

Upstream sequences of the *myogenin* gene convey responsiveness to skeletal muscle denervation in transgenic mice

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ABSTRACT

Myogenin, as well as other MyoD-related skeletal muscle-specific transcription factors, regulate a large number of skeletal muscle genes during myogenic differentiation. During later development, innervation suppresses *myogenin* expression in the fetal hind limb musculature. Denervation of skeletal muscle reverses the effects of the nerve, and results in the reactivation of *myogenin* expression, as well as of other embryonic muscle proteins. Here we report that *myogenin* upstream sequences confer tissue- and developmental-specific expression in transgenic mice harboring a *myogenin*/chloramphenicol acetyltransferase (CAT) reporter construct. Using in situ hybridization to analyze serial sections of E12.5 embryos, we found colocalization of CAT and endogenous *myogenin* transcripts in the primordial muscle of the head and limbs, in the intercostal muscle masses, and in the most caudal somites. Later in development, we observed that the expression of the transgene and endogenous *myogenin* gene continued to be restricted to skeletal muscle but decreased shortly after birth; a period that coincides with the innervation of secondary myotubes. Furthermore, denervation of the mouse hind limbs induced a 10-fold accumulation of CAT and endogenous *myogenin* transcripts by 1 day after sciatic nerve resection; a 25-fold increase was observed by 4 days after denervation. Interestingly, we observed that the accumulation of CAT enzyme activity lagged considerably with respect to the increase in CAT transcripts. Our results indicate that the *cis*-acting elements that temporally and spatially confine transcription of the gene during embryonic development, and that mediate the responses to innervation and denervation of muscle, lie within the upstream sequences analyzed in these studies.

INTRODUCTION

Skeletal muscle differentiation proceeds through a sequential series of events beginning with the commitment of pluripotential mesodermal cells to the myogenic lineage, and followed by the

differentiation and fusion of myoblasts to form multinucleated embryonic myotubes. Differentiation of myoblasts is accompanied by the transcriptional activation of a large repertoire of skeletal muscle-specific genes [see ref. 1], including those coding for the subunits of the nicotinic acetylcholine receptor (nAChR). Myogenic differentiation occurs autonomously of the nerve, however, later in development innervation regulates the expression of skeletal muscle properties [see refs. 2, 3]. Nerve-derived chemical factors modify the distribution of pre-existing nAChRs [4, 5], and may also increase the expression of receptor genes locally during synapse formation and maturation [6,7]. The chemical signals between the nerve and muscle, which are mediated through nAChRs, are converted to electrical currents that depolarize the entire myotube. Accompanying innervation, a selective group of genes are down-regulated in the extrajunctional nuclei of myofibers, including those coding for nAChR subunits [8–10] and adhesion molecules [11]. Several lines of evidence demonstrate that down-regulation of receptors is due to nerve-derived electrical activity. Denervation of muscle or blocking of nAChR transmission with specific neurotoxins result in the reaccumulation of nAChR subunits and their mRNAs throughout the muscle fiber [12–14]. Furthermore, electrical stimulation of denervated muscle with extracellular electrodes represses receptor expression [15, 16]. The major regulatory mechanism accounting for the activity-dependent repression of gene expression is transcription, as initially demonstrated by nuclear run-on assays [17]. The use of reporter constructs in transgenic mice [18, 19] and transfected cells [20, 21] have demonstrated that upstream sequences of the nAChR α and δ subunit genes confer the transcriptional regulation by denervation and electrical activity.

The family of skeletal muscle-specific basic/helix-loop-helix (bHLH) factors, consisting of MyoD [22, 23], *myogenin* [24, 25], Myf-5 [26] and MRF-4/Herculin/Myf-6 [27–29], contributes to the transcriptional activation of muscle genes during differentiation of cultured cells. However, the function of each individual factor in vivo is yet not clearly understood, since muscle development is apparently unaffected in mice made deficient in *myf-5* [30] and MyoD [31] by gene targeting, whereas mice with a targeted mutation in *myogenin* have severe muscle deficiency [32, 33]. The bHLH myogenic factors mediate

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transactivation by interacting with a common *cis*-acting element, known as an E box [34], present in the enhancers of many skeletal muscle genes [see ref. 1]. Mutations of the E box element in enhancers present in the genes coding for creatine kinase [35–37], myosin light chain [38], troponin I [39, 40] and nAChR [41, 42] inactivate transcription in differentiated myocytes. The transactivation of receptor genes in response to denervation may also be mediated by these myogenic factors. Transgenic mice harboring reporter genes driven by the upstream regulatory regions of the nAChR α [18] and δ [19] genes, which contain E boxes essential for transcription, activate reporter activity after denervation. Chahine *et al.* have recently reported that a 102 bp sequence of the nAChR δ subunit, containing an E box necessary for myotube expression, confers regulation by electrical activity in transfected cultured myotubes [20]. On the other hand, the myogenic factors are also regulated by innervation. We previously reported that innervation down-regulates expression of the *myogenin* gene during fetal muscle development [43]. Denervation of adult muscle results in a dramatic reaccumulation of *myogenin* transcripts that temporally precedes the expression of nAChR mRNAs [43–45]. Furthermore, electrical stimulation of denervated rat muscle was shown to repress *myogenin* expression [43, 44]. Thus, these results strongly suggest, but do not provide direct evidence, that regulation of *myogenin* by trans-synaptic signals may reside proximal to the initial events of skeletal muscle innervation and participate in the re-establishment of embryonic muscle properties in denervated adult muscle.

