Vol. 86, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1979 Pages 478-484

ISOLATION AND TRANSLATION OF ELASTIN mRNA FROM CHICK AORTA

William Burnett and Joel Rosenbloom

Center for Oral Health Research and Department of
Histology and Embryology
School of Dental Medicine, University of Pennsylvania
Philadelphia, Pa. 19104

Received November 27, 1978

SUMMARY

mRNA was isolated from 15-day chick embryo aortae by digestion of the tissue at 40° with Proteinase K in 1% sodium dodecyl sulfate followed by chromatography on oligo(dT)-cellulose columns. The mRNA was translated using a reticulocyte lysate system and the tropoelastin immunoprecipitated using a specific antiserum prepared against insoluble elastin. The precipitated product was characterized by polyacrylamide gel electrophoresis and a major peak corresponding to a molecular weight of approximately 70,000 daltons was observed. No higher molecular weight product was observed.

In vertebrates, the protein elastin is a vital component of major blood vessels, imparting to them their elastic, rubber-like quality. In the highly insoluble elastin, the polypeptide chains are extensively cross-linked by desmosines and other cross-linkages (1,2) derived from the enzymatic oxidation of lysine residues (3-5). A single polypeptide, designated tropoelastin, with a molecular weight of about 70,000 appears to be a soluble intermediate in the biosynthesis of the fiber (6-9) although recent evidence suggests that a larger molecular weight precursor may also exist (10,11). The larger species of 100,000-135,000 daltons may not have been observed generally because of rapid proteolytic cleavage to the 70,000 dalton tropoelastin (12). More recently, Rhyanen, et al. (13) have isolated polysomes from aortae and allowed them to complete translation *in vitro*. The major product observed had a molecular weight of 70,000 and appeared to be similar or identical to tropoelastin. The result suggests that tropoelastin is the initial product of translation, although it is possible that rapid proteolytic cleavage of the product occurred. This possibility is particularly troublesome since a protease may be tightly associated with the precursors and this protease may cleave the high molecular weight species to tropoelastin (12). This protease could bind to nascent chains and be extracted along with the polysomes.

In an attempt to avoid these problems and to characterize the primary product in elastin biosynthesis, we have isolated elastin mRNA from chick aortae and translated it in a rabbit reticulocyte lysate system. Most of the product which could be precipitated with specific antiserum to elastin had a molecular

0006-291X/79/030478-07$01.00/0

weight of 70,000 as measured by polyacrylamide gel electrophoresis and no higher molecular weight species was observed.

MATERIALS AND METHODS

Isolation of mRNA. Aortae were dissected from forty eight 15-day chick embryos as previously described (8). Loose connective tissue was removed, the aortae were cut along their long axes, rinsed in ice cold Krebs medium to remove blood and placed immediately in liquid nitrogen. The tissue (about 0.8 g) was homogenized in 20 ml of 10 mM Tris·HCl (pH 7.5) buffer containing 1% SDS[1], 5 mM EDTA, 65 µg/ml Proteinase K (Beckman) as described by Rowe, et al. (14) 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 hr to digest any ribonucleases which might be present (15). After the incubation, 20 ml of 1 M NaCl was added, and the sample was centrifuged at 13,000 x g for 10 min at room temperature to remove any insoluble material.

