Elastin Biosynthesis in Chick Embryonic Lung Tissue. Comparison to Chick Aortic Elastin†

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ABSTRACT: The synthesis of elastin was studied in chick embryonic lung and compared to that found in embryonic aortic tissue. Messenger ribonucleic acid (mRNA) was isolated from lung and aortic tissues and translated in an mRNA-dependent rabbit reticulocyte lysate. The results demonstrate that both tissue RNA preparations direct the synthesis of two elastin proteins possessing molecular weights of 70 000 and 73 000, which are immunoprecipitable with antibody directed against chick aortic tropoelastin. Organ culture of embryonic lungs and aortas followed by extraction of the [^{3}H]valine-labeled proteins with urea in the presence of reducing and alkylating reagents revealed the presence of two immunoreactive elastin proteins similar to those synthesized in the cell-free system. Limited tryptic and chymotryptic peptide mapping of the two elastin proteins synthesized in aortic organ cultures revealed a strong homology between the proteins with only minor detectable differences. The 70 000-dalton elastin protein was isolated from lathyritic chick lungs and shown to be very similar to, if not identical with, the 70 000-dalton protein isolated from chick aortas, in amino acid composition, molecular size, and immunoreactivity. Automated sequencing of [^{3}H]valine-labeled tropoelastin (70 000-dalton species) isolated from aorta and lung organ cultures demonstrated identical positions of valine residues in the NH_2-terminal region of both proteins. The results from these studies demonstrate that elastin synthesis in two unique embryonic tissues involves the production of two distinct polypeptide chains, referred to in this paper as tropoelastin a and b. the 70 000-dalton protein (tropoelastin b) is similar in electrophoretic behavior to conventional tropoelastin and appears to be identical in aortic and lung tissues as judged by amino acid analyses, electrophoretic migration, high-pressure liquid chromatography, and and automated sequence analyses.

During the last 10 years there have been many reports in the literature concerning the structure and biosynthesis of aortic elastin. It is now well established by several laboratories that tropoelastin is the soluble precursor to insoluble elastin (Rucker et al., 1977; Rosenbloom & Cywinski, 1976; Narayanan et al., 1976; Uitto et al., 1976). Additionally, Burnett & Rosenbloom (1979) and Burnett el al. (1980) have demonstrated that the cell-free translation of chick aortic mRNA[1] results in a 70 000-dalton protein identified as tropoelastin by immunoprecipitation.

Although the importance of elastin in pulmonary function has been well documented (Pierce et al., 1961; Turino et al., 1968; Johanson & Pierce, 1972), little is known concerning elastin biosynthesis in lung tissue. Much of the research effort into lung elastin has thus far been directed toward studying lung insoluble elastin and its degradation associated with pulmonary obstructive diseases such as emphysema (Eriksson, 1965; Horwitz et al., 1976).

Recently, our laboratory has reported on the cell-free translation of chick aortic mRNA and a comparison of those translation products with proteins synthesized in chick aortic organ cultures (Foster et al., 1980a). This study revealed that the initial elastin gene products consist of two polypeptide chains of 73 000 and 70 000 daltons. The existence of the two elastin proteins was also confirmed in organ cultures. The present investigation was undertaken to examine the precursor forms of elastin in lung tissue via the isolation and translation of lung elastin mRNA and the identification of soluble forms of elastin in lung organ culture. Parallel with these studies, tropoelastin b was purified from lathyritic chick lung and compared to tropoelastin b obtained from aortic tissue.

Experimental Procedures

Materials. L-[2,3-^{3}H]Valine (23 Ci/mmol) and L-[5-^{3}H]proline (38 Ci/mmol) were purchased from Schwarz/Mann. L-[^{35}S]Cysteine (570 Ci/mmol) and L-[^{35}S]methionine (682 Ci/mmol) were obtained from New England Nuclear. Guanidine hydrochloride (Gdn·HCl) and diethyl pyrocarbonate were purchased from Sigma Chemical Co. Micrococcal nuclease (*Staphylococcus aureus*, 15 000 units/mg) was purchased from Boehringer-Mannheim Biochemicals. Heat-killed, formalin-fixed *S. aureus* was prepared in our laboratory according to Kessler (1975).

Isolation of Tropoelastin b from Lathyritic Chicks. A total of 200 1-day-old chicks were raised on a diet of commercial starting feed for 1 week. They were then fed a diet of the starting feed supplemented with 0.1% (w/w) α-aminoacetonitrile hydrochloride and 0.05% ϵ-aminocaproic acid for an additional 8 days. The chicks were sacrificed and tropoelastin b was isolated from aortic and lung tissue as previously described (Foster et al., 1975). Major blood vessels were removed from the lungs prior to homogenization. The only exception was that fresh MalNEt (0.1 M) and DFP (0.001 M) were added after every precipitation step to ensure sufficient concentrations of active proteolytic inhibitors. At the

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; NaDodSO$_4$, sodium dodecyl sulfate; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium ethylenediaminetetraacetate; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*′-tetraacetate; Cl_3-CCOOH, trichloroacetic acid; MalNEt, *N*-ethylmaleimide; DFP, diisopropyl fluorophosphate; DEAE, diethylaminoethyl; CM, carboxymethyl; mol wt, molecular weight; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DMAA, dimethylallylamine; DITC, diisothiocyanate; PTH, phenylthiohydantoin derivatives of amino acids; Gdn·HCl, guanidine hydrochloride.

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end of the isolation procedure, the lung tropoelastin was judged impure by $NaDodSO_4$–polyacrylamide gel electrophoresis so that another purification step was added to achieve homogeneity. Further purification was obtained by dissolving (1 mg/mL) the final product of the lung sample which contained tropoelastin plus contaminants in the original phosphate extracting buffer with appropriate proteolytic inhibitors. The sample was heated at 37 °C for 20 min, and the resulting coacervate was collected by centrifugation at 10000*g* for 30 min. The resulting pellet was redissolved in H_2O at 4 °C, dialyzed extensively against H_2O at 4 °C, and lyophilized.