In order to elucidate the molecular pathways linking the effects of muscle depolarization with changes in muscle gene transcription, we have begun to characterize the regulatory elements in the *myogenin* gene. To this end, we have generated transgenic mice expressing the CAT reporter gene driven by *myogenin* upstream sequences, in order to determine if this region contains the regulatory elements that confine *myogenin* expression to the skeletal muscle lineage in the embryo, and later repress or activate its expression in response to innervation or denervation, respectively. Recent reports on LacZ-transgenes have exclusively focused on the *myogenin* sequences necessary for embryonic expression. Herewith we report, that transcription was found to be the major mechanisms regulating the temporal and tissue-specific expression of *myogenin* during embryonic and later development, and that the regulatory *cis*-elements reside in the sequences analyzed. We demonstrate that *myogenin* upstream sequences confer skeletal muscle-specific expression in myoblasts derived from different embryonic lineages, as well as confer the down-regulation of the gene during innervation and its upregulation in response to denervation. Thus, the *cis*-acting elements that direct *myogenin* expression in response to nerve-derived electrical activity reside within the construct analyzed. Characterization of *myogenin* upstream sequences that mediate nerve-dependent responses represents the first step towards identifying the transcriptional elements that connect depolarization events at the plasma membrane with the down-stream regulation of skeletal muscle target genes.

MATERIALS AND METHODS

DNA constructs

A chloramphenicol acetyltransferase (CAT) expression vector, driven by the *myogenin* 3.7 kb upstream sequence, was constructed by initially subcloning the Pst I (–184bp) to Hae III (+18) fragment into the unique Pst I/Xba I (blunted) sites

of pCAT-basic (Promega, Madison, WI). The genomic upstream Pst I fragment, extending from position –3.7 kb to –184, was then inserted into the Pst I to generate the construct containing *myogenin* sequences extending from –3.7 kb to +18. The linearized insert used for microinjection into embryos was obtained by digestion with SphI and Bam HI, which resulted in a fragment devoid of poly-linker or plasmid sequences. The insert was isolated after digestion by centrifugation on sucrose gradients, the fractions of interest were pooled after being monitored for purity on agarose gels, dialyzed against sterile deionized water, and diluted for injection. The fragment contained the 3.7 kb upstream sequence of the *myogenin* gene, CAT sequences, and the SV40 polyadenylation signal plus the small t-antigen intron.

Transgenic mice

The transgenic mice were generated by DN_X (Princeton, New Jersey). Fertilized (C57BL6 × SJL) F2 hybrid mouse eggs were injected and reimplanted into the oviducts of pseudopregnant Swiss Webster female mice, essentially as described previously [46]. Transgenic founders were identified by slot blot analysis of tail DNA using a CAT probe. The 6 founder lines obtained were mated to wild-type C57BL6 (NIH-DCT, Maryland) to propagate the lines; positive progeny were identified using slot-blot analysis. Most of the experiments were carried-out on F2 and F3 generation mice from lines 5-1 and 2-3, since these mice yielded the larger number of offspring. All the experiments presented were done in at least two independent lines of mice.

In situ hybridization

For the developmental studies, wild-type C57BL6 females in estrus were placed in the cage with transgenic males at approximately 4 pm; the light cycle set for darkness from 7:00 pm to 5:00 am. Mice showing vaginal plugs upon inspection the next morning were placed in separate cages, and the embryos considered to be 0.5 days *post coitum* (p.c.). For the embryonic studies using *in situ* hybridization histochemistry, the mothers were sacrificed with CO₂ at day 12.5 p.c., the embryos were removed from the chorionic sacs, measured and staged to confirm their embryonic age [47], and embedded in Tissue-Tek media (Miles) using dry ice. Serial parasagittal and transverse 16 μ m fresh frozen sections were obtained for *in situ* hybridization, as described previously [48], from 1 'negative' and 4 transgenic embryos. Briefly, paraformaldehyde-fixed alternate sections were hybridized (50% deionized formamide, 4 × SSC, 1 × Denhart's, 0.25 mg/ml yeast tRNA, 0.5 mg/ml sheared single stranded salmon sperm DNA, 10% dextran sulfate, 50 mM DTT) with either *myogenin* or CAT oligonucleotides (45mers) that were 50–60% in GC content, and end-labelled with ³⁵S-dATP (NEN) by incubation with terminal transferase (Boehringer, Mannheim) using standard methods. The sections were hybridized in humidified dishes at 37°C for 18–20 hours in a solution containing 1.5 × 10⁶ cpm/ml. Slides were washed at high stringency in 2 × SSC/50% formamide at 43–45°C, further washed, dried, exposed to Hyperfilm (Amersham) for 20 days, and then dipped in Kodak NTB-3 emulsion and stored dark for 4 weeks at 4°C. They were then processed in Kodak solutions of dektol and regular fix at 15°C, stained in 0.5% methyl green, dehydrated and coverslipped in Permount (Fisher). The antisense oligonucleotide probes for *myogenin* and CAT hybridized to the same bands as cDNA on Northern blots (data not shown). As a negative control, the sections were also hybridized with an

antisense oligonucleotide specific pro-opiomelanocortin (POMC) transcripts.

Denervation experiments

The denervation studies were done essentially as previously described [43]. The crural muscles of Metaphane anesthetized 3 to 6-month-old mice were denervated by the unilateral removal of 2–3 mm of the sciatic nerve at the upper thigh. At different times after surgery, the hind limb muscles were used either to isolate total RNA or make tissue extracts for CAT assays.

Northern blots and CAT assays

Expression of the transgenes during post-partum development was analyzed on Northern blots containing total RNA isolated from the hind limb muscles of 2 days, 5 days, 2 weeks, 4 weeks and up to 8 month-old mice. Adult positive transgenic mice were identified by slot blotting of tail DNA probed with a [³²P]-labelled CAT fragment [46]. In order to identify the transgenic preweaned pups that were going to be used to generate the hind limb muscle RNA, muscles from the fore and hind limbs (stored in liquid nitrogen) were collected during dissection, and

extracts made from the fore limb were assayed for CAT expression. Total RNA was isolated from the muscles of positive mice using guanidine thiocyanate and ultracentrifugation on cesium chloride gradients. RNA was quantitated spectrophotometrically, and the integrity and relative amounts in each sample used were also assessed by ethidium bromide staining of ribosomal RNA on agarose gels. The RNA was fractionated by electrophoresis on 1.5% agarose gels containing 2.2 M formaldehyde, and then the gels were electroblotted onto Nytran membranes (Schleicher & Schuell, Keene, NH). The blots were sequentially probed, quantitated, stripped and rehybridized with different cDNAs. The probes used for hybridization were a 1.5 kb fragment from a mouse myogenin cDNA [25], a 1.5 kb EcoRI fragment containing CAT + SV40 polyadenylation sequences excised from the pCATBasic vector, and a rat α skeletal actin 3' noncoding cDNA fragment (kindly provided by M. Crow) that were [³²P]-labelled by random priming (specific activity $\geq 10^8$ dpm/ μ g of DNA). In the developmental studies we were unable to identify probes that could be used for normalization of the signals, since actin is known to be expressed at higher levels in mitotically active cells, expression of glycolytic and

