MOLECULAR WEIGHTS OF SEPARATED RABBIT α- AND β-GLOBIN MESSENGER RNAs

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1. Introduction

Several authors [1–3] have shown that translation of the 'supernatant' messenger RNA from rabbit reticulocytes in vitro results in an excess of α-globin over β-globin chains. We have previously demonstrated that rabbit reticulocyte polysomal messenger RNA, which codes for roughly equal amounts of the two globin chains in vitro, exhibits two major zones in polyacrylamide gel electrophoresis in formamide. The molecular weight of the more rapidly migrating RNA species was estimated to be 202 000 and that of the more slowly migrating RNA species was estimated to be 227 000 [4]. Since rabbit α-globin chains contain five fewer amino acids than rabbit β-globin chains, we suggested that the smaller RNA may represent the α-globin messenger and the larger RNA the β-globin messenger. But the size difference (25 000) is considerably more than necessary (4800) to code for the additional number of amino acids. We present evidence in this report that the 'supernatant' messenger, which we confirm by translation in vitro is enriched by a factor of 2–3 as compared with polysomal messenger in α-globin messenger activity, displays one major zone in polyacrylamide gel electrophoresis in formamide. The mobility of this RNA is identical to that of the smaller RNA species in polysomal messenger. The mol. wt. of rabbit α-globin messenger RNA is therefore 202 000. Conversely, the larger polysomal RNA species must represent β-globin messenger, and its mol. wt. is therefore 227 000.

2. Methods

2.1. *Preparation of post-ribosomal 'supernatant' messenger RNA*

Ribosomes, including native subunits, were removed from a rabbit reticulocyte lysate by 553 000 *g* hr centrifugation. The supernatant was carefully removed and the RNA extracted using the method of Lee et al [5]. The RNA was washed twice with 70% ethanol, dried in vacuo and the polyadenylated RNA isolated by oligo dT-cellulose chromatography [6]. This RNA was concentrated 10-fold by lyophilisation and precipitated with 2 vol of ethanol. After overnight storage at −20°C the RNA was recovered by low speed centrifugation, washed twice with 70% ethanol, dried in vacuo and dissolved in water or formamide.

2.2. *Protein synthesis in vitro*

Pre-incubated Krebs II ascites S30 was prepared as described by Mathews and Korner [7]. 200 μl incubation mixtures contained: 80 μl ascites S30, 20 mM Tris–HCl buffer, pH 7.5, 80 mM KCl, 3 mM magnesium acetate, 1 mM dithiothreitol, 10 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 1 mM ATP, 0.2 mM GTP, 100 μM of all naturally occurring L-amino acids except those added in radioactive form, 2.0 μCi [^{14}C]leucine (331 mCi/mmol), or 40 μCi [^{3}H]-leucine (58 Ci/mmol), or 110 μCi [^{35}S]methionine (200 Ci/mmol), and RNA or water as indicated in the figure legends. The mixtures were incubated for 1 hr at 37°C. When the next step was carboxymethyl cellulose

chromatography, 50 mg rabbit globin were added as carrier and the proteins were precipitated with 0.15 N HCl in acetone. If the products were to be analysed by tryptic digestion, then the mixtures were made 50 mM EDTA, pH 7.0, 1% methionine and 5 μg/ml pancreatic RNase, and incubated for a further 10 min. at 37°C. Further details of the product analyses are given in the figure legends.

2.3. *Formamide gel electrophoresis*

The preparation and conditions for electrophoresis and staining of RNA in formamide gel cylinders has already been described [4]. For direct comparison of polysomal messenger RNA and 'supernatant' messenger RNA, gel slabs of 4% polyacrylamide in formamide were made and the RNA samples were subjected to electrophoresis at 115 V, 10 MA, for 4.5 hr.

