Hemoglobin Messenger Ribonucleic Acid Translation in Cell-Free Systems from Rat and Mouse Liver and Landschutz Ascites Cells†

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ABSTRACT: Globin mRNA extracted from both mouse and rabbit reticulocytes can be faithfully translated by cell-free systems derived from rat and mouse liver and from mouse Landschutz ascites cells. This translation is not dependent upon the addition of reticulocyte-specific factors. Analysis of the product synthesized in the liver system in the presence of rabbit globin mRNA shows that both α and β chains of globin are synthesized and that there is an excess production of α chains. The 14S messenger ribonucleoprotein complex which is released from rabbit reticulocyte polysomes after EDTA treatment also stimulates protein synthesis in the liver system. Its activity is similar to that of the naked mRNA.

In bacteria, the translation of classes of mRNA (mRNA) or of the individual cistrons of bacteriophage RNA may be controlled by messenger-specific initiation factors (Dube and Rudland, 1970; Revel *et al.*, 1970; Berissi *et al.*, 1972). The importance of this regulatory mechanism in eucaryotes is not yet clear. On the one hand, several mRNAs can be translated in heterologous systems: myeloma and lens mRNA in the reticulocyte lysate (Stavnezer and Huang, 1971; Berns *et al.*, 1972); globin, lens, and myeloma mRNAs in a cell-free system from Krebs II ascites cells (Mathews *et al.*, 1971, 1972; Housman *et al.*, 1971; Brownlee *et al.*, 1972); and globin mRNA in intact *Xenopus* oocytes (Lane *et al.*, 1971). Additional factors, such as initiation factors, from the same source as the messenger, are not obligatory.

On the other hand, Heywood (1969, 1970), working with cell-free systems from the chick, has reported that translation of myosin mRNA by reticulocyte ribosomes is dependent on the addition of factors from muscle ribosomes. Factors from reticulocyte ribosomes were ineffective in promoting myosin mRNA translation but were much more active than the muscle factors in promoting globin mRNA translation. This implies the existence of messenger-specific initiation factors which are not universally present in different tissues. This idea has received further support from recent experiments which suggest that extracts of rabbit liver and Landschutz ascites cells may also lack factors essential for globin mRNA translation (Prichard *et al.*, 1971; Cohen, 1971).

We have reexamined this question using crude cell-free systems from rat and mouse liver and from mouse Landschutz ascites cells. In contrast to the previous reports, we show that rabbit and mouse globin mRNA can be faithfully translated without any requirement for additional reticulocyte components. The mRNP[1] particle, which is obtained from polysomes by dissociation with EDTA, is also translated in the mouse liver system. The proteins with which the mRNA is complexed do not significantly increase its template activity. The implications of this result are discussed.

Methods

Liver Extracts. Mouse and rat liver extracts were prepared by a modification of the method of Munro *et al.* (1964). The liver was rinsed, chopped, and homogenized in 2.5 volumes of a solution containing 0.25 M sucrose–25 mM Tris-HCl (pH 7.6)–75 mM KCl–5 mM magnesium acetate–6 mM 2-mercaptoethanol. After centrifugation at 30,000*g* for 10 min, the turbid region at the top of the supernatant was discarded. The upper two-thirds of the remainder constituted the liver S-30.[2]

Preincubation was carried out for 30 min at 37° in the presence of 1 mM neutralized ATP–0.1 mM GTP–10 mM creatine phosphate–0.5 mg/ml of creatine kinase–all 20 naturally occurring amino acids at 150 μM each. Addition of these substances diluted the S-30 by less than 5%. The preincubated S-30 was passed through a column of Sephadex G-25 (coarse) equilibrated with a solution containing 25 mM Tris-HCl (pH 7.6)–50 mM KCl–4 mM magnesium acetate–6 mM 2-mercaptoethanol. It was distributed in small volumes, rapidly frozen, and stored in liquid nitrogen.

