: the glandular stage, approximately 95 days after conception; the canalicular stage, 95–120 days after conception; the saccular or alveolar stage, >120 days after conception (Meyrick & Reid, 1977).

Estimation of Elastin Content in Lung. Minced whole lung was lyophilized, weighed, suspended in 10 volumes of 0.1 N NaOH, and heated at 98 °C for 45 min (Lansing et al., 1952). Extraction of the insoluble residue was repeated twice, except that the duration of extraction was reduced to 20 min. The alkali-insoluble residue was washed successively with 98 °C H_2O, 50% ethanol, absolute ethanol, and 1:1 acetone/ether. After the sample was dried at 150 °C overnight, the weight of insoluble residue was determined for duplicate samples.

Incubation of Lung Explants. Lungs were dissected under sterile conditions and immersed in phosphate-buffered saline (PBS) at 4 °C, and the visceral pleura and hila were removed. The lungs were freed of major airways and major blood vessels and the tissue was minced into small pieces with scissors.

The incubation procedure was essentially the same as described by Bradley et al. (1974). Minced lung (~500 mg wet weight) was suspended in 5 mL of valine-free Dulbecco–Vogt minimum essential medium, equilibrated with 95% O_2 and 5% CO_2, containing 25 mM Hepes, pH 7.4, 50 μg/mL, sodium ascorbate, 0.05% glutamine, 10 μg/mL Aprotinin, 50 μg/mL β-aminopropionitrile, 100 μg/mL penicillamine, 100 μg/mL streptomycin sulfate, and 100 units/mL penicillin; minces were incubated for 45 min at 37 °C on a rotary shaker bath. The medium was then aspirated and discarded, and fresh medium containing [^{14}C]valine (10 μCi/mL) was added; the incubation was continued for an additional 90 min unless stated otherwise. [^{14}C]Valine was used as a marker for tropoelastin production since elastin is substantially enriched in this amino acid. All incubations were carried out in quadruplicate.

Determination of DNA in Lung Explants. Duplicate samples of lung adjacent to those used for culture experiments were homogenized in PBS and used for DNA determinations. The pellet from 0.3 N $HClO_4$ precipitation of the homogenate was suspended in 1 N $HClO_4$, heated to 70 °C for 30 min, and cooled to 4 °C. After centrifugation, DNA was determined in the supernatant by the method of Burton (1956) using calf thymus DNA as a standard.

Extraction of Labeled Proteins in Lung Explants. At the end of the incubation, culture media and lung explants were separated by centrifugation at 10000*g* at 4 °C for 15 min. All subsequent procedures were carried out at 4 °C. The lung explants were washed in PBS, centrifuged, suspended in 5 mL of extraction solution, and homogenized twice with a Polytron homogenizer (Brinkmann, Westbury, NY) for 20 s at high speed. The homogenates were then extracted for 24 h by gentle agitation and centrifuged at 10000*g* for 30 min. Prior to immunoprecipitation of tropoelastin produced by lung explants, the resulting supernatant was dialyzed against 1% Triton X-100, 0.1% deoxycholate, and 20 mM ε-aminocaproic acid (Eichner & Rosenbloom, 1979). The dialyzed extract was centrifuged at 10000*g* for 20 min, and this supernatant was used to quantitate the ^{14}C-labeled tropoelastin by using the radioimmune assay as described below. The [^{14}C]valine incorporation into protein was determined by Cl_3CCOOH precipitation (Davidson et al., 1981). This extraction procedure routinely extracted about 90% of the labeled proteins. [^{14}C]Valine incorporation into total proteins was taken to be the sum of the acid-precipitable radioactivity in tissue extract plus medium and expressed per unit of DNA.

Affinity Chromatography of Tropoelastin. For isolation of tropoelastin for use as a standard in radioimmune assay, 20 mg of α-elastin, isolated from sheep nuchal ligament (Davidson et al., 1981), was coupled to 2.2 g of CNBr-activated Sepharose 4B (Pharmacia) according to instructions supplied by the manufacturer with 62% efficiency. After equilibration of this support on a small column with borate-buffered saline (5 mM boric acid, 1.25 mM borax, 150 mM NaCl, and 0.02% NaN_3, pH 8.0) at 23 °C, 5 mL of anti-α-elastin antiserum (Davidson et al., 1981) was applied; the antiserum was cycled through this column 5 times. The column was then washed extensively with borate-buffered saline, and elastin antibodies were eluted with a total of 5 mL of 0.2 M glycine hydrochloride, pH 2.3. After the amount of antibody eluted was estimated (by the extinction coefficient of the eluate at 280 nm), bovine serum albumin was added to 1 mg/mL, and the eluate was dialyzed against 0.5 M NaCl and 0.1 M $NaHCO_3$. Approximately 0.75 mg of antibody in 40 mL of 1% bovine serum albumin, 0.5 M NaCl, and 0.1 M $NaHCO_3$ was then coupled to 2.2 g of CNBr-activated Sepharose 4B as described above. Samples containing [^{14}C]-valine-labeled tropoelastin, prepared from 7-day-postnatal lung explants, were then diluted in borate-buffered saline containing 1 mM phenylmethane sulfonyl fluoride and applied to anti-α-elastin–Sepharose column at 4 °C. After at least 2 h of equilibration, unbound proteins were eluted with 20 column volumes of borate-buffered saline, and the specifically bound tropoelastin was eluted with 5 × 5 mL of 0.2 M glycine hydrochloride, pH 2.3. Nonspecifically bound proteins were removed from the column with 3.5 M KSCN in borate-buffered saline. Purified tropoelastin was concentrated by lyophilization, dissolved in H_2O, and applied to PD-10 column (Sephadex G-25, Pharmacia) equilibrated with 1% Triton X-100, 0.1% deoxycholate, and 20 mM ε-aminocaproic acid. The fraction eluting in the void volume was used as a standard for the radioimmune assay.

