Myosin Synthesis and Specificity of Eukaryotic Initiation Factors†

Arthur W. Rourke and Stuart M. Heywood*

ABSTRACT: In order to determine the extent of myosin synthesis in a heterologous cell-free amino acid incorporating system, procedures were developed to purify the 200,000 molecular weight myosin subunit (coded for by a 25–27S mRNA) free of the low molecular weight components. Proteolytic digests of this large subunit were subsequently analyzed by two-dimensional electrophoresis and by ion-exchange chromatography. Radioactive peptides from *in vitro* synthesized myosin corresponded with both ninhydrin-positive areas on the myosin fingerprint and with the ion-exchange column elution profile of a proteolytic digest of radioactive myosin synthesized *in vivo*. These results suggest that myosin mRNA is read with a high degree of fidelity in heterologous cell-free amino acid incorporating systems. The synthesis of myosin requires initiation factors obtained from muscle ribosomes. Several experiments were performed suggesting that in the presence of homologous factors both myosin and hemoglobin I synthesis have nearly identical K^+ and Mg^{2+} optima and that at high mRNA concentrations the requirement for homologous factors may be less stringent. Initiation factor 3 from muscle ribosomes (IF3m) is found to be required for myosin synthesis while initiation factors 1 and 2 (IF1, IF2) from either erythroblasts or muscle ribosomes are effective.

The use of heterologous cell-free amino acid incorporating systems has recently been used to identify an increasing number of eukaryotic mRNAs (Heywood, 1969, 1970a; Stavnezer and Huang, 1971; Rhoades *et al.*, 1971; Cohen, 1971; Lockard and Lingrel, 1971; Mathews *et al.*, 1971; Prichard *et al.*, 1971; Hausman, 1971). A number of these reports have implicated message specific initiation factors in the translation of these mRNAs (Heywood, 1969, 1970a; Prichard *et al.*, 1971; Cohen, 1971), while others have shown no requirement for specific factors. Factors obtained from muscle ribosomes have been shown to be necessary for the translation of RNA isolated from the large myosin-synthesizing polysomes found in embryonic chick muscle (Heywood, 1969, 1970b). This RNA has been identified by a number of criteria using both homologous and heterologous cell-free systems as the mRNA coding for the synthesis of the large subunit of myosin (Heywood and Nwagwu, 1969). Evidence is presented here that this 25–27S RNA can order the proper sequencing of amino acids into the 200,000 molecular weight subunit of myosin with a high degree of fidelity. In addition, evidence is presented that suggests that initiation factor 3 (IF3)[1] has a high degree of preference for mRNA obtained from the same differentiated tissue.

Experimental Section

Methods. Salt-washed ribosomes (S-ribosomes) were prepared from 14-day embryonic chick muscle and adult chicken erythroblasts as previously described (Heywood and Nwagwu, 1969; Heywood, 1969). Rabbit reticulocyte lysates were prepared according to Evans and Lingrel (1969) followed by dialysis of the 20,000*g* supernatant against incubation buffer (MIB), 0.15 M KCl, 0.005 M $MgCl_2$, 0.02 M Tris-HCl (pH 7.6), 0.006 M β-mercaptoethanol, and 10% glycerol. This lysate contained 1 mg/ml of ribosomes. The enzymes for the reconstituted cell-free amino acid incorporating systems were prepared by homogenizing 14-day embryonic chick muscle in one-half volume of 0.02 M Tris-HCl (pH 7.4), 0.005 M $MgCl_2$, and 0.01 M β-mercaptoethanol. The 10,000*g* supernatant was filtered through nylon cloth and subsequently centrifuged at 240,000*g* for 3 hr. The middle third of the supernatant was then dialyzed against incubation buffer.

Unfractionated initiation factors and those fractionated by DEAE-cellulose chromatography were prepared as previously described (Heywood, 1970a). Plastic tubes, pipets, and columns were used in handling the factors due to their propensity to bind to glass. Freshly prepared factors and enzymes were used. The addition of factors to salt-washed ribosomes (S-ribosomes) was normally done prior to lowering the salt concentration. The factor–ribosome preparation was then dialyzed against MIB.

Myosin mRNA and globin mRNA were prepared as previously described (Heywood and Nwagwu, 1969; Heywood, 1970a). Normally either 4 μg of globin mRNA or 8 μg of myosin 25–27S RNA containing an estimated 0.1 μg of myosin mRNA was added to the incubation mixtures. tRNA was prepared from adult chicken muscle by the method described by Von Ehrenstein (1968).

