DIFFERENTIAL TRANSLATION OF α- AND β-GLOBIN MESSENGER IN A CELL-FREE SYSTEM

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1. Introduction

Although the function of initiation factors in eukaryotic protein synthesis has been elucidated in some detail [1,2] there remains considerable uncertainty as to the extent to which there is a stringent requirement for messenger-specific factors. On the one hand, it has been found that messenger RNA from various mammalian and avian sources can be translated correctly in cell-free systems [3–10] and in *Xenopus* oocytes [11] without the need for supplementation with initiation factors from the same cells as the mRNA. In other cases, however, homologous initiation factors appeared to be required [12] and factor specificity was also demonstrated in competition experiments using globin mRNA and Mengo virus RNA [13]. Recently, an initiation factor with specificity for protein synthesis directed by encephalomyocarditis (EMC) viral RNA but not by globin mRNA has been purified from ascites tumour cells [14].

A number of reports in the literature indicate that there are differences in the efficiency of translation of the α- and β-globin messenger in some cell-free systems. Thus, the ascites system generally synthesizes an excess of β-globin [6, 15, 16] even though the globin mRNA is derived from reticulocytes which are known to contain more mRNA for α-globin than for β-globin [17]. In a cell-free system from liver, however, α-globin chains are produced in excess [9]. The possibility that these contradictory observations might be explained by the existence of different initiation factors for α- and β-globin mRNA prompted us to investigate in more detail the conditions for the translation of globin mRNA in a cell-free system from Krebs II ascites cells. We have found that the proportion of α/β globin synthesized in this system is markedly altered by changes in the concentration of either mRNA or initiation factors. Our results show that the translation of the messenger RNA's for the two globin chains is controlled independently.

2. Experimental

2.1. *Materials*

ATP, GTP, creatine phosphate and creatine phosphokinase were purchased from Boehringer Mannheim GmbH (Mannheim, German Federal Republic). Dithiothreitol was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). L-[^{14}C]valine (280 Ci/mol) and L-[4, 5-^{3}H]isoleucine (11 Ci/mmol) were from the Radiochemical Centre (Amersham, Bucks., U.K.).

2.2. *Methods*

Pre-incubated Krebs II ascites S 30 was prepared as described by Mathews and Korner [18] from a stock of cells kept at the Imperial Cancer Research Fund Laboratories, Lincolns Inn Fields, London, W.C.2 and kindly donated by Dr. A.E. Smith.

Rabbit globin mRNA was prepared by sodium dodecyl sulphate–phenol/chloroform extraction [19] of the 14 S mRNP particle released from reticulocyte polysomes by treatment with EDTA [20].

Reticulocyte initiation factors were prepared by extracting rabbit reticulocyte ribosomes with 0.5 M KCl as described by Miller and Schweet [21]. In an attempt to remove the α-globin mRNA present in such factors the standard factor preparation (1 ml) was

added to 0.2 g DEAE-Sephadex equilibrated in 0.5 M KCl and allowed to stand for 30 min at 0°C. After this time, the resin was removed by centrifugation through a glass-wool plug in a 2 ml Gillette disposable syringe placed in a centrifuge tube. This factor preparation (DEAE-factors) has been shown to retain only 10% of the mRNA content of the control factors (unpublished observations) but also to have altered properties in the ascites cell-free system (see figs. 4 and 5).

Cell-free incubation mixtures (50 μl) contained 15 μl pre-incubated ascites S 30, 20 mM Tris–HCl (pH 7.5), 3 mM $MgCl_2$, 85 mM KCl, 1 mM ATP, 0.2 mM GTP, 1 mM dithiothreitol, 10 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 0.12 μmol/ml of each of 18 protein amino acids (except valine and isoleucine), 0.08 μCi L-[^{14}C]valine (280 Ci/mol), 1.5 μCi L-[4, 5-^{3}H]isoleucine (11 Ci/mmol), and globin mRNA at a final concentration of 0–60 μg/ml. Where indicated, reticulocyte factors in 0.2 M KCl were added, the amount of additional KCl being adjusted accordingly. The reaction mixtures were incubated for 30 min at 37°C, then for a further 15 min at 37°C in 0.3 M NaOH. Protein was precipitated with cold 10% w/v trichloroacetic acid and filtered onto Whatman glass fibre discs. Samples were counted in a Packard 574 liquid scintillation spectrometer using 2,5-diphenyloxazole (PPO) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene (dimethyl POPOP) in toluene. The efficiency was 80% for ^{14}C and 15–20% for ^{3}H.

