# 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.

M YOGENIC 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 isozymes 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

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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 nondenaturing 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 (61).

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 NaHCO$_3$ was replaced by 6.6 mM KHCO$_3$ 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 H$_2$O$_2$ and diaminobenzidine to produce a brown reaction product at the site of bound antibodies (28). Coverslips were mounted on cultures with 7.5% gelatine, 50% 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-chloronaphthol (42), which yielded a purple reaction product.

### Myosins

Myosin was 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 0-2.5 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 protease according to the procedure of Cleveland et al. (16) 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 contained 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-
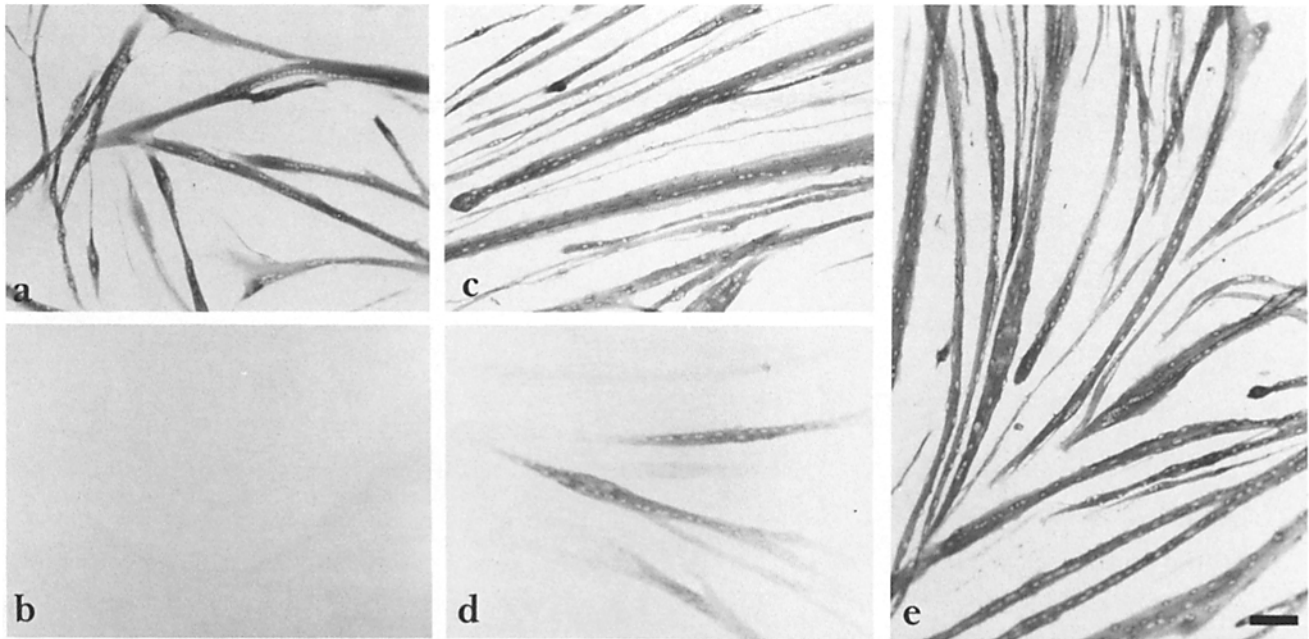
*Figure 1.* Presence of the 2E9 epitope in muscle cell cultures. 5 d (*a* and *b*) and 9 d (*c*, *d*, and *e*) chicken embryonic muscle cell cultures were stained with monoclonal antibody AG19 (*a* and *c*), which reacts with all skeletal MHCs, 2E9 (*b* and *d*), which recognizes only the neonatal MHC, 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.

