CONTROL OF INNERVATION-DEPENDENT EXPRESSION OF THE ACETYLCHOLINE RECEPTOR DELTA SUBUNIT GENE

by

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B.S., Biochemistry
University of Minnesota, 1986

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the
Degree of

Doctor of Philosophy

at the

Massachusetts Institute of Technology

February 1993

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January 29, 1993

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ABSTRACT

Acetylcholine receptors (AChRs) are highly concentrated at vertebrate neuromuscular synapses, and this accumulation of AChRs is induced by contact with motor neurons. One pathway to concentrate AChRs at synaptic sites involves the redistribution of pre-existing AChR protein. This pathway is triggered by agrin, a molecule produced by motor neurons and secreted into the synaptic basal lamina. AChR mRNA is also highly concentrated at synaptic sites, indicating that local synthesis also contributes to the localization of AChR protein. I show that this additional pathway for accumulating AChR protein involves local transcription of AChR genes by myofiber nuclei that are near the synapse. The signal for this transcriptional pathway is located in the synaptic basal lamina. In addition to inducing AChR gene expression in synaptic nuclei, innervation leads to the inactivation of AChR expression in nuclei throughout the muscle fiber. Transcription of AChR genes in non-synaptic nuclei is repressed by propagated electrical activity in the muscle fiber membrane. I show that 181 bp of 5' flanking DNA from the AChR delta subunit gene are sufficient to confer electrical activity-dependent gene expression. An understanding of how AChR genes are regulated by innervation is likely to require knowledge of the steps required to activate AChR genes initially during myogenesis. I show that 148 bp of 5' flanking DNA from the AChR delta subunit gene contains two regulatory elements that control muscle-specific gene expression. One *cis*-acting element is a binding site for basic helix-loop-helix proteins. This site has a dual role, since it is important for activation of the delta subunit gene in myotubes and for repression in myoblasts and non-muscle cells. The other *cis*-acting element is an enhancer that is required for expression of the delta subunit gene in muscle but does not confer muscle-specificity. Both of these *cis*-acting elements are contained within the region that confers synapse-specific and electrical activity-dependent gene expression, and these results are consistent with the idea that the same *cis*-acting elements may also be important for regulation by innervation.

Thesis Supervisor: Dr. Steven J. Burden
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to my Mother and Father,

Ada B. Simon and Sidney Simon

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Steven Burden, for his enthusiasm, encouragement, and expert advice during my years at MIT. I am endebted to Steve for teaching me to be focused in my thinking and ambitious in my goals. I have enjoyed my years in the Burden Lab.

Embryo injections to produce the transgenic mice described in Chapter 2 were done by my collaborator, Dr. Peter Hoppe (Jackson Laboratories). I identified the founder mice, established transgenic lines, and studied expression of the transgene. Our collaboration was a productive and pleasurable experience.

Chapter 3 is primarily the work of my collaborator, Dr. Emma Dutton. My contribution consisted of constructing some of the plasmids, supplying transgenic mice, and being involved intellectually in the design of the experiments. Special thanks go to Emma for encouraging me to include this work in my thesis dissertation.

With regard to experimental reagents, I am endebted to Andrew Lassar, Cornelius Murre, Woodring Wright, Nadia Rosenthal, Tau-Mu Yi, Mickey Hu, Norm Davidson, Tom Genetta, and Tom Kadesch.

I would like to thank the members of my thesis committee: Brent Cochran, Gerald Fischbach, Leonard Guarente, and Rudolph Jaenisch. Their interest and advice is much appreciated.

To past and present members of the Burden lab: Thanks for many interesting discussions and a lot of good times.

To friends and family: Your support has been a source of strength.

Finally, extra-special thanks go to Helen Lin for invaluable moral support and encouragement.

ABBREVIATIONS USED

AChR	acetylcholine receptor
ARIA	acetylcholine receptor inducing activity
bHLH	basic helix-loop-helix
bp/Kbp	base pairs/kilobasepairs
C	celcius
CAT	chlorampenicol acetyltransferase
cDNA	complementary DNA
CGRP	calcitonin gene-related peptide
CK	creatine kinase
cpm	counts per minute
c-terminal	carboxy terminal
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E1	E-box 1
E2	E-box 2
E3	E-box 3
EDTA	(ethylenedinitrilo)tetraacetic acid
FB	fibroblast
FBP	c-fos basal promoter
GST	glutathione S-transferase
HEB	Hela E-box binding protein
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanolsulfonic acid
hGH	human growth hormone
hr	hours
Ig	immunoglobulin
KDa	kilodalton
M/mM/μM	Molar/millimolar/micromolar
MB	myoblast
min	minutes
ml/μl	milliliter/microliter
MLC	myosin light chain
μm	microns
mRNA	messenger RNA
MT	myotube
N-CAM	neural cell adhesion molecule
ng	nanograms
PBS	phosphate buffered saline
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
TBE	Tris borate EDTA
TMR-αBGT	tetramethylrhodamine-coupled α-bungarotoxin
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TTX	tetrodotoxin
V	volts
ZEB	zinc-finger E-box binding protein

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 1

General Introduction

Introduction

The formation of neuromuscular synapses involves a series of inductive interactions between motor neurons and the myofibers they innervate. Specialized structures and molecules develop in both the nerve terminal and the synaptic region of the myofiber, though less is known about the differentiation of the nerve terminal. Motor neuron axons stop growing when they contact their target myofibers and develop specialized active zones for release of the neurotransmitter acetylcholine. The motor neuron, in turn, triggers morphological and biochemical specializations in the synaptic region of the myofiber. The postsynaptic membrane becomes highly folded, and many different molecules become concentrated at the synaptic site, including integral membrane proteins: acetylcholine receptors (AChRs), voltage-gated Na^+ channels, neural cell adhesion molecule (N-CAM); membrane-associated proteins: 43K protein, dystrophin, dystrophin-related protein (DRP), ankyrin; and extracellular matrix proteins: acetylcholinesterase, S-laminin, A-laminin, type IV collagen (α3 and α4 chains), and heparin sulfate proteoglycan. The AChR is the best studied of these, and the regulation of AChR expression has become a model for understanding the signalling mechanisms involved in postsynaptic differentiation.

The acetylcholine receptor

The AChR is the most thoroughly characterized ligand-gated ion channel. Binding of the neurotransmitter, acetylcholine, to AChRs is transduced rapidly into an increase in permeability of the postsynaptic membrane to cations. The influx of cations (primarily sodium ions) through the AChR channel is driven by an electrochemical gradient and results in the depolarization of the postsynaptic membrane. This depolarization is propagated as an action potential along the myofiber membrane, causing muscle contraction.

The AChR was the first ion channel whose subunit composition and amino acid sequence was determined. A key factor in the purification of AChRs was the availability of a rich source for the receptor. The electric organ of the electric ray, Torpedo, is a rich source of AChRs because the innervated faces of the electric organ plasma membranes are densely packed with synapses. The purification of AChRs from membrane fractions of the electric organ was also aided by affinity purification columns containing α-neurotoxins, such as α-bungarotoxin, which bind tightly and specifically to AChRs. The purified Torpedo receptor is a pentamer (α_2, β, γ, δ) consisting of four different glycosylated subunits with apparent molecular masses ranging from 40 to 65 kDa.

The purification of the Torpedo AChR made it possible to isolate cDNAs for the receptor subunits and to determine their complete amino acid sequences (Noda et al 1983). Partial amino acid sequences from the amino-termini of the purified AChR subunits (Raftery et al., 1980) were used to design DNA oligomers to probe a Torpedo electric organ cDNA library. Amino acid sequences deduced from the isolated cDNAs showed that the subunits of the AChR are homologous and are probably derived from a common ancestral gene. Confirmation that these cDNAs actually encode the AChR was obtained by injecting mRNAs made from cDNA templates into Xenopus oocytes, which resulted in the appearance of receptors that could be activated by acetylcholine and which bound α-bungarotoxin (Mishina et al., 1984). Mammalian homologues, which are approximately 80% identical in amino acid sequence to Torpedo subunits, were isolated from muscle cDNA libraries by low stringency hybridization using Torpedo subunit probes. In mammals, an additional subunit was discovered (ε), which replaces the γ-subunit postnatally (Takai et al., 1985; Mishina et al., 1986).

AChRs are also expressed by neurons in autonomic ganglia and by neurons in the central nervous system. Neuronal AChRs are encoded by different genes than muscle AChRs and have distinct pharmacological properties. Neuronal AChRs consist of only two classes of subunits, α and β, which are distinct from, but closely related to muscle

AChR subunits (Whiting et al., 1986; Boulter et al., 1986; Boulter et al., 1987; Deneris et al., 1988; Nef et al., 1988; Schoepfer et al., 1988). Seven different α−subunits (α2-α8) and three different β-subunits (β2-β4) have been identified. The stoichiometry of the receptor is $\alpha_2\beta_3$ (Anand et al., 1991; Cooper et al., 1991), suggesting that neuronal AChRs, like muscle AChRs, are pentamers. The α7 and α8 subunits differ from the other subunits, since they form homomeric receptors and bind α-bungarotoxin (Couturier et al., 1990; Schoepfer et al., 1990). Numerous in situ hybridization analyses have shown that each of the neuronal AChR subunits is expressed in discrete regions of the central nervous system. Thus, the individual subtypes in this family of receptors may not be functionally identical and may have distinct roles in particular regions of the central nervous system.

Development of neuromuscular synapses

The formation of neuromuscular synapses begins a week before birth in rodents, but it takes several weeks before all of the morphological and molecular characteristics of adult synapses are evident. This process occurs in several stages: myogenesis and motor neuron outgrowth, synaptogenesis, synapse maturation, and elimination of polyneuronal innervation.

Myogenesis

Skeletal myofibers are multinucleated cells that form by the fusion of precursor myoblasts, which originate in the somites. The axial muscles are derived from myoblasts which differentiate within a region of the somite called the myotome, while the muscles of the limbs develop from myoblasts that migrate away from the somite to the lateral regions of the embryo. Myoblasts that form the axial and limb muscles appear to represent distinct myogenic lineages, as shown by fate mapping of embryonic quail somite cells transplanted into embryonic chick somites (Ordahl and Le Douarin, 1992). These studies showed that axial muscles are derived from cells in the medial half of

newly formed somites, whereas muscles of the limbs are derived from cells in the lateral half. Limb muscle myogenesis occurs in successive migrations of distinct myoblast populations, which express different fast- and slow-type myosin heavy chain isoforms, followed by myofiber formation and growth (Miller, 1992). The initial migration, which occurs at embryonic day (E)13-E14 in rodents, consists of embryonic myoblasts that fuse rapidly to produce primary myofibers. Secondary fibers, which make up the majority of adult fibers, form on the surface of primary fibers and are derived from fetal myoblasts. Fusion of fetal myoblasts is not synchronous; secondary fibers grow by fusion of fetal myoblasts into the growing myofibers.

Motor neuron outgrowth

Motor neurons are generated in the ventricular epithelium of the embryonic spinal cord. As soon as they arise, however, motor neurons migrate away from the ventricular epithelium, become part of a motor column (Hollyday and Hamburger, 1977), and extend axons. For the most part, axons make appropriate connections from the outset by navigating along a stereotyped route (Landmesser, 1978). In chick embryos, when a section of neural tube spanning 3-4 segments is reversed in the anterior-posterior axis, motor neurons succeed in innervating muscles appropriate to their original location (Lance-Jones and Landmesser, 1980). Thus, guidance cues, rather than passive channeling, seem to be important in motor neuron axon pathfinding.

Synaptogenesis

In the developing rat embryo, motor neuron axons first enter muscle masses at E13 when myoblasts are fusing to form multinucleated myotubes. AChR expression is induced during myotube formation, and by E14-E15 AChRs are present diffusely over the surface of newly forming myotubes at a density of several hundred/μm^2 (Bevan and Steinbach, 1977; Steinbach, 1981). The first contacts between motor neurons and myofibers are made, and synaptic potentials can already be measured in the myofiber membrane

(Diamond and Miledi, 1962; Dennis et al., 1981; Chow and Poo, 1985). Soon after nerve-muscle contact, patches of extracellular matrix form in the synaptic cleft. Eventually, a continuous layer of extracellular matrix, called the basal lamina, ensheaths the myofiber (Kelly and Zacks, 1969).

Soon after nerve-muscle contact, two proteins that are essential for neuromuscular transmission accumulate at the primitive synapse: AChRs and acetylcholinesterase, the enzyme that hydrolyzes acetylcholine. By E16, AChRs are concentrated in an aggregate beneath the nerve terminal at a density of ~2500/μm^2 (Mathews-Bellinger and Salpeter, 1983), and AChRs are still present diffusely throughout the myofiber. Patches of acetylcholinesterase are present in the basal lamina in the synaptic cleft (Hall and Kelly, 1971; Betz and Sakmann, 1973; McMahan et al., 1978; Rubin et al., 1979; Sanes and Chiu, 1983). Between E16 and birth, myofibers become multiply innervated by axons that converge at the single AChR aggregate (Redfern, 1970). The density of AChRs in the synaptic plaque increases to ~10,000/μm^2 (Mathews-Bellinger and Salpeter, 1983). The basal lamina becomes a continuous sheath around the myofiber (Mathews-Bellinger and Salpeter, 1983), and the distribution of acetylcholinesterase more closely follows the distribution of AChR aggregates in the synaptic region (Steinbach, 1981).

Synapse maturation

Synaptic structure and function continue to change postnatally. The half-life of AChRs has been measured by labelling receptors with [^{125}I] α-bungarotoxin, and these experiments indicate that rat embryonic and fetal AChRs have a half-life of ~1 day (Berg and Hall, 1975; Steinbach et al, 1979; Reiness and Weinberg, 1981; Steinbach, 1981). Between E18 and birth, the half-life of AChRs in the synaptic region increases to ~10 days, while the half-life of non-synaptic AChRs remains at ~1 day (Michler and Sakmann, 1980; Reiness and Weinberg, 1981; Steinbach, 1981). Synaptic AChR clusters also become progressively more resistant to disperal by denervation (Slater, 1982), low

Ca^{2+}, or elevated KCl (Bloch and Steinbach, 1981; Braithwaite and Harris, 1979). During the first two postnatal weeks, non-synaptic AChRs decline in number, and synthesis of non-synaptic AChRs is suppressed by myofiber electrical activity (see below). AChRs persist in the synaptic region, and are ~1000-fold more concentrated in the synaptic region of the myofiber than in the non-synaptic region. Synaptic folds develop, and AChRs are concentrated at the tops of these folds, while Na^+ channels and ankyrin are concentrated at the bottoms and sides. In addition, synaptic AChR channel properties are altered by a subunit switch (ε for γ) that produces the adult form of the receptor (Mishima et al., 1986; Gu and Hall, 1988), which has a shorter mean channel open time and a higher unit conductance than the fetal AChR (Fischbach and Schuetze, 1980).

Elimination of polyneuronal innervation

All but one of the nerve terminals innervating a myofiber are eliminated during the first few postnatal weeks. Synapse elimination occurs by retraction of nerve terminals, rather than by motor neuron cell death (Brown et al., 1976; Betz et al., 1979). The process of retraction has been studied in reinnervated adult mouse muscle (Rich and Lichtmann, 1989). Following nerve damage, adult muscle becomes multiply innervated by axons that converge at the original synaptic region. As in developing muscle, multiple innervation is unstable, and all but one of the nerve terminals are eliminated. By repeatedly viewing the same nerve terminal in living animals during the period of synapse elimination, Rich and Lichtmann showed that the retraction of nerve terminals is preceded by the elimination of AChRs from the postsynaptic site, suggesting that loss of postsynaptic structures may have a role in synapse elimination (Rich and Lichtmann, 1989).

The elimination of nerve terminals is a competitive process and recent studies with Xenopus nerve-muscle cocultures have suggested a possible mechanism (Lo and

Poo, 1991; Dan and Poo, 1992). In these studies, the effect of electrically stimulating two neurons that innervate the same muscle cell was examined. Stimulation of only one of the neurons causes a decrease in the strength of the synapse made by the other neuron. Synchronous stimulation has no effect, whereas asynchronous stimulation suppresses one or both of the synapses. These and further experiments suggest that a nerve terminal that fires in synchrony with the postsynaptic myofiber is stabilized, whereas a nerve terminal that does not fire in synchrony with the myofiber is destabilized. This type of competitive mechanism would explain why all but one of the nerve terminals at developing synapses are eliminated, because each neuron would tend to stabilize itself while destabilizing its neighbors, and slight variations in initial synaptic strength would be amplified until only one terminal remains.

Mechanism of AChR accumulation at synapses

Several lines of evidence indicate that early synaptic AChR clusters are induced by nerve terminals. AChR clusters appear shortly after, but never before nerves enter developing muscle in Xenopus embryos (Chow and Cohen, 1983). In the developing chick embryo, AChR clusters are first detected on myotubes when nerve trunks are positioned close to developing muscle masses (Dahm and Landmesser, 1991). These early AChR clusters are detected in chick myotubes that are within approximately 200 μm of the nerve trunk, but nerve-muscle contact does not seem to be required, suggesting that a diffusible factor induces these clusters (Dahm and Landmesser, 1991). Following nerve branching, AChR clusters become progressively more correlated spatially with positions of nerve-muscle contact (Dahm and Landmesser, 1991). In vitro, embryonic chick myotubes can form spontaneous AChR clusters in the absence of innervation. Nerve-muscle coculture experiments, however, indicated that motor neurons show no tendency to form synapses at pre-existing AChR clusters mapped by electrophysiological methods (Frank and Fischbach, 1979). Experiments with frog somite muscle cells prelabelled with

fluorescent-labelled α-bungarotoxin demonstrated that contact with motor neurons causes a fraction of the non-synaptic AChRs to redistribute to newly formed synapses (Anderson and Cohen, 1977). This redistribution of AChRs is now known to be induced by a protein called agrin (Nitkin et al., 1987), which is made by motor neurons and secreted into the synaptic basal lamina.

Agrin and the synaptic basal lamina

Studies on regenerating muscle showed that the synaptic basal lamina contains a signal that can induce the clustering of AChRs (Burden et al., 1979; Bader, 1981; McMahan and Slater, 1984). In these experiments, myofibers were damaged to induce myofiber degeneration and at the same time the muscle was denervated. Both the myofiber and the nerve terminal degenerate, but the myofiber's basal lamina sheath remains intact. Myofibers that regenerate within the original basal lamina sheath in the absence of reinnervation accumulate AChRs at the original synaptic sites, indicating that a signal for AChR clustering is associated with the synaptic basal lamina.

The signal in the basal lamina that induces AChR clustering has been identified as agrin (Nitkin et al, 1987), a glycoprotein originally isolated from Torpedo electric organ and later shown to be concentrated in the synaptic basal lamina (Reist et al., 1987). Soluble agrin causes clustering of AChRs on cultured myotubes (Godfrey et al., 1984), and local application of agrin causes AChRs to cluster at the site of application (Campenelli et al., 1991). Although both motor neurons and myofibers synthesize agrin, blocking experiments with antibodies specific for nerve or muscle agrin indicate that nerve-derived agrin is responsible for inducing AChR clusters (Reist et al., 1992). There are different isoforms of agrin which differ in AChR clustering activity, and motor neurons, but not muscle contain isoforms that induce AChR clustering. The different isoforms of agrin in muscle and motor neurons are a result of alternative splicing, and the

clustering activity of nerve-derived agrin results from the inclusion of exons encoding 8 or 11 amino acids (Tsim et al., 1992; Ruegg et al., 1992; Ferns et al., 1992).

Synapse-specific transcription

In addition to agrin-mediated clustering of pre-existing AChRs, there is evidence that AChRs are locally inserted into the postsynaptic membrane (Role et al., 1985). Moreover, there is evidence that AChR polypeptides are synthesized locally in the synaptic region, since the mRNAs encoding the different subunits of the AChR are highly concentrated at synaptic sites in adult myofibers (Merlie and Sanes, 1985; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990). Chapter 2 of this dissertation describes studies that examine the mechanism of accumulation of AChR δ-subunit mRNA at synaptic sites. The conclusion reached from these studies is that transcription of the AChR delta subunit gene in innervated muscle is confined to nuclei that are situated at the synaptic site. Thus, synaptic nuclei are transcriptionally distinct from nuclei elsewhere in the multinucleated myofiber, and the synaptic site provides a signal that acts locally to activate transcription of AChR genes in nuclei that are positioned close to the synaptic site.

AChR inducing molecules

A signal that induces synapse-specific transcription of AChR genes is associated with the synaptic basal lamina. In an experimental paradigm similar to the one used to show that synaptic basal lamina contains AChR clustering activity, synapse-specific transcription occurs in nuclei that are near original synaptic sites in regenerated, denervated myofibers (Goldman et al., 1991; Jo and Burden, 1992; Brenner et al., 1992). Agrin, which induces the clustering of pre-existing AChRs, does not appear to be a good candidate for the transcriptional signal, since soluble agrin does not increase AChR synthesis in cultured myotubes (Godfrey et al., 1984). It remains to be determined, however, if local application of agrin can stimulate transcription in nearby nuclei.

Two proteins found in motor neurons, ARIA (AChR inducing activity) and CGRP (calcitonin-gene-related peptide), are known to induce the synthesis of AChR subunits, but the exact roles of these proteins in synapse formation is not yet clear. ARIA, a 42 kDa protein originally purified from embryonic chick brain (Usdin and Fischbach, 1986), stimulates transcription of the chick AChR α-subunit gene (Harris et al., 1988). Treatment of mouse myotubes with ARIA, however, primarily increases ε-subunit mRNA levels, and only slightly increases α-, γ-, and δ-subunit mRNA levels (Martinou et al., 1991). This data is consistent with the idea that ARIA may have a role in the postnatal induction of ε-subunit gene expression.

CGRP, a neuropeptide present in the nerve terminals of a subpopulation of motor neurons (Takami et al. 1985a; Matteoli et al., 1988), increases both surface and total AChR levels (Fontaine et al., 1986; New and Mudge, 1986) and stimulates transcription of the chick α-subunit 3-fold (Fontaine et al., 1987; Osterlund et al., 1989). The action of CGRP may be mediated by cAMP, since CGRP activates adenylate cyclase and elevates intra-cellular cAMP (Takami et al., 1986; Kobayashi et al., 1987; Laufer and Changeux, 1987). During chick development, the number of CGRP immunoreactive motor neurons peaks during the period of initial neuromuscular contact (Villar et al., 1989), and the specific activity of CGRP-binding sites on embryonic chick muscles is highest during the time when AChR numbers are maximal (Roa and Changeux, 1991). These results are consistent with the idea that CGRP may be important for induction of AChRs at synapses.

Electrical activity dependent regulation

Although local signals regulate the synthesis of synaptic AChRs, the synthesis of non-synaptic AChRs is regulated by propagated electrical activity in the myofiber membrane. The decline in non-synaptic receptors that normally occurs in chicks can be prevented by chronic in ovo paralysis (Burden, 1977). Moreover, denervation of adult muscle results

in the reappearance of non-synaptic receptors (Axelsson and Thesleff, 1959). Direct electrical stimulation of denervated muscle with extracellular electrodes prevents the reappearance of non-synaptic AChRs, indicating that myofiber electrical activity, rather than neural factors, is key to suppressing non-synaptic AChRs (Lomo and Rosenthal, 1972).

Nuclear run-on experiments and transgenic mice indicate that electrical activity controls AChR synthesis by a transcriptional mechanism (Tsay and Schmidt, 1989; Merlie and Kornhauser, 1989; Simon et al., 1992), but the important cis-elements have not yet been identified. Chapter 2 of this dissertation describes studies with transgenic mice which show that 1.8 Kbp of 5' flanking DNA from the AChR δ-subunit gene confers transcriptional regulation by electrical activity. Chapter 3 describes an in vitro culture system of primary myoblasts for studying regulation of gene expression by electrical activity. These studies show that 181 bp of AChR δ-subunit 5' flanking DNA confers regulation by electrical activity.