3. Results

3.1. *Polyacrylamide gel electrophoresis and in vitro translation of polysomal mRNA*

On polyacrylamide gel electrophoresis in formamide rabbit globin mRNA separates into two distinct bands. (see fig. 2 in our previous publication [4]). Analysis of gel scans such as that shown in fig. 1 reveals that the two bands are present in a 1:1 ratio. Housman [8], has demonstrated by hybridization studies that α- and β-globin mRNA prepared from polysomes are in a 1:1 ratio and we have made the simplest assumption that one of the bands is α messenger RNA, the other β messenger RNA.

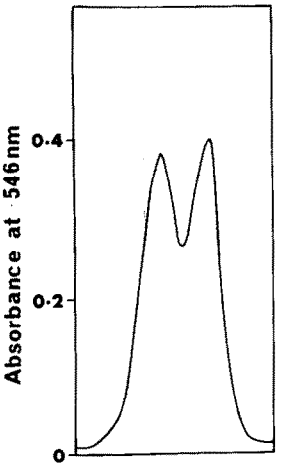


Fig. 1. Densitometry of polysomal messenger RNA separated on polyacrylamide gels in formamide and stained with pyronin Y. Cylindrical gels containing fractionated polysomal messenger RNA resembling those presented in an earlier publication [4] were scanned at 546 nm using the Gilford gel scanner.

Because the ascites cell free system does not translate the messengers for α- and β-globin with equal efficiency [9] it was necessary to establish the efficiency with which each messenger RNA was translated. To do this polysomal mRNA was translated using the ascites cell-free system. The products were chromatographed on carboxymethyl cellulose (fig. 2) and the amounts of synthesised α- and β-globin measured (table 1). The ratio of α- to β-globin was found to be 0.55 to 1. Assuming there are equal amounts of α- and β-messenger then the α messenger is synthesised at about half the efficiency of β messenger.

The assumption was made that the efficiency of translation of each messenger (α or β) is the same whatever the source of mRNA (i.e. polysomal or supernatant).

3.2. *Translation of 'supernatant' mRNA*

The concentration of RNA (i.e. RNA concentration in the incubation mixture) affects the relative efficiencies of α and β globin translation [10]. Thus at low RNA concentrations more α- and β-globin is synthesised and at high RNA concentrations much more β- than α-globin is synthesised. For this reason it was important to assay the supernatant mRNA at the same RNA concentration that had been used for the polysomal mRNA.

Fig. 2 shows carboxymethyl cellulose chromatography of the protein products of two such parallel incubations. The results are summarised in table 1. The supernatant mRNA is translated into 1.5 times as much α- globin as β-globin. Taking into account the efficiencies of translation of the two messengers it is deduced that in 'supernatant' mRNA there are 2.7 α-messengers for every 1 β-messenger.

Product analysis was also performed by tryptic digestion of the in vitro synthesised ^{35}S-labelled proteins. The peptides were separated on paper by high voltage electrophoresis. An autoradiograph of the dried paper is shown in fig. 3. The results indicate that polysomal mRNA was translated into α and β specific peptides in the ratio 0.9:1 whereas 'supernatant' mRNA