Radioimmune Assay of Tropoelastin Produced by Lung Explants. Rabbit anti-α-elastin antiserum, prepared against sheep nuchal ligament α-elastin, was evaluated for its cross-reactivity with sheep lung α-elastin by Ouchterlony immunodiffusion and by quantitative immunoprecipitation. Ouchterlony immunodiffusion was carried out by using an anti-α-elastin antiserum and sheep nuchal ligament, lung and

[2] Abbreviations used: EDTA, ethylenediaminetetraacetic acid tetrasodium salt; RIA buffer, buffer for radioimmune assay; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

aorta α-elastins as antigens. The diffusion gel matrix consisted of 1% agarose in PBS. Immunodiffusion between the center well and surrounding wells (all 2 mm in diameter) was carried out at 4 °C overnight.

Immunoprecipitation of the [^{14}C]valine-labeled tropoelastin produced by lung explants was carried out as described previously (Davidson et al., 1981) with minor modifications. Aliquots (500 μL) of the tissue extract, which had been dialyzed against 1% Triton X-100, 0.1% deoxycholate, and 20 mM ε-aminocaproic acid, were mixed with 500 μL of RIA buffer and incubated with 3 μL of anti-α-elastin antiserum or preimmune serum at 4 °C overnight. For the radioimmune assay of medium proteins, 500 μL of culture medium was mixed with 500 μL of RIA buffer containing 1% Triton X-100. *Staphylococcus aureus* cells ["Pansorbin", Calbiochem; 200 μL, 10% (v/v) solution], previously washed with a detergent solution (Kessler, 1975), were then added to the reaction mixture. The subsequent procedures were as previously described (Davidson et al., 1981). For elimination of the contributions due to nonspecific precipitation of labeled proteins, the amount of the radioactivity in an immunoprecipitate was determined by subtracting the radioactivity obtained with preimmune serum from that obtained with anti-α-elastin antiserum.

For evaluation of the accuracy of this quantitative immunoprecipitation, [^{14}C]valine-labeled lung tropoelastin, purified by affinity chromatography, was used in a radioimmune assay as described above. The supernatant obtained after the sedimentation of tropoelastin–antibody–Pansorbin complex was made up to 1% $NaDodSO_4$ and 1% β-mercaptoethanol, heated to 95 °C for 5 min, and applied to a PD-10 column previously equilibrated with 0.1% $NaDodSO_4$ and 0.1% β-mercaptoethanol. The fraction eluting in the void volume was lyophilized, dissolved in water, and applied to the discontinuous polyacrylamide slab gels as described below.

Quantitation of Tropoelastin Production by Lung Explants. Relative tropoelastin production by the lung explants was expressed as the ratio of [^{14}C]valine incorporation into tropoelastin (tissue tropoelastin and medium tropoelastin) to total [^{14}C]valine incorporation by using methods described above. For quantitation of the absolute rate of tropoelastin production by lung explants, the specific activity of the pool of free [^{14}C]valine in the explants was quantitated by homogenizing portions of the labeled lung explants in 10 volumes of 1% picric acid. Other procedures were the same as described by Breul et al. (1980). A Beckman Model 119C amino acid analyzer was used to isolate and quantitate valine in the lung-free valine pool. Specific activity of the intracellular free valine was expressed as disintegrations per minute of [^{14}C]valine per nanomole of valine. The rate of tropoelastin production was then calculated as follows: rate of tropoelastin production [molecules/(cell·h)] = [(total [^{14}C]valine incorporation into tropoelastin, dpm/g of DNA) × (DNA content of sheep cell, 6.2×10^{-12} g/cell) × (Avogadro's number, 6.02×10^{23} molecules/mol)]/[(specific activity of intracellular [^{14}C]valine, dpm/mol) × (valine content of tropoelastin, 119 mol/mol) × (labeling time, h)] (Vendrely, 1955; Enesco & Leblond, 1962; Sandberg et al., 1969; Smith et al., 1972; John & Thomas, 1972; Paz et al., 1976). Labeling time was 1.5 h in all experiments except for the time course study. The rate of tropoelastin production was expressed as the number of molecules of tropoelastin produced per cell per hour.

