Maturation of the Acetylcholine Receptor in Skeletal Muscle: Regulation of the AChR \( \gamma \)-to-\( \epsilon \) Switch

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During the development of the mammalian neuromuscular junction, acetylcholine receptors (AChRs) become localized to the postsynaptic muscle membrane. As this process nears completion, the fetal form of the receptor, containing a \( \gamma \) subunit (composition \( \alpha_2 \beta_2 \gamma_0 \)) is gradually replaced by an \( \epsilon \) subunit-containing adult form (\( \alpha_2 \beta_2 \epsilon_0 \)). To understand how this transition is controlled, we compared the expression and regulation of the AChR \( \gamma \) and \( \epsilon \) subunits in developing, adult, and cultured muscles. Immunostaining with subunit-specific antibodies showed that replacement of \( \gamma \) subunit- by \( \epsilon \) subunit-containing AChRs occurs largely during the first postnatal week in fast-twitch muscles, and occurs homogeneously throughout individual endplates. In the slow-twitch soleus, however, this transition is delayed, and in the multiply innervated slow fibers of extraocular muscle, \( \gamma \) subunit expression persists into adulthood. The transcriptional bases of the AChR subunit transition, and of these intermuscular variations, were demonstrated in mice bearing transgenes containing promoter elements from the AChR \( \gamma \) and \( \epsilon \) subunit genes, each coupled to a nuclear-localized \( \beta \)-galactosidase (nlacZ) reporter. We show that transgene expression is stimulated by the nerve-derived inducer of AChR expression, ARIA, in myotubes cultured from \( \gamma \)-nlacZ as well as \( \epsilon \)-nlacZ mice. However, the expression of \( \gamma \)-nlacZ, but not \( \epsilon \)-nlacZ, is increased by treatment of myotubes with TTX, and the ARIA sensitivity of \( \gamma \)-nlacZ is dependent on the electrical state of the myotube. Thus, the promoters of the \( \gamma \) and \( \epsilon \) subunit genes may integrate ARIA- and activity-dependent signals in different ways to generate their complementary patterns of expression.

INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is the ligand-gated ion channel that mediates synaptic transmission at the skeletal neuromuscular junction. AChRs are present at high concentrations (\( \sim 10^7 \mu m^{-2} \)) in the postsynaptic membrane, but are nearly absent (\(< 10^5 \mu m^{-2} \)) from the remaining 99% of the muscle fiber surface (Fambrough, 1979). At least four processes contribute to the generation of this striking localization. First, myotubes activate expression of the AChR subunit genes as part of the myogenic program. Second, after the arrival of the nerve terminal, some AChRs migrate in the plane of the membrane to synaptic areas, where they become anchored to a subsynaptic cytoskeleton. Third, nerve-derived factors such as acetylcholine receptor-inducing activity (ARIA) enhance transcription of the receptor genes in the small subset of nuclei that directly underlie the synapse. Finally, nerve-induced muscle activity suppresses AChR subunit gene expression in extrasynaptic nuclei, thereby enhancing the selectivity of expression by the synapse-associated nuclei. Thus, both transcriptional and posttranscriptional processes contribute to the exclusive synaptic localization of AChRs seen in mature muscle. Recent progress has been made in identifying pathways by which muscle transduces these signals, and responsive elements within the receptor subunit genes (reviewed in Hall and Sanes, 1993; Bowe and Fallon, 1995; Chu et al., 1995b; Duclet and Changeux, 1995).

Additional regulatory complexity arises during development in mammalian muscle. AChRs were originally isolated from the electric organs of eels and rays, where it was determined that they were composed of four distinct subunits in the stoichiometry \( \alpha_2 \beta_2 \gamma_0 \) (Karlin, 1980; Conti-Tronconi and Raftery, 1982). In studies of neonatal mammalian muscle, however, two types of channels were detected that differed in their conductance and mean open time.

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Finally, we used cultured muscle cells to compare the ARIA Vectors were constructed that contained 0.75, 3.0, or 10 kb of site located at the start of the CAT reporter gene in the plasmid. Antibodies

METHODS

Antibodies

Antiserato the AChR γ subunit were produced against a peptide, which replaces the γ subunit postnatally to yield receptors of the composition αβδε (Mishina et al., 1986; Witzemann et al., 1987; Gu and Hall, 1988; Martinou and Merlie, 1991). The γ and ε subunit genes are differentially regulated not only during development, but also following denervation: γ-containing receptors appear again throughout the membrane of denervated muscle, but ε-containing receptors remain confined to synaptic areas (Goldman and Staple, 1989; Brenner et al., 1990). Thus, different AChR subunits are clearly regulated in different ways: the α, β, and δ subunits are present throughout development, the γ subunit is present at early stages and after denervation, and the ε subunit appears late in development and is only found at synapses.

Not much is known about factors which influence the replacement of the AChR γ subunit by the ε subunit. It is not clear, for example, whether the same neural factors, such as ARIA, induce transcription of both γ and ε subunit genes by synaptic nuclei, or how individual nuclei down-regulate the γ subunit gene while continuing to transcribe the remaining subunits. Moreover, transgenic analysis, which has provided insight into the transcriptional regulation of the AChR α, δ, and ε subunit genes (Merlie and Kornhauser, 1989; Klarsfeld et al., 1991; Sanes et al., 1991; Simon et al., 1992; Gundersen et al., 1993; Tang et al., 1994), has not yet been applied to the γ subunit gene. Finally, although the existence and overall time course of the subunit switch have been well documented, no examination has been made of possible differences among muscles or fiber types with regard to precise timing. Such analysis might give further insight into mechanisms by which this process is regulated.

In the present work we have addressed some of these questions. First, we used antibodies against the AChR γ and ε subunits to examine the timing of the subunit switch in different muscles, to seek variations in this timing among endplates within a single muscle, and to determine the distribution of each subunit within individual endplates. Second, we generated and analyzed transgenic mice in which regulatory sequences from the γ subunit gene directed expression of reporter genes in order to assess the transcriptional basis of the patterns of expression of this gene. Third, we characterized a set of fibers in extraocular muscle which maintain expression of the γ subunit into adulthood. Finally, we used cultured muscle cells to compare the ARIA and activity dependence of γ subunit gene expression to that previously described (Chu et al., 1995a) for the ε subunit gene.

