

The MyoD family of myogenic factors is regulated by electrical activity: isolation and characterization of a mouse Myf-5 cDNA

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ABSTRACT

A full-length cDNA coding for a homolog of the human Myf-5 was isolated from a BC3H-1 mouse library and characterized. The clone codes for a protein of 255 amino acids that is 89%, 88% and 68% identical to the human, bovine and *Xenopus myf-5*, respectively. The mouse Myf-5 cDNA (mmyf-5), as well as sequences coding for MyoD, myogenin and Mrf-4, were used to probe Northern blots to analyze the effects of innervation on the expression of the MyoD family of myogenic factors. Mouse myf-5, MyoD and myogenin mRNAs levels were found to decline in hind limb muscles of mice between embryonic day 15 (E15) and the first postnatal week, a period that coincides with innervation. In contrast, Mrf-4 transcripts increase during this period and reach steady-state levels by 1-week after birth. To distinguish if the changes in myogenic factor expression are due to a developmental program or to innervation, mRNA levels were analyzed at different times after muscle denervation. Mmyf-5 transcripts begin to accumulate 2 days post-denervation; after 1 week levels are 7-fold higher than in innervated muscle. Mrf-4, MyoD and myogenin transcripts begin to accumulate as soon as 8h after denervation, and attain levels that are 8-, 15- and 40-fold higher than found in innervated skeletal muscle, respectively. The accumulation of these three mRNAs precedes the increase of nicotinic acetylcholine receptor α subunit transcripts, a gene that is transcriptionally regulated by MyoD-related factors *in vitro*. Using extracellular electrodes to directly stimulate *in situ* the soleus muscle of rats, we found that 'electrical activity' *per se*, in absence of the nerve, represses the increases of myogenic factor mRNAs associated with denervation.

INTRODUCTION

Skeletal muscle differentiation proceeds through a sequential series of events beginning with the commitment of pluripotential mesodermal cells to the myogenic lineage. The committed

myoblasts proliferate, migrate, and differentiate to form multinucleated myotubes which later, during maturation, are innervated by motoneurons and sensory fibers. Differentiation is accompanied by the transcriptional activation of genes and accumulation of mRNAs coding for contractile proteins (e.g. ref 1,2), metabolic enzymes (3,4), and neurotransmitter receptors (5,6,7). A family of genes coding for factors that commit mesodermal cells to the myogenic lineage, presumably by activating transcription of skeletal muscle-specific genes, has been identified (reviewed in 8,9,10). Complementary DNAs coding for 4 of these factors have been isolated from several species, these include homologs for MyoD (11,12,13,14), myogenin (15,16), Myf-5 (17,18,19) and Mrf-4 (20,21,22). A gene locus known as *myd* (23), which has not yet been isolated and characterized, can also convert transfected 10T_{1/2} cells into myoblasts. Proteins of the 'MyoD family' contain a conserved region predicted to conform to a helix-loop-helix structural domain necessary for dimerization (24) and a stretch of basic amino acids necessary for DNA binding (25). These nuclear proteins have been shown to bind to the regulatory elements of the creatine kinase (26,27), myosin light chain (28), troponin I (29) and nicotinic acetylcholine receptor (nAChR) genes (30,31), and to transactivate their expression. Although transcription of creatine kinase, myosin light chain and nAChR genes is activated during myoblast differentiation, their expression is regulated differently during skeletal muscle maturation. Expression of creatine kinase and myosin light chain transcripts is maintained during muscle innervation, whereas the nerve represses the levels of embryonic nAChRs and its mRNAs in extrajunctional regions of the muscle (32–34). The reduction in nAChR gene transcription during maturation is due to innervation, because denervation overcomes the repressive effects of the nerve resulting in reactivation of transcription (35,36) and accumulation of receptor transcripts (ref. 6,7).

