

Isolation and Characterization of the Mouse Acetylcholine Receptor Delta Subunit Gene: Identification of a 148-bp *Cis*-acting Region That Confers Myotube-specific Expression

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Abstract. We have isolated the gene encoding the delta subunit of the mouse skeletal muscle acetylcholine receptor (AChR) and have identified a 148-bp *cis*-acting region that controls cell type-specific and differentiation-dependent gene expression. The 5' flanking region of the delta subunit gene was fused to the protein-coding region of the chloramphenicol acetyltransferase (CAT) gene and gene fusions were transfected into C2 mouse skeletal muscle cells. Both transiently and stably transfected cells were assayed for CAT gene expression. Deletions from the 5' end of the mouse delta gene demonstrate that 148 bp of 5' flanking DNA is sufficient to confer cell type-specific and differentiation-dependent expression: CAT activity is present in transfected myotubes, but not in transfected

3T3 cells or 10T1/2 cells. Moreover, the level of CAT expression in myotubes transfected with constructs containing 148 bp of 5' flanking DNA from the delta subunit gene is identical to that in myotubes transfected with constructs containing 3.2 kb of 5' flanking DNA and similar to expression from the SV-40 early promoter. Increased CAT activity in myotubes is a result of an increased rate of transcription from the delta subunit promoter, since CAT RNA levels are also 35-fold more abundant in myotubes than myoblasts. In contrast, the SV-40 early promoter is similarly active in all cell types. Thus, 148 bp of 5' flanking DNA from the delta subunit gene contains all the information required for cell type-specific and differentiation-dependent expression of the AChR delta subunit.

THE skeletal muscle acetylcholine receptor (AChR)¹ is a ligand-gated channel composed of four structurally related subunits. The four subunits assemble into a heteropentamer (alpha₂, beta, gamma, and delta) which binds acetylcholine and thus initiates a permeability change in the myofiber membrane that ultimately couples release of neurotransmitter to myofiber contraction (Fambrough, 1979; Karlin, 1980; Anderson, 1987).

The availability of probes to identify and purify the skeletal muscle AChR has resulted in a rather thorough description of developmental and physiological events that control AChR expression (Lomo and Westgaard, 1975; Fambrough, 1979; Salpeter, 1987). These studies demonstrate that synaptic factors and electrical activity can regulate expression of AChRs and provide a framework for more detailed understanding of the mechanisms that synaptic factors and electrical activity use to regulate AChR expression. Moreover, the wealth of information regarding biosynthesis, assembly, and regulation of the skeletal muscle AChR and the magnitude of the changes in AChR expression that are elicited by changes in myofiber electrical activity make analysis of AChR expression a favorable system to elucidate the steps

and mechanisms that couple synaptic factors and electrical activity to expression of neuronal products.

The four AChR subunits are encoded by separate genes (Nef et al., 1984; Heidmann et al., 1986) and these genes are activated coordinately during embryonic development (Baldwin et al., 1988). AChRs are expressed during muscle differentiation as myoblasts withdraw from the cell cycle and fuse to form multinucleated myotubes (Patterson and Prives, 1973). Subsequent to this initial expression in primary myotubes, the quantity of AChR and AChR subunit transcripts decreases as myofibers are innervated and acquire an appropriate pattern and intensity of electrical activity (Burden, 1977; Klarsfeld and Changeux, 1985).

In innervated adult muscle the quantity of AChR and AChR transcripts is low and increases 50–100-fold in myofibers that have been deprived of electrical activity by denervation or by pharmacological antagonists of synaptic transmission or nerve/muscle action potentials (Miledi and Potter, 1971; Berg et al., 1972; Lomo and Westgaard, 1975; Merlie et al., 1984). Thus, regulation of AChR expression during development and by electrical activity is controlled largely by the availability of transcript encoding each of the AChR subunits; during myogenesis and after denervation of adult muscle an increase in the level of AChR mRNA is accompanied by a similar increase in the level of AChR protein (Merlie et al., 1984; Evans et al., 1987).

1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; CAT, chloramphenicol acetyltransferase; HGH, human growth hormone.

To understand how expression of AChR is controlled both during development and by electrical activity, we have isolated the gene encoding the delta subunit of the AChR and have identified a *cis*-acting regulatory region that is sufficient to control appropriate expression of the delta subunit gene during muscle differentiation.

Materials and Methods

Materials

Enzymes were purchased from New England Biolabs (Beverly, MA) and Boehringer Mannheim Biochemicals (Indianapolis, IN). The human growth hormone (HGH) immunoassay kit was purchased from Nichols Institute (San Juan Capistrano, CA). [¹⁴C]chloramphenicol and ³²P-labeled nucleotides were purchased from New England Nuclear (Boston, MA). DME and G418 were purchased from Gibco Laboratories (Grand Island, NY) and serum was purchased from HyClone Laboratories (Logan, UT). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Boehringer Mannheim Biochemicals.

Isolation of the Mouse AChR Delta Subunit Gene

A genomic library was constructed from mouse (AJ) liver DNA partially digested with Mbo I and cloned into EMBL3 phage DNA (Frischauf et al., 1983). 400,000 phage from the unamplified library were screened with a mouse AChR delta subunit cDNA clone kindly provided by Drs. K. Mixer-Mayne and N. Davidson (Caltech, Pasadena, CA) (LaPolla et al., 1984). The ³²P-labeled nick-translated probe hybridized to three plaques. DNA was isolated from the purified positive phage. The mouse DNA was characterized by Southern blotting and DNA sequencing (Sanger et al., 1977). One phage contains the gene encoding the AChR delta subunit and the other two phages contain the gene encoding the AChR gamma subunit.

Characterization of the mouse AChR gamma subunit gene is not presented in this study.

