Translation of Chick Aortic Elastin Messenger Ribonucleic Acid. Comparison to Elastin Synthesis in Chick Aorta Organ Culture†

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ABSTRACT: Studies were undertaken to define the molecular size of the elastin primary gene product. Translation of chick aortic messenger ribonucleic acid (mRNA) in an mRNA-dependent reticulocyte lysate resulted in the synthesis of two major proteins of 70 000 and 73 000 molecular weights. Both proteins were shown to be soluble forms of elastin by isotope incorporation, immunoprecipitation, collagenase and cyanogen bromide sensitivity, and two-dimensional gel electrophoresis. The 70 000-dalton protein behaves similarly to authentic tropoelastin in sodium dodecyl sulfate gel electrophoresis. There was no evidence for a high molecular weight form of soluble elastin, although procollagen chains were indirectly identified among the aortic mRNA-directed translation products. The same molecular size proteins were also seen in organ cultures of chick embryonic aortas labeled with [^{3}H]valine. However, the 73 000-dalton protein was not extractable in a neutral salt buffer but was found only if the aortas were extracted with urea in the presence of reducing and alkylating reagents. The results from these studies suggest that elastin is first synthesized as two distinct polypeptide chains which differ slightly in size and overall charge. The possibility that these two proteins may associate posttranslationally to form a dimer prior to secretion is postulated to explain the existence of a putative proelastin molecule seen in other systems.

Investigations into the biosynthesis of elastin have centered primarily on the identification of tropoelastin in aortic organ cultures (Murphy et al., 1972; Smith et al., 1975; Rucker et al., 1975), aortic cell suspensions (Uitto et al., 1976; Rosenbloom & Cywinski, 1976), and smooth muscle cell cultures (Abraham et al., 1974; Narayanan et al., 1976). The majority of the data thus far accumulated would suggest that elastin is first synthesized as a 68 000–75 000-dalton protein which is secreted into the extracellular matrix and rapidly incorporated into insoluble elastin fibers via lysine-derived cross-links (Siegel et al., 1970). More recently, we have reported on the isolation of a high molecular weight protein from lathyritic chick aortas which was shown to be both chemically and immunologically related to tropoelastin (Foster et al., 1976a, 1977). The finding of a similar, large elastin-like protein was confirmed in cultures of rabbit aortic smooth muscle cells (Foster et al., 1978). These observations led us to hypothesize that the high molecular weight protein was the primary gene product and a precursor to tropoelastin. Since that time, Rucker et al. (1977) and Heng-Khoo et al. (1979) have confirmed the presence of a high molecular weight elastin-like protein in chick embryonic aorta cultures and copper-deficient chick aortas. These authors further point out that the putative proelastin is very labile and its demonstration necessitates short-term pulse experiments and the presence of significant amounts of proteolytic inhibitors.

Despite these findings, the concept of a proelastin molecule remains controversial. The inability to consistently demonstrate the existence of a proelastin molecule in in vitro systems actively synthesizing elastin has generated a need for examination of the initial elastin gene product in a system devoid of potential processing enzymes. The present study describes the cell-free translation of aortic elastin mRNA[1] and a comparison of the translation products to those synthesized in aortic organ culture.

Experimental Procedures

Materials. L-[2,3-$^3H_2$]Valine (23 Ci/mol) 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. Guanidinium thiocyanate was purchased from MC/B Manufacturing Chemist, Inc., and cesium chloride (optical grade) was from Schwarz/Mann. Collagenase (form III) was purchased from Advance Biofactors Corp., and micrococcal nuclease (*Staphylococcus aureus*, 15 000 units/mg) was from Boehringer-Mannheim Biochemicals. Heat-killed, formalin-fixed *S. aureus* was a gift of Dr. Richard Meagher.

Preparation of RNA. In order to prevent RNase contamination, all glassware was heated at 190 °C for 16 h and all solutions were autoclaved. These sterile techniques were employed throughout RNA isolation and translation procedures. Aortas were removed from 17-doz 16-day chick embryos (white leghorn) and immediately frozen in liquid N_2 and powdered with a mortar and pestle (Benveniste et al., 1973). The isolation of RNA from the aortic tissue was performed by a modification of the procedure according to Przybyla et al. (1979). The powdered tissue was suspended (3 g/20 mL) in 5 M guanidinium thiocyanate, 50 mM EDTA, 50 mM Tris, (pH 7.5), 2% Sarkosyl, and 5% β-mercaptoethanol and homogenized with a Brinkman polytron at high speed for 2 min. The homogenate was layered onto 5 mL of 5.7 M cesium chloride and 100 mM EDTA and centrifuged at 180000*g* for 17 h at 20 °C. The pellet was redissolved in sterile H_2O and the RNA precipitated overnight by the addition of 0.1 volume

† From the Department of Biochemistry, University of Georgia, Athens, Georgia 30602. *Received September 7, 1979.* This work was supported by the National Institutes of Health Grant HL 22208, the Council for Tobacco Research 1179, and the National Foundation March of Dimes 5-209. J.A.F. is an Established Investigator of the American Heart Association.

[1] Abbreviations used: mRNA, messenger ribonucleic acid; NaDodSO$_4$, sodium dodecyl sulfate; RNase, ribonuclease; EDTA, disodium ethylenediaminetetraacetate; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*′-tetraacetate; Cl_3AcOH, trichloroacetic acid; MalNEt, *N*-ethylmaleimide; DFP, diisopropyl fluorophosphate; DEAE, diethylaminoethyl; CM, carboxymethyl; M_r, molecular weight; CNBr, cyanogen bromide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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of 2 M potassium acetate (pH 5.0) and 2.5 volumes of 95% ethanol at −20 °C. The RNA was collected by centrifugation at 10000*g* for 1 h at −20 °C, redissolved in H_2O, and precipitated by the addition of 2 volumes of 4.5 M sodium acetate (pH 6.0) overnight at −10 °C. The final precipitate was recovered by centrifugation at 10000*g* for 1 h at −10 °C, redissolved in 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 17-doz chick embryo aortas averaged 10–12 mg (assuming 20 A_{260} units/mg of RNA).

