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Cardiac Actin Is the Major Actin Gene Product in Skeletal Muscle Cell Differentiation In Vitro

WILLIAM BAINS,[1,2] PHYLLIS PONTE,[1] HELEN BLAU,[2] AND LARRY KEDES[3]*

The MEDIGEN Project, Department of Medicine,[1] and Department of Pharmacology,[2] Stanford University School of Medicine and Veterans Administration Medical Center,[3] Palo Alto, California 94304

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We examined the expression of α-skeletal, α-cardiac, and β- and γ-cytoskeletal actin genes in a mouse skeletal muscle cell line (C2C12) during differentiation in vitro. Using isotype-specific cDNA probes, we showed that the α-skeletal actin mRNA pool reached only 15% of the level reached in adult skeletal muscle and required several days to attain this peak, which was then stably maintained. However, these cells accumulated a pool of α-cardiac actin six times higher than the α-skeletal actin mRNA peak within 24 h of the initiation of differentiation. After cells had been cultured for an additional 3 days, this pool declined to 10% of its peak level. In contrast, over 95% of the actin mRNA in adult skeletal muscle coded for α-actin. This suggests that C2C12 cells express a pattern of sarcomeric actin genes typical of either muscle development or regeneration and distinct from that seen in mature, adult tissue. Concurrently in the course of differentiation the β- and γ-cytoskeletal actin mRNA pools decreased to less than 10% of their levels in proliferating cells. The decreases in β- and γ-cytoskeletal actin mRNAs are apparently not coordinately regulated.

Many multigene families contain members whose expression is specific to muscle. During myogenesis, the expression of these families is modulated to increase the expression of the genes which code for proteins specific to muscle and to decrease expression of other family members. The behavior of the actin gene family provides a useful model. During differentiation of muscle tissue, the amount of mRNA coding for β- and γ-cytoskeletal actins present in all nonmuscle cells (30) is reduced, whereas the sarcomeric actin mRNAs are synthesized in large amounts (10, 26, 27). The major actin proteins present in adult vertebrate heart and skeletal muscle tissues are α-cardiac and α-skeletal actin, respectively (hereinafter called "cardiac" and "skeletal" actin, respectively), and these two isotypes are only expressed in striated muscles (31).

During both development and regeneration, muscle tissue is formed from mononucleated precursor cells. These myoblasts are present in adult muscle as satellite cells positioned between the basement membrane and the sarcolemma of the differentiated, multinucleate muscle fiber (17). During muscle growth and development after muscle injury and tissue dissociation and culture in vitro, these precursor cells proliferate extensively (1, 4, 6, 14). Satellite cells fuse to form multinucleate muscle fibers, which express muscle-specific proteins (3, 5, 34) and are considered a determined precursor cell population for muscle. Both myoblast cell lines (24, 34) and clonal populations of primary cells (4, 5, 6) are available for study. These cells may be induced to differentiate readily in culture and have been the focus of many studies on the regulation of multigene families during myogenesis. Such studies have produced the unexpected result that differentiating muscle cells from adult muscle do not express the same pattern of gene activity as the tissue from which they were derived. Instead, primary cells and myoblast cell lines derived from fetal or adult muscle exhibit a fetal pattern of myosin light chain (28, 32), myosin heavy chain (33), troponin T, I, and C (29), and tropomyosin (16) syntheses. Recent studies support the existence of developmentally regulated isoforms of actin in skeletal muscle. Cardiac actin mRNA is not confined to the heart but is present, together with skeletal actin mRNA, in developing murine skeletal muscle (18) and in adult human skeletal muscle (12). These results suggest that the actin gene family, like the myosin gene family, possesses members subject to developmental modulation.

A major problem in the study of the sarcomeric actins has been the extreme similarity of the actin protein sequences and of the DNA sequences of the protein-coding regions of their genes. This has made quantitation different actin mRNAs extremely difficult. Consequently, the mRNAs for skeletal and cardiac actin were not quantitated by Minty et al. (18) or by Schwartz and Rothblum (26), because their cloned probes contained coding region sequences, and thus they could not adequately discriminate between the mRNAs for the two sarcomeric actins. However, many investigators have shown that the 3′ untranslated regions (3′UTRs) of actin mRNAs can be used as isotype-specific probes (8, 21, 27). We have used such isotype-specific probes to detect and quantitate the presence of skeletal actin in human heart muscle and cardiac actin in human skeletal muscle (12). As shown by us and others, the isotype-specific 3′UTR sequences unique to specific actin isotype mRNAs are shared across vertebrate species (19, 21, 22).