mRNA was isolated from the supernatant by oligo(dT)-cellulose chromatography in SDS (16). The column was equilibrated with 0.5% SDS, 0.5 M NaCl, 4 mM EDTA, 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, 10 mM Tris·HCl (pH 7.5) to remove the SDS, and finally with 15 ml of 0.5 M NaCl to remove the EDTA. The mRNA was then eluted with double distilled H_2O. The isolated mRNA was heated to 60° for 5 min, then cooled in ice water. It was stored at -70° 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 and Anderson (17). The reticulocytes were washed three times with buffer containing 0.14 M NaCl, 50 mM KCl, 5 mM $MgCl_2$, and then lysed by the addition of an equal volume of a 1 mM solution of dithiothreitol (pH 7.0). The lysate was centrifuged at 15,000 x g for 20 min to remove insoluble matter and the supernatant stored in 1 ml aliquots at -70°. The isolated mRNA was translated by modification of the procedure of Rowe et al. (14). The lysate was made 25 mM with hemin and treated with micrococcal nuclease (18) which reduced the endogenous translation to 1-2% of the untreated lysate. Because of the sensitivity of tropoelastin to proteolysis, various protease inhibitors were tested in the translation mix (TLCK, TPCK, PMSF, ε-ACA, Aprotinin and pancreatic trypsin inhibitor). Only ε-ACA did not inhibit translation and it was used at a final concentration of 20 mM. It was found necessary to optimize each batch of lysate with respect to K^+, Mg^{++} and spermidine concentrations. One lysate batch was used in all the experiments reported here with the ion concentrations listed below. NaCl was found to be inhibitory at all concentrations tested up to 40 mM and not used. Each assay tube of 125 µL contained 50 µl of treated lysate, 10 mM N-2-hydroxyethylpiperzine-N'-2-ethanesulfonic 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 (Cal Biochem.) 40 µCi/ml [^{3}H]valine (New England Nuclear) and 20 mM ε-ACA. [^{3}H]Valine was used since valine is found in relatively large amounts in elastin. Incubations were routinely carried out in plastic Falcon tubes at 26° for 30-180 min. 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 NEM, 10% µg/ml PMSF, 50 µg/ml TLCK, 50 µg/ml TPCK, 40 mM ε-ACA.

[1]Abbreviations used : SDS, sodium dodecyl sulfate; TLCK, tosyllysyl chloromethyl ketone; TPCK, toluene sulfonylphenylalanyl chloromethyl ketone; PMSF, phenymethane sulfonyl fluoride; ε-ACA, ε-aminocaproic acid; NEM, N-ethylmaleimide; TCA, trichloroacetic acid.

Total incorporation of the [^{3}H]valine was determined by precipitating 20 µL of the reaction mix onto a Whatman #3 filter disc with cold 5% TCA. Residual [^{3}H]valyl-tRNA was decylated by immersing the filters in hot (90°) 5% TCA for 5 min. The filters were again washed several times with cold 5% TCA and dried in a 150°C oven. The discs were placed in counting vials and the precipitated material was solubilized by adding 0.4 mL NCS (Amersham) and incubating overnight at 37°C. The samples were then counted in an Intertechnique scintillation counter. Elastin-related polypeptides were immunoprecipitated by a double antibody procedure using antiserum prepared in sheep against insoluble elastin as described previously (19). Two µL of the specific antiserum were added to 50 µL of the reaction mix and the samples incubated 2 hr at 16°. Then 50 µL of rabbit anti-sheep serum (Cappel Laboratories) were added and the incubation continued for 16 hr at 16°. The immunoprecipitates were isolated by centrifugation through layered sucrose-detergent solutions in plastic micro-centrifuge tubes (14). The samples were frozen, the tips cut off and the immunoprecipitates dissolved and counted as above. Control experiments had shown previously that labeled tropoelastin could be precipitated quantitatively by this procedure.

Acrylamide Gel Electrophoresis in SDS. Immunoprecipitates were dissolved in 120 µL of 0.01 N HCl containing phenol red indicator, the solutions carefully neutralized with 0.1 N NaOH and then made 0.2% with SDS and 0.2% with β-mercaptoethanol. The samples were placed in a boiling water bath for 3 min and then subjected to polyacrylamide gel electrophoresis as previously described (8).

RESULTS AND DISCUSSION

The mRNA was isolated by a procedure which avoids both the isolation of polysomes and the repeated phenol extractions commonly used. Forty eight aortae yielded approximately 50 µg of an mRNA fraction in a volume of 1 ml, as determined by absorbance at 260 nm. The A_{260}/A_{280} ratio was always between 2.1 and 2.2 indicating that little, if any protein was eluted with the mRNA. As yet, the mRNA has not been characterized further and may be contaminated with other types of RNA. When it was used in the treated reticulocyte lysate system it produced a significant stimulation of incorporation of [^{3}H]valine into total acid precipitable protein (Table I). More significantly, incorporation into protein which was immunoprecipitable with antiserum prepared against insoluble elastin and which has been shown previously to cross-react with tropoelastin was increased many fold over the background precipitation.