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 quantitate 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 immunocytochemically 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
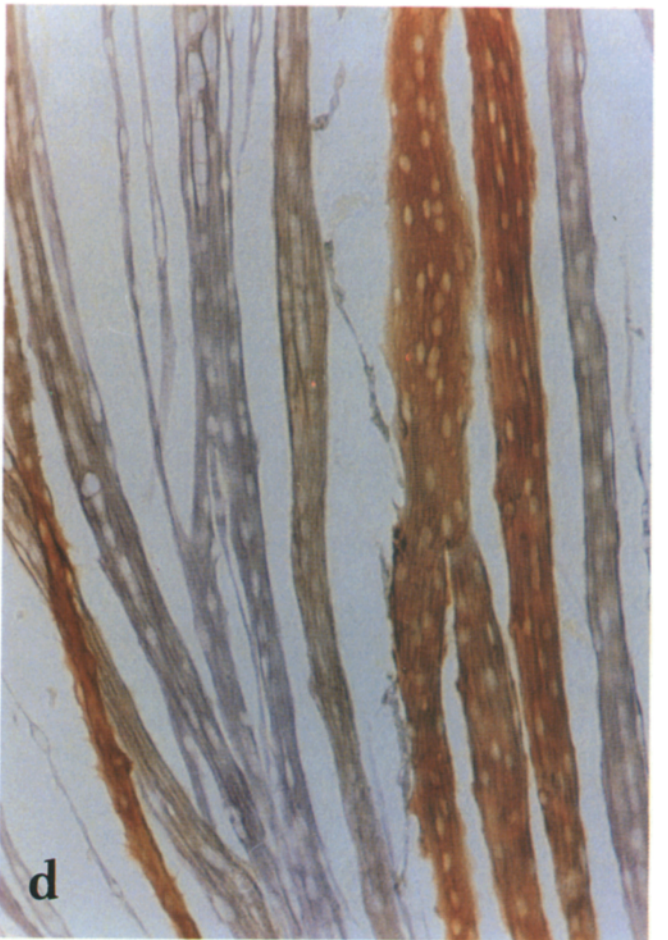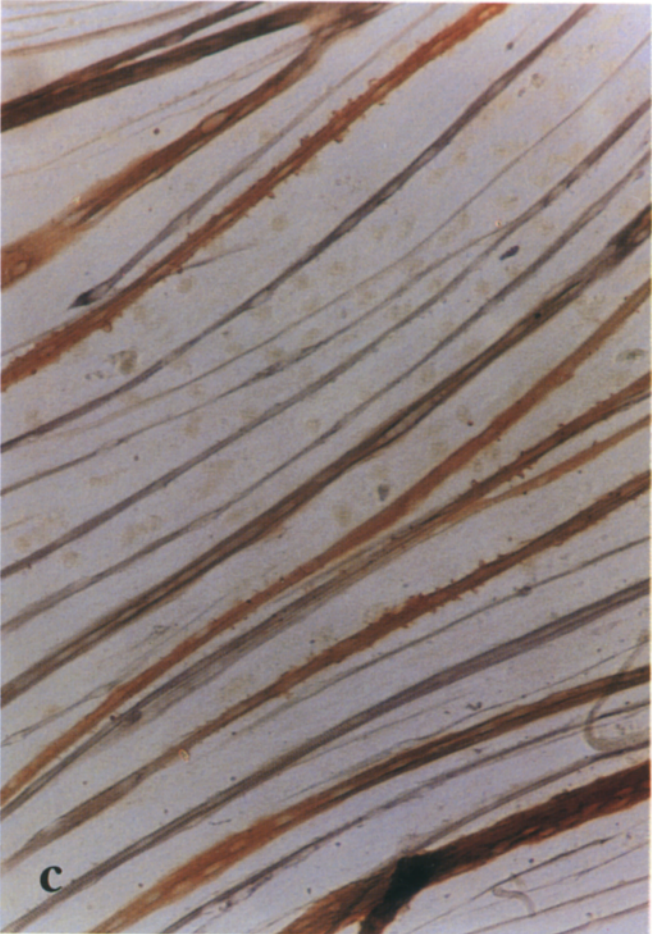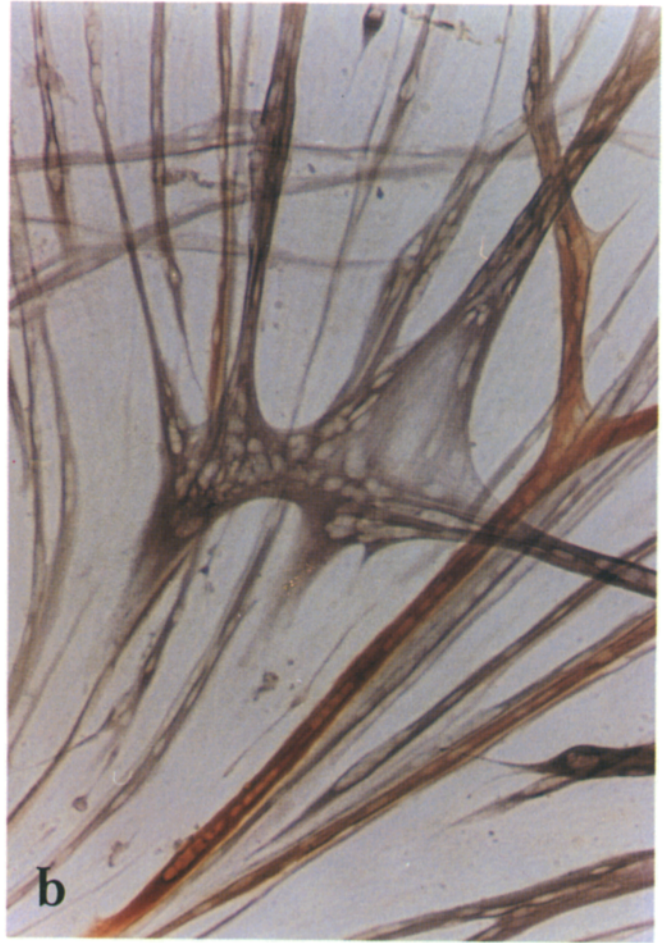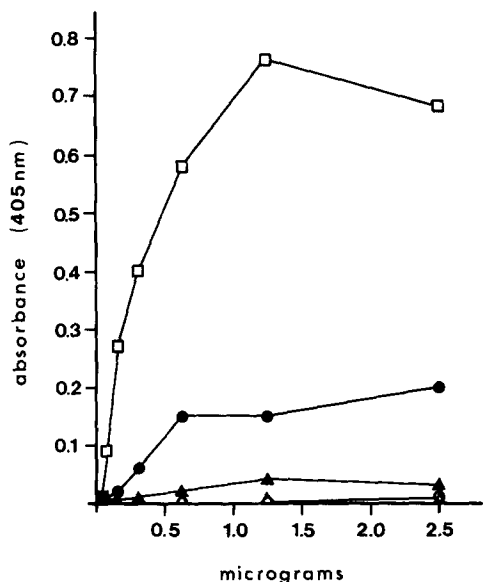
*Figure 3.* Reaction of tissue culture and PM myosins with 2E9 antibody in ELISA. Microtiter plates coated with 0–2.5 μg of myosin purified from 12-d embryonic PM (*open circle*), 20-d neonatal PM (*open square*), adult PM (*open triangle*), and 7- (*solid triangle*) and 14-d muscle cultures (*solid circle*) were reacted with 2E9 antibody. Bound antibody was detected with biotinylated rabbit anti–mouse IgG and avidin-biotin-peroxidase conjugate. Each point represents the average of duplicate determinations. 