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# Expression of MyoD in Cultured Primary Myotubes Is Dependent on Contractile Activity: Correlation with Phenotype-Specific Expression of a Sarcoplasmic Reticulum $Ca^{2+}$-ATPase Isoform

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Myogenic determination factors (MDF) have been implicated in the establishment and maintenance of the fast or slow phenotype in skeletal muscle, with MyoD favoring the fast and myogenin favoring the slow phenotype. Accordingly, contractility-induced changes in muscle phenotype should be accompanied by a change in the MyoD/myogenin ratio. Some reports show such changes, but limitations inherent to *in vivo* studies complicate interpretation of these data. Here we tested whether a relationship can be found between contractility, MDF expression, and the expression of phenotype-specific muscle proteins in a simple *in vitro* system of cultured primary myotubes. We show that contractions reduce the MyoD/myogenin ratio by specifically repressing MyoD mRNA expression. This is accompanied by a selective repression at a pretranslational level of the expression of fast-type sarcoplasmic reticulum $Ca^{2+}$-ATPase. These *in vitro* results support a phenotype-determining role of MDFs as a function of contractile activity and show that cultured myotubes can be a useful model for the analysis of the molecular mechanism of such regulation of muscle phenotype. 

The phenotype of skeletal muscle fibers is among other things characterized by the velocity of contraction and relaxation, determined by the expression pattern of specific muscle gene isoforms. A major determinant of the phenotype of skeletal muscle fibers is the type of innervation. The activity pattern of a fast motor-nerve, i.e., few and short bursts of high frequency, stimulates the expression of proteins associated with the fast phenotype. Sustained contractile activity at a low frequency, imposed by a slow motor nerve, has the opposite effect, i.e., slow type isoforms are expressed at the expense of fast type isoforms (1-5). It has been shown that the contractile activity induced by the nerve, rather than a neurotrophic factor, is responsible for this effect (1,6,7). Furthermore, a change in the level of contractile activity of an adult muscle will induce a shift in the established phenotype, coordinately affecting many muscle proteins. For instance, the expression of fast-type isoforms of muscle proteins is actively repressed when chronic contractile activity is imposed on a fast muscle (1,3).

The coordinate expression of phenotype-specific proteins at different levels of contractile activity, suggests a common regulatory mechanism. Several lines of evidence suggest that a regulatory role may be played by myogenic determination factors (MDF), a family of muscle specific transcription factors. MDF's are involved in myogenic differentiation, but are also considered to play a role in determining the phenotype of adult skeletal muscle fibers. In particular, the relative expression of MyoD and myogenin is suggested to be a determinant of muscle phenotype. Relatively high levels of expression of MyoD were observed in fast fibers,

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but only low levels in slow fibers, whereas the opposite was found for myogenin (8,9). The relative expression of MyoD and myogenin may also be involved in changes in muscle phenotype mediated by contractility, as was suggested by experiments involving mice that are myotonic due to a dysfunction of the skeletal muscle chloride channel, which results in trains of action potentials and contractions in response to a single neural stimulus. It was demonstrated that the expression of myogenin was increased in these animals whereas the expression of MyoD was repressed (10). This was accompanied by transformation to a slow phenotype with repression of muscle proteins associated with the fast phenotype, such as the fast-type isoform of the sarcoplasmic reticulum (SR) $Ca^{2+}$-ATPase protein (SERCA1) and type II myosin heavy chain isoforms (11,12). Furthermore, the conversion of the phenotype by denervation or cross-innervation was accompanied by changes in the relative expression of MyoD and myogenin. However, the results from different studies are not unequivocal (9,13-15).

When studying the possible involvement of myogenin and MyoD in contraction-mediated changes in muscle phenotype using whole muscle *in vivo*, these studies are hampered by the fact that skeletal muscles typically consist of a mix of different fiber types and by the likely involvement of secondary factors that accompany cross-innervation or denervation. The use of an *in vitro* model may overcome this limitation. We previously showed that in cultured primary myotubes, in line with *in vivo* results, contractile activity depressed the expression of SERCA1, although expression of the slow-type isoform (SERCA2a) was not monitored in that study (16). The present study was designed to investigate whether the effect of contractility on SERCA1 expression *in vitro* is isoform-specific, and whether such an effect can be correlated to changes in the relative expression of MyoD and myogenin.