Isolation of RNA. RNA was isolated from previously frozen sheep lungs at three different stages of fetal development: the glandular stage (68 days), the canalicular stage (107 days), and the alveolar stage (147 days). Histologic examination of the lungs at these ages showed the typical morphology of each stage. Total cellular RNA was extracted and isolated by an adaptation of guanidine hydrochloride procedures (Tolstoshev et al., 1981a; Davidson et al., 1981). An aliquot of the homogenate in guanidine hydrochloride was used for the determination of RNA and DNA (Davidson et al., 1981). The recovery of RNA was approximately 50% as calculated by the recovery of ^{3}H-labeled ribosomal RNA (Miles Biochemicals); there was no difference in the recoveries of RNA from lungs obtained at different stages of development.

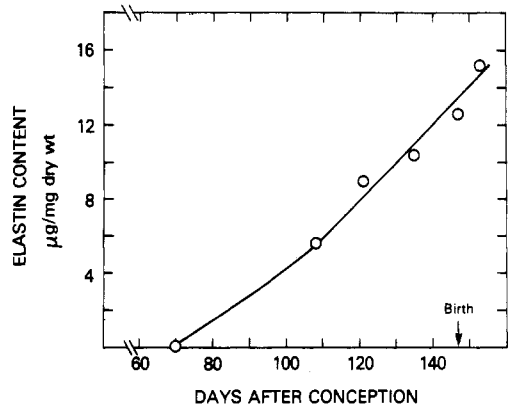

FIGURE 1: Elastin accumulation in developing lung. Insoluble elastin content of lungs was determined by the Lansing procedure (Lansing et al., 1952). Each data point represents the average of duplicate samples; the values are expressed in terms of the dry weight of the tissue.

Translation of Elastin mRNA. Functional elastin mRNA in lung RNA was translated in a mRNA-dependent rabbit reticulocyte system (Davidson et al., 1981). The reaction mixture (90 μL) containing 3–9 μg of total lung RNA was incubated at 30 °C for 45 min. Under these conditions, protein synthesis was proportional to the amount of RNA added. An equal volume of a solution containing 4% Nonidet P-40, 4% deoxycholate, and 30 mM EDTA, pH 7.5, was added to the reaction mixture at 4 °C. Over 90% of the total Cl_3CCOOH-insoluble radioactivity of the original reaction mixture was recovered in the supernatant. For immunoprecipitation of elastin precursor in the cell-free products, 60 μL of the supernatant obtained as above was mixed with 180 μL of RIA buffer (to decrease the detergent concentrations) and incubated with 3 μL of anti-α-elastin antiserum or preimmune serum at 4 °C overnight; other procedures for radioimmune assay were the same as described above.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Labeled proteins synthesized by lung explants were prepared as described by Eichner & Rosenbloom (1979) and were evaluated by using discontinuous $NaDodSO_4$–polyacrylamide gel electrophoresis as described previously (Davidson et al., 1981).

Results

Changes in Lung Insoluble Elastin Content during Development. At the glandular stage of development, the elastin content of sheep lung was extremely low (Figure 1), even though elastic fibers could be recognized morphologically (data not shown). However, the elastin content of lung increased continuously throughout the period of lung development, more than doubling in the last 50 days of gestation. These biochemical estimates of lung elastin content are consistent with the apparent increase in elastic fibers in bronchi, vascular tree, and alveolar interstitium during the same period.[3]

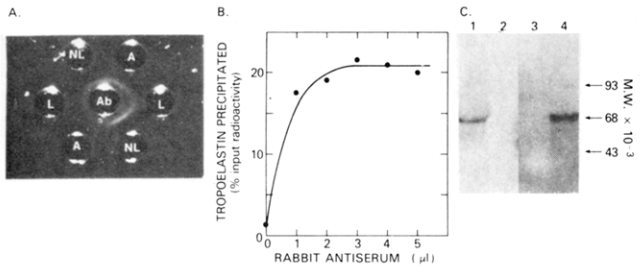

FIGURE 2: Characterization of the anti-α-elastin antiserum used to quantify lung tropoelastin production and lung elastin mRNA activity. The antiserum was prepared against sheep nuchal ligament α-elastin. (A) Ouchterlony immunodiffusion analysis. The center well contained 5 μL of rabbit anti-α-elastin antiserum. The outer wells contained α-elastin (2 μg) from nuchal ligament (labeled "NL"), α-elastin (2 μg) from lung (labeled "L"), and α-elastin (2 μg) from aorta (labeled "A"). (B) Ability of the anti-α-elastin antiserum to precipitate [^{14}C]valine-labeled tropoelastin produced by lung explants. A constant volume (500 μL) of culture medium derived from 2-day-old postnatal lung explants was reacted with an increasing volume of anti-α-elastin antiserum. The total amount of serum in each reaction was adjusted to 5 μL with preimmune serum; Pansorbin (350 μL of a 10% solution) was subsequently used as an adsorbent to quantitatively precipitate the IgG present. (C) Efficiency of immunoprecipitation of [^{14}C]-valine-labeled tropoelastin evaluated by gel electrophoresis. [^{14}C]-Valine-labeled tropoelastin was purified by affinity chromatography from tissue extracts of 7-day-old postnatal lung explants as described under Materials and Methods. [^{14}C]Valine-labeled tropoelastin (10000 dpm) was incubated with antiserum or preimmune serum and precipitated. The postprecipitate supernatant and precipitated proteins were compared by slab gel electrophoresis. (Lane 1) Supernatant following addition of preimmune serum; (lane 2) supernatant following addition of anti-α-elastin antiserum; (lane 3) proteins precipitated with preimmune serum; (lane 4) proteins precipitated with anti-α-elastin antiserum. Molecular weight (M.W.) standards indicated by arrows were phosphorylase *a* (M_r 93000), bovine sreum albumin (M_r 68000), and ovalbumin (M_r 43000).