Preparation of RNA. In order to prevent RNase contamination, all glassware was heated to 190 °C for 16 h. Where appropriate, materials were treated with a freshly prepared solution of diethylpyrocarbonate (0.5% in sterile H_2O). Solutions were autoclaved where possible, and the more heat-labile solutions were sterilized through a 0.2-μm membrane filter. Sterile techniques were employed throughout RNA isolation and translation procedures. The isolation of RNA from lung tissue was based on the technique of Strohman et al. (1977). Lungs from 20 dozen chick embryos (White Leghorn) were placed into 6 M Gdn·HCl (1–3 g of tissue/19 mL). Potassium acetate (2 M, pH 5.0) was added to make a 5% solution. The suspension was homogenized with a Brinkman polytron at high speed for 2 min at room temperature and then precipitated by the addition of 0.5 volume of 95% ethanol at −20 °C overnight. The precipitate was centrifuged for 1 h at 16000*g* at −20 °C, and the resulting pellet was resuspended at room temperature in 6 M Gdn·HCl (0.5 volume of the original extracting volume of 6 M Gdn·HCl) and 5% 0.5 M EDTA (pH 7). The mixture was vortexed thoroughly, followed by precipitation with addition of 0.25 volume of 2 M potassium acetate (pH 5.0) and 0.5 volume of 95% ethanol at −20 °C. After storage overnight at −20 °C, the precipitate was centrifuged for 10 min at 16000*g* (−20 °C), and the pellet was dried under a stream of N_2 to ensure complete removal of ethanol. The pellet was then resuspended in 20 mM EDTA, pH 7 (0.15 volume of the original extracting volume of 6 M Gdn·HCl), and extracted with 2 volumes of chloroform–butanol (4:1). The aqueous and organic phases were separated by centrifugation at 10000*g* for 10 min, and the aqueous phase was carefully removed. The organic phase was then extracted twice with 20 mM EDTA, pH 7 (same volume as described above). The aqueous layers were pooled and precipitated by the addition of 0.1 volume of 2 M potassium acetate (pH 5.0) and 2.5 volumes of 95% ethanol at −20 °C. After storage overnight at −20 °C, the pellet was collected by centrifugation at 16000*g* for 1 h (−20 °C), dried under a stream of N_2, and resuspended in sterile H_2O (0.15 volume of the initial extracting volume). The RNA was precipitated by the addition of 2 volumes of 4.5 M sodium acetate (pH 6) and stored overnight at −10 °C. The precipitate was centrifuged for 1 h at 16000*g* (−10 °C), and the pellet was washed with 95% ethanol at −20 °C. The washed RNA pellet was dried under a stream of N_2, dissolved in sterile H_2O, and stored precipitated at −20 °C in 0.1 volume of 2 M potassium acetate and 2.5 volumes of 95% ethanol. The yield of total RNA from lung tissue averaged 2–3 mg/g of tissue weight (assuming 20 A_{260} units/mg of RNA).

As a comparison, aortic RNA was isolated from the same chick embryos. In contrast to the above isolation procedure described for lung RNA, aortic RNA was isolated by extraction in 5 M guanidinium thiocyanate followed by cesium chloride gradient centrifugation (Foster et al., 1980a). It should be noted that we have used both RNA isolation procedures on lung and aortic tissues. The guanidinium thiocyanate methodology is more rapid and works very well for aortic tissue. However, the lung tissue, probably because of its greater complexity, necessitates more extensive precipitation steps to obtain pure RNA preparations.

The integrity of the isolated RNA was examined on denaturing, 99% formamide gels according to the method of Pinder et al. (1974) before it was used in further experiments. Prior to translation, the precipitated RNA was centrifuged at 10000*g* for 1 h at −20 °C, dried under a stream of N_2, and dissolved in H_2O. The RNA solution was heated at 70 °C for 1 min and quickly immersed in an ice bath just prior to addition to translation assays.

Preparation of Lysate. Rabbit reticulocyte lysates were prepared according to the procedure of Evans & Lingrel (1969). The lysate was rendered mRNA dependent by using micrococcal nuclease, by a modification of the method of Pelham & Jackson (1976) as previously described (Foster et al., 1980a).

Translation Conditions. The translation assay was prepared to a total volume of 62 μL as previously described (Foster et al., 1980a). Briefly, the final concentrations of compounds in the translation assay were 75 μM glycine, valine, proline, and alanine, and 25 μM each of all other amino acids, 20 mM Hepes (pH 7.6), 80 mM KCl, 1.3 mM $Mg(C_2H_3O_2)_2$, 1 mM ATP, 0.2 mM GTP, 12 mM creatine phosphate, 10 μg/mL creatine phosphokinase (115 units/mg), 0.2 mM spermidine, and 0.065 μM DFP. The concentrations of radioisotopes per assay were either 130 μCi of L-[2,3-^{3}H]valine and L-[5-^{3}H]-proline or 65 μCi of L-[^{35}S]methionine and L-[^{35}S]cysteine. In all cases where a radioactive amino acid was used, that particular unlabeled amino acid was omitted from the master mix.

The incorporation of isotope in the translation assay was determined by removing 2-μL aliquots at various time points and determining radioactive protein by the method of Pelham & Jackson (1976). The dried Cl_3CCOOH precipitates were counted in a Beckman LS7000 scintillation counter. The scintillation fluid consisted of 0.4% Omnifluor (w/v), 2.6% NCS tissue solubilizer (v/v), and 0.4% H_2O in toluene.