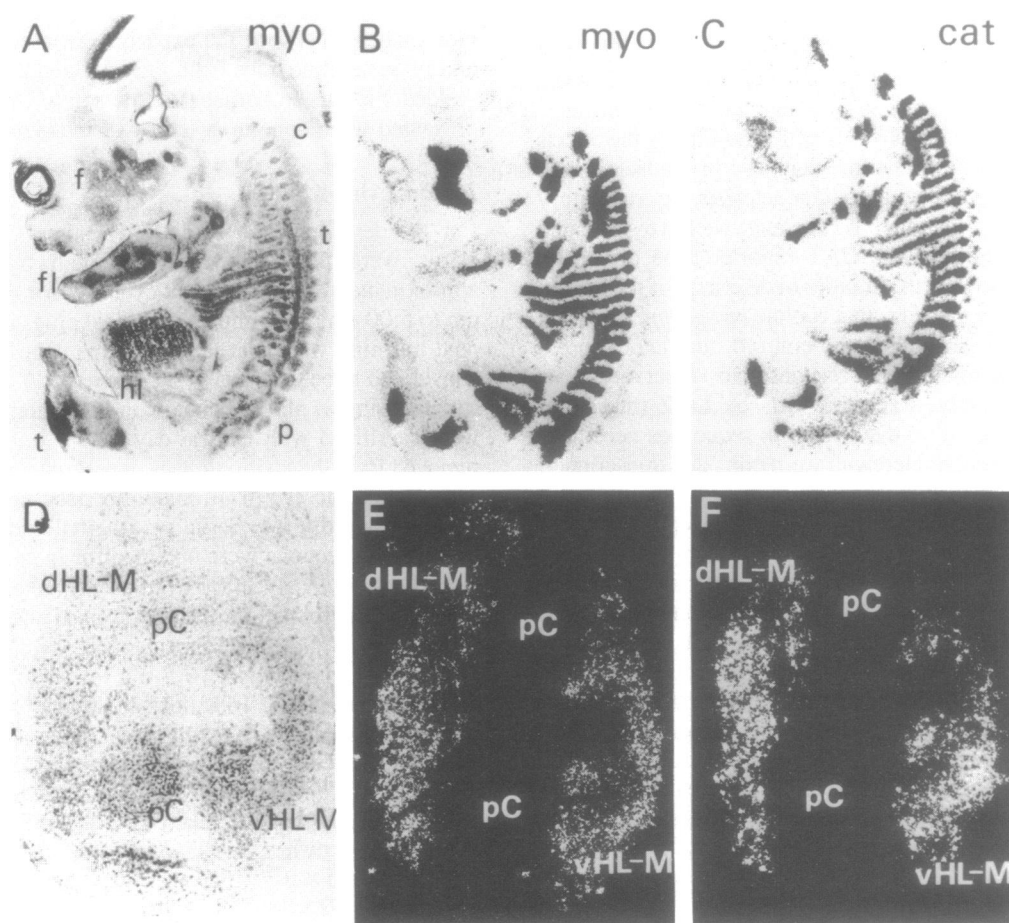


Figure 1. *Myogenin* and CAT expression co-localize in muscle precursor cells of *myogenin*-CAT transgenic embryos. Consecutive parasagittal sections of E12.5 transgenic embryos (A–C) or transverse sections through the hind limbs (D–F) were stained with methyl green (A, D), or hybridized with specific oligonucleotides to *myogenin* (B, E), or CAT (C, F). A–C, Cells in the facial (f), cervical (c), trunk (t), pelvic region (p), tail (t), and fore- and hind-limbs (fl, hl) regions that stained with the dye are marked in panel A. The autoradiograms showed in panels B and C were obtained by direct exposure of the sections to hyperfilm β -max (Amersham) for 20 days; magnification is 12 \times . E–F, Dark-field photomicrographs of consecutive transverse sections through the hind limbs of E12.5 transgenic mice hybridized with *myogenin* or CAT probes. In the hind limb, labeling of both probes is confined to the dorsal and ventral mesenchymal muscle masses (dHL-M, vHL-M), and was absent in the pre-cartilage mesenchyme (pC). These sections were dipped in emulsion and exposed for 4 weeks; magnification 90 \times .

oxidative enzyme genes are differentially regulated during muscle maturation, and our attempts to visualize Sp1 transcripts with the limited amounts of RNA used were unsuccessful. Hybridization was carried out at 42°C in 6×SSC, 5×Denhardt's, 1% SDS, 10 mM EDTA, 100 µg/ml salmon sperm DNA, and 50% formamide. Blots were washed with 0.1×SSC, 1% SDS at 65°C. Quantitation was done using the Molecular Dynamics PhosphoImager (Sunnyvale, CA). The signal per band varied linearly with respect to the amount of RNA on the blots (data not shown).

The muscle extracts used to measure CAT activity were made essentially as previously described [49]. Briefly, the muscle samples were sonicated in 0.25 M Tris-Cl (pH 8.0) on ice for 20 sec using a Branson Sonifier 450 (setting 4, output 50%). Samples were centrifuged at 15,000g for 5 minutes (4°C), and the supernatant collected for analysis. The assays were performed with 0.02–10 µg of protein (adjusted to remain in the linear range of the assay), at 37°C for 1h, and the activity determined using thin-layer chromatography. Protein concentrations were determined using the BCA kit from Pierce (Rockford, IL).

RESULTS

Six transgenic founders harboring the *myogenin*-CAT fragment were obtained, of which 4 transmitted the trait in the germline. Transgenic lines 5-1 (approximately 40 copies) and 2-3 (approximately 30 copies) were used because mice from these lines generated the large number of progeny needed for these studies.

