Purification of Messenger Ribonucleic Acids for Fast and Slow Myosin Heavy Chains by Indirect Immunoprecipitation of Polysomes from Embryonic Chick Skeletal Muscle†

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ABSTRACT: Fast and slow myosin heavy chain mRNAs were isolated by indirect immunoprecipitation of polysomes from 14-day-old embryonic chick leg muscle. The antibodies were prepared against myosin heavy chains purified by NaDodSO$_4$–polyacrylamide gel electrophoresis and were shown to be specific for fast and slow myosin heavy chains. The RNA fractions directed the synthesis of myosin heavy chains in a cell-free translation system from wheat germ. Several smaller peptides were also synthesized in lower concentrations. These probably are partial products of myosin heavy chains, since they are immunoprecipitated with antibodies to myosin heavy chains. Immunoprecipitation of the translation products with the antibodies to fast and slow myosin heavy chains showed the RNA preparations to be ~94% enriched for fast myosin heavy chain mRNA and ~84% enriched for slow myosin heavy chain mRNA with respect to myosin HC type. Peptides having slightly different mobilities on NaDodSO$_4$–polyacrylamide gels were immunoprecipitated by antibodies to fast and slow myosin heavy chains.

Contractile and regulatory proteins in skeletal muscle are known to exist in multiple polymorphic forms (Huszar, 1972; Starr & Offer, 1973; Masaki, 1974; Burridge & Bray, 1975; Whalen et al., 1976, 1978; Patrinou-Georgoulas & John, 1977; Gauthier & Lowey, 1977; Dhoot & Perry, 1979). In several cases, the expression of polymorphic forms has been shown to follow a characteristic pattern during embryonic development and during the postnatal period (Masaki & Yoshizaki, 1974; Rubinstein et al., 1977; Gauthier et al., 1978; Dhoot & Perry, 1979). Furthermore, alterations in the contractile function of the muscle may markedly affect the synthesis of different polymorphic forms (Close, 1972). Thus, a study of control of transcription of messenger RNA coding for individual molecular myofibrillar proteins may elucidate not only developmental mechanisms but also the effects of physiological conditions on gene expression.

At least three polymorphic forms of myosin heavy chain (HC)[1] have been rigorously demonstrated, i.e., fast and slow skeletal and cardiac myosin HC (Masaki, 1974; Flink, et al., 1979). Immunologic procedures indicate that all three molecular forms may be present during early embryonic periods both in skeletal and in cardiac tissue (Masaki & Yoshizaki, 1974; Masaki & Kinoshita, 1974; Gauthier et al., 1978). Moreover, an embryonic form of skeletal myosin HC has recently been documented (Rushbrook & Stracher, 1979; Whalen et al., 1979). Evidence has also been presented for additional forms of cardiac myosin HC in embryonic heart (Silver & Strohman, 1977), in the atrium and ventricle (Long et al., 1977; Sartone et al., 1978), in hearts of thyrotoxic rabbits (Flink & Morkin, 1977; Flink et al., 1979), and in hypertrophied hearts (Shiverick et al., 1976; Thomas & Alpert, 1977; Lompre et al., 1979). The actual number of molecular species of myosin HC may be considerably greater than those currently demonstrated, analogous to the multiple forms of vitellogenin (Wahli et al., 1979) and actin (McKeown et al., 1978; Kindle & Firtel, 1978) shown by cloning procedures.

Fast and slow myosin HC's are present in various adult skeletal muscles in different proportions (Arndt & Pepe, 1975; Gauthier & Lowry, 1977) and display a characteristic pattern during embryonic development and the postpartum period (Masaki & Yoshizaki, 1974; Gauthier et al., 1978). Expression of one or another form appears to be related at least in part to the nature of nerve innervation (Close, 1972), since cross innervation experiments or administration of repetitive stimuli to fast muscle markedly changes the type of myosin LC's (Sreter et al., 1974, 1973) and myosin HC's (Rubinstein et al., 1978) present. These stimuli alter the expression of genes for many other muscle proteins, resulting in a muscle of completely changed physiologic properties (Sreter et al., 1975b).

We have purified mRNAs for fast and slow myosin HC from chick embryonic skeletal muscle with the long-term goal of examining the changes in transcription of muscle proteins caused by altered physiologic stimuli. Antibodies were produced to myosin HC purified from anterior (ALD) and posterior latissimus dorsi (PLD) muscle, which predominantly contain slow and fast myosin HC, respectively (Arndt & Pepe, 1975).[2] The specificity of these antibodies was characterized; they were then used to immunoprecipitate polysomes and to characterize the product synthesized in a wheat germ cell-free translation system in response to addition of mRNAs. Im-

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[1] Abbreviations used: HC, heavy chain; ALD, anterior latissimus dorsi; PLD, posterior latissimus dorsi; EM, 17-day-old embryonic chick leg muscle; BSA, bovine serum albumin; DMAPN, dimethylaminopropionitrile; DOC, sodium deoxycholate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin G; LC, light chain; NaDodSO$_4$, sodium dodecyl sulfate; Cl$_3$AcOH, trichloroacetic acid; Temed, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; OAc, acetate.

[2] Avian PLD is classified as a fast-twitch muscle, and ALD is classified as a slow-tonic muscle. The myosin HC's prepared from these two sources are referred to as fast and low myosin HC, respectively.

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munoprecipitation of polysomes resulted in the considerable purification of mRNAs for myosin HC. Furthermore, preparations of fast and slow myosin HC mRNAs were enriched 94 and 84%, respectively, relative to the myosin HC isoenzymes. These mRNAs may be used in the preparation of cDNA and in the identification of clones which can be used as probes for the quantitation of the mRNAs.

Materials and Methods

Materials. Sodium deoxycholate and ultrapure grades of sucrose (RNase free), ammonium sulfate, and urea were obtained from Schwarz/Mann. $NaDodSO_4$, acrylamide, bis(acrylamide), Temed, and ammonium persulfate were purchased from Bio-Rad. The following products were obtained from the sources indicated: Chloramine-T, Gallard Schlessinger; agarose (Seakem), Marine Colloids, Inc.; DMAPN, Aldrich; Scintisol, Isolab, Inc.; GTP (lithium salt), P-L Biochemicals; creatine phosphokinase, Boehringer-Mannheim; leupeptin, Peninsula Laboratories; CNBr-activated Sepharose 4B, Pharmacia; Noble agar, Difco. L-[^{3}H]Leucine (58–60 Ci/mmol), L-[U-^{14}C]leucine (348 mCi/mmol), and L-[^{35}S]-methionine (>500 Ci/mmol) were purchased from Amersham/Searle. L-[^{35}S]Methionine (>500 Ci/mmol, translation grade) was obtained from New England Nuclear, as was carrier-free Na^{125}I (50 mCi in 0.1 N NaOH). Goat antirabbit IgG antibody (IgG fraction) was obtained from Miles or Cappel Laboratories.

Preparation of Myosin. Myosin from the leg muscle of 17-day-old chick embryos (EM myosin) was prepared according to procedure II of Paterson & Strohman (1970) and purified further by chromatography on DEAE-Sephadex A-50 (Richards et al., 1967). The chromatography was modified as follows. In contrast to the original procedure, myosin was dialyzed against 90 mM NaCl, 40 mM $Na_4P_2O_7$, pH 7.5, and 1 mM DTT and applied to a column equilibrated with the same buffer, and the myosin was then eluted with a linear salt gradient from 0.09 to 0.5 M NaCl.

Myosin from the ALD and PLD muscles of adult chickens was prepared according to the method of Shiverick et al. (1975).

Electrophoretic Purification of Myosin HC. Myosin was dialyzed overnight at room temperature against 10 mM NaP_i, pH 7.1, 0.1% $NaDodSO_4$, and 0.1% 2-mercaptoethanol and stored at −20 °C. Prior to electrophoresis, the solution was made 2% in $NaDodSO_4$ and 1% in 2-mercaptoethanol. The sample was heated for 3 min at 100 °C, incubated for 60–90 min at 45 °C, and then mixed with 0.28 volume of glycerol–2-mercaptoethanol–0.06% bromphenol blue (5:1:1).

The initial electrophoresis of myosin was done on 0.5% agarose–3% acrylamide gels containing $NaDodSO_4$. The gel solution was prepared as described by Peacock & Dingman (1968), with the exception that the buffer in the gel (electrophoresis buffer) was 50 mM NaP_i, pH 7.1, and 0.1% $NaDodSO_4$. Gels were polymerized in 1.6 × 9 cm glass gel tubes for 30 min in a water bath at 15–20 °C and for an additional 60 min at room temperature. A sample well was formed by polymerizing of the gel with a 1.4-cm Plexiglas rod inserted into the gel solution to a depth of 2.4 cm (Lee & Sinsheimer, 1974). The resulting sample well ensured a flat upper surface and prevented the sample from running down the side of the tube between the gel and the glass.