Direct electrical stimulation of denervated muscle results in the inactivation of AChR genes within 10 to 60 minutes (Huang et al., 1992). This effect does not require new protein synthesis, and there is evidence that a rapid increase in protein kinase C activity couples membrane excitation to AChR gene inactivation (Huang et al., 1992). In contrast, the activation of AChR genes following denervation requires new protein synthesis and occurs over a period of days (Tsay et al., 1990; Neville et al., 1991). These results suggest that denervation triggers the synthesis of transcription factors that activate AChR transcription and that protein kinase C rapidly inactivates transcription factors following electrical stimulation.

Muscle-specific gene expression

An understanding of the mechanisms that regulate AChR expression following innervation may require knowledge of the steps required to activate AChR genes during

myogenesis. In particular, *cis*-acting sequences that are important for regulation during myogenesis may also be targets for regulation by innervation. Chapter 4 describes studies which identify the two *cis*-acting sequences that control muscle-specific expression of the AChR δ-subunit gene. One of these elements is an E-box, a binding site for basic helix-loop-helix transcription factors. The E-box has a dual role: it is important for activation of gene expression in myotubes and for repression in myoblasts and non-muscle cells. The other element is an enhancer that is required for full gene expression in myotubes, but is not muscle-specific. It remains to be determined whether these elements have a role in regulation of gene expression by innervation.

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Chapter 2

Spatial Restriction of Acetylcholine Receptor Delta Subunit Gene Expression to Subsynaptic Nuclei

SUMMARY

Acetylcholine receptors (AChRs) and the mRNAs encoding the four AChR subunits are highly concentrated in the synaptic region of skeletal myofibers. The initial localization of AChRs to synaptic sites is triggered by the nerve and is caused, in part, by post-translational mechanisms that involve a redistribution of AChR protein in the myotube membrane. I have used transgenic mice that harbor a gene fusion between the murine AChR delta subunit gene and the human growth hormone (hGH) gene to show that innervation also activates two independent transcriptional pathways that are important for establishing and maintaining this non-uniform distribution of AChR mRNA and protein. One pathway is triggered by signal(s) that are associated with myofiber depolarization, and these signals act to repress delta subunit gene expression in nuclei throughout the myofiber. Denervation of muscle removes this repression and causes activation of delta subunit gene expression in nuclei in non-synaptic regions of the myofiber. A second pathway is triggered by an unknown signal that is associated with the synaptic site, and this signal acts locally to activate delta subunit gene expression only in nuclei within the synaptic region. Synapse-specific expression, however, does not depend upon the continuous presence of the nerve, since transcriptional activation of the delta subunit gene in subsynaptic nuclei persists after denervation. Thus, the nuclei in the synaptic region of multinucleated skeletal myofibers are transcriptionally distinct from nuclei elsewhere in the myofiber, and this spatially restricted transcription pattern is presumably imposed initially by the nerve.

INTRODUCTION

In adult myofibers, acetylcholine receptors (AChRs) are virtually confined to the small patch of membrane directly beneath the presynaptic nerve terminal (Salpeter, 1987). This arrangement is established during development in a series of steps that begin when the developing motor nerve interacts with embryonic myotubes and causes a redistribution of

a fraction of AChRs that were already present on the myotube membrane (Anderson and Cohen, 1977; Ziskind-Conhaim et al., 1984; Role et al., 1985). Thereafter, AChRs are excluded from non-synaptic regions of the myofiber membrane and they remain concentrated at the synaptic site (Burden, 1977).

The maintenance of a high concentration of AChRs at the synapse requires replacement of AChRs at synaptic sites (Fambrough, 1979), and there is evidence that this replacement of synaptic AChRs is accomplished by local insertion of AChRs into the postsynaptic membrane (Role et al., 1985). In addition, there is evidence that AChR polypeptides are synthesized locally in the synaptic region, since the mRNAs encoding the different subunits of the AChR are highly concentrated at synaptic sites in adult myofibers (Merlie and Sanes, 1985; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990).

The mechanisms responsible for accumulation of AChR mRNAs at synaptic sites are unclear, but could involve selective stabilization of AChR mRNAs in the synaptic region, transport of AChR mRNAs to the synaptic region, or selective transcription of AChR genes by the subset of nuclei that are within the synaptic region.

Transgenic mice were constructed that contain a gene fusion between 1.8 kbp of 5' flanking DNA from the murine AChR delta subunit gene and the human growth hormone (hGH) gene, to determine whether *cis*-acting elements in the AChR gene could restrict expression of hGH to the synaptic region of skeletal myofibers. Because hGH is processed in intracellular organelles prior to secretion, and because these organelles are closely associated with nuclei, I could infer the nuclear source of hGH transcription by studying the spatial distribution of intracellular hGH by immunocytochemistry. I show here that hGH in these transgenic mice is restricted to the perinuclear region of nuclei within the synaptic region of the myofiber, and I conclude that transcription of the endogenous AChR delta subunit gene is confined to nuclei that are situated at the synaptic site. These data indicate that synaptic nuclei are transcriptionally distinct from

nuclei elsewhere in the multinucleated skeletal myofiber and that the synaptic site provides a signal that acts locally to activate transcription of certain genes in nuclei that are positioned close to the synaptic site.

MATERIALS AND METHODS

Construction of transgenic mice

Embryo injections were done by Peter Hoppe at Jackson Laboratories (Bar Harbor, ME). C57BL/6J females were mated with LT/Sv males, and zygotic male pronuclei were injected with a gene fusion between the murine AChR delta subunit gene (-1,823/+25) (Baldwin and Burden, 1988) and the hGH gene (Selden et al., 1986). The embryos were transferred to B6/SJL F1 pseudopregnant females (Wagner et al., 1981). Five founder mice (δGH1-5), which had integrated the transgene into their germ line, were the source of five different transgenic lines. All integrations are autosomal, and they range from a few copies to more than ten copies of the transgene. Mice from each transgenic line express hGH in skeletal muscle, although the absolute level of hGH expression and the degree of muscle-specific hGH expression differs among the five lines (Table 2.1). An additional founder mouse was apparently sterile and another founder mouse contained the transgene but did not express hGH in any tissue; I did not establish lines from these mice. hGH (20-100 ng/ml) is detectable by radioimmunoassay in the serum of newborn transgenic mice.

Histology

Immunofluorescence. Adult mice were fixed by perfusion (4% formaldehyde in 0.9% sodium chloride with 0.2 M sucrose), and dissected muscles were fixed further by immersion in fixative for 1 hr. The tissue was rinsed with 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.3 (PBS), washed with 20 mM Tris, 150 mM sodium chloride, pH 7.3, permeabilized with PBS-NP40 (0.5% NP40) and incubated with

antibodies to hGH (DAKO, Carpinteria, CA) (1/500 in PBS-NP40) for 3-6 hr. at room temperature. The tissue was washed with PBS for 0.5 hr., incubated with fluorescein-labelled goat anti-rabbit IgG (Cappel, Malvern, PA) and tetramethylrhodamine-coupled alpha-bungarotoxin (TMR-α-BGT) in PBS-NP40 for 3-6 hr. at room temperature, washed with PBS for 0.5 hr. and fixed in 100% methanol at -20° C. Muscles, or individual dissected myofibers, were mounted whole (in 20% glycerol, 80 mM sodium bicarbonate, pH 9, with 10 mg/ml *p*-phenylenediamine) and were viewed with filters selective for either fluorescein or rhodamine (Burden, 1982). My attempts to detect hGH with enzyme-coupled antibodies were unsuccessful; lack of hGH staining was probably due to poor penetration of these reagents into muscle tissue. Most experiments were performed on diaphragm, intercostal, extensor digitorum longus and soleus muscles; the pattern of hGH staining was indistinguishable among the different muscles. Staining for hGH in muscle that was fixed only by immersion, and not by perfusion, was capricious; lack of hGH staining under these conditions was probably due to secretion of hGH during the time required for dissection of deeper muscles prior to their fixation. Because of the low level of hGH expression in lines δGH4 and δGH5 and the reduced fertility of line δGH3, I restricted the immunocytochemical analysis to lines δGH1 and δGH2.

In situ hybridization. Muscles were fixed in 4% formaldelhyde in PBS for 1 hr., stained for cholinesterase and dehydrated. Single myofibers were dissected in a drop of water on silated microscope slides, allowed to dry overnight at 40° C and processed for *in-situ* hybridization (Kintner and Melton, 1987). The ^{35}S-labelled anti-sense delta subunit RNA probe (1.9 kbp long) was synthesized with SP6 polymerase from SP65-delta cDNA (LaPolla et al., 1984). Exposures were for 4-5 days.

RNA analysis

RNA was isolated from tissue with guanidinium thiocyanate as described (Chomczynski and Sacchi, 1987). The levels of delta subunit, skeletal muscle actin and hGH mRNAs were measured by RNase protection (Baldwin and Burden, 1988), were quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA), and were corrected for non-specific hybridization by subtracting the value for protection with tRNA. The ^{32}P-labelled RNA probes extended from nucleotide 1 to 582 in the hGH gene (Selden et al., 1986), nucleotide 234 to 730 in the delta subunit gene (Baldwin and Burden, 1988), and nucleotide 1258 to 1661 in the actin gene (Hu et al., 1986).

RESULTS

AChR delta subunit mRNA is highly concentrated at synaptic sites

mRNAs encoding the four subunits of the AChR are enriched at synaptic sites (Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990). The distribution of delta subunit mRNA in a single isolated myofiber is illustrated in figure 2.1, which shows that delta subunit mRNA is concentrated at the synaptic site, whereas nuclei are present throughout the myofiber.

AChR delta subunit gene is transcribed selectively in subsynaptic nuclei

Accumulation of delta subunit mRNA at synaptic sites could be caused by transcriptional and/or post-transcriptional mechanisms. In order to determine whether *cis*-acting elements in the delta subunit gene restrict expression to the synaptic region of skeletal myofibers, transgenic mice were constructed that contain a gene fusion between 1.8 kbp of 5' flanking DNA from the delta subunit gene and the hGH gene (Materials and methods).

Growth hormone is normally expressed and secreted by somatotroph cells of the anterior pituitary (Herlant, 1964), but the hormone can be expressed and secreted by a

variety of non-pituitary cell types in transgenic mice (Palmiter et al., 1983; Trahair et al., 1989). Cells that have a regulated secretory pathway contain the hormone in secretory granules and in the golgi apparatus, whereas cells that have only a constitutive secretory pathway contain the hormone largely in the golgi apparatus (Trahair et al., 1989; Roth et al., 1990). Since myofibers are not regarded as secretory cells and are thought to possess a constitutive but not a regulated, secretory pathway (Kelly, 1985; Burgess and Kelly, 1987), I expected intracellular hGH to be found in the golgi apparatus of myofibers. Further, because the golgi apparatus is closely associated with nuclei, and because mRNAs do not diffuse over long distances in myotubes (Ralston and Hall, 1989a,b; Pavlath et al., 1989; Rotundo, 1990), I reasoned that the nuclear source of hGH transcription could be inferred by studying the spatial distribution of golgi that contain hGH.

I examined the intracellular distribution of hGH in skeletal myofibers from two lines of transgenic mice containing the gene fusion between the delta subunit and hGH genes using antibodies against hGH and immunofluorescence. Figure 2.2 shows that hGH expression is restricted to the synaptic region of myofibers, and that intracellular hGH is perinuclear and, by light microscopy, appears to be associated with the golgi apparatus.

The perinuclear region of nuclei immediately beneath the postsynaptic membrane are labelled intensely, and labelling is diminished in the perisynaptic region (10-50 μm from the synaptic site). hGH is not detectable in non-synaptic regions of normal muscle, although nuclei are found throughout the myofiber (figure 2.1). The synaptic localization of hGH is not due to a localized secretory pathway, because transgenic mice that carry the muscle creatine kinase promoter fused to the hGH gene, express hGH throughout entire myofibers (S.A. Jo, E.K. Dutton, and S.J. Burden, unpublished results). These results show that *cis*-acting sequences in the transgene are sufficient to confer synapse-specific expression of hGH in innervated skeletal muscle.

My data indicate that the transgene is transcribed at an enhanced rate in subsynaptic nuclei. Although the transgene encodes 25 nucleotides of mRNA from the 5' untranslated region of the delta subunit gene, this sequence is neither conserved between delta subunits genes from other species (chicken δ, Wang et al., 1990; Xenopus δ, Burden, unpublished), nor between genes encoding other AChR subunits (human α, Noda et al., 1983; chicken α, Klarsfeld et al., 1987; mouse γ, Crowder and Merlie, 1988); therefore, these 24 nucleotides are not likely to be critical for localizing delta subunit and hGH mRNAs to synaptic sites. Thus, my data indicate that myofiber nuclei that are positioned close to the synaptic site transcribe the endogenous delta subunit gene at a higher rate than nuclei elsewhere in the myofiber.

Synaptic activation persists in the absence of the nerve

Synaptic specializations, including accumulations of AChR and mRNAs encoding AChR subunits, persist at synaptic sites after denervation (Fambrough, 1979; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Witzemann et al., 1991). In order to determine whether synapse-specific transcription of the delta subunit gene was maintained after denervation, I examined the distribution of hGH in denervated muscle. Figure 2.3 shows that hGH is concentrated at synaptic sites in muscle that was denervated for 4 days. Like hGH at normal synaptic sites, hGH at denervated synaptic sites is perinuclear and is apparently associated with the golgi apparatus. Because hGH mRNA has a half-life of 2-20 hours (Diamond and Goodman, 1985), most of the hGH that is detected at denervated synaptic sites was probably transcribed and translated after denervation. Thus, enhanced transcription of the delta subunit gene at synaptic sites does not require the continuous presence of the nerve.

Denervation causes an increase in the turnover of synaptic AChRs and an increase in the level of AChR protein and AChR mRNA in perisynaptic regions (Salpeter, 1987; Goldman and Staple, 1989), and these changes could be caused by an increase in the rate

of AChR transcription. Consistent with this idea, the level of hGH in synaptic and perisynaptic regions appears greater in denervated than in innervated muscle (figures 2.2 and 2.3). These results are consistent with the idea that electrical activity suppresses transcription of the delta subunit gene throughout the myofiber and that enhanced transcription at synaptic sites in denervated muscle is caused by the removal of electrical activity-dependent repression and the persistence of synapse-specific activation.

Myofiber electrical activity has an important role in repressing AChR expression, and denervation of skeletal muscle causes an increase in the abundance of AChRs and mRNAs encoding AChR subunits in non-synaptic regions (Fambrough, 1979; Evans et al., 1987). Delta subunit mRNA levels increase 10-20 fold after denervation (figure 2.4), and this increase can be prevented, or reversed, by direct electrical stimulation of denervated muscle (Goldman et al., 1988; Witzemann et al., 1991). I measured delta subunit and hGH mRNA levels in innervated and denervated muscle to determine whether electrical activity regulates the delta subunit gene by transcriptional mechanisms. Figure 2.4 shows that denervation of muscle causes a 10-20 fold increase in endogenous delta subunit mRNA levels and a similar increase in hGH mRNA levels. Thus, this 1.8 kbp from the delta subunit gene contains the *cis*-acting regulatory elements that confer electrical activity-dependent gene regulation.

Non-synaptic nuclei in denervated muscle are heterogeneous, and expressing nuclei are colocalized with AChR clusters

Although denervation causes an increase in the level of AChRs, most AChRs in non-synaptic regions of denervated myofibers are distributed diffusely and are not detectable with TMR-α-BGT (Fambrough, 1979). However, a small fraction of these non-synaptic AChRs aggregate, and these AChR clusters can be detected with TMR-α-BGT (Ko et al, 1977). I do not detect hGH by immunofluorescence in most non-synaptic areas of denervated muscle; I suspect that it is present, but that immunofluorescence is

insufficiently sensitive to detect this low level of expression. However, hGH is detectable at non-synaptic regions where AChR clusters are found (figure 2.5). The intensity of hGH staining at these non-synaptic AChR clusters is similar to that observed at synaptic sites, and non-synaptic hGH, like synaptic hGH, is perinuclear and is apparently associated with the golgi apparatus. These results indicate that non-synaptic nuclei in denervated myofibers are transcriptionally heterogeneous, since the delta subunit gene is transcribed at different rates in different non-synaptic nuclei (see also Fontaine and Changeux, 1987; Berman et al., 1990). Further, these results indicate that the processes of clustering AChRs and enhancing AChR transcription are linked, and that colocalization of AChR transcription and AChR clusters does not require a signal from the nerve.

Signal and pathway appear shortly after synapse formation

Accumulation of AChRs at developing synapses is an early event in synapse formation and occurs several days before birth in rodents (Frank and Fischbach, 1979; Dennis, 1981; Slater, 1982). However, synaptic structure and function continue to be modified during the next few weeks, and the full complement of adult synaptic properties are not acquired until about one month after birth (Dennis, 1981; Slater, 1982; Schuetze and Role, 1987). In order to determine at what point synaptic nuclei become distinct, I examined the distribution of hGH in muscle from newborn mice. Figure 2.6 shows that hGH is concentrated at synaptic sites in newborn muscle. Like synaptic staining in adult muscle, synaptic hGH staining in developing muscle is perinuclear. However, synaptic staining in newborn muscle appears less intense than in adult muscle. Thus, synaptic nuclei become transcriptionally distinct early during synapse formation and before postjunctional folds have developed, polyneuronal innervation has been eliminated, or AChR channels with short open-times have appeared (Schuetze and Role, 1987).

Myogenin is not expressed specifically in subsynaptic nuclei

Myogenic basic-helix-loop-helix (bHLH) proteins are important regulators of muscle gene expression during myogenesis, and their targets include the genes encoding AChR subunits (Piette et al., 1990). Previous studies have shown that the levels of myogenin and MyoD1 mRNA are high in muscle from newborn mice and decrease during the following two weeks (Duclert et al., 1991; Eftimie et al., 1991). Further, the levels of myogenin and MyoD1 mRNA, like AChR mRNA, are regulated by innervation, since they increase following denervation (Duclert et al., 1991; Eftimie et al., 1991). I sought to determine whether myogenic bHLH proteins might be involved in synapse-specific expression of the AChR delta subunit gene, and I used an antibody against myogenin to determine whether myogenin is concentrated in subsynaptic nuclei. I restricted my analysis to myogenin, because antibodies against MRF4 and myf-5 are not available, and I could not detect staining in newborn or adult muscle with available antibodies to MyoD1 (Tapscott et al., 1988). Figure 2.7 shows that myogenin is expressed in nuclei throughout developing myofibers in newborn muscle, but that myogenin is not detectable in innervated, adult muscle. These results are consistent with the data for myogenin mRNA and support the idea that myogenin could have a role in coupling changes in myofiber electrical activity to changes in AChR gene expression (Duclert et al., 1991; Eftimie et al., 1991). However, because myogenin is not concentrated at synaptic sites, either in newborn or adult muscle (figure 2.7), these data do not support the idea that myogenin has a central role in activating AChR genes selectively in subsynaptic nuclei.

DISCUSSION

This study demonstrates that transcription of the AChR delta subunit gene in innervated, adult muscle is confined to nuclei that are situated within the the synaptic region. These results indicate that synaptic nuclei are transcriptionally distinct from nuclei elsewhere in the multinucleated skeletal myofiber and that the synaptic site provides a signal that acts

locally to activate transcription of a select set of genes in nuclei that are positioned close to the synaptic site.

The restricted spatial distribution of AChR gene expression indicates that a synaptic signal, which is presumably provided by the motor neuron, exerts its effect over a highly circumscribed region of the myofiber. hGH is highly concentrated immediately beneath the postsynaptic membrane, and expression of hGH is less abundant 10-50 μm from the synaptic site and is not detectable further than 50 μm from the synaptic site. These distances are similar to those measured for spread of signalling information in other transduction systems (Lamb et al., 1981; Matthews, 1986), and these data indicate that the skeletal myofiber need not have unique mechanisms to restrict the spatial flow of information. The spatial influence of the synaptic signal may be restricted further by repressive signals provided by electrical activity, and this additional influence may explain why hGH is readily detectable in the perisynaptic region of denervated myofibers. Although my results are consistent with the idea that transcriptional activity decreases exponentially from the synaptic site, my immunofluorescence methods were not quantitative, and diffusion of RNA or rapid translocation of organelles associated with the golgi apparatus could also produce a gradient of intracellular hGH. Nevertheless, my data provide an estimate of the distance over which the synaptic signal exerts its effect.

Spatial restriction of delta subunit gene expression does not depend upon the continuous presence of the nerve, since hGH remains concentrated at synaptic sites following denervation. Thus, either the nerve-derived signal that is responsible for activation of the delta subunit gene in subsynaptic nuclei remains at synaptic sites for at least several days following denervation, or the effect of this signal persists after the nerve is removed. In either case, the transcriptional machinery associated with activation of the delta subunit gene persists in the subsynaptic nuclei of adult muscle and does not require the continuous presence of the nerve.

AChRs can cluster at non-synaptic sites on denervated myofibers and on embryonic myotubes (Ko et al., 1977; Vogel et al., 1972; Fischbach and Cohen, 1973), and the mechanisms that regulate the formation of these non-synaptic clusters have served as a model for formation of AChR clusters at synapses (Schuetze and Role, 1987). I show here that AChR accumulation at non-synaptic sites *in vivo* is associated with regions of increased delta subunit transcription. Thus, there appears to be heterogeneity among the non-synaptic nuclei, since a small number of nuclei in non-synaptic regions of denervated muscle express the delta subunit gene at an enhanced rate, similar to synaptic nuclei. Because my results show that highly expressing nuclei need not be positioned at the synaptic site, colocalization of AChR transcription and AChR clusters does not require a signal from the nerve.

I do not know whether there is a causal relationship between increased density of surface AChRs and increased AChR transcription; however, since the expressing non-synaptic nuclei are not randomly distributed but are associated in small groups, it seems unlikely that random activation of individual nuclei causes the appearance of surface AChR clusters. It is not clear what triggers the formation of these synaptic specializations at non-synaptic sites; however, it is possible that a pathway, which is normally activated by the nerve at synaptic sites, can be activated independent of the nerve and lead to all specializations normally found at the synaptic site, including both surface clustering of AChRs and nuclear activation of AChR genes.

At present I have little information regarding the nature of the signal from the nerve that activates AChR gene expression at synapses. The signal could be released by the nerve and stably maintained in the synaptic basal lamina, like agrin, the extracellular matrix molecule that causes AChRs to cluster at synaptic sites (Nitkin et al., 1987). Nevertheless, agrin itself does not appear to be a likely candidate for the transcriptional signal, since agrin causes a redistribution of surface AChRs that is not accompanied by an increase in AChR synthesis (Godfrey et al., 1984). Alternatively, the signal may be

released from the nerve and be required only transiently to activate a pathway or to assemble a structure, and the signal may not be provided constitutively (Brenner et al., 1990). Identification of the synaptic signal and its receptor will be important steps in understanding how nerve-muscle signalling is mediated, and in understanding how transcription is activated.

Recent reports indicate that the signal for synapse-specific transcription is, in fact, contained in the synaptic basal lamina (Goldmann et al., 1991; Jo and Burden, 1992; Brenner et al., 1992). In the study by Jo and Burden, myofibers in δGH1 mice were denervated and damaged, and expression of hGH was studied in new fibers that regenerated within original basal lamina sheaths. Synapse-specific transcription was re-induced in these regenerated myofibers, in the absence of innervation (Jo and Burden, 1992). Thus, the synaptic basal lamina is the most likely source for the signalling molecule that stimulates transcription of AChR genes (figure 2.8).

