The 5'-flanking region of the mouse muscle nicotinic acetylcholine receptor β subunit gene promotes expression in cultured muscle cells and is activated by MRF4, myogenin and myoD

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ABSTRACT

The expression of the nicotinic acetylcholine receptor (AChR) in vertebrate striated muscle is regulated both during development by nerve-evoked muscle activity and by local factors released or associated with the nerve ending. The expression pattern of AChR is achieved by coordinate regulation of four embryonic subunit mRNAs, α , β , γ and δ . We have taken the approach of identifying the similarities and differences among cis-acting regulatory elements of AChR genes to gain a better understanding of these mechanisms. Thus, to begin to define DNA sequences necessary for the transcriptional regulation of the mouse β AChR gene, we have analyzed its 5'-flanking region. Primer extension and RNAase protection analyses showed that transcription initiates at one major and two minor sites, all of which are close to the translational initiation site. Using plasmids in which segments of the 5'-flanking region were linked to the bacterial chloramphenicol acetyltransferase (CAT) gene, we have demonstrated that 150 bp of the 5'-flanking region is active in C2 myotubes but not C2 myoblasts or NIH3T3 fibroblasts. This region contains a putative binding site for myoD, and when linked to CAT was transactivated by the muscle regulatory factors myoD, myogenin, and MRF4. Thus, a 150 bp sequence of the β -subunit gene contains information necessary for developmental specificity and responsiveness to myogenic factors.

INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is a multisubunit protein consisting of five subunits with the stoichiometry of $\alpha_2\beta\gamma\delta$ in fetal and neonatal skeletal muscle and $\alpha_2\beta\epsilon\delta$ in adult muscle (reviewed in 1–4). Although four of the subunit genes map to at least three different loci (5), their expression is tissue

specific and coordinately regulated during skeletal muscle development. The four subunit mRNAs found in fetal muscle (α , β , γ and δ) are down regulated by nerve-evoked muscle activity, and all five mRNAs are more highly concentrated in the region of the sarcoplasm nearest the neuromuscular junction and much less abundant in extrasynaptic areas of adult innervated muscle (6–9). Nuclear run on experiments have shown that, in differentiating muscle cells (10) and in muscle tissue after denervation (11), the increase in mRNA levels is caused by transcriptional activation. Thus, the coordinated changes of these four mRNAs are apparently regulated at the transcriptional level.

In spite of the coordinate nature of regulation, there are some subtle, yet interesting differences in β -subunit gene expression. β mRNA levels in differentiated C2 myotubes and adult skeletal muscle are lower than those of the α , γ and δ subunit genes (12, 13). Furthermore, upon denervation of rat hindlimb muscle, the β mRNA level increases less than the other subunit mRNAs (7, 12, 14). In neonatal rat muscle, the developmental profile of β mRNA also differs from that of the α , γ and δ subunits, where β mRNA is less abundant immediately after birth, increases transiently and finally decreases (8). Thus, it seems likely that subtle differences in expression in the β subunit gene may be due to slightly different regulatory mechanisms governing the transcription, processing, or stability of the RNA.

To study control of transcription of AChR genes, cis-acting elements necessary for tissue specific and developmental expression have recently been defined in the chick α and δ genes (15–17), the mouse α , γ , and δ genes (18–20), and the rat γ gene (21). In addition, Crowder and Merlie have defined several DNAase I hypersensitive sites in the mouse γ -subunit gene locus (22, 23). Although exhibiting little sequence identity generally, all of the AChR gene promoters that have been sequenced to date have putative binding sites for recently described myogenic factors. These factors are encoded by a family of genes, myoD (24), myogenin (25, 26), myf5 (27), and MRF4/herculin (28, 29),

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which, when individually introduced into many non-myogenic cells, convert them to cells with characteristics of skeletal muscle myoblasts. Not surprisingly, these proteins have recently been shown to bind to regulatory regions of skeletal muscle specific genes, including creatine kinase (30, 31), myosin light chain (32), and chick AChR α -subunit (33). We have shown that the chick and mouse α subunit promoters are particularly well activated by MRF4 (18), and this result is in direct contrast to the behavior of other muscle specific promoters, including troponin I, myosin light chain, and muscle creatine kinase genes (34).