In vitro amino acid incorporation was carried out in MIB. The following were added to each 0.5 ml of final volume: 1.0 mg of S-muscle or S-erythroblast ribosomes containing 0.2 mg of unfractionated initiation factors or 0.04 mg of each of the DEAE-cellulose fractionated initiation factors, 2.5 mg of S-240 enzyme fraction, 2 μmoles of ATP, 0.5 μmole of GTP, 7.5 μmoles of creatine phosphate, 100 μg of creatine kinase, 50 μg of tRNA, and 0.25 nmoles each of 20 amino acids containing 2 μCi of a uniformly labeled L-[^{14}C]amino acid mixture. Globin was synthesized utilizing S-muscle ribosomes and myosin was synthesized in systems using S-erythroblast ri-

† From the Genetics and Cell Biology Section, Biological Sciences Group, University of Connecticut, Storrs, Connecticut 06268. *Received January 31, 1972.* Supported by a grant from the National Institutes of Health (HD 03316).

* Recipient of National Institutes of Health Career Development award (GM 18,904). To whom correspondence should be addressed.

[1] To comply with standard nomenclature, our EF_1 is changed to IF1, EF_2 to IF2, and EF_3 to IF3. In addition, tissue origin of factors will be denoted with subscript *m* for muscle and *er* for erythroblasts (IF3m, initiation factor 3 derived from muscle tissue).

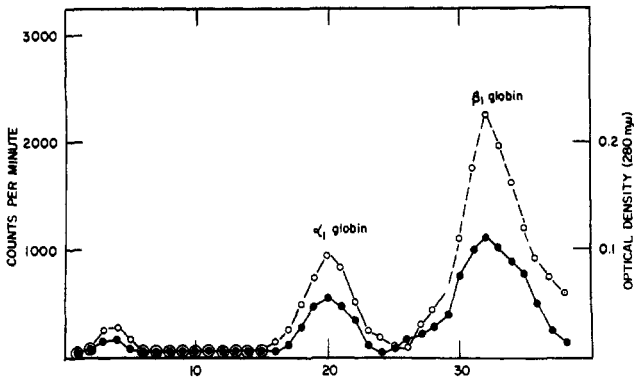

FIGURE 1: Chromatographic separation of α and β chains of globin derived from chicken hemoglobin I. The column was Bio-Rex 70 equilibrated with 10% formic acid and developed as described by Moss and Thompson (1969). ^{14}C-Labeled hemoglobin I was synthesized in a cell-free system as described in Methods using S-muscle ribosomes with unfractionated IFer. Carrier hemoglobin I was added to the products of the reaction mixture and hemoglobin I purified by the procedures of Moss and Thompson (1969). The radioactive α_1- and β_1-globin chains were obtained from three reaction mixtures. (○) Cpm and (●) optical density.

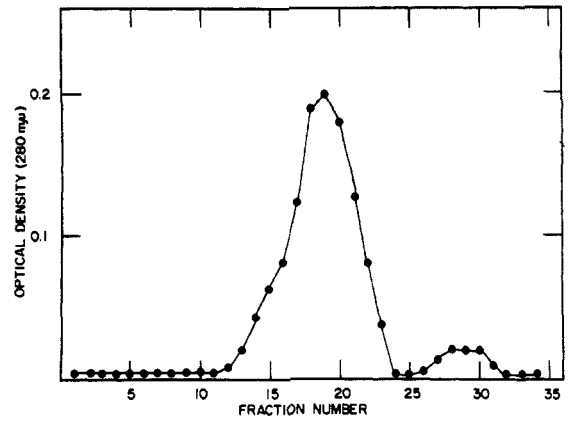

FIGURE 3: Sepharose 4B chromatography of *N*-ethylmaleimide, guanidine hydrochloride treated myosin. The column was prepared as described in Methods. After application of DEAE-Sephadex purified myosin (Figure 2) the 200,000 molecular weight subunit was eluted free of low molecular weight components. 1.7-ml fractions were collected. Fractions 16–22, containing the 200,000 molecular weight subunit of myosin, were pooled and subsequently used for peptide analysis.

