Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 364-372

CONSTRUCTION, IDENTIFICATION AND CHARACTERIZATION OF A CHICK ELASTIN cDNA CLONE

William Burnett, Kyonggeun Yoon and Joel Rosenbloom

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

Received January 14,1981

SUMMARY

mRNA was isolated from thoracic aortas of 16-day chick embryos by digestion of the tissue at 40° with Proteinase K in 1% sodium dodecyl sulfate followed by chromatography on oligo(dT)cellulose columns. Blunt-ended heteroduplex molecules consisting of one strand of mRNA and one of cDNA were formed using AMV reverse transcriptase and S1 nuclease. The duplexes were tailed with dCTP and hybridized to the plasmid pBR322 which had been restricted with PstI and tailed with dGTP. The recombinant plasmids were used to transform E. coli C600. Ampicillin sensitive and tetracycline resistant colonies were screened by in situ hybridization with partially purified elastin mRNA that had been terminally labeled with ^{32}P. Colonies which hybridized with the probe were selected for further identification by hybrid selected translation using the nuclease-treated reticulocyte lysate system. mRNA recovered from hybridization to DNA of one clone, pWB1, markedly stimulated incorporation of [^{3}H]valine into protein which was immunoprecipitable with affinity-purified anti-elastin antibody. When the immunoprecipitated product was subjected to polyacrylamide gel electrophoresis, a major peak of 70,000 daltons characteristic of tropoelastin was observed. Restriction enzyme analysis of the recombinant plasmid demonstrated that the insert was approximately 240 bases. This is the first report of the construction of a plasmid containing sequences complementary to elastin mRNA.

The connective tissue protein elastin is largely responsible for maintaining the elasticity of major blood vessels and lung tissue. 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 by a copper-requiring enzyme (3-5). Sandberg et al. (6) prepared a soluble protein called tropoelastin from the aortas of copper-deficient pigs and this protein consisted of a single polypeptide chain with an estimated

Abbreviations used: DTT, dithiothreitol; PIPES, 1,4-piperazinediethane sulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; SDS, sodium dodecyl sulfate; PVP, polyvinylpyrrolidone.

0006-291X/81/060364-09$01.00/0

molecular weight of about 70,000 and an amino acid composition which closely resembled that of insoluble elastin except for the absence of cross-links. A significant portion of porcine tropoelastin has been sequenced, although the isolated, sequenced peptides have not been ordered (7). A similar protein has been isolated from the aortas of copper-deficient or lathyritic chicks (8, 9). A variety of studies (10-16) have shown that tropoelastin is a soluble intermediate in the biosynthesis of the insoluble fiber, and cell-free translation of elastin mRNA has demonstrated that it is the primary translation product (17-19).

Further analysis of the regulation of elastin synthesis as well as sequence determination of the polypeptide chain would be facilitated by the possession of DNA sequences complementary to elastin mRNA. We report here the construction, identification and partial characterization of a cDNA clone to elastin mRNA isolated from embryonic chick aorta.

MATERIALS AND METHODS

Preparation of cDNA. mRNA was isolated from thoracic aortas of 16-day chick embryos by digestion of the tissue with proteinase K in sodium dodecyl sulfate and the poly(A+)mRNA recovered by chromatography on oligo(dT)-cellulose as previously described (17). mRNA used for a probe was further purified by centrifugation on a 5-20% sucrose gradient and the 16-35 S fraction taken and reisolated by oligo(dT) chromatography. The mRNA was used as a template for the synthesis of cDNA by AMV reverse transcriptase (a gift from J. Beard), essentially using the procedure described by Schibler, et al. (20). The 0.5 ml reaction mix contained 10 µg mRNA, 0.8 mM each of NTP (plus 50 µCi [^{32}P]dCTP), 50 mM Tris·HCl, pH 8.3, 50 mM NaCl, 10 mM $Mg(OAc)_2$, 1 mM DTT, 10 µg/ml oligo(dT), 100 µg/ml bovine serum albumin, and 50 units of reverse transcriptase. The mix was incubated for 1 hr at 42°, extracted with phenol-chloroform (1:1), and then passed through a 1 x 10 cm Sephadex G-75 column. The excluded peak of radioactivity was lyophilized and redissolved in 100 µl of 0.3 M NaCl, 4.5 mM $ZnCl_2$, 30 mM Na(OAc) pH 4.6. One thousand U of S1 nuclease (Boehringer-Mannheim) was added and the reaction proceeded for 30 min at 37°. The reaction mix was then extracted with phenol/chloroform, passed through a Sephadex G-75 column, and the excluded peak was lyophilized.