To determine whether coordinate regulation and unique aspects of AChR gene expression correlate with similarity or differences in the promoter elements, we have examined the possible role of the 5' flanking region of the previously uncharacterized mouse β gene in tissue specific and developmental regulation. To accomplish this, we have identified the β transcription start site within a previously described genomic sequence (35). A chloramphenicol acetyltransferase (CAT)-fusion gene incorporating 150 bp of the mouse β gene 5' flanking sequence was prepared and shown to express in muscle (C2), but not fibroblast (NIH3T3) cell lines. Our main result is the demonstration that the β promoter is transactivated by MRF4 as well as myoD and myogenin in NIH3T3 fibroblasts.

MATERIALS AND METHODS

RNA isolation

Total RNA was isolated by differential precipitation (36) with modifications described in Frail *et al.* (10). Poly(A⁺) mRNA was further purified by chromatography on oligo(dT) cellulose (37, 38).

RNAase protection

RNAase protection was performed as described by Melton *et al.* (39), with modifications described in a technical bulletin from Promega Biotech (Madison, WI). To map the cap site for the β -subunit gene, g β 81 in pBS(+) (35) was cut with SacII, treated with T4 polymerase to make blunt ends, and then cut with EcoRI. The isolated 1.04 kb fragment containing exon 1, intron 1, and 856 bp of upstream sequence was subcloned into the SmaI and EcoRI sites of pBS(+) (Stratagene). This construct, g β 81 Δ 1, linearized with HindIII, was transcribed in the presence of [α -³²P] UTP according to Promega Biotech's transcription protocol. The radiolabeled antisense RNA probe was hybridized overnight at 45°C to 20 μ g of total RNA isolated from differentiated BC3H1 cells, differentiated C2 cells, mouse brain, or 20 μ g of wheat germ tRNA (Sigma Chemical Company, St. Louis, MO), and subsequently digested with 5 μ g/ml of RNAase T1 (Sigma) and 40 μ g/ml of RNAase A (Sigma) for 30 min at 20°C. Samples were then treated with 50 μ g of proteinase K for 15 min at 37°C and subsequently extracted with phenol-chloroform. Ammonium acetate was added to a final concentration of 2.0 M and the RNA was precipitated with the addition of 2.5 volumes of ethanol. Samples were washed once with 70% ethanol, redissolved in 70% formamide, and denatured at 100°C for 5 min. The sizes of the RNAase protected products were determined on an 8% sequencing gel and visualized by autoradiography.

Primer extension

A 30-mer oligonucleotide primer from nucleotides 72 through 101 in the mouse AChR β -subunit cDNA (13) was end labeled

with T4 polynucleotide kinase and hybridized to 10 μ g of poly(A⁺) mRNA from differentiated BC3H1 cells or mouse liver in 100 mM TRIS, pH 7.5, 1.0 mM EDTA at 75°C for 30 min. The solution was then adjusted to 10 mM TRIS, pH 7.5, 0.1 mM EDTA and hybridization continued for an additional 2 hr at 68°C. The primer was extended with MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in the presence of 0.5 mM each of dATP, dCTP, dGTP, and dTTP for 30 min at 37°C. The sizes of the primer extended products were determined by comparison to a sequence ladder and visualized by autoradiography.

Construction of mouse β -AChR-CAT expression plasmids

A fragment containing 891 bp of 5'-flanking DNA and part of the first exon of the β -subunit gene was obtained by PCR of the plasmid used for RNAase protection, g β 81 Δ 1, and two 18-mer oligonucleotides from -856 through -839 and from 17 through 34 of the mouse AChR β -subunit gene (35). The fragment was ligated into the HindIII site of pUC9CAT (40) to obtain p β B (fig. 2). A deletion of this plasmid was obtained by digesting the PCR fragment with PstI, and ligating the smaller 150 bp fragment into the HindIII site of pUC9CAT resulting in p β A. Both constructs were confirmed by restriction analysis and DNA sequencing.

Cell culture

C2 cells (41) were propagated in DMEM plus 20% fetal calf serum (growth medium) at subconfluent densities, and differentiation was induced by switching the cells to DMEM plus 10% horse serum (differentiation medium). NIH3T3 cells were propagated in DMEM plus 10% fetal calf serum.