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The distribution of AChRs was assessed by an immunofluorescent method. For most studies, muscles were frozen unfixed in liquid nitrogen-cooled isopentane, and cross-sectioned at 5–6 μm in a cryostat. For longitudinal sections, muscles were fixed for 20 min in phosphate-buffered saline (PBS) containing 1% paraformaldehyde, rinsed briefly, and sunk in 25% sucrose/PBS before freezing. These ‘thick’ sections were cut at 16–40 μm depending on the age of the animal, and mounted on Superfrost/Plus positively charged slides (Fisher Scientific, Pittsburgh, PA).

All sections were incubated with primary antibody at 4°C overnight, washed extensively, and then reincubated for 2–4 hr in a mixture of fluorescein-conjugated goat anti-rabbit antibody (Boehringer Mannheim, Indianapolis, IN) plus either rhodamine-conjugated α-bungarotoxin, or rhodamine-conjugated goat anti-mouse antibody (Boehringer Mannheim). Primary antibodies were diluted in 1% bovine serum albumin (BSA) in PBS, while secondary antibody solutions were further supplemented with 10% normal goat serum or 5% normal mouse serum, to reduce background. For staining with anti-AChRγ, sections were fixed for 10 min with methanol at –20°C, and then treated with 50 mM ethylamine-HCl (pH 11) for 5 min at room temperature as recommended by Gu and Hall (1988), before incubation with primary antibody.

Plasmid Construction

Vectors were constructed that contained 0.75, 3.0, or 10 kb of contiguous sequence upstream of the transcriptional start site of the mouse AChR γ subunit gene. Genomic sequences were obtained from previously described genomic clones (Crowder and Merlie, 1988) as follows: a 1.1 kb HindIII fragment from clone Ch. 28 b–4, containing sequences spanning the transcriptional start site, was subcloned into the HindIII site of pgEM 1 (Promega, Madison, WI). To generate γ 0.75–CAT sequences from the 5′ end of this fragment to the internal Ban site were inserted into the HindIII site located at the start of the CAT reporter gene in the plasmid pUC9CAT (Donoghue et al., 1988). The Ban site is located 19 bp downstream of the AChR γ subunit transcriptional start site. A
2.3-kb HindIII genomic fragment was inserted upstream of γ0.75-CAT to produce γ3.0-CAT. To generate γ10-CAT, a 7.0-kb BamHI to BgII genomic fragment from clone L47.1 δ A-1 was inserted upstream of γ3.0-CAT. The γ10- nlacZ plasmid was constructed separately: this same 7.0-kb BamHI to BgII genomic fragment was cloned into a BamHI/BgII-digested cloning vector pSL1180 (Pharmacia, Uppsala, Sweden) to produce pSL1180-G10.2. The 3.0-kb BgII–BamHI fragment from genomic clone Ch 28 γ8 was directionally subcloned into pSL1180-G10.2, downstream of the 7.0-kb fragment, producing pSL1180-P3, with a full 10 kb of γ subunit gene upstream sequence. Finally, a Smal–SphI fragment containing the nlacZ reporter cassette from pmlAcF (Kapur et al., 1991) was directionally subcloned into pSL1180-P3 to generate γ10-nlacZ.

Cell Culture

C2 myogenic cells and 3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured and transfected with supercoiled DNA using the calcium phosphate method as described previously (Donoghue et al., 1988; Prody and Merlie, 1991). pSV– β-galactosidase (Promega) was cotransfected to control for transfection efficiency.

Primary muscle cultures from γ10– nlacZ mice were prepared by minor modification of the method described in Chu et al. (1995a). Briefly, P0–P2 pups were dissected and muscle tissue trypsinized individually for each, and the resulting cell suspension was distributed across 8–12 wells of a gelatin-coated 12-well dish, in Dulbecco’s modified Eagle’s medium containing 10% horse serum and 5% newborn calf serum. Approximately 400,000 cells were plated per well. After 3 days, cells were switched to media containing 2% horse serum to promote fusion. ARIA (HRG/127–244) Holmes et al., 1992; a generous gift of Dr. Mark Sliwkowski, Genentech) was added to a final concentration of 3 mM, 24 hr after cultures were switched to fusion media. Cells were stained after 3 to 7 days in fusion media. Where indicated, 5 μM TTX was added to the media during the 48 hr prior to staining.

Transgenic Mice

Transgenic mice were produced using standard procedures (Hogan et al., 1994). The following restriction enzymes were used to excise the reporter constructs from vector sequences: γ0.75–CAT, HindIII and BamHI; γ3.0–CAT, BgII and BamHI; γ10–CAT, BamHI; and γ10–nlacZ, Smal and SphI. Transgene-positive animals were identified by PCR of genomic DNA from a tail biopsy (Harley and Merlie, 1991). For the CAT–containing transgenes, founder mice were assayed between 5 and 7 weeks of age. The sciatic nerve was severed under anesthesia, and muscles from both innervated and denervated hindlimb were harvested after 5 days. Generation of the γ3500–nlacZ mice has been described previously (Sanes et al., 1991).

Reporter Gene Assays

Muscle extracts were prepared as described by Merlie and Kornhauser (1989). CAT activity was determined as described by Gorman (1985) using 100 μg of muscle extract, or cell lysates containing equal amounts of β-galactosidase activity. [14C]acetylated chloramphenicol products were quantified by scintillation counting or by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA). In transfected cells, quantification of β-galactosidase activity was performed using ONPG as the substrate (Chu et al., 1995a).

For lacZ histochemistry, whole embryos or dissected tissues were fixed for 1 hr in 2% paraformaldehyde/PBS. Cultured muscle cells were fixed for 5 min in 2% paraformaldehyde plus 0.2% glutaraldehyde in PBS. X-gal staining solution was adjusted to pH 7.0 but was otherwise identical to that described in Sanes et al. (1991).

RESULTS

Timing of the Subunit Switch

Previous studies have determined that substitution of the AChR ε subunit for the γ subunit occurs during the first two postnatal weeks in rodents (Fischbach and Schuetze, 1980; Siegelbaum et al., 1984; Witzemann et al., 1987; Gu and Hall, 1988; Martinou and Merlie, 1991). We began the present study by defining more precisely the timing of this subunit switch in mouse, and asking whether the process occurred simultaneously in all muscles. Individual muscles were taken from embryos and postnatal animals at several ages, and cryostat sections from each were costained with α-bungarotoxin and antibodies which selectively recognized either the γ or the ε AChR subunit. Bungarotoxin binds to the AChR α subunit, and thus labels both γ- and ε-containing receptors equally, allowing staining for each subunit to be compared to the total population.

