A monoclonal antibody, 2B6, has been prepared against the embryonic myosin heavy chain of rat skeletal muscle. On solid phase radioimmunoassay, 2B6 shows specificity to myosin isoforms known to contain the embryonic myosin heavy chain and on immunoblots of denatured contractile proteins and on competitive radioimmunoassay, it reacts only with the myosin heavy chain of embryonic myosin and not with the myosin heavy chain of neonatal or adult fast and slow myosins or with other contractile or noncontractile proteins. This specificity is maintained with cat, dog, guinea pig, and human myosins, but not with chicken myosins. 2B6 was used to define which isoforms in the developing animal contained the embryonic myosin heavy chain and to characterize the changes in embryonic myosin heavy chain in fast versus slow muscles during development. Finally, 2B6 was used to demonstrate that thyroid hormone hastens the disappearance of embryonic myosin heavy chain during development, while hypothyroidism retards its decrease. This confirmed our previous conclusion that thyroid hormones orchestrate changes in isoforms during development.

Like most contractile proteins, the myosin heavy chain exists in a number of polymorphic forms which show specificity among muscles (1, 2) and among muscle fibers (3, 4) with different physiological and metabolic properties. In situ two myosin heavy chains, with M₉ ≈ 200,000 each, aggregate with four myosin light chains, M₉ = 16,000–25,000, to form myosin isoforms. Various combinations of myosin heavy and light chains increase the heterogeneity of the hexameric myosin isoforms. For example, the myosin isoforms of fast twitch and slow twitch fibers contain different myosin heavy and light chains (1, 5, 6); and among the fast twitch fibers, fast red and fast white fibers contain myosins with qualitatively distinct myosin heavy chains, but similar light chains (7). In fast white muscles, however, the same heavy chain can combine with different light chain complements to form at least three distinct isoforms (8, 9). Among the slow myosins, pyrophosphate gel analyses of native isoforms (10) and gel electrophoresis of light chain components (11) suggest the existence of at least several slow isoforms. Affinity-purified, polyclonal antibodies to different isoforms have helped to localize distinct isoforms to individual muscle fiber types (3, 4, 12). More recently, gene analysis has demonstrated the existence of many distinct myosin heavy chain genes (13–15).

Developmental isoforms of the myosin heavy and light chains also exist. At a minimum, two myosin heavy chains, termed the embryonic and neonatal myosin heavy chains, sequentially precede the appearance of definitive adult fast or slow myosin heavy chains in rats (16, 17), chickens (18), humans (19), and other vertebrates. In the rat, the isoforms containing the embryonic myosin heavy chain also contain a light chain not found in later developmental stages, but which is identical to a rat atrial light chain (20). Embryonic myosin isoform(s) also contain a negligible proportion of light chain 3f when compared to adult myosin isoforms (21).

Few properties of these developmental forms of the myosin heavy chains or the isoforms into which they are assembled are known. It is not certain, for example, how many different light chain complements are combined with the embryonic or neonatal myosin heavy chains and, therefore, how many different hexameric embryonic or neonatal isoforms exist. Also, although the prolonged speed of contraction of embryonic and neonatal muscles suggests myosin isoforms with a low ATPase activity (22), histochemical staining for ATPase has shown a high, alkaline-stable ATPase for the developmentally isoforms (23). Biochemical measurements for myosin ATPase have been inconsistent. Similarly, the factors regulating the transition from embryonic to neonatal myosin are not known and the distribution of these isoforms among muscle fibers with different adult destinies is not known. The only functional property associated with embryonic myosin is that at low Mg(II) concentrations, embryonic myosin isoform(s) form smaller thick filaments than do the adult or neonatal myosin isoforms (16). Even less is known about the neonatal myosin heavy chain.

We have been interested in identifying not only the myosin isoforms which are present during the development of distinct muscle fiber types and the myosin heavy and light chains of which they are composed, but also the factors which control the transitions from isoform to isoform during development. Unfortunately, the overlapping period during which both embryonic and neonatal isoforms are present, the difficulty isolating individual fibers of developing muscle, and the absence of antibodies specific to the developmental isoforms have prevented significant progress. Hence, we have begun to produce monoclonal antibodies to developmental isoforms of rat myosins and report here the production of an antibody which reacts specifically with the heavy chain of embryonic myosin and shows little cross-reactivity with myosin heavy chains from other developmental or adult stages.