2E9 reacted strongly with the neonatal myosin but not with the embryonic or adult myosins. An intermediate reaction was obtained in wells coated with myosin from 2-wk-old muscle cell cultures, indicating that the 2E9 epitope is present in the extract.
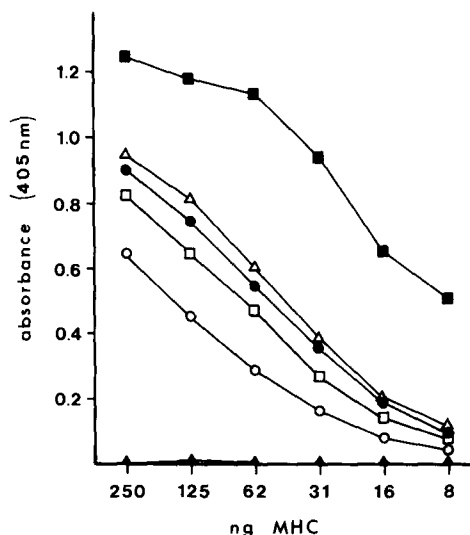
*Figure 4.* Quantitation of myosin containing the 2E9 epitope in 2-wk muscle cell cultures by a double antibody sandwich ELISA. Microtiter plates were coated with purified 2E9 antibody and incubated with myosin extracts from 2-wk tissue cultures and defined mixtures of 10-d embryonic and 20-d neonatal myosin. Embryonic MHC (*solid triangle*); neonatal MHC (*solid square*); 5% neonatal + 95% embryonic (*open circle*); 10% neonatal + 90% embryonic (*open square*); 15% neonatal + 85% embryonic (*open triangle*); and 2-wk tissue culture MHC (*solid circle*). Indicated points are the averages of duplicate determinations. The reaction curve of the tissue culture MHC fell between those of standard mixtures containing 10 and 15% neonatal MHC.