In the present study we used these probes to examine the expression of specific actin genes in the mouse skeletal muscle myoblast cell line C2C12 (2). We found that skeletal actin is only a minor differentiated product of C2C12 cells and that the major actin mRNA synthesized upon cell differentiation is cardiac actin mRNA. Furthermore, the pools of β- and γ-cytoskeletal actin mRNAs decline during myoblast differentiation, but this decline may not be coordinately regulated.

MATERIALS AND METHODS

Cloned probes. Fragments from the 3′UTR of human β- and γ-cytoskeletal actin cDNA clones were prepared as previously described (21). The cardiac actin probe was a 170-base-pair *Dde*I-*Bst*NI fragment extending from the termi-

* Corresponding author.

nator codon to nucleotide 170 in the 3'UTR of the human cardiac actin cDNA (12). The skeletal actin probe was an *Fnu4HI* DNA fragment containing the sequence between nucleotides 115 and 251 of the 3'UTR of the human skeletal actin cDNA clone pHMαA-1 (13). Both fragments were subcloned into the *SmaI* site of pHP34 (23). All cloned probes were separated from vector sequences by restriction and electrophoresis on agarose gels before self-ligation and nick translation (25). The chick actin coding region probe is the 3' coding region *KpnI* probe described by Engel et al. (11). The chick actin cDNA probe described by Cleveland et al. (8) was a gift from Don Cleveland. This probe hybridizes equally to all mammalian actin sequences under the conditions used in this study (8, 11, 13).

Cell culture. C2C12 is a subclone obtained in our laboratory (2) from a myoblast cell line derived from regenerating adult mouse skeletal muscle (34). Cells were cultured as described previously by Blau et al. (2). Briefly, mononucleated myoblasts were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 20% fetal calf serum and 0.5% chicken embryo extract and maintained at low density by frequent passaging. To induce differentiation, cells were grown to 90% confluence and then cultured for 2 days in a differentiation medium (DM) containing Dulbecco modified Eagle medium and 2% horse serum. Cytosine arabinoside was then added for a 2-day period to a final concentration of 10^{-5} M to eliminate proliferating myoblasts remaining in the culture. After a total of 4 days of culture in DM, the majority of the nuclei were contained in multinucleate, spontaneously contracting myotubes.

RNA preparation. RNA was prepared from murine tissues by the procedure of Palmiter (20). RNA was obtained from cultured muscle cell cytoplasm by the method of Brawerman et al. (7). Cells were washed three times in Tris-buffered saline, lysed in 10 mM Tris-hydrochloride (pH 7.5)–10 mM KCl–1 mM $MgCl_2$–0.5% Triton X-100–0.1% sodium deoxycholate, and homogenized with a Dounce homogenizer fitted with a loose ("B") pestle. Nuclei and cell debris were removed by centrifugation at 1,000 × *g* for 7 min at 4°C. The supernatant, containing the cytoplasm, was extracted once each with phenol, phenol-chloroform (1:1), and chloroform to remove protein. The RNA was concentrated by ethanol precipitation. RNA pellets were suspended in water. The concentration of RNA in each sample was determined spectrophotometrically and confirmed by ethidium bromide staining of RNA electrophoresed in agarose gels.

RNA electrophoresis and filter hybridization. Samples of cytoplasmic RNA (10 μg) were analyzed by electrophoresis on 1% agarose gels containing 7.5% formaldehyde, as described by Maniatis et al. (15), at 150 V for 4 to 6 h. RNA was blot-transferred onto nitrocellulose, and the filters were baked for 5 h at 80°C. The filters were prehybridized overnight in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10 mM NaH_2PO_4 (pH 7.4)–4× Denhardt solution (9)–10% dextran sulfate–50% formamide at 38°C. Hybridization proceeded for 24 h with the same buffer plus the appropriate nick-translated DNA probe. After hybridization, the filters were washed four times with 0.5× SSC at 50°C, dried, and exposed to Kodak XAR-2 X-ray film. Autoradiographs were scanned on an Optronics International P-1000 Photoscanner. After autoradiography, filters were stained with methylene blue and photographed, and the photographic negatives were scanned to determine the relative amounts of RNA in each sample that had transferred to the nitrocellulose filter.