Identification of Lung Tropoelastin. Rabbit anti-α-elastin antiserum, prepared against sheep nuchal ligament α-elastin, cross-reacted with α-elastin prepared from sheep lung and aorta (Figure 2A). A single connecting precipitin line was observed, suggesting that the α-elastin prepared from these organs have largely shared antigenic determinants. This antiserum also reacted with tropoelastin produced by lung explants and quantitatively precipitated tropoelastin under the radioimmune assay conditions utilized (Figure 2B). In contrast, preimmune serum did not precipitate lung tropoelastin (Figure 2C, lanes 1 and 3). For confirmation of the efficiency of this immunoprecipitation of tropoelastin, [^{14}C]valine-labeled lung tropoelastin purified by affinity chromatography was used in the radioimmune assay and the nonprecipitable supernatant, and precipitated proteins were evaluated by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2C). No tropoelastin was left in the supernatant obtained after the precipitation of antigen–antibody complex bound to Pansorbin (Figure 2C, lane 2), and tropoelastin was recovered in the precipitate as one major band at about M_r 70000 (Figure 2C, lane 4). In addition, no apparent degradation of tropoelastin was observed under the radioimmune assay conditions used. The recovery of tropoelastin during the procedure of radioimmune assay was greater than 96% of input radioactivity. The mobility of tropoelastin remaining in the supernatant of reactions with preimmune serum was slightly greater than that of tropoelastin precipitated with antiserum (Figure 2C, compare lane 1 with lane 4), probably due to an artifact caused by the presence of rabbit serum proteins, likely albumin.

[3] O. Kawanami, V. Ferrans, S. Shibahara, J. M. Davidson, and R. G. Crystal, unpublished results.

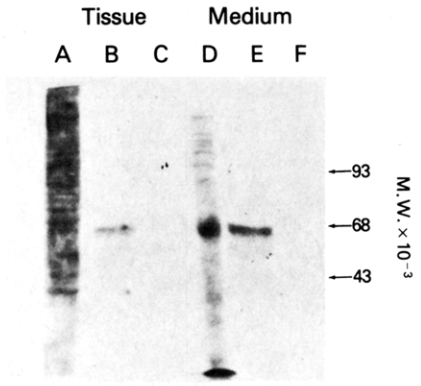

FIGURE 3: Specificity of immunoprecipitation of newly synthesized tropoelastin in extracts and culture media of lung explants. Pieces of lung of 7-day-old postnatal sheep were incubated with [^{14}C]valine at 37 °C for 90 min. The labeled proteins extracted from the tissue and those present in the media were electrophoresed on $NaDodSO_4$–polyacrylamide slab gels. Labeled tissue extract proteins are shown in lanes A–C; labeled medium proteins are shown in lanes D–F. (Lane A) Total tissue extract; (lane B) tissue extract precipitated with anti-α-elastin antiserum (derived from 40 times that amount of radioactivity of that applied to lane A); (lane C) tissue extract precipitated with preimmune serum (derived from 40 times that amount of radioactivity of that applied to lane A); (lane D) total medium proteins; (lane E) medium proteins precipitated with anti-α-elastin antiserum (derived from 5 times that amount of radioactivity of that applied to lane D); (lane F) medium proteins precipitated with preimmune serum (derived from 5 times that amount of radioactivity of that applied to lane D). Molecular weight standards indicated by arrows were phosphorylase *a* (M_r 93000), bovine serum albumin (M_r 68000), and ovalbumin (M_r 43000).

For confirmation of the specificity of immunoprecipitation for tropoelastin, lung tissue extract proteins and medium proteins were used for radioimmune assay, and the precipitated proteins were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3). One major band of immunoprecipitable protein was visualized at the region corresponding to the known molecular weight of tropoelastin (M_r ~70000) (Figure 3, lanes B and E) whereas no visible bands were observed in this molecular weight range with preimmune serum controls (Figure 3, lanes C and F). A few minor bands were sometimes observed near the top of the gel (data not shown); however, this material was precipitated by either anti-α-elastin antiserum or preimmune serum and thus was likely the result of nonspecific precipitation of labeled proteins by rabbit serum. The value of background precipitation was about 0.2% of input Cl_3CCOOH-insoluble radioactivity. For this reason, a preimmune serum control was always utilized for background subtraction when tropoelastin production was quantified. No large precursor of tropoelastin was noted in the lung explant system.

