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SYNTHESIS OF LENS PROTEIN *IN VITRO*

V. Isolation of messenger-like RNA from lens by high resolution zonal centrifugation*

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1. Introduction

Recently Lingrel described the isolation of a 9 S fraction from mouse reticulocytes which exhibited messenger activity in a cell-free system from rabbit reticulocytes [1, 2]. Also from other eukaryotic systems RNA with template activity could be isolated. Heywood described the isolation of messenger-like RNA from muscle polyribosomes. He was able to show that with this messenger and 80 S ribosomes from reticulocytes a protein could be synthesized which has the characteristics of myosin [3]. An RNA fraction isolated from myeloma appeared to function as template for the synthesis of a mouse Ig light chain in a heterologous cell-free reticulocyte system [4].

The lens is a unique tissue which predominantly produces a class of highly specific proteins: the crystallins. Like mRNA from reticulocyte [5-7] the lens messenger has been reported to be stable [8]. In the present paper a high resolution zonal centrifugation technique is described which allows the isolation of two species of lens messengers differing by a number of criteria from the bulk of ribosomal RNA and tRNA.

2. Methods

Polysomes from 2-3 months old calf lenses were prepared as described earlier [9, 10]. Polysomes from reticulocytes were prepared according to Allen and Schweet [11]. The polysomes were suspended in 0.05 M Tris-HCl, pH 7.4, to a final concentration of about 6 mg/ml, one tenth volume of 10% SDS was added and the solution was kept at 37° for 5 min. One volume of 0.05 M Tris-HCl, pH 7.4, in 12% (w/w) sucrose solution was added before pumping the sample into the zonal rotor. For the zonal centrifugation exponential gradients were applied resembling the iso-kinetic gradients described by Price [12, 13]. These isokinetic gradients can be computed according to the formula:

$$\frac{r^2}{\eta_m} (\rho_p - \rho_m) = \text{constant},$$

in which

r = the distance to the rotor centre
 ρ_p = the density of the particles
 ρ_m = the density of the medium at r
 η_m = the viscosity of the medium at r .

Zonal centrifugation was performed in a B XXX rotor of IEC. After centrifugation the pattern was automatically scanned at 260 nm using a Gilford spectrophotometer equipped with a 2 mm flow cell.

Incubations in a volume of 250 μ l were performed at 37° for 30 min. The incubation mixture contained 200 μ g ribosomes from reticulocytes, preincubated for 90 min at 37° according to Cohen [14], 700 μ g

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100,000 g supernatant protein, 2 μ g RNA fraction, 20 μ l 0.6 M KCl wash (containing the wash of 300 μ g polyribosomes, 0.05 M Tris-HCl, pH 7.4, 0.15 M KCl, 3.6 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 3.2 mM phosphoenol pyruvate, 5 μ g pyruvate kinase, 5 mM β -mercaptoethanol, 0.05 mM L-amino acids except Leu, 0.05 mM 14 C-DL Leu (specific activity 55.2 mCi/mmmole). The reaction was stopped by addition of 5% TCA. The hot TCA precipitable material was counted in a Packard liquid scintillation counter.

The base composition of the isolated RNA fractions was determined according to Katz and Comb [15].

For electron microscopy the RNA fractions were spread on carbon film in the presence of urea and carbon platinum shadowing was applied with an angle of about 15 deg.

3. Results and discussion

The difficulty in the isolation of messenger RNA from eye lens polysomes is twofold.

(a) It comprises only a small proportion of the total polysomal RNA.

(b) As the molecular weight of lens protein subunits is about 20,000 [16] the sedimentation coefficient of the corresponding mRNA can be expected to

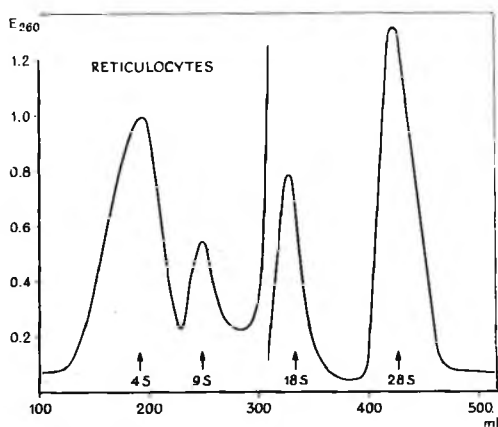


Fig. 1. Zonal centrifugation profile of polysomal RNA from reticulocytes. Samples varying in volume from 15 to 30 ml were applied. The gradient was isokinetic with the volume. A sucrose gradient from 0 to 26% (w/w) has been used. The sample was centrifuged for 5.5 hr at 50,000 rpm and 2° in a B XXX zonal rotor of an IEC centrifuge.