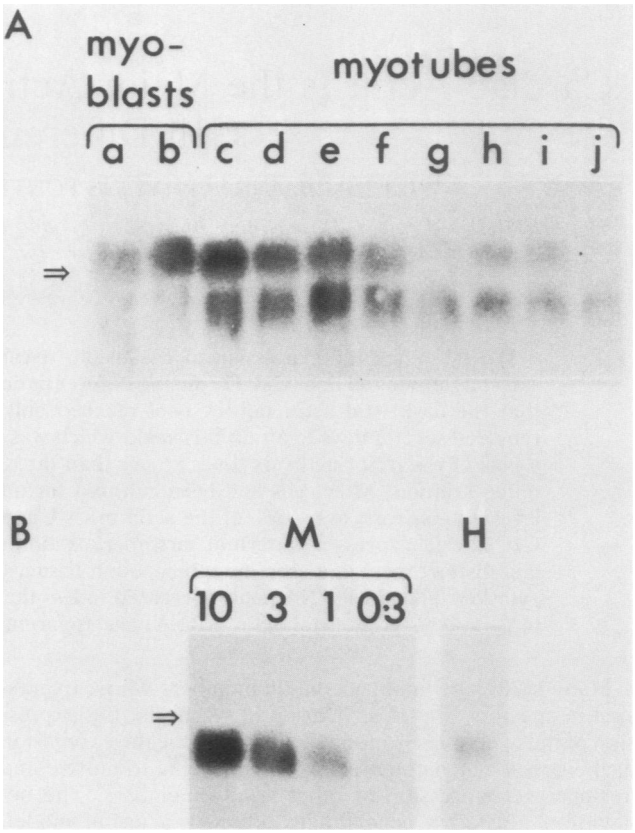

FIG. 1. (A) Autoradiograms of Northern blots of RNA from C2C12 cells hybridized to the 3' half of the chick β-actin gene. This probe hybridizes to all actin mRNAs. The autoradiograms were exposed for 6 h at −70°C. Samples are (a) proliferating C2C12 cells, (b) confluent C2C12 cells before culturing in DM, and (c to j) C2C12 cells after 6, 12, 24, 36, 48, 72, 96, and 168 h in DM. All tracks were loaded with 10 μg of total cytoplasmic RNA. Horizontal arrow indicates the migration position of 18S rRNA. (B) Autoradiograms of Northern blots of RNA from adult mouse skeletal muscle (M) and heart (H) probed with the chick β-actin gene coding probe. The four M lanes were loaded with 10, 3, 1, and 0.3 μg of skeletal muscle RNA as indicated. The H lane was loaded with 2.4 μg of heart RNA. Sections A and B of this figure are from the same Northern blot.

RESULTS

Examination of actin mRNA isotypes during differentiation. We prepared RNA from C2C12 cells which either were proliferating or had differentiated for different lengths of time. In addition, we prepared RNA from upper leg skeletal muscle and from whole heart of 8-week-old BALB/c mice. The RNA samples were electrophoresed on formaldehyde-agarose gels and blot-transferred to nitrocellulose. These filters were hybridized either with an actin-coding region probe or with isotype-specific probes to skeletal, cardiac, β-, or γ-actin mRNAs. The resulting autoradiographs are shown in Fig. 1 and 2. The hybridization pattern of the coding region probe to RNA isolated from differentiating C2C12 cells (Fig. 1A) demonstrates that the cellular actin mRNA pool changes from exclusively 18S (cytoplasmic actin mRNA) in the proliferating cells (Fig. 1A, lane a) to pre-

dominantly 16S (sarcomeric actin mRNA) in the myotubes (Fig. 1A, lanes g to j). The induction of 16S actin mRNA in C2C12 cells is very rapid, starting within 6 h of culture in DM. After the cells have been cultured for 6 h in DM, ca. 80% of the cell nuclei are still contained in mononucleated cells. The amount of sarcomeric actin mRNA in the C2C12 cells reaches a peak after cells have been cultured for one day in DM (Fig. 1A, lane e). Thus, induction of sarcomeric actin mRNA somewhat precedes morphological differentiation in this system.