Standard protein synthesis assays contained, in a volume of 25 μl, 12.5 μl of preincubated mouse liver S-30–25 mM Tris-HCl (pH 7.6)–75 mM KCl–4 mM magnesium acetate–6 mM 2-mercaptoethanol–1 mM ATP–0.1 mM GTP–10 mM creatine phosphate–0.5 mg/ml of creatine kinase–a mixture of nineteen amino acids lacking leucine (20 μM each)–5 μCi/ml of [^{14}C]-leucine. When applicable, mRNA was present at 5 μg/ml. Samples were incubated at 37° for 45 min. For estimation of amino acid incorporation, 0.5 ml of 1 M NaOH (containing 1 mg/ml of unlabeled leucine) was added and incubation continued at 37° for 15 min. Protein was precipitated for at least 1 hr at 0° with 2 ml of 10% trichloroacetic acid, filtered onto glass fiber disks, and subjected to liquid scintillation counting. For product analysis the reaction was terminated by addition of EDTA and pancreatic ribonuclease to 10 mM and 50 μg/ml, respectively, followed by incubation at 37° for 15 min.

Landschutz Ascites Cell Extracts. Cells were obtained from Dr. B. Hogan (University of Sussex). The S-30 was prepared, preincubated, and used in exactly the same way as for Krebs II ascites cells (Mathews and Korner, 1970; Mathews, 1972).

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[1] Abbreviations used are: mRNP, messenger ribonucleoprotein; S-30, 30,000*g* supernatant; RSB, reticulocyte standard buffer.

Globin mRNA and mRNP. Mouse globin mRNA, prepared as published (Williamson *et al.*, 1971) and deproteinized with phenol, was a generous gift from Dr. A. R. Williamson (Beatson Hospital, Glasgow). Rabbit globin mRNA was prepared by the procedure of Evans and Lingrel (1969) with the following modifications. (1) Anemia was induced by four daily injections of phenylhydrazine. (2) Polysomes were obtained from reticulocyte lysate by centrifugation for 2.5 hr at 60,000 rpm in the Spinco 60 Ti rotor through 8 ml of 1 M sucrose in RSB (10 mM Tris-HCl (pH 7.6)–30 mM KCl–2 mM magnesium acetate) layered over 8 ml of 2 M sucrose in RSB. (3) The 9S mRNA was isolated by centrifugation of sodium dodecyl sulfate dissociated polysomes through a linear sucrose gradient (7.5–25% in RSB), and was further purified by a second treatment with sodium dodecyl sulfate followed by a second sucrose gradient. The 14S mRNA complex was obtained by dissociation of polysomes with EDTA (Huez *et al.*, 1967) and purified on two sucrose gradients.

Electrophoresis of Tryptic Peptides. The products of 50-μl reactions containing [^{35}S]methionine (10 μM, supplemented with the other 19 amino acids at 20 μM each) were precipitated and washed with trichloroacetic acid, then washed with ethanol, ethanol–ether (1 : 1), and ether. The protein was oxidized with performic acid and digested with trypsin (1 :50, w/w) in 1% ammonium bicarbonate for 4 hr at 37°. After freeze-drying, the samples were applied to Whatman No. 3MM paper and subjected to electrophoresis at pH 6.5 for 1 hr. The dried paper was autoradiographed.

Chromatography of Globin Chains. The products of 100-μl reactions containing a [^{14}C]amino acid mixture (5 μCi/ml, supplemented with the six missing amino acids at 20 μM each) were mixed with 90 mg of unlabeled rabbit globin and added dropwise to 20 volumes of acetone–HCl (100 : 1, v/v) at −20°. The precipitate was washed in acetone, dissolved in 1 ml of starting buffer (0.02 M pyridine–0.2 M formic acid), and applied to a 22 × 1.1 cm column of carboxymethylcellulose (Whatman CM52) equilibrated in the same buffer. The column was washed with two column volumes of starting buffer and developed with a linear gradient of 400 ml of starting buffer and 400 ml of a buffer containing 0.2 M pyridine and 2 M formic acid (Dintzis, 1961). Fractions (5 ml) were analyzed for absorbancy at 280 nm and for trichloroacetic acid precipitable radioactivity (after addition of bovine serum albumin carrier). Globin labeled by incubation of intact rabbit reticulocytes with DL-[^{14}C]valine (Lingrel and Borsook, 1963) was chromatographed in the same way.