Characterization of Lung Culture System. For evaluation of the relationship between the elastin production and the increase in elastin content during lung development (Figure 1), production was characterized in a lung explant culture system. Lung explants were incubated with [^{14}C]valine in a complete medium supplemented with ascorbic acid, β-aminopropionitrile, penicillamine, and protease inhibitor to promote full hydroxylation of connective tissue proteins while retarding their cross-linking and proteolysis. The incorporation of [^{14}C]valine into tissue protein increased linearly up to 3 h (Figure 4A), and release of labeled protein into the culture medium continued to increase during a 5-h incubation (Figure 4A). In parallel with total protein production, accumulation of tropoelastin in lung tissue was linear for up to 3 h (Figure 4B) and up to 5 h in the culture medium (Figure 4B). Interestingly, although the medium radioactivity represented only

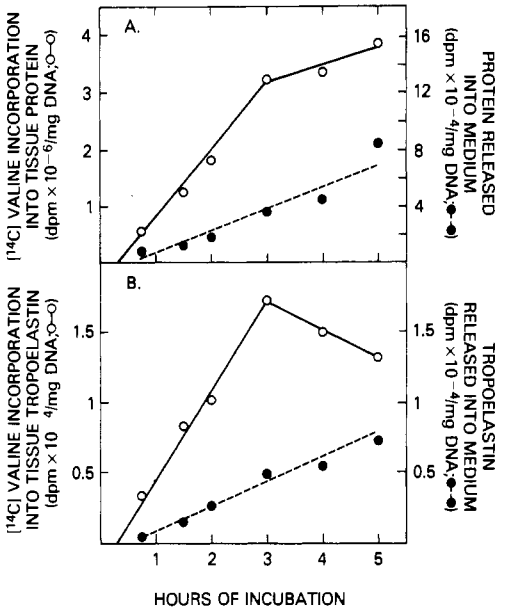

FIGURE 4: Time course of [^{14}C]valine incorporation into tissue protein by lung explants and release of newly synthesized proteins into the culture medium. Lungs of 7-day-old postnatal sheep were incubated with [^{14}C]valine for the indicated periods. Incorporation into total protein was determined by measuring Cl_3CCOOH-insoluble radioactivity present in tissue extracts and media. Tropoelastin production was quantitated by using a specific anti-α-elastin antibody as described under Materials and Methods. Each value was expressed in terms of DNA content. (A) Incorporation of [^{14}C]valine into tissue protein (○) and release of labeled protein into medium (●). (B) Incorporation of [^{14}C]valine into tropoelastin in tissue (○) and release of newly produced tropoelastin into medium (●).

1–2% of total [^{14}C]valine incorporation, the proportion of tropoelastin released into the culture medium increased from 11% to 35% of total newly produced tropoelastin in the period between 45 min and 5 h of incubation (Figure 4B).

Protein Production by Lung Explants at Different Stages of Development. When lungs of different developmental ages were incubated with [^{14}C]valine to evaluate the capacity of the tissues to produce proteins, total [^{14}C]valine incorporation (the sum of tissue radioactivity and medium radioactivity) per milligram of DNA decreased during lung development (Figure 5A). However, this apparent decrease was largely caused by the declining availability of intracellular [^{14}C]valine (Figure 5B) (due mostly to the increasing valine pool in the lung during development; data not shown). When total valine incorporation was corrected for intracellular [^{14}C]valine specific activity (Figure 5C), it was apparent that the actual rate of protein production remained generally constant during fetal development, decreasing only after birth.

Tropoelastin Production in Developing Lung. During fetal development of the lung, the rate of tropoelastin production increased about 4-fold. The rate of tropoelastin production began to increase at the canalicular stage, reached the maximum value by the alveolar stage just prior to birth, and then decreased in the early postnatal period (Figure 6A). In parallel with the change in the absolute rate of tropoelastin production, relative tropoelastin production remained low during the glandular stage, began to increase at the canalicular stage, and reached its maximum value (about 1% of total [^{14}C]valine incorporation) at the alveolar stage (Figure 6B). Although there is some data suggesting that the upper lobe of fetal lung matures faster than the lower lobe (Howatt et al., 1965; Brumley et al., 1967), no appreciable differences in tropoelastin production were observed between upper and lower lobes of developing fetal lung (data not shown).

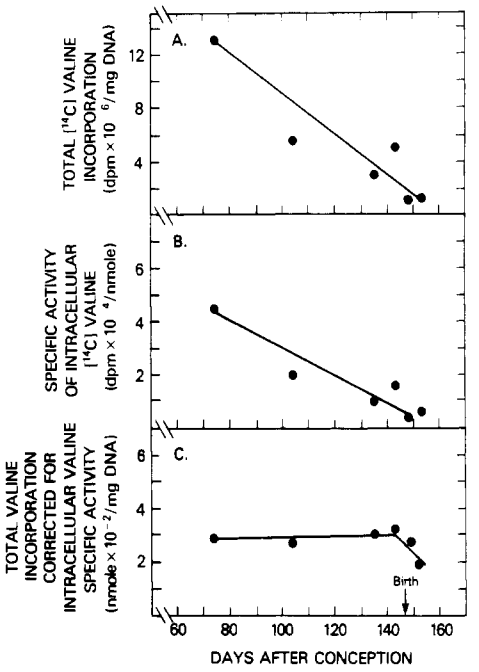

FIGURE 5: Evaluation of protein production in the developing lung. Lung explants from sheep at various ages were incubated in quadruplicate with [^{14}C]valine for 90 min, and Cl_3CCOOH-insoluble radioactivity in both tissue and medium was determined. (A) [^{14}C]Valine incorporation into total protein. Each value is the sum of tissue and medium radioactivity and is expressed in terms of DNA content. (B) Specific activity of the free [^{14}C]valine pool in tissue. (C) Protein production corrected for [^{14}C]valine specific activity.