Nylon netting (held in place with a rubber band) supported the gels, and efficient heat dissipation was achieved by submersion of as much of the gel as possible in the electrophoresis buffer. Following a preelectrophoresis at 15 mA/gel for 1 h, samples containing 0.8–1 mg of protein were layered into the sample wells. Electrophoresis was carried out at 15 mA/gel for 10.5 h or until the tracking dye had migrated along the length of the gel (5.8 cm).

A Schlieren line which, upon staining of the gel, was coincident with the leading edge of the myosin HC band was apparent during the electrophoresis. With the Schlieren line—which migrated 2.4 cm—used as a marker, a 7-mm slice of gel containing the myosin HC band was excised, leaving behind approximately 1.5 mm of the leading edge.

The myosin HC in the gel slices was purified further on 5% polyacrylamide–$NaDodSO_4$ gels. The gels were prepared in 1.6 × 9 cm gel tubes according to the method of Weber & Osborn (1969) with the following modification. The buffer in the gel (i.e., electrophoresis buffer) was changed to 89 mM Tris, 89 mM H_3BO_3, and 2.5 mM EDTA (Peacock & Dingman, 1968) with 0.1% $NaDodSO_4$. Following preelectrophoresis at 12 mA/gel for 20 min, the agarose–acrylamide gel slices were stacked (1 slice/gel) onto the surface of the 5% polyacrylamide gels. Electrophoresis was carried out at 12–20 mA/gel for 2–3 h or until the tracking dye had migrated along the length of the gel (6.7 cm).

A Schlieren line, migrating 1.4–1.7 cm, was also apparent during the second electrophoresis. On the basis of its position, a 6-mm slice of gel containing the myosin HC band was excised, leaving behind ~0.5 mm of the leading edge. Staining of the remaining fragments from both types of gels with Coomassie brilliant blue confirmed that the gels had been sectioned accurately.

Myosin HC was electroeluted from the polyacrylamide gel slices into a dialysis sac in a manner similar to that described by Stephens (1975). Electroelution was carried out at 120 V for 16–20 h in a buffer consisting of 17.8 mM Tris, 17.8 mM H_3BO_3, 0.5 mM EDTA, and 0.1% $NaDodSO_4$. It was estimated that ~50% of the protein initially loaded onto the agarose–acrylamide gels was recovered following electroelution.

Preparation of Antibodies. For the primary injection of antigen, the polyacrylamide gel slice containing ALD, PLD, or EM myosin HC was emulsified with complete Freund's adjuvant. The mixture was injected (0.2 mL/site) into the hind footpads and subcutaneously into multiple sites in the scapular region of a female New Zealand white rabbit (Erickson & Steers, 1970; Spielman et al., 1974). A booster injection consisting of a suspension of electroeluted myosin HC adsorbed onto potassium alum (Campbell et al., 1970) was given intravenously (Lazarides, 1975) 4 weeks later. Blood was drawn at 7, 10, and 14 days after the booster injection. Following a rest of 1–2 weeks, the rabbit was "boosted" again and bled on the same schedule and thereafter when necessary. Antibodies to BSA were obtained by immunization of rabbits with 5 mg of BSA emulsified with complete Freund's adjuvant, followed by intravenous booster injections of 1 mg of BSA, as described above.

Freshly drawn blood was allowed to clot at 37 °C for 1–2 h. The serum was separated from the clot by centrifugation and fractionated with ammonium sulfate at 40% saturation as described by Palmiter et al. (1971), except that the phosphate buffer was changed to 0.4 M NaCl and 0.01 M NaP_i, pH 7.5.

Rabbit IgG from the serum of unimmunized animals was used as a control for immunoprecipitations; it is referred to as nonimmune rabbit IgG.

Purification of Antibodies. Immunoadsorbents were prepared with myosin HC that was obtained on dissociation of ALD or PLD myosin with 8 M urea (Wikman-Coffelt et al., 1973a). Following dialysis of the protein against coupling

buffer [0.5 M NaCl and 5 mM triethanolamine, pH 8.5 (Bottomley & Trayer, 1975)], the protein solution was centrifuged for removal of aggregates and mixed with washed CNBr-activated Sepharose 4B (≥3 mg of protein per mL of resin) for 20 h at 4 °C. The suspension was then mixed with an equal volume of 0.5 M NaCl and 2 M ethanolamine, pH 8, for 10 h, so that unreacted groups were blocked. A column was made with the resin and washed successively with coupling buffer, with three cycles of 3–5 column volumes of 1 M NaCl and 40 mM $Na_4P_2O_7$, pH 7.5, followed by 3–5 column volumes of 1 M NaCl and 0.1 M NaOAc, pH 4.8, and finally with phosphate buffer. EM myosin HC and BSA immunoadsorbents were prepared in the same manner.

Affinity chromatography of the crude IgG fractions was done in phosphate buffer at 4 °C, essentially as described by Shapiro et al. (1974). The bound antibody was eluted with 0.4 M NaCl and 0.1 M glycine hydrochloride, pH 2.8, and dialyzed successively against 0.4 M NaCl, 50 mM NaP_i, pH 7.5, and phosphate buffer. The antibody was concentrated with an Amicon Macrosolute concentrator (B-15) or with ammonium sulfate at 40–50% saturation.

The antibodies used for polysome binding experiments or for precipitating polysomes were made RNase free by chromatography on DEAE- and CM-cellulose, as described by Palacios et al. (1972).

Ouchterlony Immunodiffusion. Immunodiffusion was done on plates made with 0.85% Difco Noble agar in 0.15 M NaCl, 40 mM $Na_4P_2O_7$, pH 7.5, 1 mM EDTA, and 0.02% NaN_3, as described by Puszkin et al. (1975).

Iodination of Myosin and Antibodies. All iodinations were based on the Chloramine-T method of Greenwood et al. (1963), as modified by McConhey & Dixon (1966). The myosin was purified on DEAE-Sephadex and did not contain DTT. Polypropylene tubes were used throughout the procedure.

To a 90-μL reaction mixture (at 4 °C) containing 50 μg of myosin, 250 μCi of ^{125}I, 0.15 M NaCl, and 40 mM $Na_4P_2O_7$, pH 7.4, was added 10 μL of freshly prepared Chloramine-T [2.5 mg/mL in pyrophosphate buffer (0.15 M NaCl, 40 mM $Na_4P_2O_7$, pH 7.5, and 1 mM EDTA)]. The reaction was incubated at 4 °C for 5 min—with intermittent mixing—and quenched by addition of 10 μL each of 0.2 M DTT and KI (20 mg/mL in pyrophosphate buffer). The [^{125}I]myosin was separated from free ^{125}I by gel filtration at room temperature on a 0.8 × 24 cm column of Sephadex G-25 (fine). Prior to use, 10 mg of BSA was passed through the column, and the column was extensively washed with pyrophosphate buffer. The [^{125}I]myosin was made 0.05% in BSA, dialyzed against pyrophosphate buffer (4 °C), centrifuged in a Brinkmann Model 5412 microfuge (12800*g*) for 10 min, and stored at 4 °C. The specific radioactivity of [^{125}I]myosin ranged from 2×10^5 to 4×10^5 cpm/μg.

The following changes were made for the iodination of antibodies. Iodination reactions were done in 0.15 M NaCl and 50 mM NaP_i, pH 7.5. When RNase-free antibodies were required, 15 mM NaCl and 10 mM NaP_i, pH 7.5, were used for gel filtration; otherwise, 0.15 M NaCl and 10 mM NaP_i, pH 7.5, were used. Five microliters of $Na_2S_2O_5$ (5 mg/mL in the appropriate buffer) was added in place of DTT.

The ^{125}I-labeled antibody was made RNase free by chromatography on DEAE- and CM-cellulose. The specific radioactivities ranged from 0.7×10^6 to 1.3×10^6 cpm/μg.

Indirect Immunoprecipitation of [^{125}I]Myosin. Immunoprecipitations were carried out in 1.5-mL conical polypropylene microfuge tubes (Starstedt). The use of glass tubes resulted in high backgrounds, probably due to the irreversible adsorption of some myosin to glass and the small quantities of [^{125}I]myosin precipitated. [^{125}I]Myosin (≤0.25 μg) was incubated with 17 μg of rabbit IgG in a total volume of 0.1 mL of 0.15 M NaCl, 40 mM $Na_4P_2O_7$, pH 7.5, 1% Triton X-100, and 1% DOC. Dilutions of the specific rabbit IgG were made with nonimmune rabbit IgG so that the total amount of IgG was kept at 17 μg. Following a 1-h incubation at 37 °C, the rabbit IgG was precipitated with goat antirabbit IgG overnight at 4 °C.