Electrophoresis on Sodium Dodecyl Sulfate–Polyacrylamide Gels. Samples from protein synthesis reactions were diluted with an equal volume of 0.01 M sodium phosphate (pH 7.0), made 1% in sodium dodecyl sulfate and 5% in 2-mercaptoethanol, and heated to 100° for 3 min. Sodium dodecyl sulfate gels (12 cm in length) containing 12.5% acrylamide and 0.34% methylenebis(acrylamide) were prepared and run as described previously (Weber and Osborn, 1969) except that siliconized tubes were used. Gels were stained with Coomassie Brilliant Blue for 2 hr and destained by diffusion (Weber *et al.*, 1972). Longitudinal slices were dried and autoradiographed.

Materials

[^{14}C]Leucine (331 Ci/mole), [^{14}C]valine (33.9 Ci/mole), [^{14}C]amino acid mixture (54 mCi/mg-atom), and [^{35}S]methionine (10–20 Ci/mmole at time of use) were purchased from the Radiochemical Centre, Amersham, Bucks. Other substances were obtained as previously specified (Mathews and Korner, 1970; Mathews, 1972).

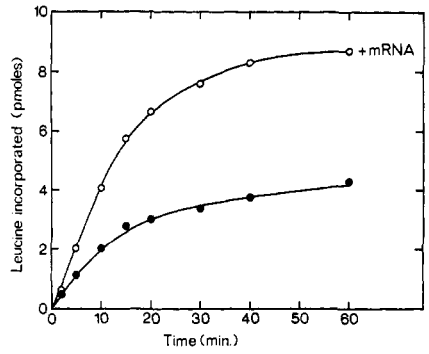

FIGURE 1: Kinetics of protein synthesis in the mouse liver system. Samples (25 μl) were removed at intervals from bulk incubations (0.25 ml) containing [^{14}C]leucine, mouse liver S-30, and (where indicated) rabbit globin mRNA at 5 μg/ml.

Results

Liver Systems. RESPONSE TO ADDED mRNA. When rabbit globin mRNA was added to a nonpreincubated mouse liver S-30, no increase in amino acid incorporation was observed, presumably because it was obscured by the high level of endogenous protein synthesis. Preincubation of the S-30 under conditions of protein synthesis (see Methods) reduced this by 90–95% and subsequent addition of mRNA elicited a stimulation with the kinetics shown in Figure 1. The maximal stimulation was threefold, achieved at a concentration of about 8 μg/ml (Figure 2). Higher levels of mRNA gave a gradual reduction in the stimulation. Essentially identical results were obtained with mouse globin mRNA in the mouse liver S-30 and with the rat liver system.

The liver systems differ from the Krebs cell system in saturating at lower concentrations of mRNA and in being inhibited by supraoptimal concentrations. The optimal efficiency of translation is roughly the same for all the systems and messengers tested: in all cases it is approximately one round per messenger added. However, it has not proved possible to reduce the endogenous incorporation of the liver systems to the low levels obtained with ascites extracts, so the relative stimulations are smaller and the background radioactivity in the product analysis is greater than previously.

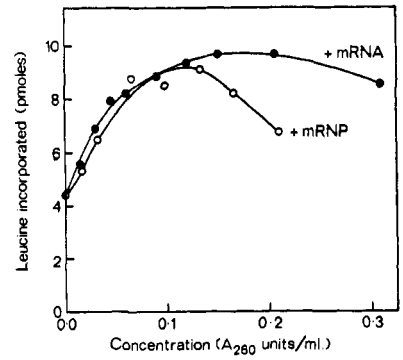

FIGURE 2: Stimulation of protein synthesis by mRNA and mRNP. Reactions (25 μl) contained [^{14}C]leucine, mouse liver S-30, and rabbit globin mRNA or mRNP as indicated. Incubations were terminated after 45 min.

