Competition between α- and β-Globin Messenger Ribonucleic Acids for Eucaryotic Initiation Factor 2†

Gianfranco Di Segni, Haim Rosen, and Raymond Kaempfer*

ABSTRACT: Synthesis of α- and β-globin was studied in a micrococcal nuclease treated reticulocyte lysate programmed with rabbit globin mRNA. The products of translation were analyzed by cellulose acetate electrophoresis under denaturing conditions. Nearly equimolar synthesis of α and β chains occurs at low mRNA concentrations, but the α/β synthetic ratio decreases drastically as more mRNA is present. Several lines of evidence suggest that direct mRNA competition for initiation factor eIF-2, the protein that binds methionyl-tRNA$_f^{Met}$, is involved in the regulation of α- and β-globin synthesis. The translational competition between α- and β-globin mRNA can be relieved by addition of highly purified eIF-2, even though total protein synthesis is not stimulated. The extent of relief by a given amount of eIF-2 declines with increasing mRNA concentration. At greater than optimal concentrations of salt, the α/β synthetic ratio declines in parallel with total globin synthesis, inhibition by KOAc occurring at higher concentrations than in the case of KCl. Addition of excess eIF-2 leads to effective relief of translational competition when it is sharpened by elevated concentrations of KCl and raises the α/β synthetic ratio fivefold (from 0.16 to 0.8). The behavior of globin mRNA translation as a function of increasing concentrations of KCl or KOAc matches exactly with that observed for the direct binding of ^{125}I-labeled globin mRNA to eIF-2, both with respect to the salt concentration giving 50% inhibition and to the displacement between the responses to KCl and KOAc. Binding of purified α-globin mRNA to eIF-2 is more sensitive to KCl than the binding of unfractionated globin mRNA. These findings suggest strongly that mRNA interacts directly with eIF-2 during protein synthesis and that α-globin mRNA has a lower affinity for eIF-2 than does β-globin mRNA.

Selective translation of certain mRNA species over other ones is often involved in the regulation of eucaryotic gene expression during differentiation or virus infection. mRNA discrimination occurs mainly at the initiation step, which involves the recognition of mRNA and its binding to ribosomes. An example of this type of regulation is found in erythroid development, where it has been shown that protein synthesis on β-globin mRNA is initiated 1.5 times more frequently than on α-globin mRNA, equimolar synthesis of α- and β-globin occurring because α-globin mRNA is 1.5 times more abundant (Lodish, 1971, 1974; Lodish & Jacobsen, 1972).

In this paper, we present evidence of competition between α-globin and β-globin mRNA for eucaryotic initiation factor 2 (eIF-2)[1]. This factor is known to reverse the block in initiation of protein synthesis observed in the absence of heme or in the presence of double-stranded RNA (Kaempfer, 1974; Clemens et al., 1975). eIF-2 forms a ternary complex with Met-tRNA$_f$ and GTP (Dettman & Stanley, 1972; Levin et al., 1973; Schreier & Staehelin, 1973) and directs the binding of Met-tRNA$_f$ to the 40S ribosomal subunit, a necessary prerequisite to the subsequent binding of mRNA (Darnbrough et al., 1973; Trachsel et al., 1977). In addition to binding Met-tRNA$_f$, eIF-2 also can bind to mRNA (Kaempfer, 1974; Hellerman & Shafritz, 1975; Barrieux & Rosenfeld, 1977, 1978; Kaempfer et al., 1978a, 1979). Specificity in the binding of eIF-2 to mRNA was shown by the finding (Kaempfer et al., 1978a) that all mRNA species tested possess a high-affinity binding site for eIF-2, including mRNA species lacking the 5′-terminal cap or 3′-terminal poly(A) moieties, while RNA species not serving as mRNA, such as negative-strand RNA (Kaempfer et al., 1978a), tRNA, or rRNA (Barrieux & Rosenfeld, 1977) do not possess such a site. Moreover, eIF-2 also appears to recognize the 5′-terminal methylated cap structure in mRNA: analogues of the cap inhibit the binding of mRNA and of Met-tRNA$_f$ to eIF-2, and addition of eIF-2 relieves the cap analogue induced inhibition of mRNA translation (Kaempfer et al., 1978b). These properties point to a role for eIF-2 in the recognition of mRNA and its binding to ribosomes, making it a protein uniquely suited for translational control.

The present study shows that eIF-2 relieves translational competition between α- and β-globin mRNA both when it is caused by a high mRNA concentration and when it is sharpened by elevated concentrations of salt. A close correlation is obtained between the results of translation experiments and those of experiments directly measuring the binding of mRNA to eIF-2. The data strongly suggest that mRNA interacts directly with eIF-2 during protein synthesis and that α-globin mRNA has a lower affinity for eIF-2 than does β-globin mRNA.

Materials and Methods

Reticulocyte Lysate. Rabbits were made anemic by five successive daily injections of 1 mL/kg of 1.25% 1-acetyl-2-phenylhydrazine in 0.14 M NaCl, 1.5 mM magnesium acetate, and 5 mM KCl and bled on the eighth day. Lysates were prepared as described by Kaempfer & Kaufman (1972). They were made 50 μg/mL in creatine kinase and 30 μM in hemin and treated with micrococcal nuclease as described by Pelham & Jackson (1976).

Cell-Free Protein Synthesis. Cell-free translation mixtures of 25 or 30 μL contained, besides mRNA, 60 vol % lysate, 120 μg/mL mouse liver tRNA, KCl or KOAc as shown, 4 mM creatine phosphate, 20 mM Hepes buffer, pH 7.5, and about 1 μCi of [^{35}S]methionine (200–700 Ci/mmol; Amersham).

† From the Laboratory for Molecular Virology, The Hebrew University-Hadassah Medical School, Jerusalem 91000, Israel. *Received December 29, 1978.* This work was supported by grants from the Chief Scientist's Office, Israel Ministry of Health, and the Israel Commission for Basic Research.

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Met-tRNA$_f$, methionyl-tRNA$_f^{Met}$; eIF-2, eucaryotic initiation factor 2; eIF-4B, eucaryotic initiation factor 4B; KOAc, potassium acetate; NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Mixtures were incubated for 45 min at 30 °C and frozen until analysis of the products. Hot trichloroacetic acid precipitable radioactivity was determined in aliquots spotted onto 1-in. square filters (Whatman No. 1 paper).