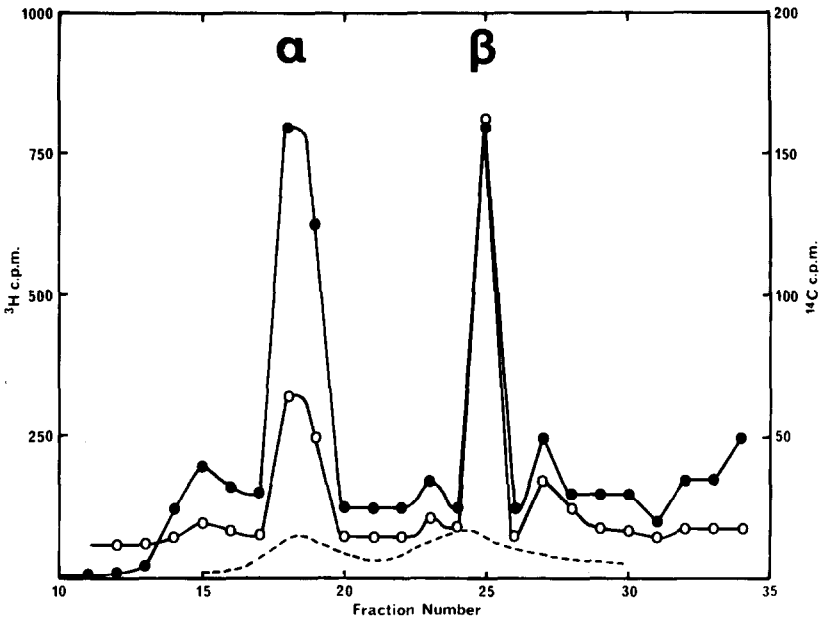
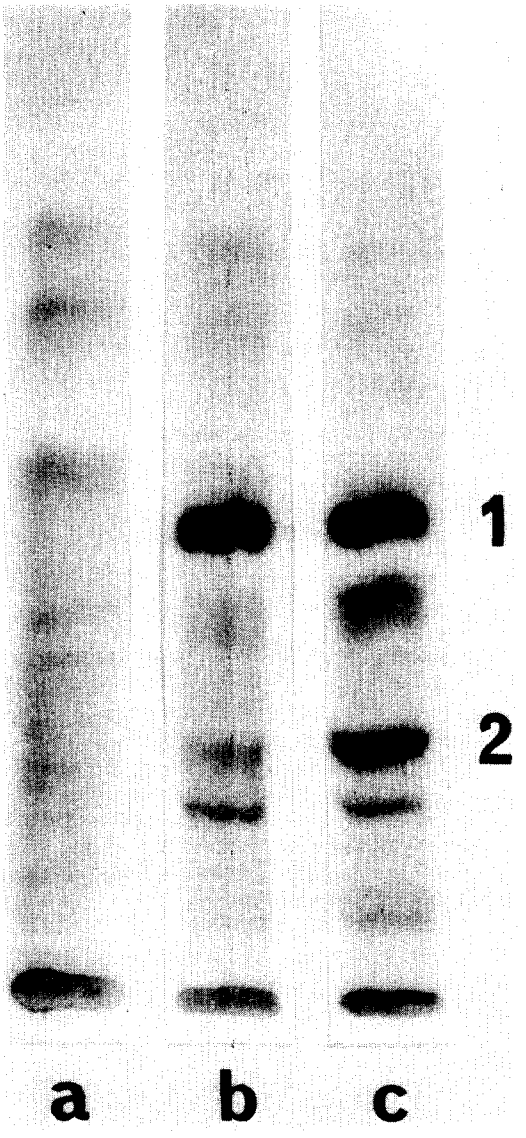


Fig. 2. Analysis of the products of translation of 'supernatant' and polysomal messenger RNA by carboxymethyl cellulose chromatography: 18 μg/ml 'supernatant' messenger RNA with [^{14}C]leucine (–•—•–) and 18 μg/ml polysomal messenger RNA with [^{3}H]leucine (o———o) were separately incubated in the ascites S30 system for 1 hr at 37°C. The two incubation mixtures were then pooled and 50 mg rabbit globin was added as carrier. The protein was precipitated with 0.15 N HCl in acetone at –20°C washed with acetone at –20°C, dried in vacuo, and dissolved in 5 ml 0.6 M formic acid, 0.06 M pyridine. The globins were separated on a carboxymethyl cellulose (Whatman CM 52) column (1.5 cm × 25 cm) using a 300 ml gradient containing 150 ml each of 0.6 M formic acid, 0.06 M pyridine, and of 3 M formic acid, 0.3 M pyridine. (– – – – –) is the elution profile at 280 nM. The column fractions were made 10% in trichloroacetic acid (TCA) and the precipitates filtered onto Whatman GF/C circles, washed once each with 5% TCA, water, ethanol and dried for 30 min at 90°C. The protein on the filter was solubilised with 1 ml Soluene (Packard) and counted for 10 min in 10 ml scintillant (6 g Permablend from Packard/1 toluene).