Figure 1B shows the hybridization of the chick coding region probe to RNA from mouse skeletal muscle and heart. The relative amounts of sarcomeric actin mRNA in heart and skeletal muscle were quantitated by densitometric scanning of these autoradiograms. The results show that the ratio of sarcomeric actin mRNA in skeletal muscle to that in heart is 2.4 to 1. Comparison of Fig. 1A and 1B shows that after 24 h in DM, the C2C12 cells contain amounts of sarcomeric actin mRNA comparable with those found in mature mouse skeletal muscle.

Skeletal and cardiac actin mRNA expression. The autoradiographs resulting from the hybridization of these RNA samples to isotype-specific 3'UTR cDNA probes are shown in Fig. 2. The results are representative of experiments performed with RNAs extracted from three separate series of differentiating C2C12 cells. Although the data presented here are from the most complete set of RNAs, the results are typical.

Essentially all of the actin mRNA in adult mouse skeletal muscle is skeletal actin mRNA (Fig. 2, panel 1a, lanes H and M), and all of the actin mRNA in adult mouse heart is cardiac actin mRNA (Fig. 2, panel 2, lanes H and M). Because we showed that the ratio of sarcomeric actin in heart to that in muscle is ca. 1:2.4, we could deduce that the amount of skeletal actin mRNA in skeletal muscle RNA is 2.4 times the amount of cardiac actin mRNA in heart RNA. These RNAs may therefore be used as standards by which the amount of cardiac and skeletal actin mRNA in C2C12 cells may be directly compared.

In Fig. 2, panels 1a and 2 show identical exposures of filters hybridized to skeletal and cardiac actin isotype-specific probes, respectively. Cardiac actin mRNA is apparent in confluent C2C12 cultures (panel 2, lane b) and is rapidly induced upon differentiation (panel 2, lane d). It reaches a peak within ca. 24 h (panel 2, lane e) and decreases as myotube maturation proceeds. α-Skeletal actin is barely detectable under the same conditions. Only upon 10-fold-longer exposure of panel 1a (shown in panel 1b), does the presence of low levels of α-skeletal actin mRNA become apparent. These results from tissue culture are in sharp contrast to those obtained with adult skeletal muscle tissue: panel 2, lane M shows only trace expression of cardiac actin mRNA, whereas panel 1a, lane M shows high levels of expression of skeletal actin.

The amounts of skeletal and cardiac actin mRNAs in C2C12 cells after culturing for selected times in DM were measured by densitometric scanning of the autoradiograms shown in Fig. 2 and quantitated by reference to the skeletal and heart muscle RNA standard (Fig. 3). The relative amounts of skeletal and cardiac actin mRNA in skeletal muscle and heart are indicated in this figure for reference. Within 12 h of the onset of differentiation, C2C12 cells begin to synthesize and accumulate a large amount of cardiac actin mRNA. By 24 h, the concentration of cardiac actin mRNA reaches a peak which is twice that found in adult heart. After 24 h, the concentration of this mRNA declines rapidly, and