The pattern of subunit substitution in the tibialis anterior is shown in Fig. 1. At Embryonic Day (E) 16, endplates stained clearly for the γ subunit, but had no detectable ε immunoreactivity (Figs. 1a and 1b). By 1 day after birth the ε subunit began to be weakly visible, although γ staining remained strong (P 1, Figs. 1c and 1d). Over the next week the strength of ε subunit staining increased while γ subunit levels declined, with staining levels being approximately equal for the two subunits between Postnatal Days (P) 3 and 5 (data not shown). By P 9, staining by anti-ε serum was intense, whereas γ immunoreactivity was barely detectable (Figs. 1e and 1f). Finally, by P 17, only the ε subunit was detectable (Figs. 1g and 1h), and this pattern was maintained into adulthood.

Next we compared the timing of this transition in a variety of muscles that varied in fiber type composition and rostrocaudal position, and that represented both axial and limb populations. Results of this comparison are summarized in Table 1. With one exception (see below), all of the muscles exhibited a pattern of gradual substitution of ε for γ. The extensor digitorum longus and sternomastoid followed a time course indistinguishable from that described above for tibialis, and less complete data for the intercostal muscles indicated a similar pattern. Diaphragm also appeared to conform to this model, with the exception that ε subunit staining was already quite strong at P 1 (Table 1). Thus no dramatic differences were found between muscles located rostrally (sternomastoid) and caudally (tibialis anterior, extensor digitorum longus), nor between limb muscles (tibialis anterior, extensor digitorum longus) and axial muscles (intercostals, diaphragm), even though these muscles

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FIG. 1. Replacement of $\gamma$- by $\varepsilon$-containing AChRs in developing muscle. Sections were doubly stained with FITC-tagged antibodies to the $\gamma$ or $\varepsilon$ subunit plus rhodamine-$\alpha$-Bungarotoxin (rBtx). Pairs of micrographs show single fields photographed with fluoresceine or rhodamine optics, to show the $\gamma$ or $\varepsilon$ subunit and all AChRs, respectively. Sections of tibialis anterior (tib.ant.) are shown for each embryonic (E) or postnatal (P) age noted (a±h), and for soleus at two of these ages (i±l). The major change in subunit composition takes place between P 1 and P 9 in tibialis, but significantly later in soleus. Bar, 20 $\mu$m.
groups have been shown to differ in numerous ways (Donoghue and Sanes, 1994).

In two muscles, however, the subunit switch exhibited a distinctly different time course. Interestingly, both of these muscles included significant numbers of slow fibers, in contrast to the primarily fast-twitch muscles described above. In the predominantly slow-twitch soleus muscle, the subunit transition was delayed by about a week: ε subunit staining was still faint at P 9, and significant γ subunit levels could be detected as late as P 21 and even occasionally at P 30 (Table 1 and Figs. 1f–1l). Most strikingly, in extraocular muscle (EOM), which contains a mixture of very fast and very slow fibers (Porter and Hauser, 1993), a subpopulation of endplates maintained high expression of the γ subunit into adulthood, while the remainder of junctions followed the typical pattern and time course of transition to ε subunit expression. This marked dichotomy led us to study EOM in more detail.

**TABLE 1** Time Course of AChR γ-to-ε Subunit Transition in Different Muscle Groups

<table>
<thead>
<tr>
<th>Muscle Group</th>
<th>Diaphragm</th>
<th>Soleus</th>
<th>EOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibialis anterior, extensor digitorum longus, sternomastoid</td>
<td>ε - γ +</td>
<td>ε - γ +</td>
<td>ε - γ +</td>
</tr>
<tr>
<td>P 1</td>
<td>ε (+) γ +</td>
<td>ε + γ +</td>
<td>ND</td>
</tr>
<tr>
<td>P 3</td>
<td>ε + γ +</td>
<td>ε + γ +</td>
<td>ε (+) γ +</td>
</tr>
<tr>
<td>P 5</td>
<td>ε + γ (+)</td>
<td>ε + γ (+)</td>
<td>ε + γ (+)</td>
</tr>
<tr>
<td>P 17</td>
<td>ε + γ -</td>
<td>ε + γ -</td>
<td>ε + γ -</td>
</tr>
<tr>
<td>P 21</td>
<td>ε + γ -</td>
<td>ε + γ -</td>
<td>ε + γ -</td>
</tr>
<tr>
<td>P 50+</td>
<td>ε + γ -</td>
<td>ε + γ -</td>
<td>ε + γ -</td>
</tr>
</tbody>
</table>

Note. Results were obtained by immunofluorescent studies such as those shown in Fig. 1. Ages examined are shown in the left column, and muscles are listed across the top. Symbols represent the strength of ε or γ subunit staining: +, strong staining comparable to Rbtx levels; (+), weak or variable staining levels; –, no staining detectable above background. EOM, extraocular muscles. ND, not determined.

**Maintained Expression of the γ Subunit in Adult Extraocular Muscle**

The extraocular muscles are a set of six small muscles which connect the globe of the eye to the orbit, and allow control of gaze in all directions. EOM receives innervation from cranial nerves (III, IV, and VI), and differs from other skeletal muscles in several distinctive physiological and biochemical features (for reviews, see Kaminski et al., 1990; Porter and Hauser, 1993). Of particular importance here, while most EOM fibers are similar to the singly innervated, fast-twitch fibers of limb and axial muscles, a substantial subpopulation of EOM fibers bears multiple simplified endplates and has slow-twitch or tonic responses (Hess and Pilar, 1963; Bach-y-Rita and Ito, 1966; Salpeter et al., 1974). We suspected that the γ- and ε-staining populations of fibers might correspond to the slow multiply innervated fibers (MIFs) and the fast singly innervated fibers, respectively. We tested this hypothesis by examining the end-plates and myosin isoforms of the fibers expressing the two different AChR subunits.