A kinetic study of incorporation into this immunoprecipitable fraction revealed that incorporation continued nearly linearly for about 90 min at 26°, after which the rate of incorporation declined and fell to zero by 150 min (Fig. 1). The initial portion of the curves were somewhat sigmoidal, possibly due at early times to synthesis of fragments of nascent hemoglobin chains, which were not immunoprecipitated. The background immunoprecipitable counts remained at a very low level throughout the incubation.

One of the main objects of this study was to develop a method by which functional mRNA for elastin could be measured. Figure 2 demonstrates that the quantity of radioactivity recovered in the immunoprecipitable fraction was a

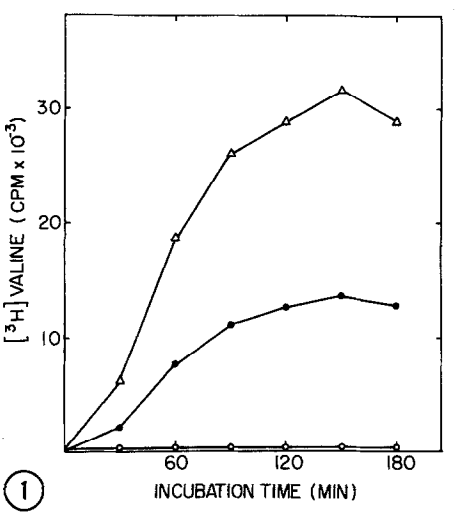
TABLE I

Incorporation of [^{3}H]Valine into Elastin by Modified Nuclease-treated Lysate[a]

Addition	TCA precipitable cpm x 10^{-3}	Elastin cpm x 10^{-3}	% of the total in Elastin
None (blank)	14.4	0.3	2
0.2 µg mRNA	24.4	7.6	31
minus blank value[b]	10.0	7.3	73
0.5 µg mRNA	39.6	18.6	47
minus blank value[b]	25.2	18.3	72

[a]Each 125 µL reaction mixture was incubated at 26° for 1 hr as described in Materials and Methods. The values represent the average of duplicate determinations which agreed within 5%. Elastin was recovered by immunoprecipitation.

[b]The blank values have been subtracted for computative purposes.



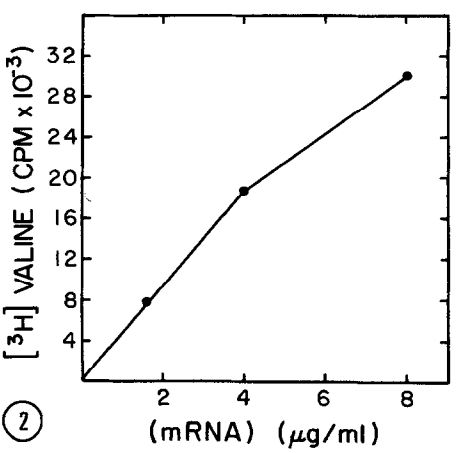


Fig. 1. Time course of incorporation of [^{3}H]valine into elastin immunoprecipitate. Assay mixtures were incubated at 26° and aliquots taken at the indicated times and the incorporated radioactivity recovered by immunoprecipitation with antiserum specific to elastin as described in Materials and Methods. The values represent the average of duplicate determinations which agreed within 5%. (o-o-o) blank; (●-●-●) 1.6 µg/ml mRNA; (Δ-Δ-Δ) 4 µg/ml mRNA.

Fig. 2. Dependence on mRNA concentration of the incorporation of [^{3}H]valine into elastin immunoprecipitate. Assay mixtures were incubated at 26° for 1 hr and the radioactivity incorporated into elastin recovered by immunoprecipitation. The values represent the average of duplicate determinations which agreed within 5%.