bosomes. When hemoglobin was synthesized, 10 μl of an S-240 from the erythroblast lysate was added to the cell-free amino acid incorporating system as a source of heme. When rabbit reticulocyte lysates were used to synthesize myosin, no additional ribosomes or S-240 were added to the incubation mixtures. After the addition of mRNA, the reaction mixtures were incubated for 30 min at 35°. Following incubation, KCl was added to a final concentration of 0.5 M and the mixtures were centrifuged at 240,000*g* for 1 hr. Carrier protein (0.5 mg of myosin or hemoglobin I) was added to the supernatant. Myosin was then precipitated by dialysis against 0.02 M Tris (pH 7.4)–0.006 M β-mercaptoethanol. Hemoglobin I was isolated and purified by the procedures of Moss and Thompson (1969), and subsequently analyzed on acrylamide gels (Heywood, 1970a). The gels were sliced into 1-mm slices and assayed for radioactivity. The globin synthesized by the 9–12S globin mRNA was found to cochromatograph with α_1 and β_1

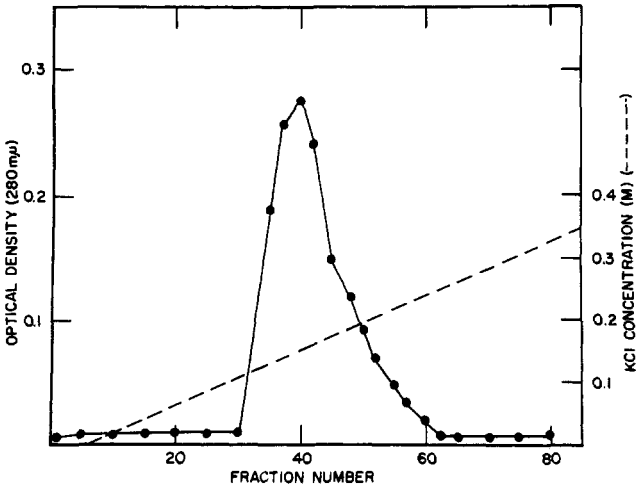

FIGURE 2: DEAE-Sephadex chromatography of myosin prepared by the procedure of Paterson and Strohman (1970). Myosin (4 mg) was applied to the column as described in Methods and eluted from the column by a 100-ml linear KCl gradient. Fractions 35–50 were collected and pooled for further analysis.

subunits of hemoglobin I under the conditions described by Moss and Thompson (1969) (Figure 1).

Carrier myosin was prepared from adult chicken muscle according to Paterson and Strohman (1970) and stored in 50% glycerol at −20°. Precipitated myosin synthesized in four reaction mixtures and containing from 3 to 5 mg of carrier myosin was resuspended in 0.02 M $K_4P_2O_7$ (pH 7.5). The myosin was applied to a 0.9 × 20.0 cm DEAE-Sephadex A-50 column. The column was equilibrated with 0.02 M $K_4P_2O_7$ (pH 7.5) until the optical density at 280 and 260 mμ returned to zero. Myosin was then eluted from the column by a linear KCl gradient (Figure 2). Fractions 35–50 were pooled and guanidine hydrochloride and *N*-ethylamaleimide were added to a final concentration of 5 and 0.004 M, respectively (Gazith *et al.*, 1970). The myosin was then concentrated against Sephadex G-200 to a 0.3–0.4% solution, dialyzed against 5 M guanidine hydrochloride (pH 7.0), and applied to a 0.9 × 60.0 cm column of Sepharose 4B equilibrated with 5 M guanidine hydrochloride. The column was poured and run with a hydrostatic head pressure of 12–15 cm and washed with quanidine hydrochloride until the optical density at 280 mμ was less than 0.03. Each column was used only once and the separation of myosin subunits was monitored at 280 mμ (Figure 3). The purity of the 200,000 molecular weight subunit of myosin (fractions 16–22, Figure 3) was routinely determined by sodium dodecyl sulfate-acrylamide gel electrophoresis (Figure 4) using the procedure of Weber and Osborn (1969).

A fingerprint analysis of the large subunit of myosin was performed using the following procedure. Fractions 16–22 from the Sepharose 4B column were pooled, concentrated against Sephadex G-200 to 2–3 mg of protein/ml, and dialyzed against 1% NH_4HCO_3 (pH 8.4). The resulting suspension was heated at 35° for 15 min and trypsin added to give an enzyme to substrate ratio of 1:100. Toluene was added to the reaction mixture to a final concentration of 0.1%. After 3.5-hr incubation at 35° trypsin was again added at the same ratio. Following an additional 6-hr digestion, α-chymotrypsin was added to give an enzyme:substrate ratio of 1.5:100. Following 8 more hr of digestion the peptide mixture was diluted threefold with 1% NH_4HCO_3 (pH 8.4) and insoluble material removed by centrifugation at 2000*g* for 10 min. The supernatent

TABLE I: The Synthesis of Myosin in Various Cell-Free Amino Acid Incorporating Systems Utilizing Ribosomes from Different Sources.[a]