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 assays.

Prior to translation, the 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 then heated at 70 °C for 1 min and quickly immersed in an ice bath.

Preparation of Lysate. Rabbit reticulocyte lysates were prepared according to the procedure of Evans & Lingrel (1966). The prepared lysate was rendered mRNA-dependent by a modification of the method of Pelham & Jackson (1976). One microliter of 75 mM $CaCl_2$, 5 μL of creatine kinase (5 mg/mL of 50% aqueous glycerol), 2.5 μL of 1 mM hemin (in 90% ethylene glycol, 20 mM Tris-HCl, pH 8.2, and 50 mM KCl), and 1 μL of micrococcal nuclease (mg/mL of H_2O) were added to 90 μL of thawed lysate. Incubation was for 10 min at 20 °C. The digestion was terminated by the addition of 1 μL of 200 mM EGTA. Undigested lysate preparations were treated in an identical manner with the exception that nuclease was not added.

Translation Conditions.[2] The regular translation assay consisted of 25 μL of reticulocyte lysate or nuclease-treated lysate, 19 μL of master mix, and 18 μL for the addition of variable amounts of RNA, H_2O, and γ-globulins. The final concentrations of compounds in the translation assay contributed by the master mix were 75 μM glycine, valine, proline, and alanine and 25 μM for 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 (62-μL total volume) were either 130 μCi of L-[2,3-$^3H_2$]valine or L-[5-^{3}H]proline or 65 μCi of L-[^{35}S]methionine or 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 examined by removing 2-μL aliquots at various time points and determining the radioactive protein by the method of Pelham & Jackson (1976). The dried Cl_3AcOH precipitates were counted in 0.4% Omnifluor dissolved in toluene by using a Beckman LS7000 scintillation counter.

Organ Culture of Chick Embryonic Aortas. Aortas were removed from 20-doz 16-day chick embryos (white leghorn) 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 aortas were transferred to fresh medium (4 doz aortas/mL) containing 1 mCi/mL L-[2,3-$^3H_2$]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 the aortas were immediately homogenized with a polytron (4 °C) in 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 (4 doz aortas/mL). The homogenate was extracted for 1 h at 4 °C and centrifuged at 15000*g* for 1 h. The phosphate buffer extract will subsequently be referred to as the NSB extract (neutral salt buffer). The supernatant was stored at 4 °C, the pellet was suspended in 2 M urea and 0.01 M Tris (pH 8.0), and the same concentrations of proteolytic inhibitors as indicated above were added. The pellet was rehomogenized with a polytron, reduced with dithiothreitol (10 mg/mL), and alkylated with MalNEt (0.1 M). Extraction was allowed to proceed for 2 h at 4 °C, after which time the extract was centrifuged for 1 h at 15000*g* and the urea supernatant stored at 4 °C. This extract will subsequently be referred to as the urea extract.

Preparation of Tropoelastin. Tropoelastin was isolated from the aortas of lathyritic chicks as previously described (Foster et al., 1975) by using α-aminoacetonitrile hydrochloride as the lathyrogen (Foster et al., 1979a). For the purpose of removing any contaminating enzyme(s) (Mecham & Foster, 1977) or glycoproteins (Abraham & Carnes, 1978a,b), the tropoelastin was chromatographed on a DEAE-cellulose column in the presence of ε-aminocaproic acid as reported previously (Mecham & Foster, 1977). The purified tropoelastin was reduced with [^{3}H]$NaBH_4$ for use as a radioactive standard in various electrophoretic techniques in this study (Foster et al., 1975).

Immunological Techniques. Antibody to purified tropoelastin was raised in rabbits by a slight modification of a procedure we have previously published (Foster et al., 1976b). The only difference in the procedure was that the rabbits were injected in hind toepads. The site of injection resulted in a faster appearance of antibody as well as a higher titer. The γ-globulins were partially purified from both immune and nonimmune sera by ammonium sulfate precipitation (Harboe & Ingild, 1973). The γ-globulins were then rendered RNase-free by using a combined DEAE- and 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 per translation assay.

Immunoprecipitation of the cell-free products was accomplished by a modification of the method of Monson & Goodman (1978) using heat-killed, formalin-fixed *S. aureus* as an immunoadsorbent (Kessler, 1975). To the translation reaction, run in the presence of 35 μg of γ-globulin, was added 0.2 volume of 5X immunoprecipitation buffer [1X = 0.5% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 1% sodium deoxycholate, 5 mM EDTA, and 2 mM unlabeled amino acid corresponding to the radioactive amino acid used for labeling]. The reaction mixture was then centrifuged at 10000*g* for 5 min to remove any particulate matter. Ten microliters of additional γ-globulin (3.5 μg/μL) was added and the mixture was allowed to sit at room temperature for 5 min, after which time 40 μL of *S. aureus* (30% suspension in 1X buffer) was added. After 5 min at room temperature, the suspension was layered onto 300 μL of 1 M sucrose in 1X buffer and centrifuged at 4 °C for 5 min at 10000*g* and the pellet was washed and centrifuged 3 times with 300 μL of 1X buffer. Radiolabeled protein was then eluted from the *S. aureus* by incubation for 20 min in 50 μL of 3% $NaDodSO_4$ and 5 M urea and centrifuged at 10000*g* for 5 min. Prior to examination on $NaDodSO_4$ slab gels, 2 μL of β-mercapto-

[2] S. R. Karr, A. Przybyla, and J. A. Foster, unpublished experiments. A complete study of optimal conditions for translation of elastin mRNA is in preparation.

ethanol was added and the sample was placed in a boiling H_2O bath for 3 min.

Immunoprecipitation of organ culture extracts was performed by incubating 20 μL of the NSB or urea extracts, 20 μL of unfractionated antisera, and 0.2 volume of 5X buffer at 37 °C for 1 h. Immunoprecipitation with *S. aureus* was performed as described above.

