Cellular and Molecular Neurobiology, Vol. 12, No. 6, 1992

Response of Myogenic Determination Factors to Cessation and Resumption of Electrical Activity in Skeletal Muscle: A Possible Role for Myogenin in Denervation Supersensitivity

Craig M. Neville,[1] Marlies Schmidt,[1] and Jakob Schmidt[1,2]

Received February 1, 1992; accepted April 10, 1992

KEY WORDS: denervation; membrane activity; acetylcholine receptor; α subunit; gene regulation; myogenin.

SUMMARY

1. We have prepared probes specific for the chicken myogenic determination genes MyoD, myogenin, myf5, and herculin and have investigated the expression of these genes in response to denervation and acute electrical stimulation in neonate chick muscle, using ribonuclease protection.

2. Upon denervation, herculin mRNA remains essentially unchanged, myf5 transcript levels approximately double, and MyoD message is up-regulated by two- to fivefold. In contrast, the message coding for myogenin, barely detectable in innervated muscle, rises dramatically (~200-fold) on the second day after nerve section; in this respect it resembles acetylcholine receptor (AChR) α-, γ- and δ-subunit mRNAs. Cohybridization experiments reveal that the increase in myogenin mRNA slightly precedes the rise in AChR α-subunit message.

3. Electrical stimulation of denervated muscle leads to an immediate decline in myogenin and AChR α-subunit mRNAs, with half-lives of less than an hour and approximately 4 hr, respectively; message stability measurements suggest that this is effected through a rapid shutdown of transcription. Messages coding for

[1] Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, New York 11794.
[2] To whom correspondence should be addressed.

0272-4340/92/1200-0511$06.50/0

MyoD, myf5, and herculin decay much more slowly, as a result of slower turnover.

4. Previous experiments have indicated the involvement of a *de novo* induced (Tsay, H.-J., Neville, C. M., and Schmidt, J., *FEBS Lett.* **274**:69–72, 1990) autocatalytic (Neville, C. M., Schmidt, M., and Schmidt, J., *NeuroReport* **2**:655–657, 1991) transcription factor in the denervation-triggered up-regulation of AChR α-subunit expression; the denervation and electrical stimulation experiments reported here are compatible with the notion that myogenin is that factor.

INTRODUCTION

The analysis of promoter regions of nicotinic acetylcholine receptor (AChR) subunit genes has revealed the presence of consensus MyoD binding sites (CANNTG motifs, E boxes). In all receptor genes investigated so far, such elements have been shown to be crucial for full promoter activity (Y. Wang *et al.*, 1988; X.-M. Wang *et al.*, 1990, 1991; Piette *et al.*, 1990, 1991; Jia *et al.*, 1992).

Members of the MyoD family of basic helix–loop–helix (bHLH) proteins (MyoD1, myogenin, myf5, herculin/MRF4/myf6) are known to initiate the myogenic program in nonmuscle cells (Olson, 1990; Tapscott and Weintraub, 1991). Since they activate a number of skeletal muscle-specific enhancers through CANNTG elements [e.g., in the MCK gene (Buskin and Hauschka, 1989; Lassar *et al.*, 1989; Murre *et al.*, 1989; Brennan *et al.*, 1990) and in the MLC1/3 gene (Donoghue *et al.*, 1988)], they may also be responsible for the skeletal muscle-specific activation of AChR subunit genes. Direct binding and indirect activation studies have confirmed this expectation for the AChR α- (Piette *et al.*, 1990), γ-, and δ-subunit genes (X.-M. Wang *et al.*, 1991).

Regulatory elements in muscle promoters have been identified by transfection of cultured muscle cells. This approach provides information on the control of developmental and tissue specificity but is not necessarily relevant for the mechanisms underlying phenotypic plasticity of mature myotubes. Although there can be little doubt that neural influences affect gene expression, giving rise to a spectrum of fiber types, and that denervation leads to very conspicuous changes in the activity of certain genes, it has not been established that this involves the same set of *trans*- and *cis*-acting elements as those active during differentiation. The use of transgenic animals carrying α-subunit promoter/reporter gene constructs has revealed that about 850 bp of upstream flanking sequence is sufficient for denervation-induced gene activation (Merlie and Kornhauser, 1989). A considerably shorter promoter region (downstream of position -116), comprising the α-subunit gene enhancer whose principal elements are a tandem pair of MyoD binding sites (Y. Wang *et al.*, 1988), retains a "robust denervation response" according to a more recent report (Merlie *et al.*, 1991).

It is thus reasonable to propose that MyoD family proteins mediate the pronounced increase in the transcriptional activity of receptor genes observed after denervation. The denervation-triggered *de novo* synthesis of a transactivator

has been postulated on the basis of cycloheximide experiments (Tsay *et al.*, 1990). The kinetics of α-subunit mRNA appearance and disappearance, following denervation and electrical stimulation, respectively, is consistent with the participation of an autocatalytic transcription factor in receptor gene control (Neville *et al.*, 1991); these lines of evidence add support to the notion that myogenic factors, which are known to activate their own genes (Olson, 1990; Tapscott and Weintraub, 1991), may be involved (Neville *et al.*, 1991). Transcripts coding for such a factor should rise before receptor subunit messages begin to appear; similarly, a decline in the expression and/or activity of a regulatory factor after resumption of electrical activity of the plasma membrane should precede the reduction in receptor subunit expression. We have therefore compared the kinetics of the appearance and loss of messages coding for AChR subunits and for putative transacting factors in innervated, denervated, and denervated-stimulated muscle.

METHODS

Animal Experiments. White Leghorn cockerels (Hall's Brothers Hatchery, North Brookfield, MA), 3 to 4 days after hatching, were anesthesized with ketamine intraperitoneally (50–100 mg/kg), and section of the sciatic nerve was performed as described previously (Shieh *et al.*, 1988). Anesthesia was maintained at a surgical level by supplementation as needed throughout the procedure. The effects of actinomycin D and electrical stimulation were measured 6–7 days after denervation. For the stimulation experiments, the protocol of Lømo and Westgaard (1975) was adapted. The denervated leg musculature was stimulated for periods of up to 10 hr in 100-Hz trains, 2-sec duration, applied once every minute. Cycloheximide experiments were performed as described (Tsay *et al.*, 1990). Briefly, the drug, at doses of 0.2 μg/g body weight, was injected intraperitoneally at 4-hr intervals. At the desired time animals were killed by decapitation, and the leg musculature below the knee was removed and frozen in liquid nitrogen. Generally, muscles from two or three animals corresponding to the same time point were pooled and total RNA processed by the guanidinium isothiocyanate procedure (Chirgwin *et al.*, 1979). RNA was quantified spectrophotometrically and checked for integrity by visualization of rRNA in agarose/formaldehyde gels. All animal experimentation followed protocols approved by the Institutional Animal Care and Use Committee.