Most studies on the expression of myogenic factors have been confined primarily to cultured cells (11,12,15–17,20,21,37) or to early stages of embryonic skeletal muscle development prior to innervation (13–15,19,38,39), focusing on the function of these factors during commitment of pluripotential mesodermal

cells and myoblast differentiation. Since we had found that myogenin and MyoD regulate nAChR receptor genes (30), we analyzed their expression during late embryonic development and were the first to report that the expression of both factors is down-regulated by innervation (40). In order to extend our observations to the other MyoD-related factors, the initial purpose of this study has been to obtain a cDNA for the murine Myf-5 (mmyf-5). The mmyf-5 cDNA, as well as a Mrf-4 probe (20), were then used to analyze myogenic factor mRNA levels during muscle maturation, and after denervation, to assess the effects of innervation on their expression. Since the nerve can modulate the expression of genes in muscles either by the release of trophic substances and/or by depolarization, we have used direct stimulation of soleus muscle with extracellular electrodes to demonstrate that electrical activity *per se* suppresses the accumulation of myogenic factor mRNAs induced by denervation.

MATERIAL AND METHODS

Animals

NIH Swiss mice were obtained from the NIH animal facility for the developmental and denervation experiments. Male Sprague-Dawley rats of about 350 g were used for the electrical stimulation experiments. All animals were handled according to the NIH Animal Care and Welfare protocol.

Isolation and characterization of a mouse Myf-5 cDNA

A mouse cDNA library made from the muscle-like cell line BC3H-1 was kindly provided by Dr. J.P. Merlie; its construction has been previously described (41). Approximately 2.4×10^5 plaques of the differentiated BC3H-1 cDNA library were screened with a human Myf-5 probe. The cDNA fragment was labeled by random priming (1×10^8 dpm/ μ g), and the membranes were hybridized at 42°C in a solution containing 35% formamide, 5×SSC, 5×Denhart's (1× is 0.02% each of Ficoll, polyvinylpyrrolidone, bovine serum albumin), 100 μ g/ml salmon sperm DNA and 1% SDS. The filters were washed in 1×SSC and 1% SDS at 55°C and exposed to X-ray film overnight. A total of 20 positive signals of different intensities were obtained. The 5 clones yielding the strongest signals were characterized further and found to contain inserts ranging between 1.8 to 2.2 kb. The insert from the largest cDNA, expressed by clone #9, was subcloned into Bluescribe (Stratagene, CA) and sequenced on both strands with Sequenase (United States Biotechnologies, OH) using oligonucleotide primers and the Sanger di-deoxy termination technique (37). The compilation of sequences and analysis of the data were done with DNASTAR.

Denervation and stimulation of skeletal muscle

The denervation, stimulation and isolation of muscle were done essentially as previously described by Eftimie *et al.* (40). For the developmental studies, embryonic day 1 (E1) refers to the day after copulation. After the embryos were removed from the chorionic sacs, they were measured and staged to confirm their embryonic age. The muscles dissected from the hind limbs of embryos from a litter were pooled and considered as a single preparation. The muscles isolated from E15 and E17 mice contained minor amounts of bone residue that were difficult to remove during dissection. To study the effects of denervation, the sciatic nerve of anesthetized 8-week-old mice was resected

unilaterally at the upper thigh. At different times after surgery total RNA was isolated from the posterior crural muscle groups. The crural muscles from approximately 6 mice were pooled for each RNA preparation.

Stimulation of adult soleus rat muscle was performed as previously described (43). Briefly, anesthetized animals (Nembutal) were denervated by bilateral removal of a 5 mm segment of the sciatic nerve. Electrodes were implanted into one of the hind limbs, and the soleus muscle was stimulated chronically in freely mobile living rats for 6 or 10 days in 100 Hz trains, 1 s duration, applied once every 100 s. The train of pulses were of alternating polarity with a strength of 10–15 mA and a duration of 0.5 ms. The contralateral denervated/unstimulated muscle served as a control. After stimulation, the soleus muscles were removed under anesthesia, frozen in liquid nitrogen, and stored at –70°C until used for RNA preparations.