Myogenin is a transcription factor that has an important role in initiating muscle differentiation (Wright et al., 1989), and the abundance of myogenin mRNA is regulated by innervation (Duclert et al., 1991; Eftimie et al., 1991). Myogenin is not detectable at synaptic sites in adult muscle, but is present in nuclei throughout newborn muscle. These data do not support the idea that myogenin has a central role in activating AChR genes selectively in subsynaptic nuclei. However, these data are consistent with the idea that myogenin has a role in initiating AChR gene expression during myogenesis and that myogenin could be involved in coupling changes in electrical activity to changes in AChR gene expression (Piette et al., 1990; Duclert et al., 1991; Eftimie et al., 1991). It will be important to determine whether any of the other myogenic bHLH proteins are concentrated in subsynaptic nuclei. In any case, the identification of the transcription factors that activate gene expression selectively in subsynaptic nuclei will be facilitated by analysis of additional transgenic lines and identification of the important *cis*-acting regulatory elements in the delta subunit gene that confer synapse-specific expression. In

this regard, additional lines of transgenic mice indicate that 181 bp of δ subunit 5' flanking DNA is sufficient for synapse-specific expression (S.A. Jo, E.K. Dutton, and S.J. Burden, unpublished results).

mRNAs encoding the alpha, beta and epsilon subunits of the AChR are also highly concentrated at synaptic sites in adult myofibers (Merlie and Sanes, 1985; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990), and it would seem likely that the genes encoding these subunits are also transcriptionally activated in the subsynaptic nuclei of adult muscle. Indeed, a recent study using transgenic mice indicates that the epsilon subunit gene is selectively transcribed by subsynaptic nuclei (Sanes et al., 1991). A previous report has shown that a transgenic mouse line that harbors a gene fusion between the chicken AChR alpha subunit 5' flanking region and a β-galactosidase reporter gene, expresses β-galactosidase in newborn muscle (Klarsfeld et al., 1991). Expression is enriched in the central region of the muscle, where most synapses are located; however, staining extends far beyond synaptic sites in newborn muscle (Klarsfeld et al., 1991). Therefore, it is unclear whether the expression pattern in newborn muscle reflects preferential expression of the transgene by synaptic nuclei as proposed (Klarsfeld et al., 1991), or whether the pattern reflects the developmental history of myotubes, in which myoblast fusion begins in the central region of the muscle (Kitiyakara and Angevine, 1963; Bennett and Pettigrew, 1974; Braithwaite and Harris, 1979). Since expression of β-galactosidase is not detectable after postnatal day 4, the authors were not able to evaluate this latter possibility. The absence of expression in adult muscle could be due to silencing of the transgene, but it is also possible that the transgene, while it contains the elements for muscle-specific and electrical activity-dependent regulation (Merlie and Kornhauser, 1989), lacks an element necessary for synapse-specific expression. In this regard, another group recently reported that mice carrying a similar chicken alpha subunit-β-galactosidase transgene, express β-galactosidase in non-synaptic as well as synaptic nuclei (Sanes et al., 1991).

Like synaptic nuclei in vertebrate skeletal myofibers, nuclei in the syncytial blastoderm of Drosophila are transcriptionally distinct (Nusslein-Volhard, 1991). For example, expression of the *tailless* gene is restricted to nuclei in the terminal regions (Pignoni et al., 1990), and expression of the *twist* gene is restricted to nuclei in the ventral region of the syncytial blastoderm (Thisse et al., 1988). Polarity is established in the dorsoventral and terminal systems by signals that are synthesized in distinct follicular cells which ensheathe the developing oocyte (Stevens et al., 1990; Stein et al., 1991). Although the dorsoventral and terminal systems use different pathways to interpret the spatial information provided to the oocyte (Anderson et al., 1985; Sprenger et al., 1989; Casanova and Struhl, 1990), in each system polar information is maintained in the egg, and the continuous presence of follicular cells is not required to establish distinct transcriptional activity in subsets of nuclei (Stevens et al., 1990; Stein et al., 1991). The results presented in this study indicate that the presynaptic nerve terminal is the source of a signal which provides spatial information to the myofiber, and that this signal acts to activate transcription of a select set of genes in nuclei that are situated near the synaptic site. It will be interesting to determine whether similar components and mechanics are used to establish transcriptionally distinct nuclei in the syncytial blastoderm and in the syncytial myofiber and whether steps involved in pattern formation in Drosophila are shared with steps involved in neuromuscular synapse formation.

Table 2.1 hGH mRNA levels in transgenic muscle and non-muscle tissue

Line #	Den. muscle	Heart	Liver	Spleen
δGH1	100	30.0	<0.5	10
δGH2	100	<0.2	<0.2	1
δGH3	100	0.4	0.3	20
δGH4	100	40.0	6.0	10
δGH5	100	2.0	90.0	10

Table 2.1 In all five lines the highest level of hGH mRNA is seen in denervated muscle, but only δGH2 shows nearly complete muscle specificity. Because there is no consistent pattern of tissue expression among these lines, it seems likely that the site of transgene integration and cryptic elements in the hGH gene (Low et al., 1989) determine the level of hGH expression in non-skeletal muscle tissue. The levels of hGH mRNA in heart, liver, spleen and denervated skeletal muscle were determined by RNase protection (Materials and methods). The amount of hGH mRNA/total RNA in denervated muscle from each line was assigned 100%, and the values of hGH mRNA/total RNA in other tissues are expressed relative to denervated muscle.

Figure 2.1. AChR delta subunit mRNA is concentrated at synaptic sites.

Mouse diaphragm muscle was stained for acetylcholinesterase, and individual myofibers were dissected and processed for *in-situ* hybridization using a ^{35}S-labelled-anti-sense delta RNA probe (Materials and methods). (A) The synaptic site on the myofiber is identified by cholinesterase staining. (B) The autoradiographic grains are concentrated at the synaptic site. The single synaptic site on another myofiber is identified by the nerve terminal and associated Schwann cells, which are seen clearly on the myofiber surface (C). The nuclei of both the myofiber and Schwann cells contribute to a cluster of nuclei at the synaptic site (D). Three to six myofiber nuclei are present at each synaptic site, and the density of myofiber nuclei in non-synaptic regions is 2.5-3 fold less. Myofiber nuclei are located at the periphery of the cell and are found throughout the myofiber (D). Bar = 7.5 μm for (A,B) and 15 μm for (C,D).

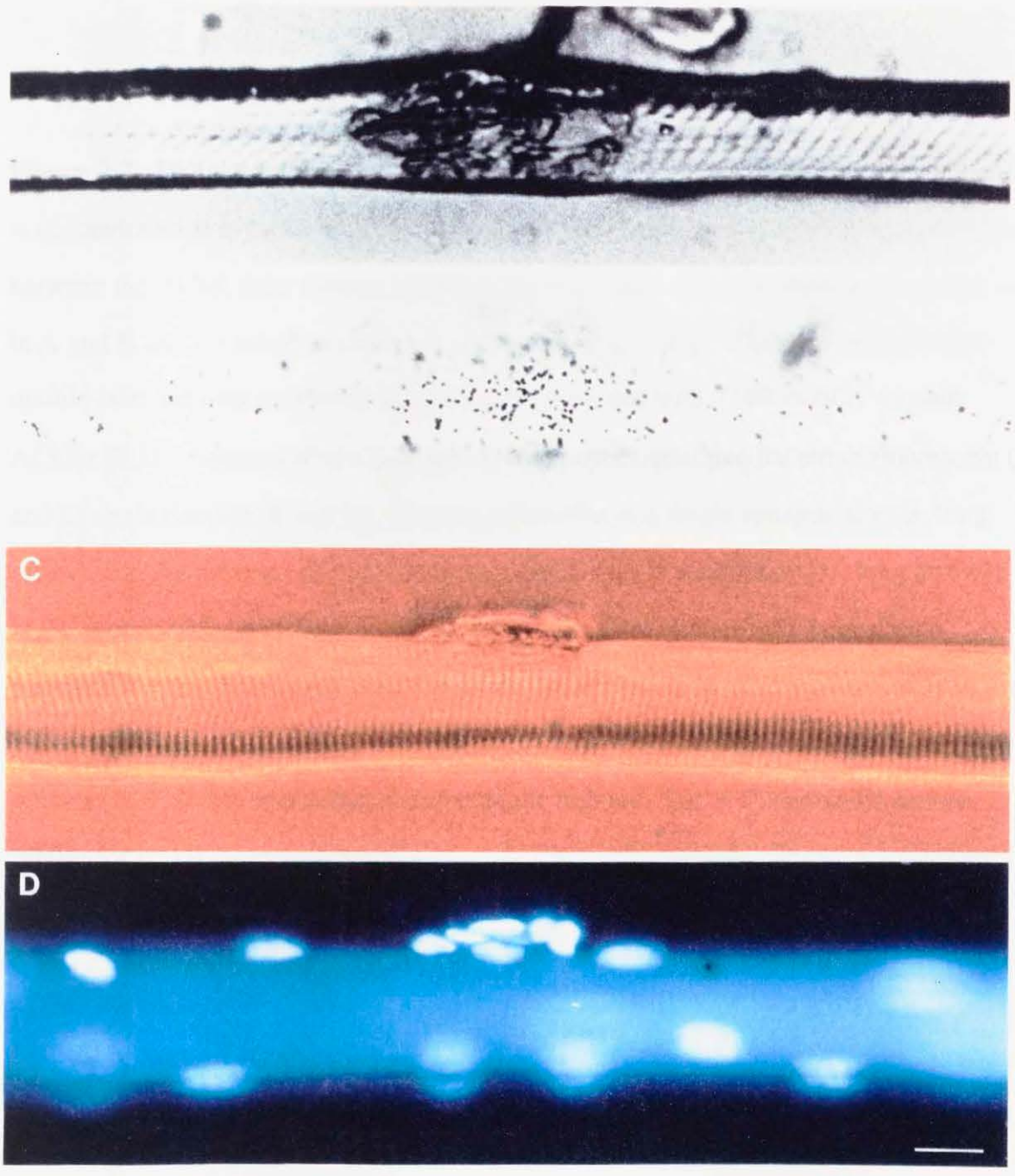

C
D

Figure 2.2. Delta subunit gene is transcribed preferentially in synaptic nuclei. hGH is concentrated at synaptic sites of muscle from transgenic mice that harbor a gene fusion between the AChR delta subunit gene and the hGH gene. Two myofibers are shown, one in A and B and the other, at lower magnification, in C and D. Single myofibers were double-labelled with antibodies against hGH (A,C) and with TMR-α-BGT to stain AChRs (B,D), mounted whole, and viewed with optics selective for either fluorescein (A and C) or rhodamine (B and D). Most myofibers have a single synaptic site (B, long arrow), and the presence of two adjacent synaptic sites is uncommon (D, long arrows). hGH is concentrated at the perinuclear region of nuclei immediately beneath the postsynaptic membrane (A,C, short arrows). Lower levels of hGH are detectable in the perisynaptic region (10-50 μm from the edge of the synaptic site) (A,C, curved arrows), whereas hGH is not detectable in non-synaptic regions. Bar = 12 μm for (A,B) and 30 μm for (C,D).

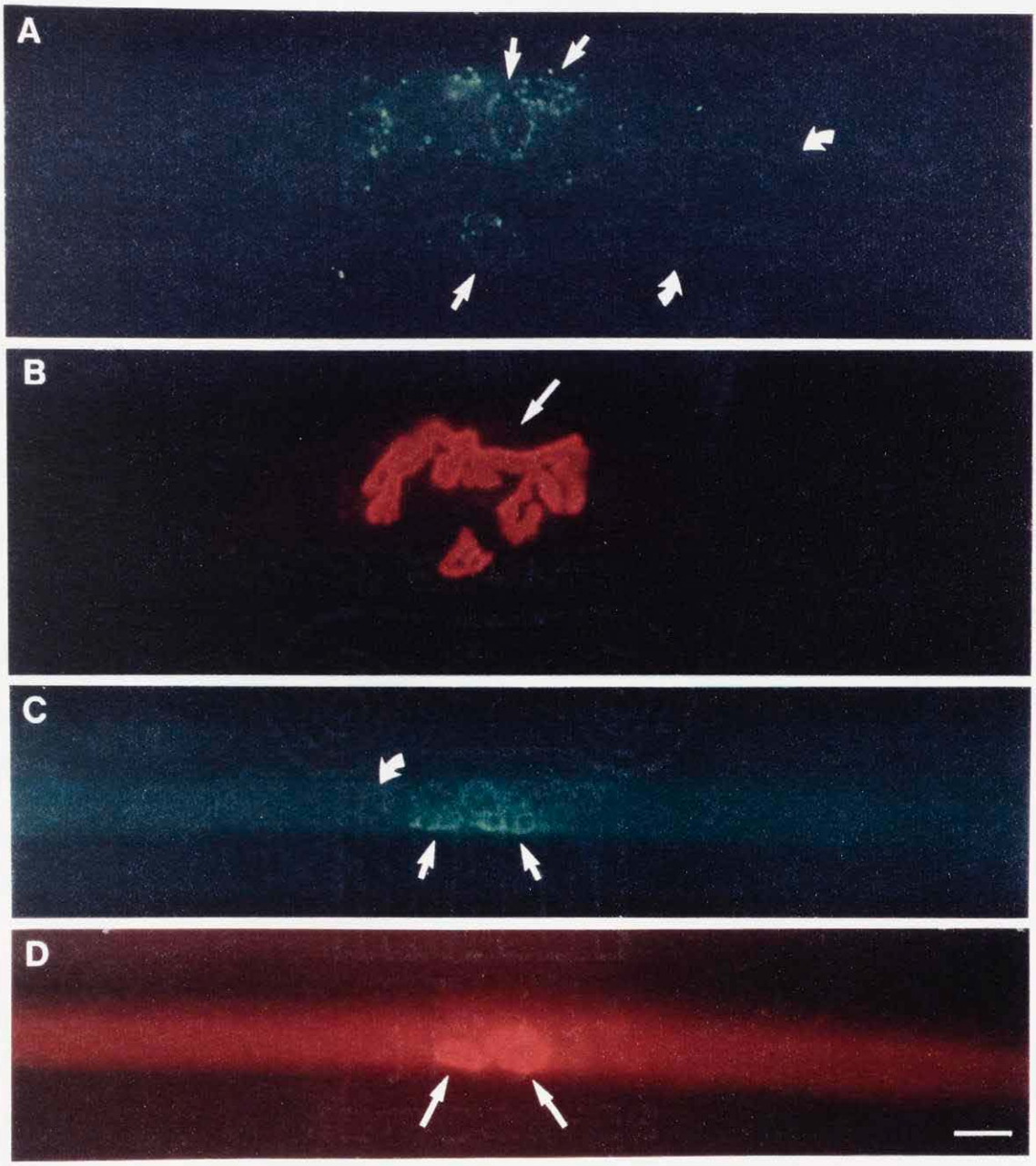

A
B
C
D

Figure 2.3. Preferential transcription of the delta subunit gene in synaptic nuclei does not require the continuous presence of the nerve. The extensor digitorum longus muscle was denervated by cutting the sciatic nerve, and single myofibers, which were denervated for 4 days, were labelled with antibodies against hGH (A,C) and with TMR-α-BGT (B,D) and mounted whole. Two myofibers are shown, one in A and B and the other in C and D. The denervated synaptic site is marked by TMR-α-BGT (B,D, long arrow). hGH is concentrated at the perinuclear regions of nuclei immediately beneath the postsynaptic membrane of denervated muscle (A,C, short arrow). hGH is readily detectable in the perisynaptic region of denervated muscle (C, curved arrow). hGH staining is more intense at synaptic sites and at perisynaptic regions in denervated muscle than in innervated muscle. Bar = 30 μm.

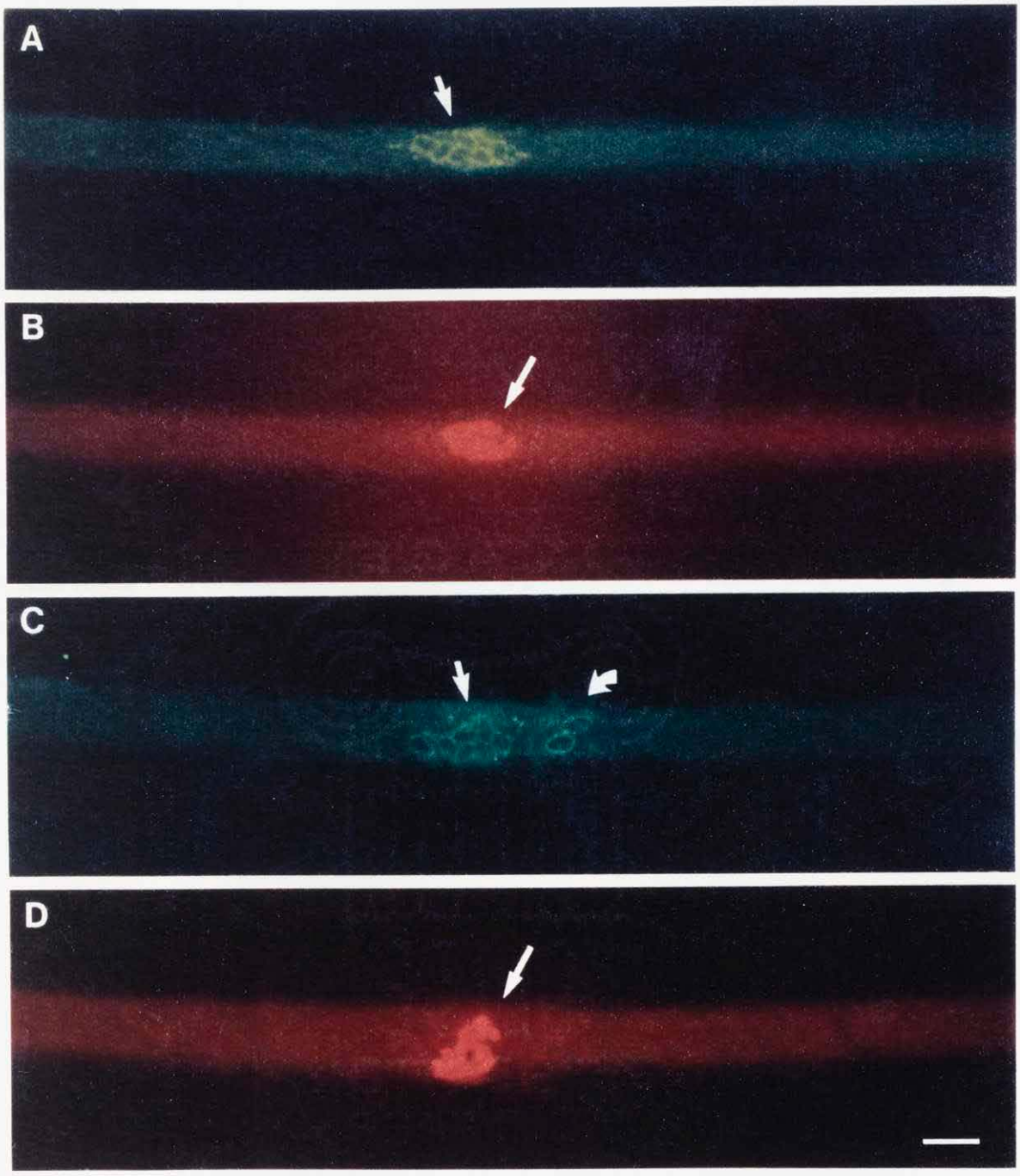

A
B
C
D

Figure 2.4. Denervation causes an increase in the rate of transcription from the delta subunit gene. (A) The level of hGH, endogenous delta subunit and actin mRNAs in innervated (Inn) and denervated (Den) muscles from line δGH2 were determined by RNase protection (Materials and methods). The position of the protected bands are indicated with arrows. (B) In four lines (δGH1-4), the level of hGH and delta subunit mRNAs, normalized to the level of actin mRNA, is 10-20 fold greater in denervated than in innervated muscle. The level of hGH expression in lines δGH4 and δGH5 is about 40 fold lower than in line δGH3. Lower left leg muscles were denervated by cutting the sciatic nerve, and innervated and denervated muscles were assayed 4 days later. The value for denervated muscle from line δGH3 was assigned 100%, and all other values are expressed relative to δGH3 denervated muscle. The abundance of actin mRNA was 20% lower in denervated than in innervated muscle.

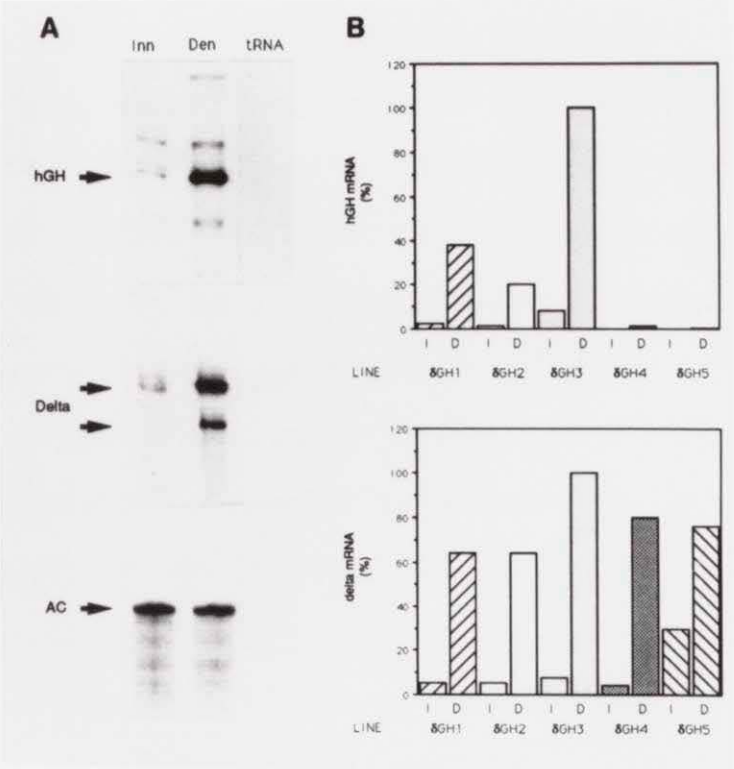

A
Inn
Den
tRNA
hGH
Delta
AC
B
hGH mRNA (%)
delta mRNA (%)
LINE
δGH1
δGH2
δGH3
δGH4
δGH5

Figure 2.5. Non-synaptic nuclei in denervated muscle are heterogeneous, and nuclei that express the delta subunit gene at an enhanced rate are positioned close to non-synaptic AChR clusters. Three myofibers are shown, one in A and B another in C and D, and a third in E and F. The myofibers were double-labelled with antibodies against hGH (A,C,E) and with TMR-α-BGT to stain AChRs (B,D,F). Non-synaptic AChR clusters were detected with TMR-α-BGT on approximately one-third of the denervated myofibers (B,D,F, long arrows), and hGH was observed in the region of each AChR cluster (A,C,E, short arrows). Non-synaptic AChR clusters are usually found further than 500 μm from synaptic sites (B,D), although they can occur adjacent to the denervated synaptic site (F, curved arrow). In some cases the hGH-positive nuclei are in the vicinity of AChR clusters (C,D), whereas in other cases, hGH-positive nuclei are found precisely underlying AChR clusters (A,B and E,F). The denervated synaptic site is readily distinguished from non-synaptic AChR clusters by its characteristic shape and size, and was identified on each myofiber that had non-synaptic AChR clusters. Bar = 12 μm for (A,B,C,D) and 30 μm for (E,F).