Myogenin upstream sequences confine transcription of the CAT gene to skeletal muscle cells during embryonic and post-natal development of transgenic mice

We have analyzed the transcription of the CAT reporter gene directed by 3.7 kb of *myogenin* upstream sequences in our transgenic mice. The expression of the endogenous *myogenin* mRNA and CAT transcripts were compared in mouse embryos using *in situ* hybridization, in order to determine if elements within the *myogenin* upstream sequences mediate the tissue-specific transcriptional regulation of the gene during early murine development. Serial fresh frozen parasagittal and transverse sections were obtained from 12.5 day p.c. embryos, and consecutive sections were hybridized with oligonucleotide probes specific to either *myogenin* or CAT transcripts. Two labelled oligonucleotides for each type of transcript were used in order to increase the signals. A methyl green-stained section and autoradiographic film exposed to the hybridized sections of the whole embryo are shown in Figure 1 A–C. It is evident from the pattern of hybridization observed throughout the embryo, that expression of *myogenin* and CAT transcripts colocalize in the head, distal and proximal parts of the limbs, and the trunk. Higher magnification of darkfield micrographs obtained from transverse sections of the 12.5 day transgenic embryos showed that the striped pattern originated from *myogenin* and CAT signals in the muscle masses that surround the precartilaginous mesenchyme (data not shown), structures that will give rise to ribs and vertebrae. Furthermore, at 12.5 days p.c., *myogenin* transcribing cells were observed in the mesenchymal muscle masses surrounding the presumptive muscle of the hind limb (Fig. 1D–F). The signals observed in these embryonic sections were specific; adjacent sections hybridized with the neural-specific probe coding for pro-opiomelanocortin (POMC) did not show

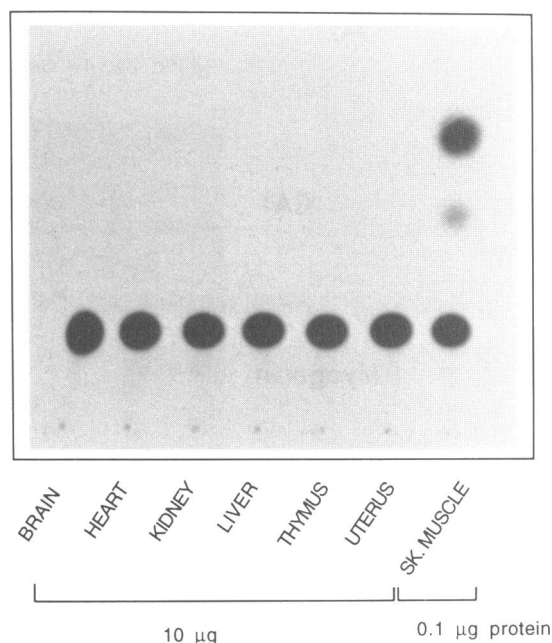


Figure 2. *Myogenin* upstream sequence confers skeletal muscle-specific transcription in adult transgenic mice. CAT activity was measured in extracts made from tissues of adult mice; the representative results above were obtained from a 6 month old transgenic animal (line 5-1). Skeletal muscle was the only tissue expressing activity above background, even when 100-fold more extract from other tissues were used in the assays.

any signal (data not shown). The regional expression of CAT and *myogenin* transcripts in the transgenic animals, as well as their temporal accumulation, is indistinguishable from the endogenous *myogenin* expression pattern previously observed in C3H wild-type 11.5 day mouse embryos [50, 51].

In order to determine if *myogenin* 5'-flanking sequences also contain the elements that restrict transcription of the gene to skeletal muscle in adult mice, we measured the levels of CAT activity in different tissues of 6 to 8-month-old mice from three independent lines. As shown on Figure 2, 0.1 µg of protein derived from skeletal muscle extract converted 40% of the chloramphenicol substrate in our assay. In contrast, CAT activity was undetectable in assays containing 10 µg of protein from brain, heart, kidney, liver, thymus and uterus extracts. Our results demonstrate that transcription is the predominant mechanism confining the expression of *myogenin* to muscle precursor cells during embryogenesis and in adult animals, and that the regulatory elements reside in the upstream sequence used in this study.

Cis-acting elements residing within the *myogenin* upstream region confer transcriptional repression during innervation of skeletal muscle

We previously showed that *myogenin* mRNA levels begin to decrease about birth and attain adult levels by approximately 3-weeks after birth [43], during a period that coincides with the innervation of secondary myotubes in the mouse hind limb. To determine if the upstream sequences of the *myogenin* gene mediate transcriptional down-regulation during development, we compared the levels of *myogenin* and CAT transcripts in the hind limb muscles of two transgenic lines during post-natal

