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Correlation of Functional Elastin Messenger Ribonucleic Acid Levels and Rate of Elastin Synthesis in the Developing Chick Aorta†

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ABSTRACT: Thoracic aortas from 8- to 16-day chick embryos were incubated in vitro for 30 min with [^{3}H]valine, and the newly synthesized, labeled proteins were subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate and analysis with affinity-purified, elastin-specific antibody. The results demonstrate that at day 8 ~21% of the incorporated [^{3}H]valine was found in tropoelastin (70 000 daltons). In the following 8 days of development, there was a significant increase in the relative incorporation into tropoelastin so that at day 16, 43% was now found in tropoelastin. mRNA was isolated from aortas of various age embryos by digestion of the tissue with proteinase K in sodium dodecyl sulfate followed by chromatography on oligo(dT)-cellulose in order to investigate possible control mechanisms. The mRNA was translated in a reticulocyte lysate system, and the incorporation of [^{3}H]valine into products which were precipitable by either trichloroacetic acid or the elastin-specific antibody was determined. When 8-day aorta mRNA was translated, 18% of the trichloroacetic acid precipitable [^{3}H]valine was also immunoprecipitable. This value increased to 36% at day 10 and 45% at day 16. The injection of 150 μg of hydrocortisone 21-phosphate into 8-day eggs produced a significant increase in both the relative rate of tropoelastin synthesized by the isolated aortas and the amount of immunoprecipitable protein synthesized in the reticulocyte lysate system in response to mRNA isolated from the aortas of the treated embryos. The close agreement between the values determined from aortas incubated in vitro and from isolated mRNA translated in a cell-free heterologous system indicates that the observed changes in tropoelastin synthesis during development and after hydrocortisone administration are governed by the elastin mRNA content of the aortas.

Elastin is a vital component of the major blood vessels and lungs of vertebrates. A single 70 000-dalton polypeptide, designated tropoelastin, appears to be a soluble intermediate in the synthesis of the protein (Sandberg et al., 1969; Murphy et al., 1972; Rucker et al., 1973; Smith & Carnes, 1973; Foster et al., 1975; Narayanan & Page, 1976; Rosenbloom & Cywinski, 1976; Uitto et al., 1976), although the existence of a larger precursor has been reported (Foster et al., 1976, 1978). After secretion from the cell, the soluble elastin chains are extensively cross-linked by the enzymatic oxidation of lysine residues, resulting in highly insoluble elastin fibers (Partridge, 1962; Franzblau et al., 1965; Miller et al., 1965; Partridge et al., 1966; Pinnell & Martin, 1968). These mature fibers are responsible for the rubberlike resilience of the aorta and other arteries.

Microscopic observations and biochemical analysis of the embryonic chick aorta indicate that elastin biosynthesis follows a characteristic developmental pattern during embryogenesis. During the first 6 days of development, the aorta appears to be composed of a homogeneous population of rounded cells, and elastin fibers cannot be detected definitively by specific histologic staining techniques. After day 7 or 8 of development, however, the cell population appears more heterogeneous, smooth muscle cells become evident, and elastin fibers become readily discernible in the extracellular matrix (Karrer & Cox, 1961; Kadar & Veress, 1974). From day 8 to 16 of development, the rate of tropoelastin synthesis in the aorta increases dramatically and intercellular elastin fibers increase in both number and size. This characteristic pattern can be altered by administration of hydrocortisone in ovo at day 8 of development so that the relative rate of tropoelastin synthesis in the 9-day embryo becomes similar to that of a 13-16-day embryo (Eichner et al., 1978).

The biochemical mechanisms involved in this rise in tropoelastin synthesis during embryogenesis or following hydrocortisone administration have not been examined to date. Recent evidence from several developmental and hormonally induced systems has demonstrated changes in the synthesis of specific proteins which closely parallel changes in the abundance of their respective mRNAs (Chan et al., 1973; Alton & Lodish, 1977; Soh & Sarkar, 1978). Proteins synthesized by freshly isolated aortas of various ages incubated in vitro were compared with the products synthesized in a rabbit reticulocyte lysate system in response to mRNA isolated from the same age aortas in order to investigate whether the