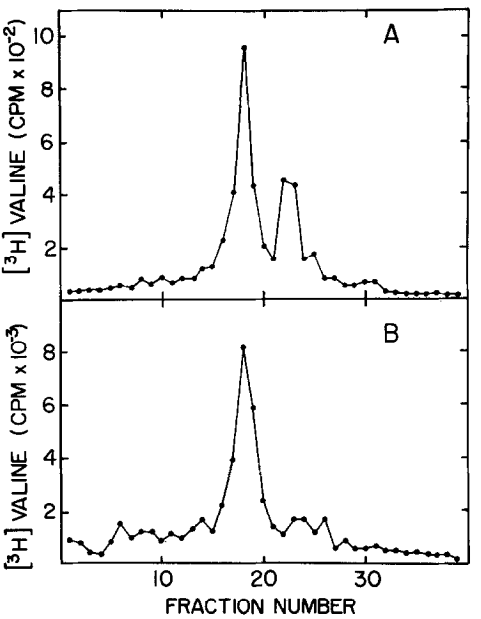


Fig. 3. (A) Polyacrylamide gel electrophoresis of elastin immunoprecipitate. The elastin immunoprecipitate from a 1 hr incubation at 26° containing 4 μg/ml mRNA was dissolved and subject to polyacrylamide gel electrophoresis as described in Materials and Methods. (B) Four thoracic aortae from 15-day old chick embryos were incubated in 1.0 ml Krebs medium for 30 min with 10 μCi [^{3}H]valine. At the end of the incubation the aortae were placed in 0.01 M sodium phosphate buffer, pH 7.4, containing 1% SDS, 20 mM NEM, 10 mM EDTA and 10 μg/ml PMSF and placed in a boiling water bath for 5 min. The extract was dialyzed against 0.1% SDS, 0.1% mercaptoethanol in .01 M phosphate and the dialyzed sample subject to polyacrylamide electrophoresis. Hydrolysis of the insoluble aortic residue showed that over 90% of the incorporated radioactivity was extracted by the hot SDS. The marker protein, bovine serum albumin (molecular weight 68,000), was located at fraction 19 and the tracking dye at fraction 39.

linear function of the mRNA concentration at least to 4 μg/ml and the assay system could probably be usefully employed to 8 μg/ml. In order to characterize the labeled protein in the immunoprecipitate, the samples were subjected to polyacrylamide gel electrophoresis in SDS (Fig. 3A). This experiment revealed that a large fraction of the radioactivity was found in a peak corresponding to a molecular weight of about 70,000 and most of the remainder was in a smaller peak of about 56,000. No significant radioactivity was found in the 100,000-130,000 dalton region. Presently we have no firm explanation for the lower molecular weight peak. It could result from incomplete translation, partially degraded mRNA, proteolytic degradation in spite of the presence of ε-ACA or antibody to an unidentified protein in our antiserum. These possibilities are under investigation.

Further examination of the data in Table I reveals that 72-73% of the total incorporated radioactivity resulting from stimulation by added aorta mRNA (the

minus blank values) was immunoprecipitable. It was of interest to compare this quantity with the incorporation of [^{3}H]valine into tropoelastin by the intact aorta. Therefore, 15-day aortae were incubated for the relatively short time of 30 min so that no cross-linking took place, under conditions in which greater than 90% of the incorporated [^{3}H]valine could be extracted into hot SDS. The labeled proteins were subjected to gel electrophoresis (Fig. 3B). Approximately 55% of the radioactivity was located in a 70,000 dalton peak shown to be tropoelastin by a number of criteria (8,13,19-20). Not included in this total are lower molecular weight polypeptides which may be related to elastin (19). Thus, from this point of view, the relative synthesis of tropoelastin in the lysate system reflects moderately well that in the intact aorta.