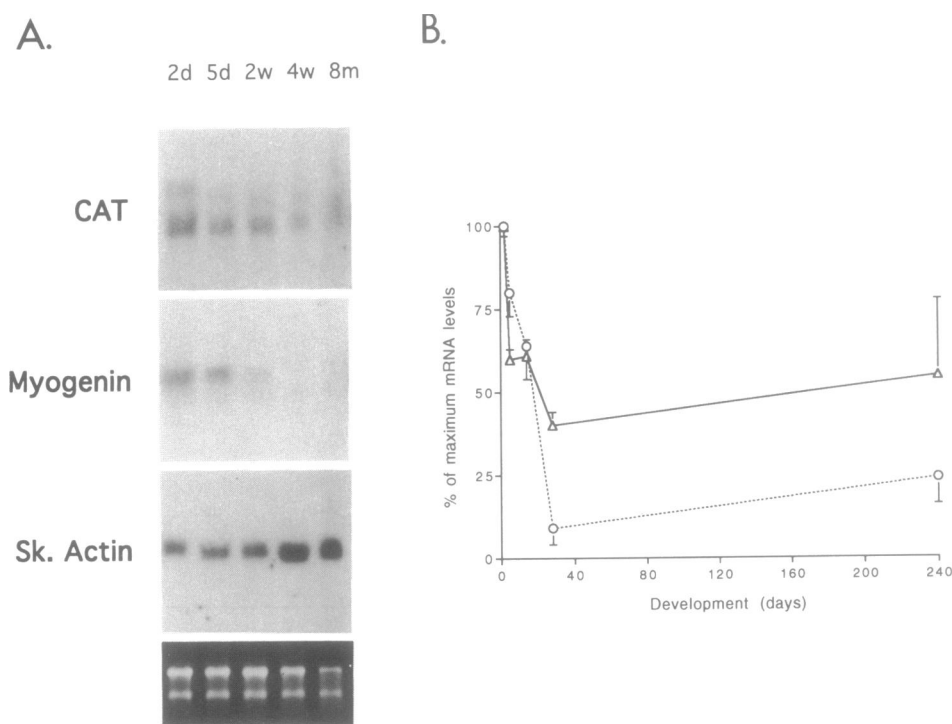


Figure 3. *Myogenin* upstream sequences mediate transcriptional repression during peri-natal development. (A) The representative Northern blots shown above contain 5 μ g of total RNA isolated from the hind limbs of transgenic mice (line 2-3) with ages extending from 2 day-old to 8 month-old. The blot was sequentially hybridized with cDNA fragments coding for CAT, *myogenin* and the α skeletal actin control. As previously reported [56], two bands were observed for CAT transcripts containing the SV40 poly-A addition site. The RNA was quantitated spectro-photometrically, and the relative amounts of RNA in each lane were checked to be approximately equal by staining of the gel with ethidium bromide (bottom panel). The blots were exposed to X-ray film for 24–48 hours. (B) Quantification of the relative levels of CAT (Δ) and myogenin (\circ) transcripts during development. Blots as those presented in panel (A) were quantitated and the data shown are the mean \pm the range of two independent experiments carried-out in mice from both transgenic lines 5-1 and 2-3.

development. The reason we compared both CAT and endogenous myogenin mRNAs in our studies, is because we were concerned that the multiple copies of the *myogenin*-CAT construct in our transgenic mice could modify the transcription rates of the gene by sequestering limiting *trans*-acting factors. CAT transcripts were used as the reporter, instead of CAT enzymatic activity, because these measurements served to assess changes in transcription more accurately. Analysis of CAT transcript levels permitted us to resolve transient changes in transcription, since the RNAs are less stable than the protein and their expression is more proximal to transcription. After analysis of a large number of mice, we found that CAT activity accumulated significantly later than its transcripts (see next section).

The developmental analysis was performed using Northern blots containing 5 μ g of total RNA isolated from the hind limbs of transgenic mice of 2 days, 5 days, 2 weeks, 4 weeks and up to 8 months of age. The blots were sequentially hybridized, quantitated, stripped and rehybridized with labelled myogenin, CAT and α skeletal actin probes (see Methods). The results of a representative experiment using transgenic mice from line 2-3 are presented in Fig. 3A. The levels of CAT and myogenin transcripts were found to decrease during development. In sharp contrast, we found that the levels of α skeletal actin mRNAs increased during maturation; expression of α actin is known to increase during myogenic differentiation and not to be repressed by innervation. We were unable to identify a probe corresponding to a transcript that remained unchanged during this period of

development to use for normalization (see Methods). Ethidium bromide staining of the ribosomal markers confirmed that the samples in each lane contained approximately equal amounts of total RNA (bottom panel). The results from independent experiments using either transgenic mice from lines 5-1 and 2-3 were quantitated, and are graphically presented on Figure 3B. The CAT and myogenin signals obtained with the RNA isolated from mice of the two transgenic lines was approximately the same. The highest mRNA levels were observed in the perinatal period, as previously reported for endogenous myogenin transcripts in wild-type mice [43], during a time that coincides with formation and innervation of secondary myotubes in the mouse hind limb [52–54]. The temporal decrease of both transcripts during development was similar. However, when compared to myogenin, the relative levels of CAT message remained higher in the adult muscle with respect to peak-levels measured on day 2 after birth. The reason for this difference is unknown; plausible explanations that could account for this result include: that multiple copies of the *myogenin*-CAT constructs may result in higher synthesis of reporter transcripts; the CAT message is more stable than myogenin mRNAs; or, that additional transcriptional regulatory elements necessary to fully repress *myogenin* during muscle maturation are absent in the upstream sequences tested. Nonetheless, the decrease in CAT RNA expression observed during development in the transgenic mice is consistent with the idea that innervation represses *myogenin* transcription.

