Adaptation of Nicotinic Acetylcholine Receptor, Myogenin, and MRF4 Gene Expression to Long-Term Muscle Denervation

Larry Adams,*‡ Bruce M. Carlson,‖§ Leslie Henderson,¶ and Daniel Goldman*‡

*Mental Health Research Institute, ‡Department of Biological Chemistry, ‖Department of Anatomy and Cell Biology, §Institute of Gerontology, University of Michigan, Ann Arbor, Michigan 48109; and ¶Departments of Biochemistry and Physiology, Dartmouth Medical School, Hanover, New Hampshire 03755

Abstract. Muscle activity alters the expression of functionally distinct nicotinic acetylcholine receptors (nAChR) via regulation of subunit gene expression. Denervation increases the expression of all subunit genes and promotes the expression of embryonic-type ($\alpha_2\beta\delta\gamma$) nAChRs, while electrical stimulation of denervated muscle prevents this induction. We have discovered that the denervation-induced increases in α, β, γ, and δ subunit gene expression do not persist in muscles that have been denervated for periods extending beyond a couple of months. However, expression of RNA encoding the ϵ-subunit remains elevated suggesting a return to expression of predominantly adult-type ($\alpha_2\beta\delta\epsilon$) nAChR in long-term denervated muscles; a finding confirmed by single channel patch-clamp analysis. Since the nAChR subunit genes are regulated by the MyoD family of muscle regulatory factors, and the genes encoding these factors are also induced following short-term muscle denervation, we determined their level of expression in long-term denervated muscle. Although MyoD and myf-5 RNA levels remained elevated, myogenin and MRF4 RNAs were induced only transiently by muscle denervation. Surprisingly, Id-1, a negative regulator of transcription, was gradually induced in denervated muscle with RNA levels peaking about two months after denervation. It is likely that this maintained level of increased Id expression, in conjunction with the returning levels of myogenin and MRF4 expression, account for the reduced level of embryonic receptors in long-term denervated muscle. These changing patterns of gene expression may have important consequences for the ability of muscle to recover function after denervation.

T HE muscle nicotinic acetylcholine receptor (nAChR)[1] is a pentameric integral membrane protein that functions as a ligand-gated ion channel. During muscle development the levels, distribution, and properties of this receptor change (for review see Brehm and Henderson, 1988; Hall and Sanes, 1993). Many of these changes are correlated with muscle innervation. Before innervation, or after denervation of adult muscle embryonic-type nAChRs are expressed throughout the muscle fiber. These receptors are composed of four different subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$. After innervation of muscle, the γ subunit is replaced by an ϵ subunit, and these adult-type receptors ($\alpha_2\beta\epsilon\delta$) are preferentially expressed at the neuromuscular junction (NMJ).

The switch from embryonic to adult-type receptors results in a change in their channel properties. Embryonic-type receptors exhibit a low single channel conductance and long mean channel open time, while adult-type receptors have a higher conductance and faster open time kinetics (for review see Schuetze and Role, 1987; Brehm and Henderson, 1988). Although the reason for the switch in receptor subtypes is not completely clear, it may arise from a requirement for long open time, embryonic-type of receptors in developing muscle (Jaramillo et al., 1988), which are detrimental to adult muscle and therefore replaced with adult-type receptors (Engel et al., 1982; Ohno et al., 1995).

Muscle denervation has been used as a model system to study the molecular mechanisms by which muscle activity regulates synaptic protein expression. Denervation of adult muscle causes a dramatic increase in the sensitivity of the fiber to ACh (Ginetzinsky and Shamarina, 1942; Axelsson and Thesleff, 1959) which is directly attributable to increases in the level of the α, β, γ, and δ subunit RNAs within 24–48 h (Goldman et al., 1988). Increases in these nAChR RNAs lead to high levels of expression of embryonic-type nAChR throughout the muscle fiber (Katz and Miledi, 1972; Neher and Sakmann, 1976; Henderson et al., 1987). In addition, the level of ϵ subunit RNA, which is

preferentially expressed in adult muscle, is increased locally at the NMJ within 7 d of muscle denervation (Witzemann et al., 1989, 1991; Gundersen et al., 1993). These changes in RNA levels have been shown to result from altered patterns of nAChR gene expression (Merlie and Kornhauser 1989; Klarsfeld et al., 1991; Simon et al., 1992; Gundersen et al., 1993). In addition, activity-dependent regulation is selective, since RNAs encoding other muscle-specific proteins, such as creatine kinase and myosin light chain, are unaffected by muscle denervation or electrical stimulation (Chahine et al., 1992; Dutton et al., 1993; Gilmour et al., 1995; Su et al., 1995).

The molecular mechanisms mediating the effects of muscle activity on nAChR gene expression are beginning to be elucidated. A calcium-dependent protein kinase C and a cAMP-dependent signaling cascade have been implicated in mediating the effects of muscle activity in chick and rat, respectively (Klarsfeld et al., 1989; Huang et al., 1992; Chahine et al., 1993). DNA sequences that ultimately respond to muscle depolarization and alter nAChR gene expression have also been identified. In the mouse δ and chick α subunit genes, DNA sequences conforming to the consensus E-box sequence (CANNTG) have been identified, and those located nearest to the transcriptional start sites have been shown to participate in mediating activity-dependent control of nAChR gene expression (Bessereau et al., 1994; Tang et al., 1994; Su et al., 1995). These elements are bound by members of the helix-loop-helix family of myogenic regulatory factors (MRFs), which include MyoD, myogenin, MRF4, and Myf-5. These proteins are thought to bind E-box sequences as heterodimers consisting of one of the above mentioned proteins complexed with a ubiquitously expressed E protein such as E47 or E12 (Weintraub et al., 1991).

In contrast to the helix-loop-helix proteins described above, one member of this family of proteins, referred to as Id, lacks a DNA binding domain (Benezra et al., 1990). Id can complex with other E-box binding proteins, but these complexes have a low affinity for DNA. Via these protein–protein interactions with other myogenic regulatory factors, Id can act to inhibit helix-loop-helix protein-dependent gene activation.