Measurement of mRNA. mRNA levels were measured by ribonuclease protection assays essentially as described (Melton *et al.*, 1984). The protected bands in the gels were quantified with an Ambis Beta Scanner.

Isolation of Chicken Myogenin cDNA. A total of 10^5 plaques from a unidirectional λZAP II cDNA library generated from mRNA derived from 1-day denervated chick leg muscle was screened with a random-primed murine myogenin cDNA probe following conditions recommended by Stratagene. Six positive plaques were chosen and purified to homogeneity. All six contained the complete coding sequence. One was sequenced in its entirety and conformed to

the sequence reported by Fujisawa-Sehara *et al.* (1990). The 3′ *Bam*HI–*Xho*I fragment of 520 bp was subcloned into pSK− for the generation of riboprobe. Multiple closely spaced bands observed in the nuclease protection assay probably result from breathing of the AT-rich hybrid during digestion.

Isolation of cDNA Partially Encoding the Chick myf5 and Herculin Messages. Two degenerate oligonucleotides, ccggatccTGCCTII(C/T)ITGGGCITGCAA-(A/G)IIITGCAA(A/G)(A/C)GIAA and ccggatccAT(A/G)TAI(C/T)(G/T)-IATIGC(A/G)I(A/T)IC(G/T)IAGIAT(C/T)TCIAC(C/T)TT, corresponding to regions shared by the four known myogenic factors were synthesized. One microgram of chick genomic DNA was amplified by the polymerase chain reaction under the following conditions: 5 cycles—95°/1 min, 37°/1 min, ramp/2 min, 72°/30 sec; 25 cycles—94°/1 min, 55°/1 min, 72°/1 sec. The flanking *Bam*HI sites generated by the primers in the resulting 210-bp product were cleaved and the fragments inserted into the M13 vector PhageScript SK (Stratagene). Individual plaques were grown up and sequenced, using Sequenase 2.0, following the manufacturer's recommendations (U.S. Biochemical), to ascertain their identities. Inserts corresponding to myf5 and herculin were PCR-amplified using the flanking universal Reverse and −20 primers and transcribed using the appropriate phage RNA polymerase to generate labeled antisense riboprobe.

Construction of Other Riboprobe Templates. The 399-bp *Hin*dIII–*Eco*RI genomic fragment of the chicken AChR α-subunit gene, containing exon 7 and flanking intron sequences (Shieh *et al.*, 1987), was cloned into the Bluescript plasmid pSK- in order to generate riboprobe. The 355-bp cDNA fragment encoding the amino-terminal portion of the chicken β-subunit gene (Moss *et al.*, 1987) was excised from pGEM4beta with *Bam*HI and cloned into pSK-. pB5, an ~500-bp *Pst*I/*Hin*dIII genomic fragment which comprises γ-subunit exon 1 and flanking sequences, and p2, a 494-bp *Pst*I/*Pst*I genomic fragment which contains portions of the 5′ untranslated sequence and exon 1 of the δ subunit, were cloned into pSK+. Finally, the 102-bp MyoD *Stu*I–*Pst*I fragment (Lin *et al.*, 1989) was subcloned into pKS+ for production of riboprobe.

RESULTS

AChR Subunit Transcript Levels Rise After a Latency Period. The denervation response of leg muscle AChR α-, γ- and δ-subunit mRNAs has been analyzed previously with chicks approximately 8 weeks old (Shieh *et al.*, 1988). The present investigation was carried out with neonate animals (denervation performed about 2–3 days after hatching). To permit a valid comparison with the time courses of myogenic factor transcripts, quantitation of these receptor messages was repeated. The β-subunit mRNA was included in the analysis. Results were largely comparable to those obtained in the earlier experiment; mRNAs specific for the α, γ, and δ subunits increased one to two orders of magnitude. In contrast, the β message, which we had not previously investigated, was upregulated by only three- to fourfold. If normalized to the maximal

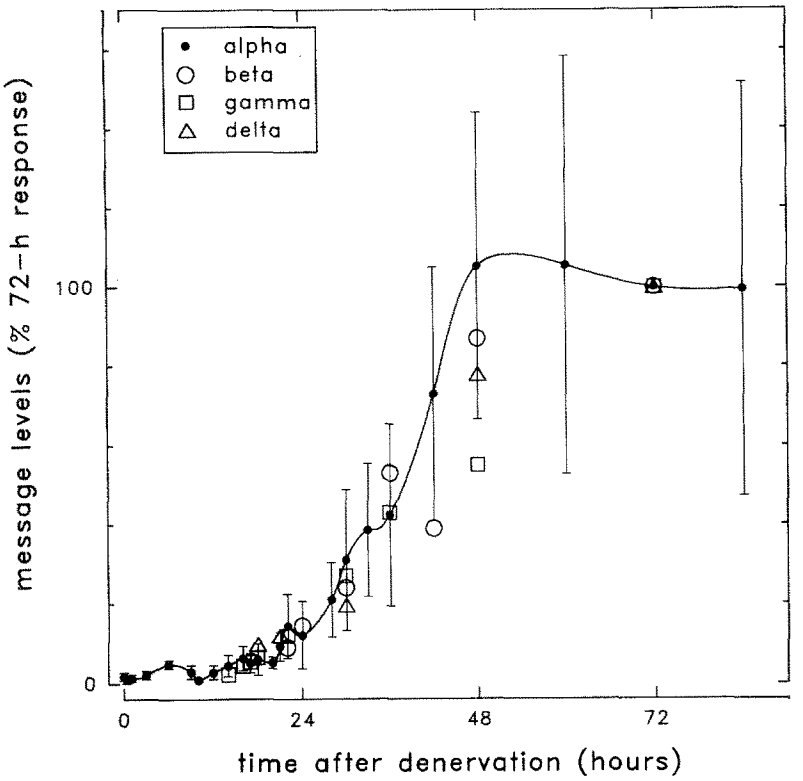

Fig. 1. Coordinate up-regulation of AChR subunit transcripts. AChR subunit messages were quantified by riboprobe analysis as described under Methods. The α-subunit data (filled circles) represent a compilation of eight experiments, with data normalized to the 72-hr value in each experiment. Mean and SD are presented for all other time points. Data from individual experiments are shown for β (open circles), γ (squares), and δ (triangles); these data are corrected for constitutive expression (about 5, 5, and 30% of maximal denervation response for the γ-, δ- and β-subunit mRNA, respectively) and scaled to the 72-hr value.

denervation response and corrected for constitutive expression, the β, γ, and δ messages follow a time course similar to that of α mRNA (Fig. 1). Data from the 12 to 36-hr interval can be fitted to a logarithmic growth curve with a doubling time of 5–6 hr.