Rocket immunoelectrophoresis of radiolabeled tropoelastin and translation products was performed by a procedure recently developed in our laboratory (Foster et al., 1979b). An agarose gel (1%) containing 1.6% antisera was made in 0.0125 M phosphate bufer (pH 7.4) and cast on a glass plate. The tray buffer was 0.05 M phosphate buffer. Electrophoresis of 5-μL radioactive samples was performed on an LKB multiphor unit with a cooling plate maintained at 6 °C with a Haake constant temperature circulator (400 V, 5 h). Tropoelastin migrates toward the cathode under these conditions. After electrophoresis, the gel was washed in 0.1 N NaCl overnight with several changes. During this washing, the gel became detached from the glass and had to be handled carefully. The gel was then washed in deionized H_2O for 1 h. Fluorography of the gel was performed essentially as described by Bonner & Laskey (1974) with the exceptions that methanol was substituted for dimethyl sulfoxide and the percentage of 2,5-diphenyloxazole was 18% (w/v) in methanol. The impregnated gel was dried onto filter paper by using a Bio-Rad slab dryer and exposed to Kodak X-Omat R film at −80 °C.

$NaDodSO_4$ 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 as described by Laemmli (1970). Organ culture samples were prepared by adding an equal volume of 2X sample buffer to 10 μL of the extracts. 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.

Two-Dimensional Gel Electrophoresis. Nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and $NaDodSO_4$ slab gel electrophoresis in the second dimension were performed according to the procedure of O'Farrell et al. (1977). The first dimension cylindrical gels were run at 400 V for 8 h (32 V h) as indicated in the figure legends. Processing of the second dimension slab gel was exactly as described above for $NaDodSO_4$ gels.

Collagenase Digestion. Ten-microliter aliquots of either the cell-free translation assay or the phosphate buffer extract of the aorta organ culture were added to 10 μL of 20 mM Hepes buffer (pH 7.5) containing 5 mM MalNEt and 10 mM $CaCl_2$. Collagenase was dissolved in 20 mM Tris (pH 7.4) and 5 mM $CaCl_2$ at a concentration of 100 units/mL. Forty microliters of the collagenase was added to the cell-free and organ samples, and incubation was performed overnight at 37 °C. Control incubations were performed by the addition of Tris buffer with no collagenase. Samples were prepared for electrophoresis by precipitation in 8 volumes of acetone.

Cyanogen Bromide Cleavage. Cyanogen bromide cleavage was performed on the mRNA-directed translation products and the urea extract from aorta organ cultures. Ten microliters of both samples was diluted to 500 μL with 70% formic acid. Five microliters of cyanogen bromide reagent (100 mg/mL of dimethylformamide) was added, and the samples were shaken gently at room temperature for 5 h. The reaction mixture was taken up in 5 mL of H_2O, dialyzed against H_2O overnight, and lyophilized. Controls included the addition and incubation with 70% formic acid but without cyanogen bromide.

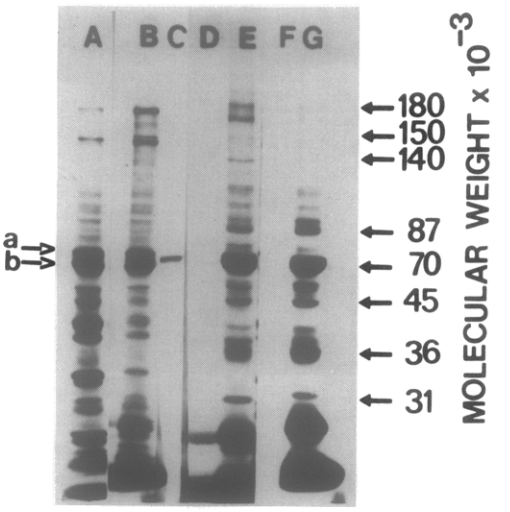

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of translation products from mRNA-dependent reticulocyte lysate with added aortic RNA labeled with [^{3}H]valine (A) and [^{3}H]proline (B). (C) Authentic tropoelastin reduced with [^{3}H]$NaBH_4$. (D and F) Digested reticulocyte lysate with no added RNA incubated with [^{3}H]valine and [^{3}H]proline, respectively. (E and G) Untreated reticulocyte lysate with no added RNA incubated with [^{3}H]valine and [^{3}H]proline, respectively. Translation time was 90 min. Lanes A and B were exposed for 2 weeks and lanes C–G for 1 week.

Results

Translation of Embryonic Chick Aortic mRNA. The molecular weight distributions of [^{3}H]valine- and [^{3}H]proline-labeled proteins directed by aortic RNA in the mRNA-dependent reticulocyte lysate are given in Figure 1. The [^{3}H]valine profile (lane A) revealed a complex pattern of proteins ranging in molecular weight from 180 000 to 10 000. There are, however, two prominent proteins (a and b) which possess apparent molecular weights of 70 000 and 73 000. Interestingly, authentic, [^{3}H]$NaBH_4$-reduced tropoelastin (lane C) migrates between these two proteins. The [^{3}H]proline profile (lane B) is similar to that of the [^{3}H]valine profile except that in addition to the prominent proteins a and b, the higher molecular weight proteins of apparent M_r 183 000 and 150 000 are more distinctive with the proline label. Close inspection of these high molecular weight proteins reveals that each of the two protein bands is comprised of a doublet with different incorporation levels of valine and proline.

