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Myosin types during the development of embryonic chicken fast and slow muscles

(myogenesis/muscle fiber/anterior latissimus dorsi/pectoralis)

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ABSTRACT **We have studied the myosin types present in developing fast and slow muscles of the chicken embryo. Myosin light chains were characterized by their mobility on sodium dodecyl sulfate/polyacrylamide gels; myosin heavy chains were identified by their reaction with antibodies specific for adult fast or adult slow myosin heavy chains. During development, the pectoralis muscle, a fast muscle in the adult, contains heavy chains and two of the three light chains characteristic of adult fast muscle myosin. However, the anterior latissimus dorsi muscle, a slow muscle in the adult, also contains fast myosin light and heavy chains during early development. Only after the time of innervation does this muscle begin synthesizing predominantly the slow myosin heavy and light chains. We hypothesize that the synthesis of fast myosin in both early fast and slow muscles is the result of the endogenous program for muscle development; initiation of the synthesis of slow myosin, however, is dependent upon exogenous factors.**

Adult muscles can be classified as "slow" or "fast" by a number of physiological, biochemical, and immunological criteria. For example, compared to fast muscles, slow muscles, have a longer twitch time, a lower maximum isometric tetanus tension, and a lower maximum isometric twitch tension (1). Similarly, the contractile proteins of these muscles are not the products of the same genes: different myosin heavy and light chains (2–5), as well as tropomyosins and troponins (6), are found in the two types of muscle.

During early development, all muscles appear to contain identical muscle fibers that are physiologically slow (1, 7–9), and only after innervation does the dichotomy between fast and slow muscles become apparent. The time of innervation correlates with a decrease in the twitch time of some muscles, which then become the definitive fast muscles of the adult; innervation changes the contraction time of other muscles very little, and they become the definitive slow muscles in the adult animal.

Inherent in these observations is the concept that the nerve determines the expression of genes in the muscle. In particular, it is argued that innervation is necessary to induce fast muscles from a common pool of slow muscle fibers (1, 7–10). In fact, the nerves innervating fast and slow muscles do differ by their frequency of firing and their after-hyperpolarization time (11). Recent evidence suggests that the frequency of nerve impulses is the inducing factor (12).

Some biochemical evidence, however, conflicts with the conclusion that nerves induce the expression of the "fast" genotype. For example, when cultured *in vitro* in the complete absence of nerves, the embryonic pectoralis muscle of the chicken exhibits the high myosin ATPase activity and the myosin light chains characteristic of the adult fast pectoralis muscle (13). In addition, light meromyosin paracrystals made from the myosin of this cultured embryonic pectoralis muscle are identical to those made from adult fast pectoralis muscle myosin and not to those from adult slow muscle myosin. Sreter *et al.* (14) demonstrated that some muscles in the fetal rabbit have fast myosin light chains and a high ATPase activity. In this case, light meromyosin paracrystals and proteolytic digests suggested that the myosin heavy chain is similar to neither adult fast nor adult slow myosin heavy chains. Whether or not the fetal rabbit muscles used in these experiments had already been innervated was not reported.

Because of the conflicting data about the influence of nerves on the "fast" genotype and because of the absence of information about the myosin in developing slow muscles, we investigated the development of both presumptive fast and slow muscles in the chicken embryo *in ovo* and used specific myosin types as markers for fast and slow muscles.

MATERIALS AND METHODS

Frozen anterior and posterior latissimus dorsi muscles from adult hens were obtained from Pel-Freez Biologicals (Rogers, AR). Pectoralis muscle was dissected from freshly killed adult hens. Column-purified myosin was prepared from these muscles as described (4).