Longitudinal sections of eye muscles were used to examine the relative distributions of ε and γ subunits along the lengths of the fibers. Singly innervated fibers generally receive their nerve contact within a single central endplate band (Hess and Pilar, 1963; Pachter et al., 1976), and these fibers, centrally located, en plaque endplates were found to have exclusively ε-containing AChRs (Figs. 2a and 2b). MIFs, by contrast, have clusters of smaller en plaque endings scattered along their lengths, extending well into the distal regions of the muscle (Hess and Pilar, 1963; Pachter et al., 1976). Such distal endplates were found to stain strongly for the γ subunit (Fig. 2c). Some of these small endplates also stained weakly for the ε subunit, but most did not (Fig. 2d). Thus it appears that the γ subunit-expressing subpopulation of fibers are indeed the MIFs that have been previously described, based on these correlations with endplate size and location.

As an independent test of the identity of the γ-maintaining fibers, we double-labeled cross sections with γ- or ε-specific antisera plus antibodies to slow or fast myosin. MIFs have been shown to have very slow contractile responses (Hess and Pilar, 1963), and are therefore expected to be slow myosin-positive. Indeed, AChR γ-containing junctions were found exclusively on fibers which stained negative for fast-twitch (or type II) myosins and positive for slow (or type I) myosins (Figs. 3b and 3c). In contrast, junctions containing the ε subunit were found on fibers staining positive for fast-twitch myosin (Fig. 3a). Thus, this correlation with slow myosin expression confirms our identification of MIFs as the γ subunit-maintaining fiber population within the EOM.

**Correlation of Subunit Composition with Endplate Complexity**

The AChR subunit switch is only one of several maturational events which occur at the neuromuscular junction.
FIG. 2. Subsets of EOM endplates differentially express the $\gamma$ or $\epsilon$ subunits. Sections were stained as described in the legend to Fig. 1, and photographed to show the $\gamma$ or $\epsilon$ subunit (left) or all AChRs (right). The top row of panels show large en plaque endings found in the central endplate band, which stain for the $\epsilon$ but not $\gamma$ subunit; the bottom row shows the small en grappe endings found in more distal muscle regions, which stain primarily for the $\gamma$ subunit. Bar, 20 $\mu$m.

during the first two postnatal weeks. Another major transformation taking place during the same period is in the geometry of the endplate itself. Neonatal endplates are simple, plaque-like contacts, which gradually become more complicated through the opening of receptor-free “holes” and growth of the total synaptic area, until the final branched shapes characteristic of the adult are formed (Steinbach, 1981; Slater, 1982; Balice-Gordon and Lichtman, 1990). Previous work has shown that individual developing muscles contain endplates of very differing morphology and maturity, indicating that this structural remodeling occurs in an asynchronous manner (Steinbach, 1981; Slater, 1982). However, from our studies of cross sections described thus far, it appeared that the level of staining for each subunit was similar at all of the endplates within a fast muscle, implying that they were undergoing the subunit transition synchronously. To examine this issue more directly, we stained thick, longitudinal muscle sections with subunit-specific antisera and rBtx. In these sections entire endplates could readily be seen, and thus easily classified by level of relative maturity. While some variation in absolute AChR $\gamma$ or $\epsilon$ subunit staining levels was seen among individual endplates viewed in this manner, these variations showed no correlation with endplate complexity: strong $\epsilon$ subunit staining was found both on undifferentiated plaques and on more branched and convoluted endplates, and $\gamma$ subunit staining was likewise found on endplates at various stages of morphological differentiation (Fig. 4). Thus, these molecular and geometric aspects of endplate maturation must be regulated independently.

These en face views also allowed us to examine whether the distribution of the $\gamma$ and $\epsilon$ subunits was homogeneous within an endplate or whether the two types of AChR might segregate into different subregions of the synaptic site. At all ages examined, staining for both $\gamma$ and $\epsilon$ subunits showed no sharp discontinuities within an endplate, and corresponded precisely to the relative density and distribution of total AChR, as assessed with rBtx (Fig. 4). This correspondence persisted even where the toxin staining was very dim (Fig. 4a, arrows). Thus, within an endplate the entire postsynaptic surface must lose $\gamma$-containing AChRs and acquire $\epsilon$-containing AChRs synchronously. This uniformity is particularly interesting in light of the previous finding that some regions of the synaptic site are destined to be lost...
altogether during synapse elimination (Balice-Gordon and Lichtman, 1993): \(\varepsilon\)-containing and \(\gamma\)-containing receptors appear to be eliminated together during such restructuring, rather than one or the other being selectively retained. This evidence indicates that a switch of localized subunit concentrations is unlikely to play a role in establishing differences between subregions of the postsynaptic endplate site. Further support for this conclusion comes from the preliminary observation that synapse elimination occurs on schedule in an AChR \(\varepsilon\) subunit-deficient mutant mouse (A.C.M., J. Mudd, J.P.M., and J.R.S., manuscript in preparation).

**Tissue-Specific and Activity-Dependent Expression of the AChR \(\gamma\) Subunit Gene**

The disappearance of \(\gamma\) subunit-containing AChRs in the postnatal period results from a decrease in total AChR \(\gamma\) subunit mRNA (Witzemann et al., 1987). To gain a better understanding of how this decrease is achieved, we used transgenic mice to examine the transcriptional regulation the AChR \(\gamma\) subunit gene in vivo.

Sequences within several hundred bases of the AChR \(\gamma\) subunit gene have been shown to direct cell type- and differentiation-specific expression of a reporter gene in cultured cells (Gilmour et al., 1991, 1995; Numberger et al., 1991; Durr et al., 1994). Accordingly, we fused a 750-bp stretch of 5' flanking sequence to a CAT reporter gene (\(\gamma\)0.75-CAT), and tested this construct by transient transfection in NIH 3T3 fibroblasts and the myogenic cell line C2. In agreement with previous reports, we found that \(\gamma\)0.75-CAT was expressed poorly in fibroblasts and undifferentiated myoblasts, but exhibited high levels of expression in C2 cells that had been induced to form myotubes (Table 2). We then proceeded to generate transgenic mice using the \(\gamma\)0.75-CAT construct.