Construction of Delta Gene-Chloramphenicol Acetyltransferase (CAT) Gene Fusions

A 2.0-kb Pvu II fragment that includes 1.8 kb of 5' flanking DNA, the first exon, and part of the first intron of the mouse delta subunit gene was subcloned in the sense orientation into the Sma I site of SP65 DNA. This DNA was linearized with Hind III and digested with Bal 31 to remove the methionine initiator codon of the delta subunit gene. T4 DNA polymerase was used to fill in the ends of the digested DNA with deoxynucleotides (Maniatis et al., 1982). The Bal 31-digested DNA was digested further with Eco RI and the fragment containing the delta subunit gene was subcloned into Eco RI and Hinc II digested SP65 DNA (SP-Pvu II, MD). A Hind III fragment which contains the CAT coding sequence and the small intron and polyadenylation site of SV-40 t antigen (Gorman et al., 1982; Laimins et al., 1982) was subcloned into the Hind III site of SP-Pvu II, MD (SP-Pvu II, MD-CAT). 5' deletions of SP-Pvu II, MD-CAT DNA were generated by digestion with appropriate restriction endonucleases or by Bal 31 digestion. An additional 1,330 bp of 5' flanking DNA was added by digestion of SP-Pvu II, MD-CAT with Sac I, removal of the 433 bp 5' Pvu II to Sac I fragment and ligation of a 1,762-bp Sac I fragment (Fig. 1). The constructs were analyzed by mapping with restriction endonucleases and by DNA sequencing. Cells were also transfected with a plasmid containing the SV-40 early promoter fused to the coding sequence of the CAT gene (SV-40-CAT) (Gorman et al., 1982) and with a plasmid (SP65) containing the CAT-coding sequence on a Hind III fragment but without a promoter (SP65-CAT).

Transfection of Mouse Cell Lines

DNA was transfected into mouse cell lines as a calcium phosphate precipitate (Wigler et al., 1979). For transient transfection assays we transfected a skeletal muscle cell line (C₂C₁₂ cells), a fibroblast cell line (NIH-3T3 cells), and a multipotential mesodermal stem cell line (C3H 10T1/2 cells) with a mixture of SP-MD-CAT plasmid DNA and plasmid DNA containing the HGH gene (Selden et al., 1986). C₂ cells were grown in DME supplemented with 15% FCS and 50 µg/ml gentamycin; 3T3 cells were grown in DME supplemented with 10% FCS and 50 µg/ml of penicillin/streptomycin; 10T1/2 cells were grown in DME supplemented with 15% FCS and 50

µg/ml of penicillin/streptomycin. Each 10-cm plate was transfected with 10 µg of SP-MD-CAT DNA and 2 µg of HGH DNA. Since differentiation and fusion of semiconfluent C₂ cells is initiated by a shift from 15% FCS to 2-10% serum, all cell lines were maintained for 2-3 d in DME containing 5% adult bovine serum after transfection. CAT expression in 3T3 cells and 10T1/2 cells could not be induced by growing cells in 10-15% FCS after transfection. Cells were harvested 2-3 d after transfection.

Transfection of cells with the HGH gene was performed to monitor the efficiency of transfection. We measured the level of secreted HGH by radioimmunoassay as described by the supplier of an HGH immunoassay kit (Nichols Institute, San Juan Capistrano, CA) and corrected for variations in transfection efficiency by expressing CAT activity relative to HGH activity. HGH activity varied less than twofold among the three different cell lines, and there was less than twofold variation in HGH activity in different experiments in any one cell line: HGH levels in transfected C₂ cells were 150-250 ng/10-cm plate, in 3T3 cells 120-180 ng/plate, and in 10T1/2 cells 140-180 ng/plate.

Stably transformed C₂ cells were isolated by cotransfecting cells with SP-MD-CAT and pSV2-neo DNA (10:1 molar ratio of SP-MD-CAT/pSV2-neo DNA) and selecting for resistance to G418 (700 µg/ml) (Southern and Berg, 1982). CAT assays were performed on pooled, stably transformed cells. Expression in stably transformed cells was normalized to total cellular protein and relative CAT specific activities in myoblasts and myotubes are presented in Table II. A single plate (100 cm) of myoblasts contains ~100 µg of protein and a single plate of myotubes contains ~750 µg of protein.

CAT Assay

Cells were washed twice in ice-cold PBS, scraped off the plate in PBS with a rubber policeman, collected by centrifugation, and resuspended in 150 µl of ice-cold 0.25 M Tris, pH 7.9. Crude cell extracts were prepared by three cycles of freezing/thawing (dry ice/ethanol, followed by heating at 60°C). Cell debris was removed by centrifugation and deacylation activity minimized by heating the supernatant for 10 min at 60°C. CAT assays were performed with 50-80% of crude extract from transiently transfected cells and 10-20% of extract from stably transformed cells as described (Gorman et al., 1982). The amount of extract in each assay from transient transfections was normalized to levels of secreted HGH (160 ng of HGH per assay). 0.4 µCi of [¹⁴C]chloramphenicol (New England Nuclear) was incubated with crude extract in the presence of 0.4 mM acetyl CoA for 2 h at 37°C. After extraction with ethyl acetate, acetylated chloramphenicol was resolved from unacetylated chloramphenicol on a TLC plate developed with 20:1 chloroform/methanol. Radioactive chloramphenicol spots were identified by autoradiography, excised from the TLC plate, and radioactivity determined by scintillation counting.

RNase Protection Assay

Total RNA was isolated from cells (Chirgwin et al., 1979) and used in RNase protection assays (Melton et al., 1984; Baldwin et al., 1988). The probe for CAT mRNA was synthesized from SP65-CAT DNA (250-bp Hind III-Eco RI fragment from the 5' end of the CAT gene subcloned into Eco RI and Hind III digested SP65 DNA) linearized with Hind III; the probe is 260 nucleotides long and protects 250 nucleotides of CAT RNA. The probe for neomycin phosphotransferase (neo) mRNA was synthesized from SP65-neo DNA (1.26-kb Hind III-Sma I fragment from pSV2-neo subcloned into Sma I and Hind III digested SP65 DNA) linearized with Pvu II; the probe is 720 nucleotides long and protects 695 nucleotides of neo RNA. The probe for mapping the transcription initiation site was synthesized from SP65-MD DNA (Pvu II-Pvu II fragment extending from nucleotide -1821 to nucleotide +234 subcloned into Sma I digested SP65 DNA) linearized with Xho II; the probe is 400 nucleotides long. The probe for mapping the transcription start site in delta-CAT gene fusions was synthesized from SP65 DNA containing the 786-bp Eco RI fragment from SP65-Xmn I, MD-CAT subcloned into the Eco RI site of SP65 DNA. This Eco RI fragment contains 499 bp of 5' flanking region and 24 bp of 5' untranslated region from the delta subunit gene, 13 bp from the polylinker of SP65 DNA, and 250 bp from the CAT gene. The DNA was linearized with Bam HI. The probe is 831 nucleotides long and protects 286-287 nucleotides of RNA (250 nucleotides of CAT RNA, 13 nucleotides encoded by the polylinker, and 23-24 nucleotides from the delta subunit gene) (Fig. 6). Preparation of ³²P-labeled probes, hybridization, digestion, and analysis of protected labeled products was as described (Melton et al., 1984; Baldwin et al., 1988).