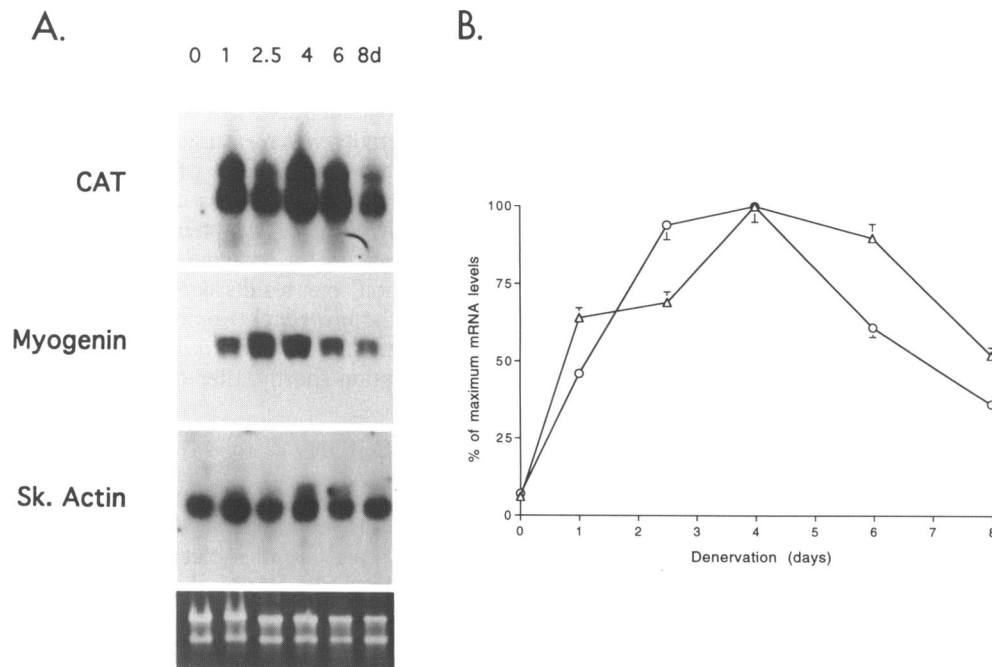


Figure 4. Denervation dramatically induces transcription of the *myogenin*-CAT transgene. (A) The Northern blots contain 5 μ g of total RNA isolated from the hind limb crural muscles of adult transgenic mice (line 5-1) that were either innervated (\circ) or denervated for 1 to 8 days. The blot was sequentially hybridized with cDNA probes coding for myogenin, CAT and α skeletal actin; the bottom panel shows ethidium bromide staining of rRNA. (B) The time-course of CAT (Δ) and myogenin (\circ) transcript accumulation was quantified from Northern blots containing RNA isolated from *myogenin*-CAT transgenes (lines 5-1 and 2-3). The data shown are the mean + \ - the range of two independent experiments, and have been normalized to skeletal α actin signals to account for any muscle degeneration that may have occurred during the course of the experiment. However, corrections for actin levels were minimal since the difference between samples was within 10% (see panel A).

The myogenin promoter fragment regulates transcriptional responses to denervation

Innervation, and more specifically nerve-elicited electrical activity, was previously shown to repress the accumulation of myogenic factor mRNAs in the adult skeletal muscle [43-44, 55]. Denervation de-represses the effects of the nerve resulting in a dramatic increase of myogenin, MyoD and MRF-4 transcripts [43-45, 55]. The increase of myogenic factor mRNAs precedes the accumulation of nAChR transcripts and the manifestation of muscle embryonic properties. To determine if the down-regulation of myogenin mRNAs during development is due to transcription, and if the regulatory sites lie within the promoter fragment, we compared the changes in CAT and myogenin expression in transgenic mice after muscle denervation. Adult mice (3-8 month old) from lines 5-1 and 2-3 were denervated by transection of the sciatic nerve at the upper thigh, RNA prepared from the crural muscles at 1, 2.5, 4, 6 and 8 days post-denervation, and hybridized sequentially on Northern blots with CAT and myogenin probes. As a control, the blots were also hybridized with an α skeletal actin probe, since expression of this gene has previously been demonstrated not to increase with denervation. Autoradiograms obtained from representative blots containing RNA isolated from transgenic 5-1 mice are shown in Figure 4A; time '0' refers to RNA isolated from innervated muscle. A dramatic increase of CAT and *myogenin* transcripts was already observed by 1 day after denervation. The accumulation of these mRNAs in response to denervation was specific, because the levels of α actin remained approximately

the same during the denervation time-course. Hybridization with a labelled CAT fragment, as well as with the two specific CAT oligonucleotides used for the in situ analysis, gave two bands on the Northern blots. This was expected since aberrant splicing of CAT transcripts containing the simian virus 40 small-t intron, not affecting the protein coding sequences, has extensively been analyzed previously [56]. The ethidium bromide staining of the gel (bottom panel), as well as the blots hybridized with actin, indicate that the lanes contained approximately equal quantities of undegraded RNA. The results obtained from independent experiments using lines 5-1 and 2-3 were quantitated, normalized to α actin signals, and plotted in Fig. 4B. Myogenin and CAT transcripts showed a similar time-course of accumulation and quantitative response to denervation, by 1d after nerve resection transcript levels were 10-fold higher than in innervated muscle. CAT transcripts peaked by 4 days after denervation when they attained levels that were 25-fold higher than found in innervated muscle. We also analyzed a transgenic line reported by Cheng *et al.* [57] that harbors a 1.56 kb myogenin/CAT construct, and found that denervation resulted in approximately a 25-fold increase of CAT transcripts after 2.5 days; the overall levels of CAT transcripts are lower in these mice (data not shown). These results demonstrate that transcriptional repression underlies the suppressive effects of nerve activity on *myogenin* expression. Furthermore, the denervation-induced activation of *myogenin* gene transcription significantly precedes the increase of nAChR α subunit transcription found in transgenic mice harboring a receptor-CAT fusion gene [18]. These results are consistent with our proposal that *myogenin* activation lies upstream of

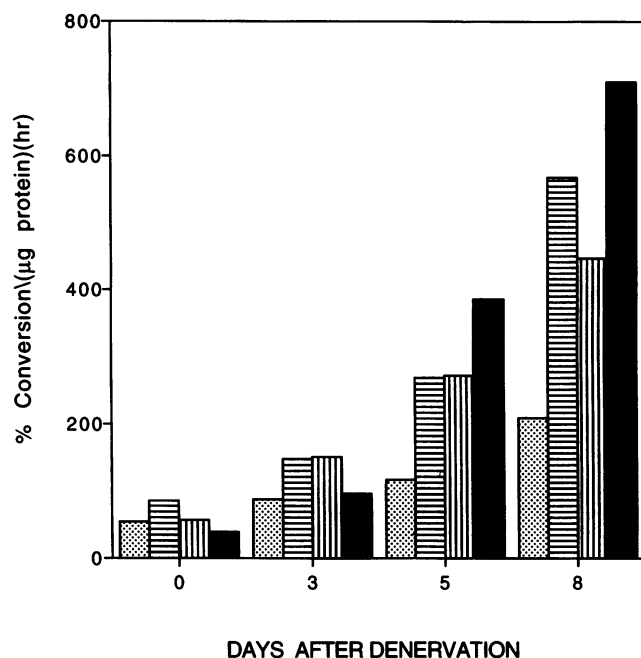


Figure 5. Comparison of CAT activity levels in different hind limb muscle groups of *myogenin*-CAT transgenes before and after denervation. Muscle extracts were prepared from the soleus (dotted), anterior tibialis (horizontal), extensor digitorium longus (vertical) and superficial gastrocnemius (solid black) muscles of adult *myogenin*-CAT transgenes (line 2-3) either before (○) or after denervation for 3, 5 or 8 days. Determinations of CAT activity were done at 37°C for 1h containing between 0.1–10 µg of protein, in order to maintain the assay within a linear range of enzyme activity. For this reason, the data is plotted as % conversion of chloramphenicol per µg of protein used in an 1h assay (see Methods). Similar results were obtained with transgenes from line 5-1, except that the overall CAT activity was approximately half of that obtained for mice from line 2-3.

denervation-induced genes, and could be one of the initial targets for activity dependent changes in muscle [43].