Transfections

DNA was transfected into all cells by calcium phosphate co-precipitation (42). C2 cells were seeded at approximately 2×10^5 cells per 10 cm plate for cells that will remain myoblasts throughout the experiment, and 5×10^5 cells per 10 cm plate for cells that will differentiate into myotubes. Cells were refed with fresh growth medium 24 hr after plating and 3 hr before transfection. For each plate 15 μ g of a CAT construct and 5 μ g of a SV40-*Lac Z* construct, pCH110, (43) were transfected. Cells were glycerol shocked 12–24 hr later with 1.5 ml of 15% glycerol in HEPES buffered saline for 2 min at room temperature, followed by washing with DMEM. The cells were then incubated in either growth medium for the myoblasts or differentiation medium for the myotubes. C2 myoblasts were harvested 24 hr, and myotubes 48 hr after glycerol shock, and cell extracts were prepared by several freeze-thaw cycles followed by centrifugation. NIH3T3 cells were transfected in the same manner except that 1×10^5 cells were seeded per 10 cm plate, DMEM plus 10% fetal calf serum was replaced after glycerol shock and the cells were harvested 48 hr after glycerol shock.

Transient transfections of myogenic factor expression plasmids with CAT reporter constructs in NIH3T3 cells were carried out as follows: NIH3T3 cells were seeded at 1×10^5 cells per ml. The transfections were carried out as described above with 15 μ g of the CAT-reporter constructs, 10 μ g of the myogenic factor expression plasmids, and 5 μ g of the SV40 *Lac Z* plasmid. After glycerol shock, cells were maintained in DMEM plus 10% fetal calf serum for 48 hr and then harvested.

Enzyme assays

Cotransfection of cells with SV40-*Lac Z* was performed to monitor the transfection efficiency in each plate, and β -galactosidase assays were done as described in Miller (44). CAT activity in cell lysates was determined as previously described (45) using volumes of cell extract containing equal amounts of β -galactosidase, and quantitation was performed by excising labeled spots from thin layer plates and scintillation counting.

RESULTS

β -Subunit gene transcription initiation sites

To begin our study of regulatory elements in the 5'-flanking regions of the mouse β subunit gene, we first identified the transcription start sites by RNAase protection and primer extension analyses. For the β -subunit gene, RNAase protection yielded three products that corresponded to three bands in the primer extension analysis indicating three start sites (fig. 1). These products were clearly present in the lanes containing BC3H1

muscle cell RNA, which was shown previously by Northern analysis to contain β -subunit RNA, and clearly absent in the tRNA and brain lanes used as negative controls. Although other bands also appear in the primer extension experiments, these did not appear in the RNAase protection experiments and are not considered authentic start sites.

Like other genes with multiple transcription initiation sites (17, 46), the mouse β -subunit gene appears to lack typical TATA and CCAAT boxes in the promoter region, and there appear to be no sequences to approximate them in the region immediately upstream of the transcription initiation site.

The 5' flanking region of the β subunit gene confers myotube specific expression

β -promoter-CAT fusions were constructed and transfected into mouse cell lines to determine whether the sequences proximal to the transcription initiation site could regulate CAT expression in C2 myotubes. Both constructs, β B (850 bp) and the shorter construct, β A (150 bp), exhibited higher CAT activity in C2

Figure 1. Identification of AChR β -subunit transcription initiation sites. (A) RNAase protection analysis. 20 μ g of total RNA from mouse brain (BRAIN), and differentiated BC3H1 cells (BC3H1), and 20 μ g of tRNA (tRNA) were hybridized to a 32 P-RNA probe extending from -856 to +186 of the β -subunit gene. 91, 87, and 82 bp nucleotide fragments are protected from RNAase digestion, corresponding to nucleotides +1, +5, and +10, respectively in the mouse β -subunit gene. Because the 91 bp fragment appeared to be the most abundant, it was designated the major transcriptional start site, and numbered +1. PBR322 cut with *Hin*FI and labeled with 35 S was run in the lane marked pBR. The sizes of the fragments are indicated. (B) Primer extension analysis. A 32 P-labeled 30-mer oligonucleotide corresponding to positions 72 to 101 of the mouse β -subunit cDNA (13) was hybridized to 10 μ g of poly A+ mRNA from differentiated BC3H1 cells and 10 μ g of yeast tRNA and extended as described in materials and methods. The extended products were 203, 199, and 194 nucleotides long, which correspond to the same nucleotides in the β -subunit gene as the RNAase protection fragments, with the 203 nucleotide product corresponding to +1. (C) Schematic showing the partial mouse AChR β -subunit gene structure and the probes used for RNAase protection and primer extension. (D) Partial sequence of the β -subunit gene. The transcriptional start sites are marked with arrows. The translational start site is at +34 (met), and intron 1 starts at +92. Putative myoD binding sites are underlined with a solid line, GC rich regions are marked with a broken line, a putative CARG box (52, 53) is indicated with a double broken line, and a SHUE box (23) is marked with a broken wavy line.