Preparation of hybrid plasmid. Complementary homopolymer tails were added to the cDNA:mRNA hybrid molecule; and to Pst I-cut pBR322 (21). Each reaction mix contained 0.1-1 µg of DNA, 1 mM $CoCl_2$, 40 mM Na PIPES pH 6.8, 0.1 mM DTT, 50 units of terminal transferase (PL Biochemicals), and 0.1 mM of the appropriate deoxynucleotide triphosphate. pBR322 was tailed with dGTP at room temperature for 20 min, and the cDNA:mRNA was tailed with dCTP at room temperature for 5 min, which resulted in tails approximately 10 nucleotides long. The reaction

was terminated by the addition of EDTA to a final concentration of 5 mM and the mix was phenol-chloroform extracted. The plasmid, which was unlabeled, was precipitated by the addition of 2.5 vol of ethanol. The tailed heteroduplex was passed through a Sephadex G-75 column, lyophilized and redissolved in 0.1 M NaCl, 2.0 mM EDTA and 10 mM HEPES pH 7.5. Equimolar amounts of plasmid and cDNA:mRNA duplexes (average length was estimated to be 1 kb by gel electrophoresis) were mixed at a final concentration of 1 μg/ml total DNA. The molecules were annealed by slowly cooling the solution from 65° to room temperature.

Transformation of E. coli C600. The E. coli were grown in 100 ml L broth until the absorbance at 550 nm was 0.3. The cells were centrifuged at 3000 g for 15 min, resuspended in wash buffer (40 mM $CaCl_2$, 10 mM $RbCl_2$, 10 mM morpholinopropanesulfonic acid, pH 6.5) and centrifuged again. The wash step was repeated and the cells were finally suspended in 10 ml of wash buffer. One-tenth μg of plasmid was added to 200 μl of cells and placed in ice for 20 min. Then the cells were heat shocked (42° for 5 min), spun down and resuspended in 50 μl of L broth. The cells were incubated for 2 hrs at 37°, then spread on agar plates containing 5 μg/ml tetracycline.

Screening of transformed colonies. One hundred fifty of the tetracycline-resistant colonies were transferred to agar plates containing 20 μg/ml of ampicillin and incubated overnight at 37°. Twenty-three of the colonies were sensitive to the ampicillin and were kept for further screening. Colony hybridization was carried out by the method of Grunstein and Hogness (22) using a ^{32}P end-labeled probe prepared in the following way. One μg aorta mRNA was partially hydrolyzed in 0.1 M NaOH at 4° for 30 min, and the solution then neutralized. ^{32}P was added by incubating the mRNA with 100 μCi of [$\gamma^{32}P$]ATP in 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 1.5 mM spermine, 5 mM DTT with 5 units of T4 polynucleotide kinase (PL Biochemicals) for 1 hr at 37°. The labeled RNA (2 x 10^6 cpm/μg) was extracted with phenol/chloroform and separated from the ATP by Sephadex G-50 chromatography. Hybridization was carried out overnight at 42° in 70% formamide, 0.4 M NaCl. Colonies which hybridized moderately well with the probe were selected for further screening.

Hybrid selected translation was performed essentially as described by Meyuhas and Perry (23). Individual colonies to be tested were grown in one liter of M-9 medium and the plasmids were amplified by the addition of uridine (1 mg/ml) and chloramphenicol (170 μg/ml). Plasmid DNA was isolated by the alkaline method of Birnboim and Doly (24) followed by equilibrium centrifugation in CsCl. Plasmid DNA (40 μg) was restricted with HinfI (BRL), phenol/chloroform extracted and precipitated with 2.5 volumes of ethanol. The DNA was dissolved in 80 μl of H_2O, placed in a boiling H_2O bath for 10 min, then cooled quickly in dry ice-ethanol. Twenty μl of 1 M Na acetate pH 4.0 was added to the DNA solution when the DBM paper was ready (25). The DBM paper was prepared from ABM paper (BRL, Inc.) as previously described (26). After the activation was complete, the papers were quickly rinsed three times with water and once with 0.2 M Na acetate pH 4.0. They were then blotted dry and placed on a piece of parafilm in a Petri dish. The DNA was added and the filters were allowed to dry under a hood. The papers were then incubated for 2 hrs at 42° and stored in 50% formamide, 0.4 M NaCl, 20 mM PIPES pH 6.8, 0.2% SDS, 1 mM EDTA, 0.02% Ficoll, 0.02% PVP, 1% glycine, 1 mg/ml yeast tRNA, 40 μg/ml poly A. The papers were incubated for 1 hr at 42° in prehybridization solution (50% formamide, 0.4 M NaCl, 20 mM PIPES pH 6.8, 0.2% SDS, 1 mg/ml tRNA and 50 μg/ml poly A). Hybridization was carried out for 24 hrs at 42° in 100 μl of the same solution plus 10 mM Vanadyl Ribonucleoside Complex (BRL) and 3 μg mRNA. The papers were then incubated for 30 min at 42° in a posthybridization solution (prehybridization solution plus 1 mM EDTA and without tRNA or poly A). They were then washed twice with 0.2 M NaCl, 10 mM PIPES pH 6.8, 0.2% SDS, 1 mM EDTA, and then incubated for 1 hr in the posthybridization solution. The hybridized mRNA was eluted in 200 μl of 95%