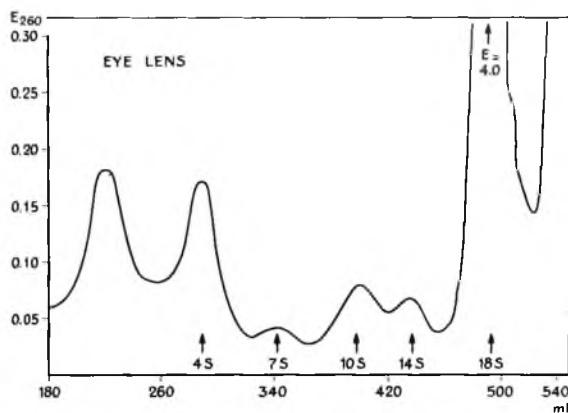


Fig. 2. Zonal centrifugation profile of polysomal RNA from lens polyribosomes. Centrifugation was performed for 15 hr at 50,000 rpm and at 2° in an exponential sucrose gradient in 0.05 M Tris-HCl, pH 7.4, (0–28% sucrose w/w). The gradient was constructed by pumping 37% sucrose in a mixing chamber containing 300 ml buffer. 50 ml of the gradient with 100 ml buffer was used as overlayer. The sample volume varied from 15 to 30 ml.

be about 11 S so that it may be masked by the bulk of ribosomal RNA.

High resolution zonal centrifugation appeared to be a very useful tool for the solution of this problem.

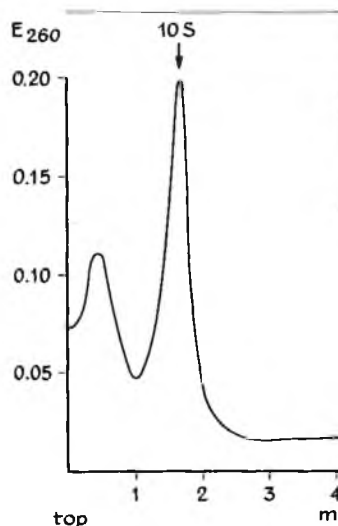


Fig. 3. Recentrifugation of the 10 S fraction. Recentrifugation of the 10 S fraction was performed for 4 hr at 6° in a SB 405 rotor of the IEC ultracentrifuge with a 5–19.1% exponential sucrose gradient.

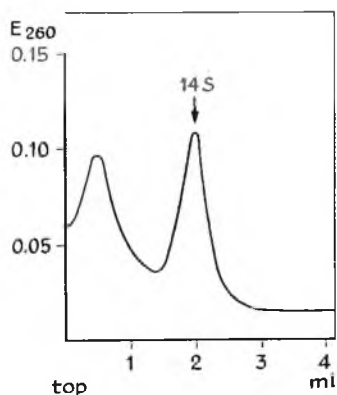


Fig. 4. Recentrifugation of the 14 S fraction. Conditions as mentioned under fig. 3.

We used exponential gradients resembling the isokinetic gradients described in the literature [13] but somewhat steeper to prevent zone broadening.

In order to check our method RNA from reticulocyte polysomes was separated (fig. 1). In fig. 2 it is clearly shown that with RNA extracted from lens polysomes even higher resolution can be achieved. The homogeneity of the lens "messenger" fractions was demonstrated by re-centrifugation in a swinging bucket rotor (fig. 3 and 4).

The activity of the fractions was tested in the reticulocyte cell-free system (table 1). It can be seen that crude initiation factors obtained from polysomes after washing with 0.6 M KCl enhance the stimulation although there is some variation in the different experiments, possibly caused by the instability of either one or more initiation factors. The addition of 18 S

Table 1
Stimulation of amino acid incorporation by RNA fractions from lens and reticulocyte polysomes.

Added fraction	Incorporation of Leu (pmoles)	
	-KCl Wash	+KCl Wash
—	2.7	3.3
18 S	3.3	3.3
9 S reticulocytes	6.5	7.6
10 S lens	7.6	12.2
14 S lens	7.6	10.7

Assay conditions as reported under Methods.

RNA instead of the messenger-like RNA species does not result in an increase of the amino acid incorporation excluding the possibility of non-specific stimulation by high molecular weight RNA.

In table 2 the base composition of the different RNA fractions isolated from eye lens polysomes is summarized. It appears, that the 18 S and 28 S fractions have values expected for ribosomal RNA whereas the 10 S and 14 S fractions exhibit a more "DNA"-like base composition.

We obtained similar results with RNA isolated from RNP particles derived from polysomes by EDTA treatment in magnesium-free medium. A striking observation was that in addition to the small and large ribosomal subunits two discrete RNP fractions could be isolated with sedimentation coefficients of about 16 S and 21 S respectively. From the 16 S particle consistently the 10 S "messenger" was obtained whereas the 14 S RNA represented the major component of the 21 S particle. The active fraction

Table 2
Nucleotide composition of messenger-like RNA.