Myogenic Factor Messages Are Differentially Affected by Denervation. The similarity of the denervation response of the receptor subunit genes is suggestive of a common transactivator. Figure 2 shows a consensus sequence, derived from important activating regions of receptor subunit promoters, which could underlie this shared regulatory behavior and which bears a striking resemblance to the preferred target sequence of both MyoD (Blackwell and Weintraub, 1990) and myogenin (Wright *et al.*, 1991). A study of the possible involvement of the MyoD family of transcription factors was therefore undertaken. Initially a species-specific probe was available only for MyoD; additional chicken-specific sequences were obtained by screening a skeletal muscle library (myogenin) and by PCR

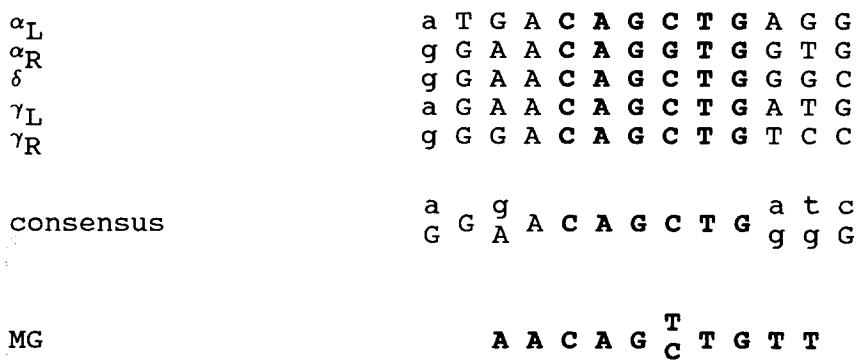

Fig. 2. Functionally significant E boxes in chick AChR genes. Functionally significant E boxes from three receptor subunit gene promoters contain a myogenin half site. α_L, upstream or left E box from α enhancer; α_R, downstream or right E box from α enhancer (Wang *et al.*, 1988); δ, E box from δ enhancer (Wang *et al.*, 1990); γ_L and γ_R, left and right E box from the γ-subunit promoter (Jia *et al.*, 1992); MG, preferred myogenin target sequence (Wright *et al.*, 1991).

amplification of genomic DNA using degenerate primers specific for myogenic determination factors (myf5 and herculin). Sequences corresponding to the basic helix–loop–helix region are shown in Fig. 3. In denervated muscle, MyoD mRNA begins to increase 1 day after nerve section. Transcript levels rise by a factor of 2 to 5, from a fairly substantial constitutive expression rate, beginning at about 15 hr after denervation. Myf5 and herculin message, which are expressed at low levels in neonate muscle, change only marginally in concentration during the 1-week postdenervation period examined in this investigation. After a 24-hr lag period, myf5 mRNA reaches levels approximately twice the control. Herculin message also rises slightly but never reaches a level more than one-third above that of innervated muscle. Myogenin transcripts also go up following denervation. Compared to MyoD, transcripts encoding myogenin are barely detectable in innervated muscle. After nerve section these low mRNA levels rise by two orders of magnitude, from approximately 5 amol/mg RNA to over 1 fmol/RNA (Fig. 4). Myogenin and receptor subunit transcripts rise in parallel. To compare better the time courses of myogenin and α messages, we resorted to hybridizing RNA samples with probes for both mRNAs simultaneously. Beginning at about 15 hr after denervation, when the rise in both messages first becomes detectable, the myogenin/α mRNA ratio transiently increases, indicating that myogenin expression precedes upregulation of α-subunit message (Fig. 5).

Electrical Stimulation Leads to a Rapid Decline in Myogenin Transcript Levels. To reverse the effects of denervation, the leg musculature was subjected to electrical stimulation 1 week after nerve section; chronically denervated muscle was used for these experiments because it takes several days for AChR subunit levels to stabilize. The denervated limb was stimulated for periods up to 10 hr, whereupon the muscle was analyzed for subunit and factor transcripts. Significant reduction in message levels, compared to unstimulated controls, was observed after 4 hr or more of imposed electrical activity for the α subunit, as reported