The observation that authentic tropoelastin did not comigrate with the two major [^{3}H]valine-labeled proteins found in the translation assay led us to explore the possibility that another unlabeled protein originating from the reticulocyte lysate itself may be interfering with the electrophoretic migration of the radiolabeled synthesized proteins. This possibility was suggested both by examining the lysate endogenous RNA products (undigested lysate, lanes E and F) which revealed a major protein in the area of tropoelastin and by noting the peculiar rounded shape of the fluorographed proteins labeled a and b (see lanes A and B). Figure 2 pictures a Coomassie Blue stained gel of nuclease-treated lysate (lane A). Also included in Figure 2 is a fluorogram of aortic mRNA

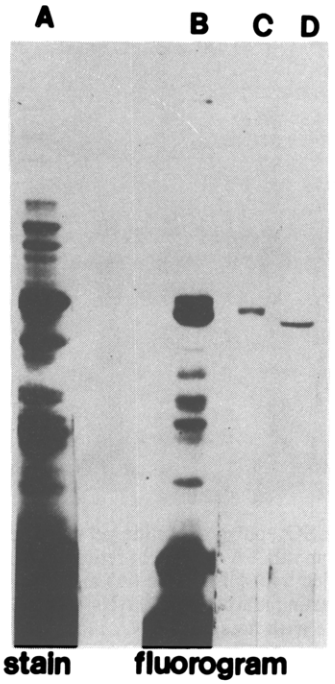

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis. (A) Coomassie Blue stain of reticulocyte lysate proteins. Fluorography of (B) translation products from digested reticulocyte lysate plus aortic RNA incubated with [^{3}H]proline, (C) authentic [^{3}H]tropoelastin, and (D) authentic [^{3}H]tropoelastin plus unlabeled reticulocyte lysate proteins. Translation time was 90 min. Exposure of lanes B–D was for 1 week.

translation products labeled with [^{3}H]valine (lane B), authentic [^{3}H]$NaBH_4$-reduced tropoelastin (lane C), and authentic [^{3}H]$NaBH_4$-reduced tropoelastin plus 2 μL of reticulocyte lysate (lane D). These results demonstrate that the 70 000-dalton protein synthesized by the aortic mRNA migrates very similarly to chemically purified tropoelastin when run together with reticulocyte lysate proteins.

The particular assignment of molecular weights in this study was based on tropoelastin (M_r 70 000) and the degradation fragments derived from tropoelastin (M_r 57 000, 45 000, and 36 000) which were obtained by incubation of [^{3}H]tropoelastin with an associated trypsin-like neutral protease (Mecham & Foster, 1977). The reason that these standards were used as opposed to globular standards was that tropoelastin exhibits anomalous behavior in the Laemmli (1970) discontinuous gel system and migrates faster than human serum albumin (M_r 66 000). In the Weber & Osborn (1969) $NaDodSO_4$ system performed in cylindrical gels, tropoelastin migrates slower than human serum albumin (Foster et al., 1975; Mecham & Foster, 1977). The assignment of M_r 70 000 and 73 000 was calculated on the immunoprecipitated proteins (see below) so that interference by the major lysate protein in electrophoretic mobility was eliminated.

A time study was performed in order to investigate the possibility that the 70 000- and 73 000-dalton proteins originate during translation from cleavage of a larger molecular weight precursor protein. The in vitro translation of aortic mRNA with [^{3}H]valine was terminated at various times (5–90 min), and the products were examined on $NaDodSO_4$ slab gel electrophoresis (data not shown). The results from this study demonstrate that the 70 000- and 73 000-dalton proteins do not arise from the initial cleavage of a higher molecular weight protein.

Figure 3 shows the labeling of aortic mRNA-directed products with [^{35}S]cysteine (lane B), [^{35}S]cysteine and [^{35}S]methionine (lane E), [^{35}S]methionine (lane G), and [^{3}H]proline (lane H). A standard [^{3}H]tropoelastin is included in lane I. The data from these experiments, together with the two-dimensional gel electrophoresis data (see below), demonstrate that both the 70 000- and 73 000-dalton proteins incorporate both methionine and cysteine. The implications of this observation are included under Discussion.

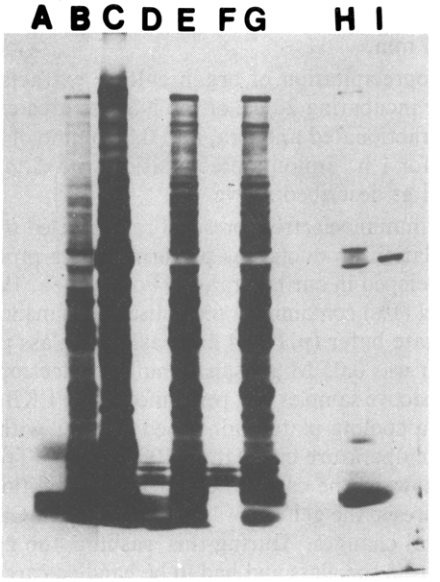

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of aortic RNA translation products using mRNA-dependent reticulocyte lysate with [^{35}S]cysteine (B), [^{35}S]cysteine and [^{35}S]methionine (E), [^{35}S]methionine (G), and [^{3}H]proline (H). Lanes A, D, and F are digested reticulocyte lysates with no added RNA incubated with [^{35}S]cysteine (A), [^{35}S]cysteine and [^{35}S]methionine (D), and [^{35}S]methionine (F). Lane C is endogenous mRNA reticulocyte products labeled with [^{35}S]cysteine and [^{35}S]methionine. Lane I is authentic [^{3}H]tropoelastin. Translation time was 90 min. Lanes A–G were exposed for 4 h and lanes H and I for 1 week.

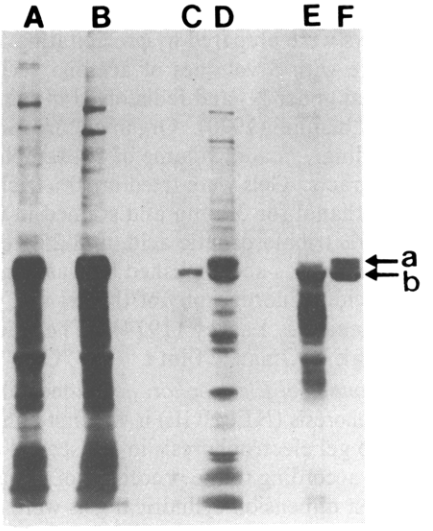

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of aortic organ culture and cell-free translation products. (A) NSB extract of chick aortas incubated for 1 h with [^{3}H]valine and first extracted with NSB. (B) Urea extract of chick aortas. (C) Authentic [^{3}H]-tropoelastin. (D) Translation products of aortic RNA with digested reticulocyte lysate incubated with [^{3}H]valine. (E) Same as (A) but with the addition of unlabeled reticulocyte lysate to the sample. (F) Immunoprecipitate of (B) with addition of reticulocyte lysate. Lanes A and B represent 10 μL of sample whereas lane E represents 5 μL of extract plus 5 μL of reticulocyte lysate. Lane E represents immunoprecipitation of 20 μL of urea extract plus 5 μL of lysate. Exposure was for 1 week.