Primer Extension

A 42-mer synthetic oligodeoxynucleotide which is complementary to nucleotides 3 to 44 in the mouse delta subunit cDNA (lambda58 in LaPolla et al., 1984) was synthesized on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The 5' end of the oligonucleotide was labeled with ^{32}P (Maniatis et al., 1982) and the labeled primer was hybridized to RNA (in 0.4 M sodium chloride, 0.1% SDS, 10 mM Pipes, pH 6.4) at 70°C for 8 h. Addition of nucleotides to the annealed primer was catalyzed by reverse transcriptase (Hu et al., 1986); the reaction products were resolved in a DNA sequencing gel.

Results

Isolation and Characterization of the Delta Subunit Gene

The gene encoding the delta subunit of the murine AChR was isolated from a murine genomic library (see Materials and Methods). The phage DNA contains ~18 kb of murine DNA including 5.5 kb encoding the entire protein coding sequence of the mouse delta subunit, ~7 kb 5' to the transcription start site and ~5.5 kb 3' to the translation termination codon. A restriction map of the murine DNA was determined by Southern blotting and a partial restriction map is illustrated in Fig. 1. Approximately 2.5 kb of DNA that contains 2 kb of 5' flanking DNA, the first two exons and two introns of the mouse delta subunit gene was sequenced (Fig. 1). The DNA sequence within the two exons is identical to the corresponding region of the delta subunit cDNA isolated from the BC3H1 mouse muscle cell line (LaPolla et al., 1984) with the exception of an arginine residue at amino acid 25 encoded by CGU rather than an alanine residue encoded by GCU (Simon, A., and S. J. Burden, unpublished data). Moreover, the positions of the exon/intron boundaries in the mouse delta subunit gene are similar to those in the delta subunit gene from chicken (Nef et al., 1984): the first intron in the mouse delta subunit gene occurs at amino acid -7 of the signal sequence and the second intron occurs between amino acids 45 and 46 of the mature protein (Fig. 1).

The transcription initiation site was assigned by primer extension and RNase protection analysis (Fig. 2). Two transcription initiation sites are detected: one transcription start site is at an A residue assigned as nucleotide +1 and a second start site is at a T residue at nucleotide +2. Thus, the delta subunit gene encodes a 54–55 bp long 5' untranslated region. Although a canonical TATA element is not present, an A/T rich region that is bounded by C-rich regions is present 30–33 bp upstream from the transcription initiation site.

221–260 bp upstream from the transcription initiation site is a tract of alternating purine–pyrimidine residues composed of 20 AC repeats. Such purine–pyrimidine repeats may be involved in recombination (Bullock et al., 1986; Treco and Arnheim, 1986) and may reflect duplication of AChR subunit genes during evolution (Noda et al., 1983). In this regard, it is noteworthy that the 5' flanking region of the mouse AChR gamma subunit gene contains a purine–pyrimidine tract composed of 8 AC repeats ~800 bp upstream from the methionine initiator codon (unpublished data).

A sequence homology with the chicken AChR alpha subunit gene is present in the delta subunit gene: 7–17 bp upstream from the cap site of the delta subunit gene is a 9/11 nucleotide match with a sequence immediately upstream

from the cap site in the chicken alpha subunit gene (Klarsfeld et al., 1987). Moreover, this homology with the chicken alpha subunit gene is repeated in the mouse delta subunit gene 111 bp further upstream (-113 to -128) where the match is longer and consists of 14/16 residues (Fig. 1). A portion of this sequence (-8 to -15) is repeated in the chicken alpha subunit gene (-92 to -98) and occurs for a third time in the mouse delta subunit gene (-108 to -115) (Fig. 1).

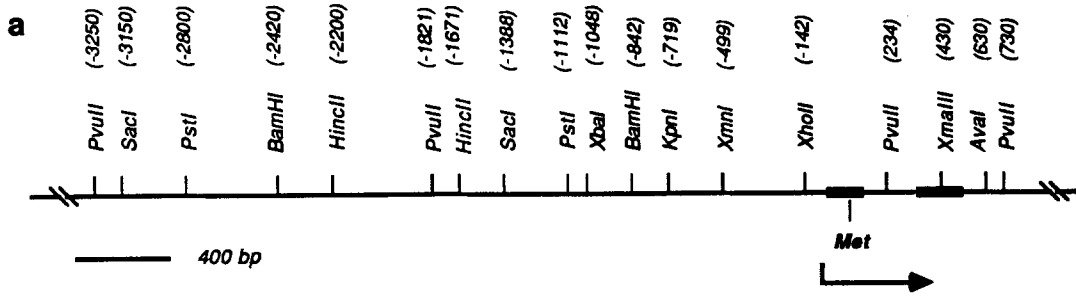
Identification of Upstream Region that Confers Myotube-specific Expression

Gene fusions between the 5' flanking region of the mouse delta subunit gene and the CAT gene were constructed and transfected into mouse cell lines (Materials and Methods). The methionine initiator codon was removed by Bal 31 exonuclease digestion to ensure that translation initiated at the methionine codon of the CAT gene. Initially, we transfected two delta-CAT gene fusions containing 3' deletions in the delta subunit gene into C2 cells and assayed for CAT activity in myotubes to determine whether promoter activity was retained in these 3' deletion constructs. The two gene fusions had identical 5' ends from the delta subunit gene (Pvu II site at -1,821), but differed in the amount of delta subunit DNA removed by Bal 31 exonuclease and thus had different 3' ends (Fig. 1). The construct with a 3' end at nucleotide -32 produced no CAT activity in C2 myotubes, whereas the construct with a 3' end at nucleotide +24 produced CAT activity in C2 myotubes that was comparable to that produced with SV-40 early promoter (Table I). Therefore, further analysis was restricted to the construct with a 3' end at nucleotide +24. Thus, all delta-CAT gene fusions include 24 bp of the 5' untranslated region and terminate 31 bp before the methionine initiator codon of the delta subunit gene.

