Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 1025-1031

TRANSLATION OF TYPE II PROCOLLAGEN mRNA AND HYDROXYLATION OF THE CELL-FREE PRODUCT

Kathryn S.E. Cheah, Michael E. Grant & David S. Jackson
Department of Medical Biochemistry, University of Manchester
Medical School, Oxford Road, Manchester M13 9PT, U.K.

Received October 31, 1979

SUMMARY: Poly(A)-rich RNA was isolated from embryonic chick sternal cartilage cells and translated in a rabbit reticulocyte-lysate messenger-dependent cell-free protein synthesizing system. The presence of mRNA for procollagen type II was indicated by the synthesis of a high molecular weight collagenous polypeptide. This component is identified as the unhydroxylated precursor of type II procollagen on the basis of its electrophoretic mobility, its susceptibility to highly purified bacterial collagenase, and its ability to act as a substrate for collagen prolyl hydroxylase.

INTRODUCTION: One of the characteristics of the differentiated state of connective tissue cells is their synthesis and secretion of specific collagen molecules of which at least five genetically distinct species are known. Regulation of the expression of the different collagen genes is basic to the development and differentiation of vertebrates and is also relevant to a number of diseases associated with defective or altered collagen molecules (for review see Ref. 1). Preliminary to any investigations of the control of collagen gene expression must be the isolation and characterization of the different procollagen mRNAs. To date, studies (1-6) have concentrated on the isolation and translation of the mRNA for procollagen I (the precursor of type I collagen). In this paper we describe the preparation and translation of a poly(A)-rich RNA fraction from chick embryonic sternal cartilage cells and provide the first demonstration of the synthesis of intact pro-α chains of type II collagen in the reticulocyte-lysate cell-free system.

EXPERIMENTAL: Matrix-free cells (5×10^9) isolated from the sternal cartilages of 17-day old chick embryos (7), were homogenised

Abbreviation: SDS, sodium dodecyl sulfate.

0006-291X/79/231025-07$01.00/0

in 20mM Tris-HCl pH 7.6 at 45°C, 1mM EDTA, 2% SDS containing 500µg/ml Proteinase K (BDH) and incubated at 45°C for 1h. Sodium acetate was added to 100mM and the mixture extracted several times with an equal volume of phenol:chloroform:isoamyl alcohol (50:50:1) at room temperature, to remove residual protein. A poly(A)-rich RNA fraction was prepared, translated in a messenger-dependent reticulocyte-lysate cell-free protein synthesizing system (8) and the translation products analysed on SDS-polyacrylamide gels (10%) as described previously (2).

The susceptibility of the translation products to digestion by highly purified bacterial collagenase was determined using the conditions described by Cheah et al (2). Translation products were also incubated with chick prolyl hydroxylase prepared by an affinity chromatographic procedure (9). The incubation was for 2h at 37°C in the presence of the appropriate cofactors (2) and the hydroxy[^{3}H]proline synthesised was determined by a specific radiochemical assay (10).

Standards of [^{14}C]proline-labelled procollagen, collagen and unhydroxylated procollagen (protocollagen) were prepared by incubating embryonic chick tendon and cartilage cells or tissue with [^{14}C]proline as described previously (2).

RESULTS AND DISCUSSION: The poly(A)-rich RNA fraction prepared from embryonic chick sternal cartilages was translated in a messenger-dependent reticulocyte-lysate cell-free system (8) in the presence of [^{3}H]proline or [^{35}S]methionine. This RNA preparation directed the synthesis of several high molecular weight polypeptides, one of which was more strongly labelled with [^{3}H]proline (Band A, Fig. 1) and had a mobility on SDS-polyacrylamide gels similar to that of unhydroxylated pro-α chains of the cartilage collagen precursor. Unhydroxylated cartilage procollagen (protocollagen II) was chosen as the appropriate standard for comparison with the translation products since the reticulocyte-lysate contains no prolyl hydroxylase activity (2) and the lack of hydroxylated proline residues has been found to influence the mobility of collagenous molecules in SDS-polyacrylamide gels. Comparison of the electrophoretic mobilities of pro-α chains, unhydroxylated pro-α chains and α-chains for collagen types I and II (Fig. 2) demonstrates that the fully hydroxylated pro-α chains migrate more slowly than their unhydroxylated counterparts, which have a mobility intermediate