Assay Systems and Ribosome Source	Factors	Myosin mRNA	Cpm[b]
Muscle ribosomes		+	1400
Muscle ribosomes		−	155
S-Muscle ribosomes		+	145
S-Muscle ribosomes	IFm	+	1250
S-Muscle ribosomes	IFer	+	185
Erythroblast ribosomes		+	20
S-Erythroblast ribosomes		+	0
S-Erythroblast ribosomes	IFm	−	0
S-Erythroblast ribosomes	IFm	+	1040
S-Erythroblast ribosomes	IFer	+	25
Rabbit reticulocyte lysate		+	5
Rabbit reticulocyte lysate	IFm	+	610
Rabbit reticulocyte lysate	IFer	+	10
Muscle (S-30)[c]	IFm	−	70
Muscle (S-30)[c]	IFm	+	900

[a] Myosin synthesis in a number of different cell-free amino acid incorporating systems. The reaction mixtures were prepared as described in Methods. Rabbit reticulocyte lysates were prepared according to Evans and Lingrel (1969). A common feature of all the amino acid incorporating systems tested is the requirement for muscle initiation factors. IFm, unfractionated KCl wash factors from muscle ribosomes. IFer, unfractionated KCl wash factors from erythroblast ribosomes. S-Ribosomes are ribosomes washed in KCl (Heywood, 1969). Myosin synthesis was determined from the radioactivity migrating with the 200,000 molecular weight subunit of myosin on sodium dodecyl sulfate–acrylamide gels (Figure 4). [b] Radioactivity migrating with the 200,000 molecular weight subunit of myosin during sodium dodecyl sulfate–acrylamide gel electrophoresis. [c] Muscle cell-free amino acid incorporating system obtained from undialyzed 30,000*g* (30 min) supernatant of 13-day embryonic chick muscle (contains 3.0 mg/ml of ribosomes). Incubation conditions were as described in Methods section. Large polysomes were removed prior to using the muscle S-30 by centrifugation through 2 M sucrose at 240,000*g* for 10 min.

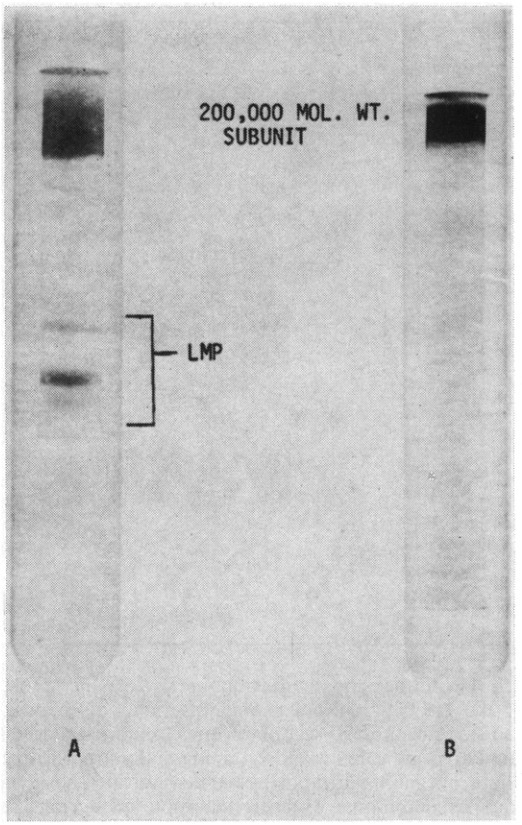

FIGURE 4: Sodium dodecyl sulfate–acrylamide gel electrophoresis of: (A) myosin purified by DEAE-Sephadex chromatography (Figure 2); (B) 200,000 molecular weight subunit of myosin obtained from Sepharose 4B chromatography (Figure 3). (A) Electrophoresis of 150 μg of myosin on a 6-cm, 5% acrylamide gel at 7 mA/tube. (B) Electrophoresis 150 μg of the 200,000 molecular weight subunit of myosin on a 10 cm, 7% acrylamide gel at 7 mA/tube. Electrophoresis was continued until the Bromophenol Blue dye front was at the end of the gel. Proteins were stained with Coomassie Brilliant Blue (Schwarz/Mann Research Laboratories). LMP, low molecular weight components of myosin.

was lyophilized and the peptides then dissolved in H_2O at 60°. The peptides were subsequently analyzed by two-dimensional electrophoresis on a 30.5 × 30.5 cm Whatman No. 3MM paper. First dimension electrophoresis was performed for 50 min at 2000 V at pH 2.0 (7.5% acetic acid–2.5% formic acid) and the second dimension for 60 min at 2000 V at 6.4 (pyridine–acetic acid–water, 10:0.4:90, v/v). The peptide map was developed using a ninhydrin–collidine stain. Each ninhydrin-positive area was cut out and assayed for radioactivity in order to determine the extent of *in vitro* myosin synthesis. Ninhydrin-negative areas were ruled off in 2 cm^2 areas and also assayed for radioactivity.