Muscle denervation has a dramatic effect on muscle-specific gene expression and muscle physiology. Short periods of denervation, up to 1 mo, results in an increase in embryonic-type nAChR and MRF gene expression (Evans et al., 1987; Witzemann and Sakmann, 1991; Voytik et al., 1993). This period corresponds to the time when the muscle can best recover its function after reinnervation. However, prolonged periods of denervation, extending beyond a couple of months, significantly reduce the ability of muscle to become functionally reinnervated (Sunderland, 1978). During this time period, the muscle fiber undergoes dramatic changes in its size, cytoplasmic content, number of nuclei and in its ability to recover function upon regeneration and/or reinnervation (B. M. Carlson, unpublished data). This decreased potential for recovery may be correlated with a concomitant change in the muscle's ability to maintain a particular pattern of muscle-specific gene expression. To determine if there is a change in muscle-specific gene expression after prolonged periods of denervation which may correlate with the diminished ability to

recover function, we assayed for nAChR, creatine kinase and MRF gene expression at various times of denervation. We report here that the changes in gene expression observed after short-term muscle denervation are not maintained in long-term denervated muscles. Most surprising was our finding that the high levels of α, β, γ, and δ subunit RNAs which typify both embryonic and adult denervated muscle, begin to return to innervated levels following 1–2 mo of muscle denervation. In contrast, the adult-type ε subunit and creatine kinase RNAs do not decrease as a result of prolonged periods of denervation. These results indicate that there is a return to predominantly adult-type nAChR gene expression in long-term denervated muscle.

Patch-clamp recordings indicated that changes in subunit gene expression are reflected in changes in both the single channel properties of the receptors and their relative levels of expression in junctional and nonjunctional membrane. In contrast to muscle fibers denervated for short periods of time which express high levels of low conductance, long open time receptors throughout the membrane, fibers denervated for greater than two months expressed predominantly the high conductance, short open time form of the receptor. Moreover, the level of ACh-induced activity was high only in recordings from junctional membrane, suggesting that a high density of receptors is limited to the subsynaptic membrane. Physiological data suggesting that high levels of nAChR expression are restricted to the previous synaptic site was also indicated by the pattern of fluorescence in these fibers stained with rhodamine-conjugated α-bungarotoxin, an irreversible ligand for the muscle nAChR (Chang and Lee, 1963).

The decreased expression of α, β, γ, and δ subunit RNAs after prolonged periods of muscle denervation corresponds to a time when myogenin and MRF4 expression are also declining and Id expression is increasing. Our data suggest that the different patterns of nAChR subunit gene expression observed in short- and long-term denervated muscle may be a consequence of the changing pattern of expression of these myogenic factors and may underlie the decreased ability of muscle to recover function as the time of denervation increases.

Materials and Methods

Muscle Denervation

RNA Isolation and RNase Protection Assay

Total RNA was isolated using the method of Chirgwin et al. (1979). RNase protection assays were carried out as previously described (Saccomanno et al., 1992; Walke et al., 1994). After hybridization of RNA with appropriate probes, RNase T2 was used to digest away single-stranded RNA. RNase-resistant hybrids were analyzed on 6% polyacrylamide, 8 M urea gels. After electrophoresis, gels were dried and exposed to x-ray film. Signals were quantitated by scanning densitometry and values normalized to either total RNA in the hybridization reaction or muscle creatine ki-

nase RNA signal. Similar results were obtained using both normalization procedures. RNase protection assays were repeated at least twice with different samples of muscle RNA. Specificity of protected bands was confirmed by hybridization of probes to tRNA which resulted in no protected fragments on the gel. In addition, probe integrity during the hybridization reaction was monitored by omitting the RNase step.

Probes

Antisense RNA probes were prepared by run-off transcription of plasmids harboring cDNA inserts that were linearized with appropriate restriction enzymes. The probes used to detect muscle creatine kinase (MCK) and nAChR α, β, γ, δ, and ϵ-RNAs, were as described previously (Chahine et al., 1993). The α-probe is 600 nucleotides, including exon and intron sequences, of which 240 nucleotides are protected by α-RNA. The β-probe is 229 nucleotides long, all of which is protected by β-RNA. The γ-probe is 560 nucleotides long, all of which is protected by γ-RNA. The δ-probe is 440 nucleotides long of which 344 bases correspond to genomic DNA 5' to the transcriptional start site and therefore only 96 nucleotides of this probe are protected by δ-RNA. The ϵ-probe is 484 nucleotides long, all of which are protected by ϵ-RNA. The MCK probe is 292 nucleotides long, all of which are protected by the MCK RNA. The myogenin probe was prepared from an EcoRI linearized BSSK(+) vector harboring a 190-bp EcoRI/SacI fragment of the mouse myogenin cDNA (Wright et al., 1989). The myogenin probe and protected fragment are ~190 nucleotides long. The MyoD probe was prepared from a HindIII linearized BSSK(+) vector harboring a 1785-bp mouse MyoD cDNA insert (Davis et al., 1987) in its EcoRI site. This generates a probe of ~500 nucleotides which is completely protected by MyoD RNA. The MRF4 probe was prepared from a SalI linearized BSSK (+) vector harboring a 400-bp PstI/SalI MRF4 mouse genomic DNA fragment (Miner and Wold, 1990). The 400-nucleotide-long MRF4 probe only protects ~100 nucleotides of MRF4 RNA due to the presense of intron sequences in this probe. The myf-5 (Braun et al., 1989) probe was prepared from an EcoRI linearized BSSK(+) vector harboring a 400-bp SacI/HindIII mouse genomic DNA fragment. Approximately 380 nucleotides of the 400-nucleotide-long myf-5 probe are protected by myf-5 RNA. The Id probe was prepared from PvuII linearized BSSK(−) vector harboring the mouse Id cDNA insert (Benezra et al., 1990). The probe is 607 nucleotides long, all of which are protected by Id RNA. Probes were purified from denaturing acrylamide gels.

In Situ Hybridization and nAChR Staining

In situ hybridization assays were performed as previously described (Goldman and Staple, 1989; Goldman et al., 1991). nAChRs were stained with rhodamine-conjugated α-bungarotoxin as previously described (Henderson et al., 1987).

Physiological Recordings

Individual muscle fibers were obtained from the flexor digitorum brevis (fdb) muscles of denervated rats. The fdb muscle was dissected and dissociated according to Henderson et al. (1987), and recordings were made in the cell-attached configuration of the patch clamp technique (Hamill et al., 1981) as previously described (Brennan and Henderson, 1993). Irrespective of the time after denervation, the original endplate was morphologically distinguishable at the time of recording, as has previously been reported (Henderson et al., 1987).

Single channel currents were acquired and analyzed as described by Brennan et al. (1992). Mean open times were estimated at $V_{pipette} = +80$ mV for distributions containing greater than 100 events, except for some nonsynaptic recordings where the frequency of events was extremely low. Slope conductances were estimated by linear regression from the current vs. voltage relationship for $V_{pipette}$ between +60 and +140 mV. Estimates of the ACh-induced activity represent the product of the open probability times the number of channels and were calculated by TAC® software (Instrutech Corp., Elmont, NY). Estimates of the relative percentages of openings corresponding to high and low conductance events, as well as levels of ACh-induced activity, were made immediately after seal formation at $V_{pipette} = +80$ mV. Statistical significance was determined using a Student's two-tailed t test. Where given, n values indicate the number of fibers.