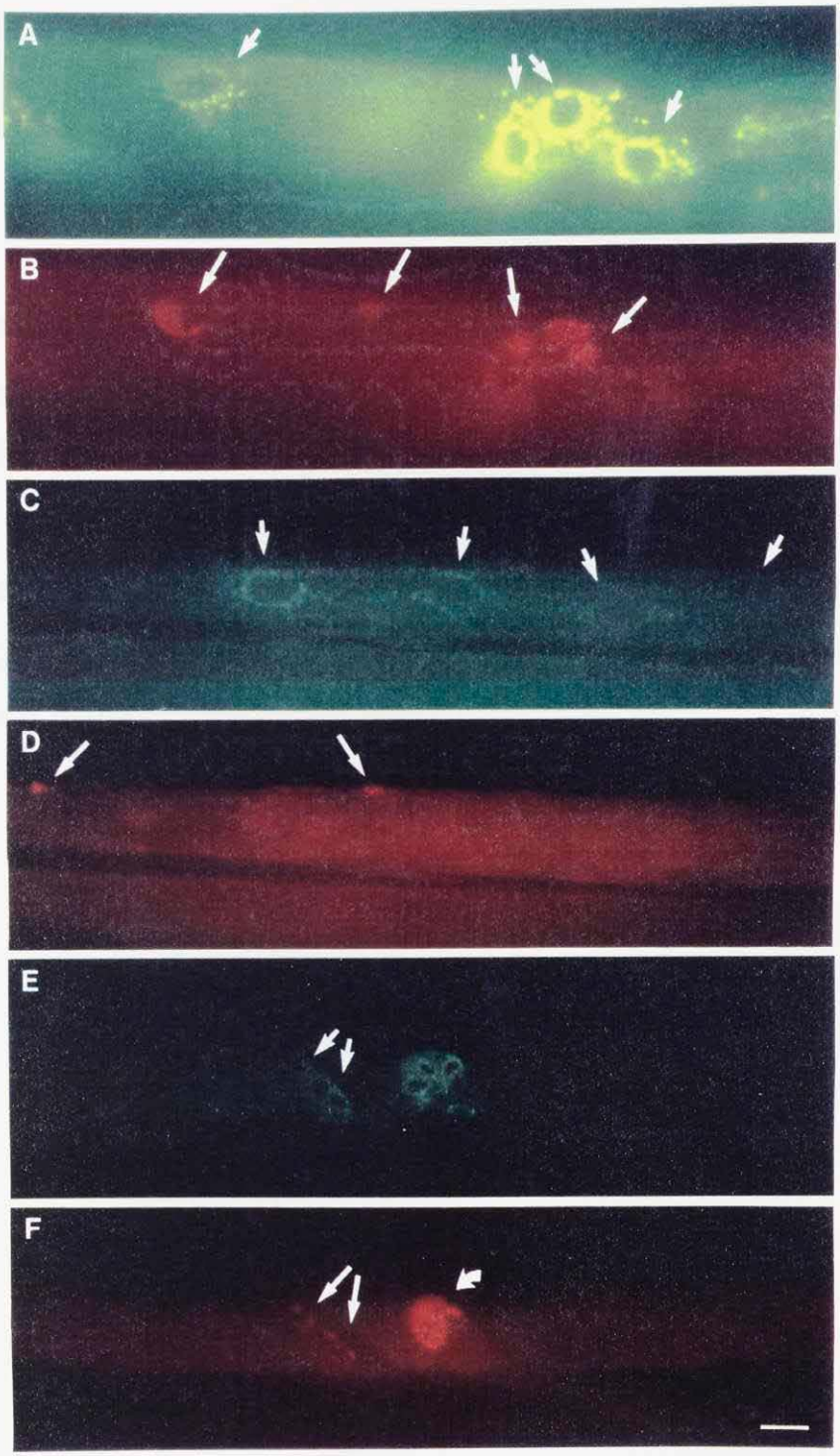

A
B
C
D
E
F

Figure 2.6. Delta subunit gene is transcribed preferentially in synaptic nuclei in newborn muscle. hGH is concentrated at synaptic sites in muscle from newborn transgenic mice. An individual myofiber from the diaphragm muscle, which was double-labelled with antibodies against hGH (A) and with TMR-α-BGT (B), was mounted whole and viewed with optics selective for fluorescein (A) and rhodamine (B). hGH is concentrated at the perinuclear region of nuclei immediately beneath the postsynaptic membrane (arrows); there are less nuclei at synaptic sites in newborn muscle than in adult muscle. Bar = 10 μm.

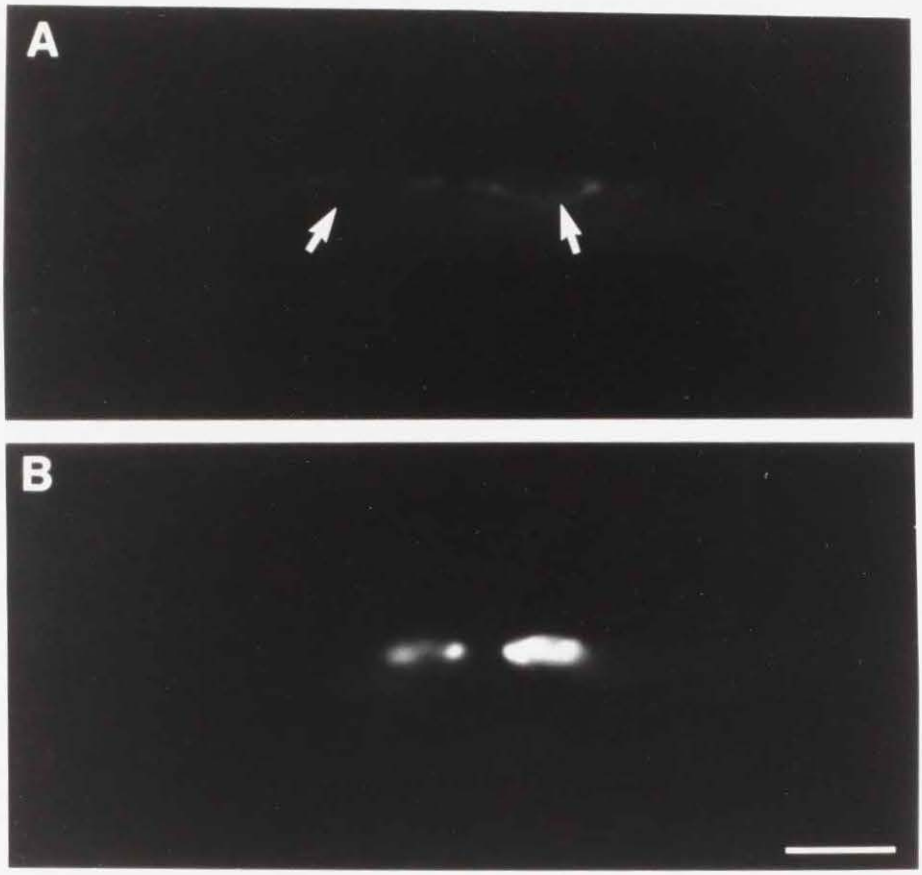

A
B

Figure 2.7. Myogenin is not expressed specifically at synaptic sites.

Diaphragm muscles from newborn rats (A,B), or adult rats (C,D) were labelled with a monoclonal antibody (F5D) against myogenin (A,C) and TMR-α-BGT (B,D). A whole mount of newborn muscle is shown in A and B, whereas a single adult myofiber is shown in C and D. Myogenin is expressed in nuclei throughout developing myofibers from newborn rat muscle, but myogenin is not concentrated at synaptic sites (A,B). The whole mount of the innervated zone of newborn diaphragm muscle contains more than one hundred myofibers, but only several dozen of the myofibers and synaptic sites are in focus. Myogenin is not detectable at synaptic sites or non-synaptic regions of innervated, adult muscle (C,D). Bar = 30 μm.

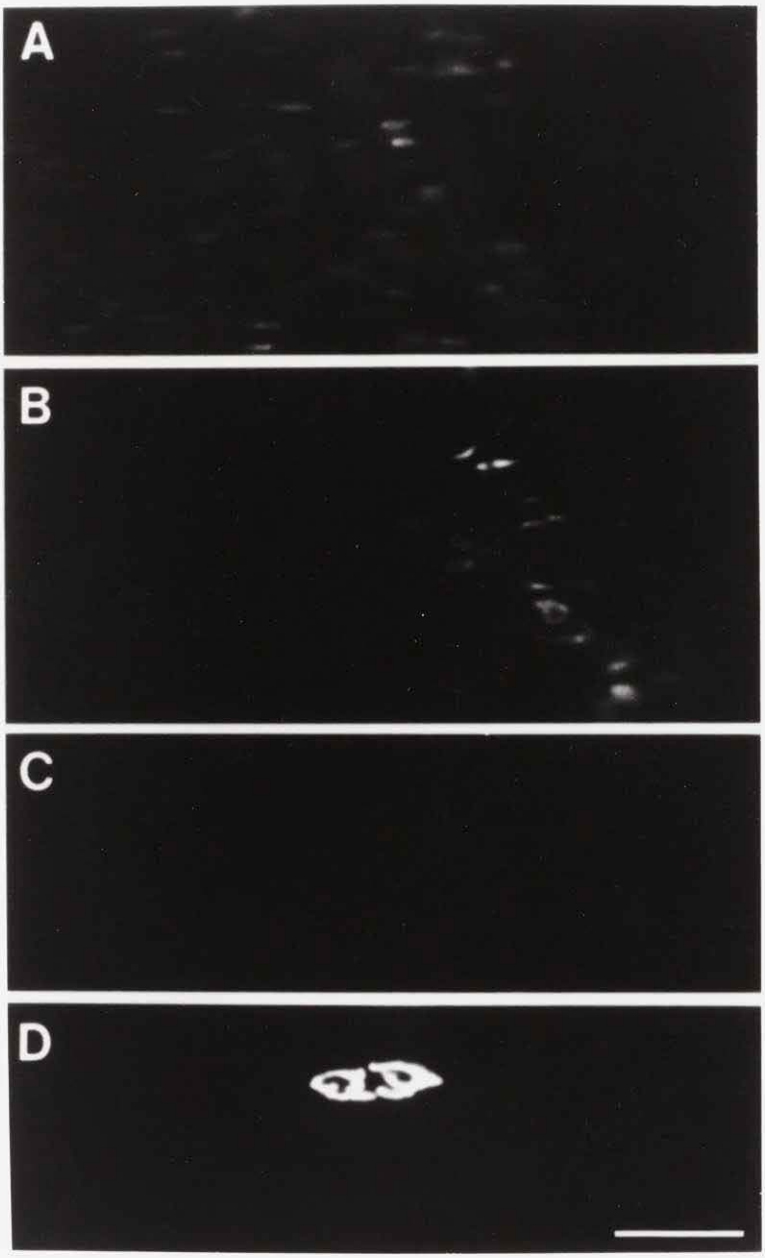
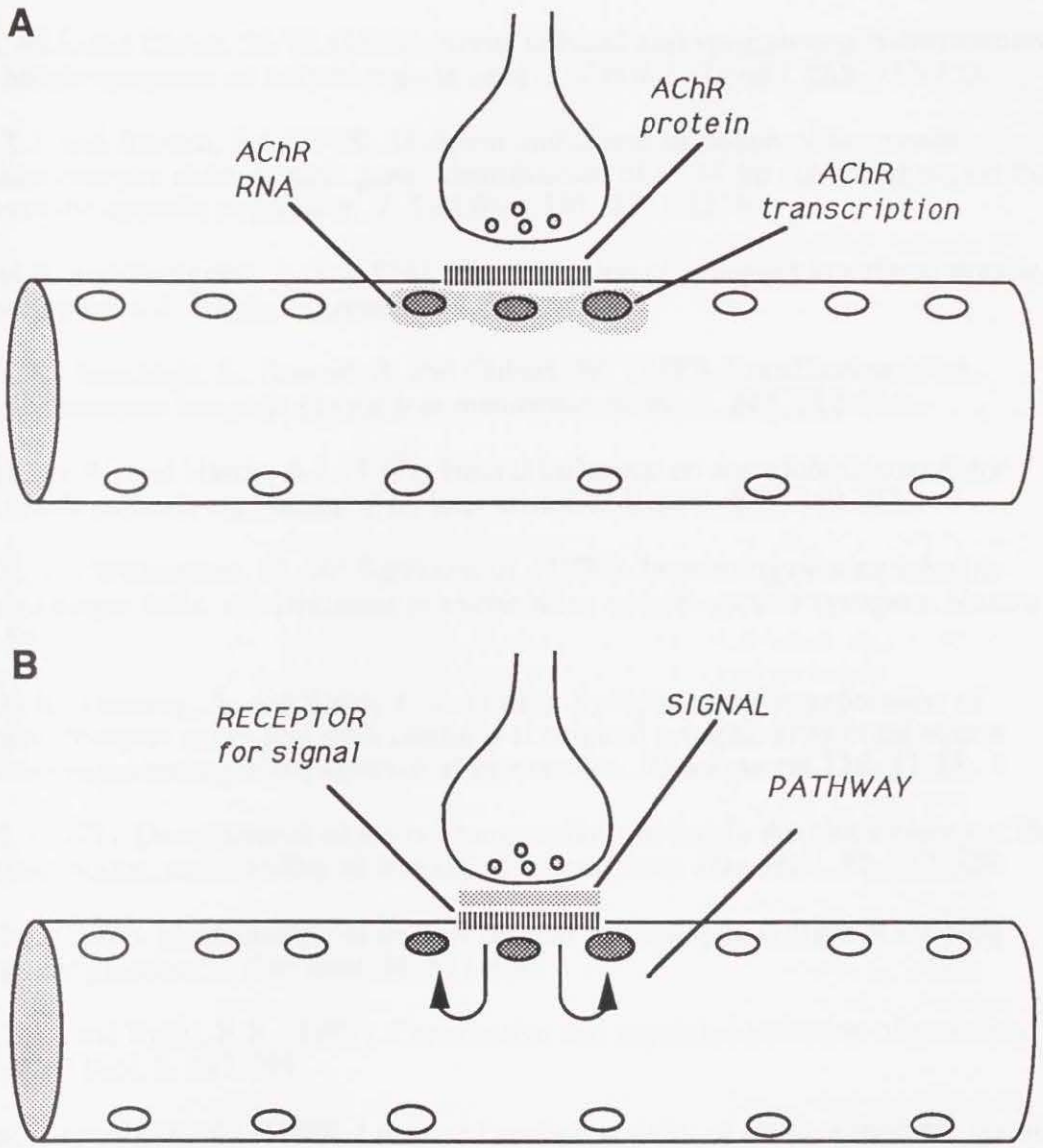

A
B
C
D

Figure 2.8. A model for synapse-specific expression of AChR genes.

(A) Transcription of AChR subunit genes is spatially restricted to the subset of myofiber nuclei that are located near the synaptic site. As a result, AChR mRNA is concentrated at the synaptic site, and AChRs are locally synthesized and inserted into the postsynaptic membrane. (B) Synpase-specific transcription is specified by a signal in the synaptic basal lamina. The signal for synapse-specific transcription is received by a receptor in the myofiber membrane, which may or may not be concentrated at the synaptic site. Activation of the receptor stimulates an intracellular signalling pathway that leads to transcription of AChR subunit genes by subsynaptic nuclei.


A
AChR
protein
AChR
RNA
AChR
transcription
B
RECEPTOR
for signal
SIGNAL
PATHWAY

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Chapter 3

Electrical Activity-Dependent Regulation of the Acetylcholine Receptor Delta Subunit Gene, MyoD and Myogenin in Primary Myotubes

SUMMARY

Expression of the skeletal muscle acetylcholine receptor (AChR) is regulated by nerve-evoked muscle activity. Studies using transgenic mice have shown that this regulation is controlled largely by transcriptional mechanisms, because responsiveness to electrical activity can be conferred by transgenes containing *cis*-acting sequences from the AChR subunit genes. The lack of a convenient muscle cell culture system for studying electrical activity-dependent gene regulation, however, has made it difficult to identify the important *cis*-acting sequences and to characterize an electrical activity-dependent signalling pathway. Emma Dutton and I developed a muscle culture system to study the mechanism of electrical activity-dependent gene expression. Gene fusions between the murine AChR δ-subunit gene and the human growth hormone gene were transfected into primary myoblasts, and the amount of growth hormone secreted into the culture medium from either spontaneously electrically active or inactive myotube cultures was measured. We show that 181 bp of 5' flanking DNA from the AChR δ-subunit gene are sufficient to confer electrical activity-dependent gene expression. In addition, we show that the rate of AChR δ-subunit gene expression is different among individual nuclei in a single myotube, but that highly expressing nuclei are not necessarily co-localized with AChR clusters. We also show that expression of MyoD and myogenin are regulated by electrical activity in primary myotube cultures and that all nuclei within a myotube express similar levels of MyoD and similar levels of myogenin.

INTRODUCTION

The skeletal muscle acetylcholine receptor (AChR) is a heteropentamer composed of four subunits (α_2, β, γ or ε, δ) that are encoded by separate genes, which are regulated similarly during development (Laufer and Changeux, 1989). AChR mRNA is expressed at high levels throughout developing muscle, but expression decreases as the myofibers are innervated, and in innervated adult muscle, AChR mRNA is largely restricted to the

single synaptic site (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman and Staple, 1989; Fontaine and Changeux, 1989; Brenner et al., 1990; Simon et al., 1992). Expression of AChR mRNA increases following denervation, and this additional AChR mRNA is found throughout the myofiber (Merlie et al., 1984; Goldman and Staple, 1989; Fontaine and Changeux, 1989). This increase in mRNA is prevented by direct stimulation of denervated muscle (Goldman et al., 1988), indicating that loss of electrical activity rather than loss of putative nerve-derived trophic factors is largely responsible for the increase in AChR expression following denervation. Studies using transgenic mice have shown that electrical activity regulates AChR gene expression by transcriptional mechanisms and that *cis*-acting elements that confer electrical activity-dependent gene expression are contained in less than one or two kbp of 5' flanking DNA from the α and δ-subunit genes respectively (Merlie and Kornhauser, 1989; Simon et al., 1992).

Little is known about the pathway that couples changes in muscle cell electrical activity to changes in AChR gene expression. Although transcriptional mechanisms are important (Tsay and Schmidt, 1989; Merlie and Kornhauser, 1989; Simon et al., 1992) transgenic mice are neither ideal for delineating the critical electrical activity-dependent regulatory sequences nor for characterizing an electrical activity-dependent signalling pathway. For these reasons, we developed a primary rat muscle cell culture system to investigate how muscle cell electrical activity regulates AChR gene expression. We show here that 181 bp of 5' flanking DNA from the AChR δ-subunit gene, which confers myotube-specific gene expression (Baldwin and Burden, 1988) and includes an enhancer and a single E-box, contains *cis*-acting elements that are sufficient for electrical activity-dependent gene regulation.

MATERIALS AND METHODS

Cell Culture

Primary rat (Schaffner and Daniels, 1982) or transgenic mouse muscle (Freerksen et al., 1986) cell cultures were prepared as described previously. Muscle tissue from fetal rats or from degenerating adult tibialis anterior muscle was dissociated, and cells were plated on Matrigel (Collaborative Research Inc., Bedford, MA)-coated dishes (Hartley and Yablonka-Reuveni, 1990). Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum and 10% horse serum. After transfection, cells were placed in differentiation medium (DMEM supplemented with 10% horse serum) to induce myoblast fusion.

Gene Fusions

Gene fusions between the mouse AChR δ gene and the hGH gene were constructed as described (Baldwin and Burden, 1988; Simon et al., 1992). Deletion of nucleotides from -500 to -182 placed activating sequences in the plasmid DNA closer to the δ-subunit promoter and caused increased expression in all cell types (Simon and Burden, unpublished). Because increased expression in myoblasts and fibroblasts obscured electrical activity-dependent regulation in myotubes, we inserted two Kbp of DNA (*Torpedo* dystrophin cDNA) (Yeadon et al., 1991) into the AChR δ (-181/+25)-hGH plasmid upstream of nucleotide -181 to minimize the effect of the activating plasmid sequences. The rabbit muscle creatine kinase (CK) gene (-1.2 kbp/+54) (17) was cloned into the p0hGH vector (Selden et al., 1986). Myoblasts were transfected using Ca^{2+}-phosphate precipitation.

hGH, CAT and RNAse Protection Assays

hGH radioimmunoassays were done according to the manufacturer's directions (Nichols Institute Diagnostic, San Juan, CA). CAT assays and RNAse protection assays were

done as described (Baldwin and Burden, 1988). The rat δ-subunit (Witzemann et al., 1990) and the skeletal muscle α-actin RNA (Hu et al., 1986) probes extended from nucleotides +361 to +900 and from +650 to +1257 respectively. Total RNA was isolated using guanidinium thiocyanate (Chirgwin et al., 1979). To control for potential variability in the number of myotubes in electrically inactive and active cultures, we normalized the amount of AChR δ mRNA to α-actin mRNA, which does not change significantly following denervation (Simon et al., 1992).

Immunofluorescence

Cells were fixed with 2% paraformaldehyde (in phosphate buffered saline) and permeabilized with 0.5% Triton X-100 (in phosphate buffered saline). AChR were labelled with tetramethylrhodamine-conjugated α-bungarotoxin (TMR-α-BGT) as described (Dutton and Olek, 1990). hGH, MyoD and myogenin were detected by indirect immunofluorescence using antibodies to hGH (Simon et al., 1992), MyoD (Tapscott et al., 1988, α-160-307) or myogenin (Wright et al., 1991), and appropriate fluorochrome-conjugated secondary antibodies.

RESULTS

AChR δ-Subunit Gene is Regulated by Electrical Activity in Primary Myotubes.

We developed a primary rat muscle cell culture system to identify *cis*-acting elements in the AChR δ-subunit gene that are sufficient to confer electrical activity-dependent gene expression. We transfected primary rat myoblasts with AChR δ-hGH gene fusions and measured the amount of hGH secreted in culture medium, which was changed daily. Multinucleated myotubes form and begin to contract spontaneously one to two days after transfection. Tetrodotoxin (TTX, 0.5 μg/ml), a sodium channel blocker, was added at the first sign of contraction to inhibit spontaneous electrical activity. Thus, we could

measure the amount of hGH secreted from electrically active and inactive myotube cultures.

Because AChR genes are expressed immediately after myoblast fusion and prior to the onset of electrical activity, we reasoned that relatively stable reporter gene products might preclude detection of subsequent down regulation of the AChR δ-subunit gene. Therefore, it was important to use a reporter gene product that would not accumulate in myotubes prior to the onset of electrical activity. Because hGH is secreted into the culture medium, which was changed and sampled daily, the amount of secreted hGH provided an accurate measure of the rate of δ-subunit transcription.

To control for variability in transfection efficiency and in the number of myotubes in each culture dish, we co-transfected cells with a gene fusion between the chloramphenicol acetyltrasferase (CAT) gene and the myosin light chain 1 (MLC) gene promoter, which is expressed specifically in myotubes (Donoghue et al., 1988), but is not regulated by electrical activity (Merlie and Kornhauser, 1989).

The amount of hGH secreted from myoblasts transfected with the AChR δ (-1,823/+25)-hGH gene fusion is low and increases 20-40 fold as myoblasts differentiate into myotubes (figure 3.1). Myotubes begin to contract spontaneously shortly thereafter, and they develop striations and often contain peripherally located nuclei; myotubes that were treated with TTX are electrically inactive and lack these morphological specializations (figure 3.3). Inactive myotubes secrete ~3-fold more hGH than active myotubes (figure 3.1). Although expression from active and inactive myotubes decreases with time, inactive myotubes continue to secrete ~3-fold more hGH than active myotubes (figure 3.1). These results indicate that the rate of AChR δ-subunit transcription is greater in electrically inactive myotubes than in active myotubes, and that *cis*-acting elements in the AChR δ-subunit gene confer electrical activity-dependent expression of hGH in primary rat myotube cultures.

To control for the possibility that the difference in the amount of hGH secreted by active and inactive myotubes might be due to differences in the properties of secretion between electrically active and inactive myotubes, we transfected myoblast cultures with a gene fusion between hGH and the CK gene, which is expressed specifically in myotubes (Jaynes et al., 1986) but is not regulated by electrical activity (Chahine et al., 1992). We found that active and inactive myotubes transfected with the CK-hGH gene fusion secrete similar amounts of hGH (figure 3.1 and table 3.1). Thus, the difference in the amount of hGH secreted between electrically active and inactive myotubes transfected with the AChR δ-hGH gene fusion is due to regulatory sequences in the δ-subunit gene.