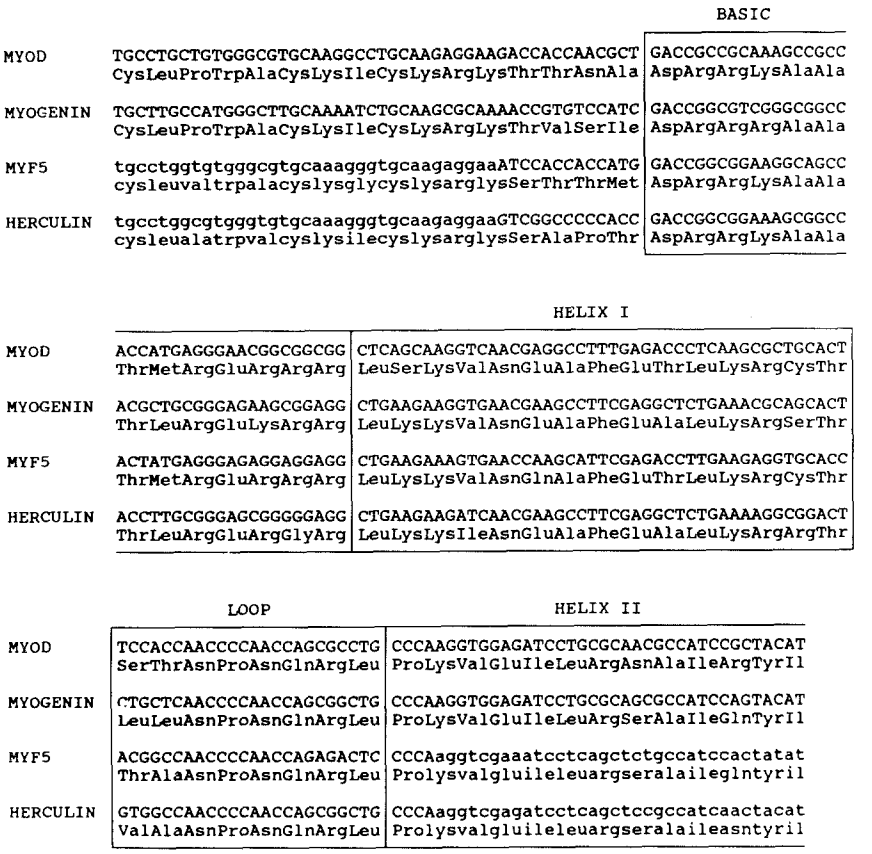

Fig. 3. Sequences of chick myogenic factor bHLH regions. Chicken genomic DNA was PCR amplified as described under Methods. The upstream oligonucleotide was designed to encode CLPWACKXCKRK, a stretch of 12 amino acids just upstream of the basic DNA binding region, of which 10 are conserved in all myogenic genes from *Drosophila* to *Homo*. The downstream primer encodes KVEILRMAIRY, a portion of helix II of the dimerization domain. Of the four genes amplified, two were found to be the previously described chick MyoD and myogenin genes (Lin *et al.*, 1989; Fujiawa-Sehara *et al.*, 1990). The other two are the chick homologues of myf5 and herculin (Braun *et al.*, 1989, 1990; Rhodes and Konieczny, 1989; Miner and Wold, 1990) based on comparison with the mammalian genes. The DNA sequences with their deduced amino acid sequences are shown. In the region flanked by the PCR primers, myf5 differs by one, and herculin by two amino acid residues from their human homologues. The sequence derived from PCR primers is shown in lowercase letters.

previously (Neville *et al.*, 1991), while messages coding for the other subunits were much less affected. When levels of factor transcripts were measured we observed a rapid disappearance of myogenin message and little effect on MyoD, myf5, and herculin mRNAs (Fig. 6).

Effect of Metabolic Inhibitors. Actinomycin D experiments were performed to test the half-life of the transcripts. It was found that in the presence of the drug, myogenin message decays with a half-time of less than 1 hr. Previously the half-life of α-subunit mRNA had been determined to be approximately 4 hr (Tsay

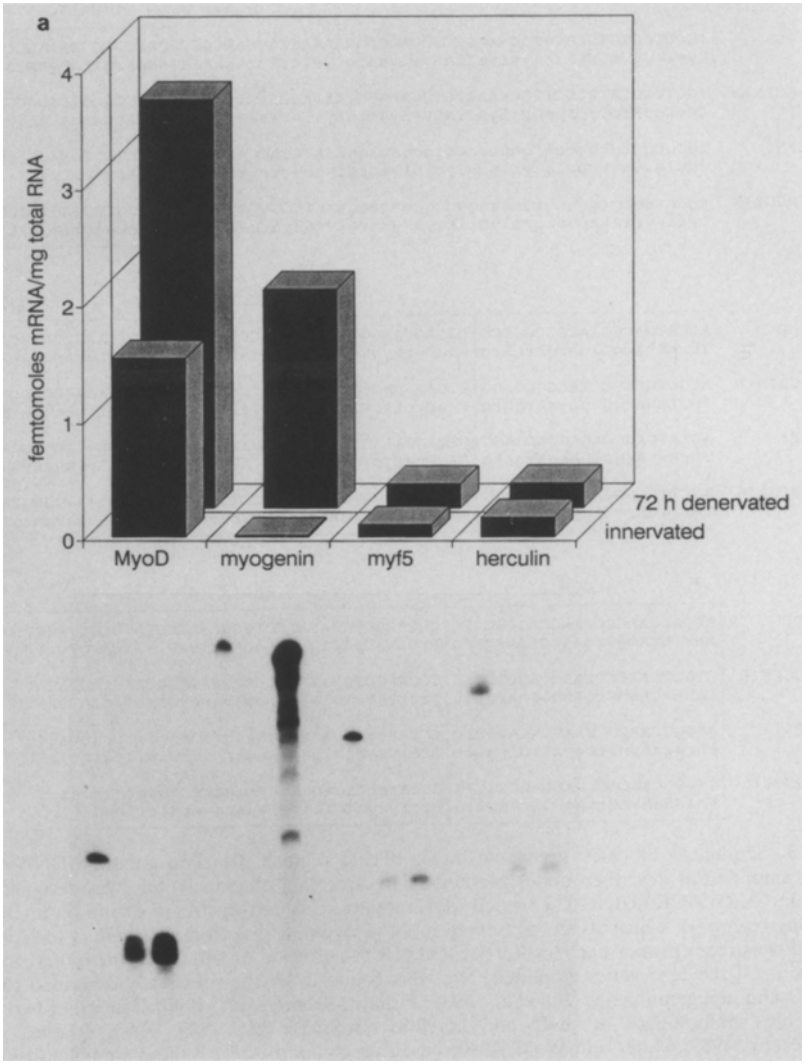

Fig. 4. Response of myogenic factor mRNAs to denervation. Samples of total RNA (30 μg) from innervated and denervated muscle were hybridized with factor (MyoD, myogenin, myf5, and herculin) riboprobe and subjected to nuclease protection. (A) Transcript quantification. Four lanes are shown for each factor (from left to right): undigested probe, probe digested in the presence of RNA from innervated muscle, 3-day denervated muscle, and yeast. Quantitative results, obtained by β scanner and computed from known specific activities of the probes used, are presented in bar graph form at the top. (B) Time course. RNA from each of the time points indicated was hybridized simultaneously with MyoD, myogenin, and myf5 riboprobe and subjected to nuclease protection. Herculin was examined separately, as the size of its protected band is the same as that of myf5. Data are presented as multiples of innervated (t_0) control. Original autoradiographic data underneath in chronological order.