## MATERIALS AND METHODS

*Materials.* Fetal calf serum (FCS), horse serum (HS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO-BRL (Gaitersburg, MD, U.S.A.). Culture plastics were from Nunc (Roskilde, Denmark). Tetrodotoxin (TTX) and 1-($\beta$-D-Arabinofuranosyl)-cytosine (AraC) were obtained from Sigma (St. Louis, MO, U.S.A.). Hybond-N nylon membranes were obtained from Amersham (Buckinghamshire, UK). [$\alpha$-$^{32}$P]dATP, [$\alpha$-$^{32}$P]dCTP, and [$\gamma^{32}$P]-ATP and intensifying screens were from Du Pont New England Nuclear (Wilmington, DE, U.S.A.) All DNA modification enzymes were obtained from Boehringer Mannheim (Mannheim, Germany). The random prime labeling kit was from Promega (Madison, WI, U.S.A.). X-ray films were obtained from Fuji (Tokyo, Japan), and laserdensitometric scanning of autoradiograms was performed on an LKB 220 Ultroscan (LKB, Uppsala, Sweden).

*Cell culture.* Primary myoblasts were isolated from 2-day old neonatal rats by trypsin digestion as described by Koningsberg et al. (17). Cells were seeded at a density of $2 \times 10^3$ cells/cm$^2$ in 25 cm$^2$ tissue culture flasks that were precoated with 0.1% gelatin. Cells were grown to confluence on DMEM containing 10% (v/v) HS and 5% (v/v) FCS. After three days, cells were switched to differentiation medium consisting of DMEM containing 10% (v/v) HS, with or without the $Na^+$-channel blocker tetrodotoxin (TTX; 10 $\mu$M) to arrest contractions (this time point is designated day 0). At day 2, the medium was replaced with DMEM + 5% (v/v) HS and 15 $\mu$M AraC (the percentage of serum was reduced to maximize contractions; AraC was included to eliminate fibroblasts from the culture). The medium was again changed at day 5. Spontaneous contractions in control cultures were sparse at day 1, but increased thereafter, eventually involving the majority of the myotubes.

*RNA isolation and Northern-blot analysis.* Total RNA was isolated using guanidium thiocyanate and subsequently electrophoresed and blotted as described previously (18). Up to 30 $\mu$g of denatured RNA were separated by electrophoresis in 0.8% agarose gels and blotted to Hybond-N nylon membranes. Blots were UV cross-linked and finally baked at 80°C for 2 hours.

Hybridizations were carried out with $^{32}$P random-prime labeled cDNA probes in 50% formamide, 5× SSPE, 5× Denhardt's solution, 1% SDS and 60 $\mu$g/ml sheared, denatured herring sperm (1× SSPE is 180 mM sodium chloride, 0.1 mM EDTA, 10 mM sodium phosphate, pH 7.4; 100× Denhardt's solution is 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) BSA) at 42°C for 16 hours. Blots were washed to a stringency of 0.1× SSPE, 0.1% SDS at 52°C. Signals of hybridizations with the cDNA probes were normalized using the 18S rRNA signal as an independent standard as described by Samarel et al. (19). The 18S rRNA oligonucleotide probe was [$\gamma^{32}$P]-ATP end-labeled using T4 polynucleotide kinase and hybridized at 52°C in 5× SSPE, 5× Denhardt's solution and 60 $\mu$g/ml sheared, denatured herring sperm DNA. The blots were washed to a stringency of 2× SSPE, 0.1%SDS at 52°C. Blots were subjected to