Cellulose Acetate Electrophoresis. Products of cell-free translation were analyzed by electrophoresis on cellulose acetate in buffer containing urea and 2-mercaptoethanol (Ueda & Schneider, 1969; Schneider, 1974; Soreq et al., 1974; Nudel et al., 1977). This is a simple and rapid technique that does not require prior purification of the globins by acid–acetone precipitation. Although several buffers can be used, we have found a discontinuous acidic buffer (Schneider, 1974) most suitable for rabbit globin. To prepare running buffer, 360 g of urea (Mallinckrodt) was dissolved at room temperature into 700 mL of solution A, containing, per liter, 10.2 g of Tris base (about 80 mM), 0.76 g of Na_2EDTA, and 3.2 g of boric acid, and the volume was adjusted to about 980 mL with additional solution. The pH was then adjusted to 6.0 with concentrated citric acid (300 g/L), and additional solution A was added to reach the final volume of 1 L. Just before placing running buffer into a Gelman Instrument Co. deluxe electrophoresis chamber, 5 mL of 14 M 2-mercaptoethanol per L was added. Diluted buffer was prepared by diluting solution A with 2 volumes of water before addition of urea and adjustment of the pH; just before use, 5 mL of 14 M 2-mercaptoethanol per L was added. Cellulose acetate membrane strips of 5 × 12 cm (Oxoid) were soaked for 15 min in the diluted buffer and then blotted lightly with filter paper before being placed in the electrophoresis chamber with each end on a wick of Whatman 3 MM filter paper that was soaked in the concentrated buffer. The membrane was tensioned with the aid of magnets. A volume of 1 μL of sample was then applied along a 6-mm track at the origin, marked with pencil dots at 3 cm from the anodal end of the membrane. To prepare a sample, 4 μL of total, incubated reaction mixture for protein synthesis, 4 μL of diluted buffer, and 2 μL of 14 M 2-mercaptoethanol were mixed and left for 30 min at room temperature. Electrophoresis was at 18 °C for 1–2 h at a constant current of 1–1.5 mA/strip (for four strips, with a current of 5.5 mA, the initial voltage was 255 V). The strips were stained for 10 min with 0.5% Ponceau S (Aldrich Chemical Co.) in 5% trichloroacetic acid, destained with 3% glacial acetic acid, and dried under an infrared lamp. Kodak RP/M X-Omat film was used for autoradiography. Films were scanned at 550 nm in a Gilford spectrophotometer. The amounts of α- and β-globin were determined from their respective peaks either by computing peak height × width at half-height or by measuring peak areas, not including the tailing beyond the β-globin peak. Using either method, we obtained very similar α/β globin synthetic ratios.

Preparation of [^{35}S]Met-tRNA$_f$ and Assay of Ternary Complex Formation. [^{35}S]Met-tRNA$_f$ was prepared, and ternary complex formation with eIF-2 and GTP was assayed, as described earlier (Kaempfer et al., 1978a).

Purification of eIF-2. All operations were carried out at 4 °C. Reticulocyte lysate was centrifuged for 3 h in a Spinco 50Ti rotor at an average of 150000*g*. The tubes contained a 0.1-mL cushion of 75% enzyme-grade sucrose in 10 mM Tris-HCl, pH 7.4, 2 mM $Mg(OAc)_2$, and 50 mM KCl. The ribosomal pellet was suspended with the aid of a glass rod into 30 volumes of 10 mM Tris-HCl, pH 7.4, 0.4 M KCl, and 10 mM 2-mercaptoethanol. The suspension was stirred for 45 min and centrifuged at 150000*g* for 3 h. The supernatant was precipitated with ammonium sulfate to 50% saturation. The pellet, collected by centrifugation for 20 min at 28000*g*, was dissolved into 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 5 mM 2-mercaptoethanol and dialyzed against 1000 volumes of the same buffer. The dialyzed preparation was centrifuged for 10 min at 2000*g* and stored in aliquots at −70 °C. This ribosomal wash fraction was applied to a 10-volume bed of DEAE-cellulose equilibrated with 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM KCl, 10 mM 2-mercaptoethanol, and 10% glycerol. The column was washed with the same buffer and developed by stepwise elution at 0.1 M and 0.22 M KCl. The peak of A_{280} material eluted at 0.22 M KCl was diluted to 0.1 M KCl with buffer A (50 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 1 mM dithioethreitol, and 10% glycerol) and applied to a phosphocellulose column about 40% the size of the DEAE-cellulose column. The column was washed with 0.2 M KCl in buffer A and stepwise eluted with 0.4 M KCl in buffer A. A linear gradient of 0.4–0.8 M KCl in buffer A was then applied, and eIF-2 was assayed as soon as the gradient was completed.

eIF-2 purified by this procedure is at least 98% pure as judged by $NaDodSO_4$—polyacrylamide gel electrophoresis and is free of detectable protein at 80000, the molecular weight assigned to factor eIF-4B; its mRNA-binding activity is completely sensitive to competitive inhibition by Met-tRNA$_f$ and GTP but not by uncharged tRNA, showing that the only mRNA-binding component in the preparation is eIF-2 itself (H. Rosen and R. Kaempfer, unpublished experiments).

Globin mRNA. Globin mRNA was prepared as described by Kaempfer et al. (1978a). α-Globin mRNA was purified by a modification of the procedure of Gianni et al. (1972). The upper two-thirds of the S-150 of a reticulocyte lysate was carefully removed to avoid contamination with ribosomes and adjusted to pH 5.1 with acetic acid. After stirring for 10 min at 0 °C and centrifuging for 20 min at 27000*g*, we dissolved the pellet into 20 mL of 0.14 M NaCl, 1.5 mM magnesium acetate, and 5 mM KCl. Buffer (10 mL) containing 0.1 M NaCl, 0.05 M sodium acetate, 0.001 M EDTA, and 1% $NaDodSO_4$ and adjusted to pH 5.1 with acetic acid was then added. The solution was twice extracted with chloroform–isoamyl alcohol (24:1), and the aqueous phase was precipitated with absolute ethanol at −20 °C. The precipitated RNA was dissolved in 0.5 M NaCl, 0.5% $NaDodSO_4$, and 10 mM Tris-HCl, pH 7.4, and subjected to oligo(dT)-cellulose chromatography.