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 *c*). Cultures were maintained for 2 wk in 12 mM [K$^+$] medium and never exhibited any significant reaction with 2E9 (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 antibod-

*Figure 2.* Differential appearance of the 2E9 epitope in maturing muscle cell cultures. Cells were fixed after 4 (*a*), 7 (*b*), 10 (*c*), and 14 d (*d*) in culture and stained with 2E9 (brown color) to show the appearance of myosin containing the 2E9 epitope, and subsequently with AG19 (purple color) to show all the myotubes. After 1 wk in culture myotubes start to accumulate myosin that reacts with 2E9 (*b*), and the number of cells that stain with 2E9 increases with time in culture (*c* and *d*). All cells that reacted with 2E9 also reacted with AG19, but the purple color is not visible because of the dark brown stain. Bars: (*a*, *b*, and *d*) 100 μm; (*c*) 200 μm.
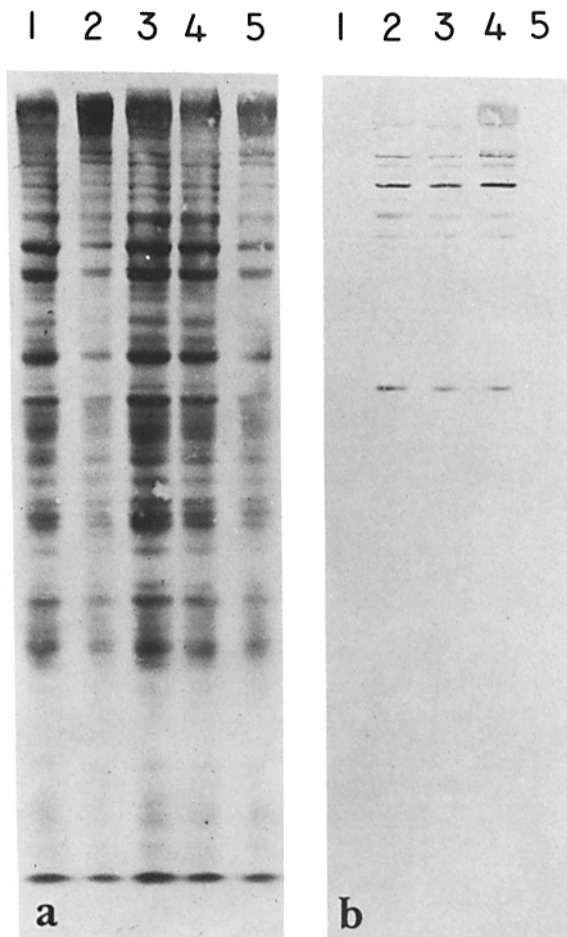
*Figure 5.* Comparison of neonatal and tissue culture MHC peptide map immunoblots reacted with 2E9 antibody. Peptide maps were prepared from SDS PAGE purified MHC of 7-d cultures (lane *1*), 2-wk cultures (lane *2*), 3-wk cultures (lane *3*), a mixture of 15% neonatal and 85% embryonic PM myosin (lane *4*), and pure embryonic PM myosin (lane *5*). Replicate gels were blotted to nitrocellulose and stained with amido black (*a*) or incubated with 2E9 antibody (*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 myosin recognized by 2E9 in cell cultures is identical to neonatal PM MHC.

ies. Using peptide maps of MHC, we previously demonstrated that chicken cell cultures expressed embryonic myosins and that we could not detect neonatal or adult pectoral MHCs (2, 5, 7). Our ability to detect the neonatal MHC in the present study is due to the sensitivity and specificity of 2E9 antibody. We have found that we can detect neonatal MHC by double antibody sandwich ELISA when it represents 1% of the total MHC present (data not shown). Therefore, this assay is at least 10-fold more sensitive than peptide mapping.

Although the amount of neonatal myosin varied from culture to culture, it always represented a small fraction of the total myosin. While in older cultures up to 50% of the myotubes reacted with 2E9 antibody, we have never found any myotubes that failed to react with EB165 antibody. This suggests that the embryonic isoform is not repressed in cultured cells as it normally is in myofibers of neonatal pectoral muscle (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 required for the expression of neonatal and adult MHC isoforms (12, 38, 44), these observations do not rule out the possibility that muscle activity is required for the switch from embryonic myosins. Denervated muscle has been shown to exhibit continued fibrillations in the absence of reinnervation (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 increasing MHC turnover (8, 23, 54, 59). Enhanced synthesis of fast myosin light chain 3 has been observed in highly contractile chick muscle cultures (41). These results are correlated with observations in vivo where muscle mass and myosin accumulation are altered by changes in muscle activity (25-27, 35, 50).

That a correlation exists between the accumulation of neonatal 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 action potential generation and spontaneous contractions. We have shown earlier that under these conditions myotubes remained quiescent and stopped accumulating myosin (8) and other contractile proteins (20). Similar results were obtained by using tetrodotoxin (54, 59), which also inhibits spontaneous 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 tetrodotoxin block spontaneous contractions via different mechanisms, it is unlikely that the inhibition of neonatal MHC expression 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 present experiments can not distinguish between these two possibilities, it is feasible to design an ELISA protocol to separately determine the $t_{0.5}$ of embryonic and neonatal MHCs in culture.

