Contractile Activity Is Required for the Expression of Neonatal Myosin Heavy Chain in Embryonic Chick Pectoral Muscle Cultures

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Abstract. The expression of neonatal myosin heavy chain (MHC) was examined in developing embryonic chicken muscle cultures using a monoclonal antibody (2E9) that has been shown to be specific for that isoform (Bandman, E., 1985, Science (Wash. DC), 227:780–782). After 1 wk in vitro some myotubes could be stained with the antibody, and the number of cells that reacted with 2E9 increased with time in culture. All myotubes always stained with a second monoclonal antibody that reacted with all MHC isoforms (AG19) or with a third monoclonal antibody that reacted with the embryonic but not the neonatal MHC (EB165). Quantitation by ELISA of an extract from 2-wk cultures demonstrated that the neonatal MHC represented between 10 and 15% of the total myosin. The appearance of the neonatal isoform was inhibited by switching young cultures to medium with a higher [K+] which has been shown to block spontaneous contractions of myotubes in culture. Furthermore, if mature cultures that reacted with the neonatal antibody were placed into high [K+] medium, neonatal MHC disappeared from virtually all myotubes within 3 d. The effect of high [K+] medium was reversible. When cultures maintained in high [K+] medium for 2 wk were placed in standard medium, which permitted the resumption of contractile activity, within 24 h cells began to react with the neonatal specific antibody, and by 72 h many myotubes were strongly positive. Since similar results were also obtained by inhibiting spontaneous contractions with tetrodotoxin, we suggest that the development of contractile activity is not only associated with the maturation of myotubes in culture, but may also be the signal that induces the expression of the neonatal MHC.

Myogenic cultures provide a model system in which the expression of muscle-specific proteins can be studied. Myotubes formed in vitro from embryonic myoblasts contain similar myofibrillar proteins and a similar ultrastructure as newly differentiated muscle cells in the embryo (18, 30, 33, 52, 64). However, while cross-striated, contractile myotubes are readily produced in culture, their ability to undergo the subsequent changes associated with muscle maturation is limited.

During late embryonic and postnatal development in vivo muscle fibers undergo a number of changes in muscle protein expression. Some proteins found in adult muscle fibers are not produced in embryonic muscle. For example, phosphorylase, a glycolytic enzyme found in adult avian pectoral muscle fibers, initially appears at hatching and increases during neonatal development (14, 19). Many skeletal muscle proteins exist as different isoforms, and maturing muscle fibers often exhibit changes in isoform expression. Skeletal muscle myosin, for example, is represented by many isoforms that are expressed at different stages of development in both avian and mammalian muscle (for review see reference 3). In chicken pectoral muscle (PM),1 three myosin heavy chains (MHCs) are expressed sequentially (1, 7, 36, 63). These isoforms are referred to as the embryonic, neonatal, and adult MHCs according to their appearance during PM development.

Attempts have been made to determine the regulation of MHC gene expression in vivo. Denervation of newborn chicken or rat muscle did not block the appearance of the adult isoform, which suggests that the nerve is not responsible for these isoform switches (12, 38, 44). Thyroid hormone has been implicated in regulating the appearance of adult fast myosin, since hyperthyroidism accelerated the transition from the neonatal to the adult fast MHC isoform in the rat (24), while hypothyroidism delayed this change (13, 24). Endogenous myogenic factors may also play a role in MHC expression, as recent studies have found that not all chicken fast muscles express these three isoforms sequentially (6, 36, 51). These observations illustrate that the complex environment of the muscle fiber in vivo makes it difficult to interpret which one of many interacting factors may regulate the expression of MHC isoforms during maturation.