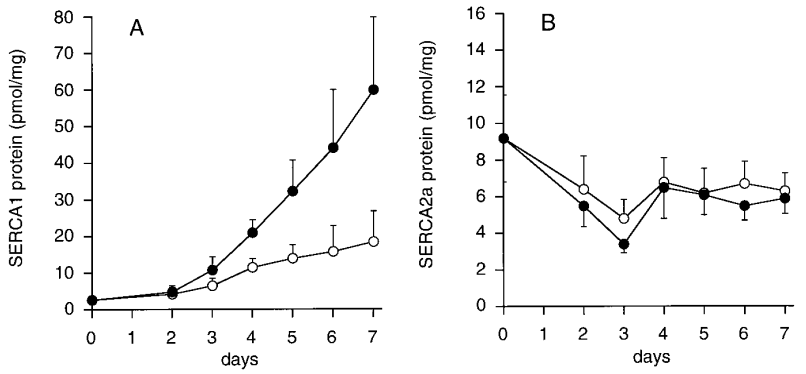


**FIG. 1.** Effects of contractile activity on the SERCA1 (A) and SERCA2a (B) protein levels. Primary rat myoblasts were isolated and grown as described under Materials and Methods. Spontaneous contractions developed from day 0 and were arrested by TTX-treatment, which was started at day 0. ○, spontaneous contractions; ●, no contractions. Points represent means ± S.E.M. of 5 experiments in duplicate, except day 2 (n=2) and day 3 (n=3). Analysis of variance revealed that contractions significantly repressed SERCA1, but did not affect SERCA2a expression.

autoradiography for up to 15 days and signals were quantified by laser-densitometric scanning. Several exposure times were used to ensure signals within the linear range of the film.

The following cDNA probes were used for Northern blot hybridizations: MyoD: a 376 bp cDNA fragment containing the last 42 bp of exon 2 and the first 334 bp of exon 3 of the rat MyoD gene; myogenin: a 380 bp cDNA fragment consisting of the last 45 bp of exon 1, all 82 bp of exon 2 and the first 253 bp of exon 3 of the mouse myogenin gene; (both MDF probes were kindly made available and checked on Northern blot for specificity by Dr. G. Molnar and Dr. N. A. Schroedl of the Alfred E. duPont Institute, Wilmington, Delaware, U.S.A.); SERCA1: a previously described +586 bp SERCA1 specific cDNA probe (18); SERCA2a: a 1000 bp fragment of the 3′ end (*Pst*I to polyA+-tail) of the rat SERCA2a cDNA subclone RH39, which was kindly provided by Dr. A.-M. Lompré (Université Paris Sud, Paris, France) (20).

*SERCA1 and SERCA2a protein analysis.* For the determination of SERCA1 and SERCA2a protein levels, cultures were washed twice with PBS and then covered with 300 $\mu$l PBS and frozen at −20°C. After thawing, the cells were harvested by scraping with a rubber policeman and stored at −20°C in separate aliquots for analysis of total protein, SERCA1 and SERCA2a. SERCA1 protein levels were determined by e.l.i.s.a. as described by Muller et al. (21) using the SERCA1 specific antibody A52, which was a kind gift of Dr. D.H. MacLennan (Banting and Best Institute, Toronto, Canada). SERCA2a protein levels were measured in a dot-blot immuno-assay as described by Muller et al. (22) employing a SERCA2a specific polyclonal antiserum kindly provided by Dr. F. Wuytack (Katholieke Universiteit Leuven, Belgium). SERCA1 and SERCA2a levels were normalized to total protein as determined by the method of Lowry et al. (23).

*Statistical analysis.* Differences between groups as a function of time or treatment were tested by analysis of variance (ANOVA) for repeated experiments and considered statistically significant for $P<0.05$.

## RESULTS

Examination of quiescent and spontaneously contracting primary myotubes revealed no overt differences in morphology between the cells, although quiescent tubes tended to be somewhat flattened compared to contracting tubes. The development of CPK-activity during the six-day period of the experiment was the same in the two groups, indicating unimpeded myogenic differentiation in both (not shown).

Whereas contractions did not affect overall myogenic differentiation, the development of SR $Ca^{2+}$-ATPase (SERCA) expression was repressed. In line with previous data (16), SERCA1 protein levels in quiescent cultures increased over 20-fold between day 0 and day 7, and contractions resulted in a reduction at day 7 to 25.4±5.4% of the level found in quiescent cells (Fig.1A).