The rapid return and the perinuclear appearance of 2E9 reactivity after cells are replaced in normal $K^+$ medium suggests either transcriptional regulation of neonatal MHC expression or an increase in neonatal MHC mRNA stability. Within a few hours of the medium change, occasional spon-
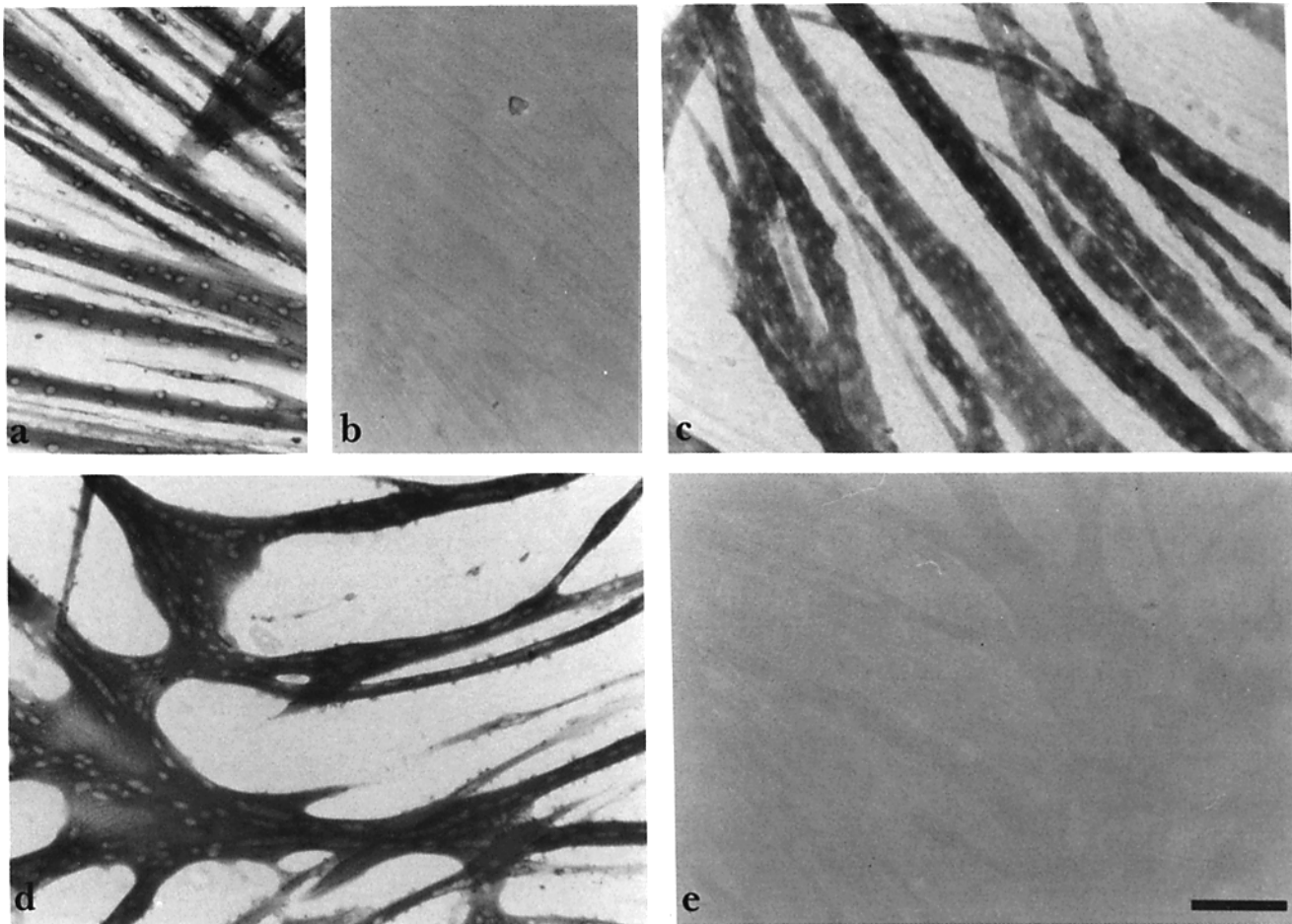
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*Figure 6.* Effect of 12 mM K⁺ medium on the accumulation of neonatal MHC in cultured cells. Cultures grown in normal medium were fixed at 5 d (*a* and *b*) and 9 d (*c*). Replicate cultures were switched from normal medium to 12 mM K⁺ medium at day 5 and fixed at 9 d (*d* and *e*). The cultures were stained with EB165 (*a* and *d*) or 2E9 (*b*, *c*, and *e*). 5-d cultures reacted with EB165 (*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 EB165 (*d*) but not 2E9 (*e*), and thus did not contain neonatal MHC. Bar, 100 μm.

taneous 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.

*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).