In muscle cell cultures derived from embryonic myoblasts, the embryonic MHC is initially expressed after terminal differentiation (5, 62). In chicken PM cultures no transition to the neonatal isoform has been observed even after several

1. Abbreviations used in this paper: MHC, myosin heavy chain; PM, pectoral muscle.
months in vitro (2, 7). These studies have relied on biochemical methods such as non-denaturing gel electrophoresis (2) and SDS PAGE peptide mapping (2, 7) to demonstrate the presence of neonatal myosin. However, because of limited sensitivity of these methods it is possible that a low level of neonatal MHC expression has not been detected. In rat primary cultures the expression of the neonatal isoform has been observed immunocytochemically with a polyclonal antibody (6). In this report we used a monoclonal antibody (2E9) specific to neonatal MHC (4) to re-examine myosin expression in differentiating chicken muscle cell cultures. We found that after 1 wk in culture, myotubes began to express neonatal MHC, and in 2-wk-old cultures it represented 10–15% of the total myosin. The expression of neonatal myosin was found to be coupled to the development of spontaneous contractile activity, since myotubes placed in culture conditions that inhibited contraction did not react with 2E9. If mature contractile cultures that had accumulated the neonatal isoform were placed in culture conditions that prevented further activity, the neonatal isoform rapidly disappeared from myotubes. This effect was reversible and neonatal MHC reappeared within 24 h after cells were returned to an environment that permitted the resumption of contractile activity. These observations suggest that the development of spontaneous contractions plays a functional role in the maturation of myotubes in culture and may be a necessary prerequisite for the expression of neonatal MHC.

Materials and Methods

Cell Cultures and Media

Myogenic cell cultures derived from 11–12-d embryonic chicken PM were prepared as described (7). Standard medium contained 88% MEM (Gibco, Grand Island, NY), 10% horse serum (Gibco and KC Biologicals, Lenexa, KS), and 2% embryo extract (43). No antibiotic or antifungal agents were used because recent observations have demonstrated that these agents depressed MHC accumulation (40). High [K+] medium was identical to standard medium except that 6.6 mM NaHCO3 was replaced by 6.6 mM KHCO3 in the MEM. The final [K+] of this medium was ~12 mM (8). In some experiments tetrodotoxin (0.16 μM) was added to standard medium.

Antibodies

The specificities of monoclonal antibody 2E9 for neonatal chicken pectoralis MHC (4) and of EB165 for embryonic and adult MHC (15) have been shown earlier. AG19 reacted with all chicken skeletal MHC isoforms. All dilutions of monoclonal antibodies were prepared from ascites fluids. A purified rabbit polyclonal anti-chicken myosin antibody was kindly provided by Dr. R. Strohman (55). This antibody reacted equally well with embryonic, neonatal, and adult PM myosins in ELISA.

Immunocytochemistry

Cell cultures were washed with PBS, fixed in 95% ethanol for 10 min, washed again, and then blocked with 2% horse serum, 0.1% sodium azide in PBS for 30 min at 37°C or overnight at 4°C. The cells were incubated with monoclonal antibodies diluted in PBS with 1% horse serum, 0.05% sodium azide (2E9 1:2,500, EB165 1:5,000, and AG19 1:10,000) for 1 h at 37°C. Bound antibody was detected by incubating the cells with biotinylated rabbit anti-mouse antibody followed by avidin-biotin-peroxidase diluted in PBS with 1% horse serum according to the instructions of the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) for 30 min at 37°C. After each reaction the cells were washed for 15 min with three changes of PBS. The cultures were finally incubated with H2O2 and diaminobenzidine to produce a brown reaction product at the site of bound antibodies (28). Coverslips were mounted on cultures with 7.5% gelatine, 30% glycerol.

In those experiments in which the same cultures were reacted with two different antibodies we used the following protocol. After the first antibody reaction, cultures were stained with diaminobenzidine as described above. The cultures were washed with PBS and bound monoclonal antibody was removed by incubating the dishes two times for 5 min in 100 mM glycine-HCl, pH 2.8 (42). This treatment does not remove the brown reaction product of the diaminobenzidine. After washing, the cultures were blocked again and reacted with a second monoclonal antibody as above except that the peroxidase reaction was carried out with 4-chlorononaphthol (42), which yielded a purple reaction product.

Myosins

Myosins were purified from cell cultures, 10 and 12 d embryonic, 20 d posthatch and adult chicken PM as described (7). The concentration of MHCs in the extracts were determined by 10% SDS PAGE (34).