181 bp of 5' flanking DNA Confer Electrical Activity-Dependent Gene Regulation.

To determine the minimal sequence sufficient to confer electrical activity-dependent expression, we prepared 5' deletions of the δ-subunit gene and transfected myoblasts with these additional gene fusions. Electrically inactive myotubes transfected with constructs containing 843 bp, 500 bp or 181 bp of 5' flanking DNA from the δ-subunit gene secrete ~3-fold more hGH than electrically active myotubes (table 3.1). These results demonstrate that 181 bp of 5' flanking DNA from the AChR δ-subunit gene are sufficient to confer electrical activity-dependent expression.

Endogenous and Transfected δ-Subunit Genes are Regulated Similarly.

Mice that carry an AChR δ (-1,823/+25)-hGH transgene express ~15 fold more hGH in denervated than in innervated muscle (Simon et al., 1992). Similarly, denervated muscle contains ~15-fold more AChR δ-subunit mRNA than innervated muscle (Simon et al., 1992). To determine whether the transfected AChR δ-subunit gene fusions and the endogenous AChR δ-subunit gene are regulated similarly in primary rat myotubes, we measured the level of endogenous AChR δ mRNA in active and inactive myotube

cultures (Materials and Methods). We found that electrically inactive myotubes express 3.45 ± 0.22 fold more endogenous AChR δ-subunit mRNA than active myotubes (n=6). Thus, the endogenous AChR δ-subunit gene and the transfected gene fusions are regulated similarly by electrical activity in primary rat myotube cultures. These results indicate that the difference in the level of δ-subunit mRNA in active and inactive myotubes is regulated largely by transcriptional mechanisms.

Heterogeneity of AChR δ-Subunit Gene Expression in Individual Myotubes.

Because hGH is processed in the Golgi apparatus prior to secretion and because the Golgi apparatus is closely associated with nuclei, the nuclear source of hGH transcription can be determined by studying the intracellular distribution of hGH with immunocytochemical techniques (Simon et al., 1992). Previous studies using transgenic mice carrying an AChR δ (-1,823/+25)-hGH gene fusion showed that hGH expression is restricted to nuclei positioned near AChR clusters (Simon et al., 1992). Because AChR clusters are found on the surface of myotubes grown in cell culture, we wondered whether nuclei near these spontaneous AChR clusters preferentially express the AChR δ-subunit gene. Myotube cultures, prepared from transgenic mice satellite cells (Freerksen et al., 1986; Simon et al., 1992), were stained with antibodies to hGH and with TMR-α-BGT to determine whether nuclei near AChR clusters preferentially express hGH. We found that the abundance of hGH is indeed greater around a subset of nuclei in primary myotubes (figure 3.2). There is a poor correlation, however, between the position of highly expressing nuclei and the site of AChR clusters (figure 3.2); importantly, AChR clusters are not necessarily found near highly expressing nuclei and highly expressing nuclei are not necessarily found near AChR clusters (figure 3.2). These results indicate that the presence of an AChR cluster is not sufficient to induce increased AChR δ-subunit transcription in nearby nuclei and that increased AChR

δ-subunit expression is not sufficient to specify the site of an AChR cluster in primary rat myotubes.

Expression of MyoD and Myogenin are Regulated by Electrical Activity in Primary Myotubes.

To determine whether this culture system might be suitable for studying other gene products that are regulated by electrical activity, we studied the expression of MyoD and myogenin in active and inactive myotube cultures. MyoD and myogenin, basic helix-loop-helix proteins, are of particular interest because they are potential regulators of AChR subunit genes (Piette, et al., 1990; Gilmour et al., 1991), and following denervation, their mRNAs increase in abundance prior to AChR mRNA (Witzemann and Sakmann, 1991; Eftimie et al., 1991; Duclert et al., 1991). We stained electrically active and inactive myotubes with antibodies to hGH, MyoD and myogenin. As expected, we found that intracellular hGH expression is greater in electrically inactive than in active myotubes (figure 3.3). Like hGH, MyoD and myogenin are also more abundant in electrically inactive than in active myotubes (figure 3.3). Thus, the level of MyoD and myogenin protein expression is regulated by electrical activity in myotubes grown in cell culture.

In contrast to the non-uniform expression of the AChR δ-subunit gene (figure 3.2), all nuclei within a myotube appear to express similar levels of MyoD and similar levels of myogenin (figure 3.3). Thus, different nuclei within a multinucleated myotube express similar levels of MyoD and similar levels of myogenin protein, but they can express the AChR δ-subunit gene at different rates. These results indicate that additional factors, including potential regulation of MyoD or myogenin activities, control AChR δ-subunit gene expression.

DISCUSSION

We developed a primary rat muscle culture system to study electrical activity-dependent gene expression, and we show here that 181 bp of 5' flanking DNA from the murine AChR δ-subunit gene are sufficient to confer electrical activity-dependent gene expression in primary rat myotubes. This is a convenient system for studying electrical activity-dependent gene expression for the following reasons: (1) an electrical activity-dependent signalling pathway can be characterized more readily in cultured myotubes than in animals, (2) neither innervation nor extracellular stimulating electrodes are required, because these primary myotubes contract spontaneously, and (3) a decrease in the rate of transcription is rapidly and accurately reflected by a decrease in hGH secretion. In addition, because hGH expression can be visualized by immunofluorescence, this system may be useful for studying synapse-specific transcription.

The magnitude of electrical activity-dependent regulation that we detect in cell culture is less than we find in transgenic mice (Simon et al., 1992). One explanation for this discrepancy is that the frequency of electrical activity, which is known to influence AChR expression (Lomo and Westgaard, 1975), may not be optimal in our cultures for repressing AChR expression. Further, since some myotubes (~25%) in active cultures do not contract, expression from these inactive myotubes is likely to decrease the measured effect of electrical activity.

We find that spontaneously active myotubes regulate transfected and endogenous genes similarly, and these results indicate that regulation of δ-subunit expression by electrical activity is controlled largely by transcriptional mechanisms. Chahine et al. have reported that 102 bp of 5' flanking DNA from the rat AChR δ-subunit gene are sufficient to confer electrical activity-dependent regulation in cultured myotubes that are stimulated with extracellular electrodes; these myotubes, however, regulate the transfected δ-subunit gene fusion less strongly (4-fold) than the endogenous δ-subunit gene (10-fold) (Chahine

et al., 1992). Although the reported difference in regulation between endogenous and transfected genes might suggest additional post-transcriptional regulation of AChR expression, it is possible that accumulation of a stable reporter product (luciferase) may account for the reported difference.

The mouse and rat δ-subunit sequences that confer electrical activity-dependent expression are 96% identical and contain a single E-box near the transcripition start site (Baldwin and Burden, 1988; Chahine et al., 1992). Thus, comparison of these two sequences provides little additional information regarding the key *cis*-acting elements and indicates the need for further functional studies.

The AChR δ-subunit gene in adult myofibers is expressed preferentially in nuclei positioned near synaptic AChR clusters and in nuclei near AChR clusters in non-synaptic regions of denervated muscle (Simon et al., 1992). These results raise the possibility that the position of highly expressing nuclei and clustering of AChR protein are causally linked. Previous studies have shown that AChR mRNAs are expressed preferentially in a subset of nuclei in cultured myotubes (Fontaine and Changeux, 1989; Bursztajn et al., 1989; Harris et al., 1989); however, because it is difficult to combine *in situ* hybridization with immunocytochemistry, it has not been possible to determine whether these highly expressing nuclei are associated with clusters of AChR protein. We show that a subset of nuclei in cultured myotubes express the AChR δ-subunit gene at a greater rate than other nuclei in the same myotube but that the position of these highly expressing nuclei is not necessarily correlated with the location of AChR clusters. The presence of nuclei which express the δ-subunit gene at a high rate would not specify the site of an AChR cluster, if these nuclei do not also express the other AChR subunit genes at a high rate; further studies will be necessary to determine whether all four AChR subunit genes are highly expressed in the same subset of nuclei. Moreover, AChR clusters in embryonic myotubes usually occur near patches of extracellular matrix that may contain signals important for AChR clustering (Sanes and Lawrence, 1983; Gordon et al., 1992), and it is possible that

highly expressing nuclei in primary cultures are not located near patches of such extracellular matrix. We do not know how transcriptional differences between nuclei in primary myotubes are established; nuclei in the synaptic region of adult muscle are also transcriptionally distinct from other myofiber nuclei (Klarsfeld et al., 1991; Sanes et al., 1991; Simon et al., 1992), and it is possible that mechanisms that distinguish subsets of nuclei in cultured myotubes are similar to mechanisms that act to selectively regulate gene expression in synaptic nuclei. Our results indicate that the formation of AChR clusters at synaptic sites is not simply a consequence of the proximity of highly expressing nuclei; rather, it appears more likely that different signals, one which activates an AChR clustering pathway and a second which activates an AChR transcriptional pathway, co-exist at synaptic sites.

There is evidence that an increase in intracellular [Ca2+] and stimulation of protein kinase C activity may be involved in electrical activity-dependent inactivation of the chick α subunit gene (Klarsfeld et al., 1989; Laufer et al., 1991; Huang et al., 1992). It is not clear how activation of protein kinase C might lead to inactivation of AChR subunit genes, but it is possible that this pathway involves post-translational modification of myogenic bHLH proteins, resulting in their inability to activate their own genes or downstream genes, including the AChR subunit genes. A single binding site (E-box) for myogenic transcription factors, which is located close (-24/-15) to the transcription start site, is contained in the 181 bp of the AChR δ-subunit gene that confer electrical activity-dependent expression (Baldwin and Burden, 1989). This E-box and an enhancer, which is located between -150 and -50 and is active in all cell types tested, are critical for maximal expression of the AChR δ subunit gene in myotubes (Chapter 4). Thus, it will be important to determine whether these elements are also required for electrical activity-dependent regulation. Moreover, it will be important to determine whether myogenic bHLH proteins are modified by electrical activity, and if so, whether such modification(s) are critical for regulation of AChR genes.

Figure 3.1. AChR δ-subunit gene is regulated by electrical activity in primary myotubes. The amount of hGH secreted from a single culture was measured on eight consecutive days, and the mean ± SE for four sister cultures is shown. Electrically inactive myotubes transfected with a δ (-1,823/+25)-hGH gene fusion secrete ~3-fold more hGH than active myotubes transfected with the same gene fusion. Electrically active and inactive myotubes transfected with a CK-hGH gene fusion secrete similar amounts of hGH. Loss of transfected DNA and/or cell loss is probably responsible for the progressive decrease in hGH expression observed in cultures transfected with either gene fusion ; nevertheless, inactive myotubes transfected with δ (-1,823/+25)-hGH secrete ~3-fold more hGH than active myotubes at all times. Squares indicate expression from myoblasts and myotubes before twitching was observed; TTX was added at the first sign of contraction.

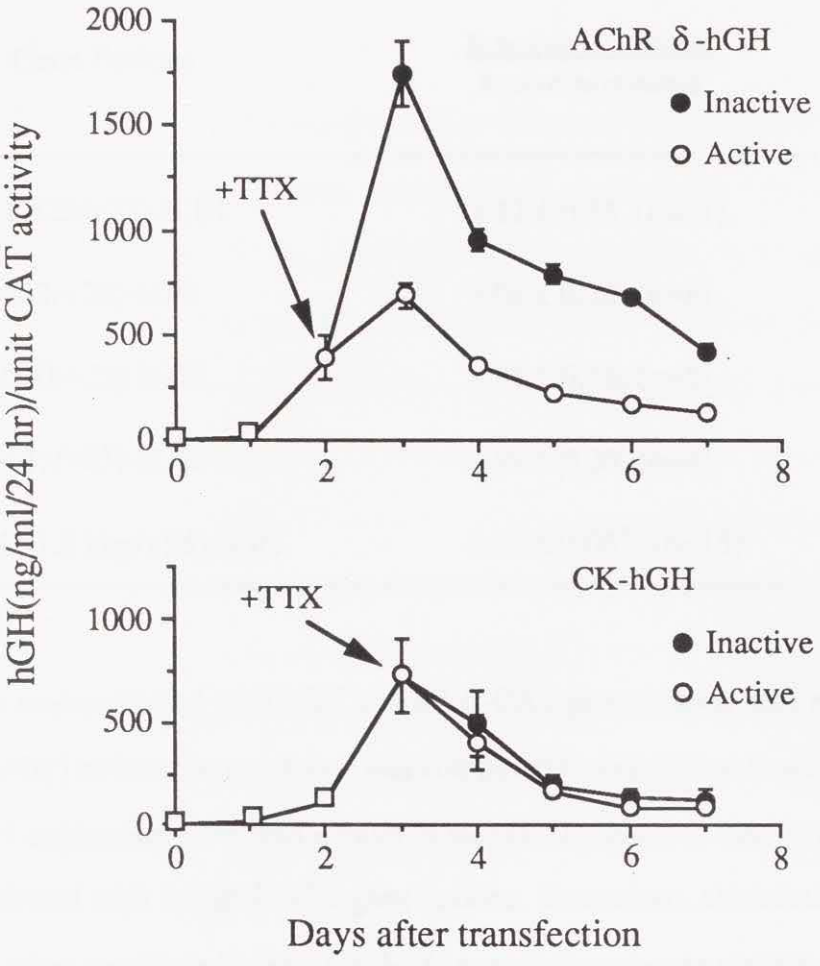

AChR δ-hGH
Inactive
Active
+TTX
2000
1500
1000
500
0
0
2
4
6
8
CK-hGH
+TTX
Inactive
Active
1000
500
0
0
2
4
6
8
hGH(ng/ml/24 hr)/unit CAT activity
Days after transfection

Table 3.1. 181 bp of 5' flanking DNA from the AChR δ-subunit gene contain *cis*-acting elements for electrical activity-dependent regulation.

Gene Fusions	Gene Expression Inactive myotubes / Active myotubes
δ(-1,823/+25)-hGH	3.22 ± 0.56, (n=11)
δ(-843/+25)-hGH	3.04 ± 0.20, (n=8)
δ(-500/+25)-hGH	3.31 ± 0.56, (n=5)
δ(-181/+25)-hGH	2.89 ± 0.29, (n=4)
CK(-1.2 kbp/+54)-hGH	1.15 ± 0.06*, (n=15)

Myoblasts were co-transfected with hGH and MLC-CAT gene fusions, and expression (hGH/CAT activity) in inactive myotubes was compared to expression in active myotubes. hGH expression is ~3-fold greater in electrically inactive than in active myotubes transfected with AChR δ-hGH gene fusions. In contrast, electrically inactive and active myotubes transfected with a CK-hGH gene fusion secrete similar amounts of hGH. Thus, the increased hGH expression in electrically inactive myotubes transfected with the AChR δ-hGH gene fusions is due to electrical activity-dependent regulatory sequences contained within the δ-subunit gene. The means ± SE are shown.

*Significantly different from AChR δ (-1,823/+25)-hGH, (Student's *t* test, p=0.01).

Figure 3.2. AChR δ-subunit gene is expressed preferentially by a subset of nuclei in individual myotubes. Myotube cultures, prepared from satellite cells derived from transgenic mice carrying an AChR δ (-1,823/+25)-hGH gene fusion, were stained with antibodies to hGH and myogenin, or with antibodies to hGH and TMR-α-BGT. Different nuclei in an individual myotube express similar levels of myogenin (A), whereas hGH expression (B) is enhanced near a subset of nuclei (arrows) in the same myotube. The position of highly expressing nuclei (C), however, is not necessarily correlated with sites of AChR clusters (D). Importantly, AChR clusters are not always associated with highly expressing nuclei (arrowhead), and highly expressing nuclei are not always associated with AChR clusters (long arrow). Because a substantial fraction of the myotube contains highly expressing nuclei (B, C), it is not clear whether co-distribution of AChR clusters (short arrows in D) and highly expressing nuclei (short arrows in C) is significant or occurs by chance. Bar = 10 μm.

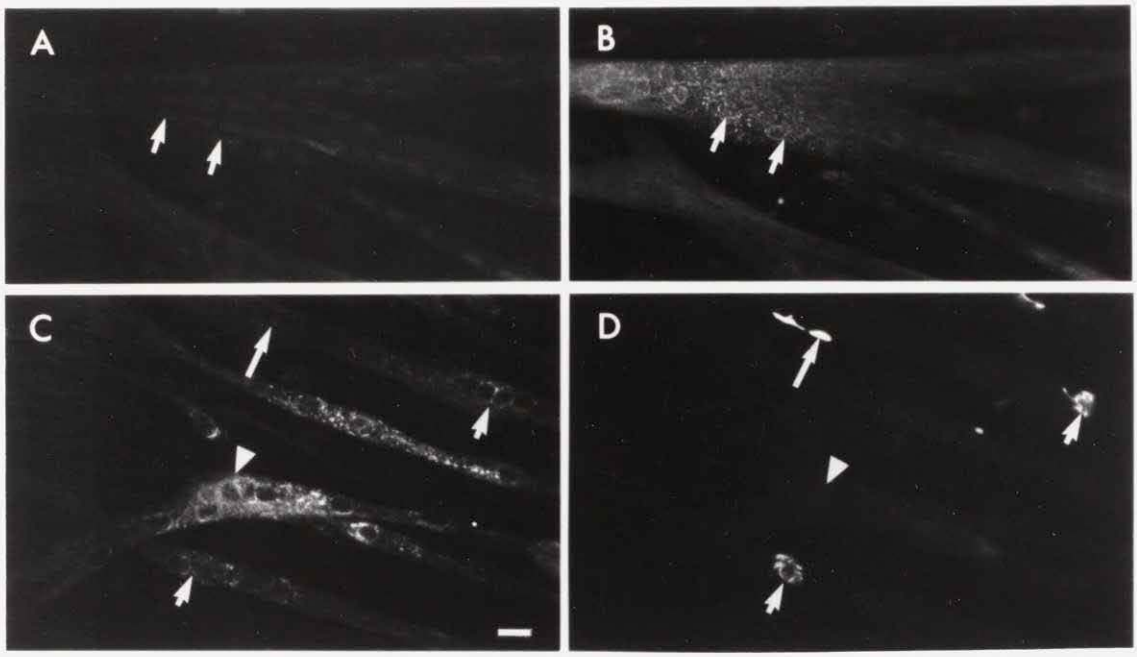
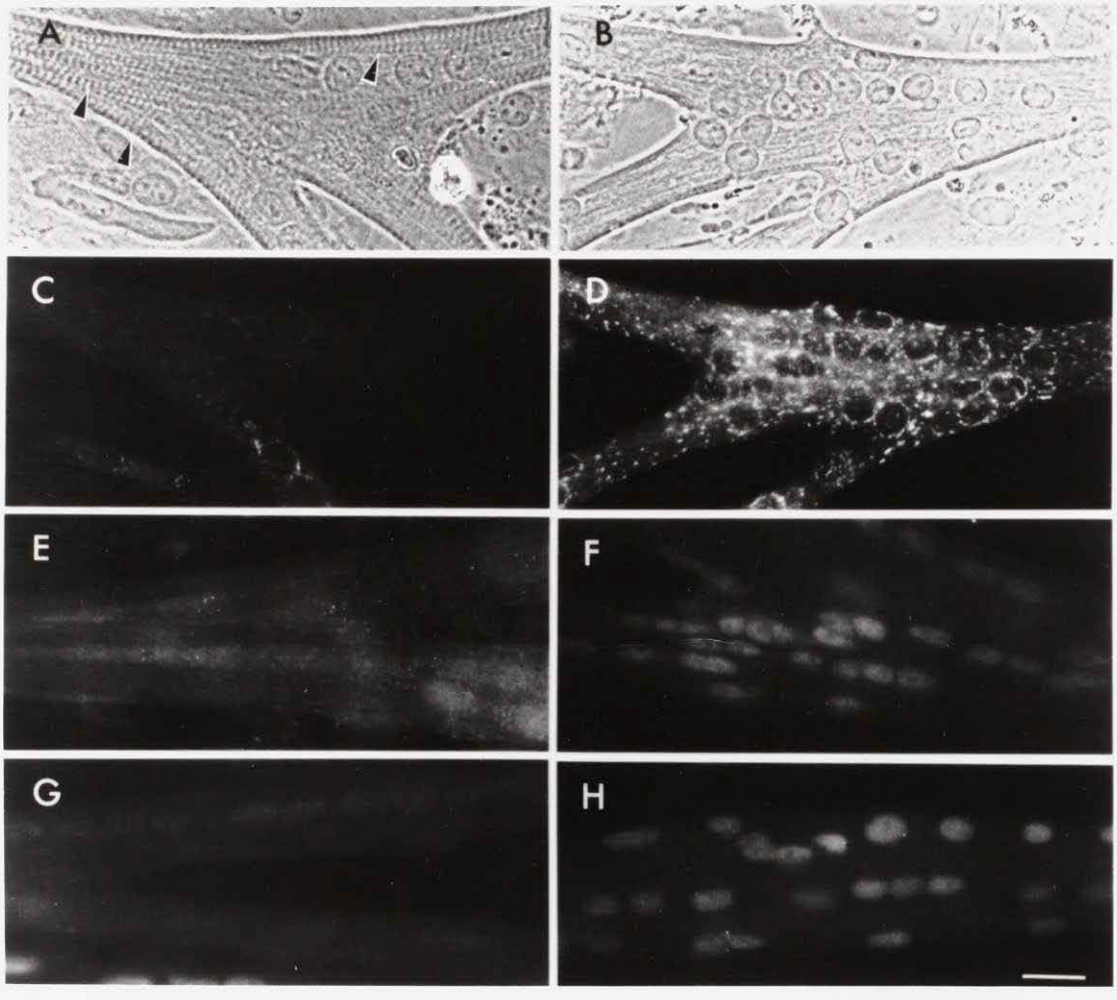

A
B
C
D

Figure 3.3. MyoD and myogenin are regulated by electrical activity in primary myotubes. Electrically active and inactive transgenic mouse or primary rat myotubes were stained with antibodies to hGH, MyoD or myogenin. Active myotubes (A) are morphologically distinguished from inactive myotubes (B), because active myotubes develop striations (arrows) and often have peripherally located nuclei. hGH expression is greater in electrically inactive (D) than active (C) myotubes. Similarly, expression of MyoD (E,F) and myogenin (G,H) is greater in electrically inactive (F,H) than active (E,G) myotubes. Bar = 10 μm.


A
B
C
D
E
F
G
H

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Chapter 4

Positive and Negative Regulation of the Acetylcholine Receptor Delta Subunit Gene Mediated by an E-box

SUMMARY

Expression of the skeletal muscle acetylcholine receptor (AChR) is induced during muscle development and is subsequently regulated by innervation: local synaptic factors activate transcription of AChR genes in the synaptic nuclei, while propagated electrical activity represses AChR transcription in nuclei throughout the remainder of the myofiber. Because both the initiation and the subsequent regulation of AChR expression are controlled by transcriptional mechanisms, an understanding of the pathways that regulate AChR expression following innervation is likely to require knowledge of the steps required to activate AChR genes during myogenesis. Thus, I sought to identify the *cis*-acting sequences that control muscle-specific expression of the AChR δ-subunit gene. I show that 148 bp of 5' flanking DNA from the AChR δ-subunit gene contains two regulatory elements that control muscle-specific gene expression. One element is a binding site for basic helix-loop-helix proteins (E-box). I show that this E-box has a dual role, since it is important both for activation of the δ-subunit gene in myotubes and for repression in myoblasts and non-muscle cells. MyoD/E2A and myogenin/E2A heterodimers bind to this E-box and may activate δ-subunit gene expression in myotubes. An additional E-box binding activity, which does not contain MyoD, myogenin or E2A proteins, is present in muscle and non-muscle cells and may be responsible for repressing δ-subunit transcription in non-muscle cells. The other *cis*-acting element is an enhancer that binds proteins in muscle and non-muscle cells. I show that this enhancer is required for expression of the δ-subunit gene in muscle, but it does not confer muscle-specificity.