*References*

1. Bader, D., T. Masaki, and D. A. Fischman. 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro*. *J. Cell Biol.* 95:763–770.
2. Bandman, E. 1984. Myosin components of the latissimus dorsi and the pectoralis major muscles in the dystrophic chicken. *Muscle and Nerve.* 7:312–326.
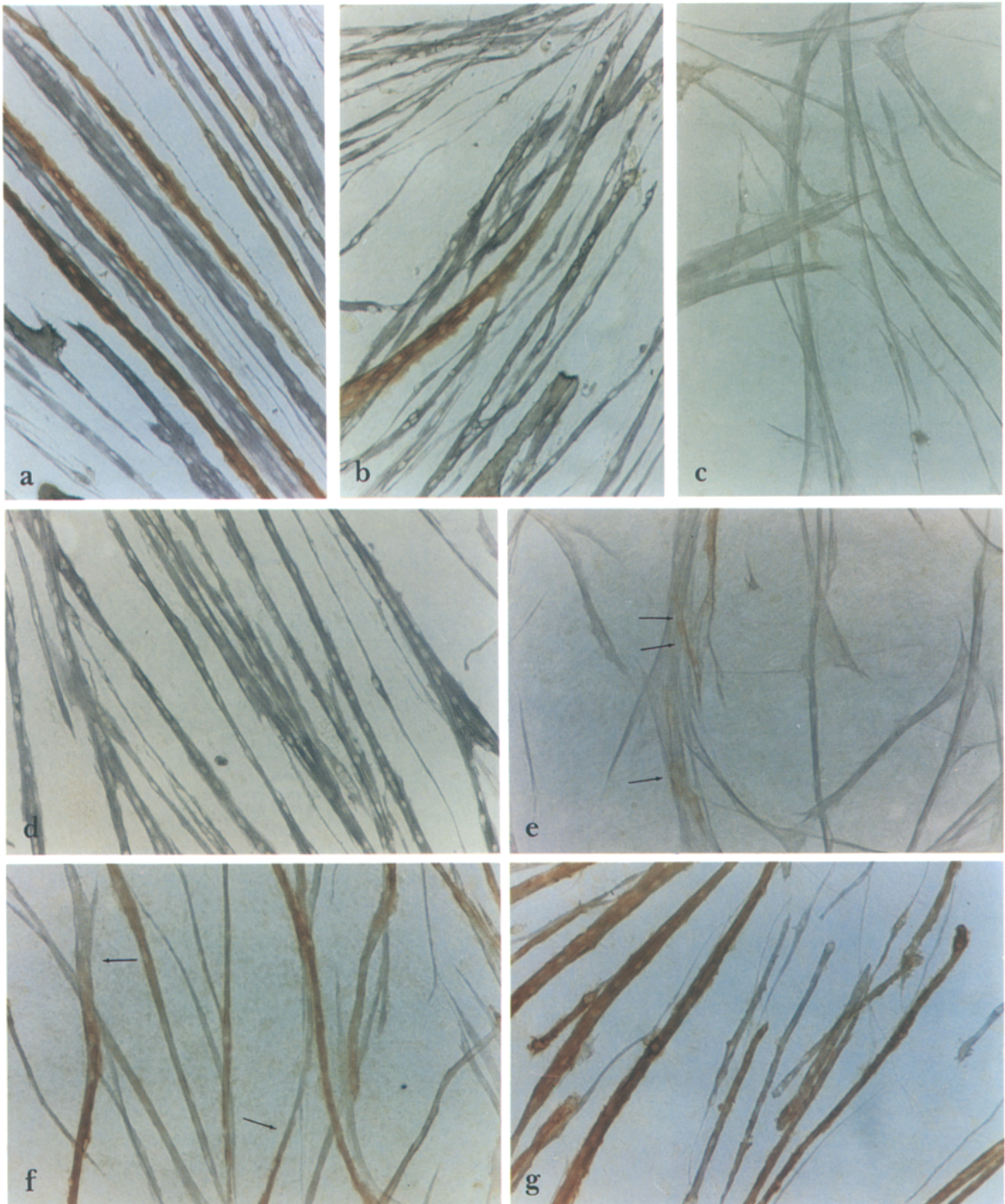
*Figure 7.* The accumulation of neonatal MHC in culture is correlated with spontaneous contractile activity. Cultures grown in normal medium for 14 d were fixed (*a*) or switched to 12 mM K$^+$ medium and fixed either 1 d (*b*) or 3 d (*c*) later. A culture was fixed after 14 d in 12 mM K$^+$ medium (*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.

3. Bandman, E. 1985. Myosin isoenzyme transitions in muscle development, maturation, and disease. *Int. Rev. Cytol.* 97:97–131.

4. Bandman, E. 1985. Continued expression of neonatal myosin heavy chain in adult dystrophic skeletal muscle. *Science (Wash. DC).* 227:780–782.

5. Bandman, E., R. Matsuda, J. Micou-Eastwood and R. C. Strohman. 1981. *In vitro* translation of RNA from embryonic and adult pectoralis muscle produces different myosin heavy chains. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 136:301–305.

6. Bandman, E., R. Matsuda, and R. C. Strohman. 1982. Myosin heavy chains from two different fast-twitch muscles have different peptide maps but identical mRNAs. *Cell.* 29:645–650.

7. Bandman, E., R. Matsuda, and R. C. Strohman. 1982. Developmental appearance of myosin heavy and light chain isoforms *in vivo* and *in vitro* in chicken skeletal muscle. *Dev. Biol.* 93:508–518.

8. Bandman, E., and R. C. Strohman. 1982. Increased $K^+$ inhibits spontaneous contractions and reduces myosin accumulation in cultured chick myotubes. *J. Cell Biol.* 93:698–704.

9. Barany, M., and R. I. Close. 1971. The transformation of myosin in cross-reinnervated rat muscles. *J. Physiol.* 231:455–474.

10. Brevet, A., E. Pinto, J. Peacock, and F. Stockdale. 1976. Myosin synthesis is increased by electrical stimulation of skeletal muscle cell cultures. *Science (Wash. DC).* 193:1152–1154.

11. Brown, W., S. Salmons, and R. G. Whalen. 1983. The sequential replacement of myosin subunit isoforms during muscle type transformation induced by long term electrical stimulation. *J. Biol. Chem.* 258:14686–14692.