2.3. *Calculation of messenger-dependent synthesis of globin α- and β-chains*

In the presence of globin messenger RNA the pre-incubated ascites cell-free system synthesizes mainly globin, but some endogenous protein synthesis also occurs and the synthesis of both globin and endogenous protein is stimulated by reticulocyte initiation factors [6]. All results reported in this paper have therefore been corrected for endogenous protein synthesis, as well as for globin synthesis due to mRNA present in the reticulocyte initiation factor preparations [6], by subtracting the incorporation of labelled amino acids into protein that was obtained in control incubations without added globin messenger and with or without initiation factors, as appropriate. When required, the radioactivity of protein samples was converted into moles amino acid incorporated assuming no dilution of the added isotope.

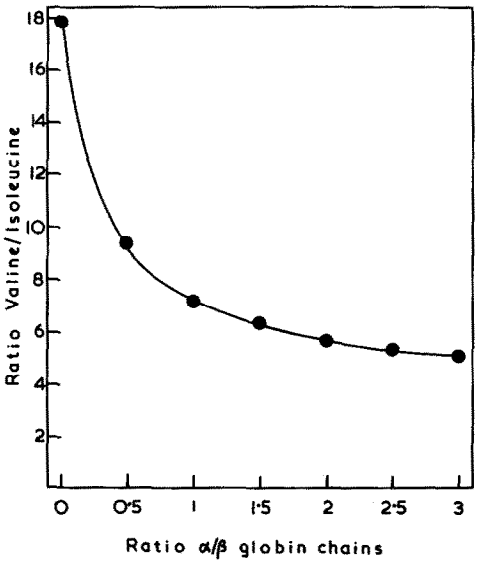

Fig. 1. Relationship between the molar ratio of valine and isoleucine and the proportion of α-chains to β-chains in rabbit globin. The valine/isoleucine ratio was calculated for different mixtures of α-chains and β-chains from the published amino acid composition of rabbit globin [22].

The α-chain of rabbit haemoglobin contains 17 leucine, 11 valine and 3 isoleucine residues whereas the β-chain contains 18 leucine, 18 valine and only 1 isoleucine residue [22]. Changes in the relative synthesis of globin α- and β-chains would be expected therefore to affect markedly the valine/isoleucine ratio of the newly synthesized globin, but have little effect on the incorporation of leucine. The theoretical valine/isoleucine ratios corresponding to α/β globin ratios ranging from 0 to 3 are shown in fig. 1. It can be seen that over most of this range changes in the proportion of α and β globin synthesized in the cell-free system should be detectable by measuring the valine/isoleucine ratio of the total protein. The validity of this method has been confirmed by the satisfactory correlation between the α/β globin ratio calculated in this way and the results obtained by labelling the globin with [^{14}C]leucine in otherwise identical cell-free incubations and separating the α- and β-chains by chromatography on carboxymethyl cellulose [23] (see fig. 2). Several α/β ratios in the range 0.3–2.4 have been verified by this technique.