ELISA

Standard ELISA was carried out as previously described (4). Briefly, microtiter plates were coated with 0.2–2.5 μg of various purified myosins. After blocking, the wells were incubated with 2E9 antibody (1:5,000) and bound antibodies were detected using the Vectastain ABC kit (Vector Laboratories, Inc.) and 2,2′azinobis(3-ethylbenz-thiazoline sulfonic acid) for the peroxidase reaction. The absorbance at 405 nm was determined after 30 min. For double antibody sandwich ELISA (37, 58), microtiter plates were coated with 1 μg of column-purified 2E9 antibody in 50 mM carbonate-bicarbonate buffer, pH 9.6 for 3 h at 37°C. The wells were blocked with 5% nonfat dry milk (31) in PBS (pH re-adjusted to 7.4) for 3 h at 37°C and incubated overnight at 4°C with myosin samples diluted in 20 mM pyrophosphate buffer, pH 7.4 containing 5% nonfat dry milk. After washing with PBS, the wells were incubated with a polyclonal anti-chicken myosin antibody diluted 1:2,000 in PBS, 1% horse serum, 0.05% sodium azide for 1 h at 37°C. Bound polyclonal antibody was detected with the Rabbit Vectastain ABC kit as described above for standard ELISA with the exception that the absorbance at 405 nm was determined after 1 h.

Immunoblots of Peptide Maps

Peptide maps were prepared by digesting 5 μg of SDS PAGE purified MHCs with 100 ng of Staphylococcus aureus V8 proteinase according to the procedure of Cleveland et al. (6) as modified by Bandman et al. (5, 7). Replicate gels were transferred to nitrocellulose sheets (56) and one was stained for protein with amido black. The other blot was reacted with 2E9 antibody (1:100) as previously described (4) with the exception that 5% nonfat dry milk was used for blocking and was present in all reactions and washes except the final rinse before the diaminobenzidine reaction (31).

Results

Maturing Myotubes Express Neonatal MHC

Chicken muscle cell cultures were grown under standard conditions and fixed and reacted with monoclonal antibodies as described in methods. As shown in Fig. 1, newly formed myotubes in 5-d-old cultures reacted with AG19 antibody which recognizes all skeletal MHC isoforms (Fig. 1 a), but not with 2E9, a monoclonal antibody which specifically recognizes the neonatal MHC isoform (Fig. 1 b). By 9 d, some myotubes clearly reacted with both AG19 and 2E9 antibodies (Fig. 1, c and d). As shown in Fig. 1 e, all myotubes contain embryonic MHC since they also reacted with EB165 which recognizes the embryonic but not the neonatal isoform.

To clearly show the differential appearance of neonatal myosin in cultured myotubes, we used a double antibody staining technique. We first reacted cultures with 2E9 antibody to label with a brown color those cells that expressed myosin containing the 2E9 epitope. Subsequently, we reacted the stained cultures with AG19 antibody to label with a pur-
Figure 1. Presence of the 2E9 epitope in muscle cell cultures. 5 d (a and b) and 9 d (c, d, and e) chicken embryonic muscle cell cultures were stained with monoclonal antibody AG19 (a and c), which reacts with all skeletal MHCs, 2E9 (b and d), which recognizes only the neonatal MHC, and EB165 (e), which reacts with the embryonic but not the neonatal MHC isoform. 5-d cells contain only myosin that does not react with 2E9 antibody (a and b). At 9 d in culture, myosin containing the 2E9 epitope is present in some myotubes (d), but all the muscle cells still contain the embryonic MHC (e). Bar, 100 μm.

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 ple color myotubes that contained myosin that was not recognized by 2E9. As shown in Fig. 2, 4-d-old cultures did not contain any cells that reacted with 2E9 (Fig. 2 a). By 7 d occasional 2E9 positive myotubes were observed (Fig. 2 b) and their number increased by 10–14 d (Fig. 2, c and d).

 Myosin that reacted with 2E9 antibody could also be detected by ELISA. Myosin was extracted from cell cultures and bound to microtiter plates. The reaction of this extract with 2E9 was compared with those of myosin extracted from embryonic, neonatal, and adult PM (Fig. 3). Although myosin from 2-wk-old cultures did not react as well as myosin from the PM of 20-d-old chicks, it clearly reacted better than myosin from embryonic or adult PM or myosin from younger cultures. This result would be expected if the neonatal isoform only represented a small fraction of the total myosin in 2-wk-cultures. However, it is not possible to accurately quantify the amount of the neonatal isoform from these curves.