Organ Culture of Chick Embryonic Lungs and Aortas. Lungs were removed from 20 dozen 16-day-old chick embryos (White Leghorns) and incubated (37 °C) for 45 min in valine-deficient Dulbecco's modified Eagle's medium, containing 10% dialyzed fetal calf serum and α-aminoacetonitrile hydrochloride (100 μg/mL). The organs were then transferred to fresh medium (5 lungs/mL) containing 0.1 mCi/mL L-[2,3-^{3}H]valine and incubated for 1 h at 37 °C in a 5% CO_2 atmosphere. At the end of the incubation period, the medium was decanted, and lungs were rinsed twice with nonradioactive media and were placed into 1 mL of 2 M urea, containing 0.01 M Tris (pH 8.0), 0.01 M chloroquine, 0.002 M EDTA, and 0.01 M DFP (4 dozen lungs/mL). The suspension was homogenized with a polytron (4 °C) at high speed for 1 min. The homogenate was flushed thoroughly with a stream of N_2, reduced for 1 h with dithiothreitol (10 mg/mL), and then alkylated with MalNEt (12.5 mg/mL). Extraction was allowed to proceed for 2 h at 4 °C, after which time the extract was centrifuged at 20000*g* for 1 h. The supernatant (referred to as the urea extract) was used for identification of elastin.

For automated sequence analysis, another group of lungs were labeled in organ culture as described above. However, after incubation, the lungs were extracted (4 dozen lungs/mL) for 1 h at 4 °C with 0.02 M phosphate (pH 7.5), 0.5 M NaCl, 0.1 M MalNEt, 0.01 M chloroquine, 0.002 M EDTA, and

0.01 M DFP. The homogenate was then centrifuged at 15000*g* for 1 h, and the supernatant was used for isolation of tropoelastin b. The phosphate buffer extract will subsequently be referred to as the NSB extract (neutral salt buffer).

For comparative studies, aortas were taken from the same embryos and incubated with [^{3}H]valine as described above. However, the aortas were first extracted with phosphate buffer for 2 h before extraction with 2 M urea in the presence of reducing and alkylating agents (Foster et al., 1980a). We have found that aortic tissue in 16-day-old embryos contains a much higher proportion of tropoelastin b which can be preferentially extracted with a neutral salt buffer (Barrineau et al., 1981). If the aortas are then extracted with urea, the tropoelastins a and b are recovered in approximately equal amounts. We have taken advantage of equal amounts of the two proteins found in the urea extracts for peptide mapping, which will be described below.

Peptide Mapping. Peptide mapping of the two tropoelastin proteins (a and b) was performed by a modification of the method of Cleveland et al. (1977) using both limited trypsin and chymotrypsin proteolysis. Aliquots (10 μL) of the [^{3}H]valine-labeled, aorta organ culture urea extract were run on a 20-slot, 8% $NaDodSO_4$ slab gel described below with the only exception that the running gel was made 1 mM in EDTA. Since a major 70 000-dalton protein found in the reticulocyte lysate serves to accentuate the separation of the a and b tropoelastins and is easily located by Coomassie Blue staining, 2 μL of the reticulocyte lysate was added to each 10-μL sample of aortic urea extract. After electrophoresis and staining of the 8% gel, 5 × 2 mm slices were cut out of the gel on the top and bottom of the stained 70 000-dalton reticulocyte protein found in the lysate. The slices were soaked for 30 min in 10 mL/slice 0.125 M Tris buffer (pH 6.8) containing 1% $NaDodSO_4$ and then stored frozen at −20 °C until used. For limited trypsin digestion, the thawed gel slices were equilibrated at 4 °C for 30 min in the above-mentioned Tris buffer containing 30% glycerol. The slices were then placed into the sample wells (10-slot wells) of a second 15% $NaDodSO_4$ slab gel and overlaid with 30 μL of the Tris–$NaDodSO_4$ buffer containing 20% glycerol and bromophenol blue. On top of this, 10 μL of Tris–$NaDodSO_4$ buffer containing 10% glycerol and varying amounts of trypsin or chymotrypsin were overlaid. Electrophoresis was performed until the tracking dye was ~3 mm above the interphase between the stacking and running gels. The power was turned off for 30 min, and then electrophoresis was resumed. Detection of tritiated peptide fragments was accomplished by fluorography as described elsewhere in the text.

$NaDodSO_4$ Slab Gel Electrophoresis. Vertical slab gel electrophoresis was performed according to the method of Laemmli (1970) using an 8% running gel. Samples from translation assays were prepared by precipitating 5–10 μL of reaction mixture with 8 volumes of acetone. The acetone pellets were dried under N_2 and redissolved in sample buffer containing β-mercaptoethanol as described by Laemmli (1970). Organ culture samples were prepared by adding an equal volume of 2× sample buffer (Laemmli, 1970) to 10 μL of the urea extract. Purified samples obtained from tissue extracts were dissolved directly into sample buffer (1 mg/mL), and 5–10 μg was applied to the sample well. Gels were fixed in 10% trichloroacetic acid and 50% ethanol for 30 min and stained in 0.2% Coomassie Blue, 10% trichloroacetic acid, and 25% ethanol for 30 min. Destaining was accomplished by washing overnight in 10% acetic acid. Fluorography of the gel was performed according to Bonner & Laskey (1974). The dried gel was exposed to Kodak X-Omat R film at −80 °C.

Preparative $NaDodSO_4$ slab gel electrophoresis was performed in order to separate tropoelastin a and b obtained from the aortic organ culture. A Hoefer Model SE 220 slab gel unit equipped with a preparative accessory kit was used. An 8% running gel and 30% plug gel was employed with a 0.1 M Tris buffer as elution buffer. Aliquots of the urea extract plus lysate were loaded into 10-slot wells, and electrophoresis was performed for 19 h at 35 MA. A dansylated albumin standard (Gray, 1967) was added to each sample to monitor the electrophoresis migration. When the dansylated protein approached the elution chamber, the elution buffer was collected in 1.5-mL fractions. After completion of electrophoresis, the radioactivity of each collected fraction was determined, and the resulting radioactive peaks were dialyzed extensively against 0.1% acetic acid and lyophilized. The samples were then analyzed on regular $NaDodSO_4$ slab gels followed by fluorography as described above.