**EXPERIMENTAL PROCEDURES**

*Protein Preparation—Actomyosins or column-purified myosins from a variety of muscles were prepared according to previously*
Monoclonal Antibody to Embryonic Myosin

Monoclonal Antibodies—Column-purified myosin prepared from bulk hindlimb muscle of newborn rats was used as the immunogen. BALB/c mice were immunized with intravenous injections of myosin emulsified in complete Freund's adjuvant. This treatment was repeated after 5 weeks. In the 9th week, the mice were boosted intravenously with 100 μg of the immunogen and 4 days later the isolated splenocytes were fused with an NS-1 myeloma cell line (30). Hybridomas were screened for anti-myosin immunoglobulin secretion by an indirect, solid phase RIA (31). Positive wells were cloned by limited dilution (32).

Solid Phase RIA—A solid phase RIA was used for screening cell line supernatants for specific immunoglobulin production and for subsequent assays (31). Proteins were immobilized in wells of polyvinyl microtiter plates (MA Bio Products) by incubating 100 μl of high salt buffer containing myosin (10 μg/ml) in each well for 2 h at room temperature. The wells were then washed five times with 1% bovine serum albumin in phosphate-buffered saline (0.05 M KH₂PO₄, pH 7.0, 0.15 M NaCl) to block any nonspecific binding. Subsequently, 25 μl of antibody solution was added to each well for 2 h. Antibody solution was either hybridoma culture supernatant or supernatant serially diluted in 0.5% bovine serum albumin in phosphate-buffered saline. After antibody solutions were discarded, the plates were washed five times with 0.5% bovine serum albumin in phosphate-buffered saline. Plates were incubated for 2 h with 30 μl/well of [³¹₀]labeled rabbit anti-mouse F(ab')₂ or goat anti-mouse μ chain, both diluted with 0.5% bovine serum albumin in phosphate-buffered saline to give activity of 20,000 cpm/well (labeled antibodies were prepared and provided by Dr. Michael Cancro, University of Pennsylvania). Plates were washed 10 times with distilled water, air-dried, and bound label assayed by γ spectrometry (Gamma Trac 1290, Tracer Analytic).

Antigen competition radioimmunoassay used column-purified myosin dissolved in high salt buffer and then serially diluted 1:2 in the same buffer. Diluted myosin samples were mixed with equal volumes of antibody solution and incubated for 2 h at 4°C. Fifty-microliter aliquots of the antigen-antibody mixtures were transferred in triplicate to wells of the microtiter plate which had been coated with myosin from newborn rat hindlimbs (see above) and incubated overnight at 4°C. Wells were washed 5 times with 0.5% bovine serum albumin in phosphate-buffered saline and bound antibody was detected with [³¹₀]labeled rabbit anti-mouse F(ab')₂, as described above. The concentration of monoclonal antibody used in this competition assay corresponded to the midpoint of the linear region of an antibody titration binding curve. This was obtained by reacting serial dilutions of each immunosorbent antibody with immobilized original antigen. Binding values for this midpoint were dilutions of 1:222 through 1:354, depending on the supernatant used. Binding data were corrected for background (generally 150-250 cpm) and bound radioactivity was expressed in per cent (B/B₀ × 100) of the radioactivity bound in the absence of competing antigen (B₀).

Immunoblots—Actomyosins or column-purified myosins were analyzed by electrophoresis on SDS-polyacrylamide gels using the buffer system of Laemmli (25). Gels were 1.0 mm thick, with a 10-20% acrylamide gradient, and were 0.05% in N,N',N'-trimethylene bisacrylamide. Gels were stained in 0.1% Coomassie Blue in 50% methanol, 10% acetic acid and destained in 10% methanol, 7.5% acetic acid. Proteins on gels were electrophototransferred to nitrocellulose paper (33) at 0.4 A for 30 min using a Transphor TE42 electrophoresis cell ( Hoefer Scientific) with 0.05 M Tris, 0.2 M glycine, 30% methanol as the transfer buffer. After the transfer, the nitrocellulose paper was incubated in wash buffer (0.05 M Tris, 0.15 M NaCl, 0.1% (w/v) bovine serum albumin, 0.05% (v/v) Nonidet P-40) overnight. The paper could be stored in this solution at 4°C until needed.