Radioiodination of mRNA. The method is based on that of Commerford (1971), with some modifications. Two plastic 5-mL syringes containing a plug of glass wool and 2–3 mL of Sephadex G-25 (fine) are mounted into the caps of two sterile, conical plastic 50-mL centrifuge tubes (Falcon). One syringe is washed 3 times with H_2O, and the other is washed with 0.2 M KCl and 20 mM Tris-HCl, pH 7.5. Washing is accomplished by centrifugation to dryness of the mounted syringes for 5 min at 1000 rpm in a cooled swinging bucket rotor (International centrifuge). The washed syringes are stored at 4 °C until use. To about 2 μg of RNA in 25 μL of H_2O, placed in a 1.5-mL microcapped polyethylene centrifuge tube, is added 25 μL of freshly dissolved 10 mM $ThCl_3$ in H_2O, followed by addition of a mixture containing 25 μL of 1 M NH_4OAc, pH 5.0, 38 μL of 0.1 mM KI, and 0.25 mCi of Na^{125}I (10–20 mCi/μg I). The entire reaction mixture is heated at 60 °C for 20 min and chilled on ice for 5 min before the addition of 125 μL of 0.1 M Na_2SO_3 in 0.1 M Tris-HCl, pH 9.0, and of 25 μL of 2 M KCl. After heating for an additional 10 min at 60 °C and cooling on ice for 5 min, we dripped the mixture onto the dry bed of the buffer-washed Sephadex syringe and centrifuged the mixture under exactly

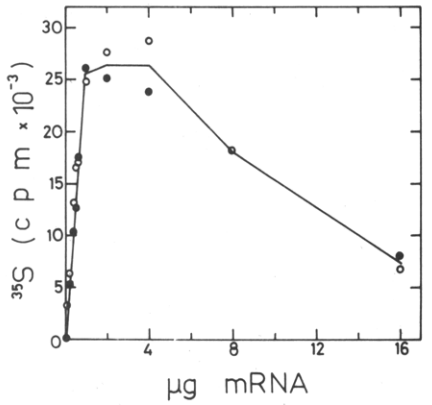

FIGURE 1: Translation of globin mRNA in the absence and presence of added eIF-2. Reaction mixtures for protein synthesis of 30 μL, containing the indicated amounts of globin mRNA, were incubated at 82 mM added KCl either in the absence (○) or presence (●) of 2 μg of added eIF-2, taken from the peak of a phosphocellulose column as illustrated in Figure 7. Aliquots of 5 μL were assayed for hot trichloroacetic acid precipitable radioactivity.

the same conditions that were used for the wash steps. The liquid collected at the bottom of the centrifuge tube is placed in the H_2O-washed Sephadex syringe which is centrifuged likewise. After removal of samples for total and trichloroacetic acid precipitable radioactivity measurement, the labeled RNA is precipitated with ethanol. All transfers involving ^{125}I are performed on ice in a hood under negative pressure. The recovery of RNA is essentially quantitative, and at least 98% of the total radioactivity recovered is trichloroacetic acid precipitable. The ^{125}I-labeled mRNA obtained in the above procedure is labeled to a relatively low specific activity, up to 2×10^6 cpm/μg. This low specific activity is important in determining the properties of the labeled RNA. The RNA is fully intact, as judged by polyacrylamide gel electrophoresis and autoradiography, and binds to eIF-2 with an affinity equal to that of the unlabeled, native RNA species, as determined by self-competition (Kaempfer et al., 1979).

Binding of mRNA to eIF-2. Reaction mixtures of 50 μL, containing KCl or KOAc as shown, 20 mM Tris-HCl, pH 7.8, 2 mM $Mg(OAc)_2$, 6 mM 2-mercaptoethanol, eIF-2, and RNA were incubated for 10 min at 25 °C and then cooled for 10 min at 0 °C before the addition of 1 mL of ice-cold buffer B (20 mM Tris-HCl, pH 7.8, 6 mM 2-mercaptoethanol, 50 mM KCl, and 2 mM $Mg(OAc)_2$). The samples were passed through 25-mm nitrocellulose filters (0.45-μm pore diameter) at a flow rate of 1 mL/min and washed 3 times with 1 mL of buffer B. Dried filters were counted by γ radiation spectrometry. For a more detailed description, see Kaempfer (1979).

Results

Figure 1 depicts the total amount of [^{35}S]methionine incorporated into protein in a micrococcal nuclease treated rabbit reticulocyte lysate programmed with increasing amounts of globin mRNA. Protein synthesis increased progressively, reaching a plateau level that is maintained up to 4 μg of mRNA per 30 μL of reaction mixture, and decreased with higher levels of mRNA. Similar results were observed by Pelham & Jackson (1976) in the same system. To analyze the products of translation, we subjected samples directly to cellulose acetate electrophoresis in the presence of urea and 2-mercaptoethanol. The autoradiogram of such an analysis is shown in Figure 2. It is seen that α-globin and β-globin are the major products of translation and are well resolved from each other. The amounts of α-globin and β-globin

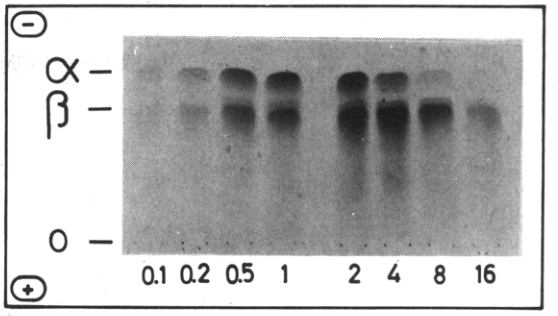

FIGURE 2: Cellulose acetate electrophoresis analysis of products synthesized in the presence of increasing amounts of globin mRNA. The autoradiogram of the electropherogram of samples from the experiment of Figure 1, incubated without added eIF-2, is shown. Numbers indicate the amounts in micrograms of mRNA present during incubation. The positions of the origin and of α- and β-globin are indicated. The minor band migrating just ahead of β-globin has not been identified. The film was exposed for 7 days.

