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# Translation of Myoglobin Messenger Ribonucleic Acid†

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ABSTRACT: Myoglobin messenger RNA (mRNA) is found in an 8–12S RNA fraction obtained from small polysomes (four–six ribosomes per polysome) and can be translated in a cell-free amino acid incorporating system. The translation product is isolated and purified to a single protein as determined by both urea– and sodium dodecyl sulfate–acrylamide gel electrophoresis. When the *in vitro* synthesized [$^3$H]myoglobin is compared to *in vivo* radioactively labeled [$^{14}$C]myoglobin, an excellent correspondence of radioactively labeled peptides is observed as judged by their elution during ion-exchange chromatography. Ribosomes obtained from white muscle, which are shown not to have endogenous myoglobin mRNA associated with them, synthesize myoglobin only when both myoglobin mRNA and initiation factors from red muscle are added to the reaction mixture. White muscle initiation factors were not effective in the translation of myoglobin mRNA. On the other hand, both red and white initiation factors were effective in translating myosin mRNA in a heterologous cell-free system. The initiation factor 3 (IF3) fraction of the initiation factors from red muscle is responsible for the selectivity of myoglobin mRNA translation. The results are discussed in terms of post-transcriptional control of protein synthesis during terminal differentiation.

The manner by which eukaryotic cells regulate both the onset of synthesis of specific proteins and the amount of the individual protein to be synthesized is a major unsolved problem in the understanding of cellular differentiation. Because embryonic muscle actively synthesizes a number of well-characterized proteins, the analysis of the synthesis of these proteins and their mRNAs[1] should ultimately reveal the control mechanisms involved in muscle differentiation. The mRNA coding for the large subunit of myosin has been isolated and partially characterized (Heywood and Nwagwu, 1969). It has been shown to be translated with a high degree of fidelity in a cell-free amino acid incorporating system using heterologous ribosomes (Rourke and Heywood, 1972) and to have a translation time of 7–8 min as expected from its large size (Morris *et al.*, 1972). A consistent feature of the translation of myosin mRNA on heterologous ribosomes has been the requirement of ribosomal factors obtained from muscle ribosomes. These cell-free amino acid incorporating systems, using erythroblast ribosomes, have, of necessity, utilized small amounts of added mRNA and, in addition, have had a high amount of endogenous mRNA activity. Under these conditions an IF3 preparation from muscle ribosomes is found to be necessary to translate myosin mRNA (Rourke and Heywood, 1972; Heywood, 1970). These results have led us to suggest that a tissue specificity involved in the recognition of mRNA during initiation of protein synthesis may exert a fine tuning in post-transcriptional regulation of gene expression in eukaryotic cells.

In order to more narrowly define the limits of mRNA selectivity, we have investigated the translation of a myoglobin mRNA fraction obtained from embryonic red muscle in a cell-free system utilizing ribosomes and ribosomal factors from red and white muscle. Myoglobin, an oxygen binding protein of about 17,000 daltons, is present in red muscle cells but is absent or present in only very low amounts in white muscle cells. Myosin, on the other hand, is present in both red and white muscle. We show here that an 8–12S RNA fraction from small polysomes contains myoglobin mRNA and is capable of directing the synthesis of myoglobin using white muscle ribosomes. While both red and

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[1] Abbreviations used are: mRNA, messenger ribonucleic acid; IF, initiation factor; MIB buffer, 0.15 M KCl, 0.005 M $MgCl_2$, 0.02 M Tris-HCl (pH 7.5), 0.006 M 2-mercaptoethanol, and 10% glycerol.

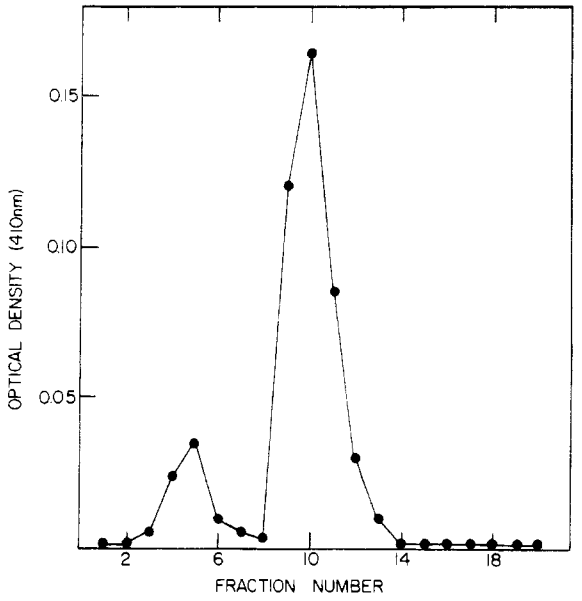


FIGURE 1: Sephadex G-100 chromatography *in vitro* synthesized myoglobin. The column was prepared and eluted as described under Materials and Methods. Fractions of 1.5 ml were collected and the optical density at 410 nm was determined. The first peak eluted contains hemoglobin. Fractions 9–12 were pooled for further purification of myoglobin.

white muscle ribosomal factors are effective in myosin synthesis on erythroblast ribosomes, myoglobin synthesis is strongly dependent on ribosomal factors isolated from red muscle—specifically the IF3 fraction. These results suggest that there may be a multiplicity of specific factors in the IF3 fraction derived from the same cell type.