Unless otherwise noted, the radioactivity of synthesized products was determined by counting on a low-background, gas-flow counter. Dried acrylamide gel slices of 1 mm gave a counting efficiency of 12%.

Materials. Chemicals for *in vitro* protein synthesis were obtained from Sigma Chemical Co. and Mann Research Laboratories. Radioactive amino acids were obtained from New England Nuclear Corp. Ribonuclease-free sucrose and optically pure guanidine hydrochloride were obtained from Mann Research Laboratories.

Results

A number of different test systems have been utilized to assay for myosin mRNA. These include unwashed muscle and erythroblast ribosomes, 1 M KCl washed muscle and erythroblast ribosomes, and muscle and rabbit reticulocyte lysate systems (Table I). Although the muscle ribosome systems appear to be the most efficient, they suffer from the presence of endogenous myosin mRNA resulting in approximately 10% of the radioactivity incorporated into myosin in the absence of added mRNA. A common feature of the heterologous systems is the requirement for the addition of initiation factors removed from muscle ribosomes by the 1 M KCl wash (IFm). The fact that S-erythroblast ribosomes with IFm are not capable of synthesizing myosin without added mRNA suggests that the factors do not contain myosin mRNA. The heterologous cell-free amino acid incorporating system utilizing 1 M KCl washed chicken erythroblast ribosomes has been the system most used because of its relative high efficiency, the opportunity to manipulate initiation factors between tissues

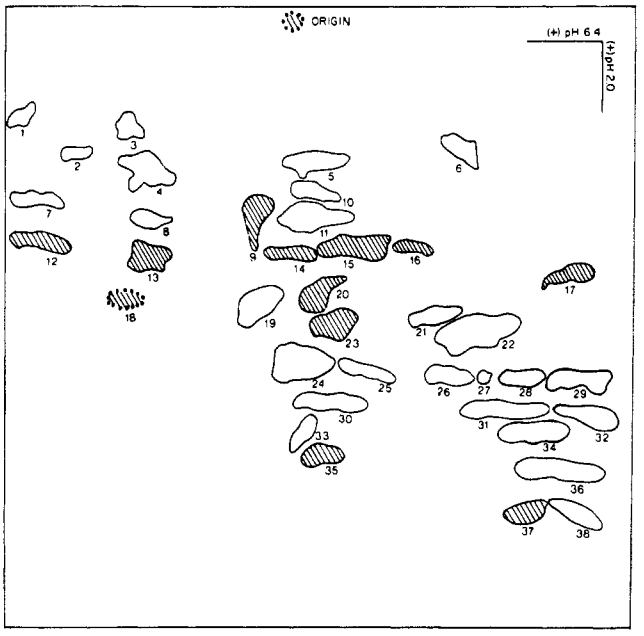

FIGURE 5: Two-dimensional electrophoresis of the proteolytic digest of the 200,000 molecular weight subunit of myosin. Proteolysis and electrophoresis were performed as described in Methods. Dotted area indicates weakly staining ninhydrin-positive area. Hashed lines over ninhydrin-positive areas indicate those peptides containing [^{35}S]methionine which is incorporated during the cell-free synthesis of myosin. Radioactivity was determined as described in legend of Table II.

TABLE II: ^{14}C Radioactivity of Ninhydrin-Positive Areas of the Myosin Fingerprint.[a]

Peptide No.	Cpm	Peptide No.	Cpm
1	115	20	134
2	96	21	67
3	90	22	178
4	135	23	110
5	80	24	250
6	35	25	170
7	153	26	90
8	87	27	50
9	130	28	90
10	82	29	122
11	117	30	130
12	112	31	230
13	92	32	151
14	150	33	150
15	87	34	80
16	47	35	110
17	82	36	100
18	71	37	60
19	160	38	30

[a] The radioactivity of the peptides from the proteolytic digest of *in vitro* synthesized 200,000 molecular weight subunit of myosin was measured following two dimensional electrophoresis (Figure 5). Ninhydrin-positive areas from the peptide map were cut out and the radioactivity determined by counting in a liquid scintillation counter using Bray's (1960) solution. Ninhydrin-negative areas from the peptide map were ruled into 2-cm^2 fractions and individually counted. These areas are found to contain no radioactivity. Cell-free amino acid incorporation was performed as described in Methods.

from the same species, and the availability of mRNA from erythroblasts as well as from muscle. Depending on the erythroblast ribosome preparation, myosin synthesis amounts to 10–40% of the total radioactivity incorporated in these reaction mixtures.

