THE ROLE OF MUSCLE AND RETICULOCYTE INITIATION FACTOR 3 ON THE TRANSLATION OF MYOSIN AND GLOBIN MESSENGER RNA IN A WHEAT GERM CELL-FREE SYSTEM

Doris S. KENNEDY and Stuart M. HEYWOOD

Genetics and Cell Biology, University of Connecticut Storrs, Connecticut 06268, USA

Received 15 November 1976

1. Introduction

Myosin mRNA can be isolated either from polysomes or nonribosomal bound mRNPs found in embryonic chick muscle [1,2]. It has been previously demonstrated that myosin mRNA obtained from either source can be translated in a reticulocyte cell-free system [1,2] and that the translation is stimulated when eukaryotic initiation factor 3 (EIF-3), isolated from muscle, is added to the reaction mixture [3]. On the other hand, rabbit reticulocyte EIF-3 has not been shown to give a similar response in this translational system. Furthermore, we have demonstrated that EIF-3 isolated from red muscle can be fractionated into several components which individually stimulate either myosin or myoglobin synthesis [3]. The specificity of EIF-3 for myoglobin synthesis found in embryonic red muscle is developmentally dependent [4]. These results have led us to suggest that EIF-3 is involved in a translational control process in muscle which selectively influences the efficiency of translation of messengers.

EIF-3 has been shown to be a large, complex protein made up of multiple subunits which are not present in stoichiometric amounts [5–7]. It has been proposed that the structural heterogeneity of this initiation factor may represent its heterogeneity in function. In this manner, a nonspecific EIF-3 could be utilized by all cellular messengers, while an alteration of a portion of the EIF-3 molecules occurring either by the addition of a modulating factor or by a chemical change such as phosphorylation, could make these altered EIF-3 molecules specific for certain messengers.

In this report we provide additional evidence in support of this hypothesis. When equimolar concentrations of myosin and globin mRNAs are added to a wheat germ cell-free system, a differential effect of muscle and reticulocyte EIF-3 is observed on their translation.

2. Materials and methods

2.1. *Wheat germ extract*

The wheat germ extract was prepared by a modification of the procedure of Roberts and Paterson [8]. Four grams of wheat germ (Niblack Foods, Inc. Rochester, NY) was extracted at 5°C by stirring gently in 12 ml of 0.02 M Hepes (pH 7.6), 0.11 M KCl, 3 mM $MgCl_2$, 6 mM β-mercaptoethanol, 0.4 mM spermine and 10% glycerol. After centrifuging at 16 000 × *g* for 20 min, the supernatant was removed avoiding both the surface layer of lipid material and the pellet. The supernatant was subsequently layered on a 1.5 × 60 cm Sephadex G-25 (medium) column equilibrated with the same buffer less the glycerol. The first one half of the milky, turbid fractions to elute were collected and frozen in liquid nitrogen in 0.2 ml aliquots. Preparations of wheat germ extracts which did not allow the completion of myosin synthesis (chain elongation) as judged by sodium dodecyl sulfate–acrylamide gel electrophoresis of the entire reaction mixture were discarded. One preparation out of three was found to prematurely terminate myosin chain elongation.

2.2. *Messenger RNA preparation*

Globin mRNA was prepared from rabbit reticulocyte lysates (supplied by Gibco Diagnostics) by

North-Holland Publishing Company – Amsterdam

oligo dT–cellulose chromatography of RNA from phenol extracted polysomes [9]. Myosin mRNA was prepared from the 70–120 S myosin mRNPs found in 13 day embryonic chick muscle as previously described [2]. Briefly, purification involved both oligo dT–cellulose chromatography and subsequent centrifugation of the 26 S myosin mRNA on 10–30% sucrose density gradients.