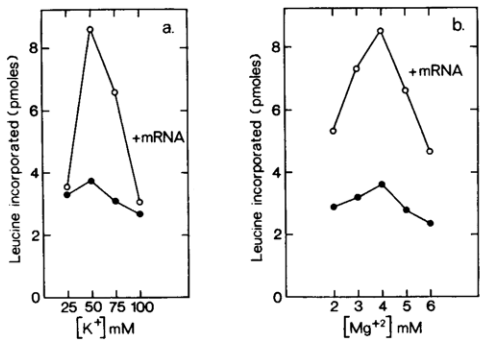

FIGURE 3: Dependence of amino acid incorporation on salt conditions. Reactions (25 ml) containing [^{14}C]leucine, mouse liver S-30, and (where indicated) rabbit globin mRNA at 5 μg/ml were incubated for 45 min. (a) Variation of [K^+] at 4 mM Mg^{2+}. (b) Variation of [Mg^{2+}] at 50 mM KCl.

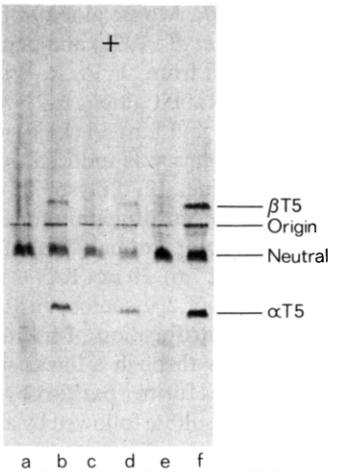

FIGURE 4: Electrophoresis of [^{35}S]methionine-labeled tryptic peptides. Incubations contained the following: (a) rat liver S-30, (b) rat liver S-30 + mouse globin mRNA (2.5 μg/ml), (c) mouse liver S-30, (d) mouse liver S-30 + mouse globin mRNA (2.5 μg/ml), (e) Landschutz ascites S-30, (f) Landschutz ascites S-30 + mouse globin mRNA (10 μg/ml). The protein was digested with trypsin and subjected to electrophoresis at pH 6.5 followed by autoradiography. The positions of the marker methionine-containing mouse globin peptides, αT5 and βT5, are indicated.

RESPONSE TO ADDED mRNP. When released from polysomes by EDTA, the globin messenger is isolated in a 14S particle complexed with two basic proteins (Huez *et al.*, 1967). It has been suggested that these proteins may play an important role in the translation or transport of mRNA (Lebleu *et al.*, 1971; Spirin, 1970). Figure 2 shows that the mRNP stimulates protein synthesis in the mouse liver system. However, the maximal effect was the same as with naked mRNA, and the mRNA was slightly more efficient in terms of absorbance units. Preliminary work (Sampson, unpublished data) indicates that 9S RNA accounts for about two-thirds of the absorbance of the mRNP preparation. When adjustment is made for this, the mRNP becomes marginally more efficient, but the difference is too small to constitute evidence for a major role in messenger translation.

Influence of Ionic Conditions. The salt concentrations were found to be critical for the translation of added mRNA. Figure 3 shows the effect of varying the potassium and magnesium ion concentrations on the endogenous synthesis and the translation of rabbit globin mRNA in the mouse liver S-30. Optimal conditions were 4 mM Mg^{2+} and 50 mM KCl in the mouse liver S-30 and were very similar (4 mM Mg^{2+} and 75 mM KCl) in rat liver extracts. These optima were very similar for different preparations from the same tissue, but differ significantly from those found for the Krebs cell system (Mathews, 1972). One generalization that may prove useful in other systems is that the most favorable conditions for translation of globin mRNA are close to those for the endogenous incorporation.

