FREE CYTOPLASMIC α-GLOBIN MESSENGER RNA APPEARS DURING THE MATURATION OF RABBIT RETICULOCYTES

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1. Introduction

The existence in mammalian reticulocytes of a free cytoplasmic nucleoprotein containing mainly α-globin messenger RNA (mRNA) was clearly demonstrated [1–4]. Such an entity was also found in duck erythroblasts [5]. In these nucleated cells, the messenger RNA of the free cytoplasmic nucleoprotein was shown to be precursor of the polysomal mRNA [5].

In the case of rabbit reticulocytes which have lost their nucleus since approx. 10 h when they appear in the peripheral blood [6], nothing is known concerning the origin of the free cytoplasmic messenger ribonucleoprotein. It could be either a precursor form of the polysomal mRNA or the result of an imbalance between chain elongation and initiation which is known to occur when reticulocytes mature [6,7]. This second hypothesis was first suggested by Bonanou-Tzedaki et al. [1].

In the present paper, we show, on basis of labelling kinetics and poly(A) content, that cytoplasmic globin mRNA of rabbit reticulocytes appears as a result of reticulocyte maturation. We also describe a new method for its isolation and we propose an explanation for its high α-globin mRNA content.

2. Material and methods

Reticulocyte polyribosomes and pH 5 precipitate of the post-ribosomal supernatant were obtained according to Gianni et al. [2].

2.1. *Buffers used*

Buffer A: 0.01 M Tris, 0.001 M EDTA, 0.4 M NaCl, 0.2% (w/v) sodium dodecylsulphate brought to pH 7.5 with HCl.

Buffer B: 0.01 M Tris, 0.001 M EDTA, 0.2% (w/v) sodium dodecylsulphate brought to pH 7.5 with HCl.

Buffer C: 0.1 M Tris, 0.015 M KCl brought to pH 7.5 with HCl.

2.2. *Globin mRNA preparation*

2.2.1. From the polyribosomes

Reticulocyte polyribosomes were submitted to a proteinase K treatment according to Mach et al. [8] and mRNA was purified directly from the total suspension by poly(U)-Sepharose affinity chromatography according to Lindberg et al. [9] except that RNA bound to the poly(U)-Sepharose was recovered by elution with a low ionic strength buffer (buffer B) at 48°C [10] instead of formamide. Globin mRNA recovered by ethanol precipitation was further purified by sucrose gradient centrifugation (40 h at 4°C in 10–20% (w/v) linear sucrose gradients made in Tris-HCl 0.01 M buffer pH 7.4 spun at 25 000 rev/min in a SW 27 Spinco rotor.)

2.2.2. From the pH 5 precipitate of the post-ribosomal supernatant

This is an adaptation of the method used for obtaining of mRNA from polyribosomal pellets. pH 5 precipitate from 400 ml of post-ribosomal supernatant [2] was suspended in 16 ml of buffer C.

The suspension was brought to 0.5% (w/v) sodium dodecylsulphate and 1600 μg of proteinase K were added. It was then gently shaken at 0°C for approximately 10 min up to a complete clarification and then for 30 min at room temperature. The suspension was then brought to 0.4 M NaCl, 0.001 M EDTA. Hybridization of poly(A) containing molecules with poly(U) was allowed to occur for 30 min at room temperature by gently shaking 2 ml of poly(U)-Sepharose in the suspension (poly(U)-Sepharose contains 300 μg of poly(U) per ml). The procedure was then as previously described.

2.3. *Fractionation of mRNA according to the length of the poly(A) segment*

Polysomal and supernatant mRNA preparations were bound separately to poly(U)-Sepharose at room temperature in buffer A. The poly(U)-Sepharose columns were then eluted with buffer B at 25°C and the mRNA which was released at that temperature was recovered. When no more material was eluted from the columns, the temperature was raised to 48°C and a second fraction of mRNA which was more tightly bound to the poly(U)-Sepharose was eluted.