Because of the small amount of material available, myosin from embryonic muscles could not be purified by the above method. Actomyosin was prepared from embryonic pectoralis muscles by one or two precipitations of a high-salt extract (15), and myosin was purified from this actomyosin by column chromatography on Bio-Gel A15 (16, 17). Even this method was not suitable for isolating myosin from the more limited quantity of embryonic anterior latissimus dorsi muscle (ALD); in addition, this method led to extensive degradation of the myosin. To overcome these problems, ALD dissected from 11-day and 20-day embryos were immediately placed in glycerine/standard salt, 1:1 (vol/vol), at −20° for 3 weeks. The solution was occasionally stirred. The muscles were then washed with standard salt and immediately extracted in 0.6 M KI/0.015 M dithiothreitol/0.01 M Tris, pH 7.0, and chromatographed on a Bio-Gel column. Adult myosins isolated by either of these methods were indistinguishable by light chain patterns and antigenicity from myosins isolated by the DEAE-Sephadex procedure.

Antibodies specific to adult fast and adult slow myosin heavy chains were a gift of I. Arndt; their preparation and specificity have been described (4). In addition to these specific antibodies, crude antiserum against ALD myosin was also used. This crude antiserum contained antibodies to both fast and slow myosins and showed two lines when run on immunodiffusion plates against posterior latissimus dorsi myosin which contains both myosin types (ref. 4; Fig. 2*a*). The line farthest from the central

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Abbreviation: ALD, anterior latissimus dorsi muscle.

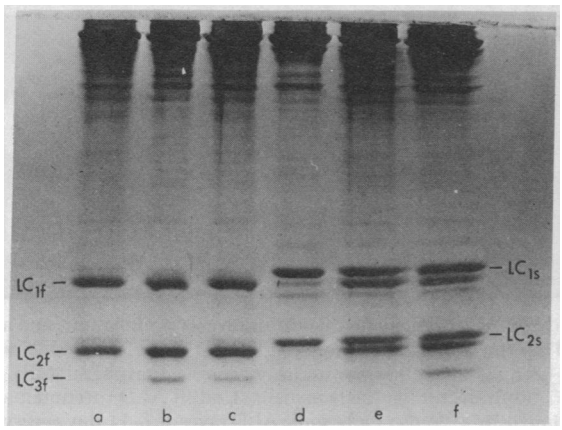

FIG. 1. Sodium dodecyl sulfate gels of embryonic and adult pectoralis myosins. Proteins were electrophoresed for approximately 4 hr at a constant current of 25 mA on slab gels containing 0.1% sodium dodecyl sulfate and 12.5% acrylamide (18). Lanes: a, 20 μg of embryonic pectoralis myosin; b, 20 μg of adult pectoralis (fast) myosin; c, 20 μg each of embryonic and adult pectoralis myosins; d, 20 μg of adult ALD (slow) myosin; e, 20 μg each of embryonic pectoralis and adult ALD myosins; f, 20 μg each of adult pectoralis and adult ALD myosins. In lanes d, e, and f the band between LC_{2f} and LC_{3f} is a degradation product of adult ALD myosin that gradually appeared during the year this sample was used.