Lyophilized goat antirabbit IgG (IgG fraction) was obtained commercially and was reconstituted in pyrophosphate or phosphate buffer. As determined with ^{125}I-labeled normal rabbit IgG, a 6:1 ratio of specific goat antibody to rabbit IgG was necessary for the complete precipitation of the rabbit IgG under the above conditions. Only the IgG fraction of the goat antirabbit IgG antibody was used, as the serum fraction sometimes contained a heat-sensitive substance that precipitated myosin nonspecifically.

The immunoprecipitates were diluted with 0.2 mL of cold wash buffer (0.15 M NaCl and 40 mM $Na_4P_2O_7$, pH 7.5) and pelleted in a microfuge for 1 min. The supernatant was aspirated off, and the pellets were washed 2 times with 0.4 mL of wash buffer. Radioactivity was measured directly in a NaI γ scintillation counter.

Preparation of Polysomes. The preparation of polysomes and RNA was done at 4 °C. The sterile technique (Palacios et al., 1972) was used to minimize RNase contamination.

Leg muscle from 14-day-old chick embryos was homogenized in polysome buffer [0.25 M KCl, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, heparin (500 μg/mL), and cycloheximide (50 μg/mL)] containing 0.25 M sucrose and 15 mM 2-mercaptoethanol, as described by Heywood et al. (1967). The homogenate was centrifuged at 9000*g* for 10 min, and then the supernatant was centrifuged at 10000*g* for 10 min. The supernatant was made 1% in both Triton X-100 and DOC and layered onto discontinuous gradients consisting of 1.5 mL of 2.5 M sucrose in polysome buffer minus heparin, overlaid with either 4 mL of 1 M sucrose or 2.5 mL of 1 M sucrose and 1.5 mL of 1.8 M sucrose, all in polysome buffer plus 1% Triton X-100 and 1% DOC. Gradients were centrifuged in a Beckman SW 41 rotor at 40000 rpm for 1 h. Polysomes banding at the 2.5 M sucrose interphase were collected in a sterile syringe, as described by Palacios et al. (1972). The polysome preparations, ranging from 20 to 40 A_{260} units/mL, were stored at −20 °C and were usually used within 1 day of preparation.

Immunoprecipitation of Polysomes. Polysomes isolated from 1.0–1.8–2.5 M sucrose step gradients were centrifuged at 16000*g* for 20 min for removal of aggregates. Thirty to forty A_{260} units of polysomes at a concentration of ≥15 A_{260} units/mL was incubated for 60 min with specific antibody (10 μg/A_{260} unit) in polysome buffer containing 1% DOC, 1% Triton X-100, and sufficient NaCl to bring the monovalent salt concentration to 0.3 M. The rabbit IgG was then precipitated by addition of twice the amount of goat antirabbit IgG antibody necessary for complete precipitation (usually to a 10- to 12-fold weight excess of specific goat antibody). The additions were such that the final concentration of polysomes was ≥10 A_{260} units/mL and the specific rabbit antibody concentration was at *least* 100 μg/mL. Immunoprecipitates were allowed to form for 2.5 h on ice and were then sedimented twice through discontinuous sucrose gradients, as described by Shapiro et al. (1974).

Isolation of RNA. The immunoprecipitates were dispersed in 0.1 M Tris-HCl, pH 9.0, 10 mM EDTA, 2% $NaDodSO_4$,

and heparin (100 μg/mL), and the RNA was extracted by the phenol–chloroform–isoamyl alcohol method of Aviv & Leder (1972). RNA from polysomes was extracted in the same way after precipitation of the polysomes with 2 volumes of ethanol at −20 °C. The RNA precipitates were washed twice with 4 M LiCl, 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, as described by Palmiter (1974), redissolved in water, and precipitated with 2.5 volumes of ethanol at −20 °C. The final pellets were dried in vacuo, redissolved in water, and stored at −20 or −70 °C.

In some instances, poly(A) RNA was isolated from total polysomal RNA by chromatography on poly(U)–Sepharose 4B (Shapiro & Schimke, 1975; Taylor & Tse, 1976).

Cell-Free Translation in a Wheat Germ System. A wheat germ S-30 extract was prepared by a modification of the method of Roberts & Paterson (1973) which incorporated the grinding and extraction of Marcu & Dudock (1974), the buffers of Kennedy & Heywood (1976), and substitution of KOAc and $Mg(OAc)_2$ for KCl and $MgCl_2$ (Weber et al., 1977). Wheat germ (General Mills) was separated from chaff and endosperm as described by Shih & Kaesberg (1973). Four grams of wheat germ was ground for 1–2 min, dry, in a mortar with an equal quantity of powdered glass. Eight milliliters of extraction buffer [20 mM Hepes, pH 7.6, 0.11 M KOAc, 3 mM $Mg(OAc)_2$, 0.2 mM spermine, 2 mM $CaCl_2$, 10% glycerol, and 6 mM 2-mercaptoethanol] was added to the powder and mixed with a spatula for 1 min. The thick paste was centrifuged at 230000*g* for 10 min, and then the supernatant was centrifuged at 30000*g* for 10 min. The supernatant (minus the lipid layer) was applied to a 1.5 × 60 cm column of Sephadex G-25 (medium) equilibrated with extraction buffer minus the $CaCl_2$ and glycerol. The first half of the turbid fractions, appearing after the void volume, was pooled, centrifuged at 30000*g* for 20 min, and stored at −70 °C in 0.2-mL aliquots. The extract contained ~50 A_{260} units/mL and had an A_{260}/A_{280} ratio of 1.86.

Cell-free translations were done in 50-μL reaction mixtures containing 20 mM Hepes, pH 7.4, 0.11 M KOAc, 3 mM $Mg(OAc)_2$, 2 mM DTT, 2.5 mM ATP, 0.5 mM GTP, 8 mM phosphocreatine, 0.5 μg of creatine phosphokinase, 0.1 mM of each unlabeled amino acid, 60 μM spermine, 10 μCi of L-[^{3}H]leucine or 15 μCi of L-[^{35}S]methionine, 15 μL of wheat germ S-30, and total RNA (<15 μg) or poly(A) RNA (<2 μg). The mixtures were incubated at 23 °C (Zehavi-Willner & Pestka, 1976) for 120–180 min. Five (^{3}H-labeled) or two (^{35}S-labeled) microliter aliquots of reaction mixture were spotted on Whatman 3MM filter disks and precipitated and washed with Cl_3AcOH, ethanol, and ether as described by Roberts & Paterson (1973). Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter, in 10 mL of Scintisol. Aliquots for $NaDodSO_4$–polyacrylamide gel electrophoresis were first precipitated with 9 volumes of cold acetone and then dissolved in $NaDodSO_4$ buffer (see below).

Immunoprecipitation of Cell-Free Translation Products. Following cell-free translation, 40 μL of the reaction mixture was mixed with 40 μL of phosphate buffer and 20 μL of 5% DOC, 5% Triton X-100, 5% BSA, and 0.05 M Met (or Leu) in phosphate buffer. The solution was centrifuged in a microfuge for 10 min, and 25-μL aliquots of the supernatant were incubated with 7 μg of rabbit IgG in a total volume of 80 μL in phosphate buffer, 1% DOC, 1% Triton X-100, 1% BSA, and 10 mM Met (or Leu). When a smaller amount of specific antibody was used, nonimmune rabbit IgG was added to bring the total amount of antibody to 7 μg. Following a 60-min incubation at room temperature, a 10- to 12-fold weight excess of specific goat antirabbit IgG antibody over rabbit IgG was added. Immunoprecipitates were allowed to form for 2–2.5 h at room temperature and were sedimented through 200-μL discontinuous sucrose gradients containing phosphate buffer, 1% DOC, 1% Triton X-100, 0.1% BSA, and 10 mM Met (or Leu), as described by Shapiro et al. (1974) and Rhoads et al. (1973). The pellets were resuspended, washed twice more over discontinuous sucrose gradients, and dissolved in $NaDodSO_4$ buffer (see below).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis of cell-free translation products and immunoprecipitates was done on slab gels in a discontinuous buffer system as described by Laemmli (1970) and by Laemmli & Favre (1973). A 3% acrylamide stacking gel was used in conjunction with various separating gels. Samples were mixed with 50 μL of $NaDodSO_4$ buffer (62.5 mM Tris-HCl, pH 6.8, 2% $NaDodSO_4$, 20 μM leupeptin, and 2.5% 2-mercaptoethanol), heated in a boiling-water bath for 3 min, and then incubated at 45 °C for 90–120 min. Prior to electrophoresis, 0.3 volume of a mixture of glycerol–2-mercaptoethanol–0.06% bromphenol blue (5:1:1) was added to the sample. Electrophoresis was carried out at 30 mA until the tracking dye migrated along the length of the gel. Gels were fixed in 10% acetic acid and 25% isopropyl alcohol and stained with Coomassie brilliant blue as described by Fairbanks et al. (1971). Gel slabs were prepared for fluorography by the method of Bonner & Laskey (1974). Exposure of Kodak RP Royal X-Omat film to the dried gel was done at −70 °C.