INTRODUCTION

The skeletal muscle acetylcholine receptor (AChR) is a multisubunit, ligand-gated ion channel which is the neurotransmitter receptor at all vertebrate neuromuscular junctions. The four different subunits (α, β, γ, δ) are encoded by separate genes which are expressed coordinately during muscle development (Laufer and Changeux, 1989). Transcription of

these genes, unlike that of most other muscle genes, is regulated subsequently by innervation: local synaptic signals activate transcription of AChR genes in myofiber nuclei that are near the synapse and propagated electrical activity represses AChR transcription in nuclei throughout the myofiber (Klarsfeld et al., 1987; Goldman et al., 1988; Goldmann and Staple, 1989; Merlie and Kornhauser, 1989; Tsay and Schmidt, 1989; Sanes et al., 1991; Simon et al., 1992)

Little is known about the signalling pathways involved in neural regulation of AChR gene expression. The AChR subunit genes are first expressed during myogenesis, when myoblasts stop dividing and fuse into multinucleated myofibers (Buonanno and Merlie, 1986; Witzemann et al., 1989), and these genes are regulated subsequently by innervation. Thus, an understanding of the mechanisms that regulate AChR expression following innervation may require knowledge of the steps required to activate AChR genes during myogenesis. In particular, *cis*-acting elements that are important for regulation during myogenesis may also be targets for regulation by innervation. Thus, I sought to identify the *cis*-acting sequences that control AChR expression during myogenesis and to determine how these elements confer muscle-specific expression.

I analyzed the 5' flanking DNA of the AChR δ-subunit gene to identify the *cis*-acting sequences that control muscle-specific expression. I show here that 148 bp of 5' flanking DNA from the AChR δ-subunit gene contains two regulatory elements that control muscle-specific gene expression. One element is an E-box, a binding site for basic helix-loop-helix (bHLH) proteins (Baldwin, 1989). I show that this E-box has a dual role, since it is important for activation of the δ-subunit gene in myotubes and for repression in myoblasts and non-muscle cells. The other element is an enhancer that is required for expression of the delta subunit gene in muscle but does not confer muscle-specificity, since this enhancer can activate expression of a heterologous promoter in muscle and non-muscle cells.

MATERIALS AND METHODS

Plasmid constructions

The -843/+25 CAT, -148/+25 CAT, -128/+25 CAT, and -107/+25 CAT plasmids (Baldwin and Burden, 1988) contain 5' flanking DNA from the AChR δ-subunit gene fused to the chloramphenicol acetyltransferase (CAT) gene. To introduce mutations into the δ-subunit 5' flanking DNA contained in -843/+25 CAT, an EcoRI fragment was first cut out of -843/+25 CAT and ligated into the EcoRI site of the M13mp18 vector to make the δ-M13 plasmid. The EcoRI fragment from -843/+25 CAT contains 843 bp of δ-subunit 5' flanking DNA and a portion of the CAT gene. Single-strand δ-M13 plasmid DNA was prepared to use as a template in oligo-directed mutagenesis. Mutagenesis was performed as specified in the instructions of a commercially available oligo-directed mutagenesis kit supplied by Amersham Corporation. Oligonucleotides were synthesized by the Biopolymers Lab at MIT. Point mutations were introduced into E-box sites with oligonucleotides containing specific mismatches or limited random mismatches. Deletion mutations were introduced with oligonucleotides which hybridized to 15 bases on either side of the region to be deleted. The mutagenized EcoRI insert was sequenced to identify mutations and to check for any second site mutations. Double-strand δ-M13 plasmid DNA was prepared, and the EcoRI fragment containing the mutated δ sequence was cloned back into EcoRI-digested -843/+25 CAT plasmid.

The -1823/+25 hGH plasmid (Simon et al., 1992) contains δ-subunit 5' flanking DNA fused to the gene encoding human growth hormone (hGH). To construct -1823/+25 hGH plasmids that contain E-box mutations, a SacI/PstI fragment containing nucleotides -843/+25, was isolated from -843/+25 CAT E-box mutants, blunted, and ligated into the HincII site of the p0hGH plasmid (Selden et al., 1986) to make -843/+25 hGH E-box mutants. A HindIII/KpnI fragment from -1823/+25 hGH was then ligated with a HindIII/KpnI fragment from -843/+25 hGH E-box mutants to create -1823/+25 hGH E-box mutants.

The -148/-53 FBP-CAT and -53/-148 FBP-CAT plasmids were constructed by ligating a blunted EcoRI/HaeIII fragment from -148/+25 CAT into the blunted Sal I site of FBP-CAT (Baldwin, 1989). The FBP-CAT plasmid contains nucleotides -56/+109 from the c-fos gene fused to CAT. The -95/-53 FBP-CAT, -53/-95 FBP-CAT, -148/-95 FBP-CAT, and -95/-148 FBP-CAT plasmids were constructed by ligating annealed oligos containing Sal I overhangs into the Sal I site of FBP-CAT. Plasmid construction was confirmed by double-strand DNA sequencing. Plasmid DNA used in transfection experiments was purified once with a Qiagen tip-500 column and once by ultracentrifugation through CsCl.

Cell culture and transfections

C2C12 cells (C2 cells) were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 15% fetal calf serum and 50 µg/ml gentamycin. NIH-3T3 cells (3T3 cells) were grown in DMEM supplemented with 10% fetal calf serum and 50 µg/ml penicillin/streptomycin. C2 cells were initially plated at a density of 6.5×10^4 cells/60mm dish for myoblast transfections, or at 2.5×10^5 cells/60mm dish for cultures that were induced to differentiate into myotubes. 3T3 cells were plated at a density of 1.0×10^5 cells/60mm dish. Transient transfections were done by a Ca^{2+}-phosphate precipitation method (Wigler et al., 1979). Precipitates were left on cells at 37°C for approximately 15 hrs (C2 cells) or 6 hrs (3T3 cells). C2 cells were glycerol shocked for 3 min at room temperature (RT) and 3T3 cells were shocked for 30 sec. C2 myoblast cultures were returned to growth medium after glycerol shock and assayed for expression 36-48 hrs later. C2 cultures assayed as myotubes were kept in growth medium after glycerol shock until the cells became confluent, then transferred to differentiation medium (DMEM supplemented with 5% horse serum). Myotubes were assayed for expression 4-5 days after glycerol shock. 3T3 were assayed for expression approximately

48 hrs after glycerol shock. All transfections were done a minimum of two times, with duplicate dishes in each experiment.

δ-CAT (3.6 μg) or δ-FBP-CAT (3.6 μg) plasmids were cotransfected with 0.36 μg of pXhGH5 (Selden et al., 1986) to normalize for transfection efficiency. The pXhGH5 plasmid contains the metallothionein promoter fused to the hGH gene (Selden et al., 1986). CAT activity was normalized to the amount of hGH secreted by these cultures. δ-hGH plasmids (3.6 μg) were cotransfected with the pSV2CAT plasmid (Gorman et al., 1982)(0.36 μg) to normalize for transfection efficiency. The pSV2CAT plasmid contains the SV-40 early promoter fused to the CAT gene. Secreted hGH levels were normalized to the amount of CAT activity in these cultures. CAT assays and hGH assays were done as previously described (Baldwin and Burden, 1988).

Because mutations in E-box 1 resulted in elevated expression of δ-CAT gene fusions in myoblasts, I used δ-hGH plasmids to determine the effect of the E-box 1 mutations on myotube expression. I used hGH as the reporter rather than CAT, because CAT is relatively stable, and CAT synthesized in myoblasts may persist in the cytoplasm of myotubes during muscle differentiation. Thus, CAT expressed in myoblasts might obscure a decrease in δ-subunit gene expression in myotubes. hGH is a useful reporter product for such experiments, since hGH is secreted into the culture medium, which can be changed and sampled daily. Thus, hGH does not persist during muscle differentiation and more accurately reflects the rate of transcription.

Antibodies

Anti-MyoD antisera (Tapscott et al., 1988) and pre-immune sera was a gift from Andrew Lassar. This anti-MyoD antiserum is directed against the carboxy terminus of MyoD (amino acids 160-307), which is downstream of the bHLH domain. Anti-E2A antisera (Murre et al., 1989) and pre-immune sera was a gift from Cornelis Murre. This anti-E2A antisera was raised against the C-terminal 430 amino acids of E12 and crossreacts with

E47, E2-5(ITF-1), as well as the related protein E2-2(ITF-2) (Murre et al., 1989; and C. Murre, personal communication). The anti-myogenin hybridoma F5D cell line was a gift from Woodring Wright (Wright et al., 1991). The F5D monoclonal antibody binds to an epitope between amino acids 144-170, which is downstream of the bHLH domain. Supernatant from hybridoma cells was concentrated 15-fold in an Amicon-100 micro-concentrator for use in gel shift experiments.

Fusion proteins

Expression vectors for glutathione S-transferase-MyoD (GST-MyoD) and GST-myogenin were a gift from Andrew Lassar (Lassar et al., 1989). Fusions proteins were induced, and subsequently purified with glutathione-agarose beads, as described (Lassar et al., 1989). GST-rZEBc fusion protein was a gift from Tom Genetta. This fusion protein contains the C-terminal 310 amino acids of ZEB, which includes three zinc-finger motifs (T. Genetta, personal communication).

Oligonucleotides

The oligonucleotides listed below were used as probes in gel-shift reactions to test for E-box 1 binding activities. The wild-type oligonucleotide consists of nucleotides -25/-14 from δ–subunit 5' flanking DNA plus two additional nucleotides added to create a 5' overhang when annealed with a complementary oligonucleotide. Oligonucleotides with mutated E-box 1 sites carry the same mutations that were introduced into δ-CAT and δ-hGH transfection plasmids.

wild-type E-box 1:	5'-CGCAGCACCTGTCC-3'
mutation 1:	5'-CGCAGAACCTTTCC-3'
mutation 2:	5'-CGCAGCACGGTAAC-3'
mutation 3:	5'-CGCAGGACCTGTCC-3'
mutation 4:	5'-CGCAGCAACTGTCC-3'
mutation 5:	5'-CGCAGCACCAGTCC-3'

Gel-shift experiments

Annealed oligos were labelled with ^{32}P by a filling-in reaction with Klenow enzyme and [α-^{32}P]dCTP. Labelled probes were purified on polyacrylamide gels. Nuclear extracts were prepared by the method of Dignam et al. (1983), as modified by Abmayr and Workmann (1990). The final concentration of KCl used to extract nuclear proteins was approximately 300 mM. Nuclear extracts were pre-incubated in the binding buffer (25 mM Hepes-KOH, [pH 7.5], 50 mM KCl, 12.5 µM $ZnSO_4$, 5% glycerol, 0.1% NP-40, 1 mg/ml BSA, 0.5 mM DTT, 50 µg/ml poly[dI-dC]) for 10 min before addition of the probe. Approximately 0.1-0.5 ng of labelled probe (~30,000 cpm) were incubated with 3-7 µg of nuclear extract in a total volume of 20 µl. The mixture was incubated at RT for 10 minutes and then placed on ice for 5 minutes before loading the reaction on the gel. Electrophoresis was carried out at 4° C in a 5% polyacrylamide gel run in 0.5X TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). The gel was run for 4-5 hrs at 200V, fixed, and dried for autoradiography.

For gel-shift experiments in which antibodies were added, the antibodies were pre-incubated with the nuclear extracts for 10 min at RT, prior to the addition of the labelled probe. 1-2 µl of immune or pre-immune serum was used in each reaction. 0.5 µl of a 15-fold concentrated monoclonal antibody supernatant was used in each reaction.

For gel shift analysis of GST-rZEBc binding, approximately 10 ng of GST-rZEBc was pre-incubated in the binding buffer (25 mM Hepes-KOH, [pH 7.5], 50 mM KCl, 12.5 µM $ZnSO_4$, 5% glycerol, 0.1% NP-40, 0.2 mg/ml BSA, 0.5 mM DTT, 50 µg/ml poly[dI-dC]) for 10 minutes at RT. Labelled probes were added, and the mixture was incubated for 15 minutes at RT before loading on the polyacrylamide gel. Electrophoresis was carried out at RT, at 150V, for 1-2 hrs. Gel shift experiments with GST-MyoD and GST-myogenin were done as described for GST-rZEBc, except 35 ng of fusion protein was used and the binding buffer consisted of 20 mM HEPES (pH7.6), 50

mM KCl, 3 mM $MgCl_2$, 1mM EDTA, 8% glycerol, 0.5% NP-40, 0.2 mg/ml BSA, and 25 μg/ml poly(dI-dC).

RESULTS

Positive and negative regulation at an E-box

148 bp of δ-subunit 5' flanking DNA is sufficient to confer muscle-specific expression (Baldwin and Burden, 1988). This region contains a single binding site for basic helix-loop-helix (bHLH) proteins at -24/-15 (E-box 1, figure 4.1)(Baldwin, 1989). Because E-boxes in some, but not all muscle-specific genes are important for their activation in myotubes, I introduced point mutations into E-box 1 (E1) of the δ-subunit gene to determine the role of this E-box on δ-subunit gene expression.

I transfected C2 myoblasts with δ-hGH gene fusions, induced myotube differentiation with low serum-containing medium and measured the amount of hGH secreted into the medium. Mutations in E1 reduce hGH expression in myotubes (3-25 fold), suggesting that this site is important for activation of the δ-subunit gene during myogenesis (figure 4.2). Two additional E-boxes termed E2 (-270/-261) and E3 (-368/-359) are present in the δ-subunit 5' flanking DNA (figure 4.1); mutation of E2 or E3, individually or together, have no effect on expression (figure 4.3). These results suggest that the position and/or sequence context of E1 distinguish it from E2 and E3 as a site for activation in myotubes.

Surprisingly, mutations in E1 elevate expression in myoblasts (5-15 fold) and fibroblasts (3-7 fold), indicating that E1 is important for repressing gene expression in these cell types (figure 4.2). Because several mutations have the same effect, it is unlikely that I inadvertently created a new site for activation in myoblasts and fibroblasts. Mutation of E2 or E3, individually or together, have no effect on expression in myoblasts (figure 4.3). These results suggest that the position and/or sequence context of E1 distinguish it from E2 and E3 as a site for repression. My experiments show that E1 has a

dual role, since it is a site for activation in myotubes and a site for repression in non-muscle cells. E1 is therefore a critical element controlling muscle-specific expression of the δ-subunit gene.

Nuclear extracts from myotubes and myoblasts contain multiple E-box 1 binding activities

My transfection experiments show that E1 is a site for activation in myotubes. To identify E1 binding activities in myotubes, I performed gel-shift experiments with myotube nuclear extracts and an E1 probe. Myotube nuclear extracts contain three E1 binding activities (complexes 1, 2, and 3, figure 4.4). The binding of these activities is specific, since point mutations in E1 eliminate or greatly reduce binding (figure 4.4).

Candidates for the activators that bind to E1 include heterodimeric complexes of myogenic and E2A bHLH proteins. These heterodimeric complexes have been shown to transactivate expression of muscle-specific genes by binding to E-boxes present in their *cis*--acting regulatory regions. Antibodies to MyoD disrupt the formation of complex 1, and a monoclonal antibody to myogenin supershifts complex 2 (figure 4.5). Both complex 1 and complex 2 are disrupted by antibodies to E2A proteins (figure 4.5). These results indicate that complex 1 contains MyoD/E2A heterodimers and that complex 2 contains myogenin/E2A heterodimers. Since MyoD/E2A and myogenin/E2A heterodimers can transactivate expression of other genes by binding to E-boxes, it is likely that these heterodimeric complexes bind to E1 in vivo and activate δ-subunit expression in myotubes. Complex 3, which is also present in myoblasts and non-muscle cells (see below), is not affected by any of the antibodies, indicating that this activity does not contain MyoD, myogenin, or E2A proteins.

My experiments suggest that heterodimeric complexes of myogenic and E2A bHLH proteins activate expression in myotubes by binding to E1. The identity of a factor which could repress expression in myoblasts and fibroblasts is less clear. E-box binding

activities have been detected in myoblasts and non-muscle cells (Brennan and Olson, 1990; Lassar et al., 1991; Murre et al., 1991; Numberger et al., 1991; Jen et al., 1992), but these activities are not well characterized. To identify potential repressor activities, I performed gel-shift experiments with myoblast nuclear extracts and the E1 probe. Myoblast extracts contain two activities that comigrate with myotube complexes 1 and 3 (figure 4.4). The presence of an additional, weaker complex was more variable. The binding of these activities is specific, since point mutations in E1 eliminate or greatly reduce binding (figure 4.4). Complex 3, but not complex 1 is also present in nuclear extracts from 3T3 cells and Hela cells (data not presented). Complex 1, as expected from my results with myotube extracts, reacts with antibodies to MyoD and E2A proteins (figure 4.5), indicating that complex 1 contains MyoD/E2A heterodimers. Complex 3 does not react with any of the antibodies tested, suggesting that this complex does not contain MyoD, myogenin, or E2A proteins (figure 4.5).

Complex 3 is a good candidate for an activity that could repress the δ-subunit in myoblasts and fibroblasts, since mutations in E1 that disrupt complex 3 binding result in elevated expression in these cell types (figure 4.2 and figure 4.4). Although MyoD/E2A heterodimers are also present in myoblasts, and could conceivably function as a repressor in these cells, MyoD/E2A is not present in 3T3 cells, yet repression of the delta subunit gene in 3T3 cells is mediated by E1. Thus, I favor the idea that complex 3 is a potential repressor in myoblasts and 3T3 cells. Complex 3 could be a bHLH protein; however, because complex 3 does not crossreact with antibodies to E2A proteins, it must be distinct from E12, E47, E2-5/ITF-1 and E2-2/ITF-2. Alternatively, complex 3 may belong to another class of DNA-binding proteins.

ZEB, a zinc-finger E-box binding protein, binds to E-box 1 in vitro.

A recently identified zinc-finger E-box binding protein, termed ZEB, was cloned from a Hela expression library based on its ability to bind to the μE5 motif (E-box) of the IgH

enhancer (T. Genetta and T. Kadesch, personal communication). Similar to E1 of the δ-subunit, the μE5 site is important for both activation and repression: the μE5 site is involved in repression in non-B cells and in activation in B-cells (Ruezinsky et al., 1991). Because ZEB RNA is expressed in many cell types, including myoblasts and myotubes (T. Genetta and T. Kadesch, personal communication), and because ZEB may be involved in repression of the IgH enhancer, I considered the possibility that ZEB might bind E1 and repress the δ-subunit gene in myoblasts and non-muscle cells. A glutathione-S-transferase/ZEB fusion protein (GST-rZEB$_C$), containing the carboxy-terminal region of ZEB, does indeed bind to the E1 probe, but not to mutated E1 sites (figure 4.6). At present, I do not know whether complex 3 contains ZEB; available antibodies to ZEB do not affect E-box binding activities detected with nuclear extracts from any cell line tested, including Hela cells, from which ZEB was cloned. Nevertheless, these data are consistent with the idea that ZEB may bind to E1 in vivo.

The region -148/-53 is necessary for expression in myotubes and is an enhancer that is active in all cell types tested.

Deletion analysis shows that E1 is not the only element in the δ-subunit promoter that is critical for expression in myotubes. Figure 4.7 illustrates that deletions of sequences further 5' reduce or abolish expression in myotubes: deletion of -95/-68 reduces expression 250-fold, and deletion of -67/-53 reduces expression 1000-fold (figure 4.7).

I determined whether this region had enhancer activity by testing whether -148/-53 could stimulate expression of the c-fos basal promoter (FBP). -148/-53, in either orientation, enhances expression (25-fold) of the c-fos basal promoter (FBP) in myotubes (figure 4.8). -148/-53, however, is not a muscle-specific enhancer, since it also enhances expression of the FBP (10-20 fold) in myoblasts and fibroblasts (figure 4.8). Thus, this enhancer is not sufficient to confer muscle-specific expression on a heterologous promoter. The 3' portion of -148/-53 also has enhancer activity, but the

activity of -95/-53 is weaker than -148/-53 (figure 4.8). In contrast, -148/-95 has no enhancer activity in any cell type (figure 4.8).

To determine whether myotubes, myoblasts and fibroblasts contain activities that bind to the enhancer, I performed gel-shift experiments with nuclear extracts from these cells. Multiple activities bind to -95/-53, and the pattern of binding activities is similar with extracts from all cell types (figure 4.9). These complexes result from sequence-specific interactions, since binding is competed by unlabelled -95/-53, but not by -148/-95 DNA (figure 4.9). Although binding is observed with a -148/-95 probe, this binding was considered non-specific, since it is not competed by unlabelled -148/-95 DNA (data not presented). The presence of similar enhancer-binding activities in myotubes, myoblasts, and fibroblasts is consistent with my expression data which shows that the enhancer is active in each cell type when fused to a heterologous promoter.

DISCUSSION

My experiments show that muscle-specific expression of the δ–subunit gene is controlled by two elements that are located within 148 bp of 5' flanking DNA. One element is an E-box that is located near the transcription initiation site, and the other element is an enhancer located at -148/-53. The E-box has a dual role: it is important for activation of expression in myotubes and for repression in myoblasts and non-muscle cells. Thus, this site acts as a critical cell-specificity element that restricts δ-subunit gene expression to myotubes. The enhancer is required for expression in myotubes, since small deletions in this region reduce or abolish expression. The enhancer, however, does not confer muscle-specificity, since it can activate expression of a heterologous promoter in myoblasts and fibroblasts, as well as myotubes.

Positive and negative regulation through the same site

The experiments described here indicate that positive and negative regulation of the δ-subunit gene act through the same site, E1 (figure 4.10). Neither the mechanism of activation nor repression is clear, but distinct activator and repressor activities may share a common DNA recognition site, as has been observed with homeodomain proteins (Jaynes and O'Farrell, 1988; Ohkuma et al., 1990) and steroid hormone receptors (Akerblom et al., 1988; Oro et al., 1988). This model predicts that the relative concentrations of activator and repressor activities would determine the transcriptional state of the gene. In this sense, E1 can be considered to be a genetic switch, as has been suggested for the μE5 E-box of the IgH enhancer (Ruezinsky et al., 1991). The μE5 site is important for activation of IgH expression in B-cells and for repression in non-B cells.

In myoblasts, repression through E1 may be required to supplement negative regulation mediated by Id (Benezra et al., 1990). Although MyoD and E2A proteins are expressed in myoblasts (Tapscott et al., 1988), Id, an HLH protein that lacks a basic region, can prevent functional interaction of MyoD and E2A in myoblasts by binding to E2A proteins (Benezra et al., 1990; Jen et al., 1992). The efficiency of Id, however, is not clear, and the MyoD/E2A complexes that I observe in myoblast nuclear extracts may be a consequence of incomplete titration by Id. Thus, the repressor that binds E1 in myoblasts may function to attenuate the effect of low levels of MyoD/E2A heterodimers.

Position and/or context of E-box 1 are important for its role in activation and repression

Mutation of E2 or E3 has no effect on expression in myotubes or myoblasts, indicating that these sites are not required for activation or repression. Furthermore, these E-boxes cannot functionally substitute for E1, since the effect of mutations in E1 is evident even when E2 and E3 are present. One explanation for these results is that the context of these E-boxes may be important. Although E1, E2, and E3 fit the consensus binding site for myogenic bHLH/E2A heterodimers (Blackwell and Weintraub, 1990), E2 and E3 are in

the opposite orientation of E1, and the 2 bp that flank these three E-boxes are not identical (figure.4.1). Flanking nucleotides are important in determining the DNA-binding site preferences of bHLH proteins (Blackwell and Weintraub, 1990; Sun and Baltimore, 1991; Wright et al., 1991), and in some cases, bHLH proteins are capable of distinguishing between E-box sequences that differ by only a single base pair in their flanking sequence (Hu et al., 1992). Further experiments will be required to determine whether sequences flanking E1 have a role in determining its unique properties.

