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Myoglobin Biosynthesis in the Embryonic Chick†

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ABSTRACT: The onset of myoglobin biosynthesis in the embryonic chick has been measured by the *in vitro* synthesis of myoglobin from polysomes and by measuring the myoglobin content of thigh muscle. An abrupt rise in myoglobin biosynthetic activity appears near the sixteenth day of development, and the accumulation of myoglobin in the tissue follows this directly. Myoglobin is synthesized on small polysomes containing five–nine ribosomes, and its *in vitro* biosynthesis is considerably stimulated by the addition of heme.

Myoglobin is the reddish pigmented protein found in certain muscle tissues. Its heme group combines with oxygen which can subsequently be released to the tissue to facilitate sustained contraction. Myoglobin accumulates in varying amounts in different muscle tissues; in the adult chicken the thigh muscle has large amounts which give it a dark reddish color. In an earlier study of the polysomes in developing embryonic chick thigh muscle, it was observed that the muscle remains virtually colorless until about the 17th or 18th day of embryonic development at which time a reddish pigment begins to accumulate (Heywood and Rich, 1968). This observation prompted us to study the onset of biosynthesis of myoglobin in the muscle. The analysis was carried out by isolating the polysomes in chick muscle and determining if they were active in myoglobin synthesis. Myoglobin has a polypeptide chain of mol wt 17,500, approximately the same size as that found in the hemoglobin molecule. Hemoglobin is synthesized only on polysomes containing four–six ribosomes (Warner *et al.*, 1962) and, in agreement with earlier work using an antibody method of detection (Kagen and Linder, 1969), myoglobin was found to be synthesized on polysomes in this size range. In the present report the synthesis of myoglobin has been assayed in chick thigh muscle as a function of developmental time in the egg. It is found that the polysomal protein synthesizing system for myoglobin begins to appear around the 16th day of development and rises to a rather high level within a short time period. This synthetic activity has been correlated with a somewhat later accumulation of myoglobin in the tissue. In addition we have demonstrated that *in vitro* myoglobin biosynthesis is considerably stimulated by the addition of heme, as has been previously observed in hemoglobin synthesis (Bruns and London, 1965).

Methods

Purification of Adult Chicken Leg Myoglobin. Myoglobin was extracted from adult male white leghorn chickens according to Akeson *et al.* (1960) using two passages through a carboxymethylcellulose column (CM-cellulose). In one experiment, the myoglobin was also passed through a diethylaminoethylcellulose (DEAE-cellulose) column according to Brown (1961). This last step was usually omitted since it was not necessary in order to obtain myoglobin which was judged pure by spectral and electrophoretic criteria.

Quantitative Estimation of Myoglobin in Embryonic and Adult Muscles. The methods of Akeson *et al.* (1960) were used. One to five grams of muscle were homogenized in 0.75 vol of distilled water (pH 7.0) at 2° and subsequently centrifuged for 30 min at 40,000*g* in a Sorvall RC-2B refrigerated centrifuge. The extraction was repeated and the combined supernatants were adjusted to 50% saturation with respect to ammonium sulfate (pH 7.0). The precipitate was washed once with an equal volume of 50% saturated ammonium sulfate. The suspension was allowed to stand overnight at 2° to assure complete precipitation of the myoglobin. (It was later found that only 2–3 hr was necessary.) The precipitate containing myoglobin was then suspended in 2–4 ml of 0.02 M sodium phosphate buffer (pH 6.5) to which was added 1 mg of potassium ferricyanide to assure complete oxidation of the myoglobin. The resultant clear brown solution was next dialyzed against two changes of 0.02 M sodium phosphate (pH 6.5) (100 vol for each change) for 5 hr and then chromatographed on a 2 cm × 10 cm CM-cellulose column equilibrated with the same buffer. The myoglobin ran directly through the column and was measured by its Soret absorption using an extinction coeffi-

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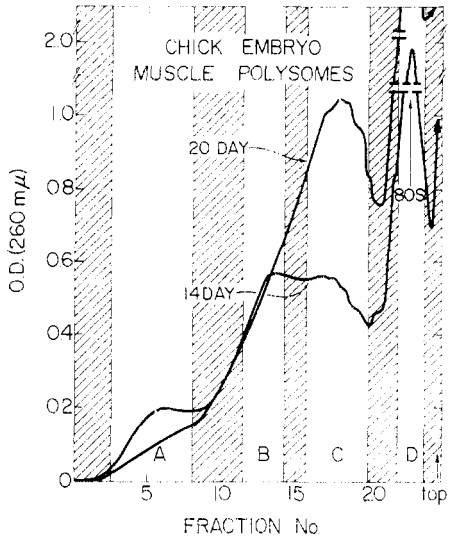