Myosin isozymes were separated on pyrophosphate slab gels as previously described (17). Prior to electrophoretic transfer of isozymes to nitrocellulose paper, the gel was soaked for 1 h in a solution containing 0.062 M Tris-HCl, 1% (w/v) SDS, 1% (v/v) d-mercaptoethanol, 10% glycerol. Transfer to nitrocellulose paper was then performed as described above except that the transfer buffer additionally contained 0.1% (w/v) SDS and the transfer occurred for 2 h. Antibody binding to proteins on the nitrocellulose paper was detected as follows. The nitrocellulose paper was incubated for 30 min in wash buffer containing 5 mg/ml normal rabbit serum. After the nitrocellulose paper was washed six times with wash buffer without normal rabbit serum, it was incubated for 1 h in hybridoma culture supernatant diluted 1:40 with wash buffer containing 5 mg/ml normal rabbit serum. The hybridoma supernatant was incubated for 1 h in affinity-purified, peroxidase-conjugated rabbit anti-mouse IgG (heavy and light chains) diluted 1:66 with wash buffer containing 2 mg/ml rabbit IgG. After six washes in phosphate-buffered saline and three washes in Tris-saline (0.5 M Tris-HCl, 0.15 M NaCl, pH 7.6), color was developed using the horseradish peroxidase color development agent from Bio-Rad. The reagent, containing 4-chloro-1-naphthol, was dissolved (0.3%, w/v) in ice-cold methanol and mixed at room temperature with 5 volumes of Tris-saline containing 0.018% (v/v) H₂O₂. Development of the purple color was stopped by rinsing with distilled water. Sera and affinity-purified antibodies used in this assay were from Cappel Laboratories.

Immunoprecipitation—To isolate the embryonic myosin isoforms from a complex mixture of isozymes, we adopted a protocol for a solid phase immunoadsorption (34). The monoclonal antibody 2P6 was isolated from the culture supernatant by (NH₄)₂SO₄ precipitation (36) and dialyzed into standard salt (120 mM KCl, 6.1 mM NaH₂PO₄, 3.9 mM KH₂PO₄, 1 mM MgCl₂).

Protein A-conjugated agarose (Bethesda Research Laboratories) was washed five times with pyrophosphate buffer (see above) and then resuspended in the same buffer. Monoclonal antibody in standard salt was incubated at room temperature. The agarose was incubated with agitation at 4°C overnight to absorb the antibody to protein A. The agarose was then washed five times with pyrophosphate buffer. Aliquots of this preparation were resuspended in equal volumes of pyrophosphate buffer and incubated with an actomyosin preparation from soleus muscles of 5-day-old rats at 4°C for 2 h. The antigen--monoclonal antibody complex bound to the agarose via protein A was separated from unbound myosin isozymes remaining in the incubation medium by centrifugation. The supernatant mixed with pyrophosphate buffer containing 50% glycerol was analyzed on a pyrophosphate gel, while the bound antigen was removed by boiling the agarose in Laemmli sample buffer (25) for 5 min. These myosins could then be analyzed on SDS-polyacrylamide gels.

Immunofluorescence—Indirect immunofluorescence was performed on 8-μm thick frozen sections of whole hindlimbs of rats at 20 days gestation. Sections were first incubated with a 1:50 dilution of 282 culture supernatant in standard salt for 1 h at 37°C, then washed three times in standard salt and incubated for 1 h at 37°C with fluorescein-labeled goat anti-mouse IgG (heavy and light chains) antibody (Cappel Laboratories) diluted 1:20 with standard salt. Sections were washed three times with standard salt, mounted in standard salt containing 25% glycerol, and examined with an Olympus fluorescence microscope with a vacuum immersion oil immersion model AH-RFL. Controls for immunofluorescence included sections stained with negative hybridoma culture supernatants and with fluorescein-labeled goat anti-mouse IgG alone.

Rat cultures established from newborn rat hindlimb muscles were grown for 6 days as described previously (22). Cultures were washed three times with phosphate-buffered saline, fixed in acetone at 20°C.
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FIG. 1. Immunoblots of actomyosin preparations from different stages of development. Actomyosins prepared from muscles containing different myosin heavy chain isoforms were run on a polyacrylamide gel containing SDS (25) and transferred to nitrocellulose (33). Nitrocellulose sheets were stained with 2B6 and peroxidase-conjugated rabbit anti-mouse IgG (heavy and light chains). Left, Coomassie Blue-stained polyacrylamide gel. Right, a duplicate of the gel on the left after transfer onto nitrocellulose and reaction with 2B6. Lanes: actomyosin from: (a) adult slow soleus muscle; (b) soleus muscles of 60-day-old rats treated with propylthiouracil from 3 days gestation; contains neonatal heavy chains; (c) 10-day-old control soleus; (d) 6-day-old cultured embryonic rat hindlimb muscle; (e) 10-day-old control fast extensor digitorum longus; (f) extensor digitorum longus of 60-day-old rats treated with propylthiouracil from 3 days gestation; (g) adult fast extensor digitorum longus muscle.

for 10 min, air-dried, and fixed in 2% paraformaldehyde in phosphate-buffered saline for 6 min. After washing three times with phosphate-buffered saline, cells were stained with antibody as described above.