High-Pressure Liquid Chromatography. The purity of the isolated lung tropoelastin was examined by gel filtration on a Waters Model 204 liquid chromatograph, using a Waters dual protein separation column, 1–125, with an isocratic system of 0.2 M Tris buffer (pH 7.0) at a flow rate of 2 mL/min. The column effluent was monitored at 230 nm. Purified chick aortic tropoelastin was used to standardize the column.

Immunological Techniques. Antiserum against chick aortic tropoelastin b was prepared in rabbits as previously described (Foster et al., 1980b). The isolated lung tropoelastin was examined for immunological identity by double immunodiffusion. For use in the cell-free system, the γ-globulins were partially purified from both immune and nonimmune sera by ammonium sulfate precipitation (Harboe & Ingild, 1973). The γ-globulins were rendered RNase free by using a combined DEAE–CM-cellulose column according to Palacios et al. (1972). Prior to the addition of γ-globulins (immune and nonimmune) to the translation assay, they were dialyzed against H_2O for 8 h, lyophilized, and redissolved in sufficient H_2O (3.5 μg/μL) to allow the addition of 35 μg of γ-globulins/translation assay.

Immunoprecipitation of the cell-free and organ culture products was accomplished by using heat-killed, formalin-fixed *S. aureus* as an immunoadsorbent as we have previously described for both cell-free and organ culture systems (Foster et al., 1980a). Immunological identity of the aortic and lung tropoelastin was examined by double immunodiffusion (Foster et al., 1976).

Amino Acid Analysis. Isolated tropoelastin samples were hydrolyzed in vacuo at 110 °C for 22 h in constant-boiling HCl. Amino acid analyses were performed on a Beckman Model 119C automated amino acid analyzer.

Automated Radiosequencing. [^{3}H]Valine-labeled tropoelastin b obtained from neutral salt buffer extracts of aortic and lung organ culture was subjected to automated sequencing on a Sequemat Mini-15 solid phase sequencer (Laursen, 1971). Tropoelastin b was immunoprecipitated from the organ culture extracts with antitropoelastin γ-globulin and *S. aureus* as described above. Following thorough washing of the antigen–antibody–*S. aureus* complex, the radiolabeled tropoelastin was recovered by vigorous shaking in 70% formic acid for 15 min. After centrifugation, the supernatant was diluted with H_2O and lyophilized. A total of 1 mg of purified chick aortic tropoelastin (Foster et al., 1975) was added to the lyophilized material to act as a carrier. The immunoprecipitate was dissolved in 200 μL of 6 M Gdn·HCl (pH adjusted to 9.8 with triethylamine), 200 μL of DMAA buffer, and 10 μL of tri-

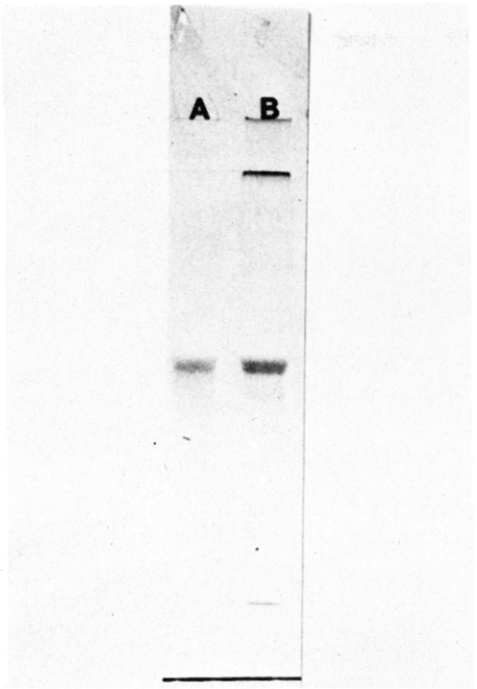

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of chick tropoelastin b purified from lung (lane A) and aorta (lane B). Detection of protein bands accomplished by staining with Coomassie Blue.

ethylamine. A total of 200 mg of DITC-activated aminopropyl glass was added and the sample vortexed (Watcher et al., 1973). Incubation was performed at room temperature for 90 min with vigorous shaking. A total of 100 μL of ethanolamine was then added to block excess DITC groups, and the sample was incubated for 1 h at room temperature with shaking. The glass beads were washed 3 times with 1 mL of DMAA and 3 times with 1 mL of methanol and dried under vacuum. Aliquots of the various washes were examined for radioactivity content in order to determine the percent coupling efficiency.

Sequencer fractions were converted to PTH-amino acids in aqueous acid and extracted into ethyl acetate to minimize background (Edman & Begg, 1967). Since only [^{3}H]valine was used as the radioactive label, the positions of valine in lung and aortic tropoelastin b were assigned by examining the organic layers of each sequencer step for radioactivity in a liquid scintillation counter.

Results

It should be noted at this point that throughout the paper we refer to the two elastin proteins identified in cell-free translations and organ culture systems as tropoelastin a and b. Tropoelastin b (70 000 daltons) behaves electrophoretically as conventional tropoelastin which has been described by a number of investigators (Smith et al., 1972; Sandberg et al., 1975; Rucker & Goettlich-Rieman, 1972; Foster et al., 1975). Further, preliminary sequencing of the NH_2-terminal region of tropoelastin b, isolated from chick aortas and lung organ cultures (see lung organ culture below), has revealed that the NH_2-terminal regions of tropoelastin b and conventional chick aortic tropoelastin are identical in terms of the positions of valine residues. Tropoelastin a, which has a slightly higher molecular weight (73 000), different p*I*, and higher cysteine content, has only recently been found in cell-free translations of aortic mRNA and aorta organ cultures (Foster et al., 1980a).

