

## A 102 base pair sequence of the nicotinic acetylcholine receptor delta-subunit gene confers regulation by muscle electrical activity

KENNETH G. CHAHINE, WADE WALKE and DANIEL GOLDMAN\*

*Mental Health Research Institute and Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109, USA*

\*To whom correspondence should be addressed

### Summary

Muscle electrical activity suppresses expression of the embryonic-type ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) nicotinic acetylcholine receptor (nAChR) genes. The molecular mechanism by which electrical activity regulates these genes is not known. One approach to this problem is to identify regions of the nAChR genes that mediate electrical regulation. Here we report results from such a study of the nAChR  $\delta$ -subunit gene. We cloned the rat  $\delta$ -subunit promoter region and created an expression vector in which this DNA controlled the expression of a downstream luciferase structural gene. The effect that muscle electrical activity had on the expression from this promoter was assayed by introducing this expression vector into electrically stimulated and tetrodotoxin (TTX)-treated rat primary myotubes, and assaying for luciferase activity. These myotubes, when stimulated

with extracellular electrodes, suppressed endogenous embryonic-type nAChR gene expression compared to those treated with TTX. Transfection of these cells with  $\delta$ -promoter-luciferase expression vectors resulted in the  $\delta$ -promoter conferring electrical regulation on luciferase expression. Additional experiments using deletions from the 5' end of the  $\delta$ -promoter region have identified an element between -677 and -550 bp that suppressed  $\delta$ -promoter activity and a minimal 102 bp sequence that promotes and regulates luciferase expression in response to muscle electrical activity. This latter sequence also contains all the necessary elements to confer tissue and developmental stage-specific expression.

**Key words.** muscle, nicotinic acetylcholine receptor, promoter, gene expression, electrical activity.

### Introduction

The muscle nAChR is a pentameric integral membrane protein that mediates communication between nerve and muscle (Schuetze and Role, 1987). Prior to innervation nAChRs are distributed throughout the muscles surface. However, after innervation they are concentrated beneath the neuromuscular junction (NMJ). The loss of receptors from extrajunctional regions of the muscle fiber is largely a result of nerve-induced muscle activity suppressing nAChR gene expression (Goldman et al., 1988; Merlie and Kornhauser, 1989). This activity-dependent regulation is reversible, since denervation of adult skeletal muscle causes these receptors to reappear throughout the muscle fiber's surface (Goldman et al., 1985; Merlie et al., 1984; Shieh et al., 1987).

The molecular mechanism by which muscle activity controls nAChR gene expression is not well understood. One approach to this problem is to identify the nAChR genes and characterize DNA sequences conferring activity-dependent regulation on their expression. This approach has resulted in the cloning of the chick  $\alpha$ -

and  $\delta$ -, and mouse  $\gamma$ - and  $\delta$ -subunit gene promoters (Klarsfeld et al., 1987; Wang et al., 1990; Gardner et al., 1987; Baldwin and Burden, 1988). However, convenient systems for characterizing activity-dependent gene expression have been lacking. To date, transgenic animals have provided the only system for studying electrical regulation of nAChR promoter activity. These studies have shown that electrical regulation of the chick  $\alpha$ -subunit promoter is mediated by sequences located within 850 bp 5' to position +20 of the chick  $\alpha$ -subunit gene (Merlie and Kornhauser, 1989).

In order to rapidly analyze nAChR promoters for activity-dependent regulation we have identified a rat primary muscle culture system whose nAChR genes respond to muscle electrical activity in a fashion similar to that observed *in vivo*. We report here the cloning of the rat nAChR  $\delta$ -subunit gene promoter and the use of this system to map a minimal 102 bp sequence that allows muscle electrical activity to regulate expression from this promoter. In addition, we show that this same DNA retains tissue and developmental stage-specific expression.

## Materials and methods

### *Cloning of the rat $\delta$ -subunit nAChR promoter*

Approximately  $5 \times 10^5$  phage from a Fisher rat genomic DNA library (Stratagene) were screened with a rat nAChR  $\delta$ -subunit,  $^{32}\text{P}$ -labelled, cDNA probe. A 7 kb fragment, containing exon 1, was subcloned into the pBS SK(+) vector (Stratagene) and characterized by Southern blot analysis for orientation and position of exon 1. A combination of restriction enzyme digestions and Southern blotting analysis resulted in the cloning of a 1.3 kb fragment of DNA containing part of the first intron and exon 1 at its 3' end. This clone is called BSSK(+) $\delta$ -1.

### *DNA sequencing*

Unidirectional deletions were generated by either exonuclease III or Bal 31 digestions of restricted DNA (Maniatis et al., 1982). Single-stranded DNA was isolated from phagemids according to manufacturers directions (Stratagene) and sequenced using the dideoxy chain termination method of Sanger et al., 1977.