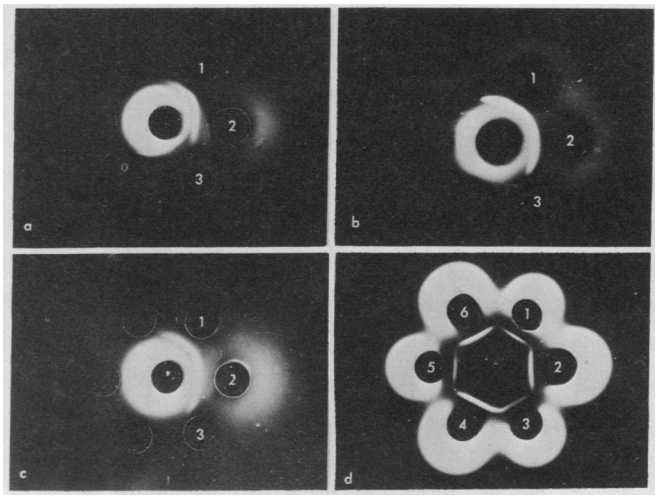

FIG. 2. Ouchterlony double-diffusion gels of embryonic and adult fast muscle myosins. (*a*) Central well: adult posterior latissimus dorsi myosin. Peripheral wells: 1, specific anti-fast myosin heavy chain antibody; 2, ALD antiserum; 3, specific anti-slow myosin heavy chain antibody. (*b*) Central well: adult pectoralis myosin. Peripheral wells: as in *a*. (*c*) Central well: embryonic pectoralis myosin. Peripheral wells: as in *a*. (*d*) Central well: specific anti-fast myosin heavy chain antibody. Peripheral wells: 1, 3, and 5, adult pectoralis myosin; 2, 4, and 6, embryonic pectoralis myosin.

antigen well is identical to the line against specific anti-fast myosin antibody in well 1. The line closest to the antigen well, representing the antigen with the lowest concentration, is continuous with the line against specific anti-slow myosin heavy chain in well 3.

Immunodiffusion was performed on hydratable microfilms (Sebia Immuno, Paris, France). Films were hydrated in deionized water for 1 hr and equilibrated overnight in 0.4 M KCl/0.03 M phosphate, pH 7.3. All protein solutions were in the same buffer. Approximately 50 μg of each protein in 20 μl of buffer was placed in the appropriate well; the gels were kept in a moist chamber at 2–4° for 48 hr. Gels were washed for 24 hr in the same buffer, stained with aqueous 1% thiazine red R for 1 hr, and destained with 1% acetic acid. The destained films were air dried, and the dried films were used as negatives for photographic prints.

Electrophoresis by the method of Laemmli (18) was performed on a vertical polyacrylamide slab gel apparatus. Gels were stained with 0.25% Coomassie brilliant blue R250 in 50% methanol/10% acetic acid for 24 hr and destained by diffusion with 15% methanol/7.5% acetic acid.

Protein was determined by the method of Lowry *et al.* (19).

RESULTS

Two muscles were examined during development: the pectoralis, which is a fast muscle in the adult chicken, and the ALD, which is a predominantly slow muscle in the adult chicken. Myosin from the adult pectoralis will be referred to as "fast myosin," and myosin from the adult ALD will be termed "slow myosin." It should be noted, however, that myosin from the adult ALD contains a small proportion of fast myosin. Myosins from embryonic pectoralis and embryonic ALD will not be classified as "fast" or "slow" until we have demonstrated their composition.

The light chains of fast and slow muscle myosins are electrophoretically distinct (2, 3). Adult fast muscle myosin contained three light chains: LC_{1f}, LC_{2f}, and LC_{3f} (Fig. 1 lane b). These had molecular weights of approximately 25,000, 18,000, and 16,000, respectively. The two light chains of slow muscle myosin, LC_{1s} and LC_{2s}, had molecular weights of approximately 27,000 and 20,000. Because the slow ALD has a small number of fast fibers (4), it contains a small quantity of fast myosin light chains (Fig. 1 lane d). When both fast and slow muscle myosins are electrophoresed together, all five light chains are apparent (Fig. 1 lane f). By this criterion, then, one can easily distinguish fast myosin light chains from slow myosin light chains.

Fast Muscle Development. Myosin was purified from 11-day embryonic chicken pectoralis muscles and electrophoresed on a 12.5% sodium dodecyl sulfate/polyacrylamide slab gel. Embryonic pectoralis myosin contained two light chains (Fig. 1 lane a); adult pectoralis myosin had three light chains (Fig. 1 lane b). From the gel containing a mixture of the embryonic and adult pectoralis myosins (Fig. 1 lane c), it is clear that the two light chains of the embryonic pectoralis myosin comigrated with LC_{1f} and LC_{2f} of the adult pectoralis myosin. In the embryonic myosin, however, there was no light chain corresponding to LC_{3f}. This confirms our previous conclusion that embryonic pectoralis myotubes in tissue culture contain only two of the three fast myosin light chains seen in the adult pectoralis muscle (17). Myosin isolated from pectoralis muscles at 20 days *in ovo* also lacked LC_{3f} (not shown).