In order to determine the fidelity by which myosin mRNA is translated, a fingerprint analysis of the myosin synthesized in the heterologous cell-free system containing S-erythroblast ribosomes and IFm has been obtained. A typical fingerprint analysis of the purified 200,000 molecular weight subunit of myosin is shown in Figure 5. Under the conditions used approximately 85% of the protein digested is resolved into peptides. This extent of enzymatic hydrolysis is consistent with previously published reports (Kielley and Barnett, 1961). When [^{35}S]methionine is the only radioactive amino acid added to the reaction mixture, 12 of the ninhydrin-stained spots contain radioactivity as indicated by the hashed lines in Figure 5. No radioactivity is found in ninhydrin-negative areas of the fingerprint. When the cell-free amino acid incorporating system includes a mixture of [^{14}C]amino acids, all of the ninhydrin-positive areas are radioactive (Table II). Radioactivity is again only found in the ninhydrin-positive areas of the fingerprint. The proteolytic digest of ^{3}H-labeled *in vitro* synthesized myosin was also compared to a similar digest of ^{14}C-labeled *in vivo* myosin by ion-exchange chromatography (Figure 6). The correspondence of each ^{3}H peak from the *in vitro* synthesized myosin with a ^{14}C peak further suggests the identity of the protein synthesized under the direction of myosin 25–27S mRNA. These results indicate that myosin mRNA is being translated with a high degree of fidelity in the heterologous cell-free system. When reaction mixtures were incubated in the absence of added myosin mRNA, no radioactivity was found to elute from the Sepharose 4B column. Therefore, subsequent proteolytic digests and radioactive analysis of control experiments were not performed.

It has been shown previously that factors removed from muscle ribosomes by a 1 M KCl wash are required for the synthesis of myosin and similarly, factors removed from erythroblast ribosomes greatly enhance hemoglobin synthesis (Heywood, 1969, 1970a). In order to determine if, under the conditions used, either myosin mRNA or globin mRNA is relatively inactive as compared to the other, the synthesis of myosin and hemoglobin I were assayed under different salt conditions. Using 5 mM Mg^{2+}, an optimal K^+ concentration of 0.1 M is observed for globin synthesis while an optimal of 0.15 M K^+ is found for myosin synthesis (Figure 7). However, globin mRNA maintains over 90% of its activity at 0.15 M K^+ (the concentration of K^+ in the incubation buffer). At 0.15 M K^+ both myosin and globin mRNA are optimally active at 5 mM Mg^{2+}. These results suggest that in the presence of their respective initiation factors, both myosin mRNA and globin mRNA show a similar dependence on salt concentrations.

The translation of globin mRNA by S-muscle ribosomes does not appear to be as completely dependent upon homologous initiation factors as does the translation of myosin mRNA on S-erythroblast ribosomes (Heywood, 1970a). These results were obtained utilizing 20 μg/ml of globin mRNA and