## Materials and Methods

*Preparation of Constituents for Cell-Free System.* Salt-washed ribosomes and ribosomal initiation factors were prepared from 19-day embryonic chick red and white muscle as previously described (Rourke and Heywood, 1972). In the results reported here, breast muscle is considered to be white muscle while only the dark muscle of the upper leg is considered to be red muscle. We would like to emphasize that fresh preparations of ribosomes and factors were used and that washing of the ribosomes was done for 5–10 min at 0° with 1.0 M KCl, 0.0025 M $MgCl_2$, 0.02 M Tris-HCl (pH 7.4), and 0.006 M 2-mercaptoethanol. Ribosomes prepared in this manner contain endogenous mRNA and are active in protein synthesis without the addition of mRNA. The fractionation of initiation factors on DEAE-cellulose was performed as previously described (Heywood, 1970) with the exception that factors in 1.0 M KCl were not dialyzed. Instead, the ribosome wash was diluted to the proper ionic conditions prior to chromatography on DEAE-cellulose. Plastic tubes, pipets, and columns were used in handling the factors due to a propensity for them to bind to glass. The addition of factors to washed ribosomes was done prior to lowering the salt concentration. The factor–ribosome preparation was subsequently dialyzed for 1 hr against incubation buffer (MIB): 0.15 M KCl, 0.005 M $MgCl_2$, 0.02 M Tris-HCl (pH 7.5), 0.006 M 2-mercaptoethanol, and 10% glycerol. Either 125 $\mu$g of unfractionated factors or 15 $\mu$g each of IF1, IF2, and IF3 was added to each 2 mg of ribosomes (Heywood, 1970).

The enzymes for the cell-free amino acid incorporating systems were prepared by homogenizing 19-day embryonic chick red muscle which had been kept for 30–40 min in cold MIB. 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, normally containing 2 mg/ml of protein, was dialyzed against MIB.

Myosin mRNA was prepared from 19-day embryonic muscle as previously described (Morris *et al.*, 1972). The myosin 25–27S RNA fraction was not purified free of 28S ribosomal RNA and 5 $\mu$g was added to the incubation mixture. Myoglobin mRNA was also obtained from 19-day embryonic red muscle polysomes by similar procedures as the myosin mRNA. Each sucrose density gradient was layered with 10,000*g* supernatant obtained from 1.5 g of red muscle. The small polysomes (see Results) were pooled from 12 sucrose density gradients, pelleted at 320,000*g* for 90 min, and subsequently resuspended in 0.05 M Tris-HCl (pH 7.2), 0.005 M EDTA, and 0.5% sodium dodecyl sulfate. After sucrose density gradient centrifugation of the polysomal RNA (see Results) the RNA sedimenting from 8 to 12 S was collected. This yields 4–6 $\mu$g of 8–12S RNA and normally 2–3 $\mu$g was added to each incubation mixture. The precise amount of myoglobin mRNA is not known for it is probable that the 8–12S fraction contains a number of species of RNA.

*Cell-Free Synthesis and Myoglobin Preparation. In vitro* protein synthesis was carried out as previously described (Rourke and Heywood, 1972) in a final volume of 0.25 ml containing 8 $\mu$Ci of tritiated amino acid mixture (New England Nuclear Corp.). The reaction mixtures were incubated for 45 min at 35°. Following incubation, 0.3 mg of carrier myoglobin was added and the volume was brought to 2 ml with cold $H_2O$. Radioactive myoglobin was subsequently isolated from the cell-free system in the following manner. To each incubation mixture solid $(NH_4)_2SO_4$ was added to a final concentration of 65%. After 30 min at 0°, the precipitate formed was discarded and solid $(NH_4)_2SO_4$ was added until 100% saturation at 0° was obtained. Myoglobin was allowed to precipitate for 18 hr. The myoglobin precipitate, collected by centrifugation at 10,000*g* for 10 min, was resuspended to 0.05 M $NaH_2PO_4$ (pH 6.0) and chromatographed on a 1 × 30 cm column of Sephadex G-100 (Pharmacia) equilibrated with the same buffer. The myoglobin, fractions 9–12 in Figure 1, was concentrated to 1-ml volume against dry Sephadex G-200 and subsequently dialyzed against 0.002 M $NaH_2PO_4$ (pH 6.4). The sample was then applied to a 0.5 × 3 cm carboxymethylcellulose column (CM-23, Whatman) equilibrated with the same buffer. Myoglobin was eluted from the column by a 20-ml linear gradient of 0.002 M $NaH_2PO_4$ (pH 6.4) to 0.05 M Tris-HCl (pH 8.2). All operations were performed at 2° and the elution myoglobin was followed at 410 nm. Myoglobin prepared in this manner migrated as a single band on sodium dodecyl sulfate and urea–acrylamide gel electrophoresis (see Results).

