COLLAGEN PRO α_1 POLYSOMES APPEAR TO SEDIMENT MORE RAPIDLY THAN PRO α_2 POLYSOMES

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1. Introduction

Although knowledge of the earliest precursor chains of the pro α_1 and pro α_2 subunits of type I procollagen is still very incomplete, the present belief is that these precursor chains differ in size <10%, with the pro α_2 precursor being the smaller [1,2].

This paper reports the unexpected finding that the polysomes that synthesize pro α_1 [I] chains are considerably larger than those that synthesize pro α_2 chains. Although the explanation for this observation is not known, it should be taken into account in attempts to construct a complete picture of the sizes, secondary structures and ribosome-loading of these mRNAs. In [3] such a difference in these polysomes was not detected (see section 4).

2. Materials and methods

2.1. *Fractionation of calvaria polysomes on sucrose gradients*

Calvaria were removed from 280–300 17-day chick embryos and were washed twice with ice-cold buffer B (250 mM KCl, 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.14) and 150 μg/ml heparin). Cells on the surfaces of the bones were lysed by exposing them for 10 min at 0°C to buffer B from which the heparin was omitted and to which was added 5.8 mM mercaptoethanol, 0.2% (v/v) Kyro EOB and 0.5% Triton X-100 [3]. The lysate was centrifuged for 10 min at 10 000 × *g* and the supernatant was applied to 15–40% (w/v) sucrose gradients in buffer B and was centrifuged in the Beckman SW-27 rotor for 2 h at 26 000 rev./min at 4°C. One gradient of each run of 6 gradients was monitored for A_{260}. Fractions of 3 ml each were collected from the bottoms of the other tubes; identical fractions were pooled and the polysomes were pelleted by centrifugation in the SW-27 rotor at 26 000 rev./min for 6.5 h at 4°C. Polysome pellets were stored at −80°C until used in the cell-free protein synthesizing system.

Membrane-bound and free polysomes from 8-day decapitated embryos were prepared by a modification of the procedure in [4].

The homologous cell-free protein synthesizing system was as in [5]. The assay for unhydroxylated collagen chains using collagen prolyl hydroxylase was in [6].

2.2. *Collagenase assay*

Following cell-free protein synthesis, samples were treated for 20 min at 37°C with 0.5 N KOH and 2.5 mM of unlabeled amino acid(s) corresponding to the labeled amino acid(s) used. One-half volume of 30 mM Tris–HCl (pH 7.5) was then added and the samples were dialyzed extensively at 4°C, first against 30 mM Tris–HCl (pH 7.5) and then against 2 changes of 0.11 M Hepes buffer (pH 7.2). Samples were homogenized in Dounce homogenizer, pre-incubated at 37°C for 60 min, and were re-homogenized. Duplicate volumes of pre-incubated samples were then incubated with or without purified collagenase (Worthington) as in [7].

3. Results

Fig.1 shows that most polysomes actively incorporating [3,4-^{3}H]proline sediment to the lower half

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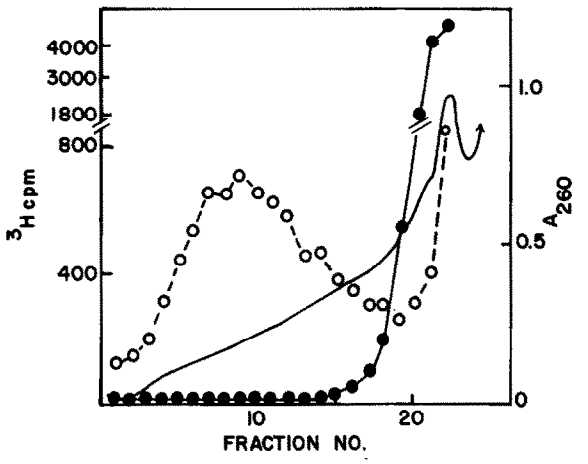