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increase in tropoelastin synthesis during development of the chick aorta could be accounted for by changing levels in functional elastin mRNA. The results demonstrate that there was a parallel increase in the rate of tropoelastin synthesis as measured in the whole aortas and in the cell-free translation system. Injection of hydrocortisone onto the chorioallantoic membrane of 8-day embryos produced a marked rise in the relative rate of tropoelastin synthesis by the aortas. Cell-free translation of mRNA isolated from the aortas of steroid-treated embryos showed an increase in tropoelastin synthesis similar to the effect seen in the whole aortas. The close agreement between the values determined from aortas incubated in vitro and from isolated mRNA translated in a cell-free heterologous system suggests that the observed changes in tropoelastin synthesis during development and after hydrocortisone administration are governed by the elastin mRNA content of the aorta.

Experimental Procedures

Materials. Oligo(dT)–cellulose (Type II) was purchased from Collaborative Research, and [^{3}H]valine was from New England Nuclear. Reagents for polyacrylamide gels were from Eastman Kodak while the reagents added to the reticulocyte lysate translation system and the protease inhibitors were from Sigma. All other chemicals were reagent grade.

Isolation of mRNA. Aortas were dissected from various age chick embryos as previously described (Murphy et al., 1972). The number of embyros used in each batch varied with the age: 175 from 8-day chicks, 150 from 9-day chicks, 100 from 10-day chicks, and 24 from 13- and 16-day chicks. Loose connective tissue was removed, and the aortas were cut along their long axes, rinsed in ice-cold Krebs medium to remove blood, and placed immediately in liquid nitrogen. The tissue (0.2–0.5 g) was homogenized in 10 mL of 10 mM Tris-HCl (pH 7.5) buffer containing 1% sodium dodecyl sulfate (NaDodSO$_4$),[1] 5 mM EDTA, and 65 μg/mL proteinase K (Beckman) (Rowe et al., 1978) except that a motorized Teflon homogenizer was used in place of a tight-fitting Dounce. The homogenate was then incubated at 40 °C for 1 h to digest any ribonucleases which might be present. After the incubation, 10 mL of 1 M NaCl was added and the sample was centrifuged at 13000g for 10 min at room temperature to remove any insoluble material.

mRNA was isolated from the supernatant by oligo(dT)–cellulose chromatography in NaDodSO$_4$ (Krystosek et al., 1975; Burnett & Rosenbloom, 1979). The column was equilibrated with 0.5% NaDodSO$_4$, 0.5 M NaCl, 4 mM EDTA, and 10 mM Tris-HCl (pH 7.5). The sample was loaded on the column and washed with the equilibration buffer until the absorbance at 254 nm returned to zero. The column was then washed, first with 100 mL of buffer containing 0.5 M NaCl, 4 mM EDTA, and 10 mM Tris-HCl (pH 7.5) to remove the NaDodSO$_4$ and finally with 15 mL of 0.5 M NaCl to remove the EDTA. The mRNA was then eluted with doubly distilled H_2O. The isolated mRNA was heated to 70 °C for 5 min and then cooled in ice water. It was stored at −70 °C and could be thawed and refrozen without loss of activity.