Adult ALD myosin also had two major light chains (Fig. 1 lane d). However, coelectrophoresis of the adult slow ALD and the embryonic pectoralis myosins revealed that the two light chains from the embryonic pectoralis are not identical to LC_{1s} and LC_{2s} from the adult slow myosin (Fig. 1 lane e).

We used antibodies specific to adult slow or fast myosin heavy chains to investigate the heavy chain complement of the pectoralis myosin during development. The Ouchterlony gel in Fig. 2*b* shows the reaction of adult pectoralis myosin with our three antibody preparations. Only the antibodies against fast myosin heavy chains—both the specific anti-fast antibodies and the anti-fast components of the ALD antiserum—reacted with the adult pectoralis myosin. Thus, this adult fast muscle, the pectoralis, contains a pure population of fast myosin heavy chains. The gel in Fig. 2*c*, with myosin isolated from 11-day embryonic pectoralis muscle in the center well, showed the identical pattern. Once again, only the antibodies against fast

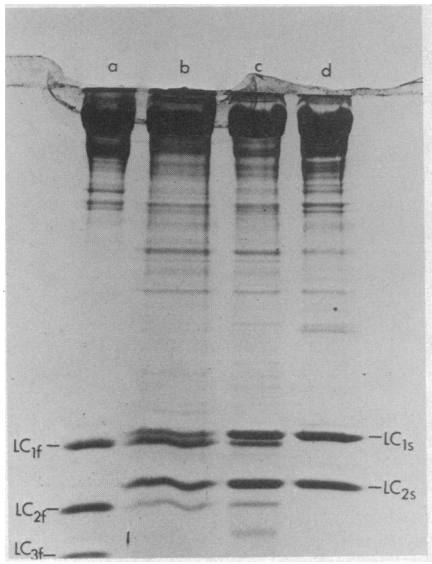

FIG. 3. Sodium dodecyl sulfate gels of embryonic and adult ALD myosins. See legend to Fig. 1 for conditions of electrophoresis. Lanes: a, 20 μg of adult pectoralis (fast) myosin; b, 20 μg of 11-day embryonic ALD myosin; c, 20 μg of 20-day embryonic ALD myosin; d, 20 μg of adult ALD (slow) myosin. The adult ALD used in this figure and in Fig. 4 is from a different preparation than the adult ALD used in Fig. 1.

myosin heavy chains reacted. In Fig. 2*d*, antibody specific to fast myosin heavy chain was in the center well, with adult and embryonic pectoralis myosins in alternating wells. The myosin heavy chains from the two muscles were antigenically identical.

Embryonic pectoralis myosin, then, contains heavy chains and two light chains identical by these criteria to those in the adult pectoralis myosin. Only the absence of LC_{3f} distinguished the embryonic from the adult pectoralis myosin. The "embryonic myosin" in this presumptive fast muscle appears to be fast myosin.

Slow Muscle Development. Similar experiments were performed with embryonic and adult ALD. In this case, the light chain and the antibody patterns were more complicated because the slow ALD contained some fast myosin.

Myosin purified from 11-day and 20-day embryonic ALD was electrophoresed on a sodium dodecyl sulfate/polyacrylamide slab gel. The light chains of the adult fast and adult slow myosins are shown in Fig. 3 lanes a and d. The myosin from 11-day embryonic ALD (Fig. 3 lane b) contained light chains with mobilities identical to those of LC_{1f} and LC_{2f} as well as of LC_{1s} and LC_{2s}. By 20 days *in ovo* (Fig. 3 lane c), the embryonic ALD myosin showed an increase in the relative proportions of LC_{1s} and LC_{2s} and a decrease in the proportions of LC_{1f} and LC_{2f} (the faint line between LC_{2f} and the expected position of LC_{3f} may be a degradation product; this line was not present when embryonic ALD myosin was isolated by other procedures). As in the embryonic pectoralis muscle, no LC_{3f} was present in the developing ALD. The adult ALD (Fig. 3 lane d) contained large amounts of slow light chains and barely detectable amounts of fast light chains.