Fig.1. Calvaria of 36 embryos were incubated in serum-free medium in the presence of α,α'-dipyridyl at 37°C to inhibit prolyl hydroxylase. After 15 min [3,4-^{3}H]proline was added (to 100 μCi/ml) and incubation was continued for 20 min. The polysomes were then isolated as described. Prior to layering on sucrose gradients, one sample was treated with RNase (Sigma) (10 μg/ml, 10 min, 37°C). After centrifugation (SW 41, 40 000 rev./min, 4°C, 60 min), 0.5 ml fractions were collected and trichloroacetic acid-precipitable radioactivity in each fraction was determined; A_{260} were recorded continuously. (——) A_{260} minus RNase; (○——○) ^{3}H cpm minus RNase; (●——●) ^{3}H cpm plus RNase.

of sucrose gradients. Since treatment of the homogenate with RNase prior to centrifugation transfers the radioactivity from the polysome to the monosome region of these gradients, these sedimentation profiles appear to represent authentic polysomes, uncontaminated with radioactive aggregated material.

In an experiment similar to that in fig.1, the collagen-synthesizing polysomes were localized on sucrose gradients. Fig.2 shows that the heavier gradient fractions are greatly enriched in collagen-synthesizing polysomes. These results, combined with those in fig.1, indicate that the great majority of collagen-synthesizing polysomes are found in the lower half of the gradient. Therefore, only the fractions of the lower part of the gradient were subjected to more detailed analyses for pro α_1 and pro α_2 ratios.

The ratio of pro α_1 to pro α_2 polysomes in each gradient fraction was calculated from the relative amounts of collagenase-digestible [^{3}H]histidine and [^{14}C]proline incorporated by these fractions during incubation in the cell-free system. This method takes advantage of the fact that α_1 chains have a much higher proline:histidine ratio (119.5) than α_2 chains (31.3) [8,9]. Since the validity of this measurement depends on the degree to which the collagenase treatment digests only collagen peptides, the specificity of the collagenase was first established (table 1). The proteins synthesized by membrane-free polysomes in the cell-free system proved to be a very suitable collagen-poor substrate for testing this specificity, since these polysomes were found to synthesize negligible collagen, as judged by their inability to act as a substrate for collagen prolyl hydroxylase. Under conditions where [3,4-^{3}H]proline-labeled pure, unhydroxylated procollagen or collagen release ~23% of their total cpm as ^3H_2O as a result of incubation with collagen prolylhydroxylase [5], the cell-free products from bound polysomes released ~8.4% of their ^{3}H whereas the peptides synthesized by free polysomes released <0.1% of their ^{3}H.

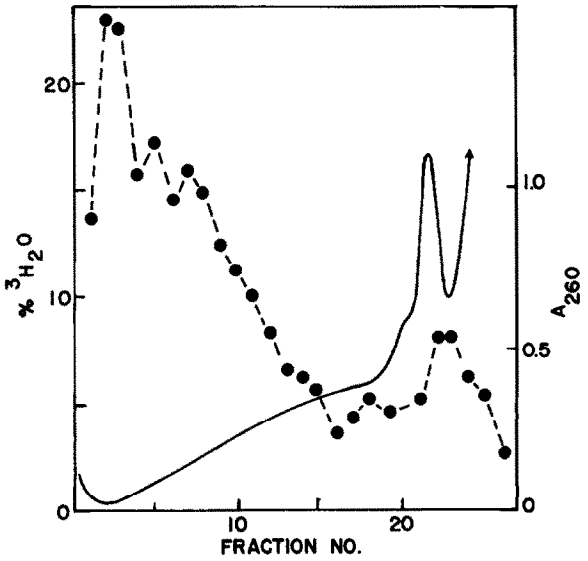

Fig.2. Protocollagen nascent chains were labeled with [3,4-^{3}H]proline in vivo in the presence of dipyridyl as in fig.1. Fractions from duplicate sucrose gradients were collected. The amounts of radioactivity in the fractions of one gradient were determined by trichloroacetic acid (TCA) precipitation, while the ^3H_2O released by collagen prolyl hydroxylase was determined for each fraction of the other gradient. The percent ^3H_2O for each fraction was calculated as follows:

$$\%\ ^3H_2O = \frac{^3H_2O\ \text{cpm}}{\text{total TCA ppt . cpm}} \times 100$$

Pure protocollagen yields ~23% ^3H_2O [5]; non-collagen proteins release <0.1% ^3H_2O (see text). (——) A_{260}; (●---●) % ^3H_2O.