formamide, 0.2% SDS, 10 mM PIPES pH 6.8 at 68° for 3 min. The elution step was repeated and the eluates were pooled. Twenty μg of yeast tRNA was added. The eluate was made 0.2 M with NaCl, and the RNA was precipitated with 1 ml of ethanol. The pellet was dissolved in 0.2 M NaCl, re-precipitated, and the last traces of ethanol were removed by evaporation in a vacuum.

Translation of mRNA. The nuclease-treated rabbit reticulocyte lysate system was used to translate the mRNA as described in detail previously (17, 18). At the end of the incubation, aliquots of the reaction mixtures were taken for total incorporation as measured by precipitability in cold 5% trichloroacetic acid and for immunoprecipitation with affinity-purified, elastin-specific antibody (17, 18).

Biosafety precautions. Construction, identification and amplification of the recombinant plasmids were carried out in compliance with NIH Guidelines for Recombinant DNA Research.

RESULTS AND DISCUSSION

Blunt-end heteroduplex molecules consisting of one strand of mRNA and one of cDNA were formed using the AMV reverse transcriptase and S1 nuclease. The duplexes were tailed with dCTP to a length of about 10 nucleotides using terminal transferase and hybridized to the plasmid pBR322 which had been restricted with PstI and tailed with dGTP. The recombinant plasmids were used to transform E. coli C600. Electrophoresis of isolated plasmids from twenty-three tetracycline-resistant, ampicillin-sensitive colonies in horizontal 1% Agarose gels showed inserts ranging in size from close to 0 to 2,000 nucleotides. To determine which, if any, of the inserts contained sequences complementary to elastin mRNA, the transformed colonies were screened by the method of Grunstein and Hogness (22). The hybridization probe was prepared by end labeling partially hydrolyzed mRNA enriched for elastin mRNA with ^{32}P, as described in Materials and Methods. Plasmids containing inserts complementary to elastin mRNA would be expected to hybridize more strongly than most other plasmids. Of the twenty-three colonies, three gave a moderately strong signal (data not shown) and were chosen for further screening.

Electrophoresis in 1% Agarose of the plasmids from the three colonies showed that they were of high purity. Most of the plasmids ran as superhelical monomers or dimers, with a small amount running as relaxed circles. No chromosomal DNA or RNA could be seen with Ethidium Bromide staining, and the intensity

TABLE 1

Translation of Hybrid-Selected mRNA[a]

Addition	TCA precipitable cpm x 10^{-2}	Elastin cpm x 10^{-2}	% of the total in elastin
Control mRNA[b]	789.2	229.9	29
pBR322	5.7	0.1	2
pWB1	20.8	20.1	97
pWB13	0	0	-
pWB14	9.0	0.2	2

[a]Each 125 µl reaction mixture was incubated at 26° for 1 hr as described previously (17, 18). Elastin was recovered by immunoprecipitation. The values represent two separate hybrid-selected mRNA isolations and translations for each clone in which the fraction of the total incorporation which was immunoprecipitable agreed within 2%, i.e. 29 ± 2%. Blank values (no mRNA addition) of 12,600 cpm for the total TCA precipitable and 35 cpm for the immunoprecipitable elastin have been subtracted from the experimental values.

[b]Carried through the hybridization procedure in formamide and recovered by alcohol precipitation.

of the plasmid bands matched the expected intensity based upon the optical density of the plasmids at 260 nm. Forty µg of plasmid DNA from each colony was cut with Hinf I and bound to DBM paper. Three µg of aorta mRNA was incubated with each paper in 50% formamide, and the hybridized mRNA was recovered and translated in a nuclease-treated rabbit reticulocyte lysate. Representative results are contained in Table I. The starting control mRNA, which was simply precipitated from formamide and translated, actively stimulated total [^{3}H]valine incorporation into protein; of which about 30% was immunoprecipitable with the affinity-purified elastin-specific antibody. mRNA recovered from hybridization to one clone, pWB1, significantly stimulated incorporation into acid precipitable protein. Furthermore, over 95% of this labeled protein was immunoprecipitable with the elastin-specific antibody.