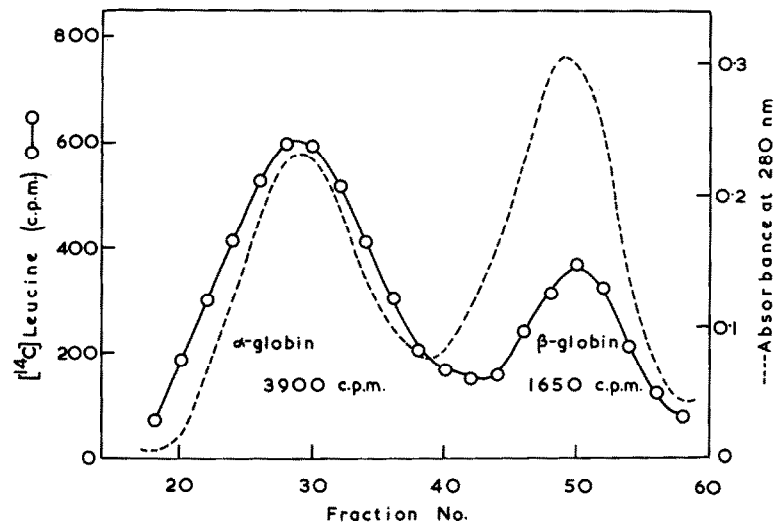

Fig. 2. Differential labelling of α-chains and β-chains of rabbit globin. Globin was labelled in the ascites cell-free system with [^{14}C]leucine under otherwise identical conditions as described in fig. 4 using rabbit globin messenger RNA at 10 μg/ml of incubation mixture in the presence of reticulocyte initiation factors (15 μl). The α-chains and β-chains were separated by chromatography on carboxymethylcellulose [23] with a gradient of formic acid (0.6 → 1.5 M)–pyridine (0.06 M → 0.15 M). The ratio of α/β globin chains as determined by measuring the radioactivity of the separated chains was 3900/1650 = 2.37 compared with a value of 2.4 obtained from the valine/isoleucine ratio (fig. 4b).

3. Results

3.1. *Ionic conditions for optimum globin synthesis*

In previous work it was shown that sufficient K^+ must be present to obtain a response to reticulocyte initiation factors when EMC RNA is used as messenger [6]. It was therefore of interest to investigate the relative amounts of α- and β-chains synthesized at different K^+ concentrations when globin mRNA was added to such cell-free incubations. Fig. 3 shows that the incorporation of both valine and isoleucine into protein is optimal at 65 mM K^+ in the absence of reticulocyte initiation factors, but when factors are added the optimum shifts to approx. 85 mM. These optima agree with the results of Metafora et al. [7]. At 65 mM K^+ the α/β globin ratio is approx. 2 in both the presence and absence of factors, but this ratio decreases as the K^+ concentration is increased, particularly when initiation factors are absent. All subsequent experiments were therefore done at the optimum [K^+] of 85 mM.

3.2. *Effect of varying the concentration of globin mRNA*

The messenger-directed incorporation of labelled valine and isoleucine into protein in the absence of initiation factors and in the presence of either a standard preparation or a preparation of factors which had been treated with DEAE-Sephadex in 0.5 M KCl is illustrated in fig. 4a, globin mRNA being added in amounts ranging from 10 to 60 μg/ml of incubation mixture. With standard initiation factors the incorporation of isoleucine reached a maximum value at 20 μg mRNA/ml. Addition of more mRNA slightly inhibited the incorporation of isoleucine, whereas that of valine increased still further. With DEAE-treated initiation factors or in the absence of factors this inhibition was not observed, but there was a less marked quantitative change in the response to globin mRNA at 10–20 μg/ml.

When the results of this experiment are replotted to show the ratio of globin chains synthesized (fig. 4b), it is evident that the synthesis of α-chains predominates at low globin messenger RNA concentrations, whereas an increase in mRNA results in a progressive decrease in the α/β globin ratio. It should be