RNA preparations and Northern blots

Total RNA was isolated from mouse and rat muscles using guanidine thiocyanate and ultracentrifugation on cesium chloride gradients. RNA was quantitated spectrophotometrically, and the integrity and relative amounts of RNA in each sample used were also assessed by ethidium bromide staining of ribosomal RNA on separate gels. 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 membranes were hybridized with EcoRI fragments from a mouse 1.7 kb MyoD cDNA (11), a 1.5 kb mouse (16) or rat (15) myogenin cDNA, the 2.2 kb mmyf-5 cDNA, the 1.3 kb rat Mrf-4 cDNA (20), a 1.6 kb mouse nAChR α subunit cDNA (44) or a plasmid containing sequence of a partial mouse skeletal α actin cDNA (45) that were 32 P-labelled by random priming to a specific activity of approximately 10^8 dpm/ μ g of DNA. Hybridization was carried out at 42°C in 6×SSC, 10×Denhardt's, 1% SDS, 10 mM EDTA, 0.1% sodium pyrophosphate, 100 μ g/ml salmon sperm DNA, and either 50% formamide or 40% formamide (when using mouse probes on rat RNA). Blots were washed with 0.1×SSC, 1% SDS at 65°C or with 0.2×SSC, 1% SDS at 60°C when using heterologous probes; there were no cross-hybridizing bands observed when these conditions were utilized. Initially, the blots were exposed to X-ray film to obtain an autoradiogram, and subsequently, the signals were quantitated directly from the blots (see below). To minimize for the variability that may occur during loading samples on the gels and during the transfer of RNA onto the Nytran membranes, the blots were stripped after quantitation and used for hybridization with other probes. The blots were stripped in 60% formamide, 1% SDS, and 10 mM Tris-Cl (pH 7.5) at 65°C, and exposed to X-ray film to assert that the probe was fully stripped. The intensity of the signals on reused blots was not affected by the stripping procedure.

Northern blot quantitation

The amount of radioactivity on the blots associated with the different bands was quantitated directly by using a Betascope 603 Blot Analyzer (Betagen Corporation, Waltham, MA). The number of counts per band varied linearly with respect to time and with respect to the amount of mRNA on the blots (data not shown). The values plotted are the average of at least three independent experiments; backgrounds obtained on each blot were subtracted.

RESULTS

Isolation and characterization of a murine myf-5 (mmyf-5) cDNA

Due to the low levels of Myf-5 mRNA in mouse muscle, we were unable to study its expression using the partial human cDNA (17) provided to us. Partly for this reason, we decided to isolate a Myf-5 murine homolog. A lambda gt11 cDNA library made from the mouse cell line BC3H-1 (kindly provided by J.P. Merlie) was screened at low stringency with an EcoR-1 fragment from the human Myf-5 cDNA as described in Methods. The largest of the three cDNAs isolated, clone 9NC, was subcloned into the Bluescribe vector and sequenced on both strands. The nucleotide sequence can be obtained from the EMBL data base under accession # X56182. The 2157 nucleotide sequence has an open reading frame (ORF) that codes for a protein of 255 amino acids; the protein is the mouse homolog of the human Myf-5 (see Fig 1). The first AUG in the ORF was chosen as the initiator methionine because the surrounding sequence (AAGGAUGG) conforms to a consensus vertebrate translation initiation site (46). Twelve bases upstream of the proposed initiator AUG there is a stop codon in the same reading frame. Most of the 5' untranslated region of the cDNA sequence shown in Fig.1 is identical to a short segment of the mmyf-5 genomic sequence reported by Ott *et.al.* (39), except that we have an extra G and a C residue 53 and 44 bases upstream of the initiator AUG, respectively. The region surrounding the G residue at position -53 was difficult to sequence on the coding strand due to a compression. The 3' non-coding region of mmyf-5 cDNA is 1152 bp long, similar in length to the bovine homolog and 467 longer than the human Myf-5. As in the case of the bovine myf-5 (18), multiple putative polyadenylation addition sites are found in the 3' noncoding region.

The nucleic acid sequences, as well as the deduced amino acid sequences obtained from myf-5 cDNAs cloned from human, mouse, bovine and *Xenopus*, are extremely well conserved. For

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Mmyf MDMTDGCQFSPSEYFYEGSCIPSPPEDEFGDQFEPVAAFGAHHKAEALQGSDDDEHVRAPTG 60
Hmyf MDVMDGCQFSPSEYFYDGSICIPSPPEGEFGDEFVPRVAAFGAHHKAEALQGSDDDEHVRAPTG
Bmyf MDMMDGCQFSPSEYFYDGSICIPSPDGEFGDEFVPRVAAFGAHHKAEALQGSDDDEHVRAPTG
Xmyf MEMVDGCHFSPEFFYDSSICIPSPPEGYTDEYEHGMSIYGAKKDLAESDDEHVRAPIG
Cons M**A**D**C**F**S**P**S**E**F**Y**S**C**I**P**S**P*******G**A**H**K**A**L**S**D**D**E**H**V**R**A**P**T**G