 To estimate the amount of myosin that contained the 2E9 epitope in 2-wk cultures that immuno cytchemically reacted well with the 2E9 antibody, we used a double antibody sandwich ELISA. In the standard ELISA method myosin extracts were used to coat microtiter plates. Since these extracts also contain proteins other than myosin, it is not possible to bind equivalent amounts of MHC to the plates to obtain a quantitative assay. In the double antibody sandwich ELISA, a constant amount of purified 2E9 antibody was bound to microtiter wells. After blocking, the wells were incubated with tissue culture myosin, and, for comparison, with defined mixtures of 10-d embryonic and 20-d neonatal myosins. For each dilution, equivalent amounts of MHCs (determined by SDS PAGE) were added to each well irrespective of the total protein concentration of the extracts. Bound MHC was detected with a polyclonal anti-myosin antibody followed by biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase. As illustrated in Fig. 4, no 10-d embryonic myosin bound to the plate. The percentage of neonatal myosin in the tissue culture extract was estimated by comparing its dilution curve with those of the standard mixtures. The data shown suggest that neonatal myosin represents between 10 and 15% of the total myosin in the tissue culture extract. Since in these cultures ~50% of the myotubes reacted with 2E9 antibody, the neonatal epitope is likely present in a minor fraction of the myosin of most of these cells.

 To confirm that the MHC recognized by 2E9 antibody in cell cultures was identical to the neonatal isoform, we reacted 2E9 with a nitrocellulose blot of SDS PAGE MHC peptide maps (Fig. 5). No reaction was observed in digests of myosin from 1-wk-old cultures (lane 1) or in digests of myosin from embryonic pectoral muscle (lane 5). However, identical bands reacted in myosin digests of 2- or 3-wk-old cultures (lanes 2 and 3) and in a mixture of neonatal (15%) and embryonic (85%) myosin (lane 4). This indicates that the 2E9 epitope is present in identical MHCs in mature cell cultures and in neonatal PM. The similar intensity of staining in the digests is good evidence that the values obtained in the ELISA described in Fig. 4 are accurate estimates of the amount of neonatal myosin present in these cultures.

 Neonatal MHC Is Not Expressed in Contraction-inhibited Cultures

 The use of K+-supplemented medium to inhibit spontaneous contractions in culture has been described (8, 23, 39). Elevating [K+] from 5.4 to 12 mM had no effect on the early development of myogenic cultures (8). However after 5 d, cultures in 12 mM [K+] stopped accumulating myosin (8). We reacted cultures grown in 12 mM [K+] medium with...
2E9 antibody to determine whether the neonatal MHC was expressed under these conditions. As shown in Fig. 6, when replicate cultures maintained in normal medium for 10 d became positive for 2E9 antibody (Fig. 6 c), no reaction was observed for cultures that had been transferred to 12 mM [K⁺] medium at 5 d (Fig. 6 e). At this time all myotubes in 12 mM [K⁺] medium still reacted with EB165 antibody (Fig. 6 d), indicating that embryonic MHC was still present in these cells. Even after 21 d, no reaction with 2E9 was observed in 12 mM [K⁺] medium (data not shown).

If cultures that had already accumulated neonatal MHC were switched to 12 mM [K⁺] medium, neonatal MHC rapidly disappeared from myotubes. As shown in Fig. 7, when 14 d-old cultures that reacted with 2E9 antibody (Fig. 7 a) were switched to 12 mM [K⁺] medium, within 72 h reactivity with 2E9 antibody had virtually disappeared (Fig. 7 d). However, if cultures were transferred back to normal medium, within 24 h myotubes began to react with 2E9 antibody and in many cases, the reaction was perinuclear (Fig. 7 e). Within 48 h, many myotubes partially reacted with 2E9 antibody (Fig. 7 f) and by 72 h after the medium change, the majority of myotubes reacted strongly with 2E9 (Fig. 7 g).

Thus, the disappearance of neonatal MHC could be rapidly reversed when cultures were replaced in contraction-permissive medium.

Addition of tetrodotoxin to normal medium also inhibits spontaneous contractile activity (54, 59, 60). Similar to 12 mM [K⁺], tetrodotoxin also inhibited the expression of neonatal myosin and induced its disappearance from mature cultures (data not shown). It is thus likely that the inhibition of neonatal MHC expression is a result of blocking spontaneous contractions, rather than a direct effect of elevated [K⁺] or tetrodotoxin on myotubes.