FIGURE 1: Sucrose gradient profile of chick embryonic skeletal muscle polyribosomes. Sedimentation was from right (top of gradient marked by arrow) to left in a 15–40% gradient in MSB buffer (0.01 M Tris (pH 7.4)–0.25 M KCl–0.01 M $MgCl_2$). Centrifugation was for 2 hr at 27,000 rpm, 3°, using a Beckman SW27 rotor in an L2-65 centrifuge. The polysomes were divided into four size classes, A–D, as shown and used in the incubation mixture as described under Materials and Methods.

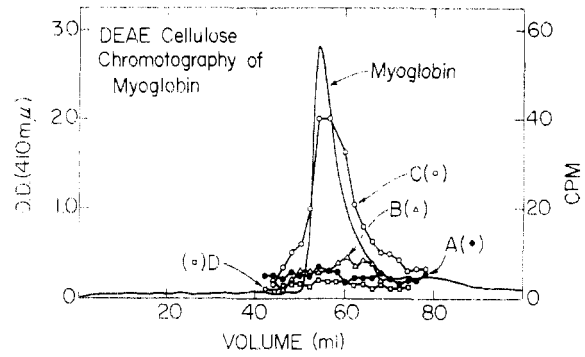

FIGURE 2: DEAE-cellulose chromatography of myoglobin. The chromatography of myoglobin marker is shown by the Soret absorbancy (solid line) together with radioactive protein from the four incubation mixtures A–D. The radioactivity in the several fractions which cochromatograph with myoglobin is shown. Only background radioactivity was found in other portions of the column eluate. The elution buffer was Tris-HCl (pH 8.6).

cient of $\epsilon^{410}_{1\%}$ 91.1, calculated from purified, adult chicken leg myoglobin. Chicken hemoglobin and cytochrome, as well as a crude extract of chicken liver, were shown not to contribute to the Soret band absorption of the column eluate. This procedure yields a 95–98% recovery of myoglobin from the starting material.

Preparation and Fractionation of Polyribosomes and in Vitro Incorporation of ^{14}C-Labeled Amino Acids into Protein. Polyribosomes were isolated from skeletal and heart muscle and fractionated into size classes according to Heywood *et al.* (1967, 1968). The *in vitro* incorporations were carried out as described by those authors, except that 0.015 M creatine phosphate and 20 μg/ml of creatine phosphokinase were used as an energy source (Adamson *et al.*, 1968). Freshly prepared hemin was also present at a concentration of 5.0×10^{-5} M (Zucker and Schulman, 1967, 1968). The effects of hemin additions were determined on cardiac polysomes. Incorporations were carried out for 2 hr at 37° and were terminated by the addition of 50 μg of pancreatic ribonuclease. Radioactive samples were prepared for counting as described previously (Low *et al.*, 1971).

Following the incubation 5–10 mg of purified chicken leg myoglobin was added to each incorporation tube which was subsequently fractionated with ammonium sulfate. The precipitate from 50 to 95% saturation was dissolved in 3 ml of 0.02 M sodium phosphate buffer (pH 6.1) containing 1 mg of potassium ferricyanide and dialyzed overnight at 2° against two changes of buffer. The solution was then chromatographed on CM-cellulose equilibrated with the same buffer (Akeson *et al.*, 1960). The main peak containing 95% of the applied Soret optical density (410 mμ) was refractionated by precipitating the material between 60 and 90% saturated ammonium sulfate. Following dialysis this was chromatographed on DEAE-cellulose, with trishydroxyaminomethane hydrochloride (Tris-HCl) (pH 8.6) according to Brown (1961). This procedure results in a myoglobin preparation with a Soret (410 mμ) *vs.* ultraviolet (280 mμ) absorbance ratio of 4.45 that migrates as a single band upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Both criteria are indicative of a pure protein (Brown, 1961).

Polyacrylamide Gel Electrophoresis. Electrophoresis in sodium dodecyl sulfate was carried out using the method of Weber and Osborn (1969) in gels containing 10% acrylamide and 0.27% methylenebisacrylamide. Gels were also run in 8.0 M urea at pH 8.6 and 4.5 using the method of Leboy *et al.* (1964). Gels containing radioactive protein were sliced and prepared for counting (Low *et al.*, 1971).