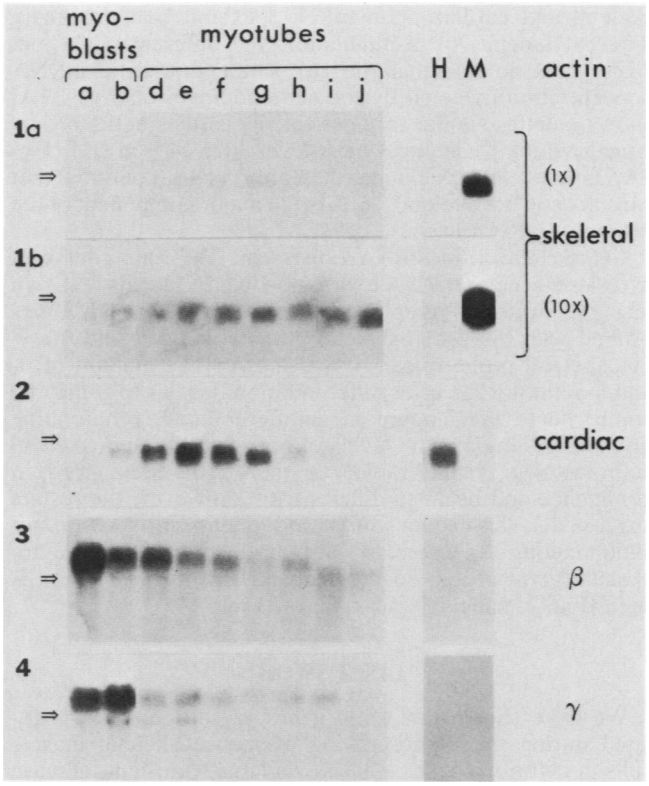

FIG. 2. Autoradiograms of Northern blots of RNA from C2C12 cells and adult mouse tissues probed with nick-translated actin gene isotype-specific probes. All panels except panel 1b (skeletal actin) were exposed for 6 h at −70°C; panel 1b (skeletal actin) was exposed for 10 times as long: 60 h at −70°C. Samples loaded are exactly as described in the legend to Fig. 1, except that all skeletal muscle tracks (M) received 10 μg of skeletal muscle RNA, and all heart tracks (H) received 3.5 μg of heart RNA. The probes were: panels 1a and 1b, skeletal actin; panel 2, cardiac actin; panel 3, β-cytoskeletal actin; panel 4, γ-cytoskeletal actin. The appearance of two hybridizing species in panels 3 and 4 is an artifact. Agarose gels can be saturated by high concentrations of rRNA in the "18S" and "28S" positions, causing RNAs which comigrate with the rRNAs to migrate anomalously. That this does not represent cross-hybridization of the β- and γ-actin cDNA probes to sarcomeric actin mRNA has been demonstrated previously (12, 21).

by 4 days, it reaches a steady level of only 10% of its maximal level.

Visual inspection of the cell cultures indicated that the rate of fusion follows a similar time course: the rate of fusion of mononucleated cells into multinucleated myotubes is maximal between 24 and 36 h after culture in DM, and fusion is essentially complete after 3 to 4 days. This pattern of gene expression is not affected by the presence of cytosine arabinoside in DM: a duplicate time course experiment performed without cytosine arabinoside in DM showed the same pattern of cardiac and skeletal actin mRNA expression (W. Bains, unpublished data). Thus, the presence of an increased proportion of mononucleated cells in these cultures does not affect the relative amounts of skeletal and cardiac actin mRNA produced.

In contrast to cardiac actin mRNA, skeletal actin mRNA accumulates more slowly (Fig. 3). It reaches a peak only after 4 days and does not change significantly thereafter. The peak pool of skeletal actin mRNA is only one-sixth as large as the peak accumulation of cardiac actin mRNA. Thus,

skeletal and cardiac actin mRNAs exhibit both markedly different kinetics of accumulation and different peak pool sizes. It is notable that the 16S sarcomeric actin mRNA concentration detected by the actin coding probe (Fig. 1A) shows kinetics similar to those for the cardiac actin mRNA concentration, reaching a peak level after 24 h in DM (Fig. 1A, lane e) and declining thereafter. This confirms that cardiac, and not skeletal, actin is the major component of the 16S actin mRNA in these cells.

Cytoskeletal actin mRNA expression. The time course of cytoskeletal actin mRNA expression in C2C12 cells is shown in Fig. 2. Autoradiographs obtained when C2C12 RNA was probed with the β-cytoskeletal probes (panel 3) and the γ-cytoskeletal probe (panel 4) are shown. The amount of β- and γ-actin mRNA after differentiation (lanes f to j) may be compared to the amount in undifferentiated, proliferating myoblasts (lane a). The levels of both β- and γ-cytoskeletal actin mRNAs decline rapidly as the C2C12 cells approach confluence and begin to differentiate. However, the results suggest that the declines of β- and γ-actin mRNAs are not simultaneous. As expected, no hybridization of these cytoskeletal actin probes to heart or skeletal muscle RNAs is seen (Fig. 2, panels 3 and 4, lanes H and M).

DISCUSSION

We have shown that actin genes are differentially regulated during the differentiation of mouse skeletal muscle cells in culture. These cells show a large, transient increase in cardiac actin mRNA, accompanied by a slower, stable accumulation of skeletal actin mRNA. Early in differentiation, the pools of β- and γ-cytoskeletal actin mRNA are reduced. The data suggest that this reduction also is not coordinately regulated.