Fig. 3 illustrates the amount of DNA from the delta subunit gene that is included in each construct. The amount of 5' flanking DNA from the delta subunit gene included in different gene fusions ranged from ~3.2 kbp to 148 bp.

The level of CAT activity produced with each construct in different cell lines is presented in Table I. CAT expression in myotubes is relatively constant with constructs containing as much as 3.2 kbp of 5' flanking DNA and as little as 148 bp of 5' flanking DNA. These same constructs produce ~100-fold less CAT activity in 3T3 cells and 10T1/2 cells (Fig. 4, Table I). Similar low level of CAT activity is detectable in cells transfected with a vector that contains the CAT protein coding region but lacks a promoter (Fig. 4, Table I) and in nontransfected cells. Thus, removal of 3 kb of 5' flanking DNA from the delta subunit gene did not result in loss of expression in myotubes nor in appearance of expression in fibroblasts or mesodermal stem cells. In contrast to the cell type-specific expression conferred by the delta subunit upstream region, the SV-40 early promoter confers equal expression of CAT in myotubes, 3T3, and 10T1/2 cells (Fig. 4, Table I).

We also transfected several CAT gene fusions (Delta, -1,821; Delta, -499; SV-40 early promoter; Fig. 3) into primary cultures of embryonic chick skeletal muscle and assayed for CAT activity in myotubes (2–3 d after transfection). The mouse delta subunit gene promoter was active in chick myotubes and produced CAT activity comparable to the SV-40 early promoter.



b

GAGCTCTTTGTACTTTCATGCTGGACCTTGTGAGCTTCTGATGGTGTCTTTGGAGCAGACATTGA
 ^-1392
 TCATTTTGTCTCATATGTGAGGGTGCAGGGGCTCTCTTAGAGACCAAGAGATGAACCTCAGGGCAT
 ^-1300
 CCTTCTTGTCTCATTGGTCTCATAGGTTGCATCATTGAGGGCAGAGTGGCAGCCTGGTCTGGCA
 ^-1200
 GGGTCTTGCATAGGTTTGGGCCACCTCCAACCTCTGCTTAGAACAAAGAAATAGGCTCAGACTA
 ^-1100
 TGGCTGCTAGTGCATGCTGCAGTGGCTCCTCTCTTGTGACAGCCCTATGGGCATCCCTTCACCCC
 ^-1000
 ATTGCTAGTCTCCCGACATCTAGATGGCCCTCCCTCCATGCCTGACGGTTCTGGCTTTCTGA
 ^-900
 TTCCTATTAGATAGAAGCAGATCACGGTCCCAGGCCATTTGAGGGTTGATTCTCTTAAGCATA
 ^-800
 CTCTTAGTGCCATCTATCCCGACCACTGACTTCTAGCCACAGTCATTACAACGGACAGGCTGAC
 ^-700
 TCTGTGTGGAAGACCCCTCTTGCCCCAGTGGATCCCTTTTCTTTCCCTTTGGTTTTGGCTACT
 ^-600
 CATAGATGCAGGAACGGTGGAGACTTCAGTTCAAATTAAGAAAGAAAAGAAATGGGCCAGTA
 ^-500
 AGGTGGCTTAGCAAAATAAGGTACCTGCTGCCGAGCCATGTGAAAGGAAAAAAATTTAACTCC
 ^-400
 TTCAAAGTTTCTCTGACTTCCACACATGCCACAACTGCCGAGAAACACACTGAGTGGATGGGTG
 ^-300
 GATGGATGGATGGATGGATGGATTTTATTATTACTTATTATTATTATTATAAATCTTTGGGGT
 ^-200
 TTTTGGAGACATGGCTTCTCTGTGTAACAACCTGGCAGTCTGGAACCTCATTCTGTAGACCAGG
 ^-148
 CTGGCCTTGAACCTCAGAGATCCTTCTCTTCCCAAGTGTGGGATTAAGCATACTGCCA
 ^-128
 CACCCAGCTTAGAGGTAATATTTTAAAGATGGGGAAAAATACACAAGTACAGGTGGAGCACT
 ^-113
 AGCAAGATGGCTCAACAGGTAACAGTTGCCCTGCTGTACAAGAAGTTTACCTCAGAACCCTCTGT
 ^-100
 AGAAAGAGAAGCAATGTGACAGGTGTACACACACACACACACACACACACACACACACACAC
 ^-80
 ACAGAGGATTCGCCCCCACCCTTGCACCCTACCCACCCAGGCCCCACCCTACCAACAATCTG
 ^-60
 TGACAATCCCTGCCTGGGATCTTTTCGTTCTGCCCTTGGCTCCTGCCCTAACTGGCAAAACCCACC
 ^-40
 CCCTCATCACCAGCTTTCAAGTATCAGATTGCGTTTCCGGCCTTCTTCTTCCAAACCCCTAAAC
 ^-20
 ACCAGCACCTGTCCCTTGCCTCATTCCACAGCAACAGGCTGAAGGGAAGACAAACCCCTAG
 ^-10
 TCAGTCAGAGGTGGGGATGGCAGGGCTGTGCTCACACTGGGGCTGTGGCTGCCCTTGTGTG
 ^-5
 TGTGgttaaggatgacataaacccttcctggggccttctactgacactggccaccgacctccetaac
 attatacctaattcccactcccactctgccccttcttaaccctagggatcccactcagggagcag

c

M.D.	TTTTCGTTCTGCCTTGGCTCCTGCCCTAACTGGCAAAACCCACCCCTCATCACCAGCTTTCAAGTAT		
	^ -128	^ -113	
	TGCCCT-GGCTCCT-C		
C.A.	TGCCCT-GGCTCCTCC		
	^ -15	^ -1	
M.D.	CAGATTGCGTTTCCGGCCTTCTTCTTCCAAACCCCTAAACCACCCAGCACCTGTCGCCCTTGCTTGCCTC		
			^ -17
			^ -7
C.A.	GT-CCCT-GCT		
			^ -16
			^ -6
M.D.	TGCCCTTGCCTCCTGCCCTAA		
	^ -128	^ -108	
	TGCCCT-G	TGCCCT--	
C.A.	TGCCCTGG	TGCCCTGG	
	^ -15	^ -8	^ -15
			^ -8
C.A.	CATGCC-TGGAA		
	^ -100	^ -90	
	TGCCCTGG		
C.A.	TGCCCTGG		
	^ -15	^ -8	