Determination of Protein Concentrations. The method of Lowry *et al.* (1951) was used.

Materials. Ribonuclease-free sucrose was purchased from Schwarz BioResearch Inc., DEAE-cellulose (DE-52) and CM-cellulose (CM-52) from Reeve Angel Inc., ribonuclease from Worthington Biochemical Corp., hemin from Eastman Organic Chemicals, ^{14}C-labeled amino acids from New England Nuclear Corp., acrylamide and methylenebisacrylamide, electrophoresis grade, from Bio-Rad Laboratories, and chemicals used in the *in vitro* incorporation from Sigma Chemical Co. All other chemicals were purchased from Mallinckrodt.

Results and Discussion

The goal of these experiments was to measure the ability of muscle polysomes to synthesize myoglobin *in vitro* as a function of the developmental stage in the chick embryo. In our initial experiments, pooled polyribosomes were collected by the myosin precipitation technique (Heywood *et al.*, 1968) and used in *in vitro* amino acid incorporation. It was shown that pooled polyribosomes from the thigh muscle of 8–14-day-old embryos did not synthesize myoglobin in detectable quantities, whereas polyribosomes from 18–20-day-old embryos as well as hatched chicks were active in myoglobin synthesis. In contrast, polyribosomes isolated from cardiac muscle at all ages studied (8–20 days *in ovo*) synthesized myoglobin.

We decided to fractionate the polyribosomes into different size classes in order to localize those polyribosomes responsible for the biosynthesis of myoglobin; in addition, this allowed us to obtain a preparation of polyribosomes more selectively involved in myoglobin biosynthesis. Thigh muscle polyribosomes were collected from sucrose gradients in four fractions, A–D (Figure 1). These were used to direct the *in vitro* synthesis of protein as described under Materials and

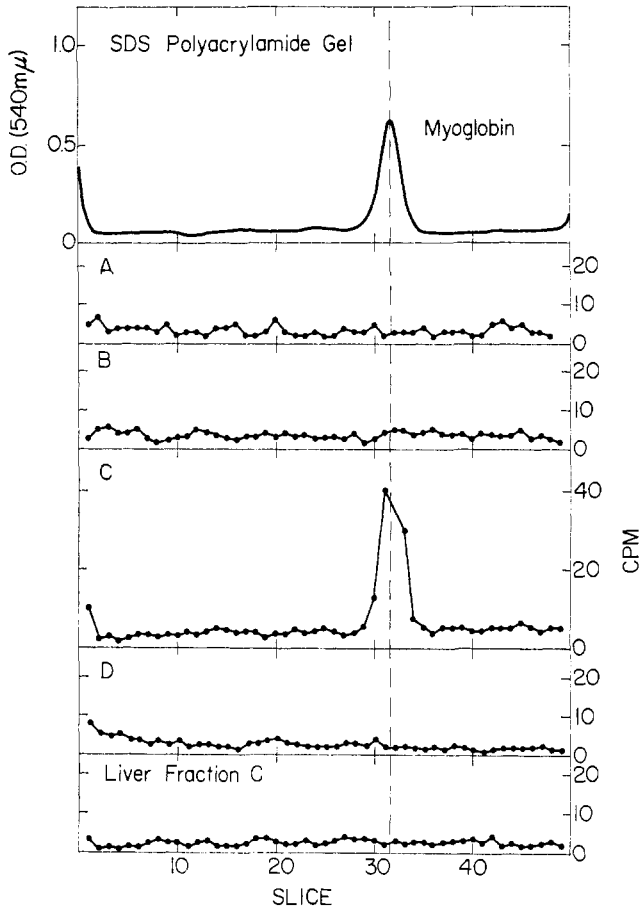

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of myoglobin. Electrophoresis was from left (top of gel marked by arrow) to right, using 10% gels, or 5 hr at 8 mA/tube. After electrophoresis the gels were frozen, sliced, and counted as described by Low *et al.*, 1971. Top panel: electropherogram of myoglobin stained with Coomassie Brilliant Blue. Bottom five panels: electropherograms of the DEAE-cellulose fractions of incubation fractions A–D, plus fraction C from liver polyribosomes (see Figure 5).

Methods. The products of polysome-directed protein synthesis from the fractions were purified for myoglobin as described under Materials and Methods. Figure 2 shows the final DEAE-cellulose chromatograms of the radioactive proteins from thigh muscle of 20-day-old embryos. The only radioactivity eluting with myoglobin is in fraction C. The polysomes in fraction C contain four–nine ribosomes (Heywood *et al.*, 1968) in agreement with Kagen and Linder (1969).