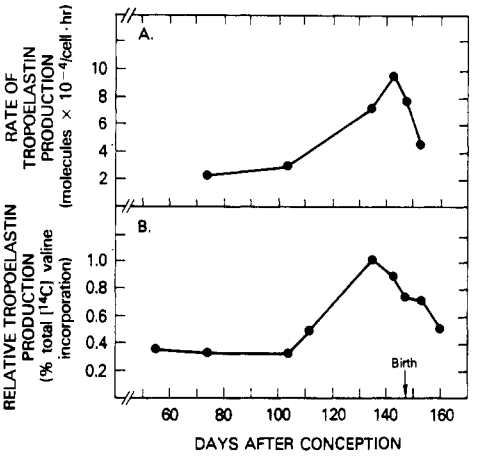

FIGURE 6: Tropoelastin production in the developing lung. Incubation conditions were the same as in Figure 5. The production of newly produced tropoelastin was quantitated by specific radioimmune assay as described under Materials and Methods. Each value is the average of four determinations. (A) Rate of tropoelastin production, expressed as tropoelastin molecules produced per cell per hour; the data were obtained from the same samples used in Figure 5 and corrected for the tissue pool of free [^{14}C]valine. (B) Relative tropoelastin production, expressed as a percent of total [^{14}C]valine incorporation.

Release of Newly Produced Tropoelastin into Culture Medium. While medium radioactivity represented only 1% of the total [^{14}C]valine incorporated into protein by the lung explants during a 90-min incubation (Figure 4A), there were perinatal differences in the release of newly produced tropoelastin into the culture medium (Figure 7). The proportion of newly produced tropoelastin released into the culture medium was relatively constant up to 135 days (~9% of total newly produced tropoelastin) and then increased rapidly to a maximum of 22% by birth. This increased release was not a general phenomenon observed for all proteins since the tropoelastin content of the medium, expressed as a percent of

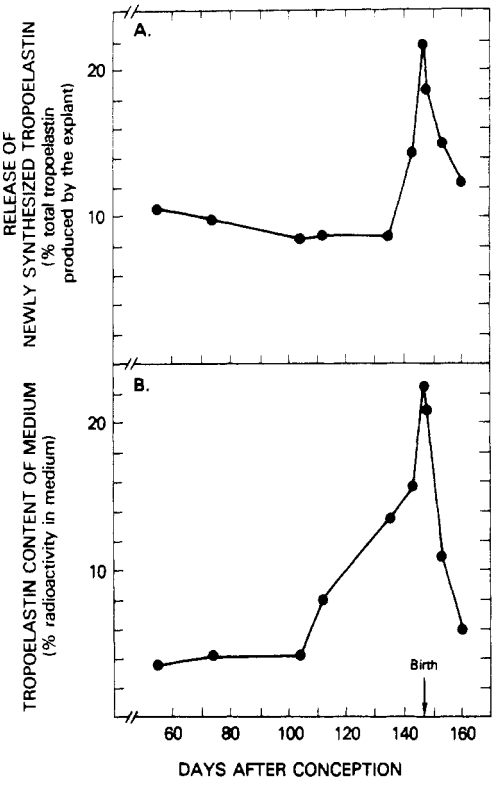

FIGURE 7: Developmental changes in the release of newly produced tropoelastin into culture medium of lung explants. Incubation conditions were the same as in Figure 5. (A) Proportion of newly produced tropoelastin that was released into the medium. (B) Tropoelastin content of medium expressed as a percent of total medium radioactivity in protein.

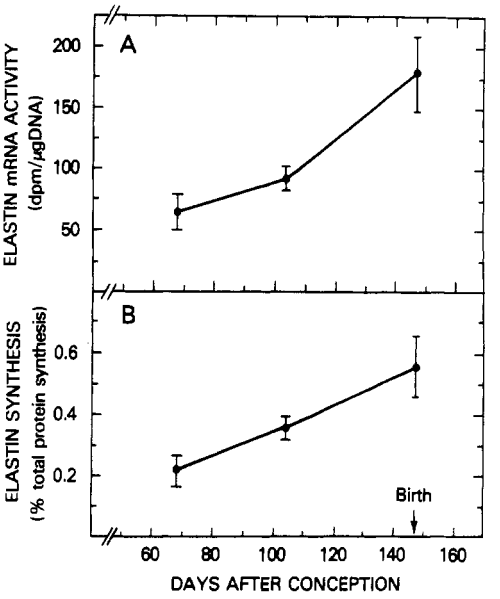

FIGURE 8: Elastin mRNA activity and relative elastin precursor synthesis during lung development. Total lung RNA was translated in a rabbit reticulocyte lysate system, and elastin precursor was identified as described under Materials and Methods. Each value is presented as mean ± standard deviation. (A) Elastin mRNA activity was expressed as the amount of elastin precursor translated per unit of DNA by calculating the product of elastin mRNA activity (disintegrations per minute of [^{3}H]elastin precursor per microgram of RNA) and the RNA/DNA ratio of the tissue; the latter generally remained constant (about 0.5) during the period of lung development examined. (B) Relative elastin precursor synthesis expressed as a proportion of total proteins synthesized in the cell-free system.