RNA	AMP	UMP	GMP	CMP	$\frac{\text{GMP} + \text{CMP}}{\text{AMP} + \text{UMP}}$
18 S + 28 S	19.4 ± 0.9	18.3 ± 0.5	32.0 ± 0.6	30.3 ± 0.7	1.65
10 S	18.9 ± 0.7	30.3 ± 1.0	28.9 ± 2.2	21.9 ± 1.1	1.03
14 S	19.2 ± 0.8	30.4 ± 0.6	30.4 ± 0.9	20.0 ± 0.4	1.02
	dAMP	dTMP	dGMP	dCMP	$\frac{\text{dGMP} + \text{dCMP}}{\text{dAMP} + \text{dTMP}}$
Calf thymus DNA [16]	27.4	29.5	22.4	20.7	0.78

The values are the averages of 3 to 6 determinations. Standard errors were calculated using the formula: $\sqrt{\sum(x - \bar{x})^2 / N \times (N - 1)}$.

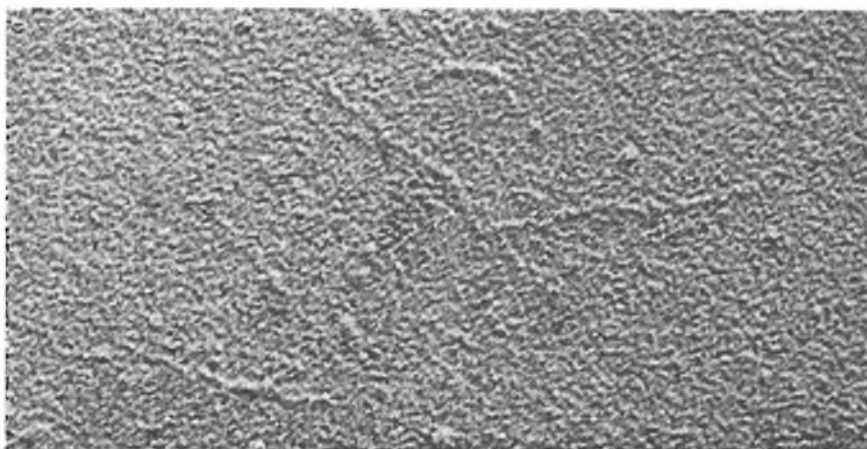


Fig. 5a. Electron micrograph of lens 'messenger' RNA fractions. Magnification 100,000. The major strand is apparently built up by shorter segments joined together.

has also been investigated by electron microscopy with different spreading techniques including simple spreading of the material on carbon films (fixation with 1% glutaraldehyde) and the Kleinschmidt [17] method for single stranded RNA [18].

The presence of stranded material could clearly be demonstrated in the 10 S fraction. The thickness of the "threads" measured in the shadowed preparations is consistent with the average thickness reported for single stranded RNA.

The length of the threads varies from 0.3 to 1 μm (fig. 5a). Occasionally longer strands have been found which possibly are resulting from association of the

smaller pieces (fig. 5b). In fact the longer filaments show different width and branching.

Whether the stimulation by these RNA fractions results in *de novo* synthesis of a specific protein has to be elucidated. These experiments are in progress. Meanwhile we were able to show that the 14 S messenger directs the synthesis of the α crystallin A_2 chain in heterologous cell-free systems.

Acknowledgements

The present investigations have partly been carried

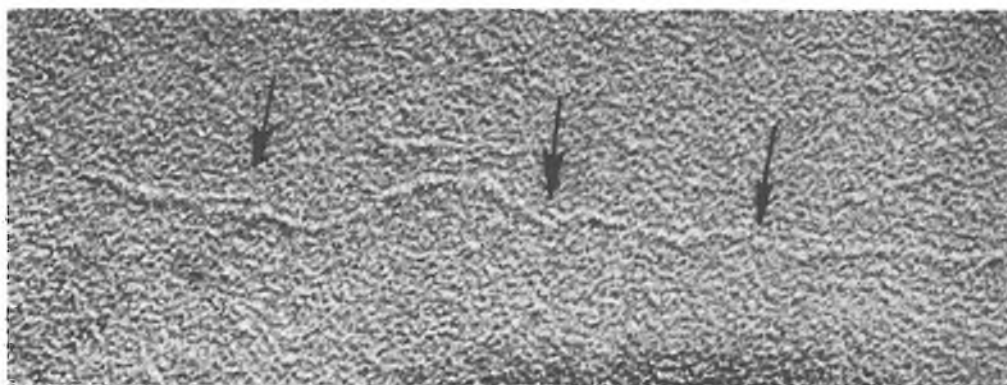


Fig. 5b. The same preparation showing a longer strand apparently built up by shorter segments. (The arrows indicate overlapping regions.)

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