Landschutz Ascites Cell System. The Landschutz S-30 has been studied less extensively. Both mouse globin mRNA and encephalomyocarditis virus RNA stimulate protein synthesis at 4 mM Mg^{2+}, with either 75 or 100 mM KCl. The lower potassium concentration was slightly more favorable for the translation of globin mRNA and was used in subsequent experiments.

Identification of the Products. The products of messenger-stimulated incorporation have been characterized as globin in three independent ways: by peptide analysis, by chromatography on carboxymethylcellulose columns, and by sodium dodecyl sulfate gel electrophoresis.

Tryptic Peptides. Mouse globin contains three methionine residues, one in the α chain (in tryptic peptide αT5; residues 32–40) and two in the β chain (in peptides βT5 and βT12; residues 41–59 and 105–120). Peptide βT12 is insoluble and remains at the origin on electrophoresis at pH 6.5 (Mathews, 1972). When tryptic digests of the cell-free products labeled with [^{35}S]methionine were subjected to electrophoresis and autoradiography, the pattern shown in Figure 4 was obtained. In the presence of mouse globin mRNA all three systems synthesized the characteristic methionine-containing globin peptides αT5 and βT5, which were absent from the controls. The neutral band, consisting of free methionine and miscellaneous peptides, was common to all samples. The reason for the high background observed with the liver S-30s was mentioned above.

Chromatography. Chromatography on columns of carboxymethylcellulose was used to confirm the authenticity of the cell-free synthesized globin, and to assess the ratio of $\alpha:\beta$ chains made. Only the rabbit mRNA-mouse liver S-30 combination has been studied in this way. The elution profile (Figure 5a) clearly shows that the messenger-directed product has the same chromatographic properties as authentic α and β chains, and that the α chains appear to be produced in a 1.5:1 excess over β chains. No radioactivity eluting in the globin region was present in the endogenous cell-free product (Figure 5b). The excess α-chain production in the liver system contrasts with the excess β-chain synthesis in the Krebs cell system and *Xenopus* oocytes (Mathews *et al.*, 1971; Housman *et al.*, 1971; Lane *et al.*, 1971), and the significance of these deviations from parity is not yet understood. As expected, globin labeled in intact reticulocytes gave a ratio close to one (0.95:1 in Figure 5c).

Sodium Dodecyl Sulfate Gel Electrophoresis. Electrophoresis of radioactively labeled cell-free products on sodium dodecyl sulfate–polyacrylamide gels gave the results shown in Figure 6. In all cases the messenger-directed products contained radioactive bands which were directly superimposable on the stained globin markers and were absent from the controls. Mouse globin gives a single band on such gels (Figure 6a). When mouse globin mRNA was added to cell-free systems from mouse liver (Figure 6f) and from Landschutz ascites cells (not shown), a single radioactive band was obtained. In contrast rabbit globin gives two separate bands (Figure