In order to check the validity of the identification by cochromatography, two additional techniques were employed. The results of electrophoresis in sodium dodecyl sulfate–acrylamide gels are shown in Figure 3. In this system, mobility is related to molecular weight. It can be seen that only products from fraction C migrate with myoglobin. No radioactive products were obtained from fractions A, B, and D after the purification as shown in Figure 3. An analogous experiment was carried out with a gel containing 8 M urea at pH 4.5. Figure 4 shows that the mobility of the product of fraction C is the same as that of purified myoglobin. A third gel analysis carried out in 8 M urea at pH 8 gave similar results.

We were unable to detect the biosynthesis of myoglobin by these methods in preparations of thigh muscle polyribosomes from 8-, 10-, 12-, and 14-day embryos even when twice as much polysomal material was used in the incorporations. In those cases, there was no radioactivity in the DEAE-cellulose

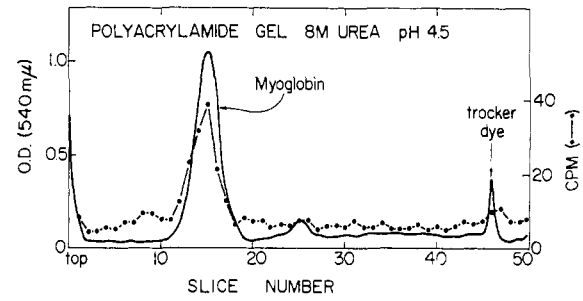

FIGURE 4: Polyacrylamide gel electrophoresis of myoglobin in 8 M urea at pH 4.5. Electrophoresis was from left to right, using 10% gels for 3 hr at 4 mA/tube. Solid line is electropherogram of marker myoglobin; after electrophoresis the gel was stained with Amido Black in 7.5% acetic acid. Dashed line is the electropherogram of the radioactive material of fraction C which cochromatographed with myoglobin in DEAE-cellulose (Figure 2).

chromatograms. Results were equivocal for 16-day embryos, but 18-day embryos clearly demonstrated some myoglobin synthesis.

As a further control, liver polyribosomes of size class C were prepared (Figure 5). In parallel *in vitro* incorporations and analyses, it was shown that these did not direct the biosynthesis of myoglobin (Figure 3).

It has been known for some time that heme participates in the regulation of globin synthesis by erythroid cells (Bruns and London, 1965). We therefore decided to determine whether or not the same was true for myoglobin. Polyribosomes of size class C isolated from cardiac muscle of 16–18-day embryos were incubated with radioactive amino acids in the presence or absence of heme, under the same conditions described for skeletal muscle polysomes. Cardiac muscle polyribosomes were used because muscle from that tissue contains more myoglobin than does thigh muscle (Kagen *et al.*, 1969). Furthermore, we have found that cardiac muscle polysomes are more active in the biosynthesis of myoglobin than are those from the 20-day-old embryonic thigh muscle.

Table I shows the radioactivity found associated with myoglobin after final purification by DEAE-cellulose chromatography. The presence of 5×10^{-5} M hemin enhanced the ability of the polyribosomes to synthesize myoglobin. It remains to be demonstrated whether or not the effect of hemin is by the same mechanism as that found in erythroid cell globin biosynthesis (Mizuno *et al.*, 1972). It is possible that the effect of heme may be nonspecific, as described by Beuzard *et al.* (1973). In this regard, it would be interesting to examine

TABLE I: Effect of 5×10^{-5} M Hemin on the Biosynthesis of Myoglobin by Cardiac Muscle Polyribosomes.[a]

Myoglobin Radioactivity (cpm)	
−Heme	+Heme
210 ± 35	418 ± 52

[a] A polysomal protein synthetic system was obtained from 20-day-old embryonic cardiac muscle and it was incubated with ^{14}C-labeled amino acids as described under Materials and Methods. Following the incorporation, myoglobin was isolated. The numbers are the mean radioactivity from three experiments (± the standard deviation) recovered as myoglobin after the final DEAE-cellulose chromatography.

