REITERATION OF DNA COMPLEMENTARY TO A CYTOPLASMIC NON-RIBOSOMAL RNA

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SUMMARY

Experimentally induced granulomas, in guinea pigs, were fractionated into a 40,000xg pellet and supernatant, which was further fractionated into a 100,000xg pellet and supernatant. The product of in vitro amino acid incorporation by the 40,000xg pellet was tentatively identified as collagen by its high proline/leucine ratio, its digestibility by bacterial collagenase and its solubility in hot trichloroacetic acid. The 100,000xg pellet incorporated leucine much more efficiently than the 40,000xg pellet and the product was insoluble in hot trichloroacetic acid.

Labeled RNA from the 40,000xg pellet formed hybrids with granuloma ($C_o t$ 1/2 = 100-150) and liver ($C_o t$ 1/2 = 8,000) chromatin DNA, indicating that genes coding for this RNA are repeated about 100-fold in granuloma and less than 5 times in liver DNA. Under conditions of poly(A) binding, 50% of this labeled RNA is retained by filters. Digestion with

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ribonuclease and ribonuclease T_1, decreases binding efficiency by 75%.

INTRODUCTION

A very interesting problem in molecular biology is that of the genomic representation of mRNA. It has been shown that eucaryotic DNA presents a considerable amount of reiterated sequences[1,2]. Only a few have been identified as genomes coding for structural ribosomal RNA[3], or as centromeric DNA[4,5]. Work on hemoglobin mRNA[6,7] and fibroin mRNA[8], has shown that these are coded for by a few or single genes, lending support to the belief that mRNA is transcribed by the unique sequences of DNA. However, DNA sequences coding for histone mRNA are reiterations of very similar genes[9].

The successful characterization of collagen mRNA[10], by showing that chick embryo RNA could direct the incorporation by rat-liver ribosomes of proline and glycine, but not leucine, into a hot-trichloroacetic acid-soluble, collagenase-sensitive product, led us to undertake the purification of this mRNA, in order to examine its genomic representation.

Collagen is synthesized on such heavy polysomes that they sediment readily through 15-30% sucrose gradients[11,12] It is assumed that the collagen synthesizing aggregate contains about 100 ribosomes[13]. Thus, one could hope to separate collagen-synthesizing polysomes from the bulk of the ribosomal population by low speed centrifugation.

EXPERIMENTAL

Granulomas were produced in guinea pigs by subcutaneous

injections of carrageenan (Irish Moss)[14]. Seven days later, the animals were killed and the granulomas removed, taking care to dissect away adhered muscle tissue. The granulomas were homogenized in 5 volumes of 0.01M Tris-HCl, pH 7.4, containing 0,14M KCl and 1.5mM $MgCl_2$. The resulting suspension was then incubated for 15 minutes at 4^{o}, after the addition of Nonidet P40 to a final concentration of 2.0%. The supernatant obtained after centrifugation at 1,000xg for 10 minutes was then centrifuged at 40.000xg for one hour. The colorless, gelatinous pellet was either used imediately or stored at -84^{o}. The supernatant was next centrifuged at 100,000xg for 120 minutes to yeld the ribosomal fraction. The pH 5 enzyme fraction was prepared from granuloma tissue as previously described for rat-liver[15]. Conditions for in vitro amino acid incorporation and radioactivity determination have also been reported[10,16].

For the hybridization experiments, both liver and granuloma nuclei were prepared[17], subjected to a concentrated salt extraction for the purification of chromatin[18], and DNA extracted from this fraction according to Church & McCarthy[19]. Labeled RNA was obtained by incubating granuloma slices with 50 mCi of ^{32}P-orthophosphate in Hank's solution enriched with 22mM glucose and 7% bovine serum for one hour at 37^{o}. After incubation, the 40,000xg pellet was isolated as above, and its RNA extracted with phenol-chloroform[20]. Hybridization under conditions of great DNA excess was carried out essentially as described by Melli et al.[21]

Estimation of poly(A) content was done using both the Millipore[22] and the poly(U) filter[23] techniques.

RESULTS AND DISCUSSION

The results shown in Table I indicate that the product of amino acid incorporation by the 40,000xg pellet presents

TABLE I

In vitro amino acid incorporation by granuloma citoplasmic fractions

SAMPLE	INCORPORATION IN NANOMOLES ^{3}H-proline	^{14}C-leucine
40,000xg pellet	0.063	0.017
40,000xg pellet + 0.5 mg collagenase	0.015	0
40,000xg pellet, washed in hot trichoroacetic acid	0.014	0.015
100,000xg pellet	0.057	0.045
100,000xg pellet, washed in hot trichloroacetic acid	0.049	0.040

The incubation mixture contained, in a final volume of 1.0 ml: 1.75 mg protein of either 40,000xg or 100,000xg pellet; 2.0 mg protein pH 5 enzyme; 4 mM $MgCl_2$; 10mM DTT; 0.6mM CTP; 0.5mM ATP; 0.5mM GTP; 6.4mM phosphoenolpyruvate; 0.2 µg pyruvate kinase; a mixture of 18 unlabeled amino acids, 0.01mM each and 0.5 µCi of ^{3}H-proline (specific activity 5Ci/mMol) and 0.5 µCi of ^{14}C-leucine (specific activity 216 mCi/mMol). Incubation was carried out at 37° and 0.12 ml aliquots were collected and delivered onto Whatman 3M paper filter disks, which were washed for radioactivity determinations[21].

a higher proline/leucine ratio than that of the 100,00xg pellet. Furthermore, it is highly susceptible to digestion by purified bacterial collagenase and readily solu-

ble in hot CCl_3COOH, suggesting that it could be collagen.