Carrier myoglobin was prepared from red leg musculature and hearts of adult chickens. The tissue was homogenized in 4 vol of cold $H_2O$ and centrifuged at 15,000*g* for 15 min. Further purification of carrier myoglobin was carried out as described above except that the Sephadex G-100 column dimensions were 1.5 × 85 cm, the CM-cellulose column dimensions were 1.0 × 15 cm, and the CM-cellulose 23 column was developed with a 200-ml linear gradient.

*Peptide Analysis of Myoglobin. In vivo* radioactively labeled myoglobin was obtained from red musculature of 7–10-day-old chicks injected with 10 $\mu$Ci of a $^{14}C$-labeled amino acid mixture (New England Nuclear Corp.). The *in vivo* labeling period was 2 hr. The heme moiety was removed from the myo-

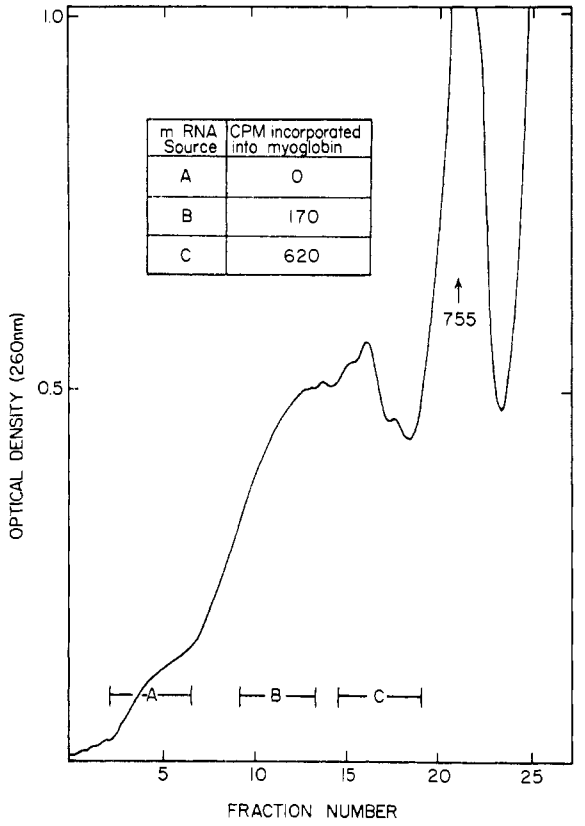


FIGURE 2: Localization of myoglobin mRNA in polysomes of 19-day chick embryo red muscle. The 10,000*g* supernatant from 1 g of leg muscle tissue, prepared as described under Materials and Methods, was layered on each of nine sucrose density gradients (27 ml, 15–40% sucrose in 0.15 M $NH_4Cl$–0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.4)) and centrifuged for 90 min at 25,000 rpm in an IEC-SB110 rotor at 2°. The material was withdrawn from the bottom of the gradient tube and the optical density was continuously recorded with a Gilford spectrophotometer. RNA was extracted from the polysomes of regions A, B, and C from a total of nine gradients as described under Materials and Methods. The 8–12S RNA from these three polysome classes was examined for myoglobin mRNA activity in cell-free amino acid incorporating systems utilizing white muscle ribosomes. The inserted table shows the total counts per minute of $^3H$-labeled amino acids incorporated into myoglobin as determined following extensive purification of myoglobin as described under Materials and Methods.

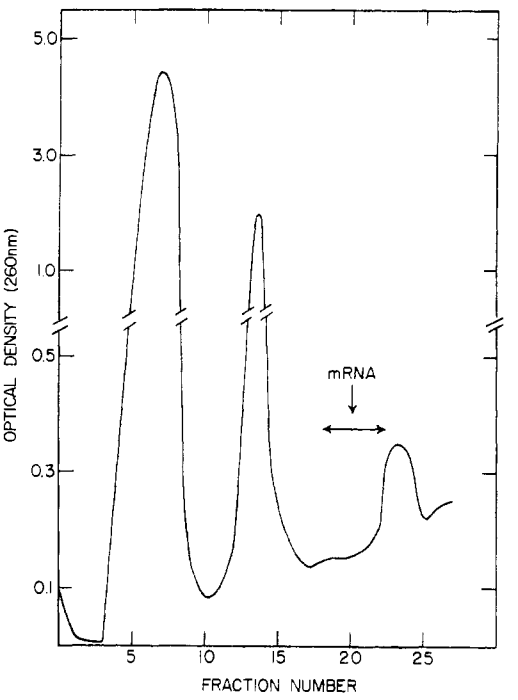


FIGURE 3: Sucrose density gradient of RNA from region C polysomes. The RNA extracted from pelleted region C polysomes of 12 gradients as described under Materials and Methods was layered on a 27-ml 10–30% sucrose density gradient containing 0.05 M Tris-HCl (pH 7.2), 0.005 M EDTA, and 0.5% sodium dodecyl sulfate. Centrifugation was for 22 hr at 23,000 rpm at 3° in an IEC SB110 rotor. The material was removed from the bottom of the gradient tube and the optical density at 260 nm was continuously recorded on a Gilford spectrophotometer. The fraction indicated above as mRNA was collected and precipitated with 2 vol of cold ethanol for use in the cell-free synthesis of myoglobin.