The levels of CAT reporter were also measured in innervated and denervated crural muscles containing different proportions of slow (type I) and fast (type II) myofibers. In these experiments CAT enzymatic activity was measured because of the greater sensitivity of this assay; it would have required a very large number of mice to isolate enough RNA from single muscles for these experiments. The steady-state levels of CAT activity in adult muscle were slightly different, with the highest levels found in the tibialis (Fig. 5). The soleus, composed of approximately equal number of I and IIA fibers in the mouse [49, 58], and the extensor digitorius longus, predominantly composed of IIA and IIB fibers [59], had similar levels of CAT activity. We obtained similar results using transgenes harboring a CAT reporter driven by 1.56kb of *myogenin* upstream sequence, previously described by Cheng *et al.* (57). These results differ from the analysis of *myogenin* mRNA steady-state levels in these two muscles in adult mice (A.B., unpublished) and rat [27], where higher levels are found in the soleus than in the extensor digitorium longus. The effects of denervation were analyzed on four crural muscles: soleus, EDL, anterior tibialis (predominantly type II) and superficial gastrocnemius (type II). Denervation resulted in the accumulation of different levels of CAT activity in the 4 muscles, where the highest relative increase was observed in the

gastrocnemius muscle by day 8 after denervation (Fig. 5). Although the levels of CAT activity increased with denervation, we were surprised by the observation that the peak levels of CAT and *myogenin* transcripts occurred at 4 days, whereas maximum CAT activity was measured at 8 days after denervation. These results warrant caution when using this reporter activity to monitor transient changes in transcription, and suggest that the assembly of the CAT protein or post-transcriptional mechanisms may regulate the enzyme activity in denervated muscle. In conclusion, our results demonstrate that *cis*-elements residing within the *myogenin* promoter fragment repress expression of the gene in response to innervation, and induce *myogenin* transcription shortly after denervation.

DISCUSSION

Myogenin, as well as the other three bHLH skeletal muscle factors, has been implicated in the transcriptional regulation of a large repertoire of skeletal muscle genes [see ref. 1]. These factors bind to a similar *cis*-element known as an E box [34]. The difference in their temporal expression during development [51, 60], as well as in response to different extracellular signals [see ref. 61], suggests that they may differentially regulate skeletal muscle gene expression. *Myogenin* regulation is complex, its expression is spatially and temporally restricted during embryonic, fetal and adult development. An elaborate regulatory network between muscle bHLH and other transcription factors has been described in cultured cells, where the factors can transcriptionally regulate each other's expression, as well as their own [see refs. 62, 63]. In addition, the effects of growth factors and innervation on skeletal muscle gene expression during development may converge on the regulation of the *myogenin* gene. As a first step in elucidating the sequences that mediate the transcriptional regulation of the gene during development and in response to innervation, we generated transgenic mice harboring a *myogenin*-CAT fusion gene. Our results demonstrate that the transcriptional regulatory *cis*-acting elements that control the spatial, temporal and tissue-specific expression of *myogenin* during embryonic and adult development reside within the upstream 3.7 kb of the gene. Furthermore, the promoter fragment contains the elements that repress *myogenin* expression during innervation, and that dramatically up-regulate transcription of the gene in response to denervation.

Regulation of *myogenin* transcription during embryonic development

The origin of myogenic precursor cells giving rise to body and head musculature differ. In chick-quail chimeras, progenitors giving rise to the muscles of the trunk and limbs were found to be expressed in the medial and lateral portions of the somites, respectively [64], whereas myogenic precursors giving rise to the head musculature, originate in the visceral arches [65]. During murine embryonic development, *myogenin* transcripts are first expressed in the rostral somitic myotomes on day 8.5 p.c. and expression is observed in more caudal somites as development proceeds; however, *myogenin* mRNAs are not detectable in the lateral somitic cells which migrate to the limbs [50]. *Myogenin* is first observed in the forming limb buds by day 11.5 [24, 50], as well as in the head musculature. The *myogenin*-CAT transgenic mice we characterized also showed expression of CAT transcripts in somites, muscle precursor cells surrounding the cartilaginous mesenchyme that give rise to the ribs, and myogenic precursors

of limb and facial musculature. Recently, the upstream sequences conferring *myogenin* regulation during embryonic development in these different muscles were shown in *myogenin-LacZ* transgenes to reside in the 133 bp 5'-flanking region [66], where a consensus sites for bHLH was found to be required for expression. Interestingly, mutation in the *myogenin* sequences of the binding site for MEF-2, another DNA binding protein present in muscle, revealed the differential expression of LacZ in cells of the myotome [66, 67]. Thus, the elements governing *myogenin* expression during embryogenesis reside in a compact region directly upstream of the gene, albeit elements residing further upstream are necessary for the proper temporal regulation. In contrast, the regulatory sequences of other muscle bHLH are organized differently with respect to location, and to the organization of regulatory elements that confer the proper temporal expression in somitic and nonsomitic mesoderm. As examples, the sequences that regulate the lineage-specific expression of *MyoD* in transgenic mice reside in an enhancer located about 15 kb upstream of the gene [68]. In the case of *myf-5*, Patapoutian *et al.* reported that the temporal expression of LacZ in visceral arches, driven by the 5 kb upstream sequence, was similar to the endogenous gene, whereas expression in somites lagged by 4 days [69]. On the other hand, *mrf-4* upstream sequences conferred proper spatial regulation in the 16.5d transgenic fetus but failed to recapitulate the early expression in somites [69]. The studies by Logan *et al.* on the analysis of the *engrailed* gene, which codes for a homeotic protein not restricted to skeletal muscle, also suggest the presence of regulatory regions that selectively direct transcription in myoblasts of different embryonic origins [70]. The results obtained with *myf-5*, *mrf-4* and *engrailed* transgenes support the idea that different modular elements direct transcription in myogenic progenitors giving rise to the head and body musculature, hitherto not evident in the case of *myogenin* regulation.