Results

Embryonic-Type nAChR RNAs Initially Increase and Then Decrease in Long-Term Denervated Muscle

It is well documented that sectioning the motor neuron results in increased expression of embryonic-type nAChRs (Katz and Miledi, 1972; Neher and Sakmann, 1976; Henderson et al., 1987) and α, β, γ, and δ subunit RNAs in skeletal muscle (Evans et al., 1987; Goldman et al., 1988; Gundersen et al., 1993; Witzemann et al., 1989). However these analyses have generally been confined to the first month of muscle denervation. In the present experiments, we have assayed for nAChR RNAs and nAChR function during a period lasting up to 1 yr after denervation.

To prevent reinnervation of the muscle fiber, we used a denervation procedure that included, in addition to sectioning the sciatic nerve, ligating the proximal and distal nerve stump and implanting the proximal nerve stump into a nearby muscle. Previous experiments, employing silver staining of segments of the distal nerve and electrical stimulation of transected sciatic nerve, have shown that this denervation procedure prevents the return of nerve fibers for a period of time extending beyond 22 mo (Carlson and Faulkner, 1988).

RNase protection assays were used to determine the level of expression of nAChR subunit RNAs in muscle that had been denervated for up to 12 mo. Each assay included a probe for a particular nAChR subunit RNA and MCK. We determined that creatine kinase RNA did not change during 12 mo of muscle denervation by assaying the level of this RNA, normalized to total RNA used in
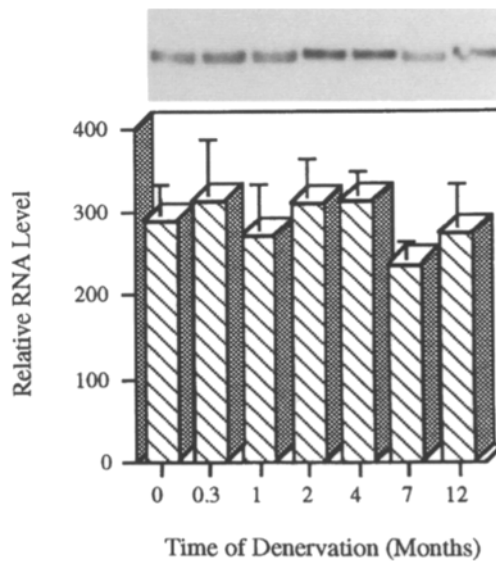

Figure 1. Long-term muscle denervation has little effect on MCK RNA levels. RNase protection assays were used to assay for creatine kinase RNA levels in muscle denervated for various lengths of time extending up to 1 yr. At the top of the graph is a representative RNase protection result. The graph represents quantitation of the MCK RNA level as a function of the time the muscle remains denervated. MCK RNA (densitometry value) is normalized to total RNA (μg) applied to the gel. Values are the average of six experiments. RNA was isolated from both gastrocnemius and tibialis anterior muscles.
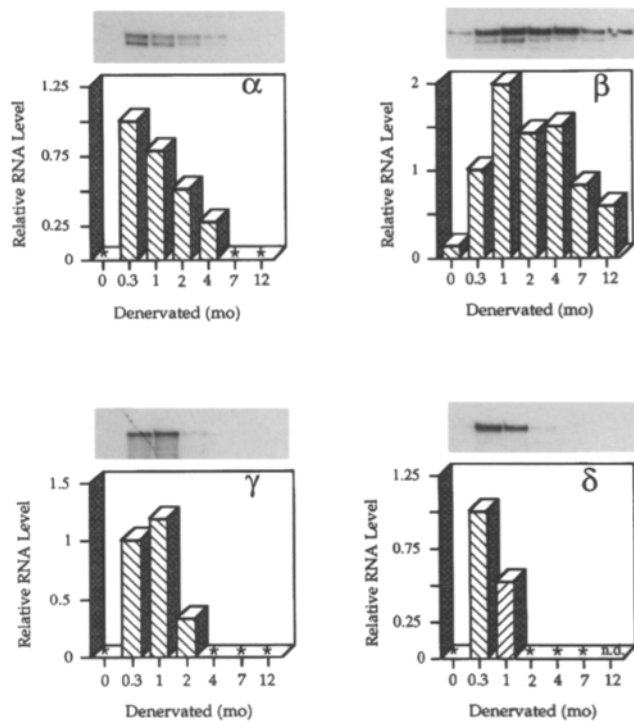
Figure 2. Adaptation of nAChR RNA levels to long-term muscle denervation. RNase protection assays were used to assay for embryonic-type nAChR RNA levels in gastrocnemius muscle denervated for various lengths of time extending up to 1 yr. At the top of each graph is a representative RNase protection result. The graph represents quantitation of the nAChR RNA level as a function of the time the muscle remains denervated. nAChR RNA is normalized to MCK RNA levels. Asterisks (*) indicate values were below detectable levels.

Figure 3. The adult-type specific ε-RNA remains elevated in long-term denervated muscle. RNase protection assays were used to assay for the embryonic-type specific γ subunit RNA and the adult-type specific ε subunit RNA in tibialis anterior muscle denervated for various lengths of time up to 7 mo. Above the graph is a representative RNase protection result. The graphs represent quantitation of γ- and ε-RNA levels as a function of time the muscle remains denervated. nAChR RNA levels are normalized to MCK RNA levels.

the hybridization reaction, in six different experiments (Fig. 1). Since muscle denervation lasting up to 12 mo had no significant effect on the expression of the MCK RNA, expression of nAChR subunit RNAs was normalized to the expression of this muscle-specific gene.

In contrast to the MCK RNA and consistent with previous reports (Evans et al., 1987; Goldman et al., 1988; Witzemann et al., 1989; Gundersen et al., 1993), RNase protection assays revealed an initial increase in nAChR RNA levels during the first month of denervation (Fig. 2). However, as denervation progressed beyond 1 mo, we were surprised to find that α, β, γ, and δ subunit RNAs specifically began to return to innervated levels. This finding was most dramatic for the γ and δ RNAs, which approached innervated levels within 2 mo after denervation. In contrast the α and β RNAs showed a more gradual decline approaching innervated levels by 7 mo after denervation. All four subunit-specific RNAs began decreasing ~1–2 mo after nerve section.