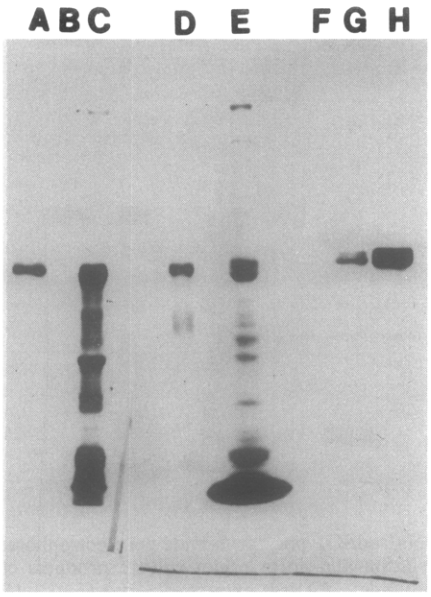

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of immunoprecipitates from aortic organ culture and aortic organ culture and aortic RNA translation products. (A) Immunoprecipitate of NSB extract of chick aortas. (B) Control of immunoprecipitate of NSB extract using nonimmune IgG. (C) NSB extract of chick aortas labeled with [^{3}H]valine (5 μL). (D) Authentic [^{3}H]tropoelastin. (E) Translation products of aortic RNA labeled with [^{3}H]proline. (F) Control immunoprecipitate of cell-free translation of aortic RNA with nonimmune IgG. (G) Immunoprecipitation of cell-free translation of aortic RNA by the addition of 70 μg of IgG after translation was complete. (H) Immunoprecipitate of cell-free translation of aortic RNA by the presence of 35 μg of IgG during translation and an additional 35 μg of IgG after translation was complete. In all immunoprecipitations *S. aureus* was used as an immunoabsorbent as described in the text. Exposure time was for 1 week.

Embryonic Aorta Organ Culture. [^{3}H]Valine labeling of chick embryonic aortas in organ culture is given in Figure 4 together with authentic [^{3}H]tropoelastin (lane C) and translation products of aortic mRNA using [^{3}H]valine (lane D). Lanes A and B picture the NSB and urea extracts of the organ cultures, respectively. The addition of 2 μL of reticulocyte lysate to the NSB extract is given in lane E. Lane F represents an immunoprecipitate of the urea extract together with 2 μL of reticulocyte lysate. Of special interest was the finding that extraction of the aorta in 2 M urea with reduction and alkylation (lane F) resulted in the appearance of two [^{3}H]valine-labeled proteins whose molecular weights resemble the major translation products obtained with aortic mRNA (lane D) and which are immunoreactive.

Figure 5 shows the precipitation of both the NSB extract of the chick aorta organ cultures and [^{3}H]proline translation products with tropoelastin antibody and *S. aureus* immunoadsorbent. Of particular interest in the cell-free system was the demonstration that both the 70 000- and 73 000-dalton protein were immunoreactive (lanes F and G) and that the translation of mRNA in the presence of added antibody (lane G) resulted in a threefold increased recovery of both proteins. It is also interesting to note that after immunoprecipitation the 70 000- and 73 000-dalton proteins migrate closer and are less distinguishable as two separate proteins than when lysate proteins are present (lane D).

Quantitation of Elastin in Organ and Cell-Free Systems. The amount of soluble elastin present in the NSB extract of the aortic organ culture was first quantitated by Coomassie Blue staining of the rocket immunoelectrophoretic plates using appropriate concentrations of purified tropoelastin. The extract of 10 doz aortas homogenized in 5 mL of phosphate buffer

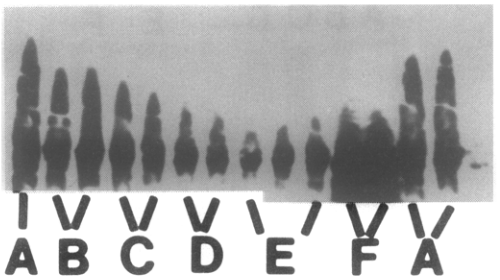

FIGURE 6: Fluorogram of rocket immunoelectrophoresis of NSB extract of chick aorta labeled with [^{3}H]valine (A–E) and aortic RNA translation products labeled with [^{35}S]methionine and [^{35}S]cysteine (F). The concentrations (micrograms per milliliter) of soluble elastin in the NSB extract samples were (A) 2.8, (B) 2.0, (C) 1.56, (D) 1.27, and (E) 0.67. The linear correlation (r) was 0.9985. 5 μL of the translation assay (F) revealed 1 μg/mL soluble elastin. Exposure time was for 2 days.

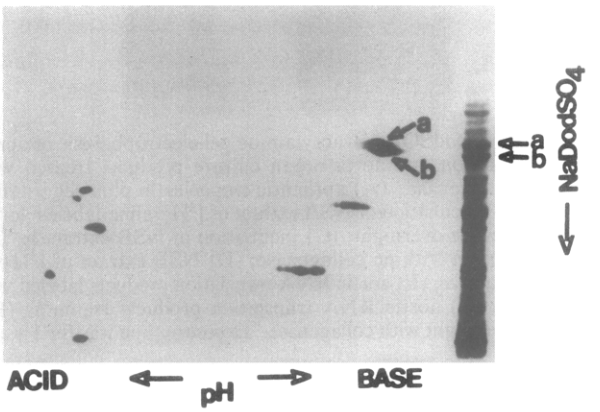

FIGURE 7: Two-dimensional gel electrophoresis of aortic RNA translation products labeled with [^{3}H]valine (90 min). First dimension electrophoresis in pH 3.5–10 ampholines was performed for 3200 V h. Exposure time was for 1 week.

was found to contain 18 μg/mL tropoelastin. The NSB extract was then serially diluted, and electrophoresis was performed with less antibody in the agarose gel, such that a significant linear correlation was obtained between rocket height and antigen concentrations of 1–3 μg/mL. Detection of radiolabeled rockets was accomplished by fluorography of the rocket plate. Five-microliter samples of cell-free translation assay were run in duplicate, and the amount of elastin present was quantitated by using the radiolabeled organ culture elastin as the standard (see Figure 6). A total of 0.88 pmol of elastin was synthesized per assay (62 μL) by using 50 μg of unfractionated aortic RNA incubated for 90 min with this system. This number includes both the 70 000-dalton and the 73 000-dalton proteins since both share antigenic determinants as well as similar charge (see Two-Dimensional Gel Electrophoresis below).