Translation of mRNA. Lysates were prepared from rabbit reticulocytes by modification of the procedure of Gilbert & Anderson (1971) and stored at −70 °C. The isolated mRNA was translated by modification of the procedure of Rowe et al. (1978). The lysate was made 25 mM with hemin and treated with micrococcal nuclease (Pelham & Jackson, 1976) which reduced the endogenous translation to 1 to 2% of the untreated lysate. ϵ-Aminocaproic acid was used at a concentration of 80 mM in the translation mix to inhibit proteolytic degradation of the tropoelastin. Lysates were optimized for K^+, Mg^{2+}, and spermidine concentrations. Each translation assay tube of 125 μL contained 50 μL of treated lysate, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0), 1.0 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 0.3 mM glycine, 0.2 mM alanine, 0.1 mM proline, 70 mM potassium acetate, 0.7 mM magnesium acetate, 0.4 mM spermidine, 100 μg/mL creatine kinase (Calbiochem), 40 μCi/mL [^{3}H]valine (New England Nuclear), and 80 mM ϵ-aminocaproic acid. Incubations were routinely carried out in plastic Falcon tubes at 26 °C for 1 h. The assays were terminated by placing the tubes in ice and adding 50 μL of a stop solution which gave the following final concentrations: 1.4% Triton X-100, 1.4% sodium deoxycholate, 4 mM EDTA, 20 mM *N*-ethylmaleimide, 10 μg/mL phenylmethanesulfonyl fluoride, 50 μg/mL tosyllysyl chloromethyl ketone, 50 μg/mL toluenesulfonylphenylalanyl chloromethyl ketone, 100 mM ϵ-aminocaproic acid. Fifty-microliter portions of the translation mixtures were added to 50 μL of 0.5 M Tris, 0.5 M NaOH, and 0.5 M H_2O_2 and incubated at 37 °C for 30 min in order to deacylate residual [^{3}H]valyl-tRNA and to decolorize the solution. Thirty-five microliters of this solution was spotted on a Whatman No. 3 filter disk and immersed in ice-cold 10% trichloroacetic acid (Cl_3AcOH). The filters were washed several times in cold 5% Cl_3AcOH, dried in a 150 °C oven, and placed in counting vials. The precipitated proteins were solubilized by adding 0.4 mL of NCS (Amersham) and incubating overnight at 37 °C.

Incubation of Aortas and Analysis of Labeled Proteins. Thoracic aortas were isolated from 8- to 16-day-old chick embryos as previously described (Murphy et al., 1972), and the aortas were slit longitudinally to facilitate diffusion through the tissue during the incubation. The aortas were washed in warm Krebs-Ringer and incubated in the same medium containing 2% fetal calf serum, 50 μg/mL ascorbate, and 100 μg/mL β-aminopropionitrile for 15 min at 37 °C in a shaking water bath. [^{3}H]Valine was then added and the incubation continued for 30 min. At the end of the incubation, the medium was removed and the aortas were homogenized with a glass–Teflon homogenizer in ice-cold 0.01 M sodium phosphate buffer, pH 7.4, containing 4 mM EDTA and 20 μg/mL phenylmethanesulfonyl fluoride. Sodium dodecyl sulfate and mercaptoethanol were added to a final concentration of 1% to the homogenates and separated media, and the samples were placed in a boiling water bath for 4 min. The samples were then dialyzed against 0.01 M phosphate buffer, pH 7.4, containing 0.1% NaDodSO$_4$ and 0.1% mercaptoethanol. After dialysis, the homogenates were centrifuged to remove insoluble matter, and aliquots of the solubilized, labeled proteins in the supernatant were counted in a scintillation counter. The insoluble residue was hydrolyzed and also counted. The solubilization procedure extracted ~90% of the labeled proteins. Aliquots of the proteins solubilized by the NaDodSO$_4$ were subjected to 5% polyacrylamide gel electrophoresis as previously described (Murphy et al., 1972).

For the purpose of preparing labeled proteins for testing with specific antibody, some incubated aortas were homogenized in a 2-mL solution containing the following reagents: 5% deoxycholate, 5% Triton X-100, 70 mM *N*-ethylmaleimide, 175 μg/mL toluenesulfonylphenylalanyl chloromethyl ketone,

[1] Abbreviations used: NaDodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

175 μg/mL tosyllysyl chloromethyl ketone, 35 μg/mL phenylmethanesulfonyl fluoride, 70 mM ε-aminocaproic acid, 14 mM EDTA, 100 μg/mL β-aminopropionitrile. The homogenized aortas were extracted for 24 h at 4 °C, the homogenate was centrifuged at 10000*g* for 45 min, and the supernatant was dialyzed against 20 mM ε-aminocaproic acid, 1% Triton X-100, and 1.0% deoxycholate. The residue was dialyzed and hydrolyzed. This procedure also extracted greater than 90% of the labeled proteins as did the extraction with $NaDodSO_4$, but this second method was necessary in order to obtain labeled immunoreactive proteins.