The transient nature of the increases in RNAs that encode subunits of the embryonic-type nAChR was in marked contrast to expression of the adult-type ε subunit RNA. Levels of ε subunit RNA were found to increase ninefold shortly after denervation and remained elevated throughout the ensuing 12 mo (Fig. 3). Note that in this particular experiment γ subunit RNA began to decrease
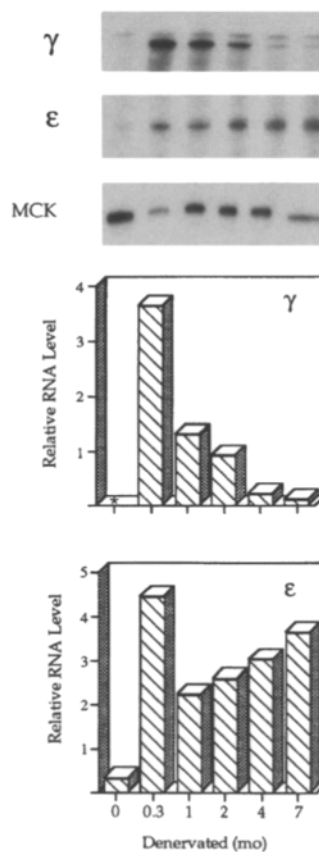
between 0.3 and 1 mo after denervation, while in the experiment reported in Fig. 2 this RNA began to decrease after 1 mo of denervation. This difference in time course likely reflects the different muscles used in these two experiments. Experiments reported in Fig. 2 used RNA isolated from the gastrocnemius muscle, while experiments reported in Fig. 3 used RNA isolated from the tibialis anterior muscle.

Prolonged Muscle Denervation Leads to a Decreased Expression of Functional Embryonic-Type nAChRs

To determine if changes in nAChR subunit mRNAs were reflected in concomitant changes in the expression of functionally distinct receptors, single channel recordings were made from denervated fdb fibers isolated from the denervated muscle. Two functionally distinct classes of AChR were present in denervated fdb fibers: high conductance (77.8 ± 2.3 pS; $n = 19$), short open time (1.56 ± 0.17 ms; $n = 20$) adult-type nAChR (ε-containing) and the low conductance (49.9 ± 1.7 pS; $n = 14$), long open time (3.13 ± 0.36 ms; $n = 15$), embryonic-type nAChR (γ-containing) channel (Fig. 4). The relative percentages of ACh-induced opening attributed to these two distinct classes were determined in both junctional and extrajunctional regions of the muscle fiber (Table I). This analysis revealed a decreased expression of embryonic-type channels as the length of time of denervation increased beyond 1 mo in both junctional and extrajunctional membrane. By 4 mo of denervation, the majority of nAChR channel openings were of the adult-type. In addition, the level of ACh-induced channel activity was 300-fold lower in recordings
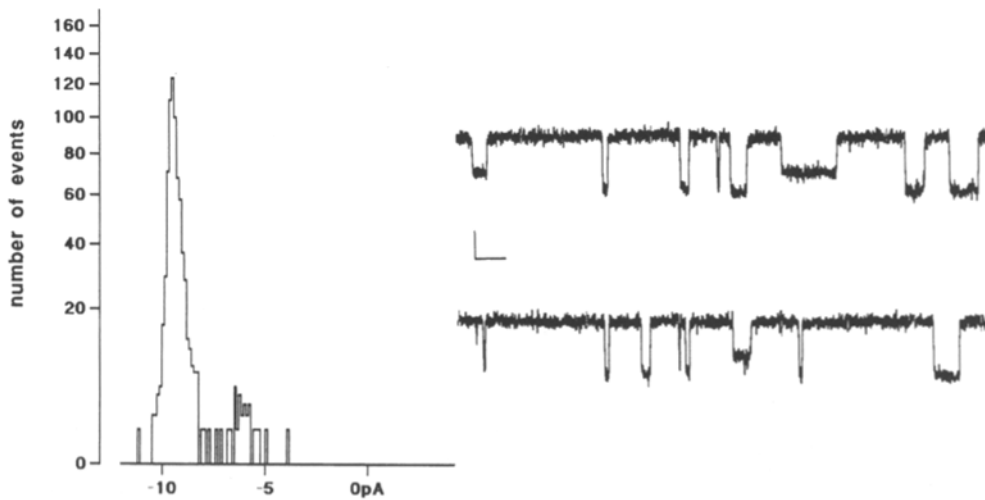
Figure 4. ACh-induced single channel events recorded from the endplate of a 4-mo denervated fdb fiber. (*Right*) Representative single channel openings elicited in a cell-attached patch from the postsynaptic membrane by 250 μM ACh. Two distinct classes of events, corresponding to the low conductance/long open time (embryonic) and high conductance/short open time (adult) forms of the nAChR were evident. (*Left*) Amplitude histogram for all events recorded at this endplate (concurrent multiple openings not shown). Two distinct amplitude classes are evident, but nearly all events fall in the distribution corresponding to the higher amplitude (adult-type) class. Bar, 5 ms; 5 pA.

from extrajunctional versus junctional membrane from long-term denervated fibers, suggesting a reversal of denervation supersensitivity.

Adult-type specific ε-RNA and nAChRs Remain Localized to Discrete Regions of the Muscle Fiber Four Months After Denervation

The adult-type specific ε-RNA is localized to the neuromuscular junction in innervated and short-term denervated muscle. This localized expression appears to be mediated by the synaptic basal lamina (Goldman et al., 1991; Brenner et al., 1992; Jo and Burden, 1992). We were interested in determining if long-term denervation affected the distribution of this RNA. In situ hybridizations revealed a localized expression of this RNA in muscles denervated for up to 1 yr (Fig. 5 *A*). In addition, we continue to detect a localized, high level of synaptic expression of nAChR protein in muscles denervated for 4 mo as revealed by fluorescent α-bungarotoxin staining (Fig. 5 *B*). These results are consistent with continued expression of ε-RNA at the

Table I. Percentage of High-Conductance Openings in FDB Fibers Denervated In Vivo

Number of days denervated	Synaptic	Nonsynaptic
0[1]	97% (42)	97% (10)
14-17[2]	8% (10)	2% (6)
19-21[2]	16% (13)	7% (6)
120-150	70% (10)	65% (8)
>210	99% (3)	67% (6)

The values represent the percentage of total openings that were due to activation of the high conductance (ε-containing) ACh receptor in synaptic and nonsynaptic recordings from flexor digitorum brevis (FDB) fibers denervated in vivo for the number of days indicated. Values in parentheses indicate the number of fibers examined. Recordings were made in the cell-attached configuration. [ACh] = 250 nM.
*Data are from Brehm and Kullberg (1987) and Brennan and Henderson (1993).
‡Data are from Henderson et al. (1987). In these experiments, ACh receptors present at the time of denervation were irreversibly blocked by α-bungarotoxin. Openings represent channels newly synthesized after denervation.

old neuromuscular junction of long-term denervated muscle and with the higher levels of ACh-induced activity in junctional than in extrajunctional recordings.