In order to characterize the immunoprecipitated protein further, it was subjected to electrophoresis on polyacrylamide gels in sodium dodecyl sulfate

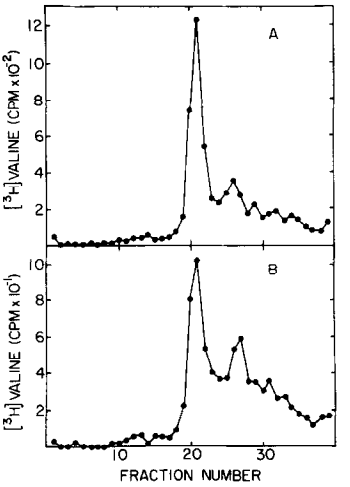
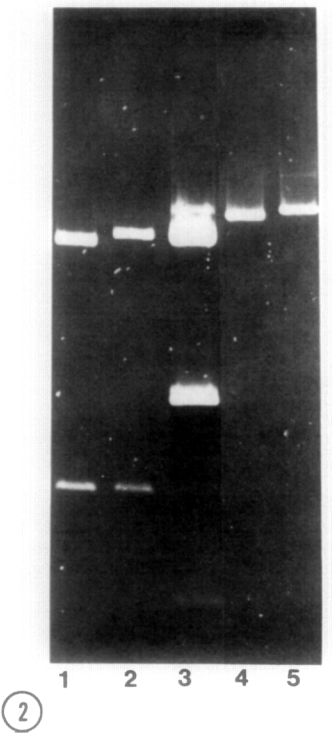


Fig. 1. Polyacrylamide gel electrophoresis of elastin immunoprecipitates. The elastin immunoprecipitates from a 1 hr incubation of the cell-free translation system at 26° containing either (A) control aorta mRNA or (B) mRNA recovered from hybridization with pWB1 DNA were dissolved and subject to polyacrylamide gel electrophoresis in SDS (17). The marker protein bovine serum albumin (68,000 daltons) was located at fraction 22.

(Fig. 1). When aorta mRNA which had been carried through the hybridization procedure and then precipitated was used in the translation system, the majority of the immunoprecipitated protein migrated as a 70,000 dalton peak characteristic of tropoelastin. A smaller amount of label was contained in lower molecular weight material, presumably shortened elastin peptides resulting from either incomplete synthesis or partial degradation. This electrophoretic pattern is very similar to that observed previously using mRNA preparations which were not exposed to hybridization conditions and recovery (17), and it suggests that there was limited degradation resulting from the hybridization procedures. The immunoprecipitated protein synthesized in response to mRNA recovered from hybridization with pWB1 yielded a similar electrophoretic pattern with a somewhat larger fraction of the material in lower molecular weight peptides. This may have resulted from partial degradation of the mRNA due to the stringent conditions necessary for elution of the mRNA from the DNA. These results firmly establish the relationship to elastin of the recombinant fragment in this plas-



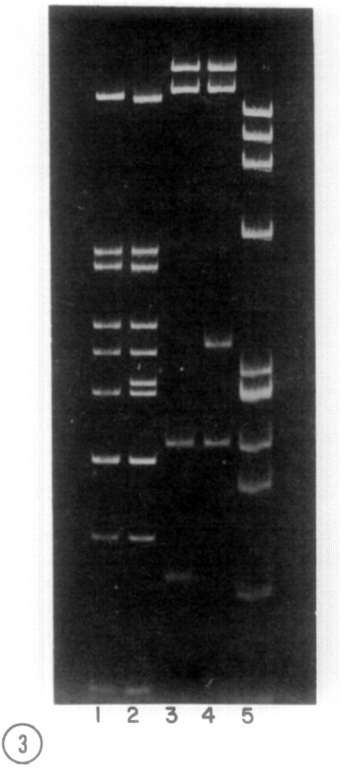


Fig. 2. Digestion with restriction endonucleases followed by 1% Agarose gel electrophoresis. 1, Pst I and Eco RI digest of pBR322; 2, Pst I and Eco RI digest of pWB1; 3, Hpa I digest of ØX174 with fragments of 3730, 1262 and 392 bases; 4, Pst I digest of pBR322; 5, Pst I digest of pWB1.