Alternatively, the position of E1 relative to the enhancer or the transcription initiation site may functionally distinguish E1 from E2 and E3. The δ-subunit gene lacks canonical TATA and CCAAT elements, although sequences are present that may serve these functions. The sequence surrounding the transcription initiation site fits the concensus for the pyrimidine-rich initiator element (Inr) (Smale and Baltimore, 1989), YAYTCYYY, in seven out of eight positions. This raises the possibility that factors bound at E1 might interact with activities bound at the Inr. In this regard, an Inr-binding transcription initiation factor, TFII-I, interacts cooperatively with an E-box binding activator, USF, a member of the bHLH-Zip family (Roy et al., 1991). TFII-I is related to USF by immunological criteria and contains repeated domains with sequences that are homologous to HLH domains (Roeder et al., 1992), suggesting that TFII-I may be capable of interacting with other proteins that have HLH domains. Thus, positive or negative regulation through E1 may result from interactions between bHLH proteins bound at E1 and general initiation factors, such as TFII-I.

Multiple E-box 1 binding activities

Myotube and myoblast nuclear extracts contain multiple E1 binding activities. I showed that complex 1, present in myotubes and myoblasts, contains MyoD/E2A, and that complex 2, present in myotubes, contains myogenin/E2A. MyoD/E2A and myogenin/E2A are likely to be required for activation of the δ-subunit in myotubes.

Complex 3 is present in myotubes, myoblasts, 3T3 cells and Hela cells, and does not contain MyoD, myogenin, or E2A proteins.

It is possible that complex 3 contains a bHLH protein that does not crossreact with antibodies to MyoD, myogenin, or E2A. HEB (Hela E-box binding factor), is a recently identified bHLH protein related to E2A and E2-2(ITF-2) (Hu et al., 1992). I do not know whether E2A antisera crossreacts with HEB, but the bHLH domains in these proteins are 80-90% identical (Hu et al., 1992). HEB can form homodimers and heterodimers with myogenin, E12, or E2-2(ITF-2) in vitro (Hu et al., 1992). If HEB is part of complex 3, its dimerization partner must be distinct from E12, E47, E2-5(ITF-1), or E2-2(ITF-2), because complex 3 is not affected by the E2A antisera. Although it is possible that complex 3 consists of HEB homodimers, Hu et al. (1992) report that HEB homodimers are not detectable by gel shift analysis with Hela nuclear extracts.

Complex 3 is unlikely to contain a member of the bHLH-Zip family of transcription factors (c-Myc, Max, USF, TFE-3, TFEB, and AP-4), which contain a bHLH domain adjacent to a leucine zipper domain (Beckmann et al., 1990; Carr and Sharp, 1990; Gregor et al., 1990; Hu et al., 1990; Luscher and Eisenman, 1990; Blackwood and Eisenman, 1991). These proteins bind poorly to E-boxes with the core sequence, CACCTG (Blackwell et al., 1990; Prendergast and Ziff, 1991; Kato et al., 1992).

My experiments with GST-rZEB$_C$ fusion protein raise the possibility that complex 3 may not be a member of the bHLH family. GST-rZEB$_C$, which contains three of the six zinc-finger motifs present in the full-length protein, binds specifically to an E1 probe. Mutations that eliminate complex 3 binding also eliminate GST-rZEB$_C$ fusion protein binding. It will be important to determine whether full length ZEB has the same binding specificity as the carboxy-terminal fragment of ZEB. Further, it will be important to determine whether overexpression of ZEB in myotubes represses δ-subunit expression.

E-box 1 is not sufficient for activation

Although many muscle-specific genes require more than one E-box for maximal expression, several genes, like the δ-subunit gene, require only a single E-box. In these genes, expression also depends on the presence of other activator sites. Expression of the human cardiac actin gene, for example, requires a CArG-box and a Sp1 site in addition to an E-box (Sartorelli et al., 1990). Similarly, the δ-subunit E1 site is necessary for maximal expression in myotubes but is not sufficient to activate expression in the absence of enhancer sequences.

The enhancer is not muscle-specific

The δ-subunit enhancer is required for expression in myotubes, but is not muscle-specific, since it can also activate expression of a heterologous promoter in myoblasts and fibroblasts. Deletion analysis indicates that the enhancer spans a relatively large region (-148/-53), suggesting that the enhancer is composed of multiple *cis*-elements, including a potential binding site for AP-2 (ACCCCACCCC) and a Sp1-like sequence (CCGGCC). Overlapping the potential AP-2 site is a sequence, CCCACCCCC (CCAC box), which is essential for muscle-specific expression of the Human myoglobin promoter and binds a 40-Kd protein (CBF40) that is present in myotubes, myoblasts, and fibroblasts (Bassel-Duby et al., 1992). Like the δ-subunit enhancer, multiple copies of the CCAC box activate expression of a heterologous promoter in both muscle and non-muscle cells (Bassel-Duby et al., 1992). Thus, the δ-subunit enhancer contains several potential binding sites for widely expressed factors, which may regulate the activity of this enhancer.

Comparison to other AChR subunit genes

The α-, β-, γ-, and δ-subunit genes contain E-boxes in their 5' flanking regions that are important for expression in myotubes (Piette et al., 1989; Gilmour et al., 1991;

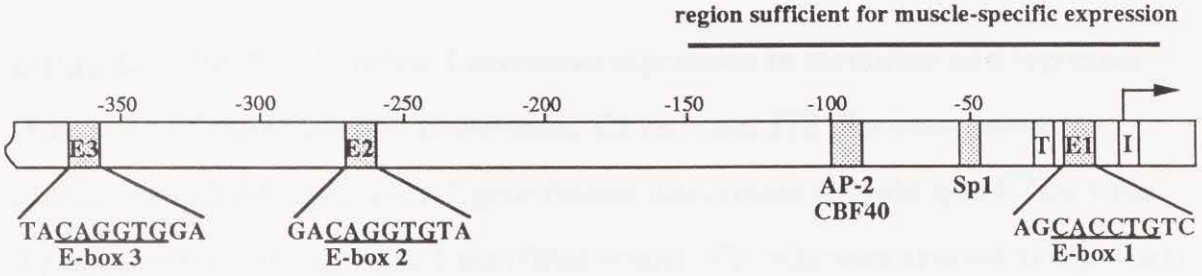
Numberger et al., 1991; Prody and Merlie, 1991). The rat ε gene, however, contains a single E-box that is not necessary for expression in myotubes, but is necessary for repression in non-muscle cells: mutation of this E-box increases expression in Hela cells and 10T1/2 cells, but does not alter expression in myotubes (Numberger et al., 1991). The reporter gene product (CAT) used in these experiments, however, is relatively stable, and it is possible that CAT synthesized in myoblasts persists in the cytoplasm of myotubes during muscle differentiation, obscuring a decrease in ε gene expression in myotubes. I have found hGH to be a useful reporter product for such experiments, since hGH is secreted into the culture medium, which can be changed and sampled daily. Thus, hGH does not persist during muscle differentiation and more accurately reflects the rate of transcription.

Role of E-box 1 and the enhancer in regulation by innervation

181 bp of δ-subunit 5' flanking DNA, which contains E1 and the enhancer, is sufficient for electrical activity-dependent expression (Chapter 2). Since myogenin and MyoD levels are regulated by electrical activity (Duclert et al., 1991; Eftime et al., 1991; Neville et al., 1991; Witzemann and Sakmann, 1991), E1 may be important for regulation by electrical activity. The presence of E-boxes, however, in many other muscle genes does not confer regulation by electrical activity. The muscle creatine kinase and myosin light chain genes, for example, are not regulated by electrical activity (Chapter 2), yet they contain E-boxes that are important for expression (Donoghue et al., 1988; Lassar et al., 1989; Wentworth et al., 1991). These genes contain additional *cis*-elements, including MEF-2 sites (Gossett et al., 1989), that may make them less dependent on levels of bHLH proteins. Alternatively, AChR genes may contain elements in addition to E-boxes that are required for regulation by electrical activity. Thus, it will be important to determine if the activities that bind to the δ-subunit enhancer are regulated by electrical activity.

Figure 4.1 Schematic summary of the position and sequence of three E-boxes present in the AChR δ-subunit 5' flanking DNA. Three binding sites for bHLH proteins, termed E-box 1 (-24/-15), E-box 2 (-270/-261), and E-box 3 (-368/359) are present in the 5' flanking DNA of the AChR δ-subunit gene. The indicated sequences of these E-boxes match the concensus for myogenic bHLH/E2A complexes (Blackwell and Weintraub, 1990). E-box 2 and E-box 3 are in the opposite orientation of E-box 1. An additional E-box (not shown) is centered at -275, but this site does not fit the concensus sequence and is not detected in binding site selection assays (Blackwell and Weintraub, 1990; Sun and Baltimore, 1991; Wright et al., 1991). Also shown are the positions of an initiator element (I), and a potential TATA element (T). Potential binding sites for AP-2, CBF40, and Sp1 are also diagrammed. 148 bp of δ-subunit 5' flanking DNA is sufficient to confer muscle-specific expression (Baldwin and Burden, 1988).

AChR delta subunit 5' flanking DNA



MyoD/E47(orE12)
Concensus binding site

A T GCACCTGTC C

Figure 4.2 Mutation of E-box 1 decreases expression in myotubes and increases expression in myoblasts and fibroblasts. C2 cells and 3T3 fibroblast cells were transfected with δ-hGH or δ-CAT gene fusions that contain the wild-type E-box 1 site (open box) or a mutated E-box 1 site (filled boxes). C2 cells were assayed as myoblasts or as myotubes. C2 myoblasts and 3T3 cells were transfected with δ-CAT gene fusions and pXhGH5 to normalize for transfection efficiency. Because E-box 1 mutations increase expression in myoblasts, I used δ-hGH gene fusions to determine the effect of these mutations in myotubes (see Materials and Methods). δ-hGH gene fusions were cotransfected with pSV2CAT to normalize for transfection efficiency. To compare the absolute expression levels in myoblasts, myotubes, and fibroblasts, wild-type δ-CAT expression was determined in all cell types, relative to pSV2CAT expression. These values are presented in the top row; the expression level in myotubes is arbitrarily set at 100. All other numbers were determined by comparing mutant expression levels to wild-type expression levels within a given cell type. E1, E-box 1; E2, E-box 2; E3, E-box 3.

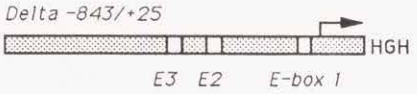
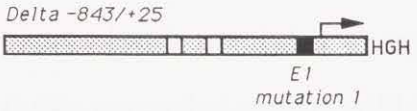
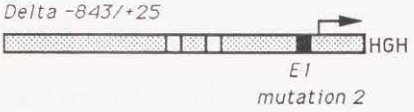
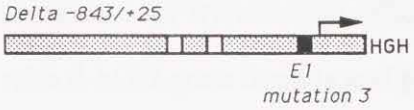
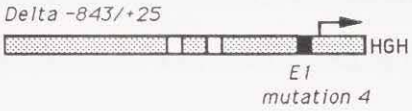
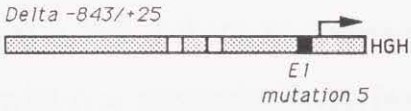
	E-box 1	Myotubes	Myoblasts	Fibroblasts
	GCACCTGTC	100	5.4	6.1
	GAACCTITC	28	83	41
	GCACGGTAA	12	43	15
	GGACCTGTC	4.2	24	18
	GCAACTGTC	36	56	21
	GCACCAGTC	4.5	39	15

Figure 4.3 Mutation of E-box 2 or E-box 3, individually or together, has no effect on expression in myotubes or myoblasts. C2 cells were transfected with δ-hGH or δ-CAT fusion genes that contain wild-type (open boxes) or mutated (filled boxes) E-box 2 and E-box 3 sites. Transfected C2 cells were assayed as myoblasts or as myotubes. C2 cells assayed as myoblasts were transfected with δ-CAT gene fusions and pXhGH5 to normalize for transfection efficiency. C2 cells assayed as myotubes were transfected with δ-hGH gene fusions and pSV2CAT to normalize for transfection efficiency. To compare the absolute expression levels in myoblasts and myotubes, wild-type δ-CAT expression was determined in these cells relative to pSV2CAT expression. These values are presented in the top row in the figure; the expression level in myotubes is arbitrarily set at 100. All other numbers were determined by comparing mutant expression levels to wild-type expression levels in myotubes or myoblasts. E1, E-box 1; E2, E-box 2; E3, E-box 3.

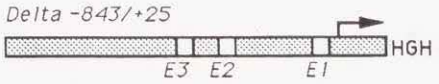
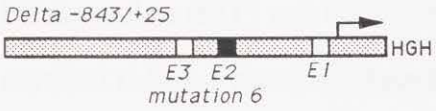
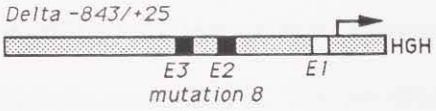
	E-box 3	E-box 2	Myotubes	Myoblasts
	ACAGGTGT	ACAGGTGG	100	5.4
	ACIGGTGT	ACAGGTGG	109	3.9
	ACAGGTGT	ACIGGTGG	67	5.2
	ACIGGTGT	ACIGGTGG	86	7.4

Figure 4.4 Nuclear extracts from myotubes and myoblasts contain multiple E-box 1 binding activities. Gel shift analysis was performed with nuclear extracts prepared from C2 myotubes (MT) and C2 myoblasts (MB). The probes contain the wild-type or a mutated E-box 1 site (see Materials and Methods). The wild-type probe contains nucleotides -25 to -14 from the δ-subunit 5' flanking DNA. 6.5 μg of nuclear extract were incubated with 0.5 ng of ^{32}P-labelled probe. Three complexes are detected with myotube nuclear extracts, labelled 1, 2, and 3 (arrows). Two complexes are detected with myoblast nuclear extracts (arrows labelled 1, and 3) that comigrate with myotube complexes 1, and 3. The presence of an additional, weaker complex was more variable. No binding was observed with mutated E-box 1 probes, except with a probe containing mutation 4, which retained approximately 10% of the binding of complexes 2, and 3.

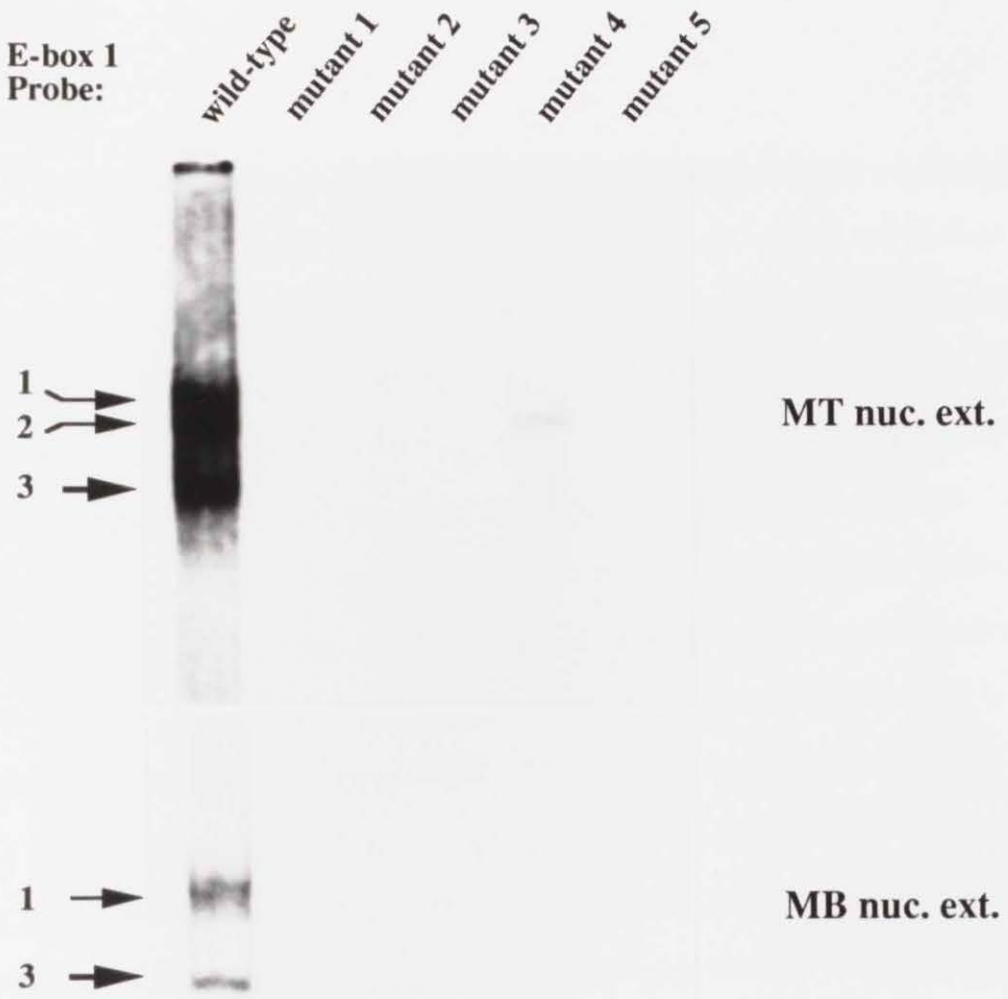

E-box 1
Probe:
wild-type
mutant 1
mutant 2
mutant 3
mutant 4
mutant 5
1
2
3
MT nuc. ext.
1
3
MB nuc. ext.

Figure 4.5 E-box 1 binding activities 1 and 2 contain myogenic bHLH proteins and E2A proteins. Gel shift analysis was performed with the E-box 1 probe and nuclear extracts (6.5 μg) prepared from C2 myotubes (MT) and C2 myoblasts (MB). Binding reactions were done in the presence or absence of antibodies to MyoD, myogenin, or E2A proteins (see Materials and Methods for a description of antibodies). Preimmune sera was used to control for the specificity of the MyoD antisera and the E2Aantisera. A monoclonal antibody to vinculin was used to control for the specificity of the monoclonal antibody to myogenin. The E-box 1 probe (0.5 ng per reaction) contains nucleotides -25 to -14 from the δ-subunit 5' flanking DNA. Complex 1 is disrupted by antibodies to MyoD and E2A, and complex 2 is supershifted by antibodies to myogenin and E2A. Complex 3 is not affected by antibodies to MyoD, myogenin, or E2A. The decrease in complex 3 by antibodies to myogenin and E2A (upper panel, myotube extracts) was not reproducible in other experiments. Arrowheads indicate supershifted complexes resulting from specific interactions between antibodies and E-box 1 binding activities.

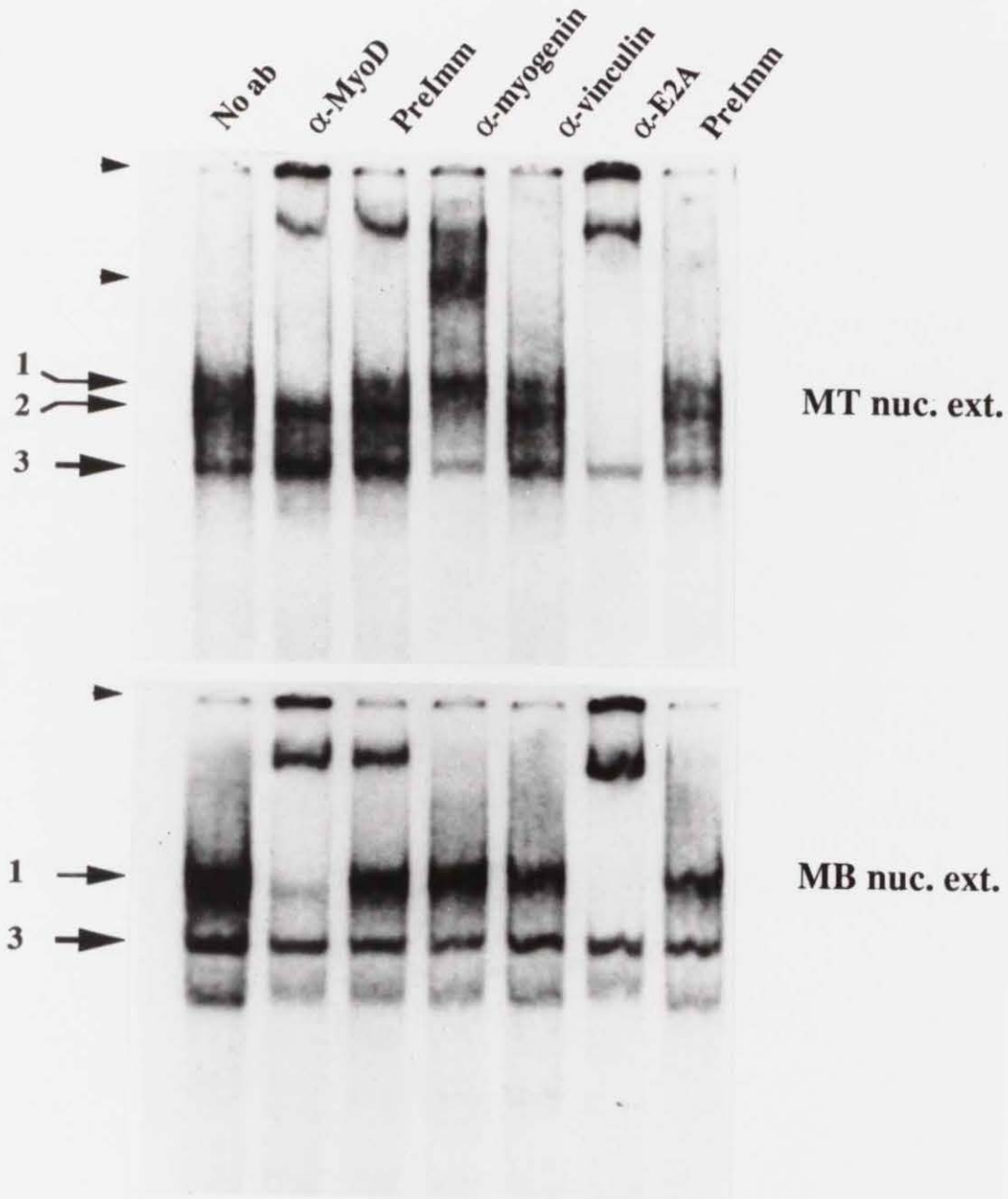

No ab
α-MyoD
PreImm
α-myogenin
α-vinculin
α-E2A
PreImm
1
2
3
MT nuc. ext.
1
3
MB nuc. ext.

Figure 4.6 Recombinant ZEB, a zinc-finger E-box binding protein, binds to E-box 1 in vitro. GST-rZEBc fusion protein (10 ng), containing the carboxy-terminal region of ZEB, was incubated with 0.2 ng of the wild-type E-box 1 probe or probes containing a mutated E-box 1 site (see Materials and Methods). The wild-type probe contains nucleotides -25 to -14 from the δ-subunit 5' flanking DNA. GST-rZEBc binds to the wild-type E-box 1 site but not to the mutated E-box 1 sites (arrow labelled rZEB). Also shown is the binding of GST-MyoD (arrow labelled rMyoD) and GST-myogenin (arrow labelled rMyogenin) to the wild-type and mutated E-box 1 sites. GST-MyoD (35ng) and GST-myogenin (35ng) bind to the wild-type site and to mutation 4, but not to the other mutated E-box sites. The position of the free probe is indicated in the GST-rZEBc panel but is not shown in the GST-MyoD panel or the GST-myogenin panel.