12. Butler-Browne, G. S., L. B. Bugaisky, S. Cuenoud, K. Schwartz, and R. G. Whalen. 1982. Denervation of newborn rat muscles does not block the appearance of adult fast myosin heavy chain. *Nature (Lond.).* 299:830–833.

13. Butler-Browne, G. S., D. Herlicoviez, and R. G. Whalen. 1984. Effects of hypothyroidism on myosin isozyme transitions in developing rat muscle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 166:71–75.

14. Cardinet, G. H., R. A. Freedland, W. S. Tyler, and L. M. Julian. 1972. Morphologic, histochemical, and quantitative enzyme study of hereditary avian muscular dystrophy. *Am. J. Vet. Res.* 33:1671–1684.

15. Cerny, L. C., and E. Bandman. 1986. Expression of myosin heavy chain isoforms in regenerating myotubes of innervated and denervated chicken pectoral muscle. *Dev. Biol.* In press.

16. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.

17. Close, R. 1969. Dynamic properties of fast and slow skeletal muscles of the rat after nerve cross-union. *J. Physiol.* 204:331–346.

18. Coleman, J., and A. Coleman. 1968. Muscle differentiation and macromolecular synthesis. *J. Cell Physiol.* 72:19–34.

19. Cosmos, E. 1966. Enzymatic activity of differentiating muscle fibers. *Dev. Biol.* 13:163–181.

20. Crisona, N. J., and R. Strohman. 1983. Inhibition of contraction of cultured muscle fibers results in increased turnover of myofibrillar proteins but not of intermediate-filament proteins. *J. Cell Biol.* 96:684–692.

21. Denny-Brown, D., and J. B. Pennybacker. 1938. Fibrillation and fasciculation in voluntary muscle. *Brain.* 61:311–334.

22. Ecob, M., G. S. Butler-Browne, and R. G. Whalen. 1983. The adult fast isozyme of myosin is present in a nerve-muscle tissue culture system. *Differentiation.* 25:84–87.

23. Friedman, B., and J. A. Powell. 1981. Modulation of myosin heavy chain turnover by spontaneous action potential generation in cultured dysgenic (mdg) muscle. *Dev. Biol.* 83:399–404.

24. Gambke, B., G. E. Lyons, J. Haselgrove, A. M. Kelly, and N. A. Rubinstein. 1983. Thyroidal and neural control of myosin transitions during development of rat fast and slow muscles. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 156:335–339.

25. Goldberg, A. L. 1967. Work-induced growth of skeletal muscle in normal and hypophysectomized rats. *Am. J. Physiol.* 312:1193–1198.

26. Goldspink, D. F. 1981. The influence of contractile activity and the nerve supply on muscle size and protein turnover. *In* Plasticity of Muscle. D. Pette, editor. Walter de Gruyter, Berlin. 524–540.

27. Goss, R. J. 1972. Regulation of Organ and Tissue Growth. Academic Press, Inc., New York.

28. Graham, R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291–302.

29. Heilig, A., and D. Pette. 1983. Changes in transcriptional activity of chronically stimulated fast twitch muscle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 151:211–214.

30. Holtzer, H., H. Sanger, H. Ishikawa, and K. Strahs. 1973. Selected topics in skeletal myogenesis. *Cold Spring Harbor Symp. Quant. Biol.* XXXVII:549–566.

31. Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.* 1:3–8.

32. Kao, C. Y. 1966. Tetrodotoxin, saxitoxin and their significance in the study of excitation phenomena. *Pharmacol. Rev.* 18:997–1049.

33. Konigsberg, I. 1963. Clonal analysis of myogenesis. *Science* 40:1273–1280.

34. Laemmli, U. K. 1970. Changes of structural proteins during assembly of the head of the bacteriophage T4. *Nature (Lond.).* 227:680–685.

35. Laurent, G. J., M. P. Sparrow, and D. J. Millward. 1978. Changes in rates of protein synthesis and breakdown during hypertrophy of the anterior and posterior latissimus dorsi muscles. *Biochem. J.* 176:407–417.

36. Lowey, S., P. A. Benfield, D. D. LeBlanc, and G. S. Waller. 1983. Myosin isozymes in avian skeletal muscles. II. Fractionation of myosin isozymes in developing chicken pectoralis muscles. *J. Musc. Res. Cell Motil.* 4:695–716.

37. MacDonald, D. J., and A. M. Kelley. 1978. The rapid quantitation of serum alpha-fetoprotein by two site micro enzyme immunoassay. *Clin. Chim. Acta.* 87:367–372.

38. Matsuda, R., D. Spector, and R. C. Strohman. 1984. Denervated skeletal muscle displays discoordinate regulation for the synthesis of several myofibrillar proteins. *Proc. Natl. Acad. Sci.* 81:1360–1364.

39. McDermott, P., M. Daood, and I. Klein. 1985. Contraction regulates myosin synthesis and myosin content of cultured heart cells. *Am. J. Physiol.* 249:H763–H769.