globin by the method of Lodish and Jacobson (1972). The resulting protein was resuspended in 2% $NH_4HCO_3$ and heated to 90° for 10 min and then lyophilized. The lyophilized protein was again resuspended in 2% $NH_4HCO_3$–0.1% toluene and preincubated at 35° for 15 min. Trypsin was added to give an enzyme:substrate ratio of 2:100. After 6 hr of incubation at 35° trypsin was again added at a ratio of 1:100. The incubation was continued for an additional 10 hr. After trypsin digestion, the myoglobin peptide fragments were lyophilized and the resulting preparation was dissolved in 1 ml of 0.2 M pyridine acetate (pH 3.0). The ion exchange resin used was Aminex A-5 (Bio-Rad Laboratories) and the column dimensions were 0.6 × 11 cm. The peptides were eluted by a 200-ml linear gradient (0.2 M pyridine acetate (pH 3.0) to 2.0 M pyridine acetate (pH 5.1) at 50° under 25 psi of pressure). Three-milliliter fractions were collected and dried in a vacuum at 80° in scintillation counting vials, and, after the addition of 0.5 ml of $H_2O$ and 10 ml of Aquasol (New England Nuclear Corp.), the radioactivity was determined for both *in vitro* labeled tritiated peptides and *in vivo* $^{14}C$-labeled peptides in a liquid scintillation counter. The counting efficiency for $^3H$ was 40% and for $^{14}C$ was 60%.

## Results

Well-developed red musculature of embryonic chick synthesizes the oxygen binding protein, myoglobin. This is readily apparent by the darkening of those muscles just prior to hatching. By using a loose fitting Dounce type homogenizer a characteristic polyribosomal profile is obtained from 19-day embryonic chick red thigh muscle (Figure 2). In addition to the myosin synthesizing polysomes sedimenting in fraction A, a characteristic peak consisting of four–six ribosomes per polysome is apparent in fraction C. This latter peak is sensitive to pancreatic ribonuclease (unpublished results) suggesting that little, if any, of the optical density in this peak is a result of the cold-formed tetramere characteristic of chicken ribosomes (Morimoto *et al.*, 1972).

Fractions A, B, and C from 12 sucrose density gradients (Figure 2) were collected and the different size classes of polysomes were pelleted by centrifugation at 320,000*g* for 90 min. The polysomal pellets were subsequently resuspended and centrifuged on a sucrose density gradient as described in the legend of Figure 3. Figure 3 shows a typical optical density profile of the RNA obtained from fraction C polysomes of 12 polysome gradients. When 8–12S RNA is collected from polysome fractions A, B, and C and added to a cell-free system, it is found that the RNA from fraction A polysomes does not direct myoglobin synthesis and fraction B RNA has some activity, while fraction C 8–12S RNA has the greatest

TABLE I: Myoglobin Synthesis Using Ribosomes and Initiation Factors from Red and White Muscle.[a]

| Muscle Source | | | | | |
|---|---|---|---|---|---|
| Ribosomes | Initiation Factors | mRNA | Total cpm | Myoglobin cpm | % Myoglobin |
| Red | Red | − | 51,280 | 580 | 1.1 |
| Red | | − | 6,320 | 40 | 0.6 |
| White | White | − | 34,190 | 22 | 0.1 |
| White | | − | 7,400 | 18 | 0.2 |
| White | Red | − | 10,400 | 20 | 0.1 |
| White | White | + | 25,010 | 50 | 0.1 |
| White | | + | 12,670 | 55 | 0.4 |
| White | Red | + | 48,000 | 3500 | 7.3 |

[a] The preparation of all components of the cell-free amino acid incorporating system is described under Materials and Methods. *In vitro* synthesized myoglobin was determined as hot $Cl_3CCOOH$-precipitable radioactivity eluting as myoglobin from carboxymethylcellulose columns.

TABLE II: Myosin Synthesis Using Initiation Factors from Red and White Muscle.[a]

| Ribosomes | IF | 26S mRNA | Myosin cpm |
|---|---|---|---|
| Erythroblast | Red | + | 1080 |
| Erythroblast | White | + | 965 |
| Erythroblast | Red | − | 38 |

[a] Myosin mRNA and unfractionated initiation factors were prepared as described under Materials and Methods. Preparation of erythroblast ribosomes and purification of the 200,000-dalton subunit of myosin synthesized *in vitro* were performed as previously described (Rourke and Heywood, 1972).

capacity to direct myoglobin synthesis (insert, Figure 2). Myoglobin, a single polypeptide chain of ~17,000 daltons, is expected to have an mRNA of about 9 S which would, in turn, have four–six ribosomes attached to it. The above results confirm this and indicate that an intact mRNA species can be obtained from small polysomes of embryonic chick red muscle. In all subsequent experiments myoglobin mRNA was obtained exclusively from the fraction C polysomes as shown in Figures 2 and 3.