2.3. *EIF-3 Preparation*

EIF-3 from both rabbit reticulocytes and 14 day embryonic chick muscle were prepared in the same manner. The rabbit reticulocyte lysate used for globin mRNA isolation was also used as the source of reticulocyte EIF-3. The reticulocyte lysate or the 10 000 × *g* supernatant from embryonic muscle (100 g tissue homogenized in 0.15 M KCl, 5 mM $MgCl_2$, 0.02 M Tris–HCl (pH 7.4)) was layered on 27 ml, 15–30% sucrose density gradients in the same buffer and centrifuged at 25 000 rev/min for four hours. The gradients were subsequently analyzed at 260 nm on a Gilford spectrophotometer and the native 40 S ribosomal subunits collected (fractions 21–23, fig.1A). The 40 S ribosomal subunits from either muscle or reticulocytes were pooled from 12 sucrose density gradients and pelleted at 340 000 × *g* for 3 h. The pellets were resuspended in 0.5 ml of 1.0 M KCl, 2 mM $MgCl_2$, 0.02 M Tris–HCl (pH 7.4) and layered on an 11 ml, 10–30% sucrose density gradient containing the 1.0 M KCl buffer. After centrifuging for 5 h at 40 000 rev/min the gradients were analyzed at 280 nm. As shown in fig.1B (fractions 7–8), muscle EIF-3 sediments as a double peak at 18–20 S. In agreement with Sundkvist and Staehelin [5] only the 18 S peak of EIF-3 is found in similar preparations from reticulocytes (not shown). Upon electrophoretic analysis, the 18–20 S muscle EIF-3 and 18 S reticulocyte EIF-3 are found to be similar in complexity (unpublished results) and are comparable to those reported by Sundkvist and Staehelin [5]. The EIF-3 fractions were pooled from three preparative gradients, dialyzed against 0.02 M Tris–HCl (pH 7.4), 1 mM EDTA, 0.15 M KCl, 6 mM β-mercaptoethanol, 5% glycerol and chromatographed on DEAE-cellulose as shown in fig.2. EIF-3 elutes as a sharp peak at 0.25 M KCl confirming our previous results [7]. EIF-3 preparations were stored for up to two weeks in liquid N_2 without loss of activity.

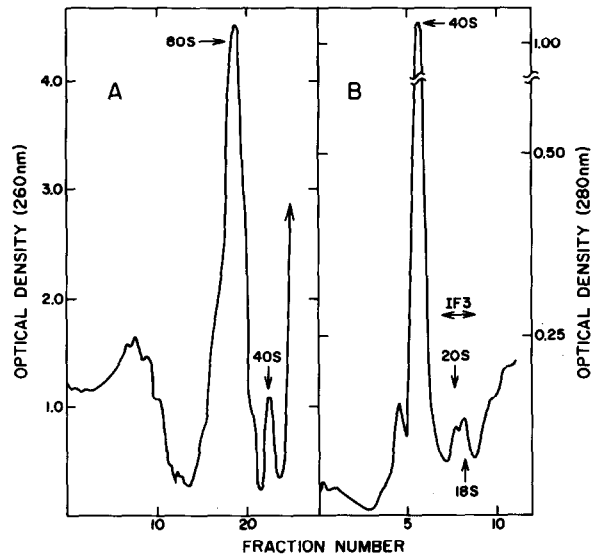

Fig.1. Preparation of muscle EIF-3 from 14 day embryonic chick muscle. (A) Sucrose density gradient separation of native 40 S ribosomal subunits. (B) Isolation of EIF-3 by high salt wash of native 40 S ribosomal subunits and sucrose density gradient analysis. Reticulocyte EIF-3 was prepared in the same manner as muscle EIF-3. Experimental details are given in Materials and methods.

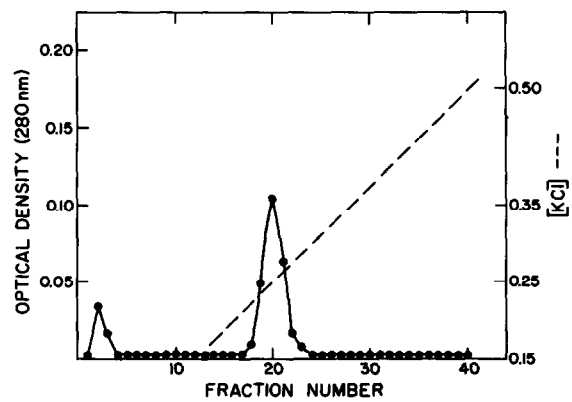

Fig.2. DEAE-Cellulose chromatography of muscle EIF-3 obtained from high salt wash of native 40 S ribosomal subunits (fig.1B). [KCl] is given in molarity. EIF-3 from both muscle and reticulocytes elutes in fractions 18–24.