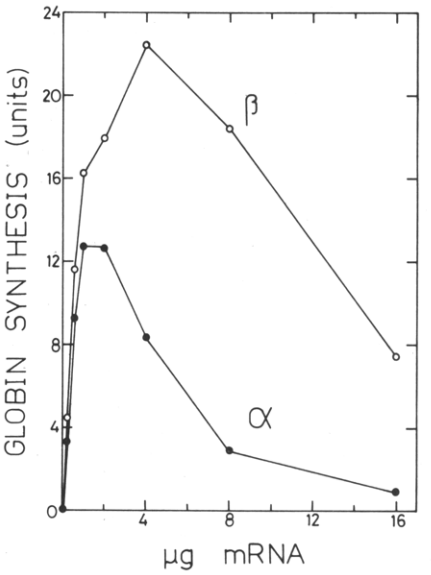

FIGURE 3: Synthesis of α- and β-globin as a function of globin mRNA concentration. The amounts of α- and β-globin synthesized in the experiment of Figure 1 were determined by densitometry of the autoradiogram of Figure 2 and quantitation of the areas under the two globin peaks and are plotted in arbitrary units. Because in the electropherogram of Figure 2 the aliquot of the sample containing 2 μg of mRNA moved as a narrower lane, the absolute amounts of α- and β-globin in this sample were normalized to the data of Figure 1. (●) α-Globin; (○) β-globin.

synthesized as a function of increasing mRNA concentration were quantitated by densitometry of the autoradiogram and are plotted in Figure 3. The amount of α chain reaches a maximum and then declines over a range of mRNA concentrations that support essentially constant synthesis of β chains. At 8 μg of mRNA per reaction mixture, synthesis of α chains has fallen to about 20%, while synthesis of β chains is near maximal. At higher levels of mRNA, β-chain synthesis also decreases, in keeping with the results of Figure 1. The ratio of α- to β-globin synthesis obtained from these data is shown in Figure 4A (lower curve). The α/β synthetic ratio is essentially constant, as long as the amount of mRNA does not exceed the saturation level of 1 μg (see Figure 1), but decreases at higher levels of mRNA. These results suggest that α-mRNA and β-mRNA compete in translation and that the competition favors translation of β-mRNA. This conclusion was reached in a number of studies employing different cell-free systems [e.g., Lodish (1971), McKeehan (1974), Temple & Lodish (1975), and Kabat & Chappell (1977)].

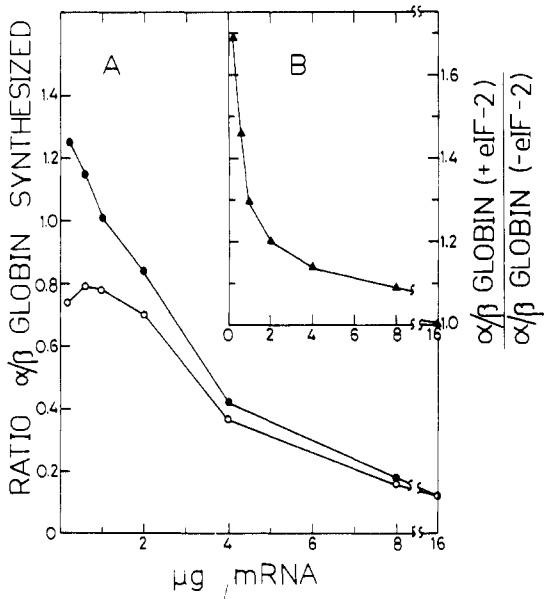

FIGURE 4: Effect of eIF-2 on the α/β synthetic ratio. (A) The α/β ratio, computed from the results of Figures 2 and 3 for the samples incubated without added eIF-2 in the experiment of Figure 1, is plotted as a function of the amount of mRNA present during translation (○). The ratio for the samples that received eIF-2 was determined likewise (●). (B) The effect of eIF-2 on the α/β ratio was determined from the data of Figure 4A, by dividing the ratio observed in the presence of added eIF-2 by that observed in its absence, and is plotted as a function of the amount of mRNA present.

In the present experiments, the effect of added initiation factor eIF-2 on the translational competition between α-mRNA and β-mRNA was studied. As seen in Figure 4A, the addition of highly purified eIF-2 to translation mixtures containing increasing amounts of globin mRNA causes a rise in the α/β synthetic ratio that is more pronounced the lower the mRNA concentration (Figure 4B). At low levels of mRNA, eIF-2 acts to raise the α/β synthetic ratio toward the ratio of about 1.5 expected from the relative content of α- and β-mRNA (Lodish, 1971; Lodish & Jacobsen, 1972). At very high levels of mRNA, the effect of eIF-2 is no longer observed.

Even though addition of eIF-2 raises the α/β synthetic ratio as much as 1.7-fold (Figure 4B), it does not stimulate total protein synthesis at any mRNA concentration (see Figure 1). Hence, eIF-2 does not limit overall protein synthesis in this system. This result must mean that in increasing the α/β synthetic ratio, eIF-2 does not act by stimulating only the synthesis of α chains. Instead, the eIF-2-dependent increase in α-chain synthesis is coupled with a concomitant decrease in β-chain synthesis. Thus, eIF-2 acts to relieve mRNA competition.

In the preceding experiments, synthesis of α chains was inhibited preferentially by increasing amounts of globin mRNA. Another variable known to influence α- and β-globin synthesis differentially is the salt concentration (Hall & Arnstein, 1973; McKeehan, 1974). We have examined the effect of salt on α- and β-chain synthesis, using both KCl and KOAc. Weber et al. (1977) and Kemper & Stolarsky (1977) have reported that KOAc supports protein synthesis at higher concentrations than KCl, apparently because of an inhibitory effect of the Cl^- ion. This finding is confirmed in Figure 5A, which depicts total protein synthesis in a series or lysates containing increasing concentrations of added KCl or KOAc. The amounts of α- and β-globin synthesized in these samples, quantitated by cellulose acetate electrophoresis analysis, are illustrated in Figure 5C. It is seen that optimal translation of both α-mRNA and β-mRNA occurs at the same salt