Immunoprecipitation of Tropoelastin. Antisera to elastin were prepared by immunizing sheep with insoluble chick aorta elastin which was isolated by the Lansing procedure as previously described (Lansing et al., 1952; Christner et al., 1976). Elastin-specific antibody was purified by isolating the immunoglobulin fraction by DEAE chromatography, absorbing the antibody onto insoluble chick aorta elastin, and then eluting it with 3 M KCNS (de Saussure & Dandliker, 1969). The eluted antibody was dialyzed rapidly against phosphate-buffered saline and frozen in small aliquots until used. Such antibody preparations have been shown to cross-react with tropoelastin and do not react with collagen or other proteins synthesized by the chick aorta (Christner et al., 1976).

Aliquots of the labeled proteins from the aortas were incubated with 3 μL of elastin antibody solution (~1 mg/mL) at 16 °C for 2 h in a total volume of 200 μL of phosphate-buffered saline. This temperature was chosen to prevent coacervation of the tropoelastin. Carrier normal sheep serum was added and then rabbit antisheep immunoglobulin (Cappel Laboratories) was added and the incubation continued at 16 °C for 24 h. Control experiments were performed to assure that maximum precipitation at equivalence was achieved with the second precipitating antisera. The precipitates were collected in 1-mL conical test tubes by centrifugation at 1500*g* for 30 min in the cold, washed with phosphate-buffered saline, and then dissolved in 200 μL of 0.5 N acetic acid. The solution was carefully neutralized with 6 N NaOH, $NaDodSO_4$ and mercaptoethanol were added to 1%, and the solution was placed in a boiling water bath for 2 min. Aliquots of the dissolved pellets as well as the supernatants and wash from the immunoprecipitation reactions were counted. Other aliquots of the dissolved pellets and supernatants were subjected to electrophoresis on polyacrylamide gels as described above.

Four microliters of the antibody solution was added to 50 μL of the cell-free translation mixture and incubated at 16 °C for 2 h. Fifty microliters of rabbit antisheep immunoglobulin containing 200 mM ε-aminocaproic acid, 20 mM *N*-ethylmaleimide, and 10 μg/mL phenylmethanesulfonyl fluoride was added, and the solution was incubated overnight at 16 °C. The immunoprecipitates were isolated by centrifugation through layered sucrose–detergent solutions in plastic microfuge tubes (Rowe et al., 1978). The tubes were frozen, and the tips were cut off and placed in counting vials. The immunoprecipitates were solubilized and counted by the same procedure used for the filter disk. Control experiments had shown previously that purified labeled tropoelastin could be precipitated by this procedure. Other immunoprecipitates were dissolved in 120 μL of 0.01 M HCl containing phenol red indicator, and the solutions were carefully neutralized with 0.1 M NaOH and then made 0.2% with $NaDodSO_4$ and 0.2% with β-mercaptoethanol. The samples were placed in a boiling water bath for 3 min and then subjected to electrophoresis on 5% polyacrylamide gel as previously described (Murphy et al., 1972).

Table I: Nucleic Acid Content of the Developing Chick Aorta[a]

age (days)	DNA (μg/aorta)	mRNA (μg/aorta)
8	1.44	0.11
9	2.30	0.17
9[b]	1.95	0.14
10	2.98	0.39
13	9.73	0.50
16	20.9	1.31

[a] Twelve aortas of each age were isolated for the determination of DNA by the method of Burton (1956). mRNA was purified as described under Experimental Procedures. The results are the average of duplicate determinations which agreed within 5%. [b] Eight-day embryos were treated with 150 μg of hydrocortisone phosphate, and 24 h later the nucleic acid content was determined.

Injection of Hydrocortisone 21-Phosphate into Eggs. Eggs were rinsed with 95% ethanol, and the shell was pierced with a sterile 25-gauge needle. From 3 to 300 μg of hydrocortisone 21-phosphate dissolved in 0.1 mL of sterile phosphate-buffered saline was given as a single injection onto the chorioallantoic membrane 24 h prior to dissection. Although hydrocortisone 21-phosphate was used in the majority of experiments because of its solubility in aqueous solution, identical results were obtained from chicks injected with hydrocortisone 21-succinate (data not shown). Control embryos were injected at the same time with 0.1 mL of sterile phosphate-buffered saline. After removal from the embryos, the aortas were incubated and treated as described above.