Cell Cultures. Muscle cell cultures were prepared from 11-day-old embryonic chick leg muscle as described by Shimada et al. (1967). Cells were grown in collagen-coated 60-mm Petri dishes, at an initial density of 3 × 10^6 cells/dish, in 3 mL of medium (85% Eagle's MEM, 10% horse serum, 5% embryo extract, 1% glutamine, and 1% penicillin–streptomycin). Cultures were incubated at 37 °C in 95% air and 5% CO_2. The medium was replaced at 48 h with fresh medium containing L-[^{14}C]leucine at 1.3 μCi/mL, and the cultures were labeled for 12–15 h. Cells were harvested and washed 3 times with Tyrode's solution.

Protein Determination and Other Conversions. Protein was determined by the microbiuret method of Itzhaki & Gil (1964), with BSA used as the standard. Generally, protein and RNA were determined with the following extinction coefficients or conversions: column-purified PLD myosin, $E_{280}^{1\%}$ = 5.05; column-purified ALD myosin, $E_{280}^{1\%}$ = 5.35 (Wu, 1969); 1 mg of IgG = 1.4 A_{280} units (Palmiter et al., 1972); 1 mg of RNA = 25 A_{260} units.

Results

Preparation of Antigens. Fast and slow myosins were isolated from the PLD and ALD muscles of adult chickens, respectively (Arndt & Pepe, 1975). On $NaDodSO_4$–polyacrylamide gels (Figure 1), ALD myosin showed only the two LC's characteristic of slow myosin, and PLD myosin showed only the three LC's characteristic of fast myosin (Sarkar et al., 1971; Sreter et al., 1974). Myosin from 17-day-old embryonic chick leg muscle, however, showed LC's corresponding to both types of myosins. The LC pattern of embryonic myosin substantiated the immunological evidence for the presence of at least two types of myosins (Masaki & Yoshizaki, 1974; Gauthier et al., 1978) and indicated a predominance of a fast type of myosin at this stage of development.

As shown in Figure 1, the best preparations of column-purified myosin still contained proteins in the 150000–170000-dalton range, supposedly from the M lines of myofibrils (Masaki & Takaiti, 1972; Etlinger & Fischman, 1973).

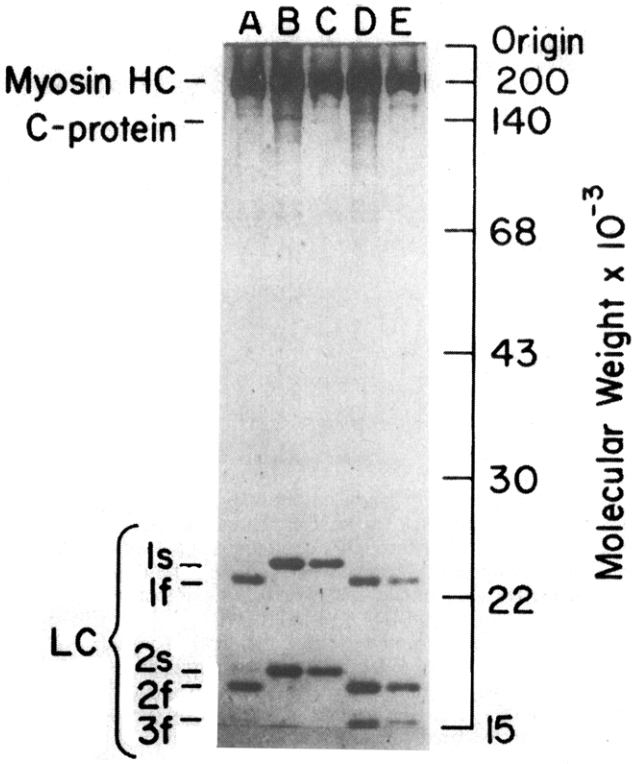

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of ALD, PLD, and embryonic leg muscle myosins. Myosin was denatured in $NaDodSO_4$ buffer, and aliquots containing 10 μg of proteins were run on a 16-cm 10% polyacrylamide–$NaDodSO_4$ slab gel as described under Materials and Methods. Identical LC patterns were obtained with up to 75 μg of LC's purified as described by Wikman-Coffelt et al. (1973b). Column-purified myosin is myosin purified by chromatography on DEAE-Sephadex. (A) Column-purified embryonic leg muscle myosin; (B) ALD myosin; (C) column-purified ALD myosin; (D) PLD myosin; (E) column-purified PLD myosin.

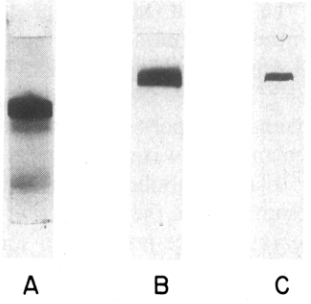

FIGURE 2: Purification of myosin HC by preparative $NaDodSO_4$–polyacrylamide gel electrophoresis. Myosin (700 μg–1 mg) was denatured in $NaDodSO_4$ and electrophoresced on (A) a 3% polyacrylamide–0.5% agarose–$NaDodSO_4$ gel. The myosin HC region was excised and electrophoresed on (B) a 5% polyacrylamide–$NaDodSO_4$ gel. A section within the myosin HC region was again excised, and myosin HC was electroeluted from the gel slices. Shown in (A) and (B) are gels that have been stained after each of the electrophoretic steps and in (C) is a 5% polyacrylamide–$NaDodSO_4$ gel of the electroeluted myosin HC.

These proteins were not removed during the conventional purification of myosin HC from myosin (i.e., denaturation with urea, guanidine hydrochloride, or $NaDodSO_4$ and fractionation by low ionic strength precipitation or gel filtration). Since pure myosin HC was required for the preparation of monospecific antibodies (either as an immunogen or for affinity purification of specific antibodies), myosin HC was purified by preparative electrophoresis (Figure 2).

Myosin was fractionated by electrophoresis on a 3% polyacrylamide–0.5% agarose–$NaDodSO_4$ gel, and a gel slice

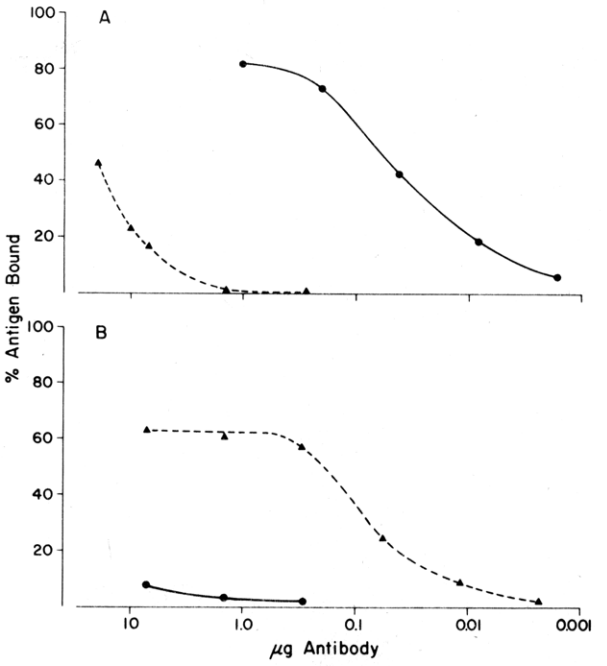

FIGURE 3: Specificity of ALD and PLD myosin HC antibodies for [^{125}I]ALD and [^{125}I]PLD myosins. Identical amounts of [^{125}I]ALD myosin or [^{125}I]PLD myosin were precipitated indirectly by (A) anti-ALD myosin HC and (B) anti-PLD myosin HC, as described under Materials and Methods. Percent [^{125}I]ALD myosin bound to antibody (●); percent [^{125}I]PLD myosin bound to antibody (▲).

containing the myosin HC was excised. To ensure the isolation of myosin HC only, we sliced the gels in such a way that part of the myosin HC band was left behind. The gel slice was placed on a 5% polyacrylamide–$NaDodSO_4$ gel, and myosin HC was purified again by electrophoresis. The myosin HC obtained following excision of the HC region and electroelution was shown to be very pure (Figure 2C).