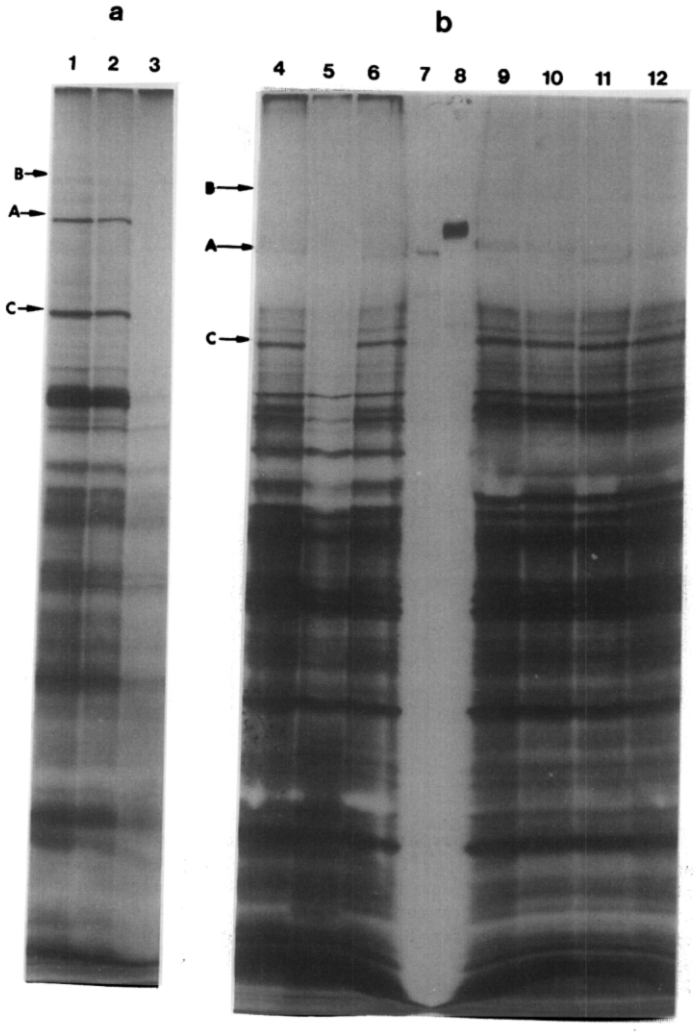

Fig. 1: Fluorograms of SDS polyacrylamide gels of translation products.

(a) Analysis of [^{3}H]proline-labelled translation products and demonstration of their susceptibility to purified bacterial collagenase. Tracks; 1, translation products; 2, control incubation with H_2O; 3, cell-free products incubated with collagenase (0.05µg/µl).

(b) Analysis of [^{35}S]methionine-labelled polypeptides and influences of bacterial collagenase and prolyl hydroxylase on translation products. Tracks; 4, translation products; 5, translation products incubated with collagenase (0.05µg/µl); 6, control incubation with H_2O; 7, unhydroxylated pro-α1(II) chains; 8, pro-α1(II) chains; 9 & 10, translation products incubated with prolyl hydroxylase (0.09 µg/µl); 11 & 12, control incubations with H_2O substituted for prolyl hydroxylase. Polypeptides referred to in the text have been designated A, B and C for reference purposes.

between pro-α and α-chains. Thus, the electrophoretic mobility of band A is in the region anticipated for the cell-free translation product of procollagen II mRNA.