Mmyf HHQAGHCLMWACKACKRKSSTMDRRKAATMRERRRLKKNVQAFETLKRCTTTNPNQRLPK 120
Hmyf HHQAGHCLMWACKACKRKSSTMDRRKAATMRERRRLKKNVQAFETLKRCTTTNPNQRLPK
Bmyf HHQAGHCLMWACKACKRKSSTMDRRKAATMRERRRLKKNVQAFETLKRCTTTNPNQRLPK
Xmyf HHQAGNCLMWACKACKRKSSTMDRRKAATMRERRRLKKNVQAFETLKRCTTTNPNQRLPK
Cons HHQAG*CLMWACKACKRKS*T*DRRKAATMRERRRLKKNVQAF*TLKRCTTTNPNQRLPK

Mmyf VEILRNAIRYIESLQELLREQVENYYSLPQGSCSEPTSPNSCSGDMPECNSPVWSRKNS 180
Hmyf VEILRNAIRYIESLQELLREQVENYYSLPQGSCSEPTSPNSCSGDMPECNSPVWSRKSS
Bmyf VEILRNAIRYIESLQELLREQVENYYSLPQGSCSEPTSPNSCSGDMPECNSPIWSRKSS
Xmyf VEILRNAIQYIESLQDLLREQVENYYSLPQSCTEPGSPMSSCSDGMSDCSSPQWSGRNS
Cons VEILRNAI*YIESLQ*LLREQVENYYSLPQSC*E**P**S**P**S**C**S**D**G**M**S**D**C**S**S**P**Q**W**S**G**R**N**S

Mmyf SFDSIYCPDVSNACAAADKSSVSSLDCLSSIVDRITSTEPSLALQDTASLSPATSANSQP 240
Hmyf TFDSIYCPDVSNVYATDKNSLSSLDCLSNIVDRITSSSQPLPIQLDASLSPVASTDSQP
Bmyf SFDSVYCPDVSNVYATDKNSLSSLDCLSSIVDRITSNQPLPIQLDASLSPVASTDSQP
Xmyf SFDNVYCSDLQTSFSSTKLTLSSLDCLSSIVDRITSSPQCSLPIPDSTPPTSSTDSLP
Cons *F**D**S**I**Y**C**P**D**V**S**N**A**C**A**A**D**K**S**S**V**S**S**L**D**C**L**S**S**I**V**D**R**I**T**S**T**E**P**S**L**A**L**Q**D**T**A**S**L**S**P**A**T**S**A**N**S**Q**P

Mmyf ATPGSSSRLLIYHVL 255
Hmyf RTPGASSRLLIYHVL
Bmyf ATPGASSRLLIYHVL
Xmyf RSPDAHDCRPIYHVL
Cons **P**P**A**A**S**S**R**L**I**Y**H**V**L
    
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Figure 1. Alignment of deduced amino acid sequences coding for Myf-5. The amino acid sequences deduced from myf-5 cDNAs isolated from mouse (Mmyf), human (Hmyf; 17), bovine (Bmyf; 18) and *Xenopus* (Xmyf; 19) have been aligned. The consensus sequences showing identity among the four proteins is shown below in bolded letters; asterisks denote residues not conserved in all the proteins. Brackets identify the basic/helix-loop-helix domains conserved in all myogenic factors.

example, the nucleic acid coding sequences of the human and mouse are 87% identical. Interestingly, the 5' and 3' untranslated regions of the mouse, human and bovine myf-5 transcripts contain stretches that are highly homologous (not shown). The conservation of these sequences suggest that they may play an important functional role. As shown in Figure 1, the amino acid sequence of the human, bovine and *Xenopus* myf-5 are 89%, 88% and 68% identical to the mmyf-5 sequence, respectively, and the four proteins contain the same number of residues. Two important myc-like structural domains, present in the other MyoD-related factors (11,15,16,20), are conserved in the amino acid sequence of mmyf-5: the helix-loop-helix motif which has been proposed to be required for dimerization (24) and the basic region that is necessary for DNA binding (25). Both regions are outlined in Fig. 1. In addition, there are stretches of amino acid identity specific to Myf-5 in the NH₂- and COOH-terminal domains of the protein that may be involved in the activation of transcription (see Discussion).