Specificity of Myosin HC Antibodies. Antibodies prepared with the electrophoretically purified myosin HC used as an immunogen were isolated and enriched 50-fold by affinity chromatography. No cross-reaction of anti-ALD myosin HC with PLD myosin or anti-PLD myosin HC with ALD myosin was observed by immunodiffusion (results not shown). Indirect immunoprecipitation of ^{125}I-labeled myosins, however, showed that both antibodies could bind the opposing antigens, except that 30–40 times more antibody was required as compared to that for the homologous antigen. For removal of cross-reacting antibodies, anti-ALD myosin HC and anti-PLD myosin HC were passed over affinity columns of the opposing myosin HC's. Following adsorption (Figure 3A), anti-ALD myosin HC still showed slight binding of PLD myosin. The amount of antibody required, however, was now 500 times more than that required to bind an equivalent amount of ALD myosin. Thus, the antigen-binding capacity, or specificity, of anti-ALD myosin HC was 500 times greater for ALD myosin than for PLD myosin. Likewise, the specificity of anti-PLD myosin HC for PLD myosin as compared to ALD myosin (Figure 3B) was at least 500-fold.

Myosin was used as the antigen for immunoprecipitation because of the difficulty of keeping myosin HC in solution in the absence of denaturants or $NaDodSO_4$. The use of myosin as the tracer did not alter the specificity of the assay for myosin HC, since the amount (and the iodination) of the contaminating proteins was minimal after purification of the myosin on DEAE-Sephadex (Figure 1).

Due to the purity of the immunogen, it was anticipated that the myosin HC antibodies would be specific for myosin HC.

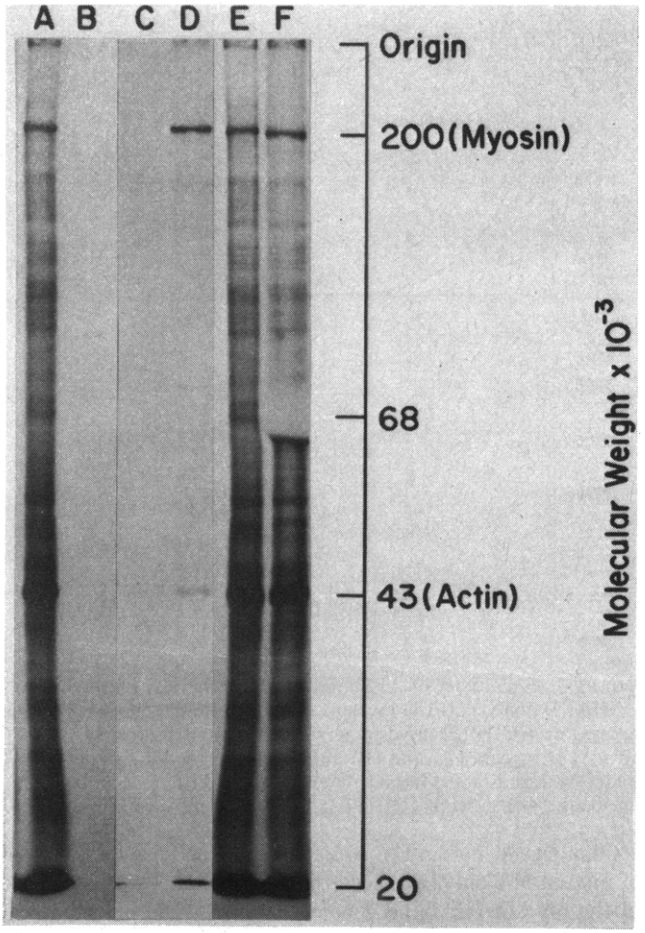

FIGURE 4: Immunoprecipitation of [^{14}C]leucine-labeled proteins from embryonic chick muscle cultures. Cultures were labeled, harvested, and washed as described under Materials and Methods. The cell pellets were resuspended in $NaDodSO_4$ buffer, heated at 100 °C for 5 min, and incubated at 45 °C for 60 min. The extract was centrifuged at 2000*g* for 10 min (room temperature), and aliquots of the supernatant were diluted at least 10-fold with 1% DOC, 1% Triton X-100, 1% BSA, and 20 μM leupeptin in phosphate buffer and centrifuged for 10 min on a microfuge. The immunoprecipitation of aliquots of the supernatant (16 000 cpm) with anti-ALD myosin HC and anti-PLD myosin HC and the washing and dissolving of the immunoprecipitates were done as described under Materials and Methods for the immunoprecipitation of cell-free translation products. Fluorography of an 8% polyacrylamide–$NaDodSO_4$ slab gel of (A and E) the [^{14}C]leucine-labeled extract (16 000 cpm), (B) the nonimmune rabbit IgG immunoprecipitate, (C) the anti-ALD myosin HC immunoprecipitate, and (D) the anti-PLD myosin HC immunoprecipitate. (F) A mock incubation of the [^{14}C]leucine-labeled extract (16 000 cpm). Lanes A, B, E, and F were exposed for 3 days while lanes C and D were exposed for 6 days.

To test this, however, we indirectly precipitated [^{14}C]-leucine-labeled extracts of muscle culture proteins (Figure 4A,E,F) with the anti-ALD and anti-PLD myosin HC prepared above. As shown in Figure 4C,D, myosin HC was the major protein precipitated. Although a light band corresponding in mobility to actin as well as very low levels of a few other bands was visible, the data demonstrated the high specificity of the antibodies for myosin HC. The precipitation of actin probably resulted from its association with myosin HC and not from antibodies against actin (Schachat et al., 1978), as it was proportional to the amount of myosin HC precipitated. The other low-level bands may have resulted from the same cause or from partial degradation of myosin HC.

The amounts of myosin HC precipitated by anti-ALD and anti-PLD myosin HC probably reflected the relative amounts of these proteins in the extract. Thus, cultured 11-day-old

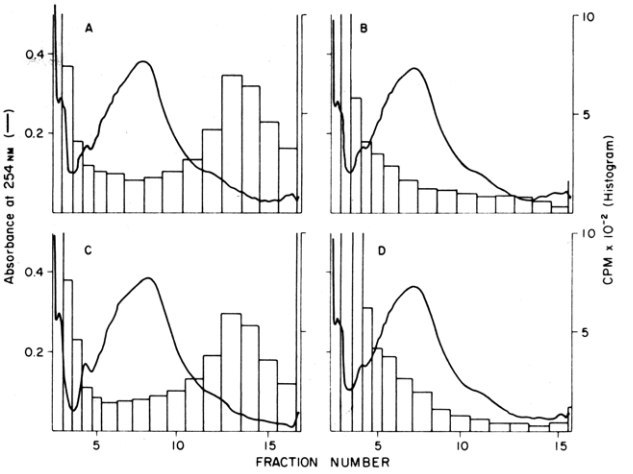

FIGURE 5: Binding of ^{125}I-labeled antibody to polysomes from 14-day-old embryonic chick leg muscle. Three A_{260} units (in 0.5 mL) of polysomes, purified through a continuous sucrose (1 M–2.5 M) gradient containing 1% Triton X-100 and 1% sodium deoxycholate, was incubated for 30 min at 4 °C with 0.24 μg of iodinated antibody as follows: (A) [^{125}I]anti-EM myosin HC; (B) [^{125}I]anti-EM myosin HC plus 2 μg of EM myosin; (C) [^{124}I]anti-EM myosin HC plus 6 μg of anti-BSA; (D) [^{125}I]anti-BSA. The samples were then layered over a 15–40% (w/v) linear sucrose gradient and centrifuged for 30 min at 286000g_{max} in a Beckman SW 41 rotor. Absorbance at 254 nm (—) was monitored with an ISCO gradient scanner, and fractions were counted for ^{125}I radioactivity (histogram). The top of the gradient is at the left of the sucrose gradient profiles.

embryonic chick leg muscle contains predominantly fast-twitch myosin, with very small amounts of slow-twitch myosin.