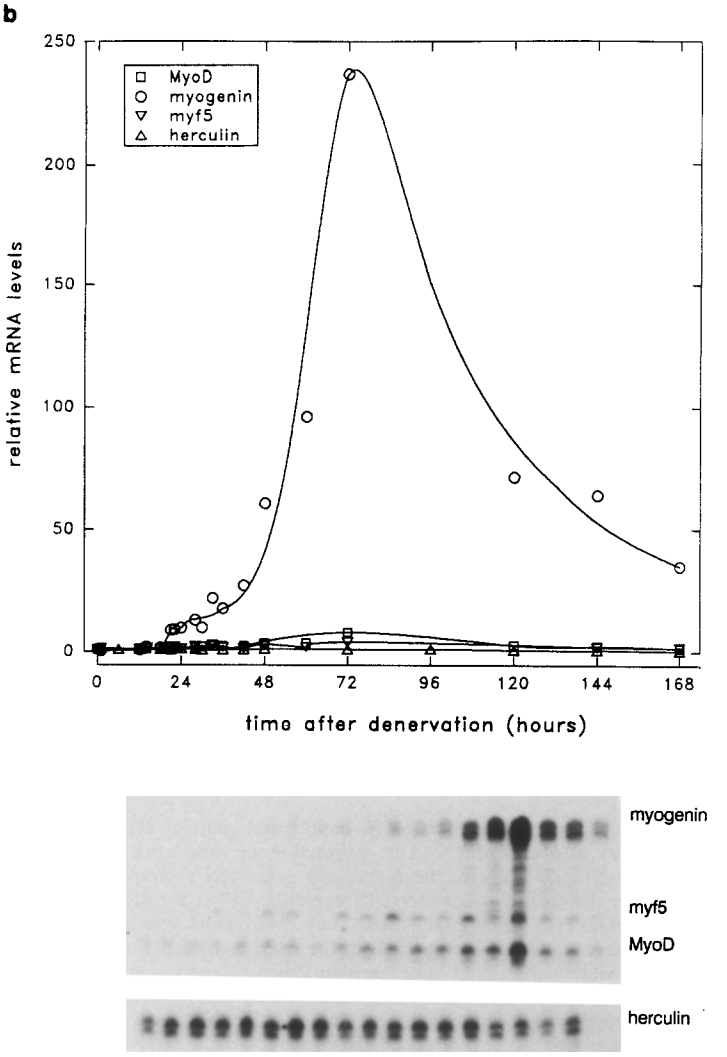

Fig. 4.—(*Contd.*)

et al., 1990). These findings suggest that electrical stimulation rapidly shuts off the activity of these genes. The resistance of other transcripts to stimulation appears to be accounted for by their stability as suggested by actinomycin D experiments (Fig. 7). Cycloheximide has previously been shown to prevent denervation changes in α-subunit message; here we report that it likewise prevents the upregulation of myogenin message. When administered either 2 hr before or 2 hr after nerve section, cycloheximide blocks the appearance of myogenin transcripts 20 hr later, while the drug only partially inhibits up-regulation when given 6 hr after surgery or later (data not shown). The results are very similar to those obtained when α transcripts are quantified in the same experimental preparations

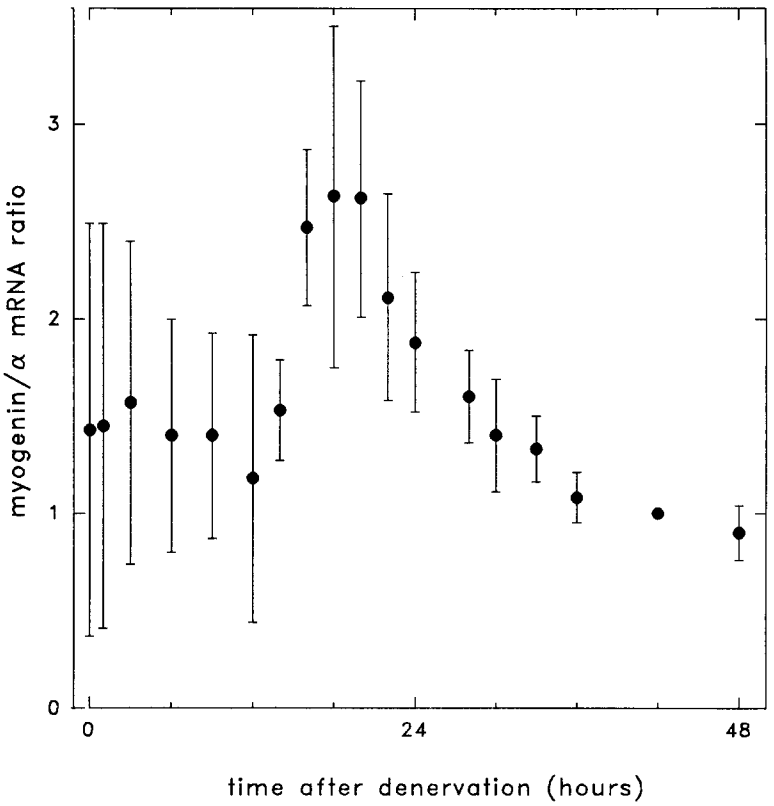

Fig. 5. Myogenin/α-subunit ratios in denervated muscle. Relative amounts of myogenin and α-subunit mRNAs as a function of time after denervation. The simultaneous quantification of messages in a given RNA sample is facilitated by differences in the size of the protected fragments: 780 nt for myogenin and 224 nt for the α subunit (the splicing intermediate of 294 nt which constitutes about 5% of the total α transcripts was disregarded in the ratio analysis). Four cohybridization experiments were carried out. For each individual experiment the myogenin/α-subunit ratios were scaled to the 42-hr value, which was arbitrarily set to equal 1. Relative values for each time point were averaged for the four experiments and are shown as mean and SD. Pronounced data scatter in the early time points results from low transcript levels.

and suggest that protein synthesis is required early after denervation in order to raise the levels of myogenin mRNA as well as receptor subunit messages.

DISCUSSION

Myogenic Factors, E Boxes, and Receptor Regulation. The majority of muscle-specific genes contains the CANNTG motifs recognized and activated by the factors of the MyoD family. The AChR subunit promoters analyzed so far also contain this element: Paired E boxes in the upstream regions of the chick α- and γ-subunit genes and a single CANNTG motif in the δ-subunit enhancer are