Long-Term Muscle Denervation Results in a Changing Pattern of MRF RNA Expression

Since long-term muscle denervation resulted in reduced expression of embryonic-type nAChRs, and because the nAChR genes are known to be regulated by MRFs (Gilmour et al., 1991; Prody and Merlie, 1992; Berberich et al., 1993; Bessereau et al., 1994; Durr et al., 1994; Tang et al., 1994), we examined MRF expression in the long-term denervated muscles. All the MRFs analyzed were induced within 10 d after muscle denervation (Fig. 6). At 10 d after denervation myogenin and MRF4 were induced ∼100- and 40-fold, respectively, while MyoD and myf-5 were induced ∼7- and 17-fold, respectively. MyoD and myf-5 expression remains high for at least 7 mo after denervation. Myogenin and MRF4 do not maintain their high level of expression beyond 1 mo after denervation, and in this respect are similar to the expression of embryonic-type nAChR RNAs.

Finally, we examined the expression of Id RNA in the denervated muscles. We were surprised to find that this RNA has a relatively slow time course of induction following muscle denervation (Fig. 6). Peak levels of Id RNA were detected ∼2 mo after denervation and these levels remained significantly above that found in innervated muscle for at least 7 mo after motor nerve section. The time period in which Id levels were maximal corresponded to the time when nAChR subunit RNA levels and levels of functional receptors were decreasing.

Discussion

Before the studies reported here, it has been presumed that embryonic-type nAChR RNA expression remained
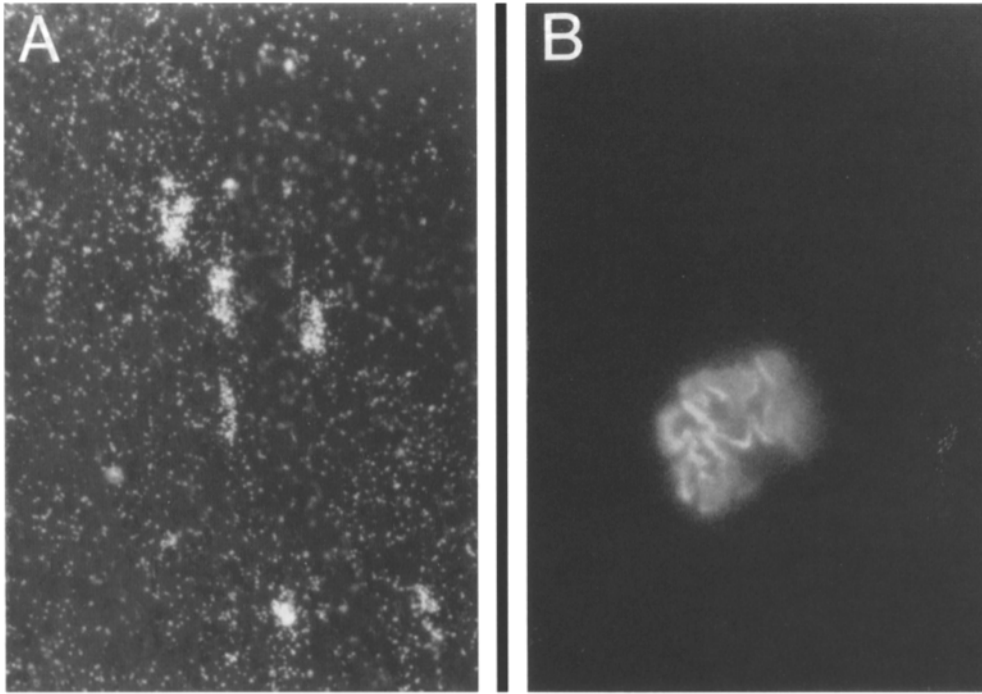
Figure 5. Localization of ε-RNA and nAChRs to discrete regions of the 4-mo denervated muscle fiber. (*A*) In situ hybridization was used to identify ε-RNA in tibialis anterior muscle fibers denervated for 4 mo. The white grains correspond to the distribution of ε-RNA (similar results were obtained at 1 yr after denervation). (*B*) Distribution of nAChR in 4 mo denervated muscle fiber. High density of synaptic receptors indicated by rhodamine α-bungarotoxin fluorescence.

elevated in denervated muscle. This is clearly not the case. After 2 mo of denervation, the predominant forms of nAChR RNAs expressed are those which encode the adult-type receptor. These changes in RNA expression are reflected in the expression of functional nAChRs on the muscle's surface. With time after denervation greater than 1–2 mo, the relative percentage of ACh-induced openings attributed to the embryonic-type receptor decreases until at times greater than 210 d, the relative percentages of openings by the adult and embryonic forms of the nAChR at the synapse are indistinguishable from those of an innervated fiber. In addition, the observation that the level of extrajunctional ACh-induced activity is quite low (suggesting that denervation supersensitivity has declined) is also consistent with a return to an "innervated condition" in long-term denervated fibers. One caveat is that the relative percentage of openings by embryonic type receptors in extrajunctional membrane (33%) does not completely return to levels characteristic of the innervated state (3%; Brehm and Kullberg, 1987; Brennan and Henderson, 1993). In addition, the overall level of ACh-induced activity at the junction in long-term denervated rat fdb fibers is approximately half that observed in innervated fibers (C. Brennan and L. Henderson, unpublished data), although this may reflect a lower overall capability of long-term denervated fibers to synthesize proteins, rather than a specific effect on nAChR expression.

Is it possible that these results derive from reinnervation of denervated muscle fibers? We do not believe this is the case for the following reasons: (*a*) a denervation procedure was used that had previously been documented to prevent the return of nerve fibers for a period of time extending beyond 22 mo (Carlson and Faulkner, 1988); (*b*) the muscle fibers retained, on an average, only 18% of their original mass consistent with their denervated state; (*c*) morphological studies showed a progressive worsening of the histological quality of the muscle fibers as the time

of denervation increased (B. M. Carlson, unpublished observation); (*d*) MyoD and myf-5 RNAs, which are regulated by muscle activity, remained elevated in the long-term denervated muscles; (*e*) at the time of dissection of the muscles there was no observable sign of reinnervation, nor did the lower leg contract upon electrical stimulation of the proximal stump of the sciatic nerve; and (*f*) using the same denervation technique, Billington (1995) has shown that the maximum tetanic force generated by 4 mo and longer denervated EDL muscles drops to 0.3% of control and remains at that level. Therefore, we conclude that the results reported here do not reflect reinnervation of the denervated muscle fibers.

