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Isolation and characterization of collagen messenger RNA*

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ABSTRACT

Chick embryo collagen-synthesizing polysomes were isolated by differential centrifugation. RNA extracted from these particles was chromatographed in oligo(dT)-cellulose columns and the mRNA thus obtained characterized as collagen mRNA by its electrophoretical mobility in acrylamide gels (equivalent to 1.05×10^6 daltons) and its effect upon a cell-free system derived from Krebs ascites tumor cells. The incorporation of ^{3}H-proline was markedly dependent upon rabbit reticulocyte initiation factors and inhibited by initiation inhibitors such as aurintricarboxilate and pyrocatechol violet. The incorporation product was characterized as collagen by its lack of tryptophan, digestibility by purified bacterial collagenase, and by its co-chromatography with unlabeled chick collagen in Sephadex G-200 and CM-cellulose columns.

INTRODUCTION

Several aspects of the expression of genes were clarified through experiments which did not require separation of mRNA from the bulk of cellular RNA. The understanding of other features of this process does however, depend on the isolation of specific messengers. The existence of polyadenylic acid sequences in most mRNA species has facilitated separation from

rRNA[1], but a second problem remains, namely, that of separating individual mRNA species from each other. For this reason, specialized tissues, where most of the protein synthetic activity is directed towards the biosynthesis of a single/few proteins, have been selected as sources of purified mRNA. Presumptive mRNA for hemoglobin[2], immunoglobulin light chains[3], ovalbumin[4], and keratin[5] have thus been isolated, and characterized.

Collagen alpha-1 and alpha-2 chains are synthesized on polysomes containing at least 30 ribosomes[6] and according to some works, no less than 100[7]. The large size of these aggregates has permitted their separation from the cell's ribosomal population by low-speed centrifugation[8-10]. In the present work, collagen mRNA has been isolated from such "giant polysomes" and separated from the bulk of rRNA by oligo (dT) - cellulose column chromatography.

The specific informational capacity of this mRNA fraction is determined by its activity upon an amino incorporating system in vitro, and by chemical characterization of the incorporation product.

MATERIALS

L-proline (3,4 ^{3}H) specific activity 35 Ci/mM) and L-tryptophan (^{14}C) (specific activity 53.5 mCi/mM) were purchased from New England Nuclear, USA.

ATP, GTP, creatine phosphate, creatine phosphokinase, dithiothreitol (DTT), RNase A, collagenase (purified in the laboratory according to the method of Peterkofsky and Diegelman[11]), cellulose Sigma Cell P 38, dicyclohexylcarbodiimide and thymidine 5-monophosphate were obtained from Sigma Chem., Co. USA.

Triton X-100 was furnished by Rohm & Haas, São Paulo, Brasil.

Aurintricarboxilic acid (ATA) was kindly offered by Dr. Harry Woods, Drug Development, Natl. Cancer Inst., Bethesda, USA.

Pyrocatechol violet (PCV) was a gift from Dr. Arthur Grollman, Dept. of Pharmacology, Albert Einstein College of Medicine, Bronx USA.

All other reagents were analytical grade. Water employed in preparing solutions was twice-distilled. All glassware and solutions were autoclaved before use.

10-Day old chick embryos were bought at nearby hatchery (Granja Tatsumi).

METHODS

Cellular fractionation

Immediately after arriving at the laboratory, eggs were broken, the embryos removed, decapitated and their bodies placed in a small volume of 0.01 M Tris-HCl buffer, pH 7,4 containing 0.14 M NaCl, 1.5 mM $MgCl_2$ and 6 mM beta-mercaptoethanol. After homogenization in five volumes of the buffer, the homogenate was centrifuged at 10,000 xg for 10 min. After removing the overlaying fat layer, the supernatant solution was decanted and Triton X-100 added to a final concentration of 2%. After 15 min at 4^{o}, the suspension was centrifuged at 23,000 r.p.m. (40,000 xg) for 60 min, in the 35 rotor of a Beckman model L3-50 ultracentrifuge.

The resultings pellets, were suspended manually in 0.045 M Tris-HCl buffer, pH 7.5, containing 7.5 mM $MgCl_2$, 0.12 M KCl and 0.077 M NaCl.

RNA purification

Total polysomal RNA was extracted by suspending the giant

polysomes in 0.01 M Tris-HCl buffer, pH 7.6, containing 0.1 M NaCl, 1 mM EDTA, 0,5% Naphthalene disulphonate and 0.5% SDS and treating the suspension with an equal volume of buffer-saturated phenol (containing 0.1% 8-hydroxyquinoline): chloroform (1:1). After gentle agitation for 5 minutes at room temperature phases were separated by centrifugation at 10,000 xg for 2 minutes and the aqueous phase re-extracted twice more. RNA was precipitated from the final aqueous phase by the addition of two volumes ethanol, after standing overnight at -20°C. The ratio A_{260}/A_{280} varied, between experiments, from 1.95 to 2.10.