Figure 1. Restriction map and sequence of the delta subunit gene. (a) Restriction map of the 5' flanking region of the mouse AChR delta subunit gene. The first two exons are indicated by bold lines; two introns follow these exons. Sites for restriction enzymes and the position of the methionine initiator codon are indicated. The direction of transcription is illustrated by an arrow; the transcription start site is number 1 and nucleotides that precede it are indicated by negative numbers. (b) Sequence of the mouse AChR delta subunit gene from the Sac I site (-1,388) to the Pvu II site (+234). The cap sites (+1 and +2) are underlined and marked with asterisks (**). The methionine initiator codon (+56 to +58) is underlined; the first intron is written in small letters. The 5' nucleotide (-148) of the shortest delta-CAT gene fusion that results in myotube-specific expression is underlined and marked with a bracket (J). The 3' nucleotide (+24) of the delta subunit gene in CAT gene fusions is underlined and marked with a bracket (J); 3' deletion to nucleotide -32 (underlined and marked with a J) results in loss of CAT expression in myotubes. A tract of alternating AC residues is underlined. The carets (^) are spaced at 50 nucleotide intervals. (c) Homology between the mouse AChR delta subunit gene and the chicken AChR alpha subunit gene. Two regions of the mouse delta subunit gene (M.D.) have homology with a region of the chicken alpha subunit gene (C.A.) that is immediately 5' to the cap site (Klarsfeld et al., 1987). A shorter portion of this sequence is repeated in the chicken alpha subunit gene and occurs for a third time in the mouse delta subunit gene. The sequences are aligned and mismatches in the sequences are indicated with a dash (-). The first transcribed nucleotide is designated as +1 and nucleotides that are 5' to the cap site are indicated with negative numbers.

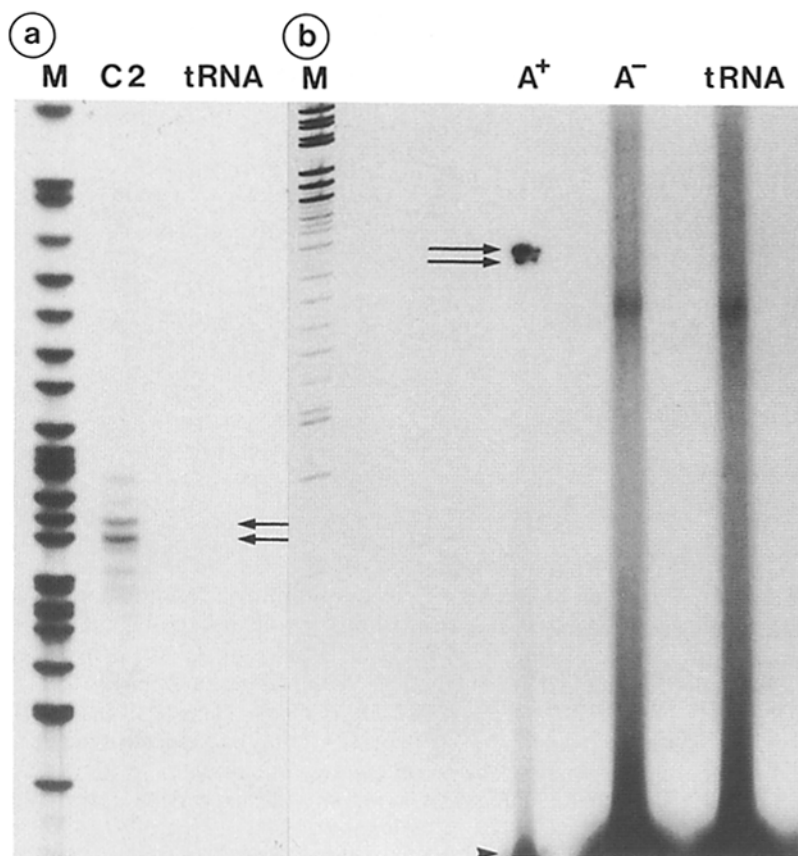


Figure 2. Identification of the transcription initiation site in the mouse AChR delta subunit gene. (a) RNase protection analysis of the transcription initiation site. 10 μ g of total RNA from C2 myotubes (C2) was hybridized to a 32 P-labeled RNA probe extending from nucleotide -142 to nucleotide +234 in the mouse AChR delta subunit gene. 107 and 105 nucleotide 32 P-labeled RNA fragments (arrows) are protected from digestion by RNase (Materials and Methods); the 3' ends of the protected fragments correspond to nucleotides +1 and +3 in the mouse AChR delta gene. Protected fragments are not detected in hybridizations with tRNA. The gel was exposed to x-ray film with an intensifying screen at -70°C for 16 h. The size of labeled products was determined by comparison to a DNA sequencing ladder (M). (b) Primer extension analysis of the transcription initiation site. A 32 P-labeled 42-mer oligodeoxynucleotide primer (Materials and Methods) was hybridized to 8 μ g of poly (A) $^{+}$ RNA from C2 myotubes, 20 μ g of poly (A) $^{-}$ RNA from C2 myotubes, or 20 μ g of tRNA, extended and analyzed as described in Materials and Methods. The extended products from C2 myotube poly (A) $^{+}$ RNA are 52 and 53 nucleotides long (arrows); the 3' end of the longer extended product corresponds to nucleotide +1 and the 3' end of the shorter product corresponds to nucleotide +2 in the mouse AChR delta subunit gene (Fig. 1). Extended products are not detected with C2 myotube poly (A) $^{-}$ RNA or with tRNA. The position of the primer is indicated with an arrowhead. The gel was exposed to x-ray film with an intensifying screen at -70°C for 48 h. The size of labeled products was determined by comparison to a DNA sequencing ladder (M).