Fig. 3. Digestion with restriction endonucleases followed by electrophoresis in 5% polyacrylamide gels. 1, Hinf I digest of pBR322; 2, Hinf I digest of pWB1; 3, Pst I and Bgl I digest of pBR322; 4, Pst I and Bgl I digest of pWB1; Hae III digest of ØX174 with fragments of 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bases (281,271 run as a doublet).

mid. Too few counts were recovered in any of the other immunoprecipitates to characterize them further.

Plasmid pWB1 was characterized by restriction enzyme analysis. The insert fragment was not released upon restriction with Pst I, so that the resulting linear plasmid migrated more slowly on 1% Agarose gels than did the parent plasmid pBR322 (Fig. 2). It was not possible to accurately estimate the size of the insert from this gel, however. pWB1 was digested with EcoRI and PstI in order to determine which PstI site had been lost. The result showed that the

site closest to the EcoRI site had been retained, but here again the size of the insert could not be accurately determined (Fig. 2). The size of the insert was determined by digesting the plasmid with Pst I and BglI and electrophoresing the fragments on 5% polyacrylamide gels (Fig. 3). The 125 base fragment found in pBR322 has been replaced by a 360-370 fragment in pWB1. Thus, the insert is approximately 240 bases. In order to characterize the insert further, the fragments resulting from HinfI digestion were also separated on a 5% polyacrylamide gel. As seen in Fig. 3, two differences were seen between pBR322 and pWB1. First, the 1.6 kb fragment of pBR322 was reduced to approximately 1.5 kb and, second, a new 300 base fragment was seen. This result indicates the presence of a HinfI site within the insert approximately 190 nucleotides from the remaining PstI site.

This is the first report of the cloning of elastin cDNA and this cloned segment will be useful in a number of respects. Only a small portion of the amino terminus of chick tropoelastin has been sequenced and it is quite difficult to obtain sufficient protein for sequencing. DNA sequencing will undoubtedly prove to be easier and more rapid. We are presently screening a chick DNA library with this cloned segment in order to obtain genomic clones, and the segment will enable us to identify larger cDNA clones as well. We have previously shown that the rate of elastin synthesis in the developing chick aorta appears to be dependent upon the level of translatable elastin mRNA (18). The cDNA clone will permit measurement of total elastin mRNA and more detailed study of the regulation of elastin synthesis.

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. Nat. Acad. Sci. U.S.A. 61, 708-716.
6. Sandberg, S. R., 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. Rucker, R. B., Gottlich-Riemann, W., and Tom, K. (1973) Biochim. Biophys. Acta 317, 193-201.
9. Foster, J. A., Shapiro, R., Voynow, D, Crombie, G., Faris, B. and Franzblau, C. (1975) Biochemistry 14, 5343-5347.
10. Murphy, L., Harsch, M., Mori, T. and Rosenbloom, J. (1972) FEBS Lett. 21, 113-117.
11. Narayanan, A. S. and Page, R. C. (1976) J. Biol. Chem. 251, 1125-1130.
12. Bressan, G. M. and Prockop, D. J. (1977) Biochemistry 16, 1406-1412.
13. Rosenbloom, J. and Cywinski, A. (1976) Biochem. Biophys. Res. Commun. 69, 613-620.
14. Uitto, J., Hoffman, H-P. and Prockop, D. J. (1976) Arch. Biochem. Biophys. 173, 187-200.
15. Smith, D. W. and Carnes, W. H. (1973) J. Biol. Chem. 248, 8157-8161.
16. Rosenbloom, J., Harsch, M. and Cywinski, A. (1980) J. Biol. Chem. 255, 100-106.
17. Burnett, W. and Rosenbloom, J. (1979) Biochem. Biophys. Res. Commun. 86, 478-484.
18. Burnett, W., Eichner, R. and Rosenbloom, J. (1980) Biochemistry 19, 1106-1111.
19. Foster, J. A., Rich, C. B., Fletcher, S., Karr, S. R. and Przybla, A. (1980) Biochemistry 19, 857-864.
20. Schibler, U., Marcu, K. B. and Perry, R. P. (1978) Cell 15, 1495-1509.
21. Maniatis, T. (personal communication).
22. Grunstein, M. and Hogness, D. S. (1975) Proc. Natl. Acad. Sci., U.S.A. 72, 3961-3968.
23. Meyuhas, O. and Perry, R. P. (1980) Gene 10, 113-130.
24. Birnboim, H. C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513-1523.
25. Alwine, J. C. (personal communication).
26. Alwine, J. C., Kemp, D. J and Stark, G. R. (1977) Proc. Natl Acad. Sci. U.S.A. 74, 5350-5353.