Two-Dimensional Gel Electrophoresis. Figure 7 pictures the two-dimensional electrophoresis of [^{3}H]valine-labeled aortic mRNA translation products. The 70 000- and 73 000-dalton proteins synthesized in the cell-free system (a and b) are both very basic and migrate closely in the first dimensional pH gradient gel. However, the 70 000-dalton protein (b) migrates slightly faster than the 73 000-dalton protein (a). Two-dimensional electrophoresis was also performed on [^{35}S]methionine- and [^{35}S]cysteine-labeled translation products, and similar results were obtained (data not shown).

Collagenase Sensitivity of Cell-Free and Organ Culture Products. The susceptibility of both the aorta organ culture and the cell-free synthesized proteins to collagenase is shown

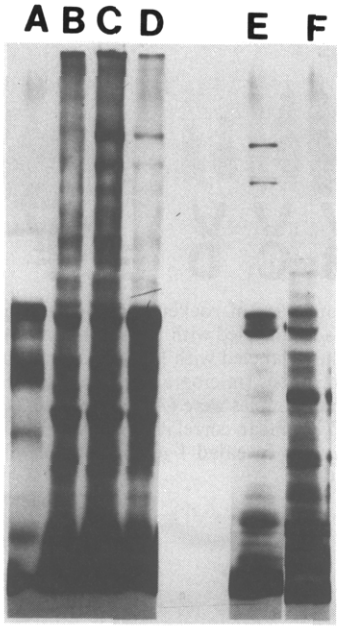

FIGURE 8: $NaDodSO_4$–polyacrylamide gel electrophoresis of aortic RNA translation and aorta organ culture products treated with bacterial collagenase: (A) authentic tropoelastin plus degradation products; (B) incubation of NSB extract of [^{3}H]valine-labeled aortas with collagenase overnight; (C) incubation of NSB extract in Tris buffer overnight with no collagenase; (D) NSB extract of [^{3}H]valine-labeled aortas; (E) aortic RNA translation products labeled with [^{3}H]proline; (F) aortic RNA translation products [same as (E)] incubated overnight with collagenase. Exposure time was for 1 week.

in Figure 8. The high molecular weight proteins found in both systems (lanes D and E) are cleaved by collagenase whereas the proteins in the area of 70 000 and 73 000 daltons are not altered. This observation together with the high level of proline incorporation and the apparent molecular weights of these proteins suggests that they are procollagens. When the NSB extract was incubated in Tris buffer without the addition of collagenase (lane C), the major [^{3}H]valine-labeled tropoelastin band is degradated to lower molecular weight material. This is also evident in the sample treated with collagenase (lane B). This observation points out the difficulty in inhibiting the enzyme(s) associated with tropoelastin (Mecham & Foster, 1977). Even though sufficient proteolytic inhibitors were added to the NSB extract of the aortas, we have continually found that incubation of the sample at 37 °C in an appropriate buffer does result in tropoelastin degradation. This may indicate either that the enzyme(s) associated with tropoelastin is very tightly bound to the substrate, thereby protecting it from inhibition, or that the enzyme is in a pro form initially and subsequentially activated upon storage and incubation at 37 °C.

Cyanogen Bromide Cleavage. The results of treatment of [^{3}H]valine-labeled translation products of aortic mRNA and the urea extract of the chick aorta organ culture are given in Figure 9. In both the organ culture and cell-free systems, the prominent [^{3}H]valine-labeled proteins of M_r 70 000 and 73 000 are not significantly altered in size by cleavage with cyanogen bromide. These data strongly suggest that the methionine residue(s) incorporated into the cell-free proteins (see Figure 3) are located very near the NH_2 or COOH termini of the polypeptide chains.

Discussion

As stated in the beginning of this report, our original goal for studying the translation of isolated aortic mRNA was to answer the critical question of the molecular size of the elastin

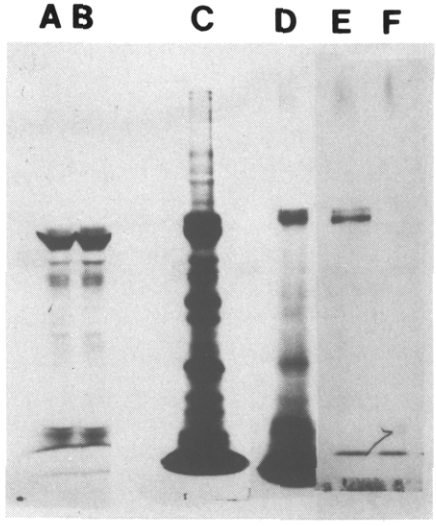

FIGURE 9: $NaDodSO_4$–polyacrylamide gel electrophoresis of aortic RNA translation and aorta organ culture products cleaved with cyanogen bromide: (A) urea extract of aortas (5 μL); (B) cyanogen bromide cleavage of aortic urea extract (5 μL); (C) aortic RNA translation products labeled with [^{3}H]valine; (D) aortic RNA translation products [same as (C)] treated with cyanogen bromide; (E) immunoprecipitate of aortic RNA translation products labeled with [^{3}H]valine; (F) control immunoprecipitation of aortic RNA translation products labeled with [^{3}H]valine using nonimmune γ-globulins. Translation was for 90 min. Exposure was for 1 week.

primary gene product. Ryhanen et al. (1978) reported on the in vitro synthesis of a 70 000-dalton protein by chick embryo aortic polysomes which was identified as tropoelastin. Burnett & Rosenbloom (1979) isolated and translated chick aortic embryo mRNA and found a major 70 000-dalton protein which was immunoprecipitable with antisera directed against chick-insoluble, cross-linked elastin. More recently, Krawetz & Anwar (1979) have reported on the translation of a 200 000-dalton protein by chick aortic mRNA. They suggest that this protein is an elastin precursor based on incorporation studies with valine, alanine, proline, and leucine.