C2 myotubes appear in culture 1-3 d after semiconfluent C2 myoblasts are grown in medium containing a low concentration of serum. Thus, transient transfection assays that result in differential expression of CAT activity in C2 myoblasts (1 d after transfection) and C2 myotubes (2-3 d after transfection) could arise as a result of accumulation of CAT during this differentiation period. To distinguish between time-dependent accumulation of CAT activity and differential rates of transcription in myotubes and myoblasts, we isolated stably transformed C2 cells (Materials and Methods) and measured CAT activity in stably transformed myoblasts and myotubes (Fig. 4, Table II). Stably transformed cells express \sim 100-fold more CAT activity in myotubes than myoblasts (Table II).

To determine whether differential CAT activity in myotubes and myoblasts is a result of increased gene expression in myotubes, we measured the quantity of CAT RNA in stably transformed myoblasts and myotubes. CAT RNA is readily detected in transfected C2 myotubes, and not detectable in transfected C2 myoblasts: stably transformed C2 cells express at least 35-fold more CAT mRNA in myotubes than myoblasts (Fig. 5). Thus, differential CAT activity in myotubes and myoblasts is a result of increased delta subunit gene expression in myotubes. In contrast to the delta subunit promoter, the SV-40 early promoter is active in myoblasts and myotubes and confers similar expression of mRNA en-

coding neomycin phosphotransferase in myoblasts and myotubes (Fig. 5).

To determine whether transcription was initiated correctly in delta-CAT gene fusions, the transcription initiation site was determined for delta (-182/+24)-CAT and for delta (-148/+24)-CAT. Fig. 6 demonstrates that transcription was initiated at nucleotides +1 and +2 in the transfected delta subunit gene. Thus, 148 bp of 5' flanking DNA from the delta subunit gene is sufficient to produce myotube-specific expression.

Discussion

This study demonstrates that a 148-bp region of the AChR delta subunit gene contains all the information required for cell type-specific and differentiation-dependent expression of the AChR delta subunit.

Although this study demonstrates that equivalent expression is obtained in C2 myotubes containing either 148 bp or 3.2 kbp of 5' flanking DNA from the delta subunit gene, it is possible that additional *cis*-acting elements can enhance or diminish expression in myotubes. Thus, *cis*-acting regulatory regions that are responsive to hormones and signals produced by electrical activity may not be contained within this 148 bp. In this regard, since stably transformed C2 cells allow one to study regulation of the delta subunit gene in

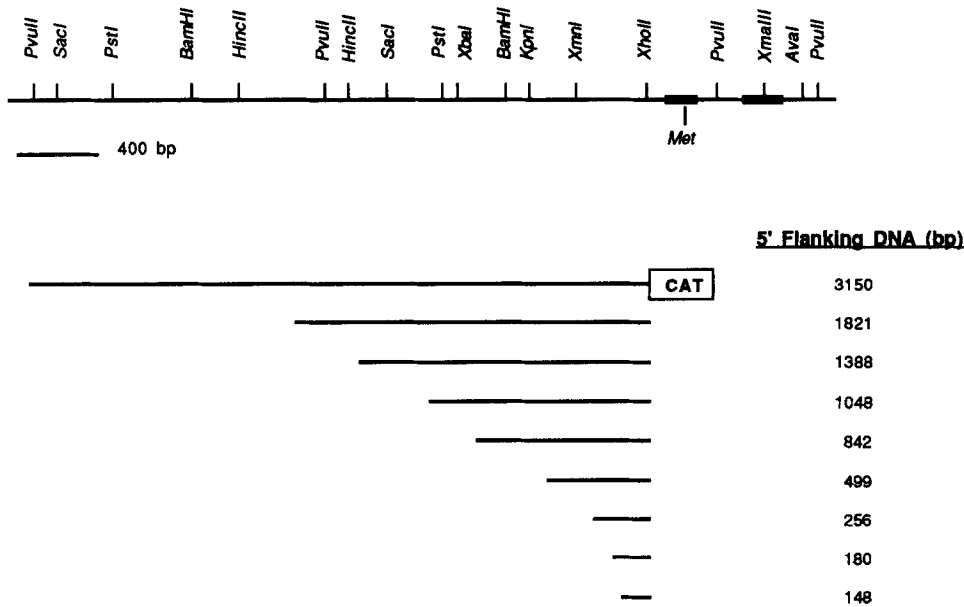


Figure 3. Mouse AChR delta subunit-CAT gene fusions. The amount of DNA from the delta subunit gene that is included in each construct is illustrated. The number of nucleotides that are 5' to the cap site is indicated for each construct.

differentiated myotubes, the stably transformed cell lines should be helpful in identifying *cis*-acting regions that are responsive to hormonal and electrical activity dependent regulators.

Nevertheless, since AChR mRNA levels increase 50-fold during differentiation of C2 cells (Yu et al., 1986) and CAT RNA is ~35-fold more abundant in transformed C2 myotubes than C2 myoblasts, it is unlikely that a potent differentiation-dependent enhancer regulates delta subunit expression and is absent from the 5' flanking region examined in this study.

Expression in myoblasts was not detected with any of the delta subunit gene constructs we tested. Thus, our data is consistent with activation of delta subunit gene expression during the transition from myoblast to myotube rather than loss of repression in myotubes. Further studies are necessary, however, to distinguish among the possibilities.

The 5' flanking region of the delta subunit gene contains a tract of alternating purine-pyrimidine residues composed of 20 AC repeats. In addition to a role in recombination (Bullock et al., 1986; Treco and Arnheim, 1986), it has been suggested that such tracts of purine-pyrimidine repeats may be transcriptional regulatory elements (Hamada et al., 1984). Since deletion of this purine-pyrimidine repeat in the delta subunit gene has no effect on CAT expression, this AC tract neither enhances nor is required for expression of the delta subunit gene in C2 myotubes.