Binding of ^{125}I-Labeled Antibodies to Polysomes. The binding of myosin HC antibodies to polysomes was tested with iodinated antibodies. [^{125}I]Anti-EM myosin HC was incubated with polysomes with 14-day-old embryonic leg muscle and sedimented through a sucrose gradient. As shown in Figure 5A, the region of the gradient (fractions 12–15) characteristic of myosin HC synthesizing polysomes (Heywood et al., 1967; Heywood & Rich, 1968) contained a peak of the radioactivity. The sedimentation of the ^{125}I-labeled antibody associated with the myosin HC synthesizing polysomes was sensitive to RNase, indicating that the antibody was associated with ribosomes or polysomes. The ^{125}I-labeled antibody appeared to be associated with specific ribosomes, since the radioactivity profile did not coincide with the absorbance profile and since another monospecific antibody (anti-BSA) did not reduce the binding (Figure 5C). Furthermore, the addition of EM myosin reduced the binding of [^{125}I]anti-EM myosin HC (Figure 5B) to the level shown with [^{125}I]anti-BSA (Figure 5D), a protein not made on these polysomes. Thus, the binding of anti-EM myosin HC to polysomes was to myosin HC nascent peptides.

The demonstration of binding of anti-EM myosin HC to specific polysomes also showed the effectiveness of the isolation procedure in purifying embryonic leg muscle polysomes from the large amount of myosin present in the cytoplasm. There was very little evidence for the nonspecific adsorption of myosin to polysomes noted by Heywood et al. (1967).

The preceding experiments showed that EM myosin HC antibodies bind to the myosin HC nascent peptides in polysomes. Similarly, [^{125}I]anti-ALD and [^{125}I]anti-PLD myosin HC antibodies also bind to myosin HC synthesizing polysomes from 14-day-old embryonic chick leg muscle (results not shown). Whether these antibodies were binding to different myosin HC nascent peptides was assessed directly by immunoprecipitation of the polysomes and cell-free translation of the isolated mRNAs.

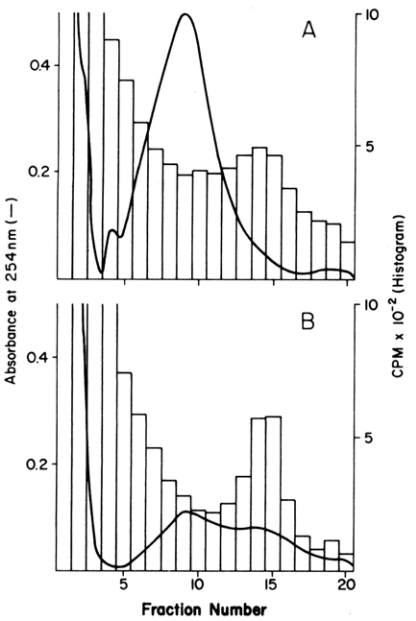

FIGURE 6: Binding of [^{125}I]anti-EM myosin HC to polysomes isolated from 1 M–1.8 M–2.5 M discontinuous sucrose gradients. Polysomes were mixed with 1.3 μg of [^{125}I]anti-EM myosin HC in a total volume of 0.2 mL of polysome buffer, 1% DOC, and 1% Triton X-100. The samples were layered onto a 5-mL 15–40% (w/v) linear sucrose gradient and centrifuged for 23 min at 42 000 rpm in a Beckman SW 50L rotor. Absorbance at 254 nm (—) and ^{125}I radioactivity (histogram) were measured as described in Figure 5. (A) One A_{260} unit of polysomes from the 1 M–1.8 M interphase; (B) 0.4 A_{260} unit of polysomes from the 1.8 M–2.5 M interphase. The top of the gradient is at the left in this figure.

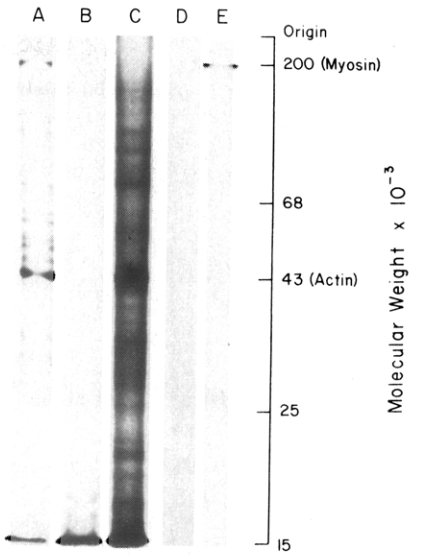

FIGURE 7: Immunoprecipitation of cell-free translation products. The translation of polysomal poly(A) RNA and the immunoprecipitation of the translation mixture with anti-EM myosin HC or nonimmune rabbit IgG were done as described under Materials and Methods. Fluorography of the [^{35}S]methionine-labeled translation products and immunoprecipitates analyzed on a 16-cm 10% polyacrylamide–Na-DodSO$_4$ slab gel was as described under Materials and Methods. (A) [^{14}C]Leucine-labeled proteins from embryonic chick leg muscle cultures. Translation products with (B) no RNA and (C) polysomal poly(A) RNA. Translation products shown in (C) immunoprecipitated with (D) nonimmune rabbit IgG and (E) anti-EM myosin HC. The samples in lanes C–E initially contained similar amounts of the translation products directed by polysomal poly(A) RNA.

Isolation of Myosin HC mRNAs. The polysome profiles shown in Figure 5 indicate that the myosin-synthesizing polysomes comprise a small fraction of the total A_{260} material. A more suitable preparation of polysomes was obtained by centrifuging of the postmitochondrial supernatant from embryonic leg muscle on a discontinuous gradient consisting of 1 M–1.8 M–2.5 M sucrose. As shown in Figure 6A, the polysomes isolated from the 1 M–1.8 M interphase contain the bulk of the A_{260} material. The preparation, however, contained very little myosin HC synthesizing polysomes as there was very little ^{125}I-labeled antibody binding in the region of the gradient where myosin HC synthesizing polysomes would sediment (fractions 13–16). In contrast, the polysomes from the 1.8 M–2.5 M interphase (Figure 6B) were considerably enriched for myosin-synthesizing polysomes, as shown by the absorbance profile and by ^{125}I-labeled antibody binding. Thus, the polysomes isolated from the 1.8 M–2.5 M interphase were used for indirect immunoprecipitation with monospecific ALD and PLD myosin HC antibodies as described under Materials and Methods.

Cell-Free Translation of mRNAs. Translation in a wheat germ system was used for assay of the mRNA activity of the RNA extracted from the immunoprecipitated polysomes. The maximum levels of synthesis (incorporation) with total RNA were comparable to that obtained with poly(A) RNA. Thus, the presence of rRNA had little effect on the activity of the system, except at very high concentrations (>300 μg/mL). Only the wheat germ S-30 extract eluting in the first half of the turbid fractions following gel filtration on Sephadex G-25 was capable of synthesizing myosin HC. The minimum concentration of magnesium necessary for the synthesis of myosin HC was 3 mM. The optimum potassium concentration was reported by Kennedy & Heywood (1976) to be 110 mM, whereas Patrinou-Georgoulas & John (1977) indicated that 160 mM potassium resulted in increased synthesis of complete myosin HC's and a decrease in the quantity of small peptides (presumably premature termination products). A concentration of 110 mM was chosen because the level of amino acid incorporation was 5 times greater at 110 mM than at 160 mM and because we did not see any significant difference in the relative amount of myosin HC synthesized at these concentrations.

The products translated by the wheat germ system were separated by NaDodSO$_4$–polyacrylamide gel electrophoresis prior to and after indirect immunoprecipitation with the myosin HC antibodies. To test the specificity of the immunoprecipitation, we used anti-EM myosin HC to precipitate the cell-free translation products synthesized with total polysomal poly(A) RNA from 14-day-old embryonic chick leg muscle. As shown in Figure 7E, anti-EM myosin HC precipitated only one major protein, which had the same mobility on NaDodSO$_4$–polyacrylamide gels as myosin HC labeled in culture (Figure 7A). Relatively low concentrations of several other proteins or peptides were also precipitated by the myosin HC antibody. These were probably incomplete peptides of myosin HC due to the relatively high level of premature termination of the wheat germ system (Benveniste et al., 1976; Hunter et al., 1977; Tse & Taylor, 1977), or they were the result of partial proteolysis. It should be noted that actin was not present in the immunoprecipitate (Figure 7E).