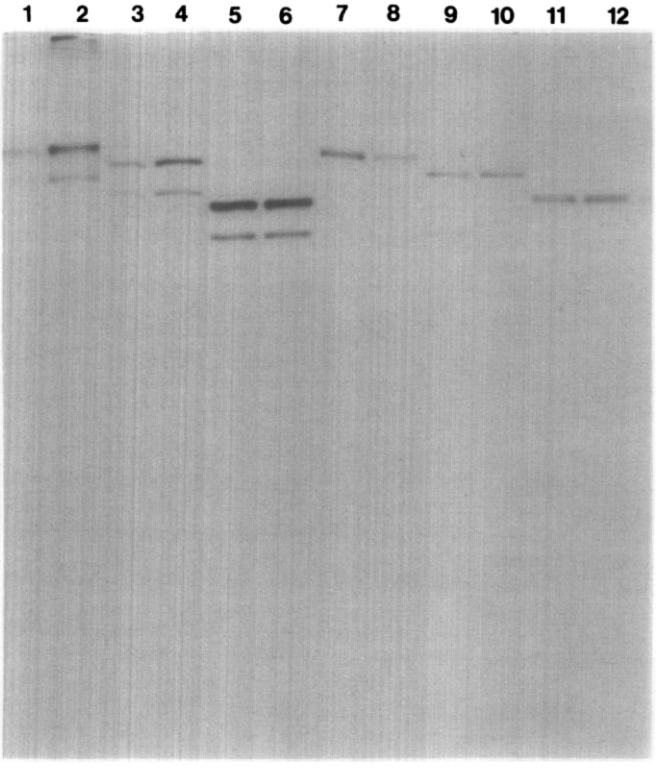

Fig. 2: Influence of hydroxylation on the electrophoretic mobilities of collagenous polypeptides. [^{14}C]Proline-labelled procollagen, collagen and protocollagen (types I and II) were analysed by electrophoresis in a discontinuous SDS/polyacrylamide slab-gel with a 3% stacking and 10% separating gel (11). Tracks 1 & 2, procollagen I; 3 & 4, protocollagen I; 5 & 6, collagen I; 7 & 8, procollagen II; 9 & 10, protocollagen II; 11 & 12, collagen II.

Determination of the susceptibility of the translation products to highly purified bacterial collagenase indicated that approximately 15% of the incorporated [^{3}H]proline was collagenase-sensitive as judged by the proportion of radioactivity rendered soluble in cold trichloroacetic acid (Table 1). Only 8% of the products were collagenase-sensitive when the experiment was repeated with [^{35}S]methionine but this result, which is analogous to data obtained in studies with chick procollagen I mRNA (2), is probably explained by the preferential labelling of collagenous molecules with [^{3}H]proline. Electrophoretic analyses indicated that band A and

Table 1 Hydroxylation of proline residues in translation products by collagen prolyl hydroxylase.

Substrate ± prolyl hydroxylase	Prolyl residues hydroxylated	Prolyl residues Collagenase-sensitive	Collagenous proline residues hydroxylated
	%	%	%
Translation products	0	13.8	0
Translation products + enzyme	4.7	16.2	29.0
Protocollagen I	0	100	0
Protocollagen I + enzyme	31.9	100	31.9
Procollagen I	40.6	100	40.6
Collagen I	50.9	100	50.9

[^{3}H]Proline-labelled translation products and [^{14}C]proline-labelled protocollagen I were incubated ± chick prolyl hydroxylase (0.09µg/µl) and the synthesis of labelled hydroxyproline determined (10). Determinations of the hydroxyproline content of [^{14}C]proline-labelled procollagen I and collagen I are included for comparison. The percentage of prolyl residues which were collagenase-sensitive was determined as the proportion of total radioactivity rendered TCA-soluble after incubation with bacterial collagenase.

$$\% \text{ collagenous proline residues hydroxylated} = \frac{\% \text{ proline residues hydroxylated} \times 100}{\% \text{ proline residues collagenase-sensitive}}$$

several others including bands B and C (Fig. 1) were degraded by collagenase.

Collagenous molecules are distinguished by their post-translational hydroxylation of peptidyl-prolyl and -lysyl residues (1) and therefore the ability of the translation products to act as substrate for purified collagen prolyl hydroxylase was investigated. The demonstration of a consequent decrease in the electrophoretic mobility of the polypeptides provides a further means of identifying collagenous components among the translation products. Only bands A and C were consistently found to have a decreased mobility after incubation of the translation products with prolyl hydroxylase and

band A migrated in a position intermediate between pro-α1(II) and unhydroxylated pro-α1(II) chains, (Fig. 1). Analyses of the hydroxylated products indicated that approximately 5% of the total [^{3}H]proline incorporated was present as hydroxy [^{3}H]proline and since approximately 15% of the products were judged to be collagenase-sensitive, it can be estimated that a third of the proline residues in the collagenous polypeptides had been hydroxylated. Procollagen polypeptides normally contain 40-45% of their imino acid content as hydroxyproline (Table 1). Type II collagen is also known to be significantly glycosylated (1) but since hydroxylation was incomplete and no glycosylation occurs during cell-free translation, band A would not be expected to migrate in gels to the same position as the fully hydroxylated and glycosylated pro-α1(II) chains unless there was also present a long leader peptide similar to the one in pre-pro-α1(I) (2,12,13).