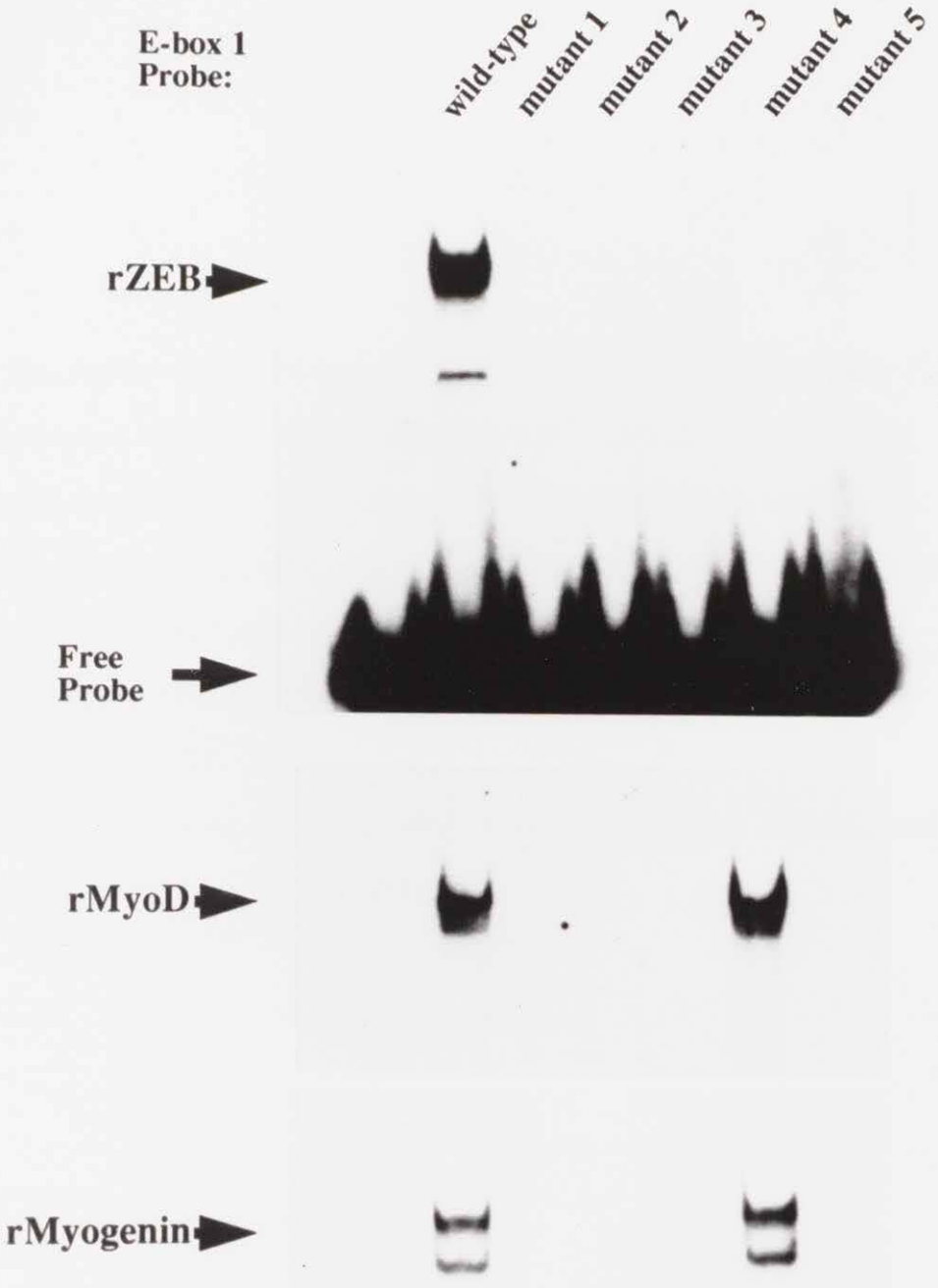

E-box 1
Probe:
wild-type
mutant 1
mutant 2
mutant 3
mutant 4
mutant 5
rZEB
Free
Probe
rMyoD
rMyogenin

Figure 4.7 The region -148/-53 is necessary for full expression in myotubes. C2 cells were transfected with δ-CAT gene fusions with the indicated 5' ends or with δ-CAT plasmids containing internal deletions in the 5' flanking DNA, and CAT expression was assayed in myotubes. C2 cells were cotransfected with pXhGH5 to normalize for transfection efficiency. Expression from intact -843/+25 CAT was arbitrarily given the value of 100, and all other values are expressed relative to the expression of -843/+25 CAT. Deletion of regions between -148/-53 reduces or virtually abolishes expression: deletion of -95/-68 reduces expression 250-fold, and deletion of -67/-53 reduces expression 1000-fold.

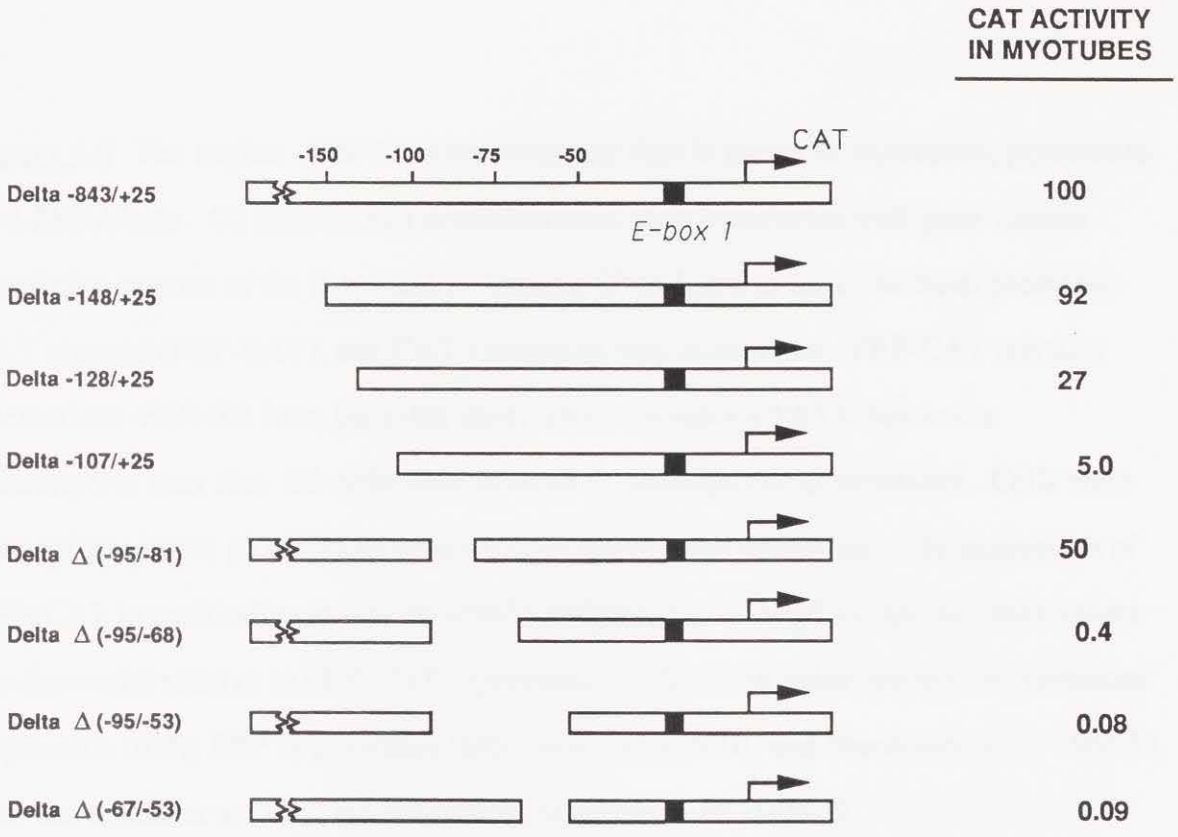

CAT ACTIVITY
IN MYOTUBES
-150
-100
-75
-50
CAT
E-box 1
Delta -843/+25
100
Delta -148/+25
92
Delta -128/+25
27
Delta -107/+25
5.0
Delta Δ (-95/-81)
50
Delta Δ (-95/-68)
0.4
Delta Δ (-95/-53)
0.08
Delta Δ (-67/-53)
0.09

Figure 4.8 The region -148/-53 is an enhancer that is active in myotubes, myoblasts, and fibroblasts. C2 cells or 3T3 fibroblast cells were transfected with gene fusions containing regions of the δ-subunit 5' flanking DNA fused to the c-fos basal promoter-CAT plasmid (FBP-CAT), and CAT expression was determined. FBP-CAT contains nucleotides -56/+109 from the c-fos gene, which includes a TATA box and a transcription start site. C2 cells were assayed as myoblasts or as myotubes. Cells were cotransfected with pXhGH5 to normalize for transfection efficiency. The expression of FBP-CAT in each cell type was arbitrarily assigned the value of 1.0 and all other values are expressed relative to FBP-CAT expression. -148/-53, in either orientation, enhances expression of the FBP in myotubes (MT), myoblasts (MB), and fibroblasts (FB). -95/-53 also has enhancer activity, but the activity is weaker than -148/-53. -148/-95 has no enhancer activity in any cell type. nd, not determined.

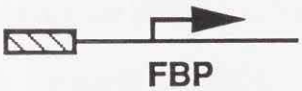
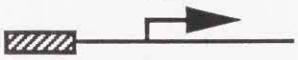
		CAT ACTIVITY		
		MT	MB	FB
δ (-148/-53)		27	11	12
δ (-53/-148)		24	19	7
		1	1	1
δ (-95/-53)		5	5	3
δ (-53/-95)		3	7	4
δ (-148/-95)		0.9	0.4	0.5
δ (-95/-148)		1	nd	nd

Figure 4.9 Nuclear extracts from myotubes, myoblasts and fibroblasts contain activities that bind to the enhancer. Gel shift analysis was performed with nuclear extracts prepared from C2 myotubes (MT), C2 myoblasts (MB), and 3T3 fibroblasts (FB). The probe (0.1 ng) contains nucleotides -95/-53 from the δ-subunit 5' flanking DNA. 3.6 μg of myotube nuclear extract, 3.0 μg of myoblast nuclear extract, or 3.0 μg of fibroblast nuclear extract, was added to each binding reaction. Included in the binding reactions was either no competitor DNA, or a hundred-fold molar excess of unlabelled -95/-53 or -148/-95 DNA. Multiple activities bind to -95/-53 (upper two arrows), and the pattern of binding activities is similar with extracts from all cell types. Binding is competed by -95/-53 but not by -148/-95. The position of the free probe is indicated.

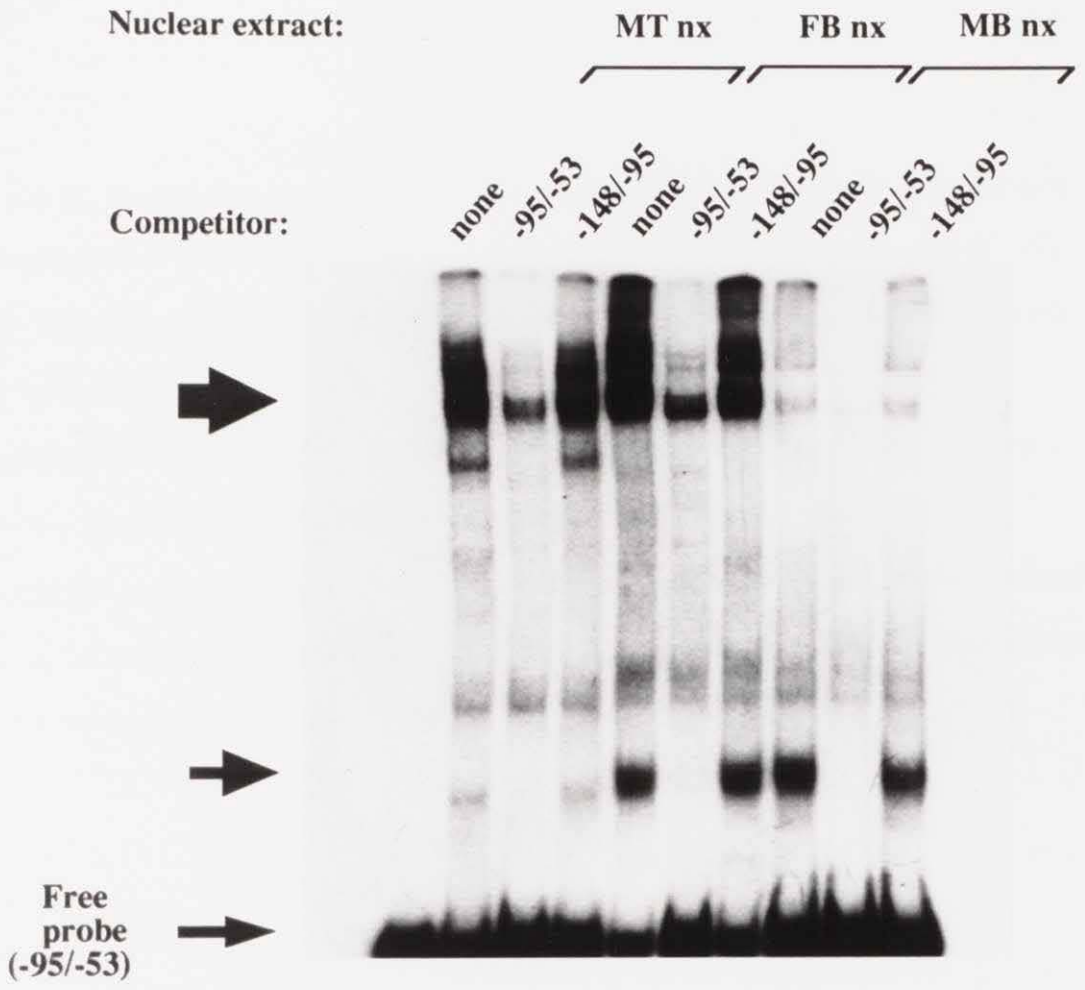
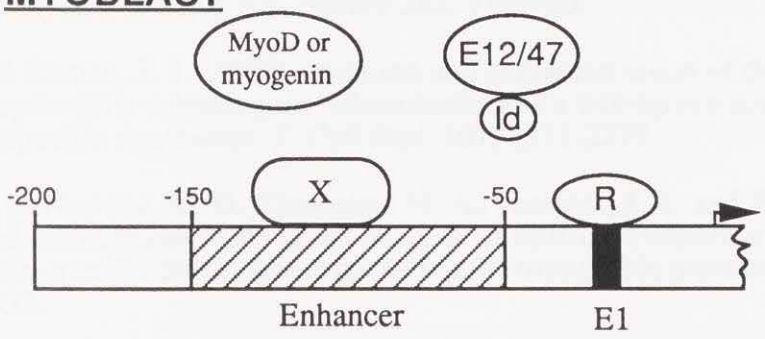

Nuclear extract:
MT nx
FB nx
MB nx
Competitor:
none
-95/-53
-148/-95
none
-95/-53
-148/-95
none
-95/-53
-148/-95
Free probe (-95/-53)

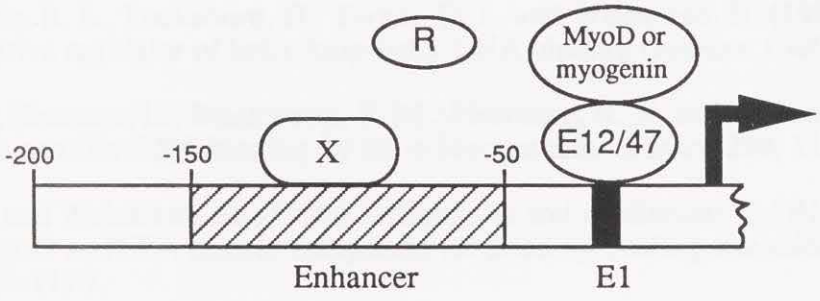
Figure 4.10 Model for muscle-specific expression of the AChR delta subunit gene

(A) Expression of the AChR δ-subunit gene is low in myoblasts and non-muscle cells. A protein (R) binds to E-box 1 (E1) and represses transcription. In addition, the Id protein binds to E2A proteins (E12 or E47) and minimizes the formation of MyoD(or myogenin)/E2A heterodimers. (B) Expression of the AChR δ-subunit gene is high in myotubes. Because Id levels are low in myotubes, MyoD(or myogenin)/E2A heterodimers are able to form. These heterodimers displace the repressor and bind to E-box 1. Both enhancer binding proteins (X) and MyoD(or myogenin)/E2A heterodimers are required for maximal transcription.

A. MYOBLAST



B. MYOTUBE



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Chapter 5

Concluding Discussion

CONCLUDING DISCUSSION

The preceding chapters address the issue of how innervation controls muscle acetylcholine receptor (AChR) gene expression. Innervation also controls the expression of other muscle proteins, including muscle contractile proteins, voltage-gated ion channels, cell adhesion molecules, and myogenic bHLH proteins. Further, the local synaptic signals and propagated electrical signals that are important for regulating muscle AChR expression may be similar to those that control expression of molecules present at neuron-neuron synapses. Here I discuss how ectopic innervation affects myosin heavy chain isoform switching, and how innervation and denervation regulate the expression of other muscle and neuronal proteins. In addition, I discuss the induction of immediate early genes in neurons in response to neurotransmitters and membrane depolarization. Finally, I consider how proteins are targeted to postsynaptic sites on neurons.

Regulation of other muscle gene products by innervation

Slow-type myofibers normally express slow isoforms of myosin heavy chain (MHC), but they turn off expression of slow MHC isoforms and turn on expression of fast MHC isoforms when innervated ectopically by nerves that normally innervate fast-type muscle (Weeds et al., 1974). This switch in MHC isoform expression can be induced by direct electrical stimulation of slow muscles at a rate characteristic of fast muscles (Salmons and Sreter, 1976; Rubenstein et al., 1978) These experiments indicate that the expression of muscle contractile proteins is influenced by the pattern and frequency of electrical stimulation (Lomo et al., 1974; Salmons and Sreter, 1976; Rubenstein et al., 1978). There is evidence that local signals are also involved in the switching of MHC isoforms following ectopic innervation. Fast MHC expression is induced by fast ectopic innervation of the rat soleus muscle (a slow muscle), but only near the ectopic synapse (Salviati et al., 1986). Thus, both propagated electrical activity and local signals can affect MHC expression.

Expression of voltage-gated Na^+ channels (embryonic type), neural cell adhesion molecule (NCAM), neural cadherin (N-CAD), myogenin, and MyoD increases following denervation. Denervated muscles also express neurotrophic factors which promote motor neuron sprouting. A common feature of these products is that they are also expressed at high levels in embryonic muscle. Thus, denervation results in an embryonic expression pattern, which may represent the default state in the absence of neural control. Some of the products expressed in denervated muscle, such as NCAM, N-CAD, and neurotrophic factors may be important for stimulating reinnervation by regenerating axons. Indeed, denervated myofibers are receptive to innervation throughout the myofiber, whereas innervated fibers are largely resistant to ectopic innervation.

Several other muscle proteins in addition to the AChR are concentrated at neuromuscular synapses, and some of these may also be expressed specifically by synaptic nuclei. At present, AChR genes are the only ones known to be transcribed specifically by synaptic nuclei; acetylcholinesterase mRNA, however, is also concentrated at synaptic sites (R.K. Lee, B.L. Jasmin, R.L. Rotundo: Soc. Neurosci. Abstr., 1992, 649.1), and it will be interesting to determine whether the acetylcholinesterase gene is locally transcribed.

Regulation of neuronal AChRs

Are neuronal AChRs regulated by innervation in the same way as muscle AChRs? Considerably less is known about the regulation of neuronal AChRs than muscle AChRs, but studies of chick and frog autonomic ganglion neurons provide a basis for comparison. Chick parasympathetic neurons do not require presynaptic input to induce AChR expression during development, since ablation of synaptic input to chick ciliary ganglion neurons does not prevent the appearance of acetylcholine-activated currents (Engisch and Fischbach, 1992). These results suggest that AChR expression in these parasympathetic neurons, as in myotubes, is initiated independently of innervation. In muscle, however,

the distribution and expression of AChRs is subsequently regulated by innervation. It remains to be determined whether innervation of ciliary ganglion neurons has subsequent effects on neuronal AChR expression.

The effects of denervation have been studied in frog and chick autonomic ganglia. Denervation of the frog cardiac ganglia results in an increase in the mean acetylcholine sensitivity of the parasympathetic neurons (Kuffler et al., 1971). Similarly, denervation of frog sympathetic ganglia also results in an increase in acetylcholine sensitivity (Dunn and Marshall, 1991). Dunn and Marshall (1985) showed that a decrease in acetylcholinesterase activity, rather than an increase in AChR expression causes an increase in acetylcholine sensitivity in the sympathetic neurons. Similarly, in cardiac ganglion neurons, the increase in acetylcholine sensitivity is not caused by a change in AChR number (Sargent et al., 1991). Thus, in both cases, expression of postsynaptic molecules is altered by denervation, but the number of AChRs is unchanged, and the level of control is not yet clear.

The effects of denervation has also been studied in neurons that innervate the ciliary muscles and sphincter muscles of the eye. Denervation of the chick ciliary ganglia causes a decrease in AChR protein and AChR mRNA (Jacob and Berg, 1987; Boyd et al., 1988). It will be important to determine whether this is caused by loss of electrical activity or by the potential loss of signals associated with synaptic sites and whether transcriptional mechanisms are involved.

Induction of immediate early genes in neurons

The expression of neuronal genes is known to be affected by neurotransmitters and by membrane depolarization. Immediate early genes, such as c-fos, are induced in neuronally differentiated PC12 cells following treatment with the cholinergic agonist nicotine or following depolarization with elevated K^+ (Greenberg et al., 1986). Induction of immmediate early genes also occurs following drug-induced generalized seizures, light pulses, peripheral sensory stimulation, direct electrical stimulation of the motor/sensory

cortex, and during kindling stimulation (reviewed in Sheng and Greenberg, 1990). Induction of immediate early genes occurs within minutes and does not require new protein synthesis. Expression of immediate early genes may induce more long term responses in neurons by activating late response genes, although direct targets of the immediate early gene products are largely unknown.

Targeting of synaptic proteins in neuronal cells

Transcription of the AChR delta subunit gene in innervated muscle is confined to nuclei that are near the synaptic site (Chapter 2). The synaptic site provides a signal that induces synapse-specific transcription, and as a consequence AChRs are synthesized preferentially in the synaptic region of the myofiber. Signals at synaptic sites on neurons may also regulate gene expression, and the signalling pathways in the synaptic region of muscle may be similar to the signalling pathways in the cell bodies of neurons. Neurons, unlike myofibers, contain a single nucleus. Thus, spatially restricted transcription is not a mechanism that neurons use to target molecules to individual postsynaptic sites.

Neurons may receive thousands of synaptic inputs, and individual synapses on the same neuron may utilize different neurotransmitters and receptors. It is not clear how particular neurotransmitter receptors are targeted to individual postsynaptic sites, but targeting of receptor protein or nerve-induced clustering of receptors are potential mechanisms. Agrin is expressed in the brain and could have a role in neuron-neuron synapse formation. Recent evidence suggests that RNA transport may also be a targeting mechanism. Central nervous system neurons can transport particular mRNAs to dendrites, and these mRNAs are translated on dendritic polyribosomes that are located near synapses (reviewed in Oswald and Banker, 1992). At present, two such transported mRNAs have been identified: mRNA encoding the cytoskeletal protein MAP2 (Garner et al., 1988) and mRNA encoding the α subunit of Ca^{2+}/calmodulin-dependent protein kinase (Burgin et al., 1990). It will be interesting to determine whether mRNAs encoding

neurotransmitter receptors are also transported to dendrites and whether the local translation of mRNAs is regulated in response to signals at synaptic sites.

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