medium radioactivity, also increased during the same period. While the increase in the tropoelastin content of the medium between 55 and 135 days reflected the rise in tropoelastin production seen during the same period (Figure 6), the mechanism responsible for the increased perinatal release is not clear; this phenomenon may reflect the increased alveolar surface area which develops during late gestation and the perinatal period. The maximum tropoelastin content of the medium reached at 147 days represented a 6-fold increase in tropoelastin content of the medium over the level at the glandular stage. Both the tropoelastin content of the medium and the release of newly produced tropoelastin into the medium decreased rapidly after birth.

Cell-Free Synthesis of Elastin Precursor with Lung RNA. For evaluation of the mechanisms for the increase in tropoelastin production during fetal lung development, total cellular RNA was isolated from lungs at three different developmental stages and translated in a rabbit reticulocyte lysate system. Anti-α-elastin antiserum reacted with elastin precursor translated in the rabbit reticulocyte lysate system and specifically precipitated an antigen with a molecular weight of about 70 000 (data not shown).

When 68-day-old (glandular), 107-day-old (canalicular), and 147-day-old (alveolar stage) fetal lungs were used for preparation of RNA, it was found that total translational activity (i.e., mRNA activity for all proteins) was not different among these three RNA preparations (data not shown). In contrast, the relative levels of translatable elastin mRNA and elastin mRNA activity, expressed as the amount of elastin precursor translated per unit of DNA, increased during fetal lung development (Figure 8). The 3-fold increase in levels of translatable elastin mRNA observed during fetal lung development was consistent with the increase in the rate of tropoelastin production of explants derived from lungs during the same period, suggesting that elastin synthesis in developing lung is, in part, controlled by the amount of functional available elastin mRNA.

Discussion

Elastin is a major connective tissue component of the adult lung, comprising 2–13% of the dry weight of the parenchyma (Pierce & Hocott, 1960; Keeley et al., 1977) and 10–30% of the connective tissue mass (Pierce et al., 1961; Fitzpatrick & Hospelhorn, 1962; Pierce & Ebert, 1965). Because of its rubber-like mechanical properties, this macromolecule plays an important role in gas exchange by influencing lung function throughout the respiratory cycle (Hance & Crystal, 1975). While the adult lung seems to contain a stable amount of elastin that turns over very slowly (Pierce et al., 1967), morphologic studies suggest that elastin accumulates rapidly in the developing fetal lung, particularly in the latter stages of gestation (Loosli & Potter, 1959; Jones & Barson, 1971; Collet & Des Biens, 1974). The present study demonstrates lung elastin accumulation during fetal maturation, presents a methodology to measure lung tropoelastin production during this period, and suggests that the increase in lung elastin content during the latter stages of fetal maturation results from changes in elastin biosynthesis modulated, at least in part, by the levels of elastin mRNA in the tissue.

Elastin Content and Tropoelastin Production in the Developing Sheep Lung. In the glandular stage of fetal lung development, elastin constitutes <0.1% of the dry weight of the whole lung; this increases more than 10-fold to approximately 1.2% by birth. Although the elastin content of adult sheep lung has not been measured, data from other species indicate that the elastin content of adult lung is much greater than that in the neonate, suggesting this macromolecule continues to accumulate from birth to the time full maturation

is reached. It is important to note that the estimation of elastin content may not be accurate for immature fetal lung since alkali-insoluble residue of immature fetal lung likely includes some of the microfibrillar component of elastic fibers in the estimation of elastin content (Keeley et al., 1977). However, two lines of evidence suggest that our data reflect the increase in "true" elastin content during fetal lung development. First, the content of alkali-insoluble residue appreciably increased during fetal lung development (Figure 1), even though the values for immature lungs may be overestimated. Second, morphological studies of fetal lung demonstrate marked developmental increase in the amount and distribution of elastic fibers[3] (Loosli & Potter, 1959; Jones & Barson, 1971; Collet & Des Biens, 1974).

Tissue obtained from sheep lung parenchyma at the different stages of development actively produces tropoelastin at an average rate of 20 000–95 000 tropoelastin molecules (lung cell)$^{-1}$ h^{-1}, representing 0.3–1.0% of all [^{14}C]valine incorporated into the tissue. From 55 to 104 days after conception, tropoelastin production is constant at a relatively low level, but it then markedly increases 3–4-fold until just before birth when it declines over a 3-week period to the same low level. This is in contrast to lung procollagen production which although much higher than tropoelastin production, decreased only slightly during fetal development[4] (Tolstoshev et al., 1981b). Interestingly, both elastin biosynthesis and procollagen biosynthesis in the developing fetal sheep lung seem to be controlled by the availability of their respective mRNA levels; in both cases, mRNA levels paralleled the level of production of the specific macromolecule.