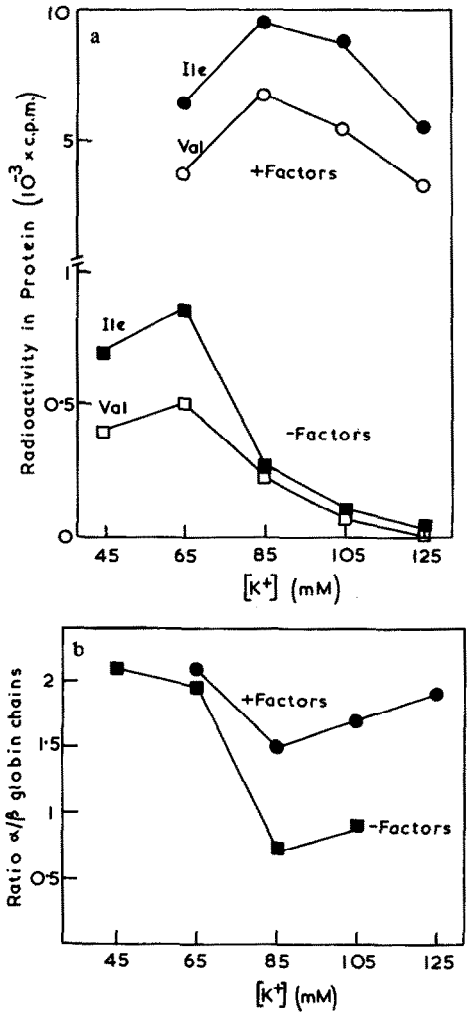

Fig. 3. Effect of potassium ion concentration on cell-free synthesis of globin chains. a) Protein was labelled with [^{14}C]valine and [^{3}H]isoleucine in the ascites cell-free system at different concentrations of KCl in the presence of rabbit globin mRNA (10 μg/ml). Reticulocyte initiation factors (10 μl) were added where indicated. b) The ratio of globin α-chains/β-chains has been calculated from the [^{14}C]valine/[^{3}H]isoleucine molar ratios derived from the ^{14}C and ^{3}H in the radioactive protein (fig. 3a) using the theoretical curve given in fig. 1.

noted also that at any given messenger RNA concentration the DEAE-treated factor preparation gave rise to a higher α/β globin ratio than that obtained with the standard factors, and that without factors the α/β globin ratio was less than 1 over the entire concentration range of mRNA.

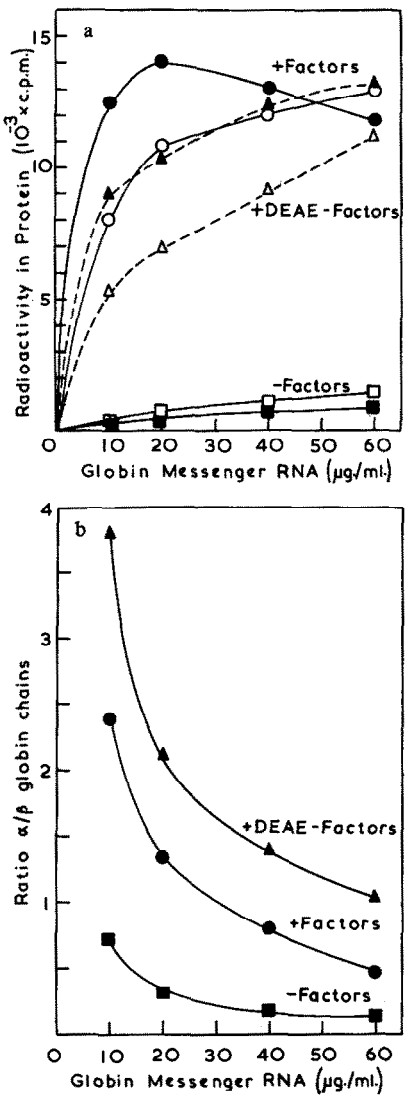

Fig. 4. Effect of globin messenger RNA concentration on cell-free synthesis of globin α-chains and β-chains. a) The ascites cell-free system was incubated at 37°C for 30 min with [^{14}C]valine and [^{3}H]isoleucine either without initiation factors or with standard factors (15 μl) or with DEAE-treated factors (15 μl). Rabbit globin mRNA was added in different amounts, as shown. The radioactivity of the protein was determined as described in the Experimental section. ○, ● With standard factors; △, ▲ with DEAE-factors; □, ■ without factors; open symbols, [^{14}C]valine; closed symbols, [^{3}H]isoleucine. b) The ratio of globin α-chains/β-chains was calculated from the ^{14}C/^{3}H ratio of the radioactive protein (cf. fig. 3b).