If slow myosin molecules (both slow heavy and light chains) were replacing whole fast myosin molecules during development, one might expect the concentration of LC_{1s} to equal that of LC_{2s} and the concentration of LC_{1f} to equal that of LC_{2f}, as seen in Fig. 1 lanes a and d or Fig. 3 lane d. Rather, in this case the concentration of LC_{1s} plus LC_{1f} approximated that of LC_{2s} plus LC_{2f}. This puzzling stoichiometry could be explained by a noncoordinated synthesis of light chains or random binding

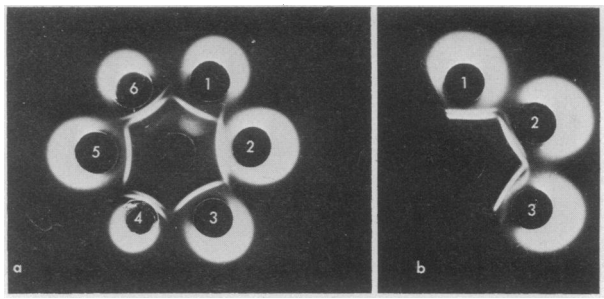

FIG. 4. Ouchterlony double-diffusion gels of embryonic and adult ALD myosins. (*a*) Central well: crude ALD antiserum, which reacts with both fast and slow myosin heavy chains. Peripheral wells: 1, 3, and 5, adult pectoralis (fast) myosin; 2, adult ALD (predominantly slow) myosin; 4, 20-day embryonic ALD myosin; 6, 11-day embryonic ALD myosin. (*b*) Central well: crude ALD antiserum; peripheral wells: 1 and 2, adult ALD myosin; 3, 11-day embryonic ALD myosin.

of light chains to heavy chains, or both. Hence, one could envision myosin molecules containing LC_{1s} plus LC_{2f} or LC_{1f} plus LC_{2s}, as well as the usual combinations of LC_{1s} plus LC_{2s} and LC_{1f} plus LC_{2f}.

During the development of the slow ALD, then, there is a progressive decrease in the proportion of fast myosin light chains accompanied by an increase in the proportion of slow myosin light chains. The antibodies to heavy chains confirm this progression. In the center well of Fig. 4*a* was the crude ALD antiserum that reacts with both adult fast and slow myosin heavy chains. Peripheral wells 1, 3, and 5 contained adult pectoralis myosin as a marker. As shown in Fig. 2*b*, this fast myosin reacts only with the anti-fast component of the crude ALD antiserum. In well 2 was adult ALD myosin; two bands are present. The prominent band, farther from the antigen well, represents slow myosin heavy chains, as has been demonstrated previously (4). The light band, closer to the antigen well, represents fast myosin heavy chains and, indeed, it shows a reaction of identity with the adult pectoralis myosin on either side. In both well 4 (20-day ALD myosin) and well 6 (11-day ALD myosin), the positions of the two bands are reversed. The fast myosin is the predominant component, and the slow myosin line is lighter and closer to the antigen well. Fig. 4*b* shows the same antibody diffused against adult ALD (wells 1 and 2) and 11-day ALD (well 3) myosins. The predominant line in the adult ALD is identical to the minor line in 11-day ALD. Conversely, the major line in 11-day ALD is antigenically identical to the minor line in adult ALD. Thus, both heavy chain components in the developing ALD are identical to both heavy chains in the adult ALD. Hence, if there is a unique heavy chain in the embryo, it either does not react with this antibody or has identical antigenicity to adult heavy chains.

These results suggest that during development the progression from fast to slow myosin in ALD involves both myosin heavy and myosin light chains.