Results

Nucleic Acid Content and Incorporation of [^{3}H]Valine into Protein in the Developing Aorta. The thoracic aorta of the 8–16-day chick embryo is a rapidly growing and differentiating structure. In order to provide a reference base by which to compare elastin synthesis in different age embryos, we have determined the nucleic acid content of the tissue during development. Table I shows that the DNA content is increasing exponentially with a doubling time of ~2 days. The total mRNA content, defined operationally as that poly(A)-containing RNA which binds to an oligo(dT) column under specific conditions, increases with embryo age at about the same rate so that the total mRNA/DNA ratio remains relatively constant, declining slightly in the older ages.

We have previously shown, using [^{3}H]glycine as a label, that the relative rate of elastin synthesis to collagen synthesis increases markedly during this developmental period of 8 days (Eichner et al., 1978). In order to focus on the substantial increase in elastin synthesis and to correlate the changes with elastin mRNA content (see below), we incubated aortas from various age embryos with [^{3}H]valine. This amino acid was chosen because it occurs in large amounts in elastin relative to other proteins and in addition is incorporated efficiently into protein in the reticulocyte lysate system used to assay for functional mRNA. The aortas were incubated for 30 min in medium containing β-aminopropionitrile so that very little cross-link formation could take place. After incubation, the aortas were extracted into a hot $NaDodSO_4$ solution and the solubilized proteins, insoluble residue, and medium from the incubation were dialyzed. The residue was hydrolyzed and counted along with the $NaDodSO_4$-soluble fraction and dialyzed medium. The results of these experiments showed that greater than 90% of the incorporated radioactivity was found in the $NaDodSO_4$ extract, and thus further studies were confined to this fraction.

These $NaDodSO_4$-solubilized, labeled proteins were subjected to polyacrylamide gel electrophoresis, and representative

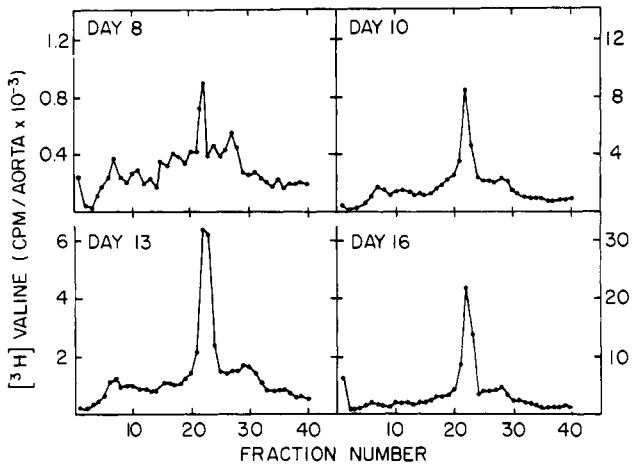

FIGURE 1: Polyacrylamide gel electrophoresis of labeled proteins extracted from embryonic aortas. Aortas from different age embryos were incubated with [^{3}H]valine as described under Experimental Procedures, and the labeled proteins were extracted and subjected to polyacrylamide electrophoresis. The gels were fractionated and counted. The marker protein, bovine serum albumin (68 000 daltons), was located at fraction 23–24.

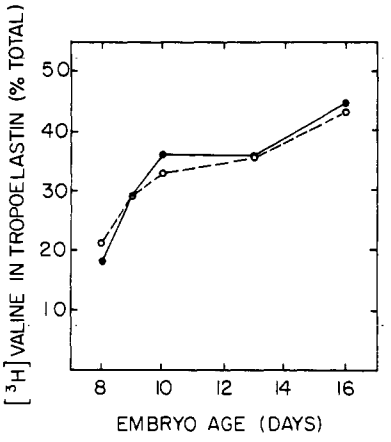

FIGURE 2: Incorporation of [^{3}H]valine into tropoelastin in comparison to total incorporation. Different age aortas were incubated with [^{3}H]valine as described under Experimental Procedures. The labeled proteins were subjected to polyacrylamide gel electrophoresis and the fraction of the radioactivity found in tropoelastin was calculated (○). mRNA was isolated from the same age embryos and translated in the treated rabbit reticulocyte lysate. The ratio of radioactivity immunoprecipitable by elastin-specific antibody to total Cl_3AcOH-precipitable radioactivity was determined (●).