White muscle, obtained from 19-day embryonic chick breast muscle, contains little, if any, myoglobin. We chose this muscle as a source of ribosomes for the cell-free synthesis of myoglobin because (1) no endogenous myoglobin mRNA would be expected to be found on white muscle ribosomes and (2) it offered the opportunity to determine if the specificity of messenger recognition factors we have observed between tissues using myosin mRNA (Rourke and Heywood, 1972; Heywood, 1970) could be observed using two different mRNAs from the same tissue (myoglobin found in red muscle and myosin found both in red and white muscle). A number of control and test reaction mixtures containing either red or white muscle 1 M KCl washed ribosomes and the ribosomal factors obtained from these ribosomes were tested for their capacity to synthesize myoglobin in the presence and absence of added mRNA (Table I). Without the addition of myoglobin mRNA to reaction mixtures containing red muscle ribosomes, ~1% of the radioactivity incorporated into protein is found in myoglobin. If initiation factors are omitted this amount decreases to one-half of this value. On the other hand, white muscle ribosomes alone or with the addition of either white or red muscle initiation factors fail to synthesize myoglobin. In addition, if a 20,000*g* lysate from white muscle is incubated with red muscle factors no myoglobin synthesis is observed (unpublished results). These results confirm the lack of myoglobin messenger activity in red muscle initiation factors as well as suggest that myoglobin mRNA is not present either associated or unassociated with ribosomes in white muscle. If, however, myoglobin mRNA is added to a reaction mixture containing white muscle ribosomes and red muscle initiation factors ~7% of the radioactivity is found in myoglobin. The addition of myoglobin mRNA to the incubation mixture in the absence of added factors or with the addition of white ribosomal initiation factors results in essentially no myoglobin synthesis. Total protein synthesis is found to increase with the addition of either white or red muscle initiation factors to the ribosomes which suggests that both factor preparations are functionally active in the cell-free amino acid incorporating system. In order to demonstrate that white muscle initiation factors are indeed effective in initiating protein synthesis they were tested in a myosin synthesizing system using erythroblast ribosomes (Table II). Both white and red muscle initiation factors are equally effective in translating myosin mRNA. These results in conjunction with those published previously (Rourke and Heywood, 1972; Heywood, 1970) suggest that a messenger recognition factor necessary for the synthesis of myosin in heterologous cell-free systems is present in both red and white muscle. However, only red muscle contains a specific factor for myoglobin synthesis. The messenger recognition factor has been identified as a factor isolated with IF3 on DEAE-cellulose and involved with the binding of mRNA to ribosomes (Heywood, 1970). In order to identify further the factor involved with the selective translation of myoglobin mRNA on white muscle ribosomes, the initiation factors from red and white muscles were fractionated into their respective IF1, IF2, and IF3 components on DEAE-cellulose (Heywood, 1970). Mixtures of these different factor preparations from both muscle types were added to the reaction mixtures and the synthesis of myoglobin determined by chromatography on CM-cellulose. As shown in Figure 4A, when IF1 (white), IF2 (white), and IF3 (red) were present, myoglobin mRNA is effectively translated; however, when IF3 (white) replaces IF3 (red) no myoglobin synthesis occurs (Figure 4B) as is also the case when both IF3 (red and white) are added in the absence of mRNA (Figure 4D). In addition, the translation of myoglobin mRNA occurs equally well in the presence of IF3 from red muscle when IF1 and IF2 from either red or white muscle are present (Figures 4A and 4C). These results suggest that the preparation of IF3 from red muscle contains a factor necessary for the translation of myoglobin mRNA that is not present in a similar preparation from white muscle while either red or white muscle IF1 and IF2 preparations are capable of supporting myoglobin synthesis. Therefore, muscle may contain a multiplicity of messenger recognition factors (IF3 fraction) and myosin mRNA and myoglobin mRNA may utilize different sets of these factors during the initiation of protein synthesis.

Thus far the assay for myoglobin synthesis has been its isolation and purification with carrier myoglobin and its final chromatographic behavior on CM-cellulose. Further analysis