myotubes than in undifferentiated C2 myoblasts and NIH3T3 fibroblasts, with a 10 to 16 fold increase in differentiated C2 myotubes over that obtained with the promoterless control plasmid pUC9CAT. Results of a representative experiment are shown in fig. 2. The rat MLC-promoter-CAT enhancer construct (MLCE) (40) displayed a similar increase of 15 fold over that of pUC9CAT, however, the 850 bp chicken AChR α -subunit

Figure 2. CAT activity of β -promoter constructs. (A) Reproduction of the thin layer chromatographic analysis of the CAT enzyme assay of C2 cells transiently transfected with the indicated constructs. CAT activity was measured as described in materials and methods. The conversion of ^{14}C -chloramphenicol to the acetylated form, determined by scintillation counting of the isolated forms, is reported at the top of each lane. MB, undifferentiated C2 myoblasts; MT, differentiated C2 myotubes. PUC9CAT represents the CAT vector with no insert (40), chick α -CAT is the 850 bp chicken α -subunit promoter fused to CAT (p α AChR CAT) as described by Klarsfeld *et al.* (47) and further subcloned by Merlie and Kornhauser (48), MLCE is a construct containing 400 bp of the myosin light chain 1 (MLC1) promoter and a 900 bp enhancer from the MLC1/3 locus in puc9CAT, as described by Donoghue *et al.* (40), and PSV2CAT contains the promoter and enhancer from SV40 as described in Gorman *et al.* (45). (B) CAT enzyme assay of NIH3T3 fibroblasts transiently transfected with the indicated constructs. (C) Diagram of CAT plasmids used to test 5' upstream regions of the β -subunit gene for promoter activity.

promoter fused to CAT (chick α -CAT) (47, 48) produced a ca. 150 fold increase. The high activity seen with β B in C2 myoblasts was peculiar to this experiment and was not observed in the four other trials. It is most likely due to an uneven distribution of cells which resulted in some cells becoming differentiated because regions of the plate were confluent. Thus, 150 bp of the 5'-flanking region of the mouse β -AChR gene contains at least some elements sufficient for tissue specific and developmental expression, and the additional 600 bp of β B may contain sequences that contribute to increased expression.

Muscle specific sequences in the β gene 5'-flanking region

Several sequences that appear in regulatory regions of other muscle specific genes also appear in the upstream region of the β gene. The β -promoter fragment contains five putative myoD binding sites, three of which are within 140 bp of the major start site (fig. 1). Other motifs include a putative CARG box (49, 50)

Figure 3. Transactivation of mouse AChR β -CAT fusions by cotransfection with myogenic factors. Transfections were performed as described in materials and methods. Cotransfections were performed with myoD, myogenin, or MRF4 cDNAs cloned adjacent to the Moloney sarcoma virus LTR promoter in the expression vector pEMSVscribe $\alpha 2$ (24). MLCE, myosin light chain promoter and enhancer in pUC9CAT (40); Ch α AChR, 750 bp of the chicken AChR gene 5' region cloned 5' of the CAT gene (47, 48), MLCP, myosin light chain promoter, but no enhancer in pUC9CAT (40). The numbers above the bars indicate the fold stimulation of the transfected CAT construct by the cotransfected myogenic factor over that by the vector, pEMSVscribe $\alpha 2$.

Figure 4. Alignment of identified control elements in the promoter/enhancer regions of the AChR subunit genes according to the position of E boxes. T, TATA box; C, CCAAT box; S, SP1 recognition site; A2, AP2 recognition site; E, E box (CANNTG). The solid black area indicates the regions determined necessary for myotube-specific expression.

at nucleotides -441 through -433, and several G-C rich regions that may also serve as SP1 binding sites (51) at -90 through -80 and -103 through -111 (fig. 4).