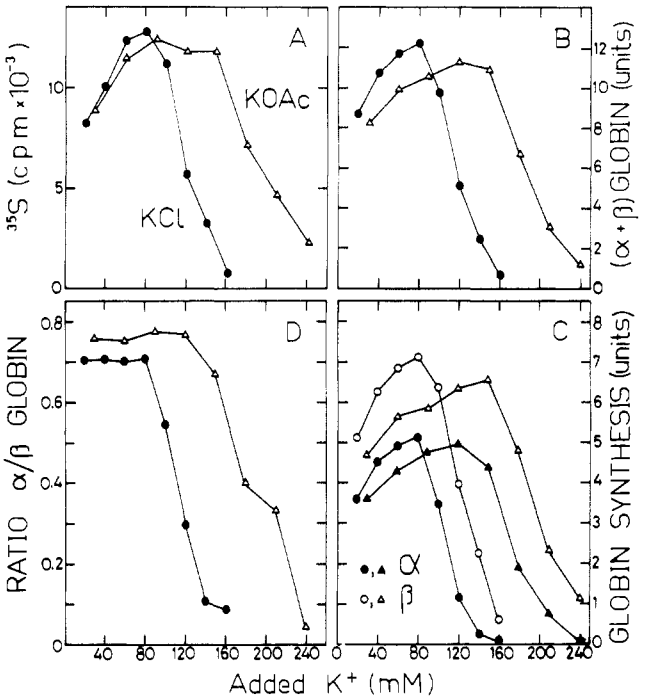

FIGURE 5: Effect of KCl and KOAc on the translation of α- and β-globin mRNA. Reaction mixtures for protein synthesis of 25 μL, containing 1.5 μg of globin mRNA, were incubated at the indicated concentrations of added KCl or KOAc. (A) Incorporation of [^{35}S]methionine into protein in 5-μL aliquots [(●) KCl; (△) KOAc]. (B) The sum of α- and β-globin synthesized [see (C)][(●) KCl; (△) KOAc]. (C) Synthesis of α- and β-globin was determined in the samples of (A), by the type of analysis as in Figures 2 and 3, and are plotted in arbitrary units [α-globin, (●) KCl and (▲) KOAc; β-globin, (○) KCl and (△) KOAc]. (D) α/β globin synthetic ratios, computed from the results of (C) [(●) KCl; (△) KOAc].

concentration, but the optimum is higher when KOAc is added. The sum of α- and β-globin synthesized (Figure 5B) matches closely in its salt dependence with overall protein synthesis (Figure 5A), showing that the quantitation of α and β chains by electrophoresis is accurate.

In spite of the fact that α- and β-globin mRNA possess the same salt optimum for translation, they differ in their sensitivity to greater than optimal concentrations of salt: synthesis of α chains decreases more drastically than that of β chains (cf. in Figure 5C the amounts of α- and β-globin synthesized at 120 mM added KCl or 180 mM added KOAc). Indeed, the α/β synthetic ratio is constant at salt concentrations up to the optimum but then decreases steeply (Figure 5D). It may be noted that for either KCl or KOAc, the salt concentration giving half-maximal inhibition of overall protein synthesis (Figure 5A), of the sum of α- and β-globin synthesis (Figure 5B), and of the ratio of α- to β-globin synthesis (Figure 5D) is identical, as is the displacement between the curves for KCl and KOAc in Figure 5A,B,C,D.

From a comparison of the behavior of globin synthesis in the presence of KCl and KOAc, it is clear that the decrease in the α/β synthetic ratio between 80 and 140 mM of added KCl is caused by the Cl^- ion and not by the K^+ ion, for no such decrease is observed when KOAc is used in the same range (Figure 5C). Weber et al. (1977) have shown that the Cl^- ion inhibits a step in initiation necessary for the binding of mRNA to ribosomes but does not significantly affect the binding of Met-tRNA$_f$ to 40S ribosomal subunits. Since, as reviewed in the introduction, eIF-2 not only binds to Met-tRNA$_f$ but can also bind to mRNA, we have asked, in the following experiments, if eIF-2 is able to overcome the inhibitory effect of Cl^- ions on translation. Figure 6 shows that

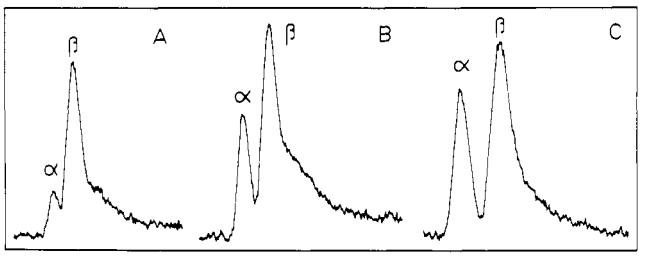

FIGURE 6: Effect of eIF-2 on the synthesis of α- and β-globin at high KCl concentration. Densitometer scans of cellulose acetate electropherograms are depicted. Reaction mixtures for protein synthesis of 26 μL contained 1.5 μg of globin mRNA, KCl to an added concentration of 130 mM, and the following amounts of added eIF-2: none (A), 1 μg (B), 1.5 μg (C). Incorporation of [^{35}S]methionine into protein was, per 3-μL aliquot, 1790 cpm in (A), 3800 cpm in (B), and 4130 cpm in (C). The α/β synthetic ratio is 0.19 in (A), 0.42 in (B), and 0.57 in (C).

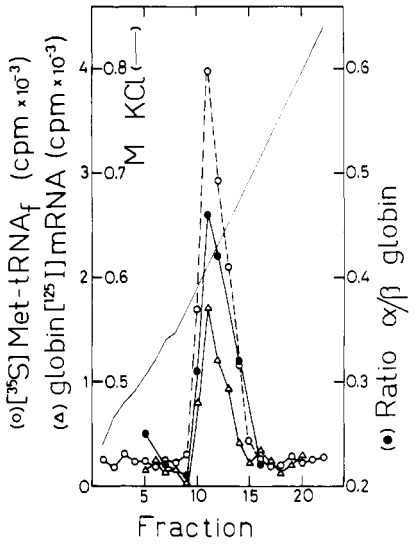

FIGURE 7: Copurification of the activity that relieves translational competition with eIF-2. Purification of eIF-2 was as described under Materials and Methods. The gradient portion of the phosphocellulose column is shown. Aliquots of 5 μL of each fraction were used to assay GTP-dependent binding of ^{35}S-labeled Met-tRNA$_f$ (input, 5500 cpm) (O). Aliquots of 0.5 μL were used to assay binding of ^{125}I-labeled globin mRNA (8×10^5 cpm/μg; input, 2600 cpm) (Δ). No background was subtracted in either assay. Aliquots of 3 μL of the fractions were added to reaction mixtures for protein synthesis of 26 μL, containing 1.5 μg of globin mRNA, that were adjusted to 130 mM of added KCl. The α/β globin synthetic ratio of the translation products was determined (●).