Translation of Enriched Fast and Slow Myosin HC mRNAs. The proteins synthesized in the wheat germ system in response to RNA which had been extracted from polysomes immunoprecipitated with ALD and PLD myosin HC antibodies were compared with products directed by total polysomal RNA by NaDodSO$_4$–polyacrylamide gel electrophoresis (Figure 8). Whereas a large number of proteins, the most prominent being actin, were observed with total polysomal

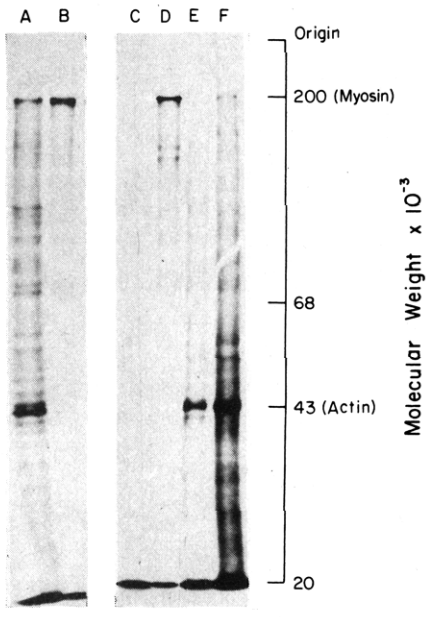

FIGURE 8: Analysis of myosin HC mRNA by cell-free translation. Fluorography of [^{3}H]leucine-labeled (A and B) and [^{35}S]-methionine-labeled (C–E) cell-free translation products analyzed on a 16-cm 8% polyacrylamide–NaDodSO$_4$ slab gel, as described under Materials and Methods. Wheat germ extracts were incubated with (A and E) total RNA from 14-day-old embryonic chick leg muscle polysomes (16 300 and 27 000 cpm, respectively), (B) total RNA from polysomes immunoprecipitated with anti-ALD myosin HC (5400 cpm), (D) total RNA from polysomes immunoprecipitated with anti-PLD myosin HC (27 000 cpm), and (C) no added RNA (27 000 cpm). As shown in Table I, the ratio of fast/slow myosin HC mRNA was 1:1 in (B) and 15:1 in (D). (F) [^{14}C]Leucine-labeled proteins from cultures of embryonic chick leg muscle (46 000 cpm). Fluorographs were exposed for 2 weeks (A and B) or for 10 h (C–F).

Table I: Fractionation of ALD and PLD Myosin HC mRNAs[a]

RNA fraction	total incorpn[b] (cpm)	radioact. (cpm) immunopptd by antibody against: PLD myosin HC[c]	ALD myosin HC[c]	PLD/ALD
total polysomal	194 000	5360	1360	4:1
polysomes pptd with anti-PLD myosin HC	81 500	15700	1040	15.1:1
polysomes pptd with anti-ALD myosin HC	74 800	7890	6420	1.2:1
polysomes pptd with anti-ALD myosin HC plus 5 mM ATP[d]	14 300	400	2180	1:5.4

[a] RNA was translated in a wheat germ cell-free translation system. Aliquots of the translation mixture were incubated with anit-PLD myosin HC, anti-ALD myosin HC, or nonimmune rabbit IgG. [^{35}S]Methionine-labeled translation products were then precipitated with rabbit IgG and goat antirabbit IgG. The precipitates were washed by sedimentation through three successive discontinuous sucrose gradients and redissolved in NaDodSO$_4$ buffer, and the radioactivity was measured. [b] Trichloroacetic acid insoluble radioactivity. [c] Nonspecific precipitation, as measured by radioactivity precipitated by nonimmune rabbit IgG, was subtracted to give the amount specifically precipitated. Nonspecific precipitation amounted to less than 1% of the total radioactivity incorporated. [d] ATP was included during the immunoprecipitation of polysomes.

RNA (Figure 8A,E), the predominant protein synthesized with the RNA extracted from the immunoprecipitated polysomes was myosin HC (Figure 8B,D). Several high molecular weight peptides were also directed by the purified mRNA, but most of these peptides were also precipitated with myosin HC antibodies (Figure 9). Since the antibodies appears to be specific for myosin HC, the precipitation of these peptides indicates that they are predominantly incomplete or partial peptides of myosin HC. The absence of actin, a major protein synthesized with polysomal RNA, showed that myosin HC mRNA was free of actin mRNA.

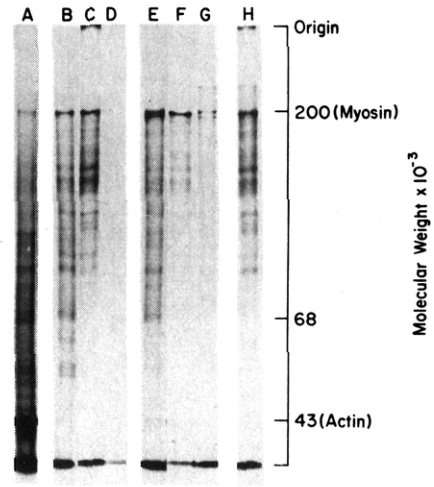

FIGURE 9: Immunoprecipitation of cell-free translation products with anti-ALD and anti-PLD myosin HC. Fluorography of the [^{35}S]-methionine-labeled translation products and immunoprecipitates analyzed on a 16-cm 6% polyacrylamide–NaDodSO$_4$ slab gel was as described previously. The radioactivity in each slot and the exposure time are indicated in brackets. (A) Translation with total polysomal RNA (28 000 cpm; 2 days). Translation with (B) PLD myosin-HC RNA (9500 cpm; 1 day) enriched for fast myosin HC mRNA (15:1 fast/slow) and indirect immunoprecipitation of the translation products with (C) anti-PLD myosin HC (1800 cpm; 6 days) and (D) anti-ALD myosin HC (400 cpm, 6 days). Translation with (E) ALD myosin HC RNA (6000 cpm; 1 day) enriched for slow myosin HC mRNA (1:1 fast/slow) and indirect immunoprecipitation of the translation products with (F) anti-PLD myosin HC (2600 cpm; 2 days) and (G) anti-ALD myosin HC (900 cpm; 6 days). (H) A mixture of (C) and (G) (2700 cpm; 6 days). 2.5 times more of the solubilized immunoprecipitates were electrophoresed in (D) and (F) than in (C) and (G). Slots containing less radioactivity were exposed for longer periods of time. However, since preflashed film was not used, the fluorographic image was not strictly proportional to the total number of disintegrations, or radioactivity times time (Laskey & Mills, 1975). Thus, the bands in the samples containing very little radioactivity (D and G) are probably fainter than would be anticipated considering the radioactivity and exposure times.

Enrichment of fast and slow myosin HC mRNA was assessed by immunoprecipitation of the cell-free translation products with anti-PLD and anti-ALD myosin HC. As shown in Table I, total polysomal RNA directed the synthesis of fast and slow myosin HC's in a ratio of ~4:1, respectively. Assuming that both myosin HC mRNAs are translated with equal efficiency, the total polysomal RNA contains 4 times more mRNA for fast myosin HC than for slow myosin HC. Immunoprecipitation of polysomes resulted in an apparent fourfold enrichment of both fast (PLD) and slow (ALD) myosin HC mRNAs. Modifying the conditions of the immunoprecipitation of polysomes by including 5 mM ATP further increased the enrichment of slow myosin HC mRNA. Thus, we were able to obtain RNA preparations of fast and slow myosin HC mRNA that were enriched ~94 and ~84% (*with respect to the type of myosin HC*), respectively.

NaDodSO$_4$–polyacrylamide gel electrophoresis of the myosin "heavy chain" proteins directed by enriched fast (15:1 fast/slow) and slow (1:1 fast/slow) myosin HC mRNA showed small differences in their mobilities. The heavy chain precipitated with anti-PLD myosin HC (Figure 9C,F) had a mobility intermediate between those of the two heavy chains

precipitated with anti-ALD myosin HC (Figure 9G). The three heavy chains could be resolved from each other when they were mixed in approximately equal amounts (Figure 9H). Furthermore, the immunoprecipitate from a cell-free translation reaction directed with the slow myosin HC mRNA, apparently containing a 1:1 ratio of both sets of heavy chains (Figure 9E), clearly separated the bands (Figure 9F,G). Immunoprecipitation of heavy chains of different mobilities demonstrated the specificity of the antibodies with respect to the type of myosin HC.

Discussion

Although the myosin HC mRNA from embryonic chick skeletal muscle has been isolated and characterized (Heywood & Nwagwu, 1969; Sarkar et al., 1973; Heywood et al., 1975), Patrinou-Georgoulas & John (1977) have recently shown that such preparations probably contain mRNA sequences for two distinct myosin HC's. By indirect immunoprecipitation of polysomes, we have isolated and fractionated two types of myosin HC mRNAs from 14-day-old embryonic chick leg muscle. Translation of each of these mRNAs in a wheat germ system indicated that substantial purification with respect to myosin HC had been achieved. Myosin HC was the predominant peptide synthesized, even though translation was carried out under conditions that were optimal for the synthesis of total proteins and were not selective for myosin HC (P. K. Umeda, unpublished experiments). The translation system, in fact, probably favors the synthesis of relatively low molecular weight proteins rather than myosin HC. The synthesis of myosin HC and the absence of most other muscle proteins indicated substantial purification of myosin HC mRNA.