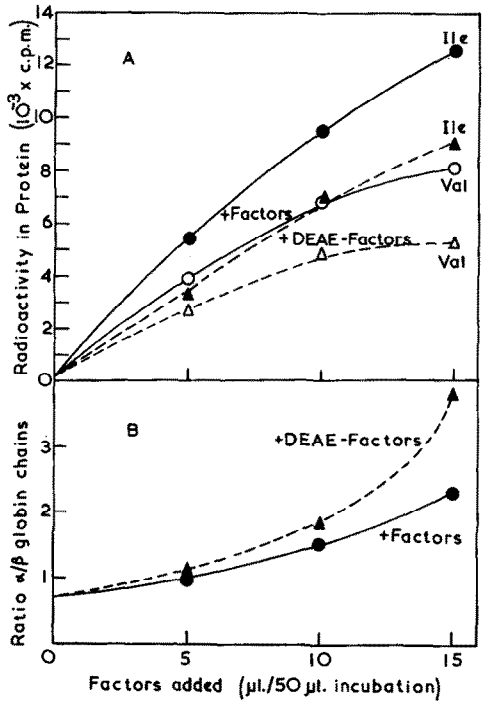

Fig. 5. Synthesis of globin α-chains and β-chains at various concentrations of initiation factors. A) The ascites cell-free system contained [^{14}C]valine and [^{3}H]isoleucine. Rabbit globin messenger RNA (10 μg/ml) was present throughout and the incorporation of amino acids into protein was determined in the presence of either standard or DEAE-treated initiation factors in varying amounts. ○, ● With standard factors; △, ▲ with DEAE-factors. B) The ratio of globin α-chains/β-chains synthesized was computed from the ^{14}C/^{3}H ratio of the radioactive protein (cf. fig. 3b).

3.3. *Translation of α and β globin mRNA at different concentrations of initiation factors*

The experiments described in the preceding section suggested that at 10–20 μg globin mRNA/ml of incubation mixture one or more of the constituents of the cell-free system became limiting, especially for the translation of the mRNA for α-globin. Since protein synthesis in the pre-incubated ascites system is strongly dependent upon initiation factors, we decided to investigate the effect of varying the amounts of initiation factors at a constant messenger concentration of 10 μg/ml (fig. 5A). The incorporation of isoleucine into protein was almost proportional to the amount of initiation factors with both standard and DEAE-treated preparations up to 15 μl per incubation, which was the maximum that could be tested. On the other hand, the incorporation of valine increased linearly only up to 10 μl of initiation factors. Fig. 5B shows that this change in the valine/isoleucine ratio with increasing factors corresponds to a 3–5-fold increase in the synthesis of α-globin relative to β-globin.

4. Discussion

Since the ribosome-associated globin messenger RNA in rabbit reticulocytes contains approx. 60% α-globin mRNA and 40% β-globin mRNA [17], we assume that our globin messenger preparation contains α-globin mRNA and β-globin mRNA in a similar ratio. Although the ascites cell-free system appears to be capable of translating the messenger correctly at the optimal potassium ion concentration (85 mM) in the presence of reticulocyte factors (fig. 3), the quantitative correlation must be coincidental as there is much variability in the relative synthesis of α and β globin under other conditions. The reason for the variability in relation to the concentration of K^+ is not known.

The experiments on the effect of messenger and factor concentration on the ratio of α/β globin synthesis (figs. 4 and 5) show clearly that the control of the translation of the α-globin messenger RNA is independent of that of the β-globin messenger. A simple explanation for these results would be the existence of two different initiation factors with specificity for the α-globin mRNA and β-globin mRNA, respectively. The β-globin-specific factor appears to be adsorbed onto DEAE-Sephadex in 0.5 M KCl (fig. 4b). It is interesting to speculate on the possible identity of this factor, which may perhaps involve an RNA molecule similar to that described by Fuhr and Natta [24]. If an α-globin-specific initiation factor was present in lesser amounts or was less efficient than the β-factor, it would be the first to become rate-limiting when the concentration of globin mRNA is increased. At high mRNA concentrations the cell-free system would therefore be unable to translate all the α-globin mRNA and the α/β globin ratio would decrease, as observed in the experiments described in fig. 4. Conversely, at constant mRNA concentration, but with the α-specific initiation factor initially more suboptimal than the β-factor, addition of factors would enable the system to translate the α-globin mRNA with in-

creasing efficiency and hence increase the α/β globin ratio, as shown in fig. 5. This interpretation would also serve to explain our earlier observations that the post-ribosomal supernatant of reticulocytes contains an appreciable amount of free α-globin mRNA [25]. At the time it was suggested that this supernatant mRNA might be present as a result of an imbalance between chain initiation and elongation and we would now propose that this imbalance is due to a deficiency of an α-globin specific factor in reticulocytes. This deficiency could be related to differences in the amounts of α- and β-factors present or to their relative affinities either for the mRNA's or the 40 S ribosomal subparticles.

We have shown that the addition of factors to a reticulocyte lysate cell-free system causes an increase in the ratio of α- to β-globin chains synthesized. On the other hand, the addition of globin mRNA to such a lysate causes a decrease in the α/β ratio (unpublished results). Thus, α-factor seems to be limiting for α-globin production, whereas β-globin synthesis is restricted by the availability of β-globin mRNA.

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