Table 1
Products of Translation of Messenger RNA

Source of messenger	Method of analysis	CPM in α-globin	CPM in β-globin	α-globin/ β-globin	α-globin/ β-globin messenger*	% α-globin messenger	% β-globin messenger
Polysomes	CMC chromatography	438	800	0.55			
Supernatant	CMC chromatography	308	205	1.5	2.7	73	27
Polysomes	Elcctrophoresis tryptic digests	25335	28097	0.9			
Supernatant	Electrophoresis tryptic digests	31862	16334	1.95	2.2	69	31

* Assuming 1:1 ratio of α:β messenger in polysomes.



gave a ratio of 1.95:1. Calculating efficiences of translation in this experiment of the two mRNAs this means that there are 2.2 α-messengers for every 1 β-messenger. These results are in good agreement with those from chromatography. Typical results are summarised in table 1. We conclude that the 'supernatant' messenger RNA contains 2–3 times more α- than β-messenger activity.

3.3. *Molecular weight distribution of RNAs in supernatant and polysomal messenger fractions*

The electrophoretic pattern of 'supernatant' RNA is shown in fig. 4a. From the densitometry trace (fig. 5) it was estimated that the ratio of the smaller species to the larger is 3.2:1. Although these values are approximate, owing to the incomplete resolution of the two RNA species, they are close enough to the calculated ratio of α- to β-messenger RNA (2.2–2.7:1) to make it probable that the smaller RNA is the messenger RNA for α-globin and the larger the messenger RNA for β-globin.

Fig. 4b is of a stained polyacrylamide gel slab in which the electrophoretic pattern in formamide of 'supernatant' mRNA and polysomal mRNA was compared. Since the band in the 'supernatant' RNA identified above as α messenger, comigrates with the faster band on polysomal mRNA it is highly probable that α-globin mRNA has a molecular weight of 202 000 and, by process of elimination, β-globin mRNA has a mol. wt of 227 000.

4. Discussion

Our results agree with previously published results in that translation of 'supernatant' messenger RNA

Fig. 3. Analysis of the products of translation of 'supernatant' and polysomal messenger RNA by fingerprinting tryptic digests: Fingerprints illustrate the products of incubation with (a) no messenger RNA; (b) 18 μg/ml 'supernatant' messenger RNA; and (c) 18 μg/ml polysomal messenger RNA. After incubation with RNase, the protein in the mixtures was precipitated with 10% TCA at 0°C. The protein was washed twice each with 5% TCA, 99% alcohol, 1:1 ether–alcohol, and finally with ether. 2 mg of rabbit globin were added to each sample. The protein was dissolved in formic acid at 100 μl/mg, two volumes of performic acid were added and the samples were oxidised at room temperature for two hr. The samples were lyophilised to remove the acid, washing with water and lyophilised to dryness before dissolving in 1% ammonium bicarbonate (100 μl/mg). Trypsin solution was added to final substrate:enzyme ratio of 50:1 by weight and the protein digested for 4 hr at 37°C. The peptides were then lyophilised, washed three times with water, lyophilising each time, then dissolved in a small volume of water. Aliquots containing approximately 20 000 cpm were applied to Whatman 3 MM paper and the peptides separated at pH 3.5 with 60 V/cm for 50 min. The labelled peptides were identified by autoradiography. Band '1' is specific to α-globin, band '2' to β-globin.