The purified mRNA also directed the synthesis of smaller amounts of several peptides having molecular weights somewhat lower than that of myosin HC. Most of these peptides were immunoprecipitated with myosin HC antibodies. The peptides are probably partial products of myosin HC, since the antibody used is highly specific for myosin HC. Immunoprecipitation of labeled muscle protein extracts with the antibody showed negligible reaction with proteins other than myosin HC. A relatively high level of incomplete synthesis of high molecular weight proteins has previously been observed in the wheat germ ribosomal system (Benveniste et al., 1976; Hunter et al., 1977; Tse & Taylor, 1977).

The similarity of the peptide patterns observed before and after immunoprecipitation of cell-free translation products directed by myosin HC mRNA provides additional evidence for the considerable purification of the myosin HC mRNA relative to other cellular mRNAs. This purification must be much greater than that indicated by the ~20% precipitation of Cl_3AcOH-precipitable radioactivity obtained with antibody to myosin HC (Table I). The results were possibly due to incomplete immunoprecipitation of myosin HC (and partial products of myosin HC) synthesized in the wheat germ system. Alternatively, very low molecular weight peptides may be synthesized that migrated with the front on gel electrophoresis and were precipitated with Cl_3AcOH but not by antibody.

We also achieved partial separation of fast and slow myosin HC mRNAs as measured by immunoprecipitation of the products translated in the wheat germ system by specific antibodies. Radioimmunoassays showed the marked specificity of the antibody reaction with respect to fast and slow myosin HC. Moreover, the antibodies immunoprecipitated myosin HC's of slightly different mobilities on $NaDodSO_4$–polyacrylamide gels, thus further confirming their specificity with respect to myosin HC types. Starting with a ratio of fast to slow myosin HC mRNA of 4:1 in 14-day-old embryonic chick leg muscle, we obtained preparations in which fast myosin HC mRNA was ~94% enriched and slow myosin HC mRNA was ~84% enriched with respect to the myosin HC type.

It has been suggested that immunoprecipitation of polysomes from muscle is difficult, if not impossible, because of nonspecific aggregation of polysomes by contaminating myosin (Heywood et al., 1968). As shown in this report, the procedure is effective when polysomes are freed of contaminating myosin by first being sedimented through a sucrose step gradient (Palacios et al., 1972). The binding of ^{125}I-labeled antibodies to heavy polysomes and the competition introduced by the addition of free myosin demonstrate the absence of significant contamination of free myosin.

Nonspecific aggregation of nascent polypeptide chains was also not apparent. Cell-free translation and immunoprecipitation of the products showed little evidence for copurification of other mRNAs. We noted, however, that some fast myosin HC polysomes often appeared to be associated with slow myosin HC polysomes. In the absence of ATP, immunoprecipitation with antibodies to slow myosin HC resulted in mRNA preparations containing considerable amounts of both myosin HC mRNAs. The improvement in the specificity of immunoprecipitation of myosin HC synthesizing polysomes after the addition of ATP may indicate interaction of nascent myosin HC chains, possibly through actin filaments. Alternatively, the high Mg^{2+} concentration (10 mM used in this procedure) may have led to a "clustering" or aggregation of ribosomes. Chelation of Mg^{2+} by ATP may have reduced the aggregation of myosin HC synthesizing polysomes.

Although only partial enrichment of the two types of myosin HC mRNAs was attained by immunoprecipitation, the preparations should be very useful in the isolation of specific probes reactive with fast or slow myosin HC mRNA. Complementary DNA transcribed by reverse transcriptase may be purified further by hybridization with purified mRNA preparations at low R_0t values (Strair et al., 1977; Gordon et al., 1978). The mRNAs may also be used to the synthesis and cloning of double-stranded cDNAs. The enrichment achieved should facilitate the isolation of cloned cDNA fragments containing sequences which correspond to fast and slow myosin HC mRNA. The clones may be identified by use of our specific antibodies for characterizing the products in the hybrid-arrest translation system (Paterson et al., 1977; Hastie & Held, 1978; Villa-Komaroff et al., 1978).

We have also noted that the two purified mRNAs for myosin HC direct the synthesis of three peptides that have slightly different mobilities, corresponding to molecular weights of about 200000. The discrimination of small amounts of labeled peptides in the Laemmli gel system is relatively fine. Thus, small differences in the mobility of different myosin HC's may be apparent even though we did not observe them with unlabeled myosin from the intact muscle. Rushbrook & Stracher (1979), however, have been able to separate fast and slow myosin HC's using a low concentration polyacrylamide gel and small amounts of protein. Antibody to fast myosin HC (PLD) immunoprecipitated a peptide with mobility intermediate between the two bands precipitated by antibody to slow myosin HC (ALD). One possibility is that the differences in the mobilities of the bands are the result of incomplete synthesis or translation. In this case, the fast myosin HC would be synthesized only as a partial peptide, whereas the slow myosin HC would be synthesized as both a complete translation product and a different partial translation product. It is more likely that the three peptides represent the primary translation products of three different myosin HC genes having

slightly different molecular weights, one which corresponds to fast myosin HC and the others which correspond to slow myosin HCs.

One of the slow myosin HC peptides may be related to a cardiac myosin HC. A band comigrating with the largest of the three peptides is directed by cardiac myosin mRNA in the wheat germ system. This peptide is specifically immunoprecipitated with ALD myosin HC antibodies (P. K. Umeda, unpublished experiments). The results are consistent with previous identification of cardiac myosin HC antigens in embryonic chick skeletal muscle (Masaki & Yoshizaki, 1974; Masaki & Kinoshita, 1974) and with the substantial homology shown by two-dimensional peptide maps of CNBr digests of cardiac and ALD myosins (Flink et al., 1979; Brevet & Whalen, 1978). Alternatively, one of the myosin HC peptides may represent an "embryonic" myosin HC (Brevet & Whalen, 1978; Huszar, 1972; Whalen et al., 1978; Sreter et al., 1975a). Our data, in any case, suggest that there are probably at least three myosin HC species in 14-day-old embryonic chick skeletal muscle.

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The Poly(adenylic acid)·Protein Complex Is Restricted to the Nonpolysomal Messenger Ribonucleoprotein of *Physarum polycephalum*†

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ABSTRACT: The distribution of poly(adenylic acid) [poly(A)]·protein complexes in the polysomal and nonpolysomal messenger ribonucleoprotein (mRNP) fractions of *Physarum polycephalum* was examined in the present study. Poly(A)-containing components released from the nonpolysomal mRNP by ribonuclease (RNase) digestion were quantitatively adsorbed to nitrocellulose filters at low ionic strength, were highly resistant to micrococcal nuclease under conditions in which free poly(A) was completely degraded, and sedimented as a 10–15S particle which was disrupted by sodium dodecyl sulfate and protease treatment. These are characteristics of the poly(A)·protein complex. In contrast, poly(A)-containing molecules released from the polysomes by RNase were refractive to nitrocellulose, were completely sensitive to micrococcal nuclease, and sedimented at 2–4 S, identical with the sedimentation exhibited by protein-free poly(A). Examination of the poly(A) sequences present in polysomal and nonpolysomal mRNP by polyacylamide gel electrophoresis showed that the former contained only very short sequences, averaging ~15 nucleotides, while the latter exhibited only much longer segments, averaging ~65 nucleotides. It is concluded that poly(A)·protein complexes are restricted to the nonpolysomal mRNP of *Physarum* and that the limiting factor in complex formation may be the length of the available poly(A) binding site.

mRNA[1] is associated with proteins in the cytoplasm of eucaryotic cells forming mRNP particles (Greenberg, 1975; Preobrazhensky & Spirin, 1978). A significant protein binding site in mRNP is the 3′-poly(A) sequence of its mRNA moiety (Blobel, 1973). The resistance of poly(A) to RNase permits the isolation of the 3′-terminal domain of the mRNP as a poly(A)·protein complex (Kwan & Brawerman, 1972; Jeffery

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[1] Abbreviations used: poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); oligo(dT), oligo(deoxythymidylic acid); mRNA, messenger ribonucleic acid; mRNP, messenger ribonucleoprotein; RNP, ribonucleoprotein; tRNA, transfer ribonucleic acid; rRNA, ribosomal ribonucleic acid; Na_2EDTA, disodium ethylenediaminetetraacetate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; Cl_3AcOH, trichloroacetic acid.

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