Table 1
Extent of digestion by purified collagenase of collagen-poor substrates labeled with a variety of amino acids

Labeled amino acid	Polysome preparation	Collagenase	TCAT[a]-precipitable dpm	% Collagenase resistant (dpm)
Proline	Membrane-free	+	78 324	86.8
Proline	Membrane-free	–	90 248	
Histidine	Membrane-free	+	115 423	94.5
Histidine	Membrane-free	–	122 103	
Leucine	Membrane-free	+	26 317	93.7
Leucine	Membrane-free	–	28 083	
Tryptophan	Total	+	9 622	103.7
Tryptophan	Total	–	9 278	

[a] TCAT, trichloroacetic acid and termic acid (5% and 0.5%, respectively)

The polysome preparations were incubated in the cell-free system with the indicated ^{3}H-labeled amino acids. The cell-free products were then incubated in duplicate with collagenase (+) or without collagenase (–) and the TCAT-precipitable radioactivity was measured. Duplicate values differend from their average by <3.4%, except in the proline-labeled samples where the (+) and (–) values differed from their respective averages by 9.4% and 6.1%

Table 1 shows that these collagen-poor peptides, after labeling with radioactive proline or histidine or leucine, are largely resistant to digestion by this collagenase preparation. Since tryptophan is missing from the triple-helical, collagenase-sensitive, portion of collagen molecules, the lack of digestion of tryptophan-labeled proteins has become a standard test for the specificity of collagenase preparations [7]. As shown in the last two lines of table 1, this collagenase preparation gave no digestion of the tryptophan-labeled proteins synthesized by total, unfractionated chick embryo polysomes. As discussed below, these data indicated that the specificity of this collagenase was sufficient to make it suitable for the measurements reported here.

When the polysomes from the 6 sucrose gradient fractions shown in fig.3 were incubated in the cell-free system with [^{14}C]proline and [^{3}H]histidine, the proline:histidine ratio in the collagenase-sensitive cell-free products did not stay constant (table 2), as would be expected if the polysomes that synthesize pro α_1 and pro α_2 chains sedimented at identical rates. As illustrated in fig.3 the proline:histidine ratio in the collagenase-digestible material increased in the heavier

4. Discussion

Before considering possible explanations for the faster sedimentation of pro α_1 polysomes, the possibility that the data can be explained by a lack of specificity of the collagenase needs to be examined.

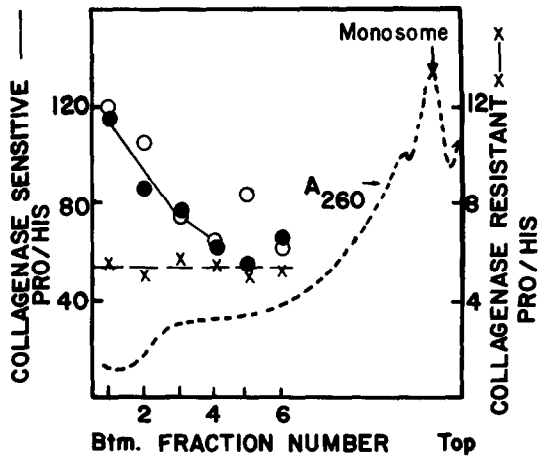

Fig.3. The proline:histidine ratio in the collagenase-sensitive

Table 2
Relative synthesis of α_1 and α_2 collagen chains by calvaria polysomes fractionated on sucrose gradients

Fraction number	Collagenase resistant (pmol Pro/pmol His)			Collagenase sensitive (pmol Pro/pmol His)			α_1/α_2
	Exp. 1	Exp. 2	Mean	Exp. 1	Exp. 2	Mean	
1.	3.6	7.7	5.65	115.8	120.9	118	202
2.	3.9	6.7	5.3	87.0	105.5	96	9.6
3.	3.8	8.0	5.9	78.2	74.6	76	3.6
4.	3.6	7.6	5.6	63.9	65.0	64	2.1
5.	3.5	7.0	5.25	56.9	84.1	70.5	2.8
6.	3.5	7.4	5.45	68.4	63.6	66	2.3

