IDENTIFICATION OF FREE CYTOPLASMIC GLOBIN mRNA OF DUCK ERYTHROBLASTS BY HYBRIDIZATION TO ANTI-MESSENGER DNA AND BY CELL-FREE PROTEIN SYNTHESIS

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Received 13 October 1972

1. Introduction

The cytoplasm of animal cells contains mRNA not associated with ribosomes which exists in the form of ribonucleoprotein (RNP) complexes [1, 2]. It has been suggested that such complexes represent a pool of mRNA intermediate between the nuclear pre-mRNA and the mRNP complex active in translation [1–4]. We have described in immature duck erythrocytes a cytoplasmic 9 S RNA not associated with ribosomes. This free cytoplasmic 9 S RNA has the same electrophoretic mobility as polyribosomal 9 S globin mRNA; its rate of synthesis and decay is consistent with, but not a proof of, a role as a precursor to polyribosomal 9 S mRNA [4].

In this paper we present direct proof for the existence of a cytoplasmic pool of genuine globin mRNA free from the ribosomes (free mRNA) by demonstrating the capacity of the free 9 S RNA to be translated into globins and to hybridize with anti-messenger DNA (amDNA) copied from globin mRNA by reverse transcriptase.

2. Methods

2.1. *Isolation of free 9 S mRNA*

20 ml of duck immature erythrocytes were lysed as previously described [4] and the postmitochondrial supernatant fractionated on a Beckman Ti 15 zonal rotor (15 to 45% linear sucrose gradient, 27,000 rpm, 14 hr, 4°). The 10 to 30 S region of the gradient, which contains the free 9 S RNA–protein complex [4, 5], was pooled, the NaCl concentration adjusted to 0.3 M, the RNP precipitated with 2 vol of ethanol, and extracted with phenol, the RNA was reprecipitated with 2 vol of ethanol in 0.1 M NaCl [6] and dissolved in buffer (0.01 M triethanolamine pH 7.4, 0.01 M NaCl).

The RNA was further fractionated on a 5 to 21.75% isokinetic [7] sucrose gradient (SW 40 Spinco rotor, 40,000 rpm, 13 hr, 2°). The 9 S region was pooled, precipitated with ethanol and redissolved in buffer (0.01 M triethanolamine, pH 7.4, 0.01 M NaCl).

2.2. *Cell-free protein synthesizing system and CM cellulose chromatography*

The template activity of the RNA was measured in a partially purified cell free system containing mouse liver ribosomes, pH 5 enzymes and rabbit reticulocyte initiation factors, according to Schreier and Staehelin [9, 10].

2.3. *Preparation of amDNA and hybridization with RNA*

Was carried out as described elsewhere [8].

3. Results

The analysis on a sucrose gradient of the RNA extracted from the 10 to 30 S free cytoplasmic ribonu-

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Abbreviations: mRNA: messenger RNA; RNP: ribonucleoprotein; mRNP: ribonucleoprotein containing mRNA; pre mRNA: RNA precursors to mRNA: amDNA: anti-messenger DNA complementary to mRNA synthesized with reverse transcriptase.

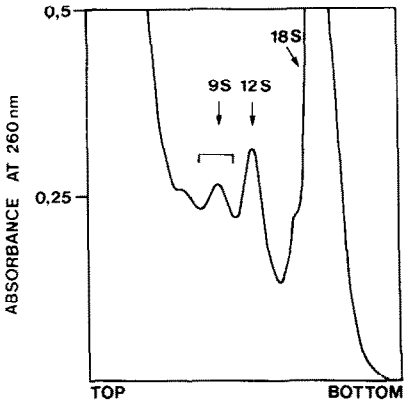

Fig. 1. Sedimentation profile of free cytoplasmic mRNA. The postmitochondrial supernatant was fractionated on a sucrose gradient according to Methods. The 10 to 30 S zone was pooled, precipitated with ethanol, phenol extracted and the RNA analyzed on the 5% to 21.75% isokinetic sucrose gradient shown (Spinco SW 40, 40,000 rpm, 13 hr, 2°).

cleoprotein particles (see Methods) is shown on fig. 1. Besides 4 S and 18 S RNA, RNA peaks in the 7 S, 9 S and 12 S regions are found. The 9 S RNA was pooled as indicated in the figure and assayed for its ability to code for duck globin in a cell-free protein synthesizing system [9, 10], and to hybridize with globin specific amDNA [8].