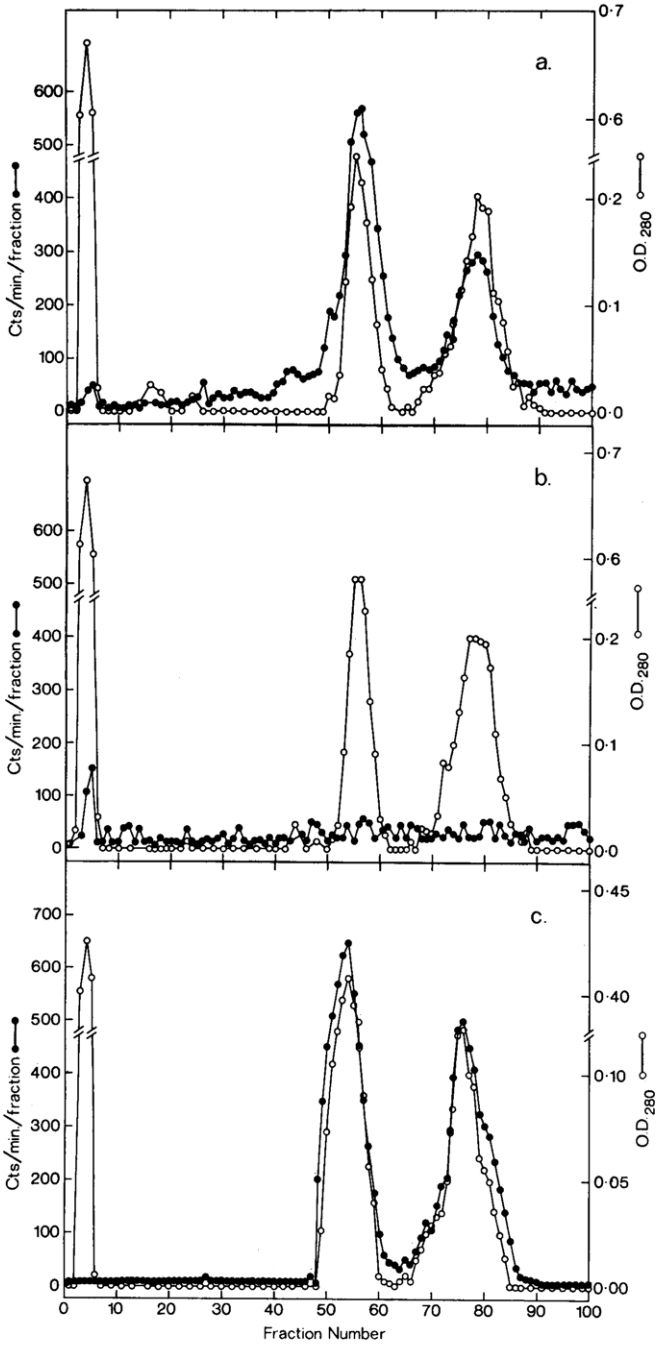

FIGURE 5: Chromatography of cell-free product on CM-cellulose. (a) Protein from reaction containing mouse liver S-30 and rabbit globin mRNA (5 μg/ml) + unlabeled rabbit globin. (b) Protein from control reaction + unlabeled rabbit globin. (c) Rabbit globin labeled by incubation of intact reticulocytes.

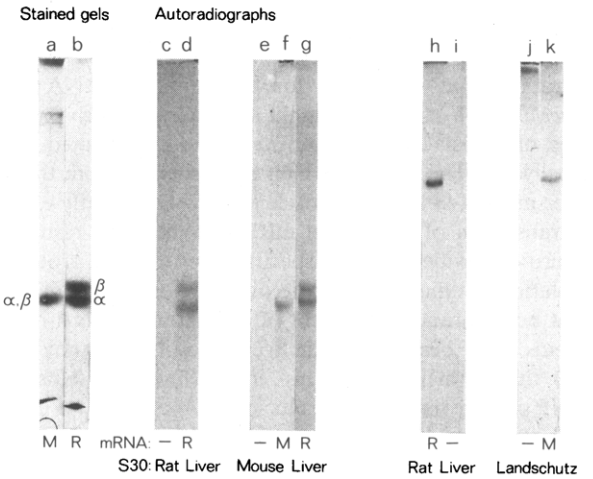

FIGURE 6: Sodium dodecyl sulfate gel analysis of cell-free products. Gels a–g were run according to Weber and Osborn (1969). Stained gels of mouse and rabbit globins are shown in a and b, respectively. Autoradiographs of [^{14}C]leucine labeled cell-free products are contained in c–g: rat liver S-30 with no mRNA (c), and with rabbit globin mRNA (d); mouse liver S-30 with no mRNA (e), with mouse globin mRNA (f), and with rabbit globin mRNA (g). Autoradiographs (h–k) are from gels run according to Swank and Munkres (1971): rat liver S-30 with rabbit globin mRNA (h), and no mRNA (i); Landschutz S-30 with no mRNA (j), and with rabbit globin mRNA (k).