2.4. *In vitro protein synthesis*

To each wheat germ incubation mixture the following were added: 50 μl wheat germ extract, 1 μg muscle tRNA, 5 mM ATP, 100 μM GTP, 40 mM creatine phosphate, 2 μg creatine kinase, 0.2 nmol each of 19 amino acids and 10 μCi [^{35}S]methionine. All solutions were made up in the Hepes wheat germ extraction buffer or H_2O to maintain the concentrations of KCl and $MgCl_2$ at 0.11 M and 3 mM respectively. These salt conditions are optimal for myosin synthesis in the system. Unless otherwise indicated, 4 μg of EIF-3, 10 μg myosin mRNA and 1 μg globin mRNA were added to the reaction mixtures. After incubation for 1 h at 25°C, 50 μg carrier myosin and 20 μg carrier globin were added to each reaction mixture. The myosin was purified by ionic precipitation and DEAE-cellulose chromatography as previously described [1]. Globin was obtained by 50–85% NH_2SO_4 precipitation of the supernatant remaining after myosin precipitation. Both proteins from the same reaction mixtures were recombined and analyzed by sodium dodecyl sulfate–acrylamide gel electrophoresis as previously described [3]. Scintillation counting efficiency was 70% for [^{35}S]methionine.

3. Results

Our previous studies involving the influence of muscle EIF-3 on myosin synthesis utilised myosin mRNA obtained from polysomes. In order to test if myosin mRNP–mRNA and myosin polysomal mRNA are translated equally well in vitro, 4 μg of each mRNA were added to both a reticulocyte and a wheat germ cell-free system. As shown in table 1, both populations of myosin mRNA are translated equally well. These results confirm those recently reported by Bag et al. [10] and suggest that chemical modifications altering the translatability of myosin mRNA are not likely the distinguishing characteristic between the stored and active populations of myosin mRNA.

When equimolar concentrations of myosin and globin mRNA are added to a wheat germ cell-free system, both are translated and the corresponding synthesized proteins show their characteristic mobility on sodium dodecyl sulfate–acrylamide gel electrophoresis (fig.3). In the absense of added mRNA, no radioactivity is incorporated into protein as determined

Table 1
Translation of myosin mRNP–mRNA and myosin polysomal mRNA in vitro

Cell-free system	Myosin mRNA	Myosin (d.p.m.)
Reticulocyte	Polysomal	2950
Reticulocyte	mRNP	3042
Wheat Germ	Polysomal	1535
Wheat Germ	mRNP	1672

Reticulocyte cell-free system was as previously described [3]. Analysis of myosin synthesis was by acrylamide gel electrophoresis as previously described [2] and indicated in this report and shown in fig.3. Myosin polysomal mRNA was prepared from 14 day embryonic chick muscle as previously described [1] but including oligo dT–cellulose chromatography.