Transcripts derived from members of the MyoD gene family are expressed differentially during late muscle development

The expression of myogenic factor transcripts during development of mouse somites and limb buds has been analyzed using *in situ* hybridization histochemistry (38,39). We have extended our studies to a later developmental period in mice, during secondary myotube formation and innervation (47,48), to evaluate the effects of the nerve on myogenic factor expression. For these studies, RNAs isolated from the hind limb muscles of E15 to 8-week-old mice were analyzed on Northern blots. Autoradiograms of the representative blots that were hybridized with myogenin, MyoD, mmyf-5 and Mrf-4 cDNA probes are shown in Figure 2A

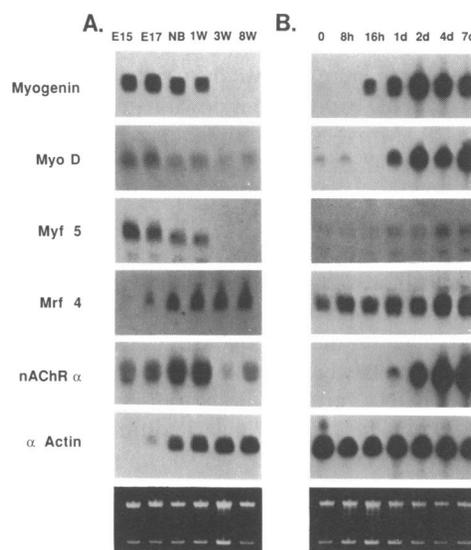


Figure 2. Regulation of myogenic factor mRNAs during skeletal muscle innervation and after denervation. A) Total RNA (10 µg) isolated from posterior crural muscle groups of mice of different ages, from E15 to 8-week-old mice, was analyzed by Northern blot. B) Northern blots containing 10 µg of total RNA isolated from innervated or denervated adult mice muscles. Posterior crural muscles were dissected from 8-week-old mice (0) or mice denervated for 8h, 16h, 1d, 2d, 4d, and 7d. RNA was quantitated spectrophotometrically, and the integrity and relative amounts of RNA in each sample used were checked by ethidium-bromide staining of ribosomal RNA on a separate gel (bottom panels). The probes used are described in Methods. The blots were exposed to X-ray film plus intensifying screens for 36 to 84 hrs.

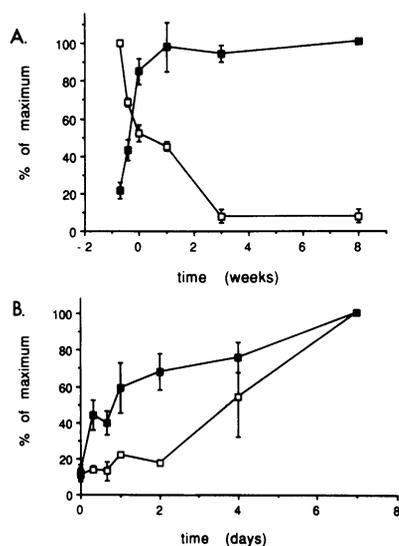


Figure 3. Quantitation of the relative amounts of mmyf-5 and Mrf-4 transcripts during development and after denervation. The relative levels of mmyf-5 (\square) and Mrf-4 (\blacksquare) transcripts were quantitated during development, *A*; and at different times after denervation, *B*. The procedure used to quantitate the signals directly from the blots are described in *Methods*. Each point represents the mean value of three independent experiments plus the standard error of the mean. The values are expressed as the % of the maximum signal. Points at time '0' represent RNA isolated at birth (panel *A*). The data in panel *B* is normalized to skeletal actin signals to account for any muscle degeneration that may have occurred during the denervation time course. All the values plotted had background subtracted.