Transactivation with myogenic factors

The presence of multiple putative myoD binding sites suggests that myoD or myoD-like proteins may be involved directly in activating the mouse β -subunit gene during myogenesis. Therefore, we tested whether myoD, myogenin, and MRF4 could transactivate the β -promoter-CAT constructs. As shown in fig. 3, myoD, myogenin, and MRF4 transactivate the cotransfected β -promoter-CAT constructs in NIH3T3 fibroblasts with a 16 to 49 fold increase over cotransfection with the PEMS β scribe α 2 control vector. Although the absolute CAT enzyme activity was higher with β A, the fold-activation with β B was consistently two to three times higher because of a higher basal CAT activity expressed by β A. Interestingly, this correlates with the number of putative myoD binding sites, five and one in β B and β A, respectively.

The β -promoter constructs were very well activated by MRF4. This behavior differs significantly from the MLCE construct included in fig. 3 for comparison and from the previously reported properties of the troponin I, muscle creatine kinase and myosin light chain 1 promoters (34). The transactivation of the β -promoter constructs by MRF4 is similar to that observed with the mouse and chicken AChR α -promoters (fig. 3 and ref. 18) and has also been observed with the mouse AChR γ and ϵ promoters (T. Sunyer, C. Prody, and J. P. Merlie, unpublished). Thus, we believe that the transactivation of the β -promoter constructs by myoD, myogenin, and MRF4 reflects physiologically relevant characteristics of the AChR gene promoters.

DISCUSSION

Our experiments define the mouse AChR β -subunit gene promoter and show that the 5'-flanking region contains elements necessary for muscle specific and developmental regulation. The lower activity of the β promoter compared with the myosin light chain 1 promoter-enhancer and chicken AChR α constructs correlates with lower levels of endogenous β mRNA, and may be due to the lack of CCAAT and TATA elements in this gene (46). More experiments will be necessary to further define sequences responsible for quantitative differences in transcription.

We have detected very limited sequence identity between the β promoter region and the other AChR subunit gene promoters and, in fact, very little sequence identity among the five subunit gene promoters. All have multiple putative myoD binding sites, however, there is little similarity among the regions outside these motifs suggesting that the helix-loop-helix class of DNA binding proteins may account for much of the specific regulation of AChR gene transcription.

Multiple putative binding sites for helix-loop-helix type transcription factors are apparent in the β promoter, and are most likely responsible for the transactivation of the β subunit gene. Surprisingly, the shorter β A construct, which has only one putative myoD binding site, was also transactivated by myoD, myogenin, and MRF4, although to a lesser extent than β B. In the case of some genes transactivated by myogenic factors, including muscle creatine kinase (52) and myosin light chain (32) two myoD binding sites appear to be required. However, the mouse δ subunit enhancer contains a single myoD binding site,

and this site seems to be sufficient for both developmental and tissue specific expression (17, 53, 54). Thus, it appears that for the β subunit gene, a single putative myoD site can lead to some transactivation with the myogenic factors.

The transactivation of the β promoter region by MRF4 is similar to results obtained with the mouse and chicken α subunit promoters (18), and is unlike those reported for the troponin I, muscle creatine kinase, and myosin light chain genes, which show very low levels of transactivation with MRF4 (34). Thus, as a class, the AChR genes may show a preference for MRF4, and further experiments will be necessary to understand how this affects the expression of AChR genes *in vivo* and whether MRF4 activation is related to any of the regulatory changes characteristic of AChR genes including control by nerve induced electrical activity (48) or neurotrophic factors (55, 56).

Binding sites for muscle regulatory factors in the AChR subunit genes appear to play a major role in their developmental regulation. The results presented here, together with the recently described ϵ promoter and enhancer sequences (21, 57) provide a preliminary description of the regions necessary for muscle specific expression for all five of the mouse muscle AChR genes. An alignment of the 5' upstream regions of these genes based on the position of the putative E boxes is shown in fig. 4. The paired E boxes of the mouse and chick α genes and mouse β and γ genes and a single E box at approximately 60 bp in the γ , δ , and ϵ genes are the only identified tissue specific elements common to three or more AChR genes. Interestingly, an upstream E box in the mouse ϵ gene matches the position of E boxes in the chick α and δ genes. It seems likely that further definition of the minimum sequence elements required for proper regulation of transcription of AChR genes will be aided by comparison of the common as well as the unique sequence-function relationships in the five promoters.

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