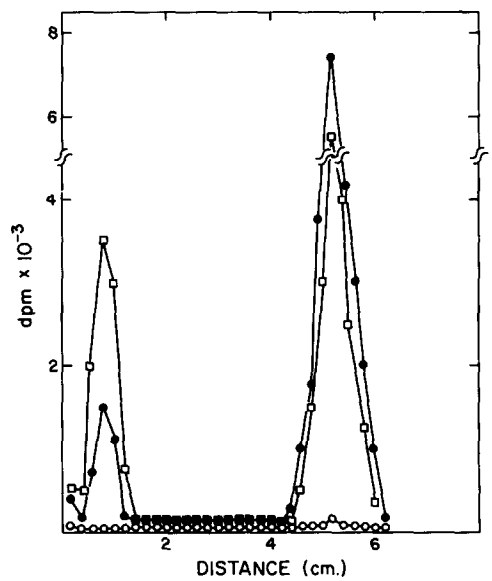

Fig.3. Sodium dodecyl sulfate–acrylamide gel electrophoresis of products of cell-free synthesis in wheat germ system. (○–○) No messenger added to reaction mixture in the presence of muscle EIF-3 (identical results are obtained if reticulocyte EIF-3 or no initiation factors are added). (□–□) 10 μg myosin mRNA and 1 μg globin mRNA with 4 μg muscle EIF-3 added to reaction mixture. (●–●) 10 μg myosin mRNA plus 1 μg globin mRNA with no added EIF-3. Cell-free synthesis and analysis of myosin and globin by purification and electrophoresis are described in Materials and methods and ref. [1]. Radioactive counting efficiency was 70% for [^{35}S]methionine.

Table 2
Specificity of muscle EIF-3 for myosin synthesis in a wheat germ cell-free system

EIF-3	Myosin (d.p.m.)	Globin (d.p.m.)	Myosin/Globin
	3350	24 455	0.14
Muscle	15 750	18 550	0.85
Reticulocyte	5325	29 000	0.18
Muscle[a]	–	19 120	–

[a]No myosin mRNA added to reaction mixture. Final volume of all incubation mixtures was 0.06 ml

by electrophoretic analysis. When 4 μg of EIF-3 from muscle is present in the wheat germ cell-free system, myosin synthesis is increased approximately three-fold while globin synthesis is slightly inhibited (fig.3 and table 2). However, if an equal amount of reticulocyte EIF-3 is added to the incubation mixture an increase in the translation of both messengers is observed. The larger stimulation of myosin synthesis compared to globin synthesis when reticulocyte EIF-3 is present is likely a result of a non-specific enhancement of translation of a less efficient mRNA by the addition of a limiting factor as predicated by Lodish [11].

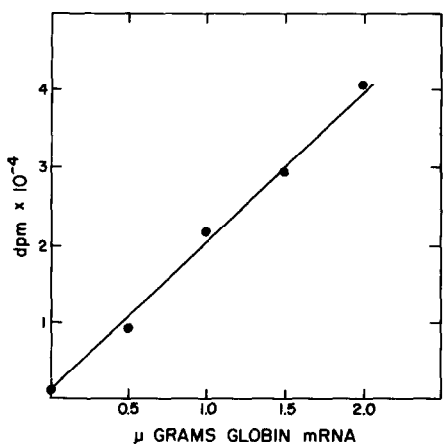

Fig.4. Effect of increasing concentration of globin mRNA on globin synthesis in the presence of 10 μg myosin mRNA and 4 μg muscle EIF-3. Analysis of globin synthesis was as in fig.3. Myosin synthesis was unaffected by increasing amounts of globin mRNA. For details of cell-free system see Materials and methods.

The addition of muscle EIF-3 to the reaction mixture in the absence of myosin mRNA also slightly inhibits globin synthesis (table 2). This suggests that the presence of this concentration of muscle EIF-3 in itself is inhibitory to globin synthesis and that the combination of muscle EIF-3 and myosin mRNA is not limiting a reactant for globin synthesis. This is verified by the demonstration that increasing amounts of globin mRNA result in a linear increase in globin synthesis when both myosin mRNA and muscle EIF-3 are present in the reaction mixture (fig.4). As shown in table 3, the addition of 1 μg of muscle EIF-3 to the reaction mixture results in a three-fold stimulation of myosin synthesis and a further increase in the amount of EIF-3 shows no additional effect on myosin synthesis. On the other hand, 1 μg of muscle EIF-3 has no effect on globin synthesis. The precise manner by which muscle EIF-3 differentially affects the translation of these mRNAs is unclear and is under further investigation.