Calvaria polysomes were fractionated as shown in fig.1 and were incubated in the cell-free system in the presence of both [^{14}C]proline and [^{3}H]histidine. The cell-free products were then incubated with and without collagenase. The incorporation of proline into the resistant peptides averaged 6.19 pmol and into the sensitive peptides 19.68 pmol; the comparable figures for histidine were 1.21 and 0.26 pmol. Since mature α_1 chains contain 2 histidines and 239 prolines [7] and α_2 chains contain 7 histidines and 219 prolines [8], the ratio of α_1/α_2 = [7 (Pro/His)−219]/[239−2 (Pro/His)] where Pro/His is the molar ratio of collagenase-sensitive proline and histidine in each fraction. Since the S30 fraction used in the cell-free system was subjected to gel filtration on Sephadex G-25, the cell-free system was assumed to contain negligible pools of proline and histidine. The precursor regions of the pro α chains were excluded from these calculations since the carboxyl-terminal 'pro peptides' are resistant to collagenase as are all but ~40 residues of the amino-terminal 'pro peptides' [1]. These should make a quantitatively unimportant contribution to the total susceptible residues, especially in view of their inefficient labeling since nascent chains carrying unlabeled amino terminal portions were the starting material in the cell-free system

(1) Even if the proteins made by the gradient fractions are assumed to experience a non-specific digestion equal to that shown in table 1 for, respectively, the proline and histidine-labeled proteins, it can be calculated that this non-specific digestion would not be capable of generating the observed increase in the proline:histidine ratio in the heavier gradient fractions.

(2) The undigested proteins, which should represent the substrate for any non-specific digestion, showed no change in their proline:histidine ratio (table 2). The reasons for the higher proline:histidine ratios in the collagenase-resistant samples of exp. 2, table 2 are not clear, but the absolute values of this ratio are not considered as reliable [illegible]

Several factors could account for the faster sedimentation of pro α_1 polysomes relative to pro α_2 polysomes.

(1) Pro α_1 mRNA may be larger relative to pro α_2 mRNA than expected from present knowledge of their immediate translation products. Preliminary evidence both in favor of [2], and against [10], a larger size for pro α_1 mRNA relative to pro α_2 mRNA has been reported.

(2) Ribosomes may be more closely packed on pro α_1 mRNA which, if true, would imply that translational control plays a role in achieving the observed [1] 2:1 rate of synthesis of pro α_1 relative to pro α_2 chains.

Other possibilities are that pro α_2 polysomes are preferentially degraded during isolation or that

effect on their relative sedimentation rates. Preferential aggregation of pro α_1 polysomes cannot be completely ruled out, but this would have to be an RNA-dependent aggregation in view of the results in fig.1.

The discrepancy between the results in [3] and ours may be caused by the use of younger embryos in his studies (14-day as compared to 17-day) or may be due to differences in the way in which the sucrose gradients were fractionated. While in [3] gradients were divided into 3 parts, we divided the lower half of our gradient into 6 parts. Our narrower cuts of the heavy region of the polysome gradient, where the collagen polysomes are known to sediment (fig.2 and [5]), should be more apt to detect differences in sedimentation rates of the two types of polysomes. Molecular hybridization experiments with DNA probes specific for pro α_1 and pro α_2 mRNAs would appear likely to provide still higher resolution of these polysomes.

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References

[1] Fessler, J. H. and Fessler, L. I. (1978) Ann. Rev. Biochem. 43, 567–603.
[2] Tolstoshev, P., Haber, R. and Crystal, R. G. (1979) Biochem. Biophys. Res. Commun. 87, 818–826.
[3] Vuust, J. (1975) Eur. J. Biochem. 60, 41–50.
[4] Bloemendal, H., Benedetti, E. L. and Bont, W. S. (1974) Methods Enzymol. 30, 313–327.
[5] Pawlowski, P. J., Gillette, M. T., Martinell, J., Lukens, L. N. and Furthmayr, H. (1975) J. Biol. Chem. 250, 2135–2142.
[6] Lukens, L. N. (1970) J. Biol. Chem. 245, 453–461.
[7] Peterkofsky, B. and Diegelmann, R. (1971) Biochemistry 10, 988–994.
[8] Miller, E. J., Lane, J. M. and Piez, K. A. (1969) Biochemistry 8, 30–39.
[9] Lane, J. M. and Miller, E. J. (1969) Biochemistry 8, 2134–2142.
[10] Rave, N., Crkvenjakov, R. and Boedtker, H. (1979) Nucleic Acids Res. 6, 3559–3567.