patterns from different age embryos are illustrated in Figure 1. By inspection of Figure 1, it is clear that as the embryo ages a progressively increasing fraction of the incorporated radioactivity is found in a 70 000-dalton peak. This fraction can be quantitated by summing the radioactivity found in the peak of the gel and expressing the values as a percentage of the total radioactivity recovered in the gel (Figure 2). In the 8-day aorta, 21% of the incorporated [^{3}H]valine was found in this peak. Thereafter, the value increased significantly, reaching 35% at day 13 and 43% at day 16. We believe this 70 000 molecular weight component to be largely tropoelastin because of its molecular weight, solubility in water–alcohol mixtures, and reactivity with elastin-specific antiserum (Murphy et al., 1972, Narayanan & Page, 1976; Rosenbloom & Cywinski, 1976; Uitto et al., 1976; Christner et al., 1976; Bressen & Prockop, 1977). However, these previous studies were carried out with materials derived from 17-day aortas and it is possible that other proteins of similar molecular weight may be present in different age embryos. Therefore, extracted

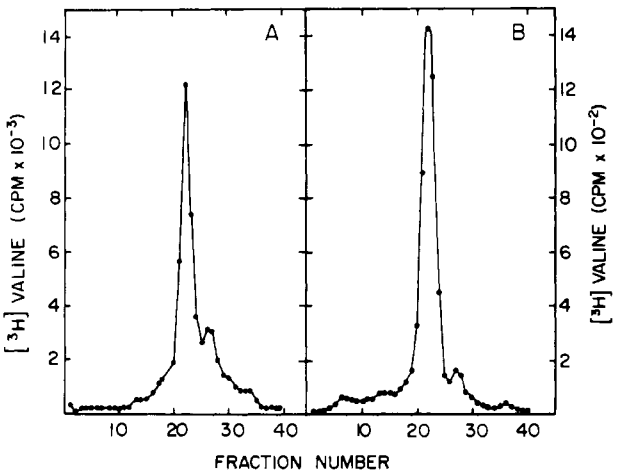

FIGURE 3: Gel electrophoresis of [^{3}H]valine-labeled protein immunoprecipitated by elastin-specific antibody. (A) mRNA from 13-day embryonic aortas was translated as described under Experimental Procedures, and that incorporated radioactivity immunoprecipitable by elastin-specific antibody was subjected to polyacrylamide electrophoresis. (B) Aortas from 13-day embryos were incubated with [^{3}H]valine, and the extracted labeled protein which was immunoprecipitable with the elastin-specific antibody was subjected to electrophoresis as described under Experimental Procedures. Marker bovine serum albumin (68 000 daltons) was located at fraction 23–24.

labeled proteins were tested with affinity-purified, elastin-specific antibody and the immunoprecipitated protein was subjected to polyacrylamide electrophoresis. A typical result using labeled protein from 13-day aortas is depicted in Figure 3B. Results from other age embryos (not shown) were very similar. With samples from all ages, the antibody precipitated proteins largely in the 70 000-dalton region with a much smaller amount of lower molecular weight polypeptides which may be derived from proteolysis of tropoelastin. In every case, ~90% of the 70 000-dalton labeled protein was immunoprecipitated; therefore, to a good approximation we can identify this component with tropoelastin.

Assay of Functional Elastin mRNA. We wished to determine whether the progressive increase in elastin synthesis observed as the embryo develops was paralleled by an increase in functional elastin mRNA. We used the nuclease-treated rabbit reticulocyte lysate as our assay system (Pelham & Jackson, 1976; Burnett & Rosenbloom, 1979). mRNA obtained from oligo(dT)–cellulose chromatography was translated by incubation in the lysate with [^{3}H]valine, and elastin-related protein was immunoprecipitated with the elastin-specific antibody. The immunoprecipitates were dissolved and subjected to polyacrylamide gel electrophoresis (Figure 3A). As was found with the labeled protein from the whole aortas, the protein in the immunoprecipitates was largely in the 70 000-dalton tropoelastin peak with a smaller quantity of lower molecular weight polypeptides.