Fig.1 shows the sedimentation profile in sucrose

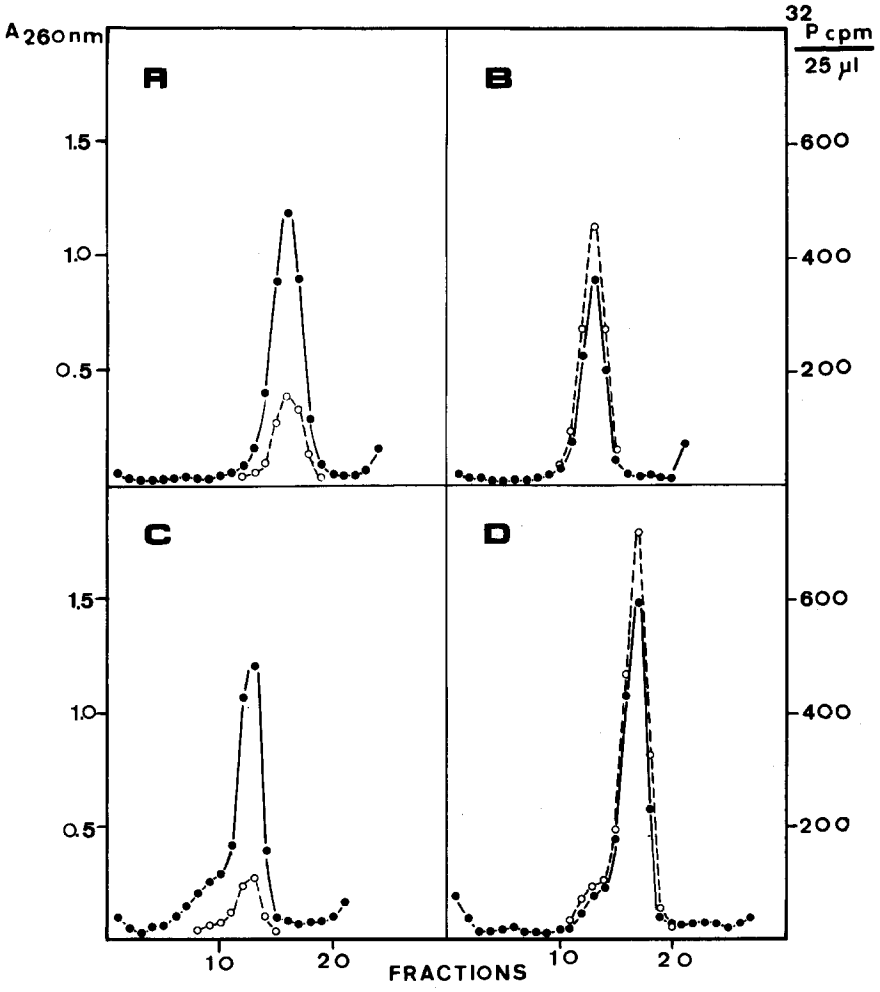

Fig.1. Sucrose gradient sedimentation of globin mRNA fractions from polyribosomes (A,B) and post-ribosomal supernatant (C,D). Fractions A and C are eluted from a poly(U)-Sepharose column at 25°C, fractions B and D are eluted at 48°C. Centrifugation was at 4°C and 25 000 rev/min for 40 h in 10–20% (w/v) sucrose gradients (made in Tris-HCl 0.01 M, pH 7.5) in a SW 27 Spinco rotor. Sedimentation direction from right to left.

gradients of mRNA fractions eluted at 25°C and 48°C from poly(U)-Sepharose.

3. Results and discussion

Fig.1A and 1B show the sedimentation profile in sucrose gradients of globin mRNA fractions from the post-ribosomal supernatant. One can see that very pure preparations can be obtained using the method that we describe above. The main advantage of the method is that it allows the treatment of a large amount of pH 5 precipitate in a single step. The mRNA bound to the poly(U)-Sepharose column may then be purified by a single centrifugation step in a few sucrose gradients.

The free cytoplasmic messenger ribonucleoprotein of rabbit reticulocytes might represent either the form in which globin messenger RNA is carried from the nucleus to the polysomes or it might be released from the polysomes as a result of reticulocyte maturation. If the first hypothesis is true, mRNA from the cytoplasmic mRNP should be younger than polysomal mRNA; if the opposite is true, it should be older.

In order to see which one of these possibilities is true, we decided to compare the specific radioactivity of free cytoplasmic and polyribosomal mRNAs obtained from reticulocytes of rabbits injected with [^{32}P]orthophosphate 15 h before their sacrifice. Reticulocytes result from the maturation of nucleated erythroid cells of the bone marrow and of the spleen. A 8 to 12 h period of time elapses between the arrest of RNA synthesis and the release of anucleated reticulocytes in the peripheral blood. In our labelling conditions, only reticulocytes which were released during the 3 to 7 h period before sacrifice contain labelled RNA. This period of time is short as compared to the life-span of rabbit reticulocytes which is about 2 days. Newly made globin mRNA molecules have thus a higher specific radioactivity than old mRNA molecules.