6b). Correspondingly, when rabbit globin mRNA was added to cell-free systems from rat liver (Figure 6d) and mouse liver (Figure 6g) two radioactive bands resulted. Further work has shown that the faster globin band contains the α chains and the slower band the β chains (Osborn and Mathews, 1972). A tracing of Figure 6g shows that the ratio of α:β chains synthesized in the mouse liver S-30 is 1.5:1, in agreement with the result from the carboxymethycellulose columns given above. Sodium dodecyl sulfate gels are known to separate proteins primarily on the basis of molecular weight (Weber and Osborn, 1969), and presumably the separation of the two chains of rabbit globin (but not of mouse) on such gels reflects a secondary effect of charge or composition.

The cell-free products have also been run on the sodium dodecyl sulfate–urea system of Swank and Munkres (1971), which gives good separation in the range of molecular weights from 2000 to 30,000. In this system both rabbit and mouse globin give a single band. Addition of globin mRNA to rat liver and Landschutz ascites cell-free systems gave rise to a single radioactive band corresponding in position to the globin marker (Figure 6h,k). There was no sign of faster moving radioactive bands resulting from the synthesis of incomplete chains.

Discussion

The data presented above demonstrate conclusively that globin mRNA is translated in preincubated S-30 extracts from liver and Landschutz ascites cells. Clearly these systems contain all the factors required for initiation, propagation, and termination of globin chains. It could be argued that the mRNA itself might contain sufficient quantities of active factors, especially as dissociation of polysomes with sodium dodecyl sulfate does not achieve complete deproteinization. However, the mouse mRNA used in these experiments was treated with phenol and would not be expected to contain any significant amount of native protein. It was just as active as preparations which had not been treated in this way. If globin mRNA specific inititation factors are required for translation of the mRNA they must already be present in these systems, which seems unlikely as none of them synthesize globin *in vivo* (though fetal liver can do so). However these experiments do not exclude the possibility that tissues contain factors specific for particular classes of messenger RNAs. Furthermore, it is possible that reticulocyte factors might enhance globin messenger translation in these systems, even though they are not obligatory: this is currently under study. In this connection, it may be significant that these systems and the

Krebs cell system produce about one globin chain for each messenger molecule added. This is a respectable efficiency and is comparable to that observed for added globin mRNA in the reticulocyte system or for bacteriophage RNA in an *Escherichia coli* extract, though it does not approach the many cycles of translation obtained in the unfractionated reticulocyte lysate. The proteins which are associated with the messenger in the 14S mRNP particle do not significantly enhance the translation of the globin mRNA. While this result does not provide evidence for a specific role of these proteins in translation, neither does it rule out the possibility since they might be a normal component of cell extracts. RNA-binding proteins have been found in liver cytosol (Stepanov *et al.*, 1971), though their relationship, if any, to the proteins of the mRNP particle remains to be elucidated.

It is significant that the translation of the globin mRNA requires different conditions in the various systems. Similarly different messengers in the Krebs system exhibit different salt optima (Mathews, 1972). This emphasizes the point that a range of conditions must be tested when studying the activity of presumptive mRNA in cell-free systems. A negative result might simply mean that unsuitable ionic conditions are being used. In addition, a range of mRNA concentrations should be tested since above a certain level extra mRNA can diminish the stimulation (see Figure 1). Finally, it is apparent that experiments with fractionated systems can lead to spurious results because of the loss or inactivation of components. On the basis of experiments with salt-washed ribosomes and factors, Cohen (1971) concluded that the Landschutz system lacks globin mRNA-specific factors; similarly Prichard *et al.* (1971) were unable to detect in liver a particular initiation factor capable of supporting globin translation. Our work with unfractionated preparations has proved that such factors are present and active in these systems. However, our experiments do not rule out the possible existence of more subtle discriminatory mechanisms. For example, factors capable of preventing the translation of particular types of mRNA *in vivo* might be lost or inactivated during the preparation or preincubation of cell extracts.

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