This unexpected pattern of sarcomeric actin gene expression is in marked contrast to that in mature skeletal muscle, in which cardiac actin mRNA is not a major actin gene product. In mature mouse skeletal muscle, cardiac actin comprises less than 5% of the sarcomeric actin mRNA pool (18). Conversely, α-skeletal actin mRNA is ≤5% of the total actin mRNA in adult rat heart (27) and mouse heart (this work). By contrast, the actin mRNA in adult human heart can be an equal mixture of cardiac and skeletal isotypes (12).

These results suggest that C2C12 cells are recapitulating a developmental or regenerative pattern of actin gene expression as they differentiate in culture. This is supported by several other lines of experimental evidence. First, we found that primary cultures of human myoblasts show similarly high ratios of cardiac to skeletal actin mRNA upon differentiation in culture (Bains, unpublished data). Minty et al. (18) also detected significant levels of cardiac actin mRNA in a teratocarcinoma-derived myoblastic cell line and in skeletal muscle during murine embryogenesis when the muscle was undergoing its greatest fractional increase in mass. These results and our studies of muscle cell differentiation in vitro support the notion that α-cardiac actin gene expression may be a characteristic of normal developing muscle, of regenerating muscle, and of muscle satellite cells which have been stimulated to differentiate in vitro.

It should be noted that most previous studies have failed to appreciate the contribution of cardiac actin gene expression to skeletal muscle myogenesis (26, 27). It is probable that these results were due to the use of actin probes which contained coding region segments and therefore were incapable of discriminating skeletal from cardiac actin mRNAs.

Thus, this unexpected pattern of actin gene expression in cultured cells and parallel findings in developing muscle point to a previously unrecognized developmental modulation in the expression of the actin gene family. Other multigene families which contain members coding for major muscle proteins have also been found to contain developmentally regulated isotypes. Thus, myosin light chain (28, 32), myosin heavy chain (16), troponin I, T, and C (29), and tropomyosin (16) all possess fetal isoforms distinct from those found in adult muscle. It is notable that it is the embryonic form of these proteins which is expressed in cultured cells, even when they are derived from adult skeletal muscle (16, 28, 29, 33; Blau, unpublished data).

Our studies only address the changes in the total cellular content of the various actin mRNAs. Thus, we cannot distinguish changes in the rate of gene transcription from alterations in processing or turnover. The kinetics of induction of cardiac actin mRNA in these cells are consistent with a predominantly transcriptional mechanism. It remains to be determined whether the rapid drop in β- and γ-cytoskeletal actin mRNA concentrations as the cells initiate differentiation and in cardiac actin mRNA concentration after cells have been cultured for 24 h in DM are due to altered rates of turnover or to altered rates of synthesis of these messages or both.

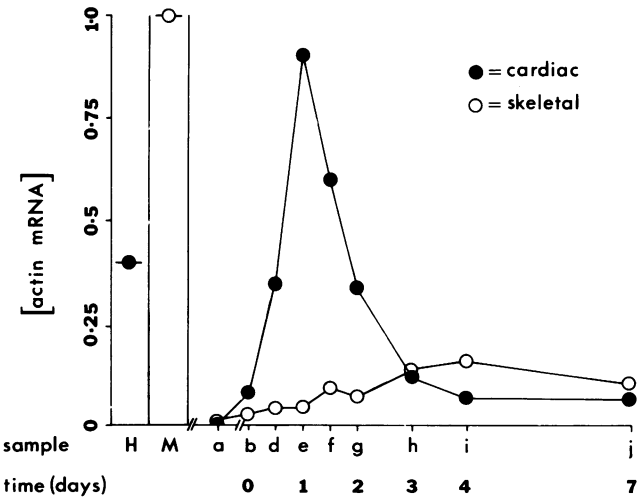

FIG. 3. Relative amounts of skeletal and cardiac actin during differentiation of C2C12 cells in vitro. RNA samples H, M, and a to j, as described in the legend to Fig. 1. Time refers to the number of days that the cells have been cultured in DM. The amount of sarcomeric actin mRNA relative to skeletal muscle α-actin mRNA has been set to a value of 1. Densitometric scans of autoradiograms of the Northern blots shown in Fig. 2 were corrected for the amount of RNA transferred to the filter (see the text) and normalized to the amount of sarcomeric actin mRNA in skeletal muscle.

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