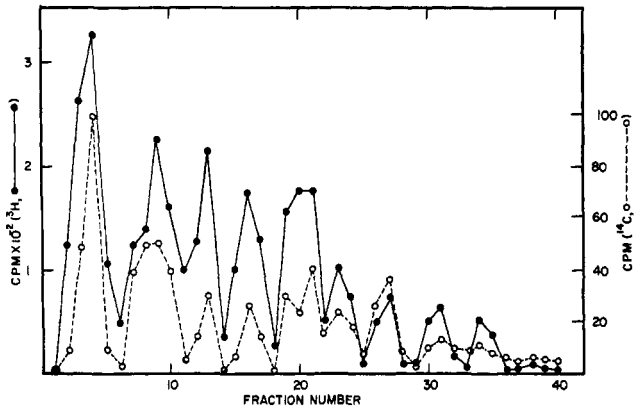

FIGURE 6: Separation of the proteolytic digest of the 200,000 molecular weight subunit of myosin by ion-exchange chromatography. Cell-free synthesis of myosin was performed as described in Methods with the exception that a [^{3}H]amino acid mixture replaced the ^{14}C-labeled amino acids. After lyophilization, the proteolytic digest of the 200,000 molecular weight subunit (10 mg of carrier myosin was added to the products of the reaction mixture) was solubilized in 0.2 M pyridine acetate (pH 2.5). The peptides were eluted by a 160-ml linear gradient (0.2 M pyridine acetate (pH 2.5) to 1.6 M pyridine acetate (pH 5.6) at 50° under 25 psi). 4-ml fractions were collected, dried, and their radioactivity determined. *In vitro*, ^{3}H-labeled myosin peptides were counted by liquid scintillation. *In vivo*, ^{14}C-labeled myosin peptides were run separately on an identical column, and the radioactivity of the eluted peptides counted on a gas-flow, low-background counter (background less than 2 cpm). The ion-exchange resin used was Aminex A-5 (Bio-Rad Lab) and the column dimensions were 0.9 × 9 cm.

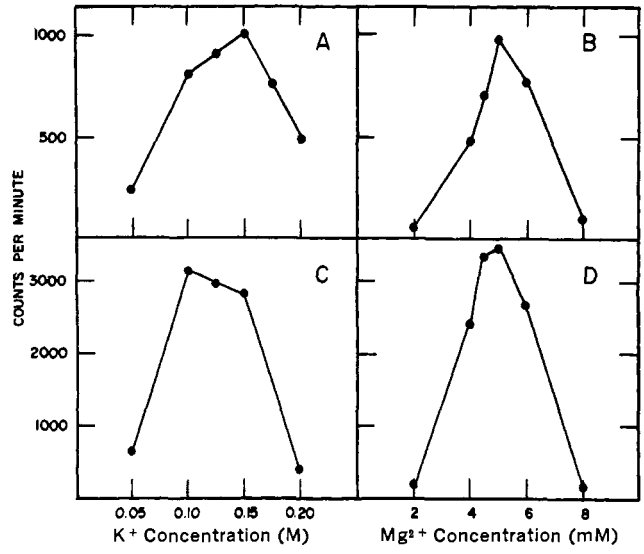

FIGURE 7: The dependence of mysoin and hemoglobin I synthesis on cation concentration. The cell-free amino acid incorporation and the isolation of the synthesized products are described in Methods. The extent of myosin and hemoglobin I synthesis was determined from the radioactivity electrophoretically migrating with the 200,000 molecular weight subunit of myosin (Figure 4) and with hemoglobin I (Heywood, 1970a) on acrylamide gels. (A and B) Myosin synthesis using S-erythroblast ribosomes, myosin mRNA, and IFm. (C and D) Hemoglobin I synthesis using S-muscle ribosomes, globin mRNA, and IFer. In parts A and C the Mg^{2+} concentration was maintained at 5 mM. In parts B and D the K^+ concentration was held at 0.15 M.

an estimated 0.1–0.3 μg/ml of myosin mRNA. If, however, the concentration of globin mRNA is lowered to an equivalent level, a definite requirement of IFer for hemoglobin I synthesis becomes evident (Figure 8).

Results obtained from the binding of ^{32}P-labeled myosin mRNA to ribosomes have implicated IF3 as the factor both involved in binding mRNA to the 40S ribosomal subunit during the formation of the initiation complex and in the recognition of specific mRNAs (Heywood, 1970a; Heywood and Thompson, 1971). Prichard *et al.* (1971) have recently demonstrated that IF3 derived from mammalian reticulocytes is necessary to translate 9S globin mRNA in liver cell-free amino acid incorporating systems. Similar results are obtained if myosin mRNA is translated by S-erythroblast ribosomes with various combinations of IF2 and IF3 obtained from muscle and erythroblast ribosomes (Table III). In the presence of IF1er, myosin synthesis requires IF3m and either IF2m or IF2er. The 40% myosin synthesis in the presence of IF2m and IF3er is likely a result of an incomplete separation of IF2m and IF3m by DEAE-cellulose chromatography. This

TABLE III: Requirement of IF3m for Myosin Synthesis in a Heterologous Cell-Free System.

Factors	Factors[a]	
	IF2m	IF2er
IF3m	1100	1025
IF3er	420	30
IF1m, IF3m	1015	

[a] Cpm incorporated into the 200,000 molecular weight subunit of myosin. [b] The specificity of IF3m for the translation of myosin mRNA. Initiation factors from muscle and erythroblast ribosomes were separated as previously described (Heywood, 1970a). The reaction mixtures are described in Methods. IF1er (40 μg) was present in all reaction mixtures. Radioactivity of the 200,000 molecular weight subunit of myosin was determined following acrylamide gel electrophoresis (Figure 4).