The consequences of expressing adult-type nAChRs in long-term denervated muscle are not known. However, it is interesting to note that the recovery of function of denervated muscle by grafting and reinnervation is ~100% of that of control muscle grafts if the muscle is grafted and reinnervated within the first two months of denervation. However, denervation lasting from 2–7 mo results in a progressive decrease in the ability of the muscle to recover function (B. M. Carlson, unpublished observation). It is possible that one component of this robust recovery of function after short-term denervation is the expression of embryonic-type nAChRs on the denervated muscles surface. Perhaps these receptors are involved in maintaining a permissive state for reinnervation. Alternatively, increased embryonic-type receptor expression may be a consequence of other events that are required for maintaining a muscle in a receptive state for reinnervation and maximal functional recovery. In this case the nAChR genes will serve as useful probes for characterizing the mechanisms leading to poor muscle recovery following long-term denervation.

To gain insight into potential mechanisms mediating the decreased expression of embryonic-type receptor genes during long-term denervation, we assayed for MRF RNA
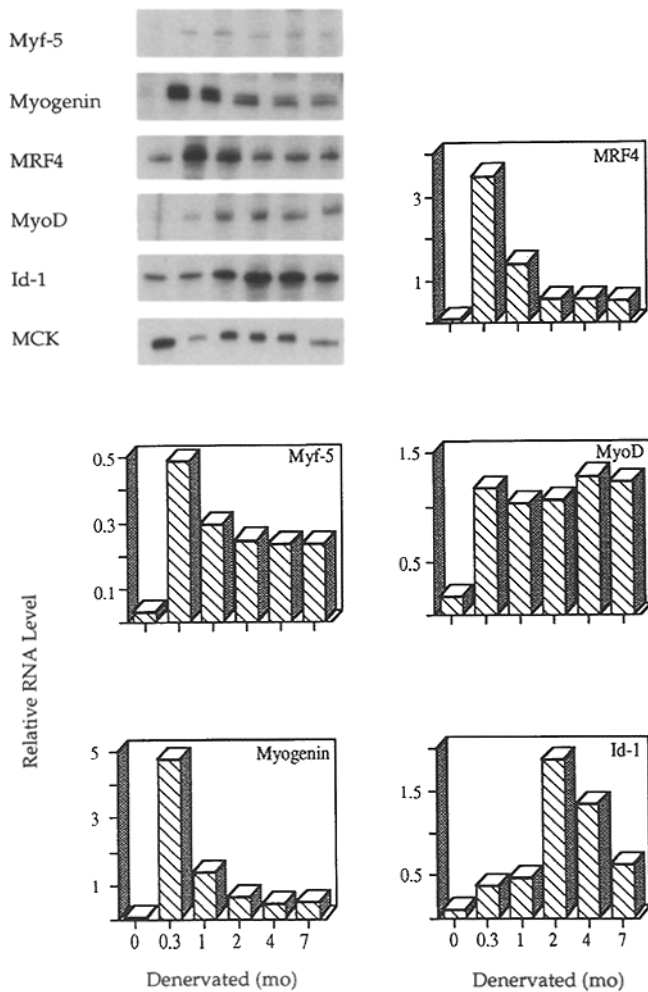
Figure 6. Regulation of myogenic factor RNA levels in short and long-term denervated muscle. RNase protection assays were used to determine the level of various myogenic factors in denervated tibialis anterior muscle. Representative RNase protection assays are shown in the upper left of the figure. Quantitation of these RNase protection assays is presented in the graphs. Muscle regulatory factor RNA levels are normalized to MCK RNA levels.

expression in these same muscle samples. We suspected that MRF expression participated in this regulation because; (*a*) these transcription factors are known to activate nAChR gene promoters in in vitro cell culture experiments (Piette et al., 1990; Gilmour et al., 1991; Prody and Merlie, 1992; Berberich et al., 1993; Durr et al., 1994); (*b*) myogenin knock-out mice fail to induce nAChR α and γ subunit RNAs (Hasty et al., 1993); and (*c*) mutations in particular E-box *cis*-acting sequences, that mediate binding of these factors to nAChR promoter DNA, affect nAChR promoter activity in vivo (Bessereau et al., 1994; Tang et al., 1994). RNase protection assays showed a complex pattern of MRF RNA expression over a 7-mo period of muscle denervation (Fig. 6). Both myogenin and MRF4 were induced shortly after muscle denervation. Consistent with previous reports (Witzemann and Sakmann, 1991; Dulcert et al., 1991; Eftimie et al., 1991; Voytik et al., 1993), we found myogenin was induced to a significantly higher level than any of the other MRFs. However, both

myogenin and MRF4 induction were transient and return to a lower level by 2 mo after denervation.

MRFs have been proposed to participate in mediating nAChR gene induction following muscle denervation (Merlie et al., 1994; Tang et al., 1994; Su et al., 1995). Our data are consistent with this possibility and indicate that the decrease in nAChR RNAs beginning ~1–2 mo after denervation may result from lower levels of myogenin and/or MRF4 expression (Fig. 6). Interestingly, in denervated chick muscle, MRF4 is not observed to increase following muscle denervation (Neville et al., 1992), yet nAChR genes are induced. There is evidence to suggest that regulation of nAChR expression in chicks does not precisely parallel that observed in mammals and amphibians. For example, the developmental change in nAChR kinetics does not occur in chick (Schuetze, 1980), and there may be species-specific differences in mechanisms governing myogenic factor, as well as nAChR gene expression (Walke et al., 1994). However, if a mechanism is conserved between birds and mammals in initiating and maintaining nAChR gene induction after muscle denervation, one would predict that myogenin is a more likely candidate for mediating this effect. In support of this hypothesis, Hasty and colleagues (1993) have shown that developmental induction of nAChR α and γ subunit RNAs is prevented in myogenin knock-out mice. However, this study also showed that δ subunit RNA is developmentally induced in myogenin-deficient mice. These data suggest that denervation paradigms may not completely recapitulate early developmental changes in MRF expression, as has previously been reported (Witzemann and Sakmann, 1991). Conversely, different nAChR subunit genes may be differentially regulated by individual MRFs.

Most interesting is our observation that Id expression gradually increases during the first 2 months after denervation (Fig. 6). This time course is consistent with the hypothesis that Id is responsible for blocking MRF activity that leads to a decline in nAChR gene expression. The effect of Id could be manifested by combining with different MRFs and therefore affecting genes differentially regulated by these MRFs. In addition, Id overexpression has recently been correlated with muscle atrophy in adult transgenic mice (Gundersen and Merlie, 1994). Denervation-induced expression of Id may be contributing to muscle atrophy as well as influencing other events related to the ability of muscle to recover function following long-term denervation. Clearly Id expression is becoming maximal in the same time frame that denervated muscle loses its ability to recover function upon reinnervation. One caveat to these interpretations is that we are assuming that MRF RNA expression reflects MRF protein levels. Although this is generally true, it will ultimately be necessary to demonstrate this correlation by assaying for MRF protein.