RESULTS

The monoclonal antibody selected for this study, 2B6, showed a high affinity for a rabbit anti-mouse F(ab')2 antibody, but did not react with a goat anti-mouse \( \mu \) chain antibody on either a solid phase RIA or on immunoblots. The reaction of 2B6 with the immunogen was the result of a specific, rather than a nonspecific, binding of a mouse immunoglobulin with the antigen, since on immunoblots the myosin did not react with negative hybridoma cell line supernatants or with a mixture of IgF, IgG, IgH, or \( \gamma G3 \) (Litton Bionetics).

The antigen used to produce the monoclonal antibody 2B6 was a mixture of native myosin isozymes purified from the bulk hindlimb muscles of the newborn rat. At this stage, these muscles contain, at a minimum, myosin isozymes with embryonic, neonatal, and adult slow myosin heavy chains (16, 17, 35), although myosins with the embryonic myosin heavy chain predominate. Choice of tissues from which to extract myosins for testing the specificity of 2B6 was difficult, since individual rat muscles do not contain pure adult fast or slow myosin isozymes, and no developmental stage contains only one of the developmental isoforms of myosin. We have used the following samples for testing our antibodies: (a) myosin rich in the embryonic myosin heavy chain was isolated from newborn rat hindlimb muscle (the original antigen); (b) neonatal myosin was isolated from gastrocnemius muscles of rats which had been made hypothyroid for 60 days after birth with propylthiouracil and a low iodine diet. These muscles contain exclusively neonatal myosin isozymes (17); (c) adult slow and fast myosins were extracted from the adult rat soleus and extensor digitorum longus muscles, respectively. Although neither of these muscles is pure fast or slow, each contains approximately 80% of either fast or slow isozymes.

Immunoblots of actomyosin preparations were used initially to determine the specificity of the antibody. Actomyosins isolated from muscles at various stages of development were electrophoresed on gradient gels containing SDS; and the proteins were transferred to nitrocellulose and reacted with the monoclonal antibody as described above. Fig. 1, lane d, shows the actomyosin from cultured rat muscle. This muscle contains predominantly the embryonic myosin heavy chain. Antibody 2B6 reacts with the myosin heavy chain, but not with other contractile and noncontractile proteins of the actomyosin preparation, including actin, tropomyosin, C-protein, or the myosin light chains. Similar immunoblots using purified C-protein, actin, and tropomyosin in high concentrations also showed no reaction with 2B6. Furthermore, 2B6 reacts only with the embryonic myosin heavy chain (Fig. 1, lane d) which is the predominant heavy chain in the cultured myosin preparation, but not with the myosin heavy chains from neonatal myosin (lanes b and f) or adult slow or fast myosins (lanes a and g). The reactivity with extensor digito-
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rum longus (lane e) and soleus (lane c) myosin heavy chains from 10-day postpartum rats indicates that some embryonic myosin heavy chain remains in those muscles at this late stage of development, a finding confirmed by pyrophosphate gels of native isozymes (17, 36) as well as by RIA. There is an occasional reaction with proteins running below the 200-kDa myosin heavy chain; this is probably due to 2B6 binding to cleavage products of the myosin heavy chain, since this reaction is seen more frequently in stored myosin samples and can be reduced by isolating myosins in the presence of protease inhibitors. The antibody does not react with the myosin heavy chain of rat ventricles, human platelets, or rabbit smooth muscle (not shown).

A solid phase RIA was used to produce a titration curve of 2B6 with the original antigen, column-purified myosin isolated from the newborn rat. One microgram of myosin was immobilized in polyvinylchloride wells and exposed first to serial dilutions of hybridoma 2B6 supernatant, and then to 125I-labeled rabbit anti-mouse F(ab')2. Bound label was measured by γ spectrometry. The titration curve obtained by plotting bound radiolabel versus serial dilutions of hybridoma supernatants showed maximum binding at a dilution of 1:10, while the midpoint of the linear region of the antibody titration curve occurred at dilutions ranging from 1:222-1:354, depending on the sample. This midpoint dilution was used for competitive RIAs.