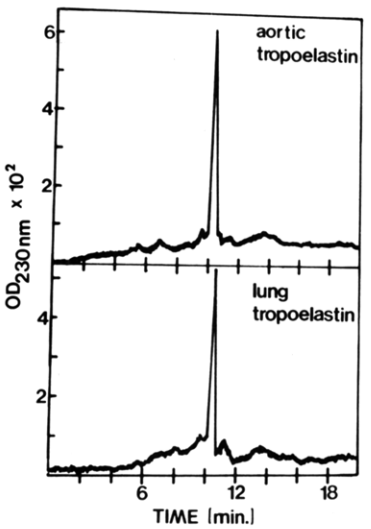

FIGURE 2: High-pressure liquid chromatograms of aortic and lung tropoelastins b on Waters dual protein separation columns (see text for experimental details).

Table I: Amino Acid Compositions of Chick Lung and Aorta Tropoelastin b[a]

amino acid	lung	aorta	amino acid	lung	aorta
Lys	33	29	Pro	135	135
His	3	2	Gly	310	314
Arg	8	7	Ala	179	186
Hyp	10	11	Val	172	179
Asp	7	6	Ile	18	19
Thr	13	12	Leu	55	50
Ser	11	9	Tyr	11	9
Glu	16	16	Phe	19	16

[a] Compositions are expressed as residues per 1000 amino acid residues.

Comparison of Chick Lung and Aortic Tropoelastin b. The $NaDodSO_4$ gel electrophoretogram of tropoelastin b purified from lathyritic chick lung and aortic tissue is given in Figure 1. Both proteins migrate as a single, homogeneous band with an apparent molecular weight of 70 000. The high-pressure liquid chromatographs given in Figure 2 confirm the homogeneity and similarity of the two tropoelastin samples, since both samples elute as one major peak in identical elution positions. The amino acid compositions of both lung and aortic tropoelastin b are given in Table I. As can be seen, both b tropoelastins possess very similar, if not identical, amino acid compositions. The yield of tropoelastin from lung tissue averaged 16 μg/g of wet weight, which is considerably lower than that obtained from aortic tissue which averaged 728 μg/g of wet weight.

Translation of Embryonic Chick Lung mRNA. The molecular weight profile of [^{3}H]proline-labeled proteins directed by lung mRNA in the mRNA-dependent reticulocyte lysate is given in Figure 3 (lane A). For comparative purposes, the translation of aortic RNA from the same batch of embryos is also included. The molecular weight distribution of [^{3}H]-proline-labeled proteins directed by the lung mRNA (lane B) ranges from 170 000 to 10 000.

Interestingly, the two prominent [^{3}H]proline-labeled proteins found in the aortic mRNA translation in the area of 70 000 daltons (lane B) are also seen among the lung mRNA products. We have previously shown that these latter aortic mRNA-directed proteins are elastin-like proteins and possess apparent molecular weights of 73 000 and 70 000. The separation of the two elastin proteins is accentuated by the

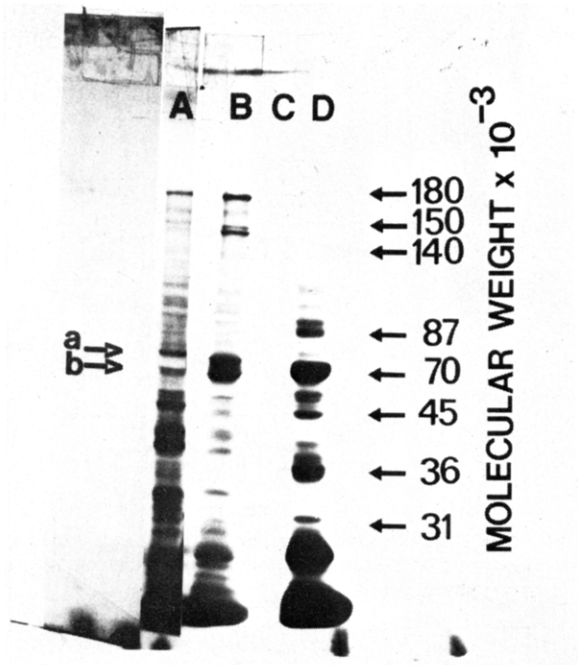

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of translation products from mRNA-dependent reticulocyte lysate with added lung RNA (lane A) and aortic RNA (lane B) labeled with [^{3}H]proline. Lane C is digested reticulocyte lysate with no added RNA incubated with [^{3}H]proline. Lane D is untreated reticulocyte lysate with no added RNA incubated with [^{3}H]proline. Translation time was 90 min. Exposure time was 1 week.

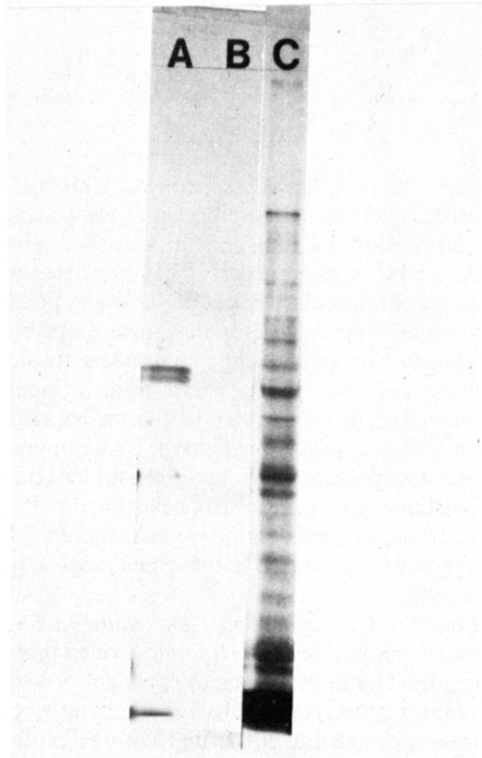

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of translation products directed by lung RNA labeled with [^{3}H]proline (lane C), immunoprecipitate of cell-free translation of lung RNA immunoprecipitated by addition of 70 μg of IgG after translation was complete (lane B), and immunoprecipitate of cell-free translation of lung RNA immunoprecipitated by the presence of 35 μg of IgG during translation and an additional 35 μg after translation was complete (lane A). Exposure time was 1 week.