In order to employ the assay for the determination of elastin mRNA content in various age aortas, it was necessary to show that there was a linear response between added mRNA and immunoprecipitable tropoelastin. Figure 4 shows that this was true up to at least 4 μg/mL with our mRNA preparations, and we worked within this range. It should be noted that the mRNA produced a many-fold increase in immunoprecipitable radioactivity over background. For example, 2 μg/mL produced a 30-fold increase. The total incorporation into protein as measured by trichloroacetic acid precipitable radioactivity also was a linear function of mRNA concentration. Therefore, we could compare immunoprecipitable radioactivity to total incorporation at any mRNA concentration within the range.

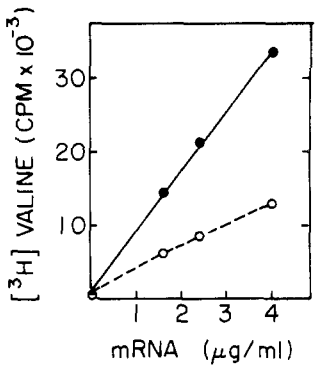

FIGURE 4: Dependence on mRNA concentration of total trichloroacetic acid precipitable incorporation and radioactivity immunoprecipitable with elastin-specific antibody. mRNA from 13-day aortas was translated as described under Experimental Procedures, and incorporation into trichloroacetic acid precipitable protein (●) and immunoprecipitable tropoelastin (○) was determined.

The functional elastin mRNA content as measured by immunoprecipitable radioactivity was determined in various age aortas. In order to compare these data to the results with the whole aorta incubations, we plotted the ratio of radioactivity in elastin to total incorporation as a function of embryo age (Figure 2). An excellent correspondence is observed between the two sets of data, in which the percentage incorporation in elastin increases from around 20% in the 8-day aorta to over 40% in the 16-day aorta.

Effect of Hydrocortisone. We had previously observed that injection of hydrocortisone 21-phosphate into 8-day eggs appeared to stimulate elastin synthesis and that 150 μg produced a maximal response. Aortas from treated embryos were incubated with [^{3}H]valine as described above and the mRNA was isolated and translated in order to investigate further this finding. As found previously with [^{3}H]glycine, hydrocortisone produced an increase in the fraction of incorporated label which was found in tropoelastin. This fraction increased from 29% in the control to 37% in the treated embryos (data not shown). Because we could not measure the intracellular specific activity with sufficient accuracy, we cannot draw any conclusions concerning the absolute rates of synthesis. When the mRNA from the treated embryos was translated, there was a corresponding increase in the fraction which was immunoprecipitable with the elastin-specific antibody. The value was 29% with the control mRNA and 41% with the mRNA from the treated embryos.

Discussion

The control of synthesis of extracellular matrix proteins is poorly understood. In the vast majority of other systems, control is ultimately exercised through adjustment of the steady-state level of mRNA, probably largely through variation in the rate of transcription (Chan et al., 1973; Alton & Lodish, 1977; Soh & Sarker, 1978). A similar regulatory mechanism appears to hold in the case of collagen synthesis in normal tendon fibroblasts in culture and in chick fibroblasts transformed with Rous sarcoma virus. Here a good correlation has been observed between the rate of procollagen synthesis and the corresponding mRNA level (Adams et al., 1977; Rowe et al., 1978). How procollagen mRNA levels are regulated is unknown at present.

The highly insoluble elastic fibers of the connective tissue matrix are synthesized through a soluble 70 000-dalton polypeptide called tropoelastin. In order to explore the previous observation that elastin synthesis varies during development of the chick aorta (Eichner et al., 1978), we developed a technique for measuring relative levels of functional elastin mRNA (Burnett & Rosenbloom, 1979). The method of mRNA purification is comparatively easy and permits rapid isolation of the polyadenylated mRNA fraction. Since the procedure does not require phenol extraction or ethanol precipitation, translatable mRNA can be isolated in several hours.