Transgenic founder animals were assayed for expression by denervating one hindlimb and measuring CAT activity in extracts of the denervated and control muscles 5 days...
TABLE 2
Analysis of γ-CAT Constructs in Transient Transfection and Transgenic Mice

<table>
<thead>
<tr>
<th>Construct</th>
<th>CAT activity in vitro (arbitrary units)</th>
<th>Transgenic expressors/total founders (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>Myoblasts</td>
</tr>
<tr>
<td>γ0.75-CAT</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>γ3.0-CAT</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>γ10-CAT</td>
<td>0.6</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Note. The left columns show expression levels of γ-CAT constructs in NIH3T3 fibroblasts and C2 myoblasts and myotubes following transfection. On the right is the total number of transgene-positive animals tested, and the number with detectable CAT activity in denervated muscle.

later. Of 19 transgene-positive founders generated, one animal exhibited a 50-fold increase of CAT activity in the denervated muscle compared to the contralateral control (Table 3). Thus, regulatory sequences within 750 bp of the AChR γ subunit gene can confer nerve activity-dependent regulation in vivo, a property which previously had only been examined in a cell culture model (Gilmour et al., 1995). The remaining 18 founders, however, did not detectably express the transgene in either innervated or denervated muscle. This low frequency of reporter expression among transgene-positive mice suggested that additional elements were required for efficient expression of this transgene in vivo. Consequently, we tested constructs incorporating additional sequences further upstream of the γ subunit gene.

Previous studies have identified DNase I hypersensitive (HS) sites surrounding the AChR γ subunit gene (Crowder and Merlie, 1986, 1988). DNase I hypersensitive sites are nuclease-accessible, nucleosome-free regions of chromatin which often demarcate regions important for transcriptional activation (for review, see Wallrath et al., 1994). The 0.75-kb promoter fragment contains only a single, developmentally regulated DNase I HS site, while a second and third site lie within 3.0 and 10 kb, respectively, 5′ of the γ subunit gene. These longer sequences were fused to the CAT reporter to generate the vectors γ3.0-CAT and γ10-CAT. Both of these genomic fragments directed cell-type and differentiation-specific expression in cell lines at levels similar to γ0.75-CAT (Table 2). Moreover, these constructs exhibited muscle-specific expression in transgenic mice, and while overall levels of expression varied considerably, a strong response to denervation was observed consistently (Table 3). In contrast to these qualitatively similar expression patterns, the proportion of expressing founder animals increased dramatically with the use of the longer sequences: from 5% with γ0.75-CAT to 67% with γ10-CAT. This result suggests that distal sequences upstream of the AChR γ subunit gene are not required for expression in vitro yet are necessary for efficient expression in transgenic mice.

Spatiotemporal Patterns of AChR γ Subunit Gene Expression in Embryos

To facilitate a more detailed analysis of the timing and distribution of AChR γ subunit expression, we generated

TABLE 3
Activity-Dependent Regulation of the AChR γ Subunit Promoter

<table>
<thead>
<tr>
<th>Animal</th>
<th>CAT activity (% conversion/100 μg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>γ0.75-CAT 59</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>γ3.0-CAT 16</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>γ3.0-CAT 51</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>γ3.0-CAT 52</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>γ10-CAT 4</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>γ10-CAT 12</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>γ10-CAT 27</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>γ10-CAT 30</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>γ10-CAT 32</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>γ10-CAT 38</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>

Note. Liver extracts and muscle extracts from innervated and 5-day denervated muscle were assayed from the transgenic mice listed in Table 1. Results are given for those mice that expressed detectable CAT. The ratio of activity in denervated muscle to that in innervated muscle is shown in the last column.
transgenic mice using a nuclear lacZ reporter gene (nlacZ). This gene has been modified by addition of a nuclear localization signal so that the β-galactosidase gene product is translocated to nuclei adjacent to its region of transcription. Constructs containing this reporter have previously been used to examine AChR α and γ subunit gene regulation, revealing the presence of transcriptionally distinct synaptic nuclei (Klarsfeld et al., 1991; Sanes et al., 1991; Gundersen et al., 1993). Because high-frequency expression of the CAT reporter gene was obtained only when long regions of 5' regulatory sequence were used, the 10-kb promoter fragment was used to direct expression of the nlacZ reporter (γ10–nlacZ) for these experiments. Five independent transgenic founders or their offspring were found to express the transgene. All of these showed similar patterns of expression, although absolute expression levels varied. Two transgenic lines were established and one of them was studied in greater detail.

Intense β-galactosidase staining of axial and limb muscle was observed in γ10–nlacZ mice by Embryonic Day 14, the earliest age examined (Fig. 5a). Analysis of whole embryos showed no staining in nonmuscle tissues, demonstrating that expression directed by the AChR γ subunit promoter was muscle-specific. Within muscles, nuclei along the entire length of the muscle exhibited accumulations of the nlacZ gene product at E 14. However, the middle of each muscle mass, representing presumptive synaptic regions, tended to exhibit higher levels of staining, and by E 16 each muscle exhibited a distinct central band of intensely stained nuclei (Fig. 5b). Costaining of muscles for acetylcholinesterase, a marker for synaptic sites, confirmed that the most intensely stained nuclei were indeed associated with endplates (Fig. 5c). These results are consistent with in situ hybridization studies of the endogenous AChR γ subunit mRNA, which showed that it is preferentially accumulated in synaptic regions starting at E 14 (Piette et al., 1993). In the γ10–nlacZ mice, muscle nuclei located extrasynaptically were still β-galactosidase positive at E 16, albeit at levels much lower than in the endplate region. Interestingly, perisynaptic nuclei present within the endplate band but not associated with synapses stained more intensely for β-galactosidase than did the distal extrasynaptic nuclei. These nuclei may possibly represent a third category of transcriptionally distinct nuclei within the muscle fiber; however, diffusion of nlacZ mRNA or protein from synaptic to perisynaptic nuclei (Ralston and Hall, 1989) cannot be ruled out as an explanation. Regardless, these results provide additional evidence that the motor neuron can induce differences in the transcriptional activity of muscle nuclei during embryonic development.