presence of a major 70 000-dalton protein found in the reticulocyte lysate used for translation (Foster et al., 1980a).

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of tropoelastin b (lane A) and tropoelastin a (lane B) obtained from [^{3}H]valine-labeled aortic organ cultures and separated by preparative $NaDodSO_4$–polyacrylamide gel electrophoresis (see text for specific details). Lane C shows the two tropoelastins prior to preparative separation. A 5-μL quantity of lysate was added to each sample. Exposure time was 4 days.

Immunoprecipitation of [^{3}H]proline-labeled proteins directed by lung mRNA is shown in Figure 4. As seen, both the 70 000- and 73 000-dalton proteins are precipitated with antibody directed against chick aortic tropoelastin. It is also important to point out that the immunoprecipitate shown in lane A was run without the addition of lysate proteins. Although the two tropoelastins migrate much closer under these conditions, one can still discern two protein bands.

In order to demonstrate that the addition of lysate to samples was not artificially separating tropoelastin into two components, we performed preparative $NaDodSO_4$ gel electrophoresis. Figure 5 presents a fluorogram of a $NaDodSO_4$ gel of tropoelastin b (lane A) and tropoelastin a (lane B) obtained from aortic organ culture which have been separated by preparative gel electrophoresis and then rerun separately on another gel in the presence of lysate. If the lysate protein were causing tropoelastin to split into two apparently different proteins, then this artifact should be evident when the separated proteins are reexamined by gel electrophoresis in the presence of the lysate. As seen in Figure 5, both tropoelastin a and tropoelastin b migrate as single homogeneous proteins with different electrophoretic mobilities.

Figure 6 shows the labeling of lung mRNA-directed proteins with [^{35}S]methionine (lane A), [^{35}S]methionine and cysteine (lane C), and [^{35}S]cysteine (lane E). The two tropoelastins (a and b) are labeled with both methionine and cysteine. The significance of the incorporation of the latter sulfur-containing amino acids will be included under Discussion.

Embryonic Lung Organ Culture. The biosynthesis of elastin in lung organ cultures was studied to determine if the cell-free translation of lung mRNA faithfully reflects elastin synthesis in intact tissue. The molecular weight distributions of proteins resulting from the [^{3}H]valine labeling of chick embryonic lungs

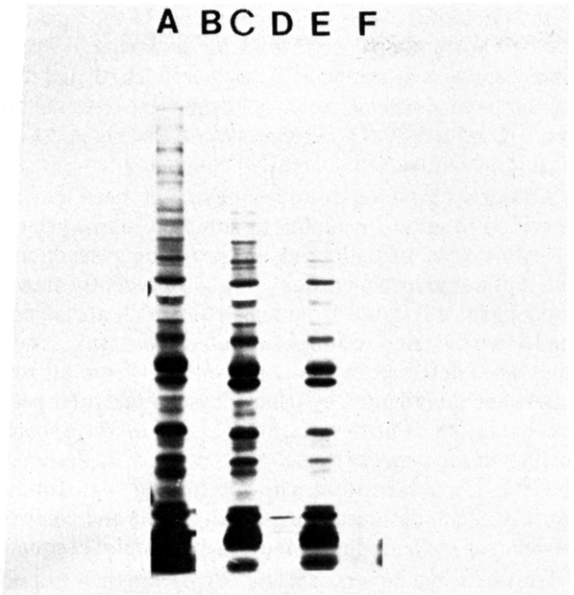

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis of lung RNA translation products using the mRNA-dependent reticulocyte lysate with [^{35}S]methionine (A), [^{35}S]methionine and [^{35}S]cysteine (C), and [^{35}S]cysteine (E). Lanes B, D, and F are digested reticulocyte lysates with no added RNA incubated with [^{35}S]methionine (B), [^{35}S]methionine and [^{35}S]cysteine (D), and [^{35}S]cysteine (F). Translation time was 90 min. Exposure time was 4 h.

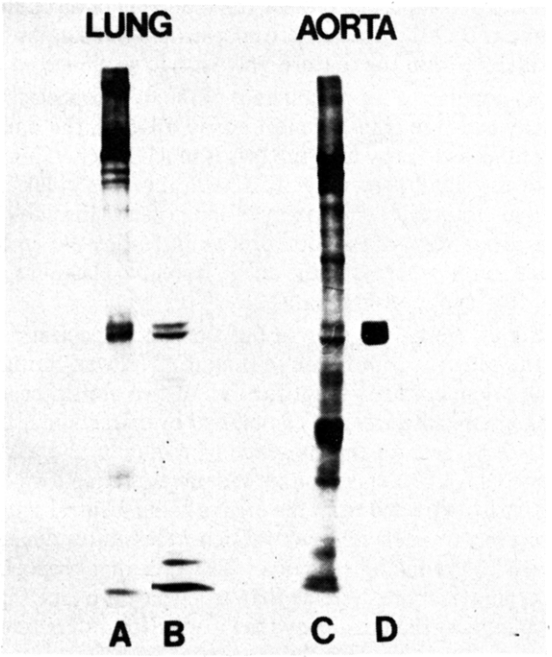

FIGURE 7: $NaDodSO_4$–polyacrylamide gel electrophoresis of immunoprecipitates from lung and aorta organ culture. (A) Urea extract of chick lungs labeled with [^{3}H]valine. (B) Immunoprecipitate of urea extract from chick lungs. (C) Urea extract of chick aortas labeled with [^{3}H]valine. (D) Immunoprecipitate of urea extract from chick aortas. Exposure time was 2 weeks for lanes A and B and 1 week for lanes C and D.