Oligo (dT)-cellulose chromatography

1,000 A_{260} units of polysomal RNA, dissolved in application buffer (0.01 M Tris-HCl, pH 7.5, containing 0.5 M KCl) were applied to a 10 ml (about 2.5 g, dry weight) oligo (dT)-cellulose column (prepared according to Gilham[12], utilizing the N,N'-dicyclohexyl-carbodiimide reaction for polymerization of 5'-TMP on cellulose). The unadsorbed material was eluted by continued washing with application buffer. The retained material (0.8 - 1.1% of applied RNA) was eluted with 0.01 M Tris-HCl buffer, pH 7.5 and precipitated by the addition of potassium acetate (final concentration 2%) and two volumes of ethanol, after standing overnight at -20°C.

Polyacrylamide gel electrophoresis of RNA

RNA samples dissolved in a buffer containing 0.04 M Tris HCl, pH 7.2, 2 mM $NaCH_3COO$, 1 mM Na_2 EDTA and 10% glycerol were analysed by polyacrylamide gel electrophoresis using 2.7% gels, crosslinked with ethylenediacrylate[13]. After a 30 min prerun at 5 mA/tube, samples (20 - 40 µg) were applied to the gels and electrophoresis was carried out for 3 hours at 5 mA/tube. Gels were scanned at 265 nm in a JOYCE LOEBL UV SCANNER.

Preparation of initiation factors and tRNA

Crude initiation factors were prepared according to the method of Schreier & Stahelin[14], and tRNA, to that of Aviv et al[15].

Preparation of Krebs II ascites tumor cells extracts[16]

Cells were propagated intraperitoneally by direct injection of freshly harvested ascitic fluid into 20 - 30 g Swiss mice. Cell extract was harvested 7 days after injection, washed three times by centrifugation (60 xg, 4 min) through cold 35 mM Tris-HCl buffer, pH 7.5, containing 140 mM NaCl, they were packed by centrifugation at 250 xg for 5 min, resuspended in 2 packed-cell volumes 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM KCl and 1.5 mM $MgCl_2$ and homogenized with 50 strokes of a tightly fitting Potter Elvehjem homogenizer equipped with a teflon pestle. The homogenate was brought to a final concentration of 30 mM Tris-HCl buffer, pH 7.5 containing 125 mM KCl; 5 mM $MgCl_2$ and 7 mM 2-mercaptoethanol, and centrifuged at 30,000 xg for 15 min. The pellet was discarded. After addition of 1 mM ATP; 0.1 mM GTP; 0.6 mM CTP; 10 mM creatine phosphate, 0.16 mg/ml creatine kinase and 25 µM (each) of amino acids, the extract was incubated at 37° for 60 min and centrifuged at 30,000 xg for 10 min. The supernatant was passed through a Sephadex G-25 column (3.5 x 25 cm) previously equilibrated with buffer containing 30 mM Tris-HCl, pH 7.5, 120 mM KCl; 5 mM $MgCl_2$, 7 mM 2-mercaptoethanol. Small aliquots were stored at -84°. Extracts used in this study contained 10 - 14 mg/ml of protein, as measured by the method of Schacterle & Pollack[17].

Assay for amino acid incorporation

Each 0.1 ml reaction mixture contained 30 mM Tris-HCl, pH 7.6, 3.6 mM $MgCl_2$, 86 mM KCl, 10 mM

dithiothreitol, 1 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 0.16 mg/ml creatine phosphokinase, 25 µM (each) of 19 nonradioactive amino acids, 0.5 Ci ^{3}H-proline or ^{14}C-triptophan (as indicated), 0.5 A_{260} units tRNA, 40 µl preincubated ascites tumor cell extract (14 mg/ml protein), 1 mg/ml rabbit reticulocyte initiation factors and mRNA as indicated. Incubation was carried out at 37° for 40 min. Reactions were stopped by the addition of 0.2 ml of 0.1 M Tris pH 10.0. Incubation was continued for 20 min and 1 ml of ice-cold 10% CCl_3COOH was added. The precipitate was left to mature for at least 60 min, collected over Whatman 3MM paper filter discs. After washing five times with 5 ml of 5% CCl_3COOH,the filters were dried and counted in a liquid scintillation counter at an efficiency of 61% for ^{3}H and 95% for ^{14}C.

For the chemical characterization of the incorporation product the reaction mixture was enlarged 20- fold and after incubation with Tris pH 10.0 was centrifuged at 105,000 xg for 120 min. The supernatant was stored at-84° until further use.