The reduction in electrophoretic mobility of band C following incubation with prolyl hydroxylase together with its collagenase-susceptibility suggest that this polypeptide could also be collagenous. Its relationship to procollagen II is unclear but it seems unlikely to be a degradation product of band A since the intensity of band C does not increase with incubation at 30°C for a further hour or two hours (cf. tracks 1 and 4 with 2, 6, and 9-12 Fig. 1). The possibility that band C arises from premature termination during translation, or is the translation product of some procollagen mRNA molecules which had been cleaved at a specific position, will require further investigation. Alternatively, band C could represent a hitherto undetected collagenous species. The nature of band B is also uncertain for it migrates more slowly than band A, pro-α1(II) (Fig. 1), or pro-α1(I) (not shown); it also appears to be tissue-specific as no similar polypeptide has been detected during the translation of chick

tendon (2) or chick artery mRNA (K.S.E. Cheah et al., in preparation). It is noteworthy that an important protein component of the cartilage extracellular matrix is the chondroitin sulphate-associated core protein, approximate mol. wt. 200000, which is relatively rich in glycine (13%) and proline (11%) (14). Thus, band B could be related to core protein on the basis of size, and the cleavage of core protein by bacterial collagenase might be expected since there is a high probability that susceptible -X-pro-gly-sequences occur within the molecule. Further work will be required to establish the identities of these other products of cell-free translation but the data reported here suggest that band A (Fig. 1) represents the complete translation product of cartilage procollagen type II mRNA.

ACKNOWLEDGMENTS

This work was supported by the Science Research Council and the Arthritis and Rheumatism Council. We are indebted to Dr. K.I. Kivirikko, University of Oulu, Finland, for his generous gift of purified chick embryo prolyl hydroxylase, and to Miss Margaret Keys for skilled technical assistance.

REFERENCES

1. Prockop, D.J., Kivirikko, K.I., Tuderman, L. & Guzman, N.A. (1979) New Eng. J. Med., 301, 13-23; 77-85.
2. Cheah, K.S.E., Grant, M.E. & Jackson, D.S. (1979), Biochem. J. 182, 81-93.
3. Lehrach, H., Frischauf, A.M., Hanahan, D., Wozney, J., Fuller, F. & Boedtker, H. (1979), Biochemistry, 18, 3146-3152.
4. Adams, S.L., Alwine, J.C., de Crombrugghe, B. & Pastan, I. (1979), J. Biol. Chem., 254, 4935-4938.
5. Tolstoshev, P., Haber, R. & Crystal, R.G. (1979), Biochem. Biophys. Res. Commun., 87, 818-826.
6. Rave, N., Crkvenjakov, R. & Boedtker, H. (1979), Nucleic Acids Res., 6, 3559-3567.
7. Dehm, P. & Prockop, D.J. (1973), Eur. J. Biochem., 35, 159-166.
8. Pelham, H.R.B. & Jackson, R.J. (1976), Eur. J. Biochem., 67, 247-256.
9. Tuderman, L., Kuutti, E.R., & Kivirikko, K.I. (1975), Eur. J. Biochem., 52, 9-16.
10. Juva, K. & Prockop, D.J. (1966), Anal. Biochem., 15, 77-83.
11. Laemmli, U.K. (1970), Nature, 227, 680-685.
12. Graves, P.N., Olsen, B.R., Fietzek, P.P., Monson, J.M. & Prockop, D.J. (1979), Fed. Proc., 38, 620.
13. Palmiter, R.D., Davidson, J.M., Gagnon, J. Rowe, D.W. & Bornstein, P. (1979), J. Biol. Chem., 254, 1433-1436.
14. Muir, H. & Hardingham, T.E. in MTP Intern. Rev. Sci. Biochemistry Series 1, Biochemistry of Carbohydrates, (Whelan, W.J., Ed.) 5, 153-222 (Butterworth's, London 1975).