Globin mRNA was thus isolated and purified from the post-ribosomal supernatant on the one hand and from polysomes on the other hand, of reticulocytes from rabbits injected with 10 mCi of [^{32}P]orthophosphate before their sacrifice. One can see in table 1 that, in our experiment, 19% of reticulocyte globin mRNA is found free in the cytoplasm and its specific radioactivity amounts to 75% of that of polyribosomal mRNA. This result constitutes a first indication that free cytoplasmic mRNA is older than polyribosomal mRNA. However, one could argue that the difference in specific radioactivity is due to the fact that free cytoplasmic globin mRNA mainly consists of α-globin mRNA which could have a different labelling kinetics than β-globin mRNA.

Table 1
Cellular distribution of globin mRNA in rabbit reticulocytes

Origin of mRNA	Proportion (%)	Specific radioactivity[a] (cpm/μg)
Polyribosomes	81	380
Post-ribosomal supernatant	19[b] (1)	286

[a] Anaemic rabbits have been injected each with 10 mCi of [^{32}P]orthophosphate 15 h before their sacrifice.
[b] Correction was made to take account that only 2/3 of the supernatant are used.

We thus decided to use another criterion to compare mRNA ages. Since it is impossible to separate α- from β-globin mRNA according to a possible difference in length of their poly(A) segments [11–13], the size distributions for the poly(A) stretches of α- and β-globin mRNAs must be roughly the same. On the other hand, it is now established that the poly(A) segment of globin mRNA decreases in size with ageing [13,14]. We have thus compared the lengths of the poly(A) stretches of free cytoplasmic and polysomal globin mRNAs.

For that purpose, we submitted the purified cytoplasmic and polysomal globin mRNA preparations to a fractionation procedure on a poly(U)-Sepharose columns which is described in Materials and methods. Globin mRNA binds to poly(U)-Sepharose at high ionic strength. At low ionic strength, part of it, with short poly(A) segments can be eluted from the column at 25°C whilst the remaining part, with longer poly(A) stretches, is released if the column is heated up to 48°C. Table 2 gives the proportions and the specific radioactivity of the fractions eluted at 25°C and 48°C in the two globin mRNA preparations.

Polyribosomal mRNA contains only 25% of material which is eluted at 25°C whilst cytoplasmic mRNA contains 55% of this material.

Table 2
Fractionation of globin mRNA on poly(U)-Sepharose

Origin of mRNA	Proportion and specific radioactivity of material eluted at 25°C from poly(U)-Sepharose	Proportion and specific radioactivity of material eluted at 48°C from poly(U)-Sepharose
Polysomes	25% (83 cpm/μg)	75% (479 cpm/μg)
Post-ribosomal supernatant	55% (116 cpm/μg)	45% (497 cpm/μg)

Cytoplasmic mRNA thus contains poly(A) segments which have an average length shorter than that of poly(A) stretches of polysomal mRNA.

We may assume that the mRNA fractions of low specific radioactivity which are eluted at 25°C from the poly(U)-Sepharose column originate from older reticulocytes and that the mRNA fractions of high specific radioactivity eluted at 48°C come from younger reticulocytes. One can deduce (table 3) that one third of globin mRNA is free in the cytoplasm in older reticulocytes whilst but 12% of it is cytoplasmic in younger reticulocytes.

It is thus proven that, in mammalian reticulocytes, free cytoplasmic mRNA represents mainly the last stage of the message life. This is in perfect agreement with our previous observations [6] showing that rabbit reticulocyte ageing is associated with a decrease in size of polysomes and thus with a decreasing efficiency of initiation of translation [7]. This probably also leads to the appearance of free message in the cytoplasm.

Most of the free message is coding for α-globin; this is in good agreement with the fact that, in reticulocytes, initiation of translation of α message is less efficient than that of β message since polysomes synthesizing α-globin are smaller in size than those synthesizing β-globin [15]. On the other hand, α message requires haemin to be translated [16]. What becomes first limiting in the initiation of translation when reticulocytes age is thus perhaps haemin.

Table 3
Cellular distribution of globin mRNA in old and young reticulocytes[a]

Cellular age	Proportion in the cytoplasm (%)	Proportion in the polysomes (%)
Young	12	88
Old	34	66

[a] The figures given are deduced from tables 1 and 2 as follows: For example, old cells contain 25% of total polysomal mRNA and 55% of total supernatant mRNA (see table 2). That means (0.25 × 0.81) + (0.55 × 0.19) of total mRNA. The proportion of cytoplasmic mRNA in old cells is thus

$$\frac{0.19 \times 0.55}{(0.19 \times 0.55) + (0.25 \times 0.81)} = 12\%$$

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