The data presented here suggests that the initial elastin precursor is the same size as tropoelastin. If a higher molecular weight "proelastin" exists, it must be subject to rapid cleavage since about 70% of the incorporation resulting from addition of mRNA was immunoprecipitable and appeared largely in a 70,000 dalton peak on SDS-5% acrylamide gels. It is possible that the antiserum did not react with the high molecular weight species but at any rate little radioactivity was seen in the 100,000-130,000 dalton region in the total translation mix (electrophoresis pattern not shown). Because there are no proteins derived from aorta cells present at the beginning of the translation assay and only trace amounts present during the assay, cleavage of a proelastin molecule would have to be carried out by a protease already present in the lysate. It seems unlikely that a protease capable of accurately cleaving proelastin to tropoelastin would be present in reticulocytes. It should be remembered, however, that low levels of proteases may be present in the lysate and/or antiserum and, therefore, this possibility cannot be ruled out entirely. We believe that this system, which represents the first report of translation of elastin mRNA, will be useful in further investigations into the biosynthesis of elastin.

Acknowledgements. This investigation was supported by the National Institutes of Health Research Grants AM-20863, AM-20553, DE-02623 and HL-21152.

REFERENCES

1. Partridge, S.M. (1962) Adv. Protein Chem. 17, 227-297.
2. Franzblau, C., Sinex, F.M., Faris, B. and Lampidis, R. (1965) Biochem. Biophys. Res. Commun. 21, 525-581.
3. Partridge, S.M., Elsden, D.F., Thomas, J., Dorfman, A., Telser, A. and Ho, P. (1966) Nature 209, 399-400.
4. Miller, E.J., Martin, G.R., Mecca, C.H. and Piez, K.A. (1965) J. Biol. Chem. 240, 3623-3627.
5. Pinnell, S.R. and Martin G.R. (1968) Proc. Natl. Acad. Sci. U.S.A. 61, 708-716.

6. Sandberg, L.B., Weissman, N. and Smith, D.W. (1969) Biochemistry 8, 2940-2945.
7. Foster, J.A., Bruenger, E., Gray, W.R. and Sandberg, L.B. (1973) J. Biol. Chem. 248, 2876-2879.
8. Murphy, L., Harsch, M., Mori, T. and Rosenbloom, J. (1972) FEBS Lett. 21, 113-117.
9. Smith, D.W. and Carnes, W.H. (1973) J. Biol. Chem. 248, 8157-8161.
10. Foster, J.A., Mecham, R.P., and Franzblau, C. (1976) Biochem. Biophys. Res. Commun. 72, 1399-1406.
11. Foster, J.A., Mecham, R.P., Rich, C.B., Cronin, M.F., Levine, A., Imberman, M., Salcedo, L.L. (1978) J. Biol. Chem. 253, 2797-2803.
12. Mecham, R.P. and Foster, J.A. (1977) Biochemistry 16, 3825-3831.
13. Ryhanen, L., Graves, P.N., Bressan, G.M., and Prockop, D.J. (1978) Arch. Biochem. Biophys. 185, 344-351.
14. Rowe, D.W., Moen, R.C., Davidson, J.M. Byers, P.H., Bornstein, P., and Palmiter, R.D. (1978) Biochemistry 17, 1581-1590.
15. Hilz, H., Wiegers, U., and Adamietz, P. (1975) Eur. J. Biochem. 56, 105-108.
16. Krystosek, A., Cawthon, M.L., and Kabat, D. (1975) J. Biol. Chem. 250, 6077-6084.
17. Gilbert, J.M. and Anderson, W.F. (1971) Meth. Enzymology 20, 542-549.
18. Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256.
19. Christner, P., Dixon, M., Cywinski, A., and Rosenbloom, J. (1976) Biochem. J. 157, 525-528.
20. Rosenbloom, J. and Cywinski, A. (1976) Biochem. Biophys. Res. Commun. 69,613-620.
21. Uitto, Z., Hoffman, H.P. and Prockop, D.J. (1976) Arch. Biochem. Biophys. 173, 187-200.