4. Discussion

The use of the wheat germ cell-free system for studying the role of eukaryotic initiation factors on protein synthesis is advantageous because a comparison of the translatability of multiple mRNAs can be made without a high endogenous messenger activity resulting from pre-initiated mRNAs. This is not the case for the reticulocyte lysate system where extraordinary procedures (preincubation or RNAase treatment) are required to lower endogenous globin synthesis. The results reported here demonstrate that

Table 3
Effect of increasing amount of muscle EIF-3 on myosin and globin synthesis

EIF-3 (μg)	Myosin (d.p.m.)	Globin (d.p.m.)	Myosin/Globin
0	3570	23 450	0.15
1	11 300	24 110	0.47
4	12 610	16 420	0.76
10	11 710	14 100	0.83

Radioactivity determined from SDS–acrylamide gel electrophoresis of products of reaction mixture as shown in fig.3 and described in Materials and methods.

exogenous EIF-3 can influence protein synthesis in a differential manner in the wheat germ system.

EIF-3 has been shown to be a large complex [5,6] consisting of multiple components as determined by denaturing gel electrophoresis. Reticulocyte EIF-3 sediments as a single peak at 18 S upon sucrose density gradient centrifugation [5] while in contrast, muscle EIF-3 shows a heterogeneity in sedimentation with two noticeable peaks occuring at 18 S and 20 S. The reason for this is unclear and is under investigation, but the possibility exists that muscle EIF-3 may exist in two forms which are separable by centrifugation.

Lodish [11] has suggested that translation is controlled simply by the efficiency of the mRNA in itself to initiate protein synthesis. The efficiency of a given mRNA for translation results from the structural aspects of the molecule. In this manner 'better' mRNAs have a higher competitive advantage over 'poorer' mRNAs. Lodish suggests that 'poorer' mRNAs could result as non-ribosomal associated mRNPs under conditions when the cell is not optimally synthesizing protein [12]. However, it is clear that in the case of myosin, the stored and polysomal forms are identical as judged by hybridization experiments using myosin cDNA [13] and the fact, as shown in this report, that both have the same translational efficiency in either the reticulocyte or wheat germ cell-free systems. Thus, the simple efficiency hypothesis of Lodish [12] does not appear to be applicable in this case.

Our previous results [1,3] and those reported here suggest that EIF-3 may be involved in the translational control of myosin synthesis. The fact that EIF-3 is heterogeneous in composition and function suggests that a non-specific EIF-3 could be competed for by all cellular messengers as predicted by Lodish [12] but if a modulator protein becomes associated with a number of EIF-3 molecules or a chemical modification occurs, these EIF-3 molecules could become specific for a mRNA or a class of mRNAs. This would allow a competitive advantage to these messengers. It will be of interest to eventually determine if these classes of mRNA code for functionally related proteins or can be related in terms of their translational efficiency. Alternatively, mRNAs coding for functionally related proteins in muscle may have messengers with similar translational efficiencies.

Acknowledgements

This work was supported by Grant No. HD 03316 from NIH and by Grant No. CA 14733 from NCI.

References

[1] Rourke, A. and Heywood, S. M. (1972) Biochemistry 11, 2061–2069.
[2] Heywood, S. M., Kennedy, D. S. and Bester, A. J. (1975) FEBS Lett. 53, 69–72.
[3] Heywood, S. M., Kennedy, D. S. and Pester, A. J. (1974) Proc. Natl. Acad. Sci. USA 71, 2428–2434.
[4] Heywood, S. M. and Kennedy, D. S. (1974) Dev. Biol. 38, 390–393.
[5] Sundkvist, I. C. and Staehelin, T. (1975) J. Mol. Biol. 99, 401–410.
[6] Freienstein, C. and Blobel, G. (1975) Proc. Natl. Acad. Sci. USA 72, 3392–3398.
[7] Heywood, S. M. (1970) Proc. Natl. Acad. Sci. USA 67, 1782–1788.
[8] Roberts, B. E. and Paterson, B. M. (1973) Proc. Natl. Acad. Sci. USA 70, 2330–2334.
[9] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.
[10] Bag, J., Jain, S., Coronado, R., Sarkar, S. ( ) Fed. Proc. 35, 1440.
[11] Lodish, H. (1974) Nature 251, 385–389.
[12] Lodish, H., Alton, T., Margolskie, J., Dottin, R. and Weiner, A. (1975) ICN-UCLA Symp. on Mol. and Cell Biology 2, 366–373.
[13] Robbins, J. and Heywood, S. M. (1976) Biochem. Biophys. Res. Commun. 68, 918–923.