(lane A) and aortas (lane C) in organ culture are presented in Figure 7. The immunoprecipitation products of both tissue extracts are also included (lanes B and D). Of special interest was the finding that lung organ culture (lane A) contains two proteins, which are immunoprecipitated with tropoelastin antibody (lane B) which is in agreement with the cell-free data (Figure 4).

Peptide Mapping of Tropoelastins a and b. The peptide mapping technique of Cleveland et al. (1977) was performed to determine the degree of homology between tropoelastins

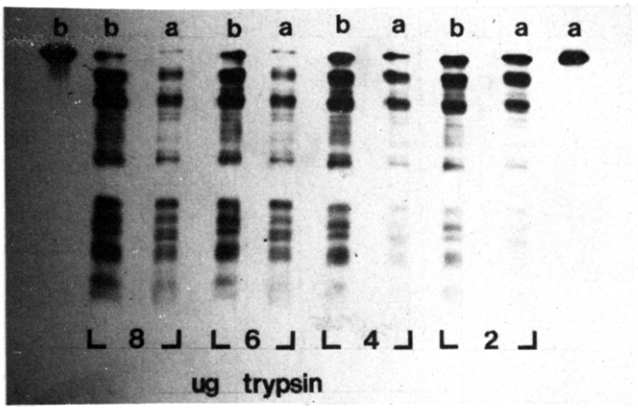

FIGURE 8: Fluorograph of peptide maps of [^{3}H]valine-labeled aortic tropoelastins a and b obtained from organ culture. Amounts of trypsin added are indicated under the various lanes. Exposure time was 3 weeks.

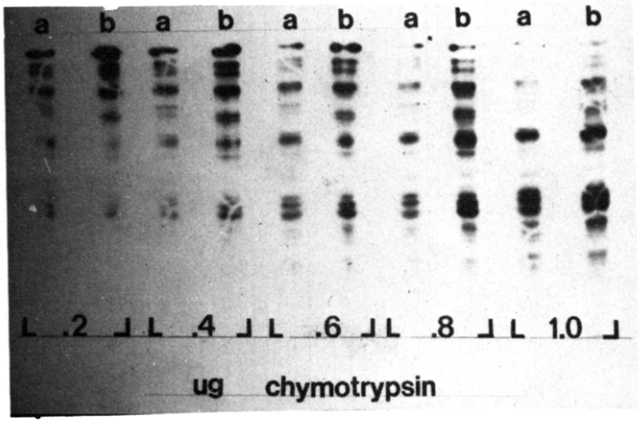

FIGURE 9: Fluorograph of peptide maps of [^{3}H]valine-labeled aortic tropoelastins a and b obtained from organ culture. Amounts of chymotrypsin are indicated under the various lanes. Exposure time was 3 weeks.

a and b. We have found that the two tropoelastins are labeled to a much higher specific activity in aortic organ cultures compared to similar cultures of lungs.

Since the fluorographic step in the Cleveland procedure is facilitated when proteins are labeled to a high specific activity and other data strongly suggest the homology of the lung and aortic tropoelastins, we have chosen to do comparative peptide mapping of tropoelastins a and b obtained from aortic cultures. Figures 8 and 9 picture the limited tryptic and chymotryptic peptide maps of the [^{3}H]valine-labeled a and b tropoelastins obtained from aorta organ cultures. As can be seen, the peptide patterns of the two proteins generated by both enzymes are similar, but, more importantly, there are obvious differences. This further corroborates our earlier finding that the two forms of tropoelastin must be somewhat similar as judged by comparable levels of valine and proline incorporation and immunoreactivity but are distinct based on two-dimensional gels, molecular weight determinations, and cysteine content (Foster et al., 1980a).

Automated Sequence Analysis. [^{3}H]Valine-labeled tropoelastin b immunoprecipitated from the NSB extracts of lung and aortic organ culture was subjected to 25 cycles of the automated sequencer. The resulting positions of valine residues in the primary sequence of the two different tissue tropoelastin b forms are given in Figure 10. Included in the figure are the positions of valine residues in purified tropoelastin isolated from lathyritic chick aortas (Foster et al., 1975). As can be seen, both the lung and aortic tropoelastins possess the same positions of valine residues in the NH_2-terminal region. The