to illustrate how this family of myogenic factors is differentially regulated during development. The signals on Northern blots using RNA from 3 independent experiments were quantitated directly using a radioanalyzing scope (see *Methods*). The relative levels of mmyf-5 and Mrf-4 mRNAs are plotted as a function of development on Figure 3A; we have previously reported how myogenin and MyoD expression is down-regulated during muscle maturation (40). Mmyf-5 transcripts are regulated in a similar fashion to myogenin and MyoD; the levels are highest at E15 and drop 10-fold by the third week after birth. In contrast, Mrf-4 transcripts accumulate after E15 and attain maximum levels by postnatal day 7 (P7). Our results obtained for Mrf-4 are in agreement with those reported by Rhodes and Konieczny (20) showing that Mrf-4 transcripts are undetectable in RNA isolated from E10 and E15 rat muscle, but are present in adult muscle. As controls, the blots were hybridized with nAChR subunit and skeletal actin cDNA probes. Both transcripts are known to accumulate coordinately during muscle differentiation, but later in development, innervation selectively down-regulates expression of receptor mRNAs. As expected, we found that during early development both nAChR and actin transcripts accumulate, and as the animals became older, receptor mRNA levels are selectively down-regulated (Fig. 2A). These results demonstrate that the transcripts coding for all the MyoD-related myogenic factors are not coordinately regulated during development, as previously shown for embryonic development (38,39), which suggested to us that the down-regulation of myogenin, MyoD and mmyf-5 may be caused by innervation.

Myogenic factor mRNAs accumulate differently after denervation

During skeletal muscle development, the expression of a repertoire of muscle-specific genes (e.g. nAChR) is initially

activated during myoblast differentiation but later repressed by innervation. The down-regulation of these genes is due to innervation and is not simply part of a program, because denervation reverses the repressive effects of the nerve and results in their renewed expression (ref. 6,7). Denervation also stimulates the proliferation of mononucleated cells located in the interstitial spaces between muscle fibers (49,50). In order to assess the effects of innervation on the expression of myogenic factor mRNAs, we measured their levels after denervation. The sciatic nerves of adult 8-week-old mice were transected, and RNA was isolated from the posterior crural muscles at different times after denervation to analyze on Northern blots. Figure 2B shows the autoradiograms of the blots hybridized with the 4 myogenic factor cDNAs. The blots were hybridized with the nAChR and actin cDNAs as controls since denervation is known to lead to the accumulation of receptor mRNAs without affecting significantly actin transcript levels (see ref. in 6,7). The signals were quantitated directly from the blots, and on Figure 3B we have plotted how the relative levels of mmyf-5 and Mrf-4 mRNAs vary at different times after denervation. We previously reported that myogenin and MyoD mRNA levels peak in 2-day denervated muscle to levels that are approximately 40- and 15-fold higher than those found in innervated muscle. Myogenin mRNAs accumulate rapidly between 8 to 16 h after denervation, and MyoD transcripts levels begin to increase sharply between 16h to 1 day post-denervation (40). The accumulation of Mrf-4 transcripts also occurs between 8 to 16h after denervation, however, the increase is not as dramatic. The highest levels observed in our experiments were attained 1 week postdenervation, 8-fold higher than those found in adult innervated muscle (Fig. 3B). The response of mmyf-5 was temporally different in comparison to the other factors; its transcripts began to accumulate significantly between 2 and 4 days after denervation. The mmyf-5 mRNA levels in 1-week-old denervated muscle were approximately 7-fold higher than those found in innervated muscle (Fig. 3B). The data presented in Figure 3B has been normalized to the skeletal actin signals to correct for any degeneration occurring during the denervation period used in these experiments; actin levels decreased only slightly on the seventh day after denervation (1.3-fold). The α and δ (not shown) subunit nAChR mRNAs also accumulated dramatically after denervation, the increase in receptor transcript levels lagged the accumulation of myogenin, MyoD and Mrf-4 mRNAs but preceded the increase of mmyf-5 (Fig. 3A).