In all of our translation studies with aortic RNA we have found no high molecular weight protein which could be identified as soluble elastin but, instead, two distinct proteins of M_r 70 000 and 73 000 which by several criteria are both soluble forms of elastin. The fact that there are two proteins synthesized in vitro could be easily overlooked in $NaDodSO_4$ gels of higher cross-link if smaller amounts of lysate proteins are present in the sample or if immunoprecipitates are examined. The major lysate protein of M_r 70 000 actually serves to accentuate the separation of the two proteins in $NaDodSO_4$ gels. The apparent small difference in molecular weight (~3000) between the two proteins is interesting in light of the current information regarding the synthesis of presecretory proteins. Blobel & Dobberstein (1975) have developed the signal hypothesis which states that proteins destined for secretion are synthesized with a short signal peptide (15–30 amino acids) on the NH_2 terminus of the nascent polypeptide chain. Cleavage of the signal peptide in vivo is a cotranslational event occurring immediately after vectoral transport of the protein is initiated and prior to complete synthesis of the protein. The 70 000- and 73 000-dalton protein seen in the translation of aortic mRNA could, in theory, represent tropoelastin (70 000 daltons) and tropoelastin plus a 3000 molecular weight signal peptide attached to the NH_2 terminus. In practice, however, there have not been any reports in the literature concerning the presence of signal peptidase activity (Jackson & Blobel, 1977) in rabbit reticulocyte lysates used

for translation of exogenous mRNAs. There are also several other experimental observations which argue against a precursor–product relationship between the 73 000- and 70 000-dalton proteins.

In all of the experiments performed, there was no evidence that the M_r 73 000 protein is a precursor to the M_r 70 000 protein which could possibly arise in the translation by cleavage of a short presecretory sequence from the NH_2 terminal. Another observation which argues against a precursor–product relationship between the 70 000- and 73 000-dalton proteins is that both proteins incorporate methionine (see Figure 3). Although we do not as yet have any chemical data on the 73 000-dalton protein, the 70 000-dalton protein possesses an electrophoretic mobility and immunological properties similar to those of chick tropoelastin, a protein which has been chemically characterized (Rucker et al., 1975; Foster et al., 1975). Authentic tropoelastin has been reported to contain no methionine residues whereas the cell-free product contains at least one methionine. This observation together with the fact that neither the 70 000-dalton protein nor the 73 000-dalton protein is altered significantly in molecular size by cyanogen bromide cleavage is consistent with the possibility that both proteins possess short leader peptides on the NH_2 terminus containing a methionine residue and/or an NH_2-terminal methionine residue.

The nature of the second protein of 73 000 M_r is somewhat puzzling in light of what is known of elastin biosynthesis. There are no data in the literature relating to a slightly higher molecular weight species of soluble elastin which would fit the description of the protein seen in the cell-free translation assay. Our data reveal that the 73 000-dalton protein is found in approximately equal concentration to the 70 000-dalton protein in translations of chick aortic mRNA. Both proteins incorporate significant amounts of proline and valine, which is indicative of an elastin-like composition (Figure 1), share antigenic determinants (Figure 5), and possess similar but slightly different basic p*I* values (Figure 7). The 73 000-dalton protein does appear to incorporate more cysteine than the 70 000-dalton component. We have obtained evidence that the 73 000-dalton component is also present in chick aorta organ culture if the tissue is extracted in 2 M urea with reduction and alkylation and is more readily identified when the sample is run in $NaDodSO_4$ slab gels together with reticulocyte lysate proteins (see Figure 4). This evidence shows that the finding of two elastin-like proteins is not an artifact of the cell-free translation but that the two proteins are also synthesized in aortic organ culture but necessitate denaturing conditions for extraction of the 73 000-dalton protein. More recently we have obtained evidence that the 73 000-dalton protein is present in both lathyritic chick and pig aortic tissue. Aortic tissue was first extracted with phosphate buffer followed by 2 M urea and lastly with 2 M urea with reduction and alkylation in order to extract this protein. The 73 000-dalton protein appears in the second urea extract using reduction and alkylation. We are currently in the process of chemically characterizing the protein. These data are consistent with the observation that the 73 000-dalton protein incorporates more cysteine than the 70 000-dalton protein, therefore possessing the potential role of forming a disulfide link to another protein within the extracellular matrix such as the microfibril (Ross, 1971).

As stated previously, we have reported on the finding of a large molecular weight protein (120 000–140 000) in lathyritic chick aortas and rabbit smooth muscle cell cultures which we postulated to be a pro form of tropoelastin. In the present study although the cell-free translation of aortic mRNA resulted in products of 180 000 daltons, these high molecular weight proteins appear to be procollagens and not a proelastin species. Further, our lysate is capable of translating skeletal muscle myosin heavy-chain mRNA which codes for a polypeptide of ~200 000 daltons.[3]

There are several possible explanations for these results. The combination of the 70 000- and 73 000-dalton proteins equals the apparent molecular weight of the putative "proelastin" molecule (Foster et al., 1978; Rucker et al., 1977). The existence of two lower molecular weight proteins could arise from either a highly specific proteolytic cleavage of a larger precursor molecule or, alternatively, the two translation proteins could represent separate gene products which may be assembled posttranslationally as a dimer for secretion into the extracellular matrix. The former possibility involving a specific proteolytic cleavage seems unlikely since the specificity of the enzyme needed is very stringent and it is difficult to imagine that the reticulocyte lysate would possess this enzyme. The time study performed corroborates this hypothesis, providing no evidence that the M_r 70 000 or 73 000 proteins originate from the cleavage of a higher molecular weight protein. Further, storage of translation products at 4 °C for over 1 month results in no apparent proteolytic activity. It is also very unlikely that the two proteins represent incomplete translation products from the same mRNA since their amounts are very constant when different RNA and lysate preparations are used and they are differentially labeled with cysteine.