The reaction of actomyosins isolated from different stages of development with 2B6 was also examined by solid phase RIA. One microgram of each actomyosin immobilized in polyvinylchloride wells was tested against a 1:10 dilution of 2B6 supernatant for maximum binding, as described above. Actomyosin from the whole hindlimb of newborn rats bound 3640 ± 460 cpm. Combined actomyosins from soleus and extensor digitorum longus muscles of 60-day-old rats treated with propylthiouracil from 3 days of gestation onward (neonatal myosin) bound 170 ± 60 cpm, while a mixture of adult soleus (slow) and extensor digitorum longus (fast) actomyosins bound 80 ± 70 cpm.

The antigen competition radioimmunoassay can be used to compare the binding of 2B6 to column-purified myosins from various stages of development. This assay depends on the ability of myosins in solution to compete with a fixed amount of immobilized newborn rat myosin for a fixed amount of monoclonal antibody. Increasing concentrations of test myosins in solution were exposed to 2B6 and the unbound 2B6 was allowed to react with 1 μg of original antigen immobilized in polyvinylchloride plates. 125I-labeled rabbit anti-mouse F(ab')2 was used to quantitate the amount of 2B6 which bound to the original antigen. When the test antigen in solution was the original newborn rat myosin, binding of 2B6 to the immobilized myosin was reduced by 50% at a myosin concentration of 12.5 μg/ml (Fig. 2). This is higher than seen for some monoclonal antibodies to adult myosins (37) and probably results from the heterogeneity of the original myosin mixture. The competitive RIA can be used to determine the fraction of reactive sites in any myosin sample compared to the number in the original antigen. This would normally be calculated as the concentration of newborn myosin at 50% inhibition of binding/concentration of test myosin at 50% inhibition. With adult fast and slow myosins, however, no concentration of myosin in solution up to 1 mg/ml produced more than a 5% inhibition of 2B6's binding to newborn rat myosin (Fig. 2). Similarly, at 1 mg/ml neonatal myosin inhibited antibody binding to newborn rat myosin by only 37.5%. If the linear portion of the neonatal myosin curve is extrapolated to 50% inhibition, the fraction of antigenic sites in the neonatal sample compared to the newborn myosin sample would be 12.5 μg/1200 μg, or 0.0104. If the myosin concentrations giving 37.5% inhibition are used, the fraction would be 6 μg/500 μg, or 0.012. Hence, neonatal myosin contains approximately 1% of the reactive sites that the original antigen contained. By both RIA and immunoblots, then, this antibody shows specificity for the embryonic myosin heavy chain and not for neonatal or adult myosin heavy chains.

This antibody shows the same specificity across species. Actomyosin was prepared from various animals in the embryonic myosin stage of their development and also from mixed muscles or combined fast and slow muscles of the adult animal. The samples were run on SDS-polyacrylamide gels, and transferred to nitrocellulose. Peroxidase staining with 2B6 (Fig. 3) reveals that the antibody reacts with the embryonic myosin heavy chain, but not with adult fast or slow myosin heavy chains, of all the mammals tested (dog, cat, rat, guinea-pig). The same specificity occurred with human muscle (not shown). The chicken shows an anomalous result. 2B6 fails to react with the embryonic myosin heavy chain of the chicken; instead, it cross-reacts lightly with the adult chicken.
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Myosin heavy chains. Solid phase RIA was used to quantitate the binding of 2B6 to column-purified myosins isolated from embryonic and adult dog diaphragm muscle. The diaphragm in the adult dog contains approximately equal amounts of fast and slow myosins, while the 65-day fetus has over 90% embryonic myosin.\(^2\) One microgram of immobilized myosin from the 65-day fetus bound 3458 ± 131 cpm, while 1 µg of adult diaphragm myosin bound 66 ± 15 cpm. Binding to adult myosin was identical to binding to tubulin, 68 ± 22 cpm.

In vivo myosin molecules aggregate into thick filaments which are the major components of the A band segment of the sarcomere. If 2B6 reacts only with the embryonic myosin heavy chain, it should stain only the A bands in embryonic muscle cells. Rat hindlimb muscles in culture contain predominantly embryonic myosin (36, 38). When these cells are fixed and reacted first with a 1:50 dilution of 2B6, then with a fluorescein-labeled goat anti-mouse IgG, a striated staining pattern is seen (Fig. 4a). Comparison of the fluorescent image with a phase micrograph of the same cell shows that the antibody is localized to the A band. In cross sections of the distal hindlimb at 20 days gestation, all fibers in all muscles react with 2B6; interstitial cells and blood vessels show no reaction, confirming that the antibody does not react with cytoplasmic, non-muscle myosins. Individual muscles in these cross sections of the 20-day embryonic limb were located and photographed. Enlargements of the areas corresponding to the fast extensor digitorum longus (Fig. 4b) and slow soleus (Fig. 4c) muscles are shown.