In summary, long-term muscle denervation results in a changing pattern of nAChR and MRF gene expression that no longer resembles the embryonic state of developing muscle fibers. It is well known that adult, innervated muscle is usually refractory to further innervation (Elsberg, 1917; Jansen et al., 1978). The changes in gene expression we observe, which in many ways come to resemble that of the innervated state, may contribute to the poor

recovery of function in long-term denervated muscle subject to reinnervation. It will be of interest to determine if interventions such as electrical stimulation of long-term denervated muscle will delay or prevent these detrimental changes in gene expression, allowing denervated muscle to retain its potential for functional recovery.

References

Axelsson, J., and S. Thesleff. 1959. A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol.* 147:178–193.

Benzezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell.* 61:49–59.

Berberich, C., I. Durr, M. Koenen, and V. Witzemann. 1993. Two adjacent E box elements and a M-CAT box are involved in the muscle-specific regulation of the rat acetylcholine receptor β-subunit gene. *Eur. J. Biochem.* 216: 395–404.

Bessereau, J. L., L. D. Stratford-Perricaudet, J. Piette, C. Le Poupon, and J. P. Changeux. 1994. In vivo and in vitro analysis of electrical activity-dependent expression of muscle acetylcholine receptor genes using adenovirus. *Proc. Natl. Acad. Sci. USA.* 91:1304–1308.

Billington, L. 1995. Reinnervation and regeneration of denervated rat skeletal muscles. Ph.D. Dissertation. University of Michigan.

Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:701–709.

Brehm, P., and R. W. Kullberg. 1987. Acetylcholine receptor channels on adult mouse skeletal muscle are functionally identical in synaptic and nonsynaptic membrane. *Proc. Natl. Acad. Sci. USA.* 84:2550–2554.

Brehm, P., and L. P. Henderson. 1988. Regulation of acetylcholine receptor channel function during development of skeletal muscle. *Dev. Biol.* 129: 1–11.

Brennan, C., and L. P. Henderson. 1993. Single channel properties of synaptic acetylcholine receptors in dystrophic muscle fibers. *Muscle & Nerve.* 16:513–519.

Brennan, C., P. B. Scotland, S. C. Froehner, and L. P. Henderson. 1992. Functional properties of acetylcholine receptors coexpressed with the 43K protein in heterologous cell systems. *Dev. Biol.* 149:100–111.

Brenner, H. R., A. Herczeg, and C. R. Slater. 1992. Synapse-specific expression of acetylcholine receptor genes and their products at original synaptic sites in rat soleus muscle fibres regenerating in the absence of innervation. *Development (Camb.).* 116:41–53.

Carlson, B. M., and J. A. Faulkner. 1988. Reinnervation of long-term denervated rat muscle freely grafted into an innervated limb. *Exp. Neurol.* 102:50–56.

Chahine, K. G., W. Walke, and D. Goldman. 1992. A 102 base pair sequence of the nicotinic acetylcholine receptor delta-subunit gene confers regulation by muscle electrical activity. *Development (Camb.).* 115:213–219.

Chahine, K. G., E. Baracchini, and D. Goldman. 1993. Coupling muscle electrical activity to gene expression via a cAMP-dependent second messenger system. *J. Biol. Chem.* 268:2893–2898.

Chang, C. C., and C. Y. Lee. 1963. Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their mode of neuromuscular blocking action. *Arch. Int. Pharmacodyn. Ther.* 144:241–257.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

Davis, R. L., H. Weintraub, and A. B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell.* 51:987–1000.

Duclert, A., J. Piette, and J. P. Changeux. 1991. Influence of innervation on myogenic factors and acetylcholine receptor α-subunit mRNAs. *NeuroReport.* 2:25–28.

Durr, I., M. Numberger, C. Berberich, and V. Witzemann. 1994. Characterization of the functional role of E-box elements for the transcriptional activity of rat acetylcholine receptor ε-subunit and γ-subunit gene promoters in primary muscle cell cultures. *Eur. J. Biochem.* 224:353–364.

Dutton, E. K., A. M. Simon, and S. J. Burden. 1993. Electrical activity-dependent regulation of the acetylcholine receptor δ-subunit gene, MyoD, and myogenin in primary myotubes. *Proc. Natl. Acad. Sci. USA.* 90:2040–2044.

Eftimie, R., H. R. Brenner, and A. Buonanno. 1991. Myogenin and MyoD join a family of skeletal muscle genes regulated by electrical activity. *Proc. Natl. Acad. Sci. USA.* 88:1349–1353.

Elsberg, C. A. 1917. Experiments on motor nerve regeneration and the direct neurotization of paralysed muscle by their own and by foreign nerve. *Science (Wash. DC).* 45:318–320.

Engel, A. G., E. H. Lambert, D. M. Mulder, C. F. Torres, K. Sahashi, T. E. Bertorini, and J. N. Whitaker. 1982. A newly recognised myasthenic syndrome attributed to a prolonged open time of the acetylcholine-induced ion channel. *Ann. Neurol.* 11:553–569.

Evans, S., D. Goldman, S. Heinemann, and J. Patrick. 1987. Muscle acetylcholine receptor biosynthesis. Regulation by transcript availability. *J. Biol. Chem.* 5:4911–4916.

Gilmour, B. P., G. R. Fanger, C. Newton, S. M. Evans, and P. D. Gardner. 1991. Multiple binding sites for myogenic regulatory factors are required for expression of the acetylcholine receptor γ-subunit gene. *J. Biol. Chem.* 266: 19871–19874.

Gilmour, B. P., D. Goldman, K. G. Chahine, and P. D. Gardner. 1995. Electrical activity suppresses nicotinic acetylcholine receptor γ subunit promoter activity. *Dev. Biol.* 168:416–428.

Ginetzinsky, A. G., and N. M. Shamarina. 1942. The tonomotor phenomenon in denervated muscle. Uspekhi Sovremennoi Biologii 15:283–294.

Goldman, D., and J. Staple. 1989. Spatial and temporal expression of acetylcholine receptor RNAs in innervated and denervated rat soleus muscle. *Neuron.* 3:219–228.

Goldman, D., H. R. Brenner, and S. Heinemann. 1988. Acetylcholine receptor α, β, γ, and δ-subunit mRNA levels are regulated by muscle activity. *Neuron.* 1:329–333.

Goldman, D., B. M. Carlson, and J. Staple. 1991. Induction of adult-type nicotinic acetylcholine receptor gene expression in noninnervated regenerating muscle. *Neuron.* 7:649–658.

Gundersen, K., and J. P. Merlie. 1994. Id-1 as a possible transcriptional mediator of muscle disuse atrophy. *Proc. Natl. Acad. Sci. USA.* 91:3647–3651.