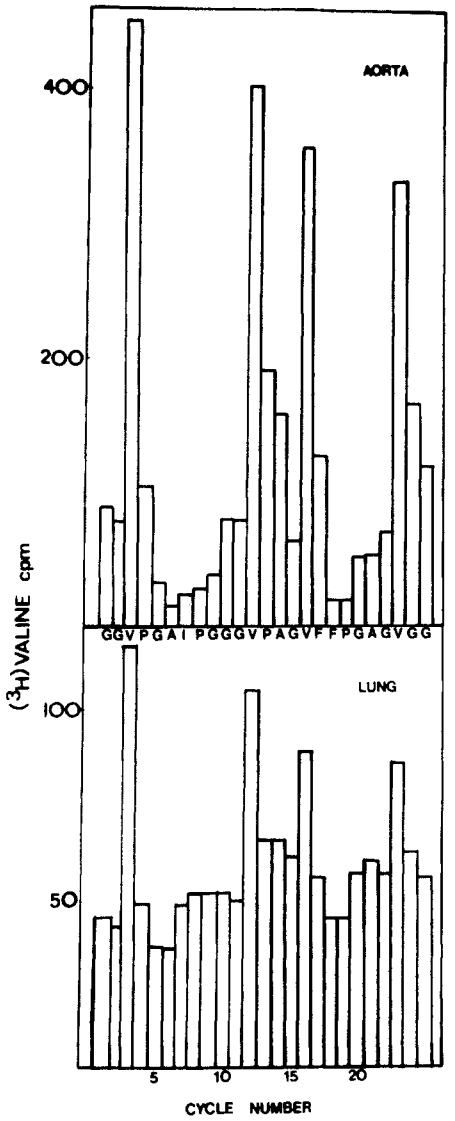

FIGURE 10: Automated Edman degradation of [^{3}H]valine-labeled tropoelastin b immunoprecipitated from NSB extracts of aorta and lung organ cultures. The NH_2-terminal sequence of conventional tropoelastin is also included (Foster et al., 1975).

data obtained are obtained are in agreement with the sequence obtained on conventional tropoelastin.

Discussion

Studies of elastin biosynthesis by both cell-free translation of lung mRNA and lung organ culture systems reveal that the initial elastin gene products of this tissue consist of two polypeptide chains of approximately 73 000 and 70 000 daltons. These data are consistent with those we have recently obtained from aortic tissue (Foster et al., 1980a). As in the case of the two aortic tropoelastins obtained in the cell-free translation system, the two lung proteins incorporate cysteine and methionine.

The presence of sulfur-containing amino acid residues in lung and aortic tropoelastin is interesting in several respects. The lack of methionine residues in the amino acid composition of insoluble and soluble elastins is often used as a criterion of purity. The finding of methionine incorporation into the primary translation products of tropoelastin mRNAs and resistance of these tropoelastins to any obvious cleavage by cyanogen bromide (Foster et al., 1980a) suggest methionine is located at the NH_2 terminal and/or in a short signal peptide (Blobel & Dobberstein, 1975) which is removed during vectorial transport of the nascent polypeptide chain. We have recently determined the primary sequence of the signal peptide of chick aortic tropoelastin b (Karr & Foster, 1981). The signal peptide is 24 amino acid residues in length and possesses one methionine residue which is at the NH_2-terminal position (Karr & Foster, 1981). Tropoelastin a also possesses a signal peptide which we are currently sequencing.

Similarly, cysteine residues have not been definitively identified in either insoluble or soluble elastin, yet the tropoelastins coded by both chick aortic and lung elastin mRNAs reveal cysteine incorporation. We have recently shown that tropoelastin b isolated from lathyritic chick aortas contains one to two cysteine residues (unpublished data). These residues were detected by omitting MalNEt from all isolation buffers and quantitating cysteine as cysteic acid after performic acid oxidation. Further, the signal peptide of tropoelastin b does not contain any cysteine residues (Karr & Foster, 1981). These data are consistent with the finding that tropoelastin b lacks cysteine residues in its signal peptide and confirms the presence of cysteine in the mature polypeptide sequence.

Tropoelastin a incorporates more cysteine than tropoelastin b in the translations of chick lung or aortic mRNAs [see Figure 6 and Foster et al. (1980a)]. Although very little chemical data are available on tropoelastin a, we have demonstrated that its extraction from both aortic and lung tissue necessitates reduction in the presence of a denaturant such as urea (Foster et al., 1980a). The fact that extraction of tropoelastin a from tissue is accomplished only by the reduction of disulfide bonds suggests that this species of tropoelastin also contains cysteine residues within the mature polypeptide sequence.

As opposed to the prominence of the two tropoelastins seen in the cell-free translation of aortic mRNA, the amount of elastin coded for by lung mRNA is much lower. This reflects both the small percentage of elastin found in chick lung, as well as the greater complexity of lung tissue. Insoluble elastin represents <3% of the total protein in 16-day-old embryonic chick lungs, whereas in the aorta, insoluble elastin represents 30–40% (unpublished data).

Significantly, the presence of the two tropoelastins found in the cell-free translation of lung mRNA was confirmed in lung organ culture. Identification of two elastin proteins in lung organ culture was accomplished by extraction of the lung with 2 M urea in the presence of reducing and alkylating reagents. As we have found with aortic tissue, tropoelastin b is easily extracted from the organs with a neutral salt buffer. However, tropoelastin a extraction necessitates denaturants as well as reducing reagents. The fact that tropoelastin a incorporates more cysteine than tropoelastin b (see Figure 6) does suggest the possibility that if may form disulfide cross-links within the extracellular matrix.

In the present study, it was also shown that tropoelastin b isolated from lathyritic chick lung and aortic tissue is identical by the criteria we have thus far examined. These include electrophoresis, high-pressure liquid chromatography, immunoreactivity, and limited sequencing of the NH_2 termini. As yet, we have not been able to isolate sufficient quantities of the 73 000-dalton protein from lathyritic aorta or lung to perform adequate chemical analysis.

More recently, we have found that tropoelastin b isolated from lathyritic pig aortic and cartilage tissues appear identical as judged by amino acid composition, molecular weight, immunoreactivity, and peptide maps (Foster et al., 1980c). These latter data, together with the present finding of a strong similarity between chick lung and aortic tropoelastin b, suggest that tropoelastin b is identical in different tissues within a particular species. As mentioned above, we do not yet possess

sufficient chemical data on tropoelastin a to draw any similar conclusion. However, in an independent study we have found that the proportion of tropoelastins a and b differs significantly between lung and aortic tissues during chick embryonic development (Barrineau et al., 1981). This finding raises the possibility that the ratio of tropoelastin b to tropoelastin a differs in various tissues and may contribute to the unique molecular organization of elastic fibers seen among different tissues.

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