Direct electrical stimulation down-regulates accumulation of myogenic mRNAs after denervation

We analyzed the levels of myogenic factor mRNAs in denervated muscle directly stimulated with extracellular electrodes in order to evaluate if electrical activity *per se* represses their expression. Electrodes were implanted into the hind limbs of rats immediately after denervation and electrically stimulated for 6 or 10 days (see *Methods*). After stimulation, RNA was prepared separately from the denervated/stimulated (D/S) and contralateral denervated/unstimulated (D) soleus muscle of each rat, as well as from innervated (I) soleus muscle from control animals. The blots containing equal amounts of total RNA were probed with cDNAs for the myogenic factors, nAChR α subunit, and α actin as a control. As shown in Figure 4, direct stimulation of skeletal muscle (lanes D/S) in absence of the nerve prevented, at least in part, the accumulation of transcripts coding for the myogenic factors after denervation (lanes D). The increases in MyoD, myogenin, mmyf-5, MRF4 and receptor mRNAs in rat

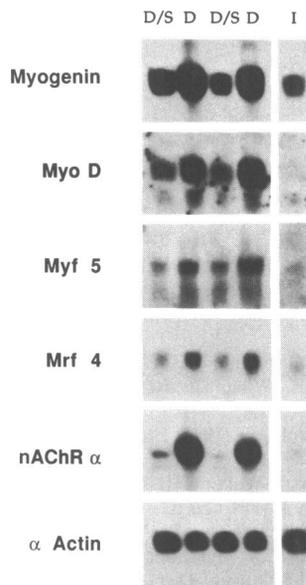


Figure 4. Electrical activity down-regulates the expression of myogenic factor mRNAs in denervated muscle. Representative Northern blots contain 10 μ gs of total RNA isolated from either innervated (I) rat soleus muscles, denervated soleus muscles that were electrically stimulated (D/S) or left unstimulated (D). The blots were hybridized with cDNA probes coding for rat myogenin (15), mouse MyoD (11), mmyf (this paper), rat Mrf-4 (20), mouse nAChR α subunit (44), and α skeletal actin (45). To assure that equal amounts of undegraded sample were being tested, RNA was quantitated spectro-photometrically and the integrity was checked by ethidium bromide staining of the ribosomal RNA.

denervated soleus muscle were approximately 7, 17, 6, 3, and 75-fold higher than in innervated muscle, respectively. However, in denervated/stimulated muscle their levels were approximately 2, 5, 3, 1.4, and 8-fold higher than those measured in innervated soleus. The effects of muscle activity on the expression of the myogenic factors is specific, because the levels of α actin mRNAs in denervated/stimulated soleus were similar, or even higher (1.5-fold), than those found in denervated muscle (Fig. 4). These experiments demonstrate that electrical activity *per se* represses the expression of transcripts coding for the myogenic factors after muscle denervation. It is important to note that the denervation-associated increases in myogenic factor mRNAs in these experiments done in rat soleus muscle can not be compared to those obtained using the crural muscle groups of mice (40, Fig. 3B), because the magnitude and temporal increases in myogenic transcripts after denervation varies between both species and different muscle types (A.B. and H.B., unpublished results).

DISCUSSION

There is a striking degree of conservation in the Myf-5 nucleic acid coding sequences of human, calf, *Xenopus* and mice. For example, the sequences in human and mice are approximately 90% identical. In addition, conservation of nucleic acid sequences extends to the untranslated regions of the mRNAs. Overall homologies of approximately 65% in the 5' nontranslated region, as well as short stretches of identity, are suggestive that translation regulatory elements may be found in this region. The 3' untranslated sequences are also conserved. It is interesting that this region in the mouse and bovine Myf-5 cDNAs are longer than that described for the human cDNA (17), and both contain

multiple polyadenylation addition sites that are located at approximately the same distance from the translation termination site (620 and 1093 bp from the amber codon). We consistently observe 2 bands of different sizes on Northern blots of mouse muscle hybridized with our mmyf-5 cDNA (see Fig. 2). If differential RNA processing occurs at the upstream and downstream polyadenylation addition sites, as has been previously described for the bovine Myf-5 transcripts (18), it could account for the two bands. Processing using the upstream site would result in transcripts resembling the human Myf-5 cDNA in length. We have not observed on the Northern blots changes in the relative intensities between the two mmyf-5 bands either in the developmental or denervation studies which could indicate regulation of RNA processing at the polyadenylation addition sites. At the protein level, the mmyf-5 has conserved sequences in the basic/helix-loop-helix domain; these motifs are present in all members of the MyoD family. In addition, there are stretches of identical amino acid sequences extending toward the NH₂- and COOH-termini that are not shared with other members of the MyoD family. In this respect it is interesting as recently reported by Braun *et al.* (51), that in contrast to MyoD (25), the human Myf-5 contains a muscle-specific transcriptional activating domain at its COOH-terminus instead of in its basic domain. The conservation of Myf-5-specific sequences at the NH₂-terminus suggests that these regions may also have important functional properties that can not be assessed in cells transfected with constructs expressing the Gal4-myf-5 fusion proteins (51).