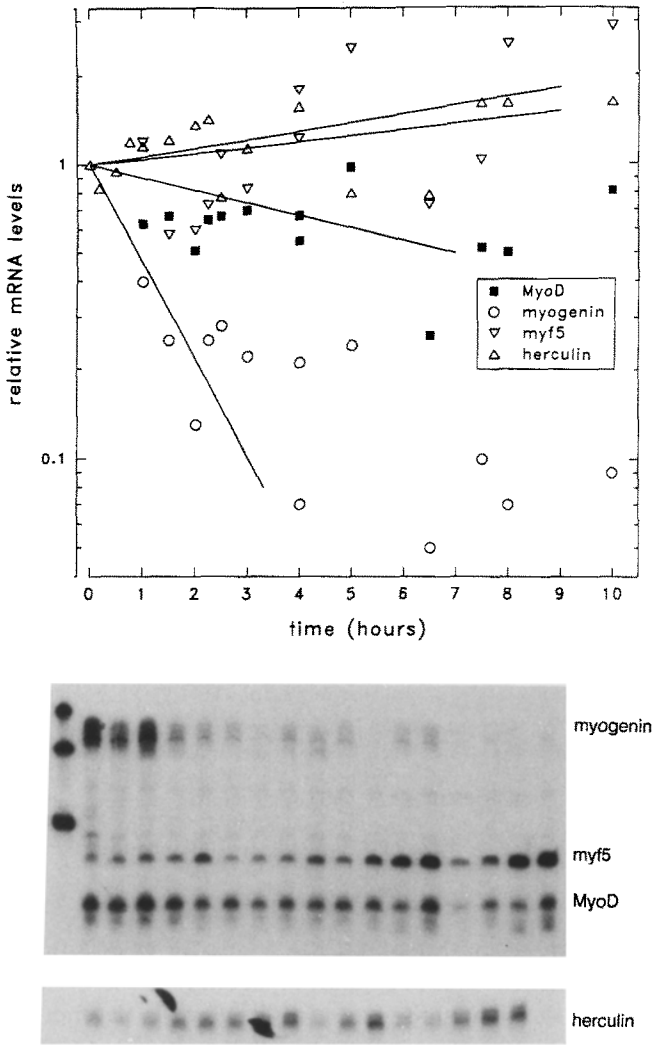

Fig. 6. Effect of electrical stimulation on the levels of myogenic factor mRNAs in chronically denervated muscle. Total RNA (30 μg) from 6-day denervated muscle that had been electrically stimulated for the indicated times was hybridized simultaneously with myogenin, myf5, and MyoD riboprobes and subjected to nuclease protection analysis. Herculin message was measured separately. Solid lines represent single-exponential fits for periods of 3 and 7 hr for myogenin and MyoD, respectively.

recognized by MyoD and myogenin, and this interaction leads, either directly or indirectly, to the activation of appropriate reporter gene constructs (Piette *et al.*, 1990; Jia *et al.*, 1992; Wang *et al.*, 1991). Although no information is currently available on the promoter of the chick β-subunit gene, it is plausible to postulate that all receptor genes are regulated by myogenic factors.

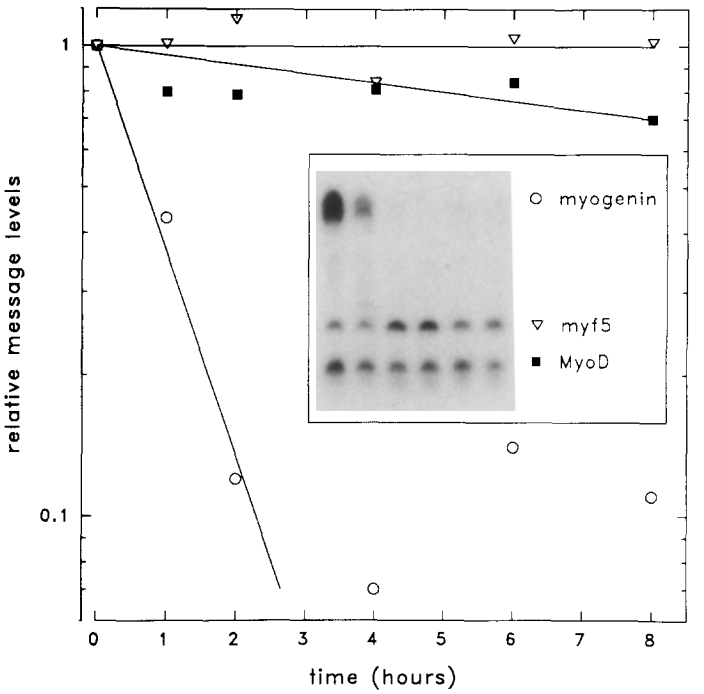

Fig. 7. Effect of actinomycin D on myogenic factor message levels. Six-day denervated chicks received intraperitoneal injections of the transcription inhibitor actinomycin D (2 μg/g body weight). Total RNA (30 μg) was obtained after the indicated survival periods, incubated simultaneously with myogenin, myf5, and MyoD riboprobes, and then subjected to nuclease digestion. The relative values obtained are shown in arbitrary units.

Direct support for a control of AChR genes by MyoD family proteins is lacking except for the observation that myogenin antisense oligonucleotides abolish expression of the γ-subunit gene in BC3H-1 cells (Brunetti and Goldfine, 1990). Circumstantial evidence is mounting, however: Recently, Duclert *et al.* (1991) studied steady-state levels of the mRNAs of myogenic factors and of the α subunit in mouse muscle, both during postnatal development and after denervation, and concluded that "myogenin and/or MyoD might mediate some of the effects of electrical activity on AChR gene expression." Eftimie *et al.* (1991) also described temporal and mechanistic correlations among levels of MyoD, myogenin, and α-subunit mRNA in mouse skeletal muscle: Changes in transcript levels of the myogenic factors precede changes in α-subunit message during development and following denervation, and the expression of all three genes in denervated muscle could be inhibited by chronic electrical stimulation. Expression of myogenic factor genes was investigated by Sakmann and his colleagues in rat muscle (Witzemann and Sakmann, 1991). Since α, γ, and δ genes are at least partly regulated by electrical activity, these authors suggest that "myogenin, being also tightly regulated by electrical muscle activity. . . , could be involved in

the regulation of extrasynaptic α, γ, and δ-subunit mRNA levels in adult muscle". At this point it may be worth pointing out that extrapolations from one species to another have to be made with caution. In the mouse, MyoD and myogenin are about equally sensitive to denervation, being up-regulated 10- to 40-fold and 30- to 40-fold, respectively (Duclert *et al.*, 1991; Eftimie *et al.*, 1991); in the rat, on the other hand, MyoD mRNA increases only 4-fold in denervated muscle, compared to a 20-fold increase in myogenin message (Witzemann and Sakmann, 1991). The dramatic up-regulation of myogenin in denervated chick leg muscle is unique, and the 50-fold weaker response of MyoD is a challenge to the expectation that these factors activate each others' expression.