**Discussion**

There have been previous studies using immunological and biochemical methods to examine MHC transitions in mammalian and avian muscle cell cultures. In rat primary cell cultures, neonatal MHC has been observed using a polyclonal antiserum (61). With the same antibody neonatal MHC was also found in mouse myotubes developing from muscle explants (22). However, in primary chicken cultures no neonatal or adult MHC was found with a monoclonal antibody that recognized these isoforms (1). The discrepancy between this latter study and our results may be due to either differences in culture conditions or sensitivity of the antibody.
myotubes that failed to react with EBI65 antibody. This sug-
gests that the embryonic isoform is not repressed in cultured
tissue, it always represented a small fraction of the
myosin. While in older cultures up to 50% of the myo-
tubes reacted with 2E9 antibody, we have never found any
myotubes that failed to react with EBI65 antibody. This sugges-
ts that the embryonic isoform is not repressed in cultured

Although the amount of neonatal myosin varied from cul-
ture to culture, it always represented a small fraction of the
total myosin. While in older cultures up to 50% of the myo-
tubes reacted with 2E9 antibody, we have never found any
myotubes that failed to react with EBI65 antibody. This sugges-
ts that the embryonic isoform is not repressed in cultured
cells as it normally is in myofibers of neonatal pectoral mus-
cle (7, 36, 63).

Our observations suggest that neonatal MHC expression
is correlated with muscle cell activity. While studies of MHC
transitions in vivo have found that innervation was not re-
quired for the expression of neonatal and adult MHC iso-
forms (12, 38, 44), these observations do not rule out the pos-
sibility that muscle activity is required for the switch from
embryonic myosins. Denervated muscle has been shown to
exhibit continued fibrillations in the absence of reinnerva-
tion (21, 45, 53), and spontaneous muscle activity is common
in chick myogenic cultures in the absence of any nerves
(33, 52).

There have been other observations that indicated that
muscle activity can effect myosin synthesis, degradation, and
accumulation in cell cultures. It has been demonstrated that
electrical stimulation or mechanical stretch of myotubes in
culture enhanced myosin accumulation by increasing the rate
of myosin synthesis (10, 57), while inhibiting spontaneous
muscle activity in vitro reduced myosin accumulation by in-
creasing MHC turnover (8, 23, 54, 59). Enhanced synthesis
of fast myosin light chain 3 has been observed in highly con-	ractsile chick muscle cultures (41). These results are cor-
related with observations in vivo where muscle mass and
myosin accumulation are altered by changes in muscle activ-
ity (25–27, 35, 50).

That a correlation exists between the accumulation of neo-
natal myosin and contractile activity can be drawn from our
observation that cultures grown in 12 mM K⁺ medium
failed to react with 2E9 antibody. Elevation of external [K⁺]
reduces the membrane potential of myotubes and inhibits ac-
tion potential generation and spontaneous contractions. We
have shown earlier that under these conditions myotubes re-
mained quiescent and stopped accumulating myosin (8) and
other contractile proteins (20). Similar results were obtained
by using tetrodotoxin (54, 59), which also inhibits sponta-
aneous contractions, but by blocking sodium channels in the
cell membrane (32). Placing cultures in tetrodotoxin also
resulted in the disappearance of neonatal myosin from our
cultures (data not shown). Since 12 mM K⁺ and tetrodo-
toxin block spontaneous contractions via different mecha-
nisms, it is unlikely that the inhibition of neonatal MHC ex-
pression is a direct effect of these agents. Thus, we propose
that the absence of activity is responsible for the inhibition
of neonatal MHC accumulation.

The inhibition of myosin accumulation induced by 12 mM
K⁺ and tetrodotoxin has been shown to be due to an elevated
rate of myosin turnover (8, 23, 54, 59). Therefore, the rapid
loss of neonatal MHC may not only result from an inhibition
of its synthesis but also from an elevated rate of breakdown.
However, it is also possible that neonatal MHC is more labile
than embryonic myosin. This would also result in its rapid
disappearance from myofibrils in culture. Although our pres-
ent experiments can not distinguish between these two pos-
sibilities, it is feasible to design an ELISA protocol to sepa-
ately determine the t₀.₅ of embryonic and neonatal MHCs in
culture.