To investigate whether the effect of contractile activity was isoform-specific, we also deter-

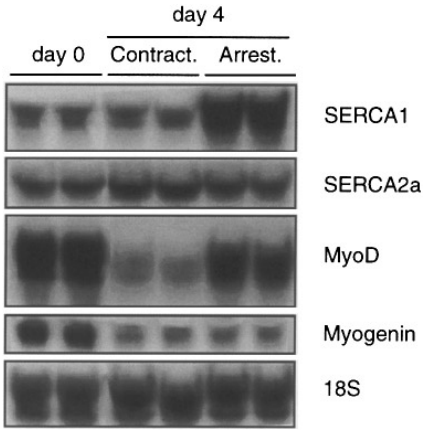


**FIG. 2.** The effect of contractile activity on SERCA and MDF mRNA expression. Cell culture was performed as described under Materials and Methods. Spontaneous contractions developed from day 0 and were arrested by TTX-treatment, which was started at day 0. The autoradiogram shows a Northern blot of total RNA isolated at day 0 and day 4 from duplicate cultures of a representative experiment. RNA was isolated, electrophoresed, and blotted as described under Materials and Methods. The same blot was successively hybridized with probes detecting the mRNA coding for SERCA1, SERCA2a, MyoD, and myogenin. Finally the blot was probed for 18S rRNA, which was used for normalization.

mined SERCA2a protein levels. The myotubes initially contained about equal levels of both SERCA isoforms, and the data in Figure 1B show that the fairly constant level of SERCA2a was not affected by contractions. If anything, a tendency was observed towards increased, rather than repressed, SERCA2a expression in contracting cultures.

An analysis of mRNA expression was then performed to test whether the selective effect of contractions on SERCA1 expression was pre-translational in nature. Using the same RNA samples, the levels of MyoD and myogenin mRNA were determined to test for a possible correlation between MDF and SERCA-isoform expression. Figure 2 shows a representative Northern blot of equal amounts of total RNA from cultures of contracting and quiescent myotubes sampled at day 0 and day 4, and probed successively for the various SERCA and MDF mRNA's. Contractions blunted the strong increase in SERCA1 mRNA between day 0 and day 4, and also reduced the level of MyoD mRNA. In contrast, the level of myogenin mRNA remained unaffected by contractions, as did the level of SERCA2a mRNA.

The time-course of the expression of the SERCA and MDF mRNA's in contracting and quiescent myotubes is depicted in Figure 3. The data in Figure 3A show that the progressive increase in SERCA1 mRNA level in quiescent cells, starting at day 2 and which underlies the development of SERCA1 protein expression seen in Figure 1, did not occur in contracting cells. At this time contractions had already induced a significant reduction of the level of MyoD mRNA (Fig. 3C). This decrease persisted and leveled off after day 3. Up to day 6 the level of MyoD mRNA in contracting cultures was on average one third of that in quiescent cultures. The levels of both myogenin and SERCA2a mRNA were insensitive to contractions throughout the experiment (Fig. 3B,D), although in the latter case mRNA levels tended to be somewhat higher in contracting cultures, in line with the SERCA2a protein data (see Fig. 1B).

## DISCUSSION

Increased contractile activity, when imposed on a fast muscle, represses the expression of fast-type isoforms of many muscle proteins and eventually leads to replacement by slow-type