Kinetic Considerations. Using run-on analysis we have previously shown that the activities of the α, γ, and δ genes change in unison after denervation. If message levels are scaled to the response on day 3 and corrected for the amounts expressed constitutively in innervated muscle, a similar time course is observed for all subunit transcripts, even including the β-subunit mRNA, which we have not previously investigated. This finding suggests that the same transactivator or a group of related factors may act on, and coordinate the expression of, individual receptor subunit genes. We have recently argued, from the asymmetry of the kinetics of α-subunit mRNA up- and down-regulation, that the α-subunit gene is regulated by an autocatalytic transactivator whose function is blocked by membrane activity (Neville *et al.*, 1991); this lends support to the possible involvement of myogenic determination factors which are known to activate their own promoters (Olson, 1990; Tapscott and Weintraub, 1991). A careful comparison of the time courses of α-subunit and myogenin messages permits an additional and independent test of the possible role of myogenin. The factor which is presumed to be limiting initially will bind to any suitable promoter and activate the corresponding gene. Only those myogenin molecules that activate the myogenin gene, however, would set the positive feedback loop in motion. Consequently, one would expect myogenin transcription to precede transiently transcription of other target genes. However, the delay may be so brief as to be imperceptible when time courses resulting from two separate experiments are compared. We therefore resorted to an experimental protocol in which the same RNA sample was hybridized with the two distinct riboprobes simultaneously. Analysis of the myogenin/α-subunit mRNA ratio at a high temporal resolution suggests that myogenin message rises earlier than α-subunit mRNA and that possibly the up-regulated myogenin gene carries the α-subunit gene along.

A striking aspect of AChR regulation is the suppression of extrasynaptic receptor by the electromechanical activity of the muscle fiber. In 1977 Hall and Reiness showed that electrical activity blocks receptor synthesis. Goldman *et al.* (1988) and Witzemann *et al.* (1991) have shown that receptor subunit mRNAs remain low despite denervation as long as the denervated muscle is chronically stimulated. More recently this has been confirmed and extended to the myogenic factors MyoD and myogenin (Eftimie *et al.*, 1991; Witzemann and Sakmann, 1991).

The present study shows that chronic stimulation is not necessary; on the contrary, plasma membrane activity very rapidly affects gene transcription. If

electrical activity of the plasma membrane mediates the influence of innervation on receptor gene expression and if, furthermore, the induction of a transactivating factor is required for the denervation response, one would expect the factor gene to be inactivated upon reinnervation (and resumption of electrical activity) as well as upon electrical stimulation of the denervated muscle. This is indeed observed for myogenin. The myogenin message has a half-life *in vivo* of less than an hour; upon electrical stimulation myogenin transcripts are depleted with a similar rate, suggesting that the myogenin gene is rapidly inactivated. In contrast, MyoD, myf5, and herculin are more stable and/or less sensitive to electrical stimulation.

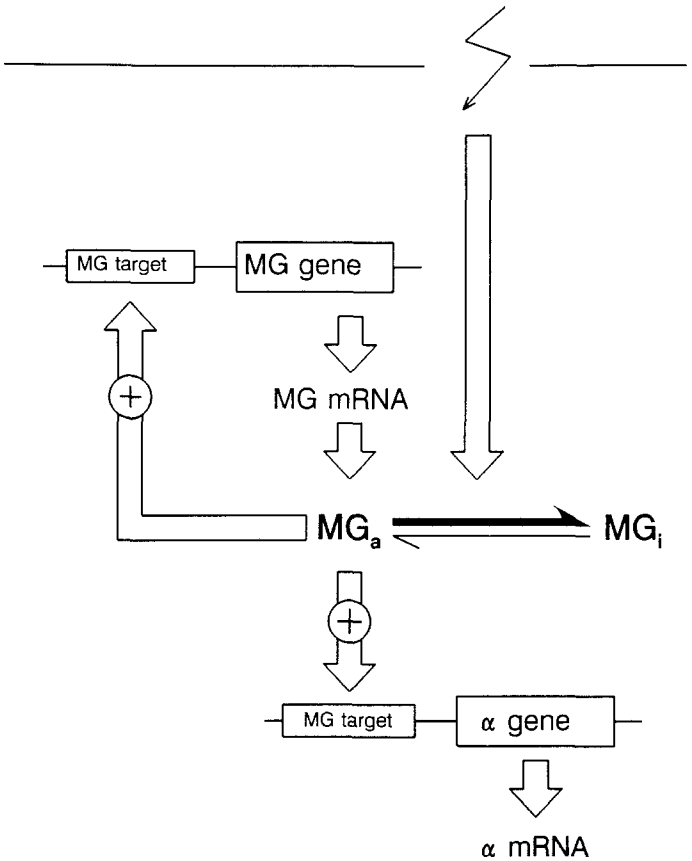

Fig. 8. Model of regulation of receptor gene transcription by plasma membrane activity. Schematic view of a mechanism that accounts for the levels of myogenin in receptor subunit mRNA in innervated and denervated chick muscle. Myogenin (MG) occurs at low levels in innervated muscle in an inactive form, MG_i. Upon cessation of membrane activity it is converted to the active form, MG_a, binds to the MG promoter, and begins to stimulate its own expression. When an adequate level of MG is attained, transcription of receptor subunit genes is initiated. Small and large rectangles denote regulatory and coding regions, respectively.

A Putative Signaling Pathway. A mechanism can be sketched that incorporates current knowledge of the functions of myogenin and of the regulatory events following nerve section: In innervated muscle, low levels of myogenin are kept in an inactive form through signals that originate in the depolarizing membrane. Perhaps protein kinase C, which (a) is rapidly mobilized in skeletal muscle by electrical stimulation (Richter *et al.*, 1987), (b) inactivates myogenin (E. Olson, personal communication), and (c) is known to inhibit AChR subunit gene expression in cultured myotubes (Klarsfeld *et al.*, 1989), acts as a mediator. Cessation of electrical activity of the sarcolemma then results in the appearance of active myogenin protein, possibly through a phosphatase-catalyzed shift to the dephosphorylated active form or through continued constitutive synthesis of the factor (which, in the absence of covalent modification, remains active). The active factor then stimulates transcription of its own gene as well as those coding for receptor subunits. This up-regulation is slow initially but then gains speed as the positive feedback loop leads to an exponential increase in factor and in factor-dependent gene activity. As soon as membrane activity is resumed, myogenin is rapidly phosphorylated and inactivated, and myogenin-dependent transcription of the AChR genes as well as the myogenin gene itself ceases (Fig. 8). It has been proposed that the autocatalytic properties of the MyoD family of transcription factors help to stabilize the differentiated phenotype (Thayer *et al.*, 1989; Olson, 1990). The present study suggests that myogenin may also contribute to, or account for, phenotype switching in the mature myofiber.

ACKNOWLEDGMENTS

We thank Eric Barnard for chick AChR β-subunit cDNA, Bruce Paterson for chick MyoD cDNA, and Eric Olson for murine myogenin cDNA. AChR α-, γ-, and δ-subunit probes originated in the laboratory of Marc Ballivet.

This research was supported by NIH Grant NS-20233 and NSF Grant BNS-8819383.