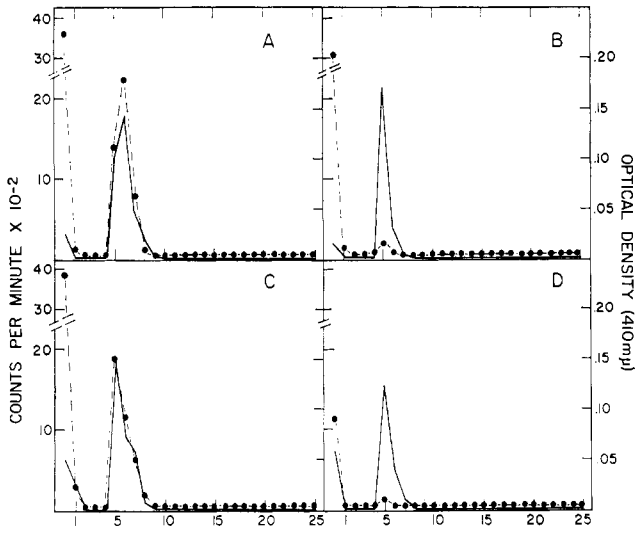


FIGURE 4: CM-cellulose chromatography of myoglobin synthesized in cell-free systems with different initiation factors. The optical density (—) was read at 410 nm and the radioactivity (●--●) was determined in a liquid scintillation counter. The cell-free amino acid incorporating systems and myoglobin purification were as described under Materials and Methods. White muscle ribosomes were used in A–D; 8–12S RNA of red muscle was added to A, B, and C. Initiation factor sources were: (A) IF1 and IF2 of white muscle and IF3 of red muscle; (B) IF1, IF2, and IF3 of white muscle; (C) IF1, IF2, and IF3 of red muscle; and (D) IF1, IF2, and IF3 of white muscle plus IF3 of red muscle.

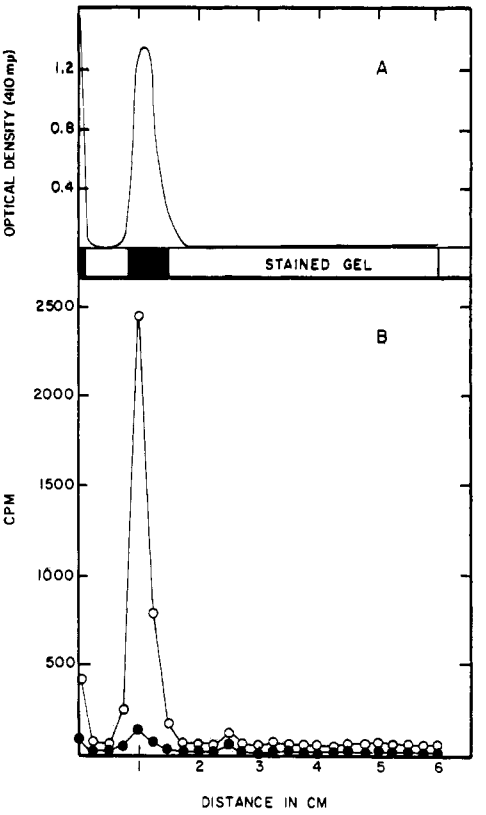


FIGURE 5: Acrylamide gel electrophoresis of purified, *in vitro* synthesized myoglobin at pH 8.6. The myoglobin was synthesized and purified as described under Materials and Methods. One-third of the purified myoglobin was applied to a 6 M urea–0.05 M Tris-acetate (pH 8.6)–15% acrylamide gel and electrophoresed for 3 hr in the cold at 8 mA/tube. The gel was scanned at 410 nm of a Gilford spectrophotometer (A, top), stained with Coomassie Brilliant Blue (A, bottom) (Schwarz/Mann Research Laboratories), and cut into 1-mm slices. Two slices were placed into each 5-ml glass scintillation counting vial and 0.2 ml of hydrogen peroxide was added. Each vial was covered and incubated 18 hr at 37° with shaking. After chilling, 4.0 ml of Aquasol (New England Nuclear Corp.) was added and the radioactivity determined (B). The radioactivity in B is corrected to represent the total amount synthesized: red factors (○--○); white factors (●—●).

of the purity of the synthesized and carrier myoglobin has been performed prior to the bulk of the myoglobin synthesis studies. Figure 5A shows both a stained urea–acrylamide gel (pH 8.5) of purified myoglobin and a scan of the gel at 410 nm. Essentially only one band of 410-nm absorbing material and one band of Coomasie Blue staining protein are apparent. On similar gels (Figure 5B) the radioactivity incorporated into myoglobin using red muscle ribosomal initiation factors is seen to migrate in the same manner as myoglobin while very little radioactivity is observed when white muscle initiation factors are added to the incubation mixture. If an identical sample with that in Figure 5B (red muscle initiation factors) is electrophoresed at pH 4.0 on urea–acrylamide gels it is also seen to migrate as a single band (Figure 6). Prior to peptide analysis, a sample of the *in vitro* synthesized myoglobin from the CM-cellulose column was tested for purity on sodium dodecyl sulfate–acrylamide gel electrophoresis and, as shown in Figure 7, both the radioactivity and protein stained band migrate as a superimposable unit. In addition, the total radioactivity applied to the gel appears in the single protein band. Therefore, by electrophoretic analysis in both urea– (pH 4.0 and 8.6) and sodium dodecyl sulfate–acrylamide gels, the myoglobin preparation obtained after the final step of purification (CM-cellulose chromatography) appears to be free from any measurable amounts of contaminating proteins.

In order to determine the fidelity by which myoglobin mRNA is translated, a peptide analysis of the *in vitro* synthesized myoglobin was compared to myoglobin isolated from chicks previously injected with a mixture of $^{14}$C-labeled amino acids. The cell-free system, as described above, contained white muscle ribosomes and unfractionated red muscle initiation factors. The myoglobin products of three reaction mixtures were pooled. As seen in Figure 8, the proteolytic digests of both the $^{3}$H-labeled *in vitro* synthesized myoglobin and $^{14}$C-labeled *in vivo* synthesized myoglobin appear identical by ion-exchange chromatography. Each peptide obtained from the *in vitro* synthesized myoglobin is superimposable with a corresponding peptide from the *in vivo* synthesized myoglobin. These results indicate that myoglobin mRNA is translated with a high degree of fidelity in the cell-free system.