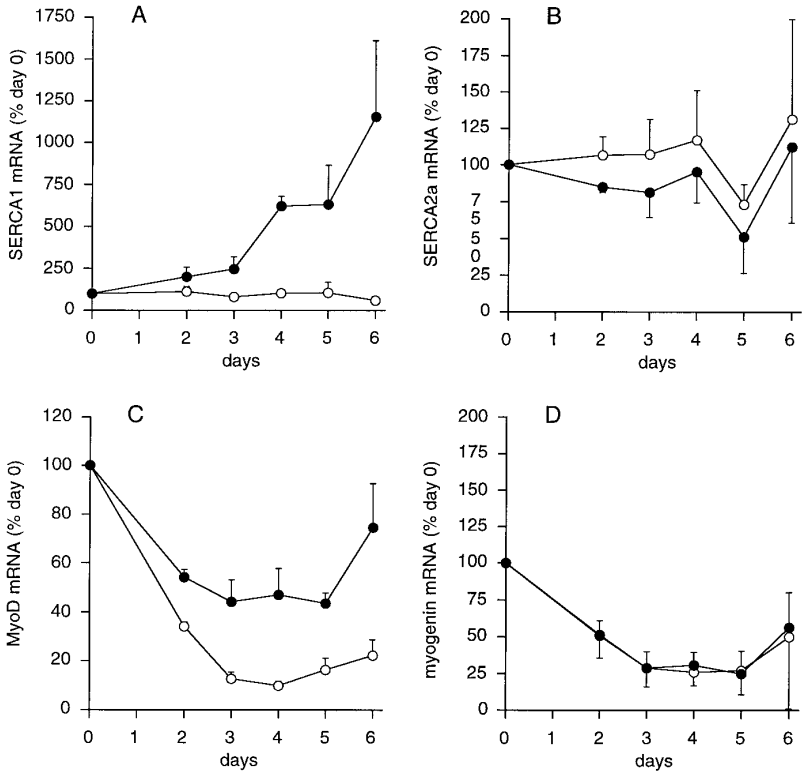


**FIG. 3.** Time course of the effect of contractions on SERCA and MDF mRNA expression. Total RNA of spontaneously contracting and TTX-arrested primary myotubes was analyzed as described in the legend to Fig. 2. Data are presented as the percentages of the levels found at day 0 and represent the means ± S.E.M. of 4 experiments, except day 2 and day 3 (n=3). ○, spontaneous contractions; ●, no contractions. Analysis of variance revealed that contractions significantly repressed SERCA1 (A) and MyoD (C) mRNA expression, whereas the expression of SERCA2a (B) and myogenin (D) mRNA was not affected by contractions.

isoforms (1,3-5). As pointed out in the Introduction, changing levels of the muscle-specific transcriptional regulators MyoD and myogenin have been implicated in the mechanism governing the coordinated expression of the multiple isoforms of muscle proteins. In general MyoD is thought to be associated with the fast phenotype and myogenin with the slow, although the mixed fiber-type composition of skeletal muscle complicates the interpretation of many studies.

Using a system of cultured primary myotubes, we now show that essential aspects of the phenotype-determining effect of contractile activity can be reproduced *in vitro*. The present results show for the first time that contractions repress expression of the fast-type, but not the slow-type isoform of SR $Ca^{2+}$-ATPase, at a pre-translational level. Furthermore, this effect correlated with the repression of MyoD expression, whereas myogenin expression was not affected. Although still circumstantial, these data strongly support the suggestion that contractile activity modulates muscle phenotype by differential regulation of the expression of MyoD and myogenin, and implicate MyoD as a fast-type specific transcriptional activator. However, the initially low levels of SERCA1 in myoblasts at high levels of MyoD (this study), and the expression of SERCA1 in the absence of MyoD in L6 muscle cells (24,25), indicate that MyoD is neither required nor sufficient for the expression of this marker of the fast phenotype. Rather, MyoD would act as a modulator of transcription and the present data suggest that a

minimal level of MyoD expression is required to stimulate expression of the SERCA1 gene. The slow-type SERCA2 gene is apparently not responsive to the changing level of MyoD expression. Although all MDF's bind to the consensus binding sequence CANNTG (E-box) in the promoter region of the target gene, the context of such binding sites may determine the efficacy of a given MDF in activating transcription. For example, the E-box-containing enhancer of the myosin light chain 1/3 gene is more efficiently activated by MyoD than by other MDF's (26-28). We recently cloned and characterized the promoter of the SERCA1 gene of the rat and localized multiple putative E-boxes (29). Comparison with the published promoter of the slow-isoform gene indicated no similarities in number or organization of these MDF binding sites, which is at least compatible with differential regulation of both isoforms by these factors (30).

In contrast to SERCA1, the expression of SERCA2a was not found to be dependent on contractile activity. Yet *in vivo*, down-regulation of SERCA1 as a result of chronically increased contractile activity is accompanied by increased expression of SERCA2a (1,4,5). The absence of an increase in myogenin expression in our system could account for this, given the suggested mechanism. However, whether myogenin is a determining factor in the expression of SERCA2a is speculative and neither confirmed nor refuted by our results. It is nonetheless clear that MyoD and myogenin are not reciprocally regulated, at least not in this system. Contraction-induced up-regulation of myogenin observed *in vivo* may thus rely on additional factors not present in cultured primary myotubes, or may require a longer time period than the relatively short period used in our *in vitro* setting.

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