Gundersen, K., J. R. Sanes, and J. P. Merlie. 1993. Neural regulation of muscle acetylcholine receptor ε- and α-subunit gene promoters in transgenic mice. *J. Cell Biol.* 123:1535–1544.

Hall, Z. W., and J. R. Sanes. 1993. Synaptic structure and development: the neuromuscular junction. *Cell.* 10(Suppl.):99–120.

Hamill, O., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391:85–100.

Hasty, P., A. Bradley, J. H. Morris, D. G. Edmondson, J. M. Venuti, E. N. Olson, and W. H. Klein. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature (Lond.).* 364:501–506.

Henderson, L. P., J. D. Lechleiter, and P. Brehm. 1987. Single channel properties of newly synthesized acetylcholine receptors following denervation of mammalian skeletal muscle. *J. Gen. Physiol.* 89:999–1014.

Huang, C. F., J. Tong, and J. Schmidt. 1992. Protein kinase C couples membrane excitation to acetylcholine receptor gene inactivation in chick skeletal muscle. *Neuron.* 9:13–20.

Jansen, J. K. S., W. Thompson, and D. P. Kuffler. 1978. The formation and maintenance of synaptic connections as illustrated by studies of the neuromuscular junction. *Prog. Brain Res.* 48:3–18.

Jaramillo, F., S. Vicini, and S. M. Schuetze. 1988. Embryonic acetylcholine receptors guarantee spontaneous contractions in rat developing muscle. *Nature (Lond.).* 335:66–68.

Jo, S. A., and S. J. Burden. 1992. Synaptic basal lamina contains a signal for synapse-specific transcription. *Development (Camb.).* 115:673–680.

Katz, B., and R. Miledi. 1972. The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.* 224:665–699.

Klarsfeld, A., R. Laufer, B. Fontaine, A. Devillers-Thiery, C. Dubreuil, and J. P. Changeux. 1989. Regulation of muscle AChR α subunit gene expression by electrical activity. Involvement of protein kinase C and Ca^{2+}. *Neuron.* 2: 1229–1236.

Klarsfeld, A., J. L. Bessereau, A. M. Salmon, A. Triller, C. Babinet, and J. P. Changeux. 1991. An acetylcholine receptor α-subunit promoter conferring preferential synaptic expression in muscle of transgenic mice. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:625–632.

Merlie, J. P., and J. M. Kornhauser. 1989. Neural regulation of gene expression by an acetylcholine receptor promoter in muscle of transgenic mice. *Neuron.* 2:1295–1300.

Merlie, J. P., J. Mudd, T. C. Cheng, and E. N. Olson. 1994. Myogenin and acetylcholine receptor α gene promoters mediate transcriptional regulation in response to motor innervation. *J. Biol. Chem.* 269:2461–2467.

Miner, J. H., and B. Wold. 1990. Herculin, a fourth member of the MyoD family of myogenic regulatory genes. *Proc. Natl. Acad. Sci. USA.* 87:1089–1093.

Neher, E., and B. Sakmann. 1976. Single-channel currents recorded from membrane of denervated frog muscles. *Nature (Lond.).* 276:799–802.

Neville, C. M., M. Schmidt, and J. Schmidt. 1992. Response of myogenic determination factors to cessation and resumption of electrical activity in skeletal muscle: a possible role for myogenin in denervation supersensitivity. *Cell Mol. Neurobiol.* 12:511–527.

Ohno, K., D. O. Hutchinson, M. Milone, M. J. Brengman, C. Bouzat, S. M. Sine, and A. G. Engel. 1995. Congenital myasthenic syndrome caused by prolonged acetylcholine receptor channel openings due to a mutation in the M2 domain of the epsilon subunit. *Proc. Natl. Acad. Sci. USA.* 92:758–762.

Piette, J., M. Huchet, A. Duclert, A. Fujisawa-Sehara, and J. P. Changeux. 1990. Localization of mRNAs coding CMD1, myogenin, and the alpha-subunit of the acetylcholine receptor during skeletal muscle development in the chicken. *Mech. Dev.* 37:95–106.

Prody, C. A., and J. P. Merlie. 1992. The 5' flanking region of the mouse muscle nicotinic acetylcholine receptor β subunit gene promotes expression in cultured muscle cells and is activated by MRF4, myogenin and myoD. *Nucleic Acids Res.* 20:2367–2372.

Saccomanno, C. F., M. Bordonaro, J. S. Chen, and J. L. Nordstrom. 1992. A faster ribonuclease protection assay. *BioTechniques.* 13:846–850.

Schuetze, S. M. 1980. The acetylcholine channel open time in chick muscle is not decreased following innervation. *J. Physiol.* 303:111–124.

Schuetze, S. M., and L. W. Role. 1987. Developmental regulation of nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* 10:403–457.

Simon, A. M., P. Hoppe, and S. J. Burden. 1992. Spatial restriction of AChR gene expression to subsynaptic nuclei. *Development (Camb.).* 114:545–553.

Su, C.-T, C.-F. Huang, and J. Schmidt. 1995. The depolarization response element in acetylcholine receptor genes is a dual-function E box. *FEBS Lett.* 366:131–136.

Sunderland, S. 1978. Nerve and nerve injuries. 2nd edition. Churchill-Livingston, Edinburgh.

Tang, J., S. A. Jo, and S. J. Burden. 1994. Separate pathways for synapse-specific and electrical activity-dependent gene expression in skeletal muscle. *Development (Camb.).* 120:1799–1804.

Voytik, S. L., M. Przyborski, S. F. Badylak, and S. F. Koneiczny. 1993. Differential expression of muscle regulatory factor genes in normal and denervated adult rat hindlimb muscles. *Dev. Dynam.* 198:214–224.

Walke, W., J. Staple, L. Adams, M. Gnegy, K. Chahine, and D. Goldman. 1994. Calcium-dependent regulation of rat and chick muscle nicotinic acetylcholine receptor gene expression. *J. Biol. Chem.* 269:19447–19456.

Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, B. Benezra, T. K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, et al. 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. *Science (Wash. DC).* 251:761–766.

Witzemann, V., and B. Sakmann. 1991. Differential regulation of MyoD and myogenin mRNA levels by nerve induced muscle activity. *FEBS Lett.* 282:259–264.

Witzemann, V., B. Barg, M. Criado, E. Stein, and B. Sakmann. 1989. Developmental regulation of five subunit specific mRNAs encoding acetylcholine receptor subtypes in rat muscle. *FEBS Lett.* 242:419–424.

Witzemann, V., H. R. Brenner, and B. Sakmann. 1991. Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. *J. Cell Biol.* 114:125–141.

Wright, W. E., D. A. Sassoon, and V. K. Lin. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD1. *Cell.* 56:607–617.