Studies on the expression of the MyoD-related factors *in vivo* have focused on the early stages of embryonic development, because it has been proposed that this family of factors are involved in committing pluripotential mesodermal cells to myoblasts by activating transcription of skeletal muscle genes (reviewed in 8–10). *In vitro*, the four MyoD-related factors have been shown to bind to similar regulatory elements (CANNTG), and to transactivate expression of, the creatine kinase (26,27), myosin light chain (28), troponin I (29), and nAChR α (30,31) and γ (30) subunit genes. It is interesting to note, that although transcription of these genes is coordinately activated during myoblast differentiation, later in development expression of receptor genes is selectively down-regulated by innervation. For this reason we felt it was important to analyze how the expression of MyoD-related factors is regulated during innervation of skeletal muscle. Our results show that the expression of the mRNAs coding for the four myogenic factors during late embryonic development, and after denervation, is not coordinately regulated. During a period that coincides with innervation of mouse secondary myotubes (47,48), the levels of myogenin, MyoD and mmyf-5 transcripts dramatically decrease. The decrease of the three transcripts is not simply part of a developmental program, because denervation induces their accumulation. Mrf-4 mRNAs, however, begin to accumulate around birth and reach steady-state levels by P7. Future studies should address the role that the nerve may play in causing the accumulation of Mrf-4 transcripts during maturation (see below).

Denervation has multiple effects, it induces rapid changes in expression of genes within the myofiber (ref. 6,7) and leads to the proliferation of interstitial mononucleated cells (49, 50). Among these are the satellite cells which contribute approximately 4% of the total number of cells in muscle and are involved in regenerating myofibers after injury. Using tritiated thymidine incorporation studies, it has been shown that the peak of cellular division occurs 4 days after denervation and that satellite cells

account for less than 10% of the dividing cell population (49,50). The changes in MyoD, myogenin, and Mrf-4 mRNA levels are large and occur hours after denervation, and since in the adult expression of MyoD-related factors is confined to skeletal muscle, it is likely that the accumulation of myogenic factors after denervation occurs predominantly in myofibers and not in the proliferating mononucleated cells. The increase in myf-5 mRNA levels are smaller and occur between 2 and 4 days after denervation, therefore these changes may happen in either proliferating cells and/or in the myofibers. The observation that Mrf-4 mRNA accumulation occurs during neonatal development but that denervation results in its increased expression seems paradoxical, and makes its regulation more difficult to understand. One possible explanation for regulation of Mrf-4 mRNA levels, is that during normal development the expression of MyoD, myogenin and Myf-5 is down-regulated by innervation while the nerve provides signals to increase the levels of Mrf-4. However, after denervation, the levels of MyoD and myogenin increase, and in turn, could possibly transactivate expression of Mrf-4. The auto- and cross-activation of the different myogenic factor genes has previously been demonstrated in C3H 10T1/2 cells (21,52). Interestingly, there is a similar puzzling regulation of the nAChR ϵ subunit mRNAs, where ϵ transcript levels increase in rat muscles around birth and denervation of adult muscle also results in further accumulation of these mRNAs (53). Interestingly, the expression of Mrf-4 transcripts during mouse development is concomitant or precedes the appearance of ϵ subunit mRNAs (54). Further studies using *in situ* hybridization and immunohistochemistry will be required to determine the localization of the four myogenic factors during innervation, and after response to nerve or muscle injury. Although in cultured cells no hormonal or cellular interactions are needed for the autonomous expression of the myogenic factors (21), the role that neural-derived factors may play in regulating myogenic factor expression and function *in vivo* needs to be investigated.

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