Postnatal Regulation of AChR γ Subunit Gene Transcription

We have previously described transgenic mice in which a 3.5-kb promoter fragment from the AChR γ subunit gene controls expression of the nlacZ reporter (γ3500-nlacZ; Sanes et al., 1991). That transgene showed a pattern of postnatal synaptic activation, and we wished to determine whether the AChR γ subunit gene showed a corresponding transcriptional down-regulation. To this end, we followed the expression of the γ10–nlacZ transgene in several muscles during postnatal development. Results from tibialis anterior are presented in Figs. 5d–5f, to facilitate comparison with immunostaining results from this muscle in Fig. 1. On the day of birth, synaptic nuclei were brightly stained and extrasynaptic nuclei were dimly stained in the majority of muscles. Over the course of the first postnatal week, extrasynaptic staining became undetectable while synaptic staining was maintained. Later, synaptic staining diminished in intensity until no positively stained fibers were seen at all by P 21. The loss of nlacZ expression lags behind the loss of γ subunit-containing AChR as detected by immunofluorescence. This delayed decrease in nlacZ expression might accurately reflect the persistence of AChR γ subunit transcription, but could also result from differential stability of nlacZ mRNA compared to AChR γ subunit mRNA, or a slower degradation rate of β-galactosidase than of AChR γ subunit protein. In any event, these results clearly demonstrate that the eventual extinction of γ subunit expression results from a postnatal repression of transcription from this gene, rather than from any change in RNA stability or other posttranscriptional regulation.

The staining pattern of other muscles was generally similar to that seen in tibialis anterior. However, consistent with the prolonged presence of AChR γ subunit immunoreactivity in the soleus, nlacZ expression in this muscle was also protracted: staining of nuclei was still apparent in soleus at P 29, whereas no staining was detectable in tibialis anterior by P 21 (data not shown). The diaphragm also exhibited intense staining for nlacZ expression in these muscles, but its pattern did not differ from that shown in tibialis anterior.
FIG. 7. ARIA- and TTX-dependent activation of the AChR \( \gamma \) subunit promoter. Primary muscle cultures were prepared from newborn mice bearing the \( \gamma10\text{-}\text{lacZ} \) transgene. (a–d) Results obtained from an experiment in which myotubes were not visibly active; (e–h) results from an experiment in which myotubes could be seen contracting. (a, e) Untreated myotubes. (b, f) Myotubes treated with 3 nM rARIA. (c, g) Myotubes treated with 5 \( \mu \)M TTX. (d, h) Myotubes treated with both rARIA and TTX. In the relatively inactive cultures, ARIA was strongly inductive, while TTX had little effect. In the active cultures, TTX produced a strong induction, while ARIA had no effect. However, in both sets of cultures, the combined effects of TTX plus ARIA were greater than the effect of either alone.
ited loss of extrasynaptic staining followed by a decrease in synaptic staining (Figs. 5g–5i), although a subpopulation of muscle fibers still exhibited synaptic staining as late as P 70 (Fig. 5j).

**ACHR Subunit Gene Transcription in Extraocular Muscle**

The discovery that AChR γ subunit protein is expressed in the EOM of adult mice (Fig. 2) prompted a more detailed examination of these muscles in γ10-nlacZ transgenic mice. For comparison, we also examined EOM from the ε3500-nlacZ mice discussed above because the initial analysis of those mice did not include study of their EOM.

Strong nlacZ staining was readily apparent in the rectus and oblique muscles of the eye in γ10-nlacZ adults, well after staining was lost from most axial and limb muscles (Fig. 6a). Although staining tended to concentrate toward the center of the muscle, a subset of fibers exhibited strong nuclear staining throughout their entire length (see arrows in Fig. 6a). When costained for acetylcholinesterase to mark synaptic sites, these intensely staining fibers were found to be en grappe endings, marking them as MIFs (Fig. 6b). Thus, the maintenance of γ-containing AChRs seen in EOM by immunohistochemistry has a basis in ongoing transcription. Curiously, while we would have predicted that the exclusively ε subunit-containing singly innervated fibers would not stain in γ10-nlacZ transgenic mice, weakly β-galactosidase-positive nuclei were often seen underlying large en plaque endplates (Fig. 6b). These fibers never showed positive nuclei outside the endplate region, however, in contrast to the MIFs, indicating that the two fiber types regulate their expression of the γ subunit in differing ways.

A different pattern was observed in the EOM of ε3500-nlacZ mice. No staining was found outside the central endplate band in these mice, consistent with the relative lack of AChR ε subunit-containing receptors in the distal endplates of MIFs. Staining in ε3500-nlacZ extraocular muscles did, however, occur in the endplate band itself, as would be expected from expression in singly innervated en plaque fibers (Fig. 6c). Thus, the staining patterns in γ10−nlacZ and ε3500-nlacZ mice indicate the existence of two transcriptionally distinct subsets of muscle fibers in EOM which together account for the dual AChR phenotype seen in these muscles.

**The AChR γ Subunit Transgene Can Be Regulated by ARIA and Activity**

The synaptic staining pattern seen in γ10−nlacZ mice provides additional support for the hypothesis that the motor neuron induces transcriptional changes in synaptic muscle nuclei (Merlie and Sanes, 1985; reviewed in Chu et al., 1995b). Recent studies have provided evidence that the neurotrophic factor ARIA (Falls et al., 1993) is one of the nerve-derived inducers of synaptic gene expression: ARIA is present at the motor endplate in vivo; and ARIA can stimulate expression of the AChR α, δ, and ε subunit genes in cultured myotubes (Altiek et al., 1995; Chu et al., 1995a; Goodearl et al., 1995; Jo et al., 1995; Sandrock et al., 1995). It is not clear, however, whether ARIA can significantly stimulate expression of the AChR γ subunit gene (Martinou et al., 1991), and the fact that γ subunit levels decrease postnatally in vivo has suggested that it might be ARIA-unresponsive. To investigate this issue we modified an assay system previously used to study the ε3500 transgene (Chu et al., 1995a) to ask whether the γ10−nlacZ transgene, which directed synapse-specific transcription in vivo, was responsive to the synaptic factor ARIA in vitro.

Primary cultures were prepared from individual γ10−nlacZ neonatal mice. Untreated muscle cultures exhibited a low but detectable level of γ10−nlacZ transgene activity in myotubes (Figs. 7a and 7e), reflecting the activation of the AChR γ subunit gene during myogenesis. To determine whether these γ subunit regulatory sequences were ARIA-responsive, we treated sibling cultures with 3 nM of a recombinant ARIA fragment for 48–96 hr before staining them for β-galactosidase. In 14 of 17 experiments, the number and intensity of transgene-expressing myotubes was at least twofold greater in ARIA-treated wells than in controls (Fig. 7b). Thus, the AChR γ subunit gene, like the AChR ε subunit gene, can indeed be induced by ARIA.