Pyrophosphate gel electrophoresis of myosins isolated from rat hindlimbs during late gestation or the first week post partum separates several isozymes. The subunit composition of these isozymes is unknown. Hoh and Yeoh (39) have analyzed the light chains of individual isozymes from newborn rabbits by excising single bands from pyrophosphate gels, eluting the myosins, and separating the subunits in SDS gels. This type of analysis of heavy and light chains can be confused by the co-migration of isozymes, as occurs frequently in chicken muscle (40). We have used immunoprecipitation combined with pyrophosphate gel electrophoresis and immunoblots to ascertain which isozymes in newborn animals contain the embryonic myosin heavy chain. For example, on pyrophosphate gels, 5-day soleus muscles contain homologous isozymes (Fig. 5, left, lane a) labeled f1–f4 in order of decreasing mobility, as well as isozymes s1 and s2 which may contain adult slow myosin heavy chains (17). Immunoblots of this pyrophosphate gel (lane a’) show that 2B6 reacts with f2, f3, and f4, but not visibly with f1. (The isozymes reacting with 2B6 were identified by staining adjacent lanes on the nitrocellulose paper with Amido Black.) Since f1 was present in smaller amounts than f2–f4, lack of reaction of 2B6 with f1 is not certain. If this myosin preparation is passed over a column containing immobilized 2B6, the myosin which fails to bind to the column contains f1, f2, and f3 in different proportions than in the original sample; f4 is absent (lane b). Now, 2B6 fails to react with any of these isozymes (lane b’). In addition, 2B6 does not react with the f1, f2, and f3 in a sample containing only neonatal myosin heavy chain. We tentatively conclude, then, that all of the f4 contains the embryonic myosin heavy chain, while all of f1 probably contains the neonatal myosin heavy chain. f2 and f3, however, represent the co-migration of isozymes containing the embryonic myosin heavy chain and those containing the neonatal myosin heavy chain.

We have also used this antibody to examine the changes in embryonic myosin heavy chain during development and some of the factors which might affect its expression. Actomyosin was prepared from the slow twitch soleus and fast twitch extensor digitorum longus muscles at various times after birth. The native proteins were stored in pyrophosphate buffer with glycerol and equal amounts of each sample were tested on the solid phase RIA. Each well, containing 1 µg of immobilized protein, was incubated with identical amounts of 2B6 and 125I-labeled anti-mouse F(ab’)_2; the results of two independent experiments are shown in Fig. 6. During normal development, both soleus and extensor digitorum longus show maximal reactivity with 2B6 at birth (in utero values were not determined). The two muscles then show different rates of decrease...
FIG. 4. Reaction of 2B6 with embryonic muscle cells. A, rat cultures established from newborn rat hindlimb muscles were grown for 6 days, and then fixed and stained with 2B6 followed by fluorescein-labeled rabbit anti-mouse IgG (heavy and light chains). Frozen sections, 8 µm thick, of whole hindlimbs of rats at 20 days gestation were similarly stained with 2B6 and a fluorescent second antibody. Individual muscles were located in the cross sections of the entire limb and photographed. Enlargements of the areas corresponding to the (B) extensor digitorum longus (future fast) muscle and (C) soleus (future slow) muscle are shown.

FIG. 5. Analysis of isozymes containing the embryonic myosin heavy chain. 2B6 was immobilized noncovalently to protein A-conjugated agarose. Myosin isolated from 5-day-old rat soleus muscles was mixed with the antibody-agarose preparation; unbound isozymes were separated from the agarose by centrifugation. Isozymes were separated on pyrophosphate gels according to Refs. 10 and 17, transferred to nitrocellulose paper, and reacted with 2B6 as described. Left, Coomassie Blue-stained isozymes on pyrophosphate gels: (a) unfractionated 5-day soleus isozymes, f1–f4; (b) isozymes which failed to bind to 2B6, f1–f3. Right, nitrocellulose paper after transfer and staining with 2B6: (a’) unfractionated 5-day soleus isozymes showing reaction of 2B6 with f2, f3, and f4; (b’) isozymes which failed to bind to 2B6; now f1–f3 are not stained by the antibody.

in embryonic myosin heavy chain. In the extensor digitorum longus, 60% of the amount at birth can be seen at 10 days, followed by a sharp drop to control levels by approximately 20 days post partum. In the soleus, however, the embryonic myosin heavy chain decreases more slowly. The amount of bound 2B6 gradually decreases to 50% of the birth level at 20 days, and then drops to control levels by approximately 35 days post partum. This less rapid decrease in embryonic myosin heavy chain in the soleus can be confirmed by analyzing the pyrophosphate gels of native myosins from the soleus and extensor digitorum longus 10 days after birth (17).