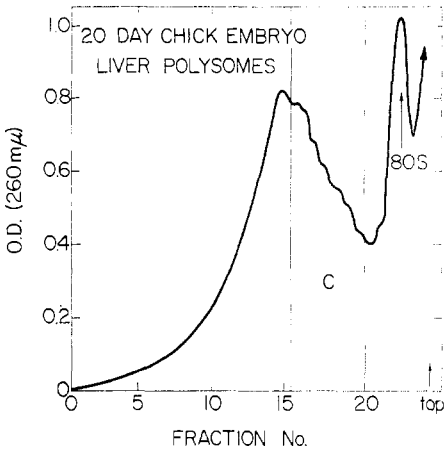

FIGURE 5: Sucrose gradient profile of chick embryonic (18 day *in ovo*) liver polyribosomes. Sedimentation was from right (top of gradient marked by arrow) to left in a 15–40% gradient in MSB buffer for 2 hr at 27,000 rpm, 3°, using a Beckman SW27 rotor in an L2-65 centrifuge. Polysomes in fraction C were collected and used in the incubation mixture as described under Materials and Methods.

the effect of hemin on polysomes synthesizing myosin and actin.

Experiments were next performed to compare the myoglobin synthetic ability of chick thigh muscle polysomes with the appearance of myoglobin during development. Figure 6A shows the myoglobin content of chick thigh muscle. Myoglobin begins to accumulate in the leg musculature of the chick after 16 days of development *in ovo*. That is the same time at which the muscle begins to darken. There is a gradual increase in myoglobin content up to the time of hatching (21 days). The concentration of this heme protein at hatching is six–seven times less than that found in adult thigh muscle, in agreement with the findings of Kagen and Linder (1968).

Figure 6B shows the changing ability of thigh polysomes to synthesize myoglobin in a standard incorporation experiment as a function of development time. There is no synthetic capacity up to 14 days; a sharp rise is seen, however, at 16–18 days. Thus, a substantial synthetic capability is expressed in this tissue over a comparatively short time period. It is interesting to note that the rate of increase of the synthetic ability appears to decline somewhat over the period 18–20 days, compared to 16–18 days. However, the accumulation of myoglobin continues steadily since the adult level is substantially greater than the last point on curve A. These two curves, A and B, are not normalized with respect to each other since different analytical methods are used to make the measurements. If the curves were normalized and one were able to quantitate the rate of myoglobin synthesis on polysomes, the synthetic activity seen in curve B should make it possible to calculate curve A directly, assuming that there is little myoglobin degradation in the embryonic chick.

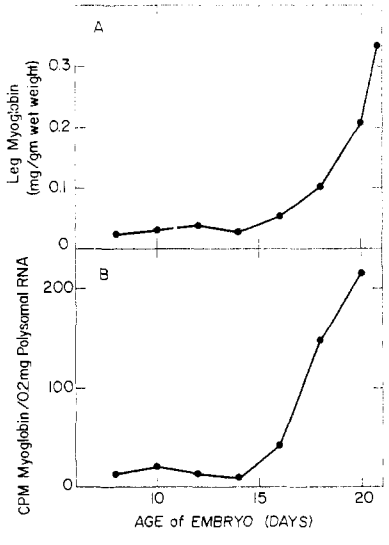

FIGURE 6: Comparison of the myoglobin synthesizing capacity of thigh muscle polyribosomes with the appearance of thigh myoglobin during development. Polysomal incubation and myoglobin isolation were as described under Materials and Methods. The myoglobin content of adult thigh muscle was found to be 1.930 mg/g wet weight.

Kagen and coworkers have studied the biosynthesis of myoglobin in duck and chicken embryos (Kagen and Linder, 1968, 1969, 1970; Kagen *et al.*, 1969). Employing an immunological assay, they have pointed out that myoglobin is synthesized very early in development in various body segments such as hind body, mid body, and cardiac regions. In our analyses we have selected an individual muscle tissue in which myoglobin synthesis is initiated at a later time during development. It is clear that myoglobin is used in the heart at an early stage of embryological development; limb demands occur only at a much later stage. While there are some advantages in immunological assays, there are also substantial hazards associated with nonspecific cross-reactivity which may occur with unfractionated materials from whole tissues (Holme *et al.*, 1971). In our work we have carried out a chemical isolation before assaying for myoglobin content or synthesis.

The abrupt appearance of myoglobin synthesizing polysomes in embryonic chick thigh tissue makes this a useful system for studying aspects of developmental biochemistry. As shown in Figure 1, there is a substantial increase in the fraction C polysomes where myoglobin synthesis occurs on comparing the 20-day to the 14-day embryo. Although this increase is undoubtedly associated with the enhanced synthesis of other small proteins, such as tropomyosin, a comparison of muscle tissue and of these polysomes at the two different time periods may lead to further insight into the control and regulation of protein synthesis in the embryo.

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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.