Next, we asked whether the AChR γ genomic sequences were activity- as well as ARIA-responsive. For this purpose, cultures were treated with the action potential blocker, TTX. In about half of the experiments (8 of 17), TTX stimulated expression of nlacZ (Fig. 7g); in the other nine experiments, TTX did not affect the number of nlacZ-positive myotubes (Fig. 7c). Responsiveness to TTX seemed to correlate with the degree of spontaneous electrical activity present prior to treatment: high levels of spontaneous twitching were observed in three separate experiments, and these three exhibited the greatest TTX-induced transgene expression. We believe that the other TTX-responsive cultures were likely to have had lower amounts of electrical activity that were missed during the short interval in which we observed them. The remaining, unresponsive, cultures may have been inactive, in which case one would not expect to observe an effect of TTX.

Because TTX could induce nlacZ expression, we wondered whether ARIA might have stimulated the AChR γ subunit promoter by suppressing electrical activity. The observation that ARIA activated the γ10−nlacZ transgene in many experiments where TTX had no effect (Figs. 7a–7c) suggested that this was not the case. As a more critical test, we compared expression levels in TTX plus ARIA-treated cultures to cultures treated with TTX alone. Even in the presence of TTX, ARIA still had an effect (Figs. 7c, 7d; 7g, 7h), as shown previously for the endogenous AChR γ subunit gene by Martinou et al. (1991). Because transgene activation by the suppression of electrical activity does not prevent ARIA from inducing expression even further, ARIA-dependent activation must involve an additional signaling pathway.

Finally, in comparing the effects of TTX and ARIA in
individual experiments, we noted that activity (as judged both by twitching and TTX-inducible gene expression) and ARIA responsiveness were inversely correlated. In fact, the three experiments mentioned above which exhibited the most spontaneous activity showed little or no response to ARIA at all (Figs. 7e and 7f). Interestingly, in these same experiments, parallel cultures which had been treated with TTX exhibited a pronounced ARIA-dependent transgene induction (Figs. 7g and 7h). These results suggest that paralysis can potentiate the ARIA-dependent induction of the AChR γ subunit gene, and/or that electrical activity inhibits ARIA-induced expression.

DISCUSSION

Here we have compared the expression and regulation of the AChR γ and ε subunit genes. Our main findings were: (1) All embryonic muscles express γ-containing AChR exclusively, most begin to express the ε subunit shortly after birth, and the majority of muscles have no detectable γ subunit-containing AChR by P 17. (2) The transition from γ to ε receptor types occurs nearly synchronously at all endplates within a fast muscle, showing no relationship to the level of geometric complexity of the individual endplate. (3) The subunit transition occurs homogeneously throughout an endplate, indicating that localized differences in receptor subunit composition are unlikely to underlie subsequent fate differences between endplate subregions. (4) The predominately slow soleus muscle shows a delay in the timing of the subunit switch, and the multiply innervated slow or tonic fibers of EOM maintain γ subunit expression into adulthood. This persistent γ subunit expression is brought about, at least in part, by differential transcriptional regulation. (5) Regulatory elements S′ to the transcriptional start site of the AChR γ subunit gene are able to direct muscle-specific, developmentally regulated, activity-dependent, and synapse-specific transcription in vivo. Synaptic expression of nlaC decreases postnatally in the γ-nlaC transgenic mice, during the same period in which synaptic nlaC expression increases in the previously described (Sanes et al., 1991) ε-nlaC mice. (6) Distal elements of the AChR γ subunit gene are required for its efficient expression in vivo but not in vitro. (7) The γ subunit gene, like the ε subunit gene (Chu et al., 1995a), is ARIA responsive, but the inductive effect of ARIA on the γ subunit gene is affected by the electrical activity level of the myofiber.

Variations in Timing of γ Elimination among Muscles

The majority of skeletal muscles studied underwent the switch from γ to ε-containing AChRs with an identical time course, resulting in weak γ subunit staining by P 9. However, in soleus this transition was strikingly slowed, and in a subset of EOM fibers γ subunit expression persisted into adulthood. These observations suggest that the rate of subunit substitution is correlated with underlying fiber type: in fast-twitch muscles, which primarily express the fast myosins type IIB and IIX (Donoghue et al., 1991), down-regulation of the γ subunit occurs quickly; in soleus, a slow-twitch muscle composed of type I and IIA fibers, the loss of the γ subunit is delayed by at least a week; and in the slow-twitch or tonic MIFs of EOM, which express myosin type I, it never occurs at all. Indeed, in other systems, some correspondence has been seen between fiber types and AChR expression: the slow-twitch muscle fibers of frogs and snakes have AChR channel types at their endplates different from those found in ordinary fast fibers (Dionne, 1989; Henderson and Brehm, 1989), implying an interrelationship between channel properties and fiber functionality.

However, fiber type per se may not correlate directly with γ subunit expression, as the γ-to-ε transition appears to be nearly synchronous at all endplates of muscles that contain large populations of both fast- and slow-twitch fibers. Instead some global properties of each muscle, such as levels and patterns of nerve activity, may override local fiber-to-fiber variations. There is evidence, for example, that innervated soleus contains higher levels of extrasynaptic AChRs than do faster muscles (Fambrough, 1979), suggesting that the slower activity pattern characteristic of this muscle may be less effective in suppressing the expression of receptor genes by extrasynaptic nuclei. Thus, this same pattern may also be less effective in turning off the synaptic expression of the highly activity-dependent AChR γ subunit, leading to the delay in developmental down-regulation observed for soleus. Other studies have confirmed that the specific pattern of nerve activity is as important to gene regulation as absolute activity levels. Direct stimulation of the soleus with a fast activity pattern can convert the contraction speed and myosin expression of that muscle to those usually seen in a much faster muscle, even when the total number of impulses is kept the same for fast and slow stimulus trains (Gorza et al., 1988). In the case of EOM, the MIFs are thought to mediate slow, sustained muscle contractions as those necessary for maintenance of eye position (Bach-y-Rita and Ito, 1966; Kaminski et al., 1990), and therefore the type of nerve stimulation they receive may be qualitatively different from any of the patterns experienced by fast-twitch muscles. This difference may help to explain their maintenance of the activity-sensitive AChR γ subunit, even while it is eventually eliminated from all other muscles.