The rapid return and the perinuclear appearance of 2E9 re-
activity after cells are replaced in normal K⁺ medium sug-
gests either transcriptional regulation of neonatal MHC ex-
pression or an increase in neonatal MHC mRNA stability.
Within a few hours of the medium change, occasional spon-

Figure 5. Comparison of neonatal and tissue culture MHC peptide
map immunobots reacted with 2E9 antibody. Peptide maps were
prepared from SDS PAGE purified MHC of 7-d cultures (lane 1),
2-wk cultures (lane 2), 3-wk cultures (lane 3), a mixture of 15%
neonatal and 85% embryonic PM myosin (lane 4), and pure em-
broynic PM myosin (lane 5). Replicate gels were blotted to nitrocel-
lulose and stained with amido black (a) or incubated with 2E9 anti-
body (b). Bound antibody was visualized as described in Materials
and Methods. Identical bands reacted in samples prepared from
2- or 3-wk tissue culture myosin (lanes 2 and 3) and the mixture
containing 15% neonatal myosin (lane 4), indicating that the myo-
sin recognized by 2E9 in cell cultures is identical to neonatal PM
MHC.
Figure 6. Effect of 12 mM K⁺ medium on the accumulation of neonatal MHC in cultured cells. Cultures grown in normal medium were fixed at 5 d (a and b) and 9 d (c). Replicate cultures were switched from normal medium to 12 mM K⁺ medium at day 5 and fixed at 9 d (d and e). The cultures were stained with EBI65 (a and d) or 2E9 (b, c, and e). 5-d cultures reacted with EBI65 (a) but not 2E9 (b) and thus contain embryonic but not neonatal MHC. At 9 d, cultures maintained in normal medium were stained by 2E9 (c), and thus had accumulated neonatal MHC, while cultures switched to 12 mM K⁺ medium reacted with EBI65 (d) but not 2E9 (e), and thus did not contain neonatal MHC. Bar, 100 μm.

Synchronous contractions can be observed and could be responsible for the reexpression of neonatal myosin. The molecular mechanism by which contractile activity may effect changes in gene expression is unclear. It has been shown, however, that changes in the pattern of activity of a muscle fiber in vivo can induce or repress the expression of myosin isoforms (11, 29, 47, 49). This phenomena is responsible for fiber-type transformation resulting from electrical stimulation (47, 48) or cross-innervation (9, 17).

The expression of neonatal myosin in post-hatch chicken muscle is coordinated with a rapid increase in size of muscle fibers, and its disappearance in mature fibers is coordinated with a cessation of muscle growth (7, 14, 19). Likewise, when muscle protein accumulation in culture is stopped by inhibiting contractile activity (8), the neonatal MHC isoform disappears. If neonatal myosin expression is a consequence of muscle cell growth, this would imply that the low level of neonatal myosin expression in vitro is the result of the inability of standard culture conditions to sustain myotube growth. Therefore, the factors responsible for determining muscle cell growth in vivo and in vitro will need to be characterized before we can fully understand the regulation of myosin isoform transitions.

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Note Added in Proof: It has recently been reported that myotubes of the mouse C2C12 line undergo a MHC transition with time in culture (Silberstein, L., S. G. Webster, M. Travis, and H. M. Blau, 1986, Cell, 46: 1075-1081).

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The accumulation of neonatal MHC in culture is correlated with spontaneous contractile activity. Cultures grown in normal medium for 14 d were fixed (a) or switched to 12 mM K⁺ medium and fixed either 1 d (b) or 3 d (c) later. A culture was fixed after 14 d in 12 mM K⁺ medium (d), while others were switched back to normal medium and fixed after 1 d (e), 2 d (f), or 3 d (g). The fixed cultures were sequentially stained with 2E9 (brown color) and AG19 (purple color) as described in Materials and Methods. Neonatal MHC disappears rapidly from cells whose spontaneous contractions are inhibited by 12 mM K⁺ medium (compare b and c with a). Reactivity with 2E9 reappears within 24 h when cells are replaced into normal medium (compare e, f, and g with d). Arrows in e show staining around nuclei at 24 h, while arrows in f show myotubes that only partially react with 2E9 at 48 h. Bar, 100 μm.
produces different myosin heavy chains. 

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