this is indeed the case. It depicts densitometer scans of the products of globin mRNA translation obtained in lysates containing an added concentration of 130 mM KCl. In these conditions there is little synthesis of α chains (Figure 6A). However, addition of increasing amounts of highly purified eIF-2 results in a strong stimulation of α-chain synthesis (Figure 6B and 6C). Synthesis of β chains also is stimulated, but to a lesser extent: the α/β synthetic ratio increases threefold between Figure 6A and 6C. It is clear from these results that eIF-2 is capable of relieving the translational competition between α-mRNA and β-mRNA observed at high KCl concentrations. The data of Figure 6 show, in addition, that eIF-2 is able to relieve the inhibition of overall protein synthesis at high KCl concentrations, in spite of the fact that at optimal KCl concentration eIF-2 does not stimulate overall synthesis (Figure 1). This strongly suggests that high salt concentrations inhibit the function of eIF-2 in protein synthesis.

Figure 7 shows that the activity that relieves competition between α-mRNA and β-mRNA at high KCl concentrations

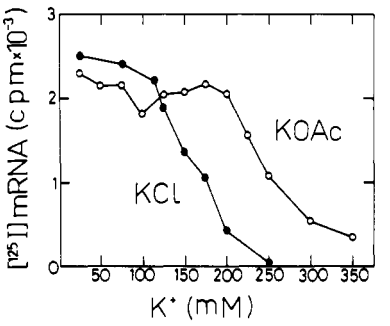

FIGURE 8: Effect of KCl and KOAc on complex formation between globin mRNA and eIF-2. mRNA-binding assay mixtures contained ^{125}I-labeled globin mRNA (1.5×10^6 cpm/μg; input, 4270 cpm), a limiting amount of eIF-2, and the indicated concentrations of KCl (●) or KOAc (O). In the presence of saturating amounts of eIF-2, 3480 cpm of mRNA were bound in the presence of 50 mM KCl. Background binding (without eIF-2) was subtracted. Background values were 156 cpm at 25 mM KCl, 102 cpm at 250 mM KCl, 133 cpm at 25 mM KOAc, and 117 cpm at 350 mM KOAc.

copurifies with eIF-2. The experiment illustrates the last step in the purification procedure described under Materials and Methods, which involves elution of eIF-2 from a phosphocellulose column by a linear gradient from 0.4 to 0.8 M KCl. Two characteristic activities of eIF-2—its ability to form a ternary complex with Met-tRNA$_f$ and GTP and its ability to bind globin mRNA, assayed by the retention of ^{125}I-labeled globin mRNA on nitrocellulose filters—are seen to coelute in a narrow peak near 0.6 M KCl. Fractions of this gradient were tested for their ability to raise the α/β globin synthetic ratio in reaction mixtures incubated at 130 mM KCl that were analyzed as in Figure 6. Whereas fractions outside the peak of eIF-2 activity supported globin synthesis to an α/β synthetic ratio of about 0.2, this ratio was increased considerably by an activity that elutes precisely in the position of eIF-2.

It was seen above that the inhibition of globin mRNA translation by high KCl concentrations, illustrated in Figure 5, can be relieved by eIF-2 (Figures 6 and 7), suggesting that the function of eIF-2 is inhibited. We have shown previously that ternary complex formation between eIF-2, Met-tRNA$_f$, and GTP is much less sensitive to increasing KCl concentrations than is complex formation between eIF-2 and mRNA (Kaempfer et al., 1978b). These findings, together with the observation of Weber et al. (1977) that high KCl concentrations block the binding of mRNA rather than Met-tRNA$_f$ to 40S ribosomal subunits, suggest that KCl may inhibit the interaction of mRNA with eIF-2 during translation. This concept is supported by the following experiment.

In Figure 8, the extent of complex formation between a limiting amount of eIF-2 and labeled globin mRNA is plotted as a function of increasing concentrations of KCl or KOAc. Binding of eIF-2 to globin mRNA decreases sharply above 115 mM KCl and reaches 50% at 160 mM. By contrast, in the presence of KOAc, binding of mRNA does not decrease perceptibly up to 200 mM and reaches 50% at about 240 mM. It is seen that, in two respects, the salt sensitivity of the interaction between mRNA and eIF-2 displays a remarkable similarity to the salt sensitivity of globin mRNA translation. First, the KOAc curve in Figure 8 is displaced to higher concentrations with respect to the KCl curve in the same way as the curves in Figure 5, the difference in concentration at 50% inhibition being about 75 mM in each case. Second, 50% inhibition of translation is observed at 115 mM of added KCl in Figure 5A. From a comparison of the KCl optimum for translation in the reticulocyte lysate, as used here, before and after gel filtration (Kaempfer et al., 1978b), we estimate the

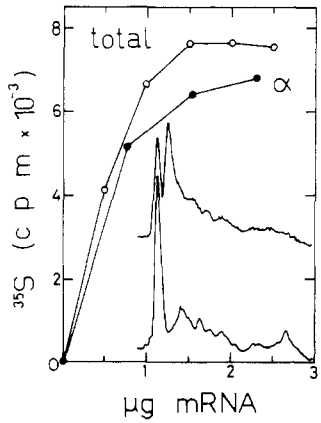

FIGURE 9: Translation of purified α-globin and total globin mRNA. Reaction mixtures for protein synthesis of 25 μL were incubated at 84 mM of added KCl with the indicated amounts of purified α-globin mRNA (●) or total globin mRNA (○), and [^{35}S]methionine incorporation into protein was determined in 5-μL aliquots. The samples containing 1.5 μg of mRNA were subjected to cellulose acetate electrophoresis, and the scans of the autoradiograms obtained are shown in the inset (top, total globin mRNA; bottom, α-globin mRNA).