The nerve-dependent transcriptional regulation of *myogenin*

The formation and innervation of myotubes in the hind limb is an asynchronous process. The wave of secondary myotube formation in the lower hind limb of the mouse, which later constitutes most of the muscle mass, begins at approximately E16 and extends to about birth [52, 53]. The innervation and retraction of multiple synapses follow the formation of the myotubes, and is a process that extends past the first post-natal week of development [54]. Due to the complexity of this process, it is difficult to assert which changes in the muscle phenotype are due to endogenous developmental programs or result from innervation. Classically, the synchronous disassociation of afferent connections to the myofibers, by denervation of adult skeletal muscle, has served to identify which changes in gene expression during development result from neuromuscular interactions. Denervation of skeletal muscle is accompanied by dramatic changes in gene expression that result in muscle re-manifesting embryonic phenotypic properties, and with the re-accumulation of nAChR transcripts at extrajunctional nuclei. The changes resulting from denervation are largely due from depriving the muscle of electrical stimuli. The artificial stimulation of denervated muscle with extracellular electrodes suppresses expression and transcription of nAChR genes [15, 16, 71] presumably in extrajunctional nuclei. The muscle b-HLH proteins have previously been shown to directly bind E box elements present in nAChR subunit promoters and transactivate reporter genes driven by receptor regulatory sequences in transfected

cultured cells [41, 42, 72–74]. Furthermore, *myogenin* mRNA expression in vivo was found to precede, and be analogous to, changes in receptor expression in response to innervation, denervation and electrical stimulation [43–45, 55]. These results lead us to propose that the effects of denervation on the re-activation of skeletal muscle gene transcription could be mediated by *myogenin* and/or MyoD. Analysis of the *myogenin* promoter was undertaken, in large part, to identify the proximal events associated with the transcriptional repression resulting from nerve-elicited electrical activity and activation caused by denervation.

The present studies represented the first steps in delineating the *myogenin cis*-acting elements responsive to regulation by the nerve. We have found that elements mediating the repression of *myogenin* transcription, as well as its upregulation in response to denervation, reside in the 5'-flanking sequence of the gene. The temporal changes of *myogenin* transcription with respect to receptor [18] strongly support that *myogenin* regulation may be proximal to the initial effects of the nerve [43]. The molecular mechanisms underlying the transcriptional responses of *myogenin* and nAChR to denervation, and to repression by nerve-elicited electrical activity, may not be the same. For example, using nuclear run-on assays, Huang *et al.* found that repression of receptor gene transcription by electrical stimulation and PKC analogs in chick muscle occurs within minutes [71], whereas the denervation-induced activation of the genes occurs within 1–2 days [17]. A possible explanation for this difference is that electrical activity may inactivate a factor already present in muscle, while transcriptional activation after denervation requires de novo protein synthesis [75, 76]. In this respect it is interesting that, in chicken skeletal muscle, calcium and protein kinase C (PKC) have been implicated in coupling the effects of electrical activity to inactivation of nAChR genes [3, 71, 77]. The inactivation of receptor gene transcription, observed within 10 min of denervated muscle stimulation, was blocked by staurosporine. Phorbol esters were also found to prevent, at least in part, the denervation-induced increase of receptor transcription [71]. Li *et al.* have recently reported that phosphorylation of *myogenin* by PKC in the DNA-binding domain prevents binding to *cis*-elements and represses the muscle differentiation program [78]. It is interesting to speculate from the experiments discussed above, that in chick muscle the electrical activity down-regulation of receptor subunit genes and the *myogenin* gene, which contain multiple E boxes in their 5'-flanking regulatory sequences, could result from the inactivation of *myogenin* by PKC phosphorylation. The upregulation of *myogenin* transcription by denervation could result from the activation of *myogenin* present in muscle and the subsequent autoregulation of the gene, as previously demonstrated in cultured cells [see 62, 63]; this process would require protein synthesis. However, this speculation should be cautiously considered at the present time, since the effects of electrical stimulation in mammalian muscle have not been linked to the PKC pathway. Chahine *et al.* recently reported that in rat primary muscle protein kinase A (PKA) related mechanisms regulate the expression of nAChR, *myogenin* and MyoD in response to electrical stimulation, and mentioned that drugs affecting the PKC pathway had little effect [79]. Interestingly, activation of adenylate cyclase and PKA activity repress the potential of myogenic bHLH proteins to control murine myoblast differentiation [80] and inhibit transcription of the *myogenin* gene [81]. The effects of cAMP and PKA on *myogenin* activity, however, may be mediated by an indirect pathway [80]. The characterization of the mouse

myogenin upstream region in transfected cultured myocytes have implicated b-HLH and MEF-2 binding sites as necessary for myotube-specific expression [82]. An intricate regulatory circuit of transactivation between myogenin and MEF-2 has been previously described (see 61, 63). The presence of binding sites for both factors in the *myogenin* upstream sequences merits further analysis of the possible role of these sites, as well as other *cis*-elements such as those for AP1, AP2, NF-1 and Oct, in regulating *myogenin* during embryonic development and later in response to innervation/denervation.

Control of *myogenin* transcription is a focal point of regulation at which autonomous factors and extracellular signals converge to modulate the process of myogenesis. The identification of *cis*-acting DNA regulatory elements that properly direct the expression of the CAT reporter gene in transgenic mice in a *myogenin*-specific manner, constitute the first steps in identifying the *trans*-acting factors that confine expression to different myogenic progenitor cells and mediate the responses to innervation. Multiple *cis*-elements and *trans*-acting factors are likely to mediate the transcriptional responses of muscle cells to exposure to serum and peptide growth factors, hormones and interactions with the nerve during development. We are currently pursuing a deletional analysis of the *myogenin* 5'-flanking region to identify smaller regions that underlie the different responses, and that could uncover separate regulatory elements that direct *myogenin* transcription in different muscle progenitors.

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