3.1. *Analysis of the in vitro synthesized proteins*

The radioactive product of the cell-free system containing the free 9 S RNA, when analyzed on SDS polyacrylamide gels, was shown to consist of a large peak of labelled material migrating in the 15,000–17,000 molecular weight range, indicative of duck globin (data not shown). To determine whether duck globin had actually been synthesized, the product was acid acetone precipitated and chromatographed on a carboxymethylcellulose column with unlabelled duck globins. The *in vitro* translation product synthesized in the presence of duck polyribosomal 9 S mRNA was analyzed in a similar manner. The result is depicted in fig. 2. Both free cytoplasmic 9 S RNA and polysomal 9 S RNA appear to have directed the synthesis of the 3 individual duck globins. This finding can be contrasted with results of experiments carried out with free mRNA from rabbit reticulocytes, where only mRNA coding for one globin chain was detected [11].

Compared with the *in vitro* product obtained with

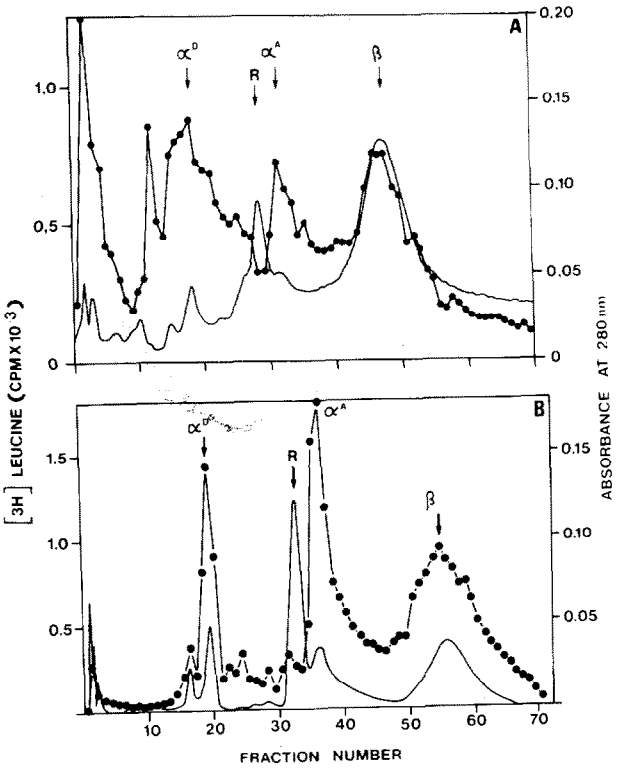

Fig. 2. Chromatographic analysis on CM 52 cellulose columns of the *in-vitro* synthesized product directed by free cytoplasmic and polyribosomal 9 S RNA. A) RNA from the 9 S region isolated on sucrose gradients (fig. 1) was added at a concentration of 0.5 moles 9 S RNA per mole of ribosome to a cell-free protein synthesizing system containing ribosomes from mouse liver, initiation factors from rabbit reticulocytes and pH 5 enzymes from rat liver as described elsewhere [9]. The reaction mixture contained per ml : 20 μmoles creatine phosphate, 3.75 units creatine kinase, 1 μmole ATP, 0.4 mole GTP, 30 μmoles of each L-amino acid, [^{3}H] leucine, 30 μmole Tris-HCl, pH 7.6, 70 μmoles KCl, 1 μmole dithiothreitol and 4.3 μmole Mg^{2+}. After 40 min of incubation at 30° the product was processed and the acetone precipitated radioactivity analyzed on a carboxymethylcellulose column (Whatman CM 52) [10, 17]. Cold duck and rabbit globin were added as markers: α^A, α^D, and β indicate the duck globin peaks; R, the rabbit globin peak. B) As A but with 9 S globin mRNA isolated from duck polyribosomes as template.

polyribosomal 9 S mRNA (fig. 2B) the product coded by the free 9 S seems to be less homogeneous and to contain other polypeptides which elute from CM cellulose over a wider range of salt concentrations. Although this makes it more difficult to quantitate the

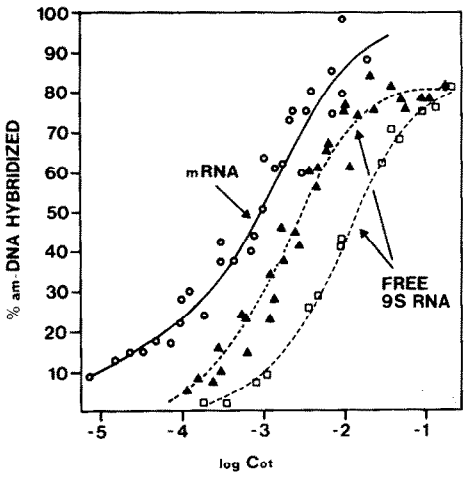

Fig. 3. Hybridization of free cytoplasmic 9 S RNA and 9 S globin mRNA with globin amDNA. Purified 9 S globin mRNA (○—○—○) and two separate preparations A (▲----▲----▲) and B (□----□----□) of free 9 S RNA were hybridized with amDNA copied on polyribosomal 9 S mRNA by avian myeloblastosis virus reverse transcriptase. The level of hybridization was determined by the single-strand specific DNAase S_1 from *Aspergillus orizae* [18] and plotted in function of log C_0t values [14]. The C_0t values and the corresponding concentrations of globin mRNA sequences are: 9 S globin mRNA: $C_0t_{1/2} = 7.5 \times 10^4$ mol. sec/l (100%); free 9 S A: $C_0t_{1/2} = 2 \times 10^{-3}$ (40%); free 9 S B: $C_0t_{1/2} = 10^{-2}$ (8%).