DISCUSSION

Many investigators believe that the nerve controls the expression of genes in the muscle (20, 21). Evidence for this concept comes from two types of experiments. First, cross innervation of a fast muscle with a nerve from a slow muscle, and vice versa, causes a reciprocal transformation of the muscles' properties—i.e., the fast muscle develops the physiological, biochemical, and histochemical properties of a slow muscle, and the slow muscle exhibits the behavior of a fast muscle (1, 7, 8). Second, during embryogenesis, all muscles contain a pool of physiologically identical muscle fibers that have the prolonged twitch time

characteristic of slow fibers (1, 7, 10, 22). Only after innervation do any physiological differences between the future fast and future slow muscles arise.

We have shown that during development a presumptive fast muscle makes a myosin similar to that of the adult fast muscle, except for the absence of LC_{3f}. This occurs even when the muscle is removed and grown *in vitro* in the absence of nerves (13, 17). In culture, the fast myosin type is even synthesized in mononucleated, postmitotic myoblasts, as well as in myotubes (23). Thus, the nerve does not determine the myosin type in this fast muscle. Whether the nerve determines the expression of LC_{3f} is unknown. Investigators have reported the absence of this light chain in the innervated pectoralis of a chicken 2 weeks after hatching (24, 25). Some change within the nerve or some other maturational event could initiate the expression of this light chain.

Before innervation, the developing slow ALD contains large amounts of fast myosin heavy and light chains. By 20 days *in ovo*, 7 days after innervation has begun (22), a larger proportion of the heavy and light chains is slow. By the adult stage, more than 90% of the myosin is slow myosin. The presence of slow myosin in the 11-day ALD, before the reported time of innervation, could indicate either that the reported time of innervation is incorrect or that some event other than innervation initiates the synthesis of slow myosin.

Similar results have been obtained with the myosin light chains of the developing rat soleus (N. Rubinstein and A. Kelly, unpublished data) and the developing rabbit soleus (26): an initial predominance of fast myosin light chains followed by an increasing amount of slow myosin light chains.

Thus, although physiological studies have shown that the rate of contraction and the force–velocity curves of embryonic muscles resemble those of adult slow muscles (1, 7), these biochemical experiments suggest that embryonic muscles contain myosin similar to adult fast myosin. We conclude, therefore, that the demonstrated correlation between myosin type and speed of contraction in adult muscles (27) cannot be extended to developing muscles. When describing developing muscles, the distinctions between "fast" and "slow" become blurred.

What causes the change in the proportion of myosin types during the development of the slow ALD? One explanation might be an asynchrony in the development of different fiber types. A sequential development of fast fibers followed by slow fibers later in embryogenesis could explain these results. Implicit in this view is the supposition that individual postmitotic myoblasts are epigenetically committed to synthesize either fast or slow myosin. Formation of different fiber types could begin at different times, or the early growth of fast fibers could be more rapid than the growth of slow fibers. Only with later development, and perhaps innervation or activity, might the slow fibers hypertrophy and outgrow the fast fibers in the ALD.

An alternative explanation is that innervation signals some muscle fibers to cease synthesizing fast myosin and to begin synthesizing slow myosin. Just as some postmitotic spinal ganglion cells have the option to switch from adrenergic to cholinergic transmitters (28), some postmitotic skeletal muscle cells may have the option to synthesize first fast and then slow myosin, depending upon exogenous influences. We have recently found evidence supporting the idea of switching options. Using type-specific antimyosin antibodies, we have demonstrated that, after chronic stimulation, individual muscle fibers of the fast extensor digitorum longus muscle of the rabbit can change from synthesizing fast myosin to synthesizing slow myosin (29).

As a result of the experiments reported in this paper and the chronic stimulation experiments, we suggest the following sequence of events. The earliest fibers in all muscles synthesize fast myosin. Under exogenous stimulus—from chronic stimulation, excessive use, or a particular type of innervation—the fiber changes its genomic programming and begins synthesizing slow myosin. The removal of this stimulus (by denervation, for example) causes a reversion to the synthesis of fast myosin (N. Rubinstein and A. Kelly, unpublished data). Thus, the expression of the fast myosin gene is an endogenously programmed feature of all muscles' development and is independent of exogenous influences, while expression of the slow myosin gene is dependent on exogenous factors.

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