We have previously shown that thyroid hormone levels control the transition from neonatal to adult isozymes (17): in the fast extensor digitorum longus, the transition from neonatal to adult fast myosin is completely blocked by hypothyroidism, while in the slow soleus, the change from neonatal to adult slow myosin is hastened by hypothyroidism. Hypothyroidism appears to prolong the disappearance of the embryonic myosin heavy chain in both the soleus and the extensor digitorum longus, although the time dependence is somewhat different between the two muscles (Fig. 6). In the hypothyroid extensor digitorum longus, the amount of 2B6 bound remains at high levels up to 25 days post partum. It then declines to about 40% of the initial value by 45 days and to control levels by 60 days post partum, at which time the extensor digitorum longus contains only neonatal myosin (17). The hypothyroid soleus follows the kinetics of the control more closely. Up to 20 days, the amount of 2B6 bound by
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The appearance of the embryonic myosin heavy chain. In the hyperthyroid soleus muscles from successive stages of development were tested for the hypothyroid soleus myosin is slightly above the values of control samples of the same age. It remains at this high level until 45 days, after which it declines to control levels by 60 days post partum. Hyperthyroidism has the opposite effect on the disappearance of the embryonic myosin heavy chain. In the hyperthyroid extensor digitorum longus, no embryonic myosin heavy chain can be detected at 10 days post partum, while in the soleus, a low but detectable level is seen at 10 days, reaching nondetectable levels by 20 days.

**DISCUSSION**

We have prepared a monoclonal antibody to the embryonic myosin heavy chain after immunization of mice with myosin isolated from the bulk hindlimb of the newborn rat. In a solid phase RIA, this antibody, 2B6, reacts with myosin samples known to be rich in embryonic myosin and shows specificity to the myosin heavy chain of the embryonic myosin isozymes by immunoblots. In addition, it binds to the A bands of embryonic muscle fibers and to the myosin isozymes on pyrophosphate gels. There is little cross-reactivity with neonatal or adult fast and slow myosin heavy chains. Using solid phase RIA, we have shown that the time course of the disappearance of embryonic myosin heavy chain differs between the developing slow soleus and the developing fast extensor digitorum longus. This time course can be modified by altering the thyroid status of the animal, although the transition from embryonic to later isozymes occurs under any thyroid conditions. All fibers in the prenatal hindlimb react with the antibody. The replacement of the embryonic myosin heavy chains with neonatal, and then adult, isoforms in different fiber types has not yet been studied.

We chose to prepare monoclonal antibodies to the embryonic myosin heavy chain since most polyclonal antibodies have shown significant cross-reactivity among myosins from different developmental stages. For example, affinity-purified polyclonal antibodies prepared against adult rabbit fast myosin reacts with rat embryonic, neonatal, and adult fast isozymes, while a companion antibody against adult rabbit slow myosin fails to cross-react with any of the other isozymes (36, 41). In other studies, affinity-purified antibodies prepared against chicken fast and slow myosins cross-reacted with rat (42) or chicken (43) embryonic myosins.

Several groups have used monoclonal antibodies to explore developmental and adult myosin heavy chains. Bader et al. (44), for example, prepared monoclonal antibodies against the myosin heavy chain of adult chicken pectoralis myosin and obtained three antibodies with different temporal specificities. Antibody MF20 bound strongly to the myosin heavy chain at all stages of development, while MP30 failed to bind to embryonic myosin heavy chain, but reacted with both neonatal and adult fast myosin heavy chains. MF14 was interpreted to bind only to adult myosin. These antibodies could be used to determine the initiation of synthesis of each isozyme during development. Similarly, Winkelmann et al. (37) prepared monoclonal antibodies which reacted with antigenic sites shared by embryonic and adult chicken pectoralis myosin. Preparation of antibodies specific to only one developmental stage has been difficult, then, with both monoclonal and polyclonal antibodies. This is surprising since the peptide maps of embryonic versus adult myosins are very dissimilar (16). One can hypothesize that the most antigenic sites are common to many myosins and may represent those parts of the molecule important for common properties, such as areas associated with filament formation or light chain binding. Although we have not established the locus at which 2B6 reacts, localization may offer a clue about what is unique and desirable about having developmental isoforms of the contractile proteins.