Whatever the cause of maintained γ subunit expression in EOM may be, this finding has clear clinical implications for our understanding of ocular myasthenia. Eye muscles are involved in the vast majority of myasthenic patients, and in many cases are the only muscles affected (Oosterhuis, 1982). Some studies have indicated that myasthenic sera may contain antibodies that react selectively with EOM but not other skeletal muscle (summarized in Kaminski et al., 1990), and antigens recognized by some sera have been found to be present exclusively at multiply innervated fibers (Oda, 1993). This evidence, combined with physiology showing that tonic fibers are often the most affected in...
ocular myasthenia, has led investigators to look at MIFs for unique AChR channel types or epitopes. Additional work demonstrating that many myasthenic sera selectively block slow but not fast channels in developing rat muscle (see Kaminski et al., 1990), and the recent detection of AChR γ subunit message in adult EOM (Horton et al., 1993) seemed to point to the AChR γ subunit as the source of this distinguishing epitope. Our finding that multiply innervated fibers of EOM are unique in expressing the γ subunit into adulthood helps explain the particular antigenicity of EOM, and may suggest new approaches to mitigating the effects of ocular involvement in myasthenic syndromes.

During preparation of this article, Kaminski et al. (1996) reported on the distribution of AChR γ and ε subunit immunoreactivity in adult rat EOM. Their main result, that the AChR γ subunit is present at en grappe endings, is consistent with ours. On the other hand, they found that AChR γ- and ε-like immunoreactivities were both present at all en grappe and most en plaque endings. In contrast, we find a predominantly complementary distribution in mouse EOM: γ but not ε at en grappe endings, and ε but not γ at en plaque endings. There are several possible explanations for these discrepancies, including species differences (rat vs mouse), age differences (we used young adults), and differences between global and orbital portions of the muscle. It is also possible that our antisera to the AChR γ subunit is less sensitive than that of Gu and Hall (1988; used by Kaminski et al., 1996), and that the antisera to the AChR ε subunit (used by both groups) reacts better with rat than mouse antigens. We cannot at present distinguish among these alternatives.

ARIA- and Activity-Dependent Regulation of AChR Subunit Expression

Many observations suggest that the nerve uses two mechanisms to induce the selective expression of AChR subunit genes by synaptic nuclei (Chu et al., 1995b). First, electrical activity represses transcription of these genes throughout the muscle fiber. Second, some secreted signal(s), such as ARIA, stimulate these genes locally in the synaptic region. Interestingly, however, expression of the five AChR subunit genes is regulated differently. The γ subunit gene is activated throughout the muscle soon after myotubes form, becomes synaptically concentrated during embryogenesis, and then disappears after birth. The ε subunit gene is activated perinatally, as γ subunit levels are declining, and is restricted to the synaptic compartment at all times. The remaining subunits, α, β, and δ, seem to combine features of the above patterns: they turn on early and then become synaptically localized, like the γ subunit, but they remain concentrated at synapses into adulthood, like the ε subunit. Thus, either the AChR subunit genes are regulated by different signals, or they respond in different ways to a common set of signals.

We have used a tissue culture system to gain insight into how these regulatory differences arise. Cultures were derived from γ10-lacZ transgenic mice, which mimicked expression of the endogenous AChR γ subunit gene in many ways. Using these cultures, we found that the AChR γ subunit promoter is highly induced by ARIA, as had been previously shown for the AChR ε subunit gene using similar cultures derived from c3500–lacZ transgenic mice (Chu et al., 1995a). Thus, the differential developmental regulation of these two genes does not arise from the selective responsiveness of one or the other to this synaptic cue. Previously, we found that ARIA could induce the AChR γ subunit gene in the presence or absence of TTX (Martinou et al., 1991). Here, we extend these findings by reporting that the magnitude of the response is dependent on the electrical state of the muscle: highly active γ10–lacZ cultures were poorly ARIA-inducible, yet these cultures could be rendered ARIA-inducible by addition of TTX. Interestingly, even in the relatively inactive cultures in which TTX had little effect on its own, TTX could still enhance the induction by ARIA (Figs. 7b and 7d). In contrast, the ε subunit gene is considerably less affected by muscle activity overall, showing very little activation in response to denervation in vivo (Witzemann et al., 1991; Gundersen et al., 1993), or to paralysis in vitro (Chu et al., 1995a). Furthermore, primary muscle cultures prepared from c3500–lacZ mice show no interdependence of ARIA and TTX treatments in regulation of this gene (G.C.C. and J.P.M., unpublished observations). These strikingly different responses of the γ and ε subunit genes to the influences of electrical activity and localized nerve-derived signals may provide a partial explanation for the fact that as the muscle matures and becomes increasingly active, expression of the γ subunit gene decreases while that of the ε subunit gene persists.

It will now be important to elucidate the mechanism by which the activity- and ARIA-dependent regulatory pathways interact. One possibility is that the γ subunit promoter/enhancer simply sums the positive ARIA induction with the repressive effects of activity. Alternatively, the respective responsive elements could directly interact; for example, sequences that regulate synapse-specific and activity-dependent expression might cooperate to bind transcriptional activators in the γ subunit promoter, even though they are capable of acting separately in the context of the ε subunit promoter (Tang et al., 1994). In this regard, it will be interesting to examine the interactions between activity and ARIA in the regulation of the other AChR subunits, α, β, and δ. One perplexing feature of synaptic development is that nerve-evoked activity turns off the transcription of many genes in extrasynaptic nuclei, while the nuclei closest to the synapse are apparently immune to such repression. The AChR γ subunit, however, shows no such immunity, and in our assay it is also unresponsive to ARIA in the presence of high activity. We predict from these results that the other AChR subunits, which do show synaptic immunity from activity-mediated repression, will remain ARIA-responsive in active muscle. Transgenic mice with synapse-specific AChR α (Klarsfeld et al., 1991) and AChR δ (Simon et al., 1992) promoters already exist, so this hypothesis should be testable by the same approach we have used here.
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