proportions of the three globin chains synthesized, it would appear that the free 9 S RNA has given rise to relatively more α^D globin than was coded for by the polyribosomal mRNA. This apparent difference between the relative abilities of the free 9 S RNA and the polysomal mRNA to code for the individual globins may be a reflection of the actual proportions of each messenger RNA present in the two RNA samples.

3.2. *Hybridization of free 9 S RNA with globin amDNA*

amDNA complementary to at least 90% of the globin mRNA sequences present in polyribosomes was prepared by transcription of highly purified 9 S globin mRNA with the reverse transcriptase of avian myeloblastosis virus [8, 12, 13]. Actinomycin D in the reaction prevented the formation of double stranded DNA. The rate of hybridization of individual preparations of free 9 S RNA with this amDNA, was plotted according to Britten and Kohne [14]. A $C_0t/2$ value was determined and used to estimate the concentration of globin coding sequences in the free 9 S RNA. The $C_0t/2$ value obtained on backhybridization of the amDNA to its pure globin mRNA template served as a quantitative standard.

The results of some of these experiments are shown in fig. 3. For comparison the C_0t curve and $C_0t/2$ value obtained with pure globin mRNA published in detail elsewhere [8] are inserted in the figure; they represent 100% coding sequence. It is evident from the data that the free 9 S preparations contained globin mRNA in amounts varying between 8 and 40%. This variation in amount may be due to the selection of the sucrose gradient fractions for the 9 S pool. Because of the relatively small amount of free RNA and low resolution of the 9 S fraction, this selection is bound to be inaccurate. In addition, apart from the globin messengers, there may be other RNA species of the same sedimentation constant which are more abundant among free than polyribosomal mRNA. The proportion of free 9 S RNA to these other RNA species seems to depend on the proportion of immature red cells [4].

Interestingly, the amDNA hybridization to free 9 S RNA levels off at 70 to 80%, about 10% lower than that obtained by back hybridization to the 9 S globin mRNA template. A relatively high representation in the free 9 S RNA of the α^D globin message, which is a very minor component in polyribosomal mRNA could lead to a considerable formation of partly mismatched amDNA/mRNA hybrids since α^A and α^D amino acid sequences are closely related but not identical. Unhybridized loops or tails interspersed with sequences of perfect complementarity would be cleaved off by the single-strand specific DNAase.

4. Discussion

Both experimental approaches reported here lead to the conclusion that the free 9 S RNA found in the cytoplasm of duck erythroblasts contains the message for the duck hemoglobins. This, together with the labeling kinetics [4] supports the idea that free mRNP complexes may represent a transient pool of mRNA prior to translation.

As pointed out earlier [2, 4] the transition from the free to the polyribosomal mRNA population may involve some pre-translational regulative control. A new indication for such a mechanism may be seen in the

apparently different representation among free 9 S RNA, compared to the polyribosomal mRNA of the messengers for the individual globin chains. The product analysis after translation as well as the hybridization kinetics of globin amDNA to free 9 S RNA indicate a different quantitative representation of the three globin mRNA's. For this we would conclude that there is some translational control mechanism which regulates the individual rates of translation of the three mRNA's. Such a mechanism operating in the living cell would be responsible for the accumulation of some of the free globin mRNA species relative to others.

Since there is little evidence that regulation could simply result from different rates of elongation of the individual chains [15, 16] such a regulative mechanism would have to operate at the level of initiation. The possibility that there is a qualitative and/or quantitative requirement of protein factors for the initiation of each type of message will be discussed elsewhere [10, 17].

Acknowledgements

We are grateful to T. Staehelin and M. Schreier for help in carrying out the cell-free protein synthesis experiments, to H. Diggelmann for transcribing 9 S mRNA into amDNA, to V. Vogt for the gift of single-strand specific DNAase S_1, to Sylvia Schmidlin and Anne-Cecile Pittet for the excellent technical assistance, and to Pierre Dubied for help in the preparation of the manuscript. We thank our colleague V. Vogt for the critical reading of this manuscript.

This work was supported by the Swiss National Foundation (Grant No 3.322.70 and 3.613.71).

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