Hybridization of the 40,000xg pellet RNA with granuloma chromatin DNA under conditions of DNA excess (Figure 1),

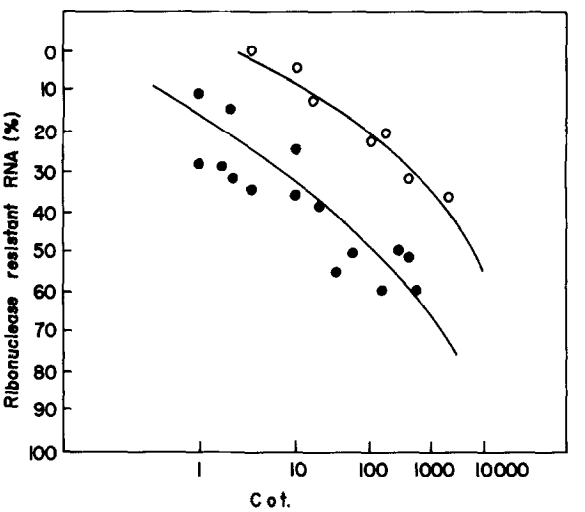

Figure 1. Time course of reassociation of granuloma DNA and liver DNA in the presence of cytoplasmic RNA fractions. Sonicated DNA, at a concentration of 5.0 mg/ml (usually 2.0 ml) was heat-denatured and 10 µg of radioactive 40,000xg pellet RNA added after cooling. At intervals, 0.1 ml of the DNA was withdrawn, delivered into 2 ml ice-cold 1 x SSC (standard saline citrate) and quickly frozen. Upon termination of the experiment one half of each sample was treated with 10 µg pancreatic ribonuclease and 50 units of RNase T_1 for 10 minutes at 37°. Both treated and untreated samples were precipitated with trichloroacetic acid in the presence of 100 µg carrier RNA. The precipitates were collected on Millipore filters.
O - O = liver DNA; ● - ● = granuloma DNA.

shows that most of the RNA is rendered ribonuclease resistant at $C_o t$ values between 10 and 1,000, and has an average $C_o t$ 1/2 (50% resistance) of 100-150, whereas when liver chromatin DNA is substituted for granuloma chromatin DNA, only 20% of the RNA anneals at $C_o t$ values smaller than 100 (Figure 1). The $C_o t$ 1/2 value for liver chromatin DNA is around 8,000.

Taking into consideration the underestimation of the $C_o t$ value brought about by the ionic strength of the experimental buffer[21] and the structural complexity of guinea pig DNA[24], its becomes evident that genes coding for a component of the 40,000xg pellet RNA are about 100-fold repeated in granuloma DNA and less than 5 times repeated in lever DNA.

It is unlikely that this hybridizing RNA is ribosomal RNA, since rRNA does not hybridize with rat liver chromatin DNA prepared in a similar way[10]. Furthermore, rRNA is coded for by a highly repetitive genome in all organisms studied[25], contrary to what was found for liver DNA (Figure 1).

It is known that most messengers contain a polyadenylic acid sequence[26], even though its function is still unclear. The examination of the 40,000 pellet RNA for its poly(A) content (Table II) shows that about 48% of input radioactivity is retained by filters under conditions in which poly(A)-containing messengers are bound. Approximately 19% of this radioactivity belongs to the poly(A) sequence itself.

Since the amount of bound radioactivity (Table II)is roughly equivalent to that rendered ribonuclease resistant under hybridizing conditions which appear to exclude rRNA (Figure 1), the results seem to indicate that conditions of excess DNA

TABLE II

Detection of poly(A) sequences in 40,000xg pellet RNA

	PERCENT RETENTION	
	Millipore(22)	Poly(U)(23)
Imput radioactivity	100	100
Filter retained radioactivity	48.3	48.1
Enzyme resistant radioactivity	9.5	9.1
KOH resistant radioactivity	0.72	1.17

Enzyme digestion was performed with 10 µg pancreatic ribonuclease and 50 units of RNase T_1, for 30 minutes at 37°. RNA was hydrolized with KOH, 0.3M final concentration, for 18 hours at 37°.

have indeed been reached and that the hybridizing RNA could, in effect, be messenger RNA, and possibly collagen mRNA, since this protein is the main translation product of the 40,000xg pellet.[27]

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