The possibility that both proteins represent separate gene products which may associate as a dimer in vivo appears to be a feasible explanation for the origin of the high molecular weight soluble elastin molecule. The published data on the putative proelastin would necessitate that a dimer would have to be stabilized by more than a disulfide linkage since the higher molecular weight species was first identified by $NaDodSO_4$ slab gel electrophoresis in the presence of mercaptoethanol. The concept of an elastin dimer is compatible with the data on elastin synthesis in smooth muscle cell cultures (Foster et al., 1978). The cell culture data would suggest that dimer formation occurs intracellularly prior to secretion. Since chick tropoelastin preparations thus far isolated contain allysine and the aldol condensation products (Foster et al., 1975), the potential for a lysyl oxidase generated cross-linked dimer analogous to β chains of collagen does exist. It is interesting to note in this regard that Abraham & Carnes (1978a,b) have recently reported on the isolation of the cross-linked dimer of elastin from copper-deficient pig aorta. We currently have no direct chemical evidence that the high molecular weight (140 000) elastin-like protein is a dimer; however, in light of the cell-free data, we are actively pursuing this possibility.

Acknowledgments

The authors thank Dr. Janet Monson for her helpful advice on the isolation of RNA and the heating of the RNA prior to translation assays.

References

Abraham, P. A., & Carnes, W. H. (1978a) *Anal. Biochem. 91*, 115.

Abraham, P. A., & Carnes, W. H. (1978b) *J. Biol. Chem. 253*, 7993.

Abraham, P. A., Smith, D. W., & Carnes, W. H. (1974) *Biochem. Biophys. Res. Commun. 58*, 597.

[3] D. Keiss and A. Przybyla, unpublished experiments.

Benveniste, K., Wilczek, & Stern, R. (1973) *Nature (London) 246*, 303.
Blobel, G., & Dobberstein, B. (1975) *J. Cell Biol. 67*, 835.
Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83.
Burnett, W., & Rosenbloom, J. (1979) *Biochem. Biophys. Res. Commun. 86*, 478.
Evans, M. J., & Lingrel, J. B. (1969) *Biochemistry 8*, 829.
Foster, J. A., Shapiro, R., Voynow, P., Crombie, G., Faris, B., & Franzblau, C. (1975) *Biochemistry 14*, 5343.
Foster, J. A., Mecham, R. P., & Franzblau, C. (1976a) *Biochem. Biophys. Res. Commun. 72*, 1399.
Foster, J. A., Knaack, D., Faris, B., Moscaritolo, R., Salcedo, L., Skinner, M., & Franzblau, C. (1976b) *Biochim. Biophys. Acta 446*, 51.
Foster, J. A., Mecham, R. P., Imberman, M., Faris, B., & Franzblau, C. (1977) *Adv. Exp. Med. Biol. 79*, 351.
Foster, J. A., Mecham, R. P., Rich, C. B., Cronin, M., Levine, A., Imberman, M., & Salcedo, L. L. (1978) *J. Biol. Chem. 253*, 2797.
Foster, J. A., Rich, C. B., Berglund, N., Huber, S., Mecham, R. P., & Lange, G. (1979a) *Biochim. Biophys. Acta* (in press).
Foster, J. A., Wu, S. C., Marzullo, M. T., & Rich, C. B. (1979b) *Anal. Biochem.* (in press).
Harbe, N., & Ingild, A. (1973) in *A Manual of Quantitative Immunoelectrophoresis* (Axelsen, N. H., Kroel, J., & Weeke, B., Eds.) p 161, Universitetsforlagel, Oslo, Norway.
Heng-Khoo, C. S., Rucker, R. B., & Buckingham, K. W. (1979) *Biochem. J. 177*, 559.
Jackson, R. C., & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5598.
Kessler, S. W. (1975) *J. Immunol. 115*, 1617.
Krawetz, S. A., & Anwar, R. A. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 300.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Mecham, R. P., & Foster, J. A. (1977) *Biochemistry 16*, 3825.
Monson, J. M., & Goodman, H. M. (1978) *Biochemistry 17*, 5122.
Murphy, L., Harsch, M., Mori, T., & Rosenbloom, J. (1972) *FEBS Lett. 21*, 113.
Narayanan, A. S., Sandberg, L. B., Ross, R., & Layman, D. L. (1976) *J. Cell Biol. 68*, 411.
O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977) *Cell 12*, 1133.
Palacios, R., Palmiter, R. D., & Schimke, R. T. (1972) *J. Biol. Chem. 247*, 2316.
Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247.
Pinder, J. C., Staynov, D. Z., & Gratzer, W. B. (1974) *Biochemistry 13*, 5373.
Przybyla, A. E., MacDonald, R. J., Harding, J. D., Pictet, R. L., & Rutter, W. J. (1979) *J. Biol. Chem. 254*, 2154.
Rosenbloom, J., & Cywinski, A. (1976) *Biochem. Biophys. Res. Commun. 69*, 613.
Ross, R. (1971) *J. Cell Biol. 50*, 172.
Rucker, R. B., Riggins, R. S., Loughlin, R., Chan, M. M., Chen, M., & Tom, K. (1975) *J. Nutr. 105*, 1062.
Rucker, R. B., Murray, J., Lefevre, M., & Lee, J. (1977) *Biochem. Biophys. Res. Commun. 75*, 358.
Ryhanen, L., Graves, P. N., Bressan, G. M., & Prockop, D. J. (1978) *Arch. Biochem. Biophys. 185*, 344.
Siegel, R. C., Pennel, S. R., & Martin, G. R. (1970) *Biochemistry 9*, 4486.
Smith, D. W., Abraham, P. A., & Carnes, W. H. (1975) *Biochem. Biophys. Res. Commun. 66*, 893.
Uitto, J., Hoffmann, H., & Prockop, D. J. (1976) *Arch. Biochem. Biophys. 173*, 187.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.