## Discussion

The sequential synthesis of specific proteins or groups of proteins during development is thought to be a result of a programmed change of the information available to the protein synthesizing constituents of the cell. Muscle cells have a number of well-characterized proteins, both fibrous and globular, which appear during terminal differentiation. Therefore, the examination of the manner by which muscle cells regulate the onset of synthesis of these proteins and the amount of the specific proteins synthesized should reveal at least in part the cellular control mechanisms involved in terminal differentiation. Other cell types which synthesize a large amount of a few proteins have generally been used to obtain eukaryotic mRNA (Lingrel *et al.*, 1971; Berns *et al.*, 1972; Rhoads *et al.*, 1971). Muscle, on the other hand, synthesizes a large number of different proteins and the amount of individual mRNA

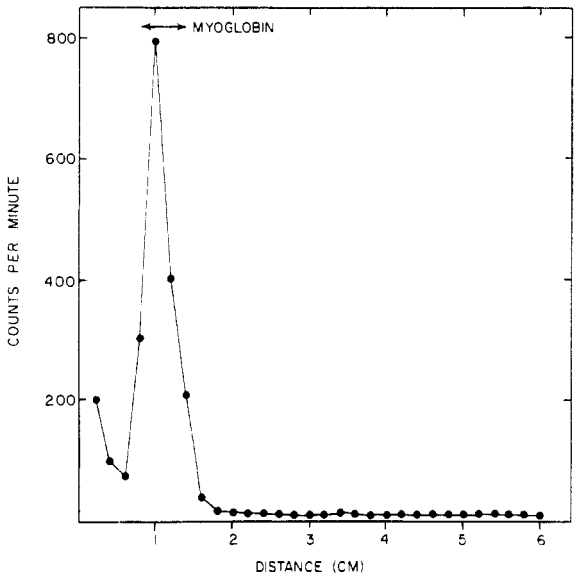


FIGURE 6: Acrylamide gel electrophoresis of purified, *in vitro* synthesized myoglobin at pH 4.0. A second aliquot of the same purified myoglobin as in Figure 5B was electrophoresed, except on a 6 M urea–0.015 M $PO_4$ (pH 4.0)–15% acrylamide gel, and the radioactivity determined as before. The radioactivity here represents only the amount (one-third of the total sample) electrophoresed on the gel.

species obtainable is significantly less. We have developed cell-free amino acid incorporating systems, dependent both on the addition of mRNA fractions and initiation factors, in which the added mRNA must compete with endogenous mRNA already attached to ribosomes. Under these conditions the addition of an IF3 fraction obtained from the same source as the mRNA is required to effectively translate the mRNA into specific protein.

Myoglobin synthesis only accounts for about 1% of the

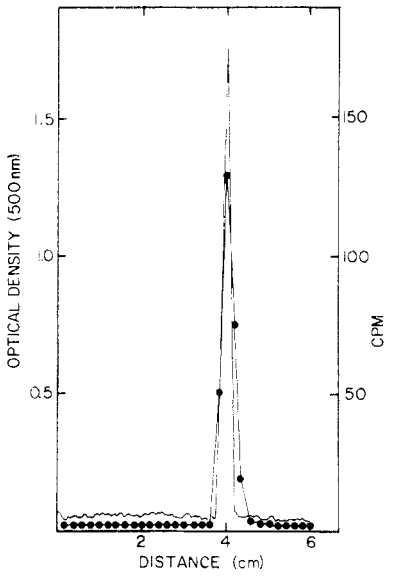


FIGURE 7: Sodium dodecyl sulfate–acrylamide gel electrophoresis of myoglobin synthesized *in vitro*. The myoglobin was synthesized and purified as described under Materials and Methods. An aliquot containing 300 cpm was applied to a 0.1% sodium dodecyl sulfate–0.05 M Tris-acetate (pH 8.6)–10% acrylamide gel and electrophoresed for 3 hr at room temperature. The radioactivity was determined as described in Figure 5: optical density (——); cpm (●—●).