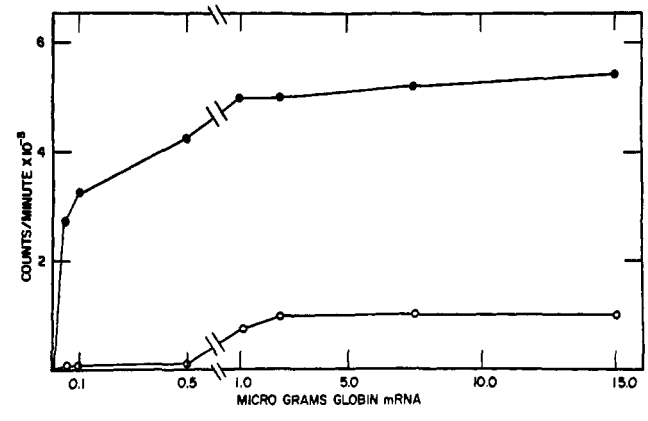

FIGURE 8: The requirement for homologous initiation factors in the synthesis of hemoglobin I at different concentrations of mRNA. Cell-free amino acid incorporation is described in Methods. Hemoglobin I was isolated and purified from the reaction products by the method of Moss and Thompson (1969). After acrylamide gel electrophoresis, the radioactivity of hemoglobin I was determined by counting 1-mm slices of the gel (Heywood, 1970a). (●) The synthesis of hemoglobin I on S-muscle ribosomes in the presence of IFer; (O) hemoglobin I synthesis in the presence of IFm.

is supported by the almost complete inability to synthesize myosin when only initiation factors from erythroblasts are added to the reaction mixture.

Discussion

Previous results have suggested that an RNA species which sediments at about 26 S, and is found associated with the large myosin-synthesizing polysomes could direct the synthesis of the 200,000 molecular weight subunit of myosin on both homologous and heterologous ribosomes (Heywood and Nwagwu, 1969; Heywood, 1969). Although this evidence strongly suggested that myosin was actually being synthesized, no information was available as to the fidelity of the translation process. The techniques developed and presented here for the complete separation of the large subunit of myosin from the low molecular weight components allow for both ion exchange chromatography and fingerprint analyses of the proteolytic digests of the large subunit of myosin and, therfore, subsequent radioactive analysis of the *in vitro* synthesized myosin. Results from such analyses suggest that myosin mRNA is translated on heterologous ribosomes in the presence of muscle initiation factors with a high degree of fidelity.

A number of reports have indicated that cell- or message-specific factors are unnecessary for the translation of heterologous mRNAs (Mathews *et al.*, 1971; Stavnezer and Huang, 1971; Rhoads *et al.*, 1971; Hausman *et al.*, 1971). With this in mind, we have further analyzed the systems employed in this laboratory. The possibility that different salt concentrations are optimal for the activity of globin and myosin mRNAs was examined. In the presence of homologous initiation factors both globin mRNA and myosin mRNA have nearly identical Mg^{2+} and K^{+} concentration optima for the synthesis of their respective proteins. It is possible that by altering salt concentration and thereby, presumably altering the structure of the mRNA, specific functions of the initiation factors may be bypassed. This remains to be examined.

It has been observed that globin synthesis occurs to a small extent in the presence of muscle initiation factors (Heywood, 1970a) while little if any myosin synthesis occurs in the absence of muscle initiation factors. The possibility that a small percentage of the mRNA was partially degraded allowing for nonspecific initiation to occur at the proper initiation site was investigated by titrating the cell-free system with decreasing concentrations of globin mRNA and assaying for hemoglobin synthesis in the presence of factors obtained from both muscle and erythroblasts. At low concentrations of mRNA (concentrations which are normally used for myosin synthesis) hemoglobin synthesis is dependent upon erythroblast initiation factors, suggesting that, at a minimum, homologous factors are required to increase the efficiency of hemoglobin synthesis.

IF3m has been observed to be required for the binding of myosin mRNA to the 40S ribosomal subunit during the formation of the initiation complex (Heywood, 1970b; Heywood and Thompson, 1971). The results reported here also suggest the specific requirement of this initiation factor for the synthesis of myosin, while IF1 and IF2 from muscle can be replaced by similar factors from erythroblasts. The multiplicity of IF3 and its role in regulation of protein synthesis is further supported by recent observations of a similar nature in prokaryotes (Berissi *et al.*, 1971; Lee-Huang and Ochoa, 1971; Grunberg-Manago *et al.* 1971; Vermeer, 1971).

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