CM-cellulose chromatography

Purified chick embryo collagen, prepared according Piez, Eigner & Lewis[18] was added to the product of a large scale incorporation experiment, obtained as above. The mixture was dissolved in 0.06 M potassium acetate buffer, pH 4.8, and extensively dialized at 4°, against the same buffer. After denaturing at 45° for 30 min, the sample was applied to a CM-cellulose column (1.5 x 10 cm) and eluted with a linear gradient from 0 to 0.15 M LiCl in the same buffer. After the end of the gradient, the column was washed with 0.5 M LiCl to remove attached materials. Temperature was kept at 40° during the

chromatography. 3 ml fractions were collected and their A_{225} and radioactivity determined.

RESULTS

Acrylamide gel electrophoretical analysis of the mRNA eluted from oligo (dT)-cellulose (Fig. 1) reveals the presence of a single peak, other than contaminating 28S rRNA and, to a lesser extent, 18S rRNA.

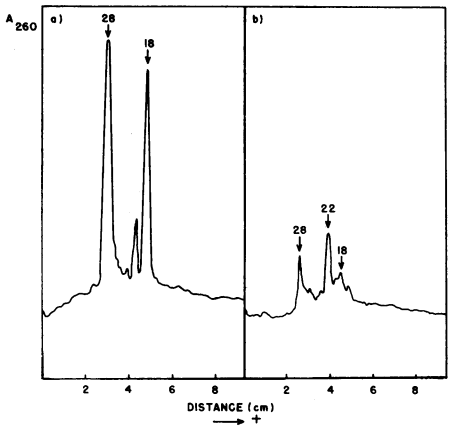

Fig. 1 - Eletrophoretical profile of RNA eluted from oligo (dT)-cellulose columns. For experimental conditions, see methods. 1a) Material that is not retained by the column (mainly rRNA). 1b) RNA bound to the column and eluted with low ionic strength buffer (mRNA fraction).

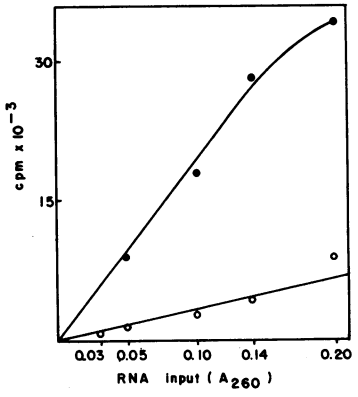

Fig. 2 - Effect of purified RNA fractions upon ^{3}H-proline incorporation by a Krebs ascites cell-free system. O - O = rRNA fraction; ● - ● = mRNA fraction.

Incorporation of ^{3}H proline by a cell-free amino acid incorporating system, is a linear function of added RNA, the mRNA fraction being much more efficient than the corresponding rRNA fraction (Fig. 2).

The incorporation is also dependent on Mg^{2+} concentration which is optimal at 3.6 mM as reported by others for the Krebs ascites tumor cell extract[2].

TABLE I

Properties of RNA-directed ^{3}H-proline and ^{14}C-tryptophan incorporation by a Krebs cell-free system[o, ≠]

Addition or omission from reaction mixture	Amino acid incorporated (pmol/0.1 ml)
Complete	90.0
-Initiation factors	1.8
-Pro, + Trp	0.45
+Pyrocatechol violet (0.2 mM)	1.8
+Aurintricarboxylate (0.5 mM)	1.9
+Collagenase (5 μg)	7.3

o experimental conditions are described in the methods section.

§ collagenase digestion was carried out for 20 min at 37° at the end of amino acid incorporation, in the presence of 0.2 mM $CaCl_2$.

≠ unless otherwise indicated, proline was used as the labeled amino acid.

Some features of the incorporation reaction are shown in Table I. The reaction is markedly dependent on initiation factors. Furthermore, initiation inhibitors such as ATA and PCV virtually abolish incorporation, indicating that the system is indeed initiating the synthesis of new polypeptide chains under the guidance of added messenger RNA. Some indication of the nature of the incorporation product is given by fact that it lacks tryptophan, which is absent from collagen[19], and is readily

digested by purified bacterial collagenase.

Even though the incorporation product could be tentatively characterized as collagen, a more rigorous characterization was achieved by examining the product of a large scale reaction mixture. Chromatography on Sephadex G-200 (Fig. 3) shows the presence of a high molecular weight protein, eluting close to the void volume, together with unlabeled carrier native chick collagen.