Four DNase I hypersensitive sites are detected near the delta subunit gene in DNA isolated from muscle cells but not fibroblasts (Crowder and Merlie, 1986). One of these hypersensitive sites is within 1 kb of the delta subunit gene 5' flanking region, whereas the other three hypersensitive sites are 3' to the translation termination codon of the delta subunit gene (Crowder and Merlie, 1986). Since 148 bp of upstream

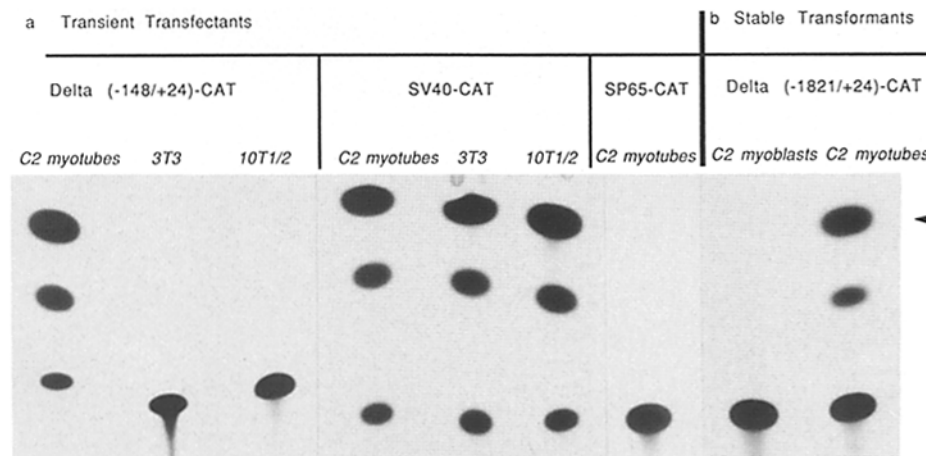


Figure 4. The mouse delta subunit promoter is active in myotubes, but not in myoblasts, 3T3 cells, or 10T1/2 cells. (a) A representative autoradiogram of TLC fractionation of the radioactive reaction products in a CAT assay from transiently transfected cells demonstrates that CAT activity is present in myotubes, but not in 3T3 cells or 10T1/2 cells transfected with delta (-148/+24)-CAT. Similar CAT activity is present in all cell types transfected with SV-40-CAT, whereas no CAT activity is evident in C2 myotubes transfected with SP65-CAT. (b) A representative autoradiogram of TLC fractionation of

the radioactive reaction products in a CAT assay from stably transformed C2 cells demonstrates that CAT activity is present in myotubes, but not in myoblasts. These C2 cells were stably transfected with delta (-1,821/+24)-CAT and with pSV2-neo DNA. The position of diacetylated chloramphenicol, resolved from monoacetylated chloramphenicol and chloramphenicol, is indicated with an arrowhead. The TLC plates were exposed to x-ray films at room temperature overnight.

Table I. Quantitation of CAT Expression in Transient Transfections

CAT gene fusion	CAT activity		
	C2 myotubes (%)	3T3 (%)	10T1/2 (%)
Delta (-3,150/+24)	100	1.4	2.0
Delta (-1,821/+24)	100	0.6	1.9
Delta (-1,288/+24)	95	2.6	1.2
Delta (-1,048/+24)	91	0.7	2.3
Delta (-842/+24)	99	1.3	1.2
Delta (-499/+24)	95	0.3	0.5
Delta (-256/+24)	88	0.3	0.3
Delta (-180/+24)	120	0.5	0.5
Delta (-148/+24)	100	0.6	0.5
Delta (-1,821/-32)	0.1	ND	ND
SV-40 early promoter	120	98	106
No promoter (SP65-CAT)	0.8	0.5	0.3

CAT activity was measured as described in Materials and Methods. The 5' and 3' nucleotides of each delta-CAT construct are indicated in parenthesis. CAT activity present in C2 myotubes transfected with the gene fusion Delta (-3,150/+24) was assigned as 100% (0.33-0.36 pmol diacetylated chloramphenicol/min per ng HGH); all other values for CAT activity are expressed relative to this value. All assays were done in duplicate; duplicate values varied by <10%. Several independent assays were performed with each construct; variation of CAT activity in independent assays was ~10% for C2 myotubes transfected with SV-40-CAT or delta-CAT and about threefold for 3T3 and 10T1/2 cells transfected with delta-CAT.

sequence from the delta subunit gene is sufficient to confer muscle-specific expression, the 3' DNase hypersensitive sites are not required for expression of the delta subunit gene in myotubes.

Since the chicken AChR alpha subunit gene is the only other AChR subunit gene in which gene expression has been analyzed at present (Klarsfeld et al., 1987), it is difficult to make meaningful sequence comparisons with other AChR subunit genes. Moreover, the region of the chicken alpha subunit gene that confers myotube-specific expression contains 850 bp of 5' flanking DNA and the essential elements within this region have not yet been delineated further. Thus, although we have identified two sequences within the 148 bp *cis*-acting element of the mouse delta subunit gene that have homology to a sequence in the chicken alpha subunit gene, we have no evidence that these sequences are important for expression.

Several other genes that are expressed selectively in vertebrate skeletal myofibers have been characterized and *cis*-acting regulatory elements that confer myotube-specific expression identified. This list includes the genes encoding creatine kinase (Jaynes et al., 1986, 1988), skeletal muscle actin (Grichnik et al., 1986; Bergsma, 1986), myosin light chains 1/3 (Billeter et al., 1988; Shirakata et al., 1988), troponin I (Konieczny and Emerson, 1987) and AChR alpha subunit (Klarsfeld et al., 1987). Nevertheless, the skeletal muscle actin gene is the only skeletal muscle gene in which this regulatory region has been delineated to less than several hundred base pairs upstream from the transcription start site (Bergsma, 1986). Although attempts have been made to identify a common sequence motif that is shared by the myotube-specific regulatory element(s) of these genes, no such sequence has been identified. Thus, it is not clear