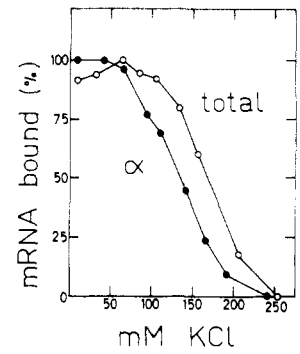

FIGURE 10: Effect of KCl on complex formation between eIF-2 and α-globin mRNA or total globin mRNA. mRNA-binding assay mixtures contained ^{125}I-labeled purified α-globin mRNA (1.5×10^5 cpm/μg; input, 1920 cpm) (●) or ^{125}I-labeled total globin mRNA (7.6×10^5 cpm/μg; input, 3850 cpm) (○), a limiting amount of eIF-2, and the indicated concentrations of KCl. In the presence of saturating amounts of eIF-2, 1380 and 2600 cpm, respectively, were bound at 50 mM KCl. Binding of 100% is 1040 cpm for α-globin mRNA and 1780 cpm for total globin mRNA. Background values were subtracted.

endogenous salt concentration in our lysate to be equivalent to about 45 mM KCl. Thus, half-maximal translation is observed at a final concentration of 160 mM KCl, precisely the concentration that gives half-maximal binding of mRNA to eIF-2. Similarly, 50% inhibition of translation is seen at 190 mM added KOAc in Figure 5, which is about 50 mM lower than the concentration of KOAc that inhibits binding of mRNA by 50% in Figure 8. Thus, there is a tight correlation between the effects of KCl and KOAc on translation of globin mRNA on one hand and on the binding of globin mRNA to eIF-2 on the other.

From the results presented so far, it is clear that α-globin mRNA competes more weakly than β-globin mRNA in translation. In particular, the differential inhibition of the translation of α- and β-globin mRNA by elevated salt concentrations and relief of this inhibition by eIF-2 suggest that the binding of α-mRNA to eIF-2 is more sensitive to salt than the binding of β-mRNA. To examine this point, α-mRNA was isolated from reticulocyte S-150 supernatant. As seen in Figure 9, on a weight basis this mRNA directed incorporation of [^{35}S]methionine into protein to a very similar extent as did total globin mRNA. The α-mRNA directed extensive synthesis of α chains but few, if any, β chains, when compared to total globin mRNA (Figure 9, inset), attesting to the purity of the α-mRNA preparation. When these preparations of mRNA were labeled with ^{125}I and their binding to a limiting amount of eIF-2 was studied as a function of increasing KCl concentration (Figure 10), a small but significant difference was observed: the binding of α-mRNA to eIF-2 is more sensitive to salt than the binding of total globin mRNA. This is precisely what would be expected if α-globin mRNA were to interact more weakly with eIF-2 than β-globin mRNA.

Table I summarizes results of an experiment in which the effect of eIF-2 on globin synthesis was studied at the same time at optimal (84 mM) and high (135 mM) concentrations of added KCl. At the KCl optimum for translation, eIF-2 did not stimulate overall protein synthesis but raised the α/β synthetic ratio 1.3-fold. At high KCl concentration, there was a fivefold increase in the α/β ratio in response to eIF-2, while overall protein synthesis increased less than twofold. Here, about 85% of the increase in total protein synthesis was due to increased α-chain formation. Clearly, the stimulatory effect of eIF-2 on the ratio of α- to β-globin synthesized is much more pronounced at high KCl concentration.

Table I: Effect of eIF-2 on Globin Synthesis at Optimal and High KCl Concentrations[a]

KCl added (mM)	eIF-2 added (μg)	total cpm	α/β	ratio (+/− eIF-2) total cpm	ratio (+/− eIF-2) α/β
84	0	26 994	1.07	0.97	1.3
84	1.5	26 106	1.40		
135	0	12 470	0.16	1.78	5.1
135	2.5	22 200	0.81		

[a] Translation mixtures of 25 μL, containing 1.5 μg of globin mRNA, were incubated in the presence of the indicated amounts of KCl and eIF-2 and analyzed by cellulose acetate electrophoresis.

Discussion

The principal new finding emerging from this study is that addition of excess initiation factor eIF-2 leads to relief of competition between α- and β-globin mRNA during translation. Such relief is observed both when competition is caused by a high mRNA concentration and when it is sharpened by a high concentration of KCl. Apparently, the function of eIF-2 in translation is inhibited by high salt concentrations because they affect the interaction of this initiation factor with mRNA. The results strongly suggest that β-globin mRNA binds more tightly to eIF-2 than does α-globin mRNA.

Translational competition between α- and β-globin mRNA was studied in the mRNA-dependent micrococcal nuclease treated reticulocyte lysate. This system gave results similar to those observed in other cell-free systems, including the regular reticulocyte lysate [e.g., Temple & Lodish (1975) and Kabat & Chappell (1977)]. In particular, extensive synthesis of β as well as α chains was observed, with an α/β synthetic ratio of approximately 1 at low mRNA concentrations, with some variation occurring between individual lysates (Figure 4 and Table I). Under standard conditions, we have never observed the deficiency in α-chain synthesis reported by Stewart et al. (1977).

The ability of eIF-2 to relieve translational competition between α- and β-globin mRNA in principle can be accounted for by two types of explanations. One is that α- and β-globin mRNA possess different affinities for eIF-2 and compete directly for this factor during initiation of protein synthesis. The other is that eIF-2 acts to enlarge the pool of 40S–Met-tRNA$_f$ complexes; as pointed out by Lodish (1974, 1976),

any increase in the rate of reactions at or before the mRNA binding step will result in a nonspecific increase in the rate of protein synthesis that leads to preferential stimulation of translation of the weakly competing mRNA species, in this case α-globin mRNA. Even though eIF-2 is responsible for the formation of 40S–Met-tRNA$_f$ complexes, several lines of evidence presented here suggest that direct mRNA competition for eIF-2 is actually involved in the regulation of α- and β-globin synthesis. First, addition of eIF-2 relieves mRNA competition without stimulating total protein synthesis at optimal salt concentration (Figure 1 and Table I). This would not be expected if eIF-2 were to influence the rate-limiting step in protein synthesis nonspecifically. This finding is consistent with direct competition of mRNA for the relieving component (here, eIF-2) but does not prove it, as has been assumed in another study (Kabat & Chappell, 1977), for the component conceivably could affect a reaction preceding the binding of mRNA, while another step at or beyond the binding of mRNA is rate-limiting in protein synthesis. Second, the behavior of globin mRNA translation as a function of increasing concentrations of KCl or KOAc (Figure 5) matches exactly with that observed for the direct binding of globin mRNA to eIF-2 (Figure 8), with respect to both the final salt concentration giving 50% inhibition and the displacement between the response to KCl and to KOAc. This striking correlation between translation data and direct mRNA-binding experiments strongly suggests that the interaction of mRNA with eIF-2 is important in determining the overall rate of initiation of translation, as well as the relative rates of initiation on α- and β-globin mRNA. This is borne out by a third line of evidence demonstrating that the binding of α-globin mRNA to eIF-2 exhibits greater salt sensitivity than the binding of unfractionated globin mRNA (Figure 10). Thus, α-globin mRNA interacts more weakly with eIF-2 than a mixture of α- and β-globin mRNA.