NOTE ADDED IN PROOF

We have recently established that PKC mediates the depolarization-triggered inhibition of acetylcholine receptor subunit genes (Huang, C. F., Tong, J., and Schmidt, J. *Neuron* **9**, in press).

REFERENCES

Blackwell, T. K., and Weintraub, H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. *Science* **250:**1104–1110.

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

Braun, T., Bober, E., Winter, B., Rosenthal, N., and Arnold, H. (1990). Myf-6, a new member of the human gene family of myogenic determination factors: Evidence for a gene cluster on chromosome 12. *EMBO J.* **9**:821–831.

Brennan, T. J., Edmondson, D. G., and Olson, E. N. (1990). Aberrant regulation of MyoD1 contributes to the partially defective phenotype of BC3H-1 cells. *J. Cell Biol.* **110**:929–937.

Brunetti, A., and Goldfine, I. D. (1990). Role of myogenin in myoblast differentiation and its regulation by fibroblast growth factor. *J. Biol. Chem.* **265**:5960–5963.

Buskin, J. N., and Hauschka, S. D. (1989). Identification of a myocyte nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. *Mol. Cell. Biol.* **9**:2627–2640.

Chirgwin, J. J., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5300.

Donoghue, M., Ernst, H., Wentworth, B., Nadal-Ginard, B., and Rosenthal, N. (1988). A muscle-specific enhancer is located at the 3′ end of the myosin light-chain 1/3 gene locus. *Genes Dev.* **2**:1779–1790.

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

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

Fujisawa-Sehara, A., Nabeshima, Y., Hosoda, Y., and Obinata, T. (1990). Myogenin contains two domains conserved among myogenic factors. *J. Biol. Chem.* **265**:15219–15223.

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

Hall, Z. E., and Reinness, C. G. (1977). Electrical stimulation of denervated muscles reduces incorporation of methionine into acetylcholine receptor. *Nature* **268**:655–657.

Jia, H.-T., Tsay, H.-J., and Schmidt, J. (1992). Analysis of binding and activating functions of the chick muscle acetylcholine receptor γ subunit upstream sequence. *Cell. Mol. Neurobiol.* **12**:241–258.

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

Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D., and Weintraub, H. (1989). MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell* **58**:823–831.

Lin, Z. Y., Dechesne, C. A., Eldridge, J., and Paterson, B. M. (1989). An avian muscle factor related to MyoD1 activates muscle-specific promoters in nonmuscle cells of different germ-layer origin and in BrdU-treated myoblasts. *Genes Dev.* **3**:986–996.

Lømo, T., and Westgaard, R. H. (1975). Further studies on the control of ACh sensitivity by muscle activity in the rat. *J. Physiol. (London)* **252**:603–626.

Melton, D. A., Krieg, P. A., Rebagliati, M., Maniatis, T., Zinn, K., and Green, M. R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* **12**:7035–7056.

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

Merlie, J. P., McKinnon, D., and Sanes, J. R. (1991). Transgenic analysis of acetylcholine receptor gene expression. *J. Cell. Biochem.* **15C**:12.

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

Moss, S. J., Beeson, D. M. W., Jackson, J. F., Darlison, M. G., and Barnard, E. A. (1987). Differential expression of nicotinic acetylcholine receptor genes in innervated and denervated chicken muscle. *EMBO J.* **6**:3917–3921.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., and Lassar, A. B. (1989). Interactions between heterologous helix–loop–helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**:537–544.

Neville, C. M., Schmidt, M., and Schmidt, J. (1991). Kinetics of expression of acetylcholine receptor α-subunit mRNA in denervated and stimulated muscle. *NeuroReport* **2**:655–657.

Olson, E. N. (1990). MyoD family: A paradigm for development? *Genes Dev.* **4**:1454–1461.

Piette, J., Bessereau, J.-L., Huchet, M., and Changeux, J.-P. (1990). Two adjacent MyoD1-binding sites regulate expression of the acetylcholine receptor α-subunit gene. *Nature* **345**:353–355.

Richter, E. A., Cleland, P. J. F., Rattigan, S., and Clark, M. G. (1987). Contraction-associated translocation of protein kinase C in rat skeletal muscle. *FEBS Lett.* **217**:232–236.

Rhodes, S. J., and Konieczny, S. F. (1989). Identification of MRF4: A new member of the muscle regulatory factor gene family. *Genes Dev.* **3:**2050–2061.

Sartorelli, V., Webster, K. A., and Kedes, L. (1990). Muscle-specific expression of the cardiac α-actin gene requires MyoD1, CArG-box binding factor, and Sp1. *Genes Dev.* **4:**1811–1822.

Shieh, B.-H., Ballivet, M., and Schmidt, J. (1987). Quantitation of an α-subunit splicing intermediate. Evidence for transcriptional activation in the control of receptor expression in denervated chick skeletal muscle. *J. Cell Biol.* **104:**1337–1341.

Shieh, B.-H., Ballivet, M., and Schmidt, J. (1988). Acetylcholine receptor synthesis rate and levels of receptor subunit mRNAs in chick muscle. *Neuroscience* **24:**175–187.

Tapscott, S. J., and Weintraub, H. (1991). MyoD and regulation of myogenesis by helix–loop–helix proteins. *J. Clin. Invest.* **87:**1133–1138.

Thayer, M. J., Tapscott, S. J., Davis, R. L., Wright, W. E., Lassar, A. B., and Weintraub, H. (1989). Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58:**241–248.

Tsay, H.-J., Neville, C. M., and Schmidt, J. (1990). Protein synthesis is required for the denervation-triggered activation of acetylcholine receptor genes. *FEBS Lett.* **274:**69–72.

Wang, X.-M., Tsay, H.-J., and Schmidt, J. (1990). Expression of the acetylcholine receptor δ subunit gene in differentiating chick muscle cells is activated by an element that contains two 16-bp copies of a segment of the α-subunit enhancer. *EMBO J.* **9:**738–790.

Wang, X.-M., Lee, Y.-S., and Schmidt, J. (1991). Analysis of E boxes in the chick muscle acetylcholine receptor δ subunit gene. *Soc. Neurosci. Symp.* **17:**243.

Wang, Y., Xu, H.-P., Wang, X.-M., and Schmidt, J. (1988). A cell type-specific enhancer drives expression of the chick muscle acetylcholine receptor α-subunit gene. *Neuron* **1:**527–534.

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

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

Wright, W. E., Binder, M., and Funk, W. (1991). Cyclic amplification and selection of targets (CASTing) for myogenin consensus binding site. *Mol. Cell. Biol.* **11:**4104–4110.