Table II. Quantitation of CAT Expression in Stably Transformed C2 Cells

CAT gene fusion	CAT specific activity	
	C2 myoblasts (%)	C2 myotubes (%)
Delta (-1,821/+24)	0.6	100
Delta (-499/+24)	0.8	100
Delta (-180/+24)	0.8	100
Delta (-148/+24)	0.9	100

CAT specific activity was determined as described in Materials and Methods. Stably transformed C2 cells were grown to semiconfluence (4 d) and either harvested as myoblasts or allowed to differentiate for 2 d and harvested as myotubes (Materials and Methods). Since we did not measure the number of integrated copies in cell lines transfected with different constructs, no attempt was made to compare CAT activity in cell lines transfected with different gene fusions. Rather, CAT-specific activity in myotubes was assigned as 100% for each construct and CAT-specific activity in myoblasts was expressed relative to the value for myotubes.

whether these genes are activated during muscle differentiation by identical factors that interact with different DNA sequences, or by unique DNA-binding proteins that interact with *cis*-acting regulatory elements of the different genes. Further analysis of the *cis*-acting region of these genes to delineate the essential sequences and identify the proteins that interact with these sequences is required to distinguish among the possibilities.

Nevertheless, this study demonstrates that a small region of the delta subunit gene is sufficient to confer myotube-specific expression and suggests that a similar analysis of gene fusions in electrically active and quiescent, stably transformed cells may identify the region(s) of the delta subunit gene that confers electrical activity-dependent regulation.

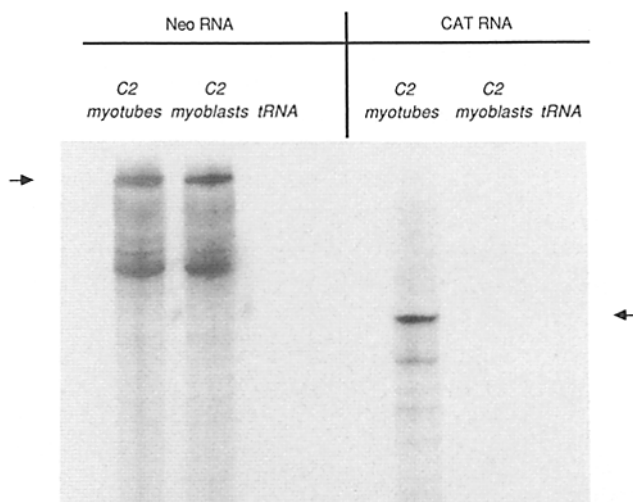


Figure 5. The delta subunit promoter is active in myotubes but not myoblasts, whereas the SV-40 early promoter is equally active in myoblasts and myotubes. 35-fold more CAT mRNA is expressed in myotubes than myoblasts whereas neomycin phosphotransferase mRNA is 1.2-fold more abundant in myotubes than myoblasts. These C2 cells were stably transformed with delta (-148/+24)-CAT and with pSV2-neo DNA. 10 µg of total cellular RNA from myoblasts and myotubes were included in each hybridization. The gel was exposed to x-ray film with an intensifying screen at -70°C for 16 h.

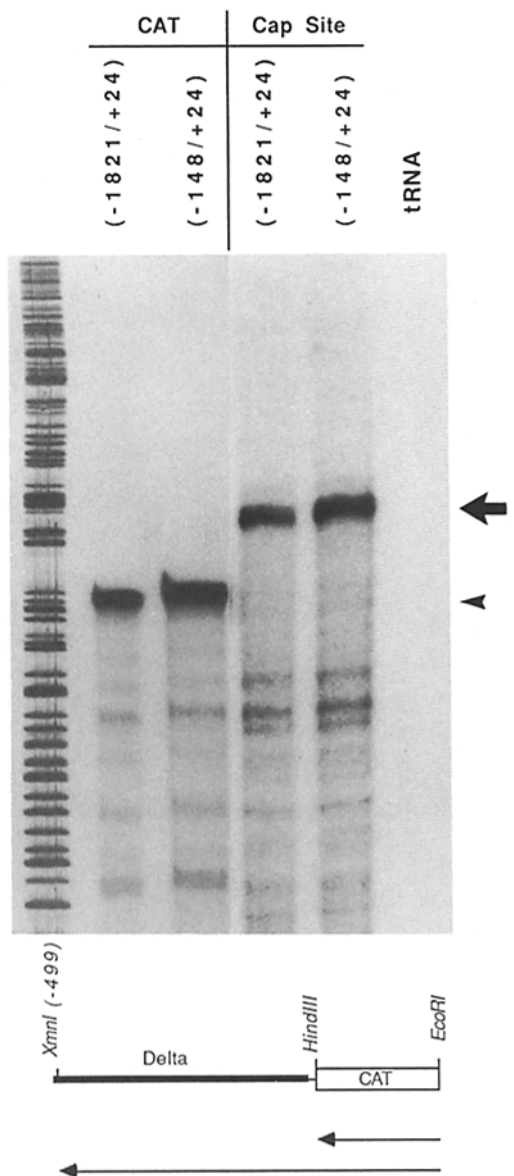


Figure 6. Transcription initiates at the same nucleotides in the transfected and endogenous delta subunit genes. The diagram illustrates the DNA template used to synthesize RNA probes. CAT RNA levels were measured by RNase protection as described (Materials and Methods; and Fig. 5); a 250 nucleotide ^{32}P -labeled RNA fragment is protected from digestion (arrowhead). The probe for mapping the cap site in delta-CAT gene fusions was synthesized from SP65 DNA containing the 786 bp Eco RI fragment from SP65-Xmn I, MD-CAT subcloned into the Eco RI site of SP65 DNA (Materials and Methods and diagram). ^{32}P -labeled RNA fragments (arrow) at 286 and 287 nucleotides are protected from digestion by RNase (Materials and Methods); the 3' ends of the protected fragments correspond to nucleotides +1 and +2 in the mouse AChR delta gene. 10 μg of RNA from C2 myotubes transfected with the indicated constructs were included in hybridizations with ^{32}P -labeled RNA probes. The size of the protected fragments was determined by comparison to a DNA sequencing ladder. The gels were exposed to x-ray film with an intensifying screen at -70°C for 16 h.

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