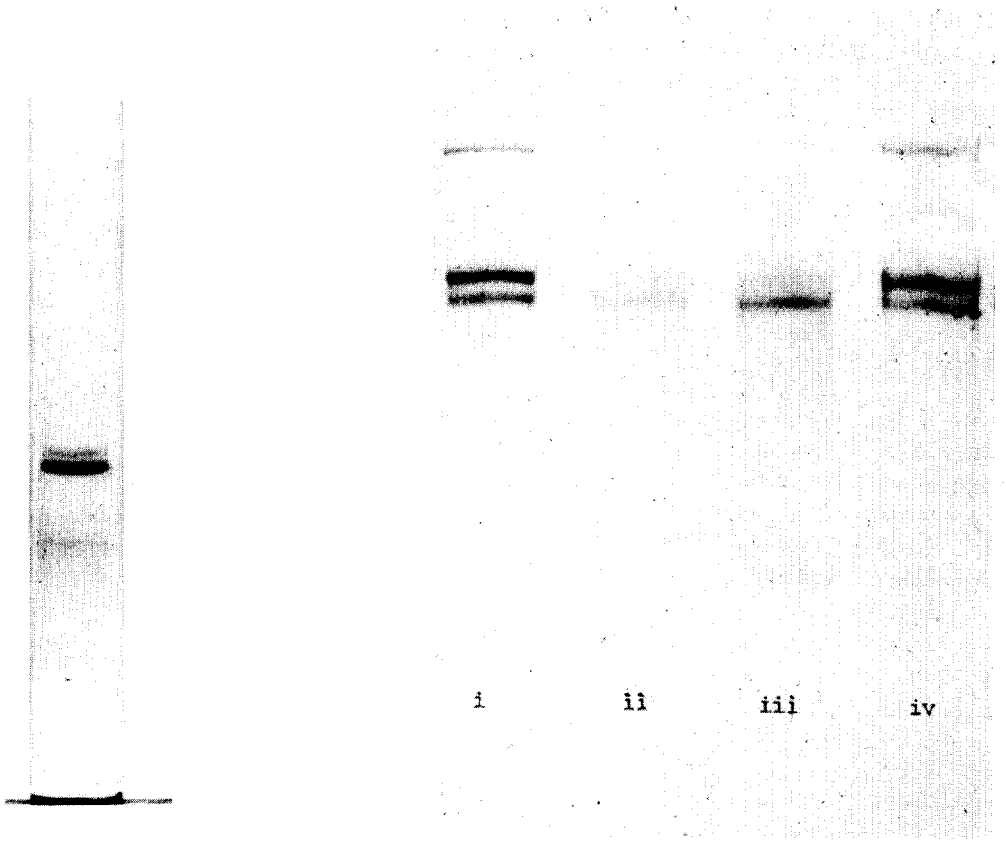


Fig. 4. Electrophoretic patterns of 'supernatant' and polysomal messenger RNA's: (a) cylindrical polyacrylamide gel in formamide separating 20 μg 'supernatant' messenger RNA. (b) Direct comparison of polysomal and 'supernatant' messenger RNA's, i and iv: each 20 μg polysomal messenger RNA, ii and iii: 'supernatant' messenger RNA. (7 and 15 μg respectively). Polysomal and 'supernatant' messenger RNA's were subjected to electrophoresis on gel slabs of 4% polyacrylamide in formamide. Both gels were stained with pyronin Y.

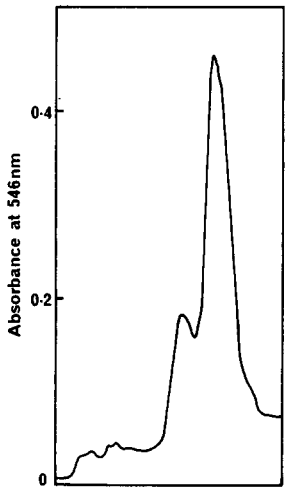


Fig. 5. Densitometry of 'supernatant' messenger RNA separated on polyacrylamide gels in formamide and stained with pyronin Y: cylindrical gels resembling those presented in an earlier publication (4) but containing 'supernatant' messenger RNA were scanned at 546 nm using the Gilford Gel Scanner.

results in the synthesis of more α- than β-globin. However, we calculate that β-globin is 29% of the total globin synthesised, whereas other authors have reported values in the range 0–20% [1–3].

Previous studies of 'supernatant' messenger RNA were unable to distinguish between the following two possibilities: (i) that both α- and β-messenger RNA are

present in the supernatant in equal amounts, but the β-messenger RNA is inactive or translated with relatively low efficiency; (ii) that there is more α-messenger than β-messenger in the supernatant and this explains the observation that in vitro translation results in more α-globin. Our results differentiate between these possibilities, indicating that the second is correct.

Acknowledgements

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