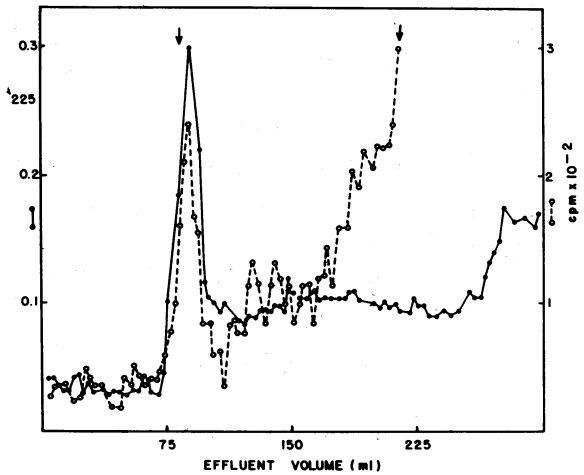

Fig. 3 - Molecular sieve chromatography of product of mRNA-directed ^{3}H-proline incorporation. A large-scale incorporation was carried out, and the product chromatographed in Sephadex G-200 together with unlabeled native chick embryo collagen, as described in the methods section. ● - ● = A_{225} ; O - O = Radioactivity (c.p.m.).

Ion exchange chromatography on CM-cellulose columns provides further evidence that the mRNA fraction determines collagen synthesis (Fig. 4). It can be seen that the radioactivity profile, follows closely that of carrier chick embryo collagen, showing that α_1 and α_2 chains were synthesized under its direction. Very little radioactivity is recovered by 0.5 M LiCl, which removes still attached material.

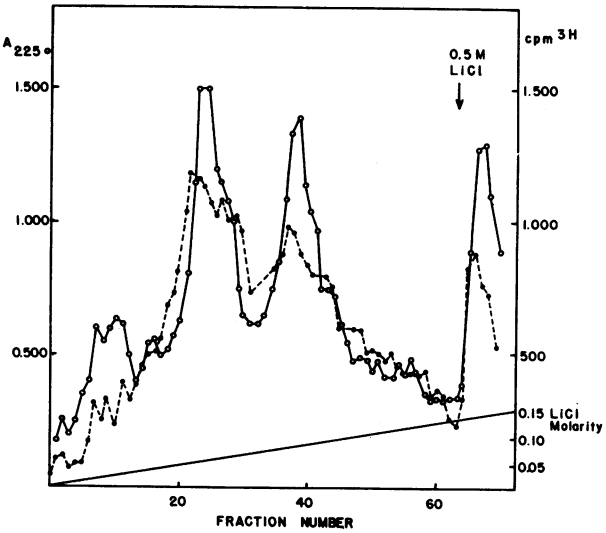

Fig. 4 - Ion-exchange chromatography of the product of mRNA-directed proline incorporation. A large scale incorporation was carried out, and the product chromatographed in a CM-cellulose together with unlabeled chick embryo collagen, as described in the methods section. O - O = A_{225}; ● - ● Radioactivity (c.p.m.).

DISCUSSION

The mobility of putative collagen mRNA (Fig. 1) agrees with the molecular weight of a polynucleotide coding for polypeptides about 1,000 amino acids long, such as collagen α_1 and α_2 chains (molecular weights 105,000 and 100,000 respectively[20]) and is in agreement with that reported by other workers[21]. The presence of a single peak, other than rRNA is also consistent with the previous demonstration that the polysomal fraction from which this RNA was isolated is almost exclusively engaged in collagen biosynthesis[10]. Contamination of this RNA by rRNA is made irrelevant by the fact that the rRNA fraction itself was much less active in directing in vitro incorporation of proline. Such contamination has also been reported by others for purification of mRNA through oligo(dT)-cellulose chromatography[2].

A very interesting finding is the absolute dependence on added initiation factors (Table I). While some authors[2,22] report independence of the system on initiation factors, it has been shown[23] that the addition of a reticulocyte ribosomal wash is highly beneficial to the in vitro translation of rabbit, human and mouse hemoglobin mRNA, as well as Q β and encephalomyocarditis viral RNA. The present results support the latter observations. The virtual abolishment of proline incorporation by the presence of initiation inhibitors such as ATA[24] or PCV[25] further suggests that the system is mostly engaged in the synthesis of newly initiated chains, under the direction of added mRNA.

Characterization of the incorporation product as collagen is suggested by its ready digestibility by purified bacterial collagenase under conditions which preclude unspecific protease activity[11]. The specificity of the enzymatic diggestion was also demonstrated previously, by its lack of effect upon the incorporation product of chick ribosomes[10].

Molecular sieve chromatography further indicates that polypeptides of a size compatible with that of collagen chains were made and ion exchange chromatography shows that the synthesized polypeptides were eluted together with unlabeled carrier α_1 and α_2 chains.

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