The methods used to quantify tropoelastin production in lung explants are specific, reproducible, and straightforward. The crux of the methodology involves the production of an anti-α-elastin antiserum and a quantitative method to extract newly synthesized tropoelastin from the tissue. The methods utilized to accomplish this were essentially the same as those developed by Eichner & Rosenbloom (1979) for study of the aorta. We have also used these techniques to quantify tropoelastin production by the developing nuchal ligament, whose relative tropoelastin production is more than 10 times that of the lung (Shibahara et al., 1980). While tropoelastin production in the developing sheep lung rises and then declines as birth approaches, relative tropoelastin production by the sheep nuchal ligament remains high even after birth. Like lung, the level of tropoelastin production in the nuchal ligament seems to be controlled, at least in part, by the levels of available elastin mRNA in the tissue (Davidson et al., 1981).

Interestingly, the release of newly produced tropoelastin into the culture medium of the lung explants changed rapidly during the perinatal period. Although the mechanism responsible for this phenomenon is not known, it did not appear to be related to cross-linking of the newly synthesized tropoelastin, as the exclusion of cross-linking inhibitors from the cultures had no appreciable effect on either tropoelastin production or the release of newly synthesized tropoelastin into the culture medium (data not shown).

Mechanisms Controlling Tropoelastin Production in Developing Fetal Lung. Evaluation of elastin mRNA activity in developing fetal sheep lung demonstrated that it generally paralleled the increase in tropoelastin production by the tissue during the same period. It seems likely, therefore, that the level of elastin production in this tissue is regulated, at least in part, by the level of available elastin mRNA in lung. In all systems studied, control of elastin production appears to be regulated by the level of translatable elastin mRNA. Besides the developing sheep lung evaluated here, tropoelastin production in the developing sheep nuchal ligament (Davidson et al., 1981) and the developing chick aorta (Burnett et al., 1980) seem to be controlled at the mRNA level. In this context, the production of connective tissue components other than elastin has also been shown to be paralleled by the mRNA specific for that component. For example, procollagen production in the developing fetal sheep lung and tendon (Tolstoshev et al., 1981b) and the chick calvarium (Moen et al., 1979) appears to be regulated by the levels of procollagen mRNA in these tissues.

The measured increase in tropoelastin production and elastin mRNA levels in the maturing fetal lung may be the consequence of either a small change in elastin mRNA levels of many types of cells or a large shift in elastin mRNA levels in a smaller subpopulation of specialized cells. Alternately, it may reflect a change in the population of cells present, with increases in elastin mRNA levels (averaged per cell) reflecting a shift toward cells that contain elastin mRNA. Tropoelastin is known to be synthesized by endothelial cells (Carnes et al., 1979; Cantor et al., 1980) and by mesenchymal cells such as smooth muscle cells (Abraham et al., 1974; Faris et al., 1976; Narayanan et al., 1976) and certain types of fibroblasts such as those found in the ligamentum nuchae (Jones et al., 1980; Mecham et al., 1981). Preliminary histological examination in our laboratory has demonstrated that elastic fibers are located in the extracellular matrix adjacent to endothelial cells, smooth muscle cells and fibroblasts.[3] However, the lung is a very complex tissue composed of various types of cells (Kuhn, 1976), and little information exists concerning the populations of endothelial cells and mesenchymal cells in developing lung. For these reasons, identification of the precise site of tropoelastin synthesis will require further study, possibly employing techniques such as immunohistochemistry or in situ nucleic acid hybridization.

The sensitivity and precision of the techniques employed in this study should make it possible to critically evaluate the modes and extent of elastin production and accumulation in both developing systems and in pathological disorders of lung connective tissue.

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Transcription of the Major *Drosophila* Heat-Shock Genes in Vitro†

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ABSTRACT: Active eukaryotic genes are more accessible to some proteins that bind DNA than are inactive genes. In order to probe the accessibility of the *Drosophila* heat-shock genes, we have isolated nuclei from *Drosophila* tissue culture cells and have used these nuclei as templates for *Escherichia coli* RNA polymerase. With nuclei isolated from cells that had not been heat shocked, the synthesis of heat-shock RNA was not detected by hybridization to a DNA clone containing sequences from the major heat-shock region. In contrast, approximately 0.22% of the RNA synthesized in nuclei isolated from cells that had been previously heat shocked hybridized to the heat-shock clone. The synthesis of heat-shock RNA was DNA dependent, was sensitive to rifampicin and to actinomycin D, and represented a 70-fold enrichment over random transcription of the *Drosophila* genome. Transcription showed an extraordinary preference for a region 5′ distal to the structural gene. These results demonstrate that preferential transcription by the bacterial RNA polymerase is indicative of the active state of *Drosophila* genes.

Evidence from numerous sources has documented the structural differences between actively transcribed and inactive chromatin. In some cases, transcriptionally active or inactive chromatin can be distinguished by gross morphology. Three striking examples are the less compact chromatin associated with active genes in the lampbrush chromosomes of amphibian oocytes, in the ribosomal RNA genes of *Oncopeltus* (Foe, 1977), and in the giant puffs of the insect salivary gland chromosomes (Lamb & Daneholt, 1979). Enzymatic probes of chromatin structure have also indicated structural differences between active and inactive chromatin: active chromatin is more accessible to some proteins that bind DNA. DNase I preferentially digests globin genes that are active in erythrocyte nuclei but does not preferentially digest the inactive

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