The relative amount of functional elastin mRNA in the aortas of various age embryos was determined by isolating and translating the polyadenylated mRNA and comparing the immunoprecipitable [^{3}H]valine to the trichloroacetic acid precipitable label. The relative rate of tropoelastin synthesis in the whole aorta was determined by comparing the amount of [^{3}H]valine in the tropoelastin peak of NaDodSO$_4$–polyacrylamide gels to the total label in the gels. This method was chosen because of the rapidity with which any proteolytic degradation could be inhibited after the incubation of the aortas. The results demonstrate that a rapid increase in the relative rate of tropoelastin synthesis occurs between day 8 and day 10 followed by a more gradual increase up to day 16. We use the functional terms "relative rate of tropoelastin synthesis" and "relative amounts of elastin mRNA" to represent the percentage of total incorporated [^{3}H]valine which could be recovered as tropoelastin. These values do not represent milligrams of tropoelastin per milligram of total protein since tropoelastin is valine-rich compared to other proteins being synthesized and whose valine content cannot be estimated. Nevertheless, because of the striking correspondence between the changing mRNA levels and the observed changes in tropoelastin synthesis (Figure 2), we conclude that the developmental changes in the rate of tropoelastin synthesis are due largely to changes in the level of elastin mRNA.

Glucocorticoids have been shown to alter the synthesis of other proteins in the embryonic chick (Kato, 1959; Piddington, 1967). In our experiments, the administration of 150 μg of hydrocortisone 21-phosphate at day 8 caused the aortas examined 24 h later to decrease slightly in wet weight and to contain 10–15% less DNA compared to the controls (Table I). The NaDodSO$_4$–polyacrylamide gel pattern of proteins synthesized by the treated aortas resembled that of normal 13–16-day aortas rather than that of 9-day embryos. Again the effect appeared to be due to changes in the relative level of elastin mRNA. Whether this hormonally induced change reflects mechanisms operative during normal development remains to be determined.

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Purification and Characterization of Winter Flounder Antifreeze Peptide Messenger Ribonucleic Acid†

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ABSTRACT: The serum of winter flounder contains a group of small antifreeze peptides which lower the freezing point of their body fluids during the winter months. The poly(A)-containing mRNA coding for these peptides has been isolated from livers of the winter specimens. When the isolated antifreeze mRNA was analyzed by a denaturing polyacrylamide gel electrophoresis, at least two distinct bands ~450 nucleotides in length are visible. In a wheat germ cell-free protein synthetic system these mRNAs direct the synthesis of small peptides which can be precipitated by antisera against purified winter flounder antifreeze peptides. Full-length cDNA was synthesized from the isolated antifreeze mRNA by avian myeloblastosis reverse transcriptase. From the RNA excess hybridization kinetic analysis, there are probably three different mRNAs coding for the antifreeze peptides. Using the radioactive cDNA probe, it was estimated that 1% of the total RNA in liver of a January specimen is antifreeze mRNA. RNA from a summer specimen showed no significant hybridization even at high concentrations of RNA. These results indicate that the control of antifreeze peptide biosynthesis relies at least in part on the synthesis or degradation of translatable mRNA.

Fishes inhabiting ice-laden seawater often encounter the danger of freezing during the winter. Many families of cold-adapted fishes have evolved antifreeze compounds, either glycopeptides or peptides, in their body fluids to protect them from freezing (DeVries et al., 1970; Duman & DeVries, 1974a; Raymond et al., 1975). It was proposed that the antifreeze compounds might bind to the surface of ice crystals and prevent the water molecules from joining the ice lattice, thus lowering the freezing point of a solution (Raymond & DeVries, 1977; DeVries & Lin, 1977).

Three small antifreeze peptides have been isolated from the serum of winter flounder (Duman & DeVries, 1976; DeVries & Lin, 1977). They have very similar amino acid compositions and sequences. Peptide 3, which has a molecular weight of 3300, is composed of 24 alanine, 5 aspartate, 4 threonine, 1 serine, 2 leucine, and 1 lysine (DeVries & Lin, 1977). In addition to these amino acids, peptides 1 and 2 contain arginine and are probably slightly larger in size. There is a seasonal variation in the concentration of these peptides in the serum, with the highest concentration observed in January and the lowest in the summer months (Petzel et al., 1979). Acclimation studies have indicated that the biosynthesis of these peptides is probably regulated by both temperature and photoperiod (Duman & DeVries, 1974b), but the molecular mechanism underlying this regulation is not known.

Using an in vitro cell-free protein synthetic system, it was shown that livers of winter flounder collected in January contain translatable mRNA of a major peptide; livers from summer specimens do not contain such mRNA. This peptide

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