40. Moss, P. M., D. H. Spector, C. A. Glass, and R. C. Strohman. 1984. Streptomycin retards the phenotypic maturation of chick myogenic cells. *In Vitro.* 20:473–478.

41. Moss, P. M., J. Micou-Eastwood, and R. C. Strohman. 1986. Altered synthesis of myosin light chains is associated with contractility in cultures of differentiating chick embryo breast muscle. *Dev. Biol.* 114:311–314.

42. Nakane, P. K. 1968. Simultaneous localization of multiple antigens using the peroxidase-labeled antibody method: a study of pituatary glands of the rat. *J. Histochem. Cytochem.* 16:557–560.

43. O'Neill, M. C., and R. C. Strohman. 1969. Changes in DNA polymerase activity associated with cell fusion in cultures of embryonic muscle. *J. Cell Physiol.* 73:61–68.

44. Obinata, T., O. Saitoh, and H. Takano-Ohmuro. 1984. Effect of denervation on the isoform transitions of tropomyosin, troponin T and myosin isozyme. *J. Biochem.* 95:585–589.

45. Purves, D., and B. Sakmann. 1974. The effect of contractile activity on fibrillation and extrajunctional acetylcholine-sensitivity in rat muscle maintained in organ culture. *J. Physiol.* 237:157–182.

46. Rubinstein, N., K. Mabuchi, F. Pepe, S. Salmons, J. Gergely, and F. Sreter. 1978. Use of type-specific antimyosins to demonstrate the transformation of individual fibers in chronically stimulated rabbit fast muscles. *J. Cell Biol.* 79:252–261.

47. Salmons, S., and F. A. Sreter. 1976. Significance of impulse activity in the transformation of skeletal muscle type. *Nature (Lond.).* 263:130–34.

48. Salmons, S., and G. Vrbova. 1969. The influence of activity on some contractile characteristics of mammalian fast and slow muscles. *J. Physiol.* 201:535–549.

49. Seedorf, K., U. Seedorf, and D. Pette. 1983. Coordinate expression of alkali and DTNB myosin light chains during transformation of rabbit fast muscle by chronic stimulation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 158:321–324.

50. Shear, C. R. 1981. Effects of disuse on growing and adult chick skeletal muscle. *J. Cell Sci.* 48:35–54.

51. Shelton, G. D., and E. Bandman. 1985. Unusual fast myosin isozyme pattern in the gastrocnemius muscle of the chicken. *J. Musc. Res. Cell Motil.* 6:435–446.

52. Shimada, Y., D. A. Fischman, and A. A. Moscona. 1968. The fine structure of embryonic chick skeletal cells differentiated *in vitro*. *J. Cell Biol.* 35:445–453.

53. Smith, J. W., and S. Thesleff. 1976. Spontaneous activity in denervated mouse diaphragm muscle. *J. Physiol.* 257:171–186.

54. Strohman, R. C., E. Bandman, C. R. Walker, and D. Spector. 1981. Regulation of myosin accumulation by muscle activity in cell culture. *J. Musc. Res. Cell Motil.* 2:269–282.

55. Strohman, R. C., P. S. Moss, J. Micou-Eastwood, and D. Spector. 1977. Messenger RNA for myosin polypeptides: isolation from single myogenic cell cultures. *Cell.* 10:265–273.

56. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* 76:4350–4353.

57. Vandenburgh, H. 1983. Cell shape and growth regulation in skeletal muscle: Exogenous versus endogenous factors. *J. Cell. Physiol.* 116:363–371.

58. Voller, A., D. E. Bidwell, and A. Bartlett. 1976. Enzyme immunoassays in diagnostic medicine: theory and practice. *Bull. Wld. Hlth. Org.* 53:55–65.

59. Walker, C. R., and R. C. Strohman. 1978. Myosin turnover in cultured muscle fibers relaxed by tetrodotoxin. *Exp. Cell Res.* 116:341–348.

60. Walker, C. R., and B. W. Wilson. 1976. Regulation of acetylcholinesterase in cultured muscle by chemical agents and electrical stimulation. *Neurosci.* 1:191–196.

61. Whalen, R. G., L. B. Bugaisky, G. S. Butler-Browne, and C. Pinset. 1984. Control of myogenesis and myosin isozyme transitions in rat muscle cells. *In* Experimental Biology and Medicine. A. Wolsky, editor. S. Karger, Basel, New York. 155–164.

62. Whalen, R. G., K. Schwartz, P. Bouveret, S. M. Sell, and F. Gros. 1979. Contractile protein isozymes in muscle development: identification of an embryonic form of myosin heavy chain. *Proc. Natl. Acad. Sci.* 76:5197–5201.

63. Winkelmann, D. A., S. Lowey, and J. L. Press. 1983. Monoclonal antibodies localize changes on myosin heavy chain isozymes during avian myogenesis. *Cell.* 34:295–306.

64. Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA.* 61:477–483.