Fourth, eIF-2 relieves the inhibition of total protein synthesis and the sharpened competition between α- and β-globin mRNA, occurring at elevated KCl concentration (Figures 6 and 7 and Table I). Since high concentrations of Cl^- ion inhibit initiation of translation by preventing primarily the binding of mRNA to 40S initiation complexes, while binding of Met-tRNA$_f$ to 40S subunits is only slightly inhibited (Weber et al., 1977), it seems likely that the ability of eIF-2 to relieve the inhibitory effect of KCl is based on its action at the mRNA-binding step rather than at the step involving 40S–Met-tRNA$_f$ complex formation. Indeed, KCl directly inhibits the binding of mRNA to eIF-2 at concentrations that hardly affect the formation of ternary complexes between eIF-2, Met-tRNA$_f$, and GTP [Figure 8 and Kaempfer et al. (1978b)].

The simplest interpretation of the findings reported here is that eIF-2 relieves translational competition by interacting directly with α- and β-globin mRNA rather than by affecting the binding of Met-tRNA$_f$. While α- and β-globin mRNA could compete for free eIF-2 molecules, it is more likely that they compete for eIF-2 molecules located in 40S–Met-tRNA$_f$ complexes. In that case, any increase in the formation of 40S–Met-tRNA$_f$ complexes would lead to relief of competition between α- and β-globin mRNA, because it would increase the number of eIF-2 molecules available for mRNA competition.

When mRNA is not in excess, the stimulatory effect of eIF-2 on the ratio of α/β globin synthesized is much more pronounced at high KCl concentration. This is to be expected, for at optimal concentrations of KCl the ratio is close to 1 to begin with and hence can increase only to the value of about 1.5 observed when the rate of initiation no longer limits translation (Lodish, 1971). At high concentrations of KCl, the initial α/β synthetic ratio is much less than 1 and thus can be stimulated over a greater range. On the other hand, it is clear that eIF-2 is far more effective in stimulating the α/β synthetic ratio when this ratio is depressed by a high KCl concentration than when it is depressed by a high concentration of mRNA (Figures 4 and 6 and Table I). This indicates that KCl and mRNA influence the function of eIF-2 in a different manner. KCl affects the quality of the interaction between eIF-2 and mRNA (see Figures 8 and 10), presumably by affecting the charge and/or the conformation of one or both of these components. By contrast, a high concentration of mRNA is not expected to affect the mRNA–eIF-2 interaction per se but is expected to change the relative binding of individual mRNA molecules in a competitive way.

Additional support for the concept that different mRNA species compete directly for eIF-2 comes from a study of the translational competition between Mengo virus RNA and globin mRNA in the mRNA-dependent reticulocyte lysate (H. Rosen, G. Di Segni, and R. Kaempfer, unpublished experiments). The competitive inhibition of globin mRNA translation by Mengo virus RNA is relieved by addition of excess eIF-2. In direct RNA-binding competition experiments, it was found that Mengo virus RNA has a 30-fold higher affinity for eIF-2 than does globin mRNA. This is also reflected by the finding that binding of Mengo virus RNA to eIF-2 is much more resistant to increasing salt concentrations than binding of globin mRNA. That study and the present one, therefore, provide strong evidence that the binding of mRNA to eIF-2 has biological significance by establishing a direct correlation between the ability of an mRNA species to compete in translation and its affinity for eIF-2.

A correlation between the ability to relieve translational competition and to bind mRNA differentially was also reported for the initiation factor eIF-4B in studies of competition between encephalomyocarditis RNA and globin mRNA (Golini et al., 1976; Baglioni et al., 1978). Kabat & Chappell (1977) also reported that eIF-4B can relieve translational competition between α- and β-globin mRNA in a reconstituted cell-free system, but correlation of translation studies with direct mRNA-binding experiments, in this case, has not been shown.

The results reported in this paper cannot be explained by assuming a contamination with eIF-4B. This is shown, first, by the fact that the activity relieving translational competition elutes from phosphocellulose precisely in the position of eIF-2 (Figure 7); eIF-4B elutes at much lower salt concentration (Schreier et al., 1977). Second, as will be reported elsewhere, eIF-2 purified by our procedure is at least 98% pure as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis and is free of detectable protein at 80 000, the molecular weight assigned to eIF-4B; moreover, its mRNA-binding activity is completely sensitive to competitive inhibition by Met-tRNA$_f$, provided GTP is present, but not by uncharged tRNA, showing that the only mRNA-binding component in the preparation is eIF-2 itself (H. Rosen and R. Kaempfer, unpublished experiments).

It seems likely that both eIF-2 and eIF-4B, and possibly other initiation factors that recognize mRNA, can effect translational control by discriminating between different mRNA species according to their affinity. It is possible that the same features of mRNA are recognized by several initiation factors at individual stages of initiation and that each of these factors contributes to the process of mRNA selection.

In furnishing evidence that eIF-2 has mRNA-discriminating properties, the present study emphasizes the importance of eIF-2 in translational control.

During erythroid development, the synthesis of the constituents of hemoglobin (heme and different globin chains) is exactly coordinated. Initiation of protein synthesis in reticulocyte lysates is dependent upon the continued supply of heme (Waxman & Rabinovitz, 1966; Zucker & Schulman, 1968). In the absence of heme, the activity of eIF-2 is lost, and protein synthesis can be restored by the addition of an excess of this initiation factor (Kaempfer & Kaufman, 1972; Kaempfer, 1974; Raffel et al., 1974; Clemens et al., 1975). Thus, eIF-2, through its property of heme dependence, may allow the coordinate synthesis of heme and of globins during erythroid development. The results of the present study indicate that, in addition, eIF-2 may allow the coordinate synthesis of α- and β-globin by serving as a target for competition between α-globin and β-globin mRNA. Thus, eIF-2 may have a dual function in translational control of hemoglobin synthesis.

Acknowledgments

We thank Ruth Israeli and Sarah Knoller for excellent assistance.

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