Control of adult myosin isoforms has been a subject of investigation for many years. Two factors have been described in detail. First, fast and slow isoforms are partially controlled...
by fast and slow motoneurons. Cross-innervation experiments in which motoneurons to fast and slow muscles are severed and forced to reinnervate the opposite muscle types result in fast isoforms in previously slow muscles and slow isoforms in previously fast muscles (45). Moreover, chronic stimulation of adult fast muscles at frequencies inherent to slow motoneurons results in a complete transition from fast to slow isoforms within individual muscles fibers (12, 46). Thyroid hormones also play a role in determining myosin types in adult muscles. Hypothyroidism results in an increase in slow myosins, while hyperthyroidism gives a rise in fast isoforms (47–49). This thyroid effect is independent of innervation (47, 48).

Until recently, little was known about the control of transitions between isoforms during development. Rubinstein and Kelly (23) demonstrated that innervation was important for the synthesis of slow myosin light chains, but not for the synthesis of fast light chains. It is the transition from neonatal to adult slow myosin which is blocked by denervation of a slow muscle (17); the slow muscle instead switches from neonatal to an adult fast myosin. The neonatal to adult fast myosin transition is not qualitatively affected by denervation, nor is the transition from embryonic to neonatal myosin in either fast or slow muscles. On the other hand, thyroid status affects the neonatal to adult fast myosin transition (45). The neonatal to adult fast myosin which is blocked by denervation of a fast or slow muscles. On the other hand, thyroid status affects the neonatal to adult fast myosin transition in the developing fast muscles of the rat hindlimb (17). In the hypothyroid neonate, the transition from neonatal to adult fast myosin is completely blocked and the muscle contains neonatal myosin at least until 60 days post partum. Hyperthyroidism results in a precocious transition to adult fast myosin. The transition from neonatal to adult slow myosin is accelerated by hypothyroidism and retarded by hyperthyroidism, but is otherwise unaffected.

In this paper, we demonstrate that hypothyroidism retards the disappearance of embryonic myosin heavy chain in both the developing fast and slow muscles, while hyperthyroidism accelerates its disappearance. Thyroid status does not, however, block the transition and, therefore, must not be the critical factor for the switch from embryonic to neonatal myosin. Thyroid levels, then, appear to orchestrate coordinated and complementary changes in myosin isoforms during development. For example, in hyperthyroid animals, a precocious appearance of adult fast myosin (17) is accompanied by a precocious disappearance of embryonic myosin. In the absence of thyroid hormones, this coordinated relationship among the developmental forms of myosin breaks down. For example, since hypothyroidism prolongs the disappearance of embryonic myosin, it hastens the increase in definitive adult slow myosin, at 2 to 3 weeks post partum the hypothyroid soleus contains large amounts of both embryonic and adult slow isoforms (17). The role of innervation in the kinetics of embryonic myosin is not completely resolved. In the absence of innervation, such as in tissue culture, embryonic myosin is still synthesized. In vitro, denervation does not prevent the subsequent course of the embryonic to neonatal isozyme transition, although the effect of denervation on the initiation of neonatal myosin synthesis has not been examined.

Peptide mapping of myosins from different stages of rat (16) or chicken (18) development have revealed the existence of at least three stages of myosin synthesis characterized by unique myosin heavy chains. Each stage, however, may be characterized by more than one myosin heavy chain. For example, Lowey et al. (50) have demonstrated unique peptide maps for two myosin heavy chains isolated from the chicken pectoralis muscle at 11 days in vivo, and neither heavy chain corresponded to adult fast or slow myosin heavy chains.

Similarly, Umeda et al. (15) used cDNA clones prepared against the myosin heavy chain mRNAs of chicken muscle to demonstrate at least two mRNA species in embryonic muscle. If the rat system is analogous to the chicken system, our monoclonal antibody may be reacting with an antigenic site common to several embryonic myosin heavy chains. These could be differentially distributed among the f₂, f₃, and f₄ isoforms.

In conclusion, the use of monoclonal antibodies against developmental isoforms of myosin may help distinguish the cues responsible for changes in myosin heavy chain development. may provide data needed to interpret the findings of complementary DNA and mRNA studies of myosin heavy chain genes, and may offer clues to the function of these developmental forms of the myosin heavy chain.

REFERENCES

Monoclonal Antibody to Embryonic Myosin