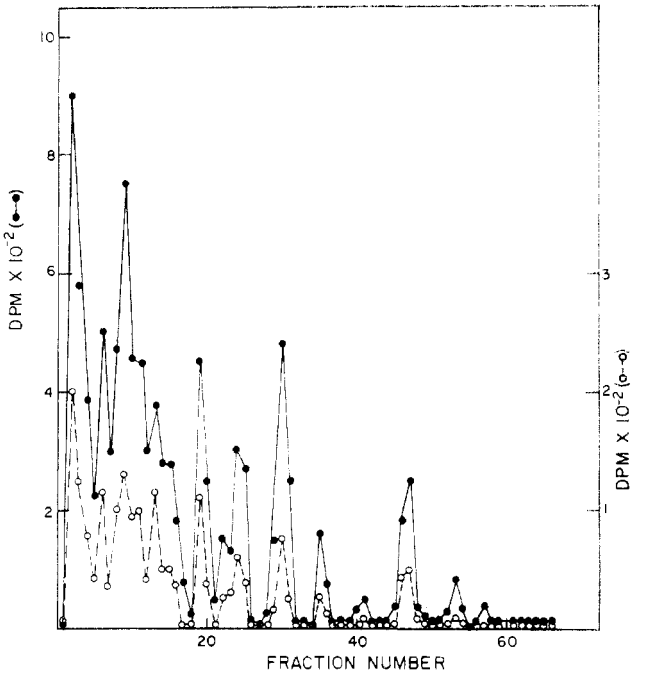


FIGURE 8: Separation of the proteolytic digest of myoglobin by ion-exchange chromatography. The cell-free synthesized, $^3$H-labeled myoglobin (●–●) and the *in vivo*, $^{14}$C-labeled myoglobin (O--O) were prepared as described under Materials and Methods. The proteolytic digest of myoglobin was solubilized in 0.2 M pyridine acetate (pH 3.0) and applied to a 0.6 × 11 cm, Aminex A-5 (Bio-Rad Laboratories) ion-exchange column. The peptides were eluted with a 200-ml linear gradient (0.2 M pyridine acetate (pH 3.0) to 2.0 M pyridine acetate (pH 5.1), in 3-ml fractions, dried, and counted in a liquid scintillation counter as described under Materials and Methods.

total protein synthesis in terminally differentiating red muscle cells (data presented here and unpublished results using late stage muscle cultures). Even given this low percentage of total synthesis, it has been possible to extract an mRNA fraction from polysomes consisting of four–six ribosomes per polysome which directs the synthesis of myoglobin when added to a cell-free amino acid incorporating system. The translation of myoglobin mRNA is accomplished with a high degree of fidelity as judged by the identical manner that peptides from both *in vivo* and *in vitro* synthesized myoglobin elute during ion-exchange chromatography. Therefore, it seems likely that additional mRNAs for muscle specific proteins will be isolated and subsequently translated in cell-free systems. This will eventually enable a detailed analysis of the transcription and translation of a number of mRNAs from one cell type.

The addition of the 8–12S RNA fraction to the heterologous cell-free system containing white muscle ribosomes is required for the synthesis of myoglobin. Also, the ratios of radioactivity in the peptides obtained from *in vivo* and *in vitro* synthesized myoglobin are nearly constant. These facts indicate that the cell-free system is initiating the synthesis of myoglobin utilizing the added mRNA. Since the RNA fraction contains other species of the same size class (mRNAs coding for proteins of 15,000–25,000 daltons), it is not possible at this time to determine the number of molecules synthesized per myoglobin mRNA. This information will have to await the purification of myoglobin mRNA.

In addition to the isolation of an mRNA fraction which can faithfully be translated into myoglobin, an important observation reported here is the requirement of red muscle IF3 fraction for this synthesis of myoglobin on white muscle

ribosomes. This finding is made even more interesting by the fact that both red and white muscle initiation factors are effective in translating myosin mRNA in a heterologous cell-free system. These results suggest that a factor necessary for the translation of myosin mRNA is present in both red and white muscle, but a factor is lacking in white muscle and present in red muscle for the translation of myoglobin mRNA. Previous findings using myosin mRNA (Rourke and Heywood, 1972) and those reported here concerned with myoglobin synthesis suggest that this factor is in the IF3 fraction removed from ribosomes by a high salt wash. This factor has been implicated both with the binding of messengers to ribosomes (Heywood and Thompson, 1970) as well as the recognition of specific mRNA. Therefore, we are led to suggest that there may be a multiplicity of specific factors in the IF3 fraction derived from the same cell type. In addition to our results, several other reports have suggested that ribosomal factors are involved in a messenger selection process in eukaryotes (Ilan and Ilan, 1971; Lebleu *et al.*, 1972; Fuhr and Natta, 1972).

A control mechanism, such as a messenger selection process, operating during the initiation of protein synthesis provides, at a minimum, a fine tuning in post-transcriptional regulation of gene expression in eukaryotic cells. Such a mechanism could not only regulate the onset of synthesis of specific proteins but also influence the amount of specific proteins to be synthesized. In addition, it is unlikely that each mRNA is regulated by a specific factor. Therefore, a useful model for post-transcriptional control of gene expression would entail a relatively small number of specific factors, each of which would recognize different sets of mRNAs whose translation products are functionally related, *i.e.*, myofibrillar proteins, histones, mitochondrial enzymes, etc. In this manner, the process of terminal differentiation of muscle may involve not only the appearance of myofibrillar protein mRNAs in the cytoplasm but also the appearance of a factor which endows specificity of cellular IF3 toward these mRNAs.

A discussion concerning the relationship of our finding with others using different experimental systems with regard to the presence or lack of specificity in mRNA translation has been published (Heywood, 1973).

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