### *Expression vector constructs*

$\delta$ -gene sequences were subcloned into the pXP1 or pXP2 expression vectors 5' to the luciferase reporter gene (Nordeen, 1988). Internal controls were the mouse creatine kinase (MCK) promoter (Jaynes et al., 1986) driving chloramphenicol acetyltransferase expression (Luckow and Schutz, 1987) (MCKCAT), the SV40 promoter (Gorman et al., 1982) driving  $\beta$ -galactosidase expression (SV40 $\beta$ gal) or the CMV promoter (Boshart et al., 1985) driving CAT expression.

### *Cell culture and electrical stimulation*

Primary rat muscle cell cultures were prepared as previously described (Goldman et al., 1991). Cells were plated on collagen coated culture dishes at a density of 2000 cells/mm<sup>2</sup> and grown in 20 mM HEPES, 2% chick embryo extract, 10% fetal calf serum, 10% horse serum, and 25  $\mu\text{g}/\text{ml}$  gentamycin. Cultures were placed in a 37°C incubator with 8% CO<sub>2</sub>. Generally, by day 4 myotubes have formed and the cultures were treated with 3  $\mu\text{g}/\text{ml}$  cytosine arabinoside for 24 hours to inhibit fibroblast proliferation. Cultures were either treated with TTX (10  $\mu\text{M}$ ) or stimulated on day 5 using previously described conditions (Goldman et al., 1988). Briefly, stimulating electrodes were immersed in the culture medium and the muscles stimulated chronically for 3-7 days in 100 Hz trains, 1 sec duration, applied once every 100 sec. Stimulus pulses within trains were of alternating polarity, their duration was 0.5 msec. and their strength was 8 mA.

The mouse muscle cell line, C2C12, was cultured as previously described (Evans et al., 1987).

NIH 3T3 cells were grown in DMEM supplemented with 10% calf serum and 25  $\mu\text{g}/\text{ml}$  gentamycin.

### *Transfections; luciferase, CAT and $\beta$ -galactosidase assays*

Transfection of muscle cells in culture was performed by CaPO<sub>4</sub> precipitation of DNA as previously described (Graham and Van der Eb, 1973), with the addition of a 2 min glycerol shock after 4 hours (Parker and Stark, 1979). Cells were harvested 48-72 hours later. Luciferase, chloramphenicol acetyltransferase (CAT) and  $\beta$ -galactosidase assays were performed as previously described (Brasier et al., 1989; Neumann et al., 1987; Bradford, 1976).

### *RNA isolation*

RNA from cell culture and tissue was isolated using the guanidinium isothiocyanate procedure as previously described (Chirgwin et al., 1979).

### *Primer extension assay*

A 25 nucleotide long oligodeoxynucleotide complementary to a portion of exon 1 (+48 to +72) of the  $\delta$ -subunit gene (see Fig. 1) was used for primer extensions as previously described (Maniatis et al., 1982).

### *RNase protection assay*

RNase protection assays were carried out as previously described (Goldman and Staple, 1989). The delta clone BSSK $\delta$ -1087/+96 linearized with *Pvu*II generates a 440 bp probe which spans most of exon 1 and extends 344 bp upstream of the transcriptional start site. The muscle creatine kinase clone, BSSKII(+) MCK, is a 292 nucleotide long *Bgl*II/*Hind*III fragment corresponding to nucleotides 857-1149 of the mouse muscle creatine kinase subclone pMCKm36 (Jaynes et al., 1986). The MCK probe protects two fragments in the RNase protection assay that reflects slight sequence differences between mouse and rat creatine kinase RNAs.

## Results

### *Analysis of the $\delta$ -subunit genes 5' flanking sequence and identification of the transcriptional start site*

The  $\delta$ -subunit promoter region was isolated from a rat genomic library as described in Materials and methods. DNA sequencing revealed subclone BSSK(+) $\delta$ -1 is 1,317 nucleotides long and contains part of the first intron and all of exon 1 at its extreme 3' end (Fig. 1). The nucleotides corresponding to the start site of transcription were mapped by RNase protection and primer extension experiments (Fig. 2). The primer extension experiments showed denervated muscle and primary rat myotubes initiate  $\delta$ -subunit gene transcription at the A and T residues marked with an asterisk at positions +1 and +2, respectively, in Fig. 1. In addition, denervated muscle also initiated transcription at position -1. Consistent with these results were those obtained with RNase protections. Since RNase A only cleaves 3' to pyrimidine residues and the probe is an antisense sequence, the high relative molecular mass band on the gel is consistent with transcripts starting at nucleotide -1 (RNase cleavage 3' to the C residue complementary to nucleotide -5 in Fig. 1) and the intermediate band is consistent with transcripts starting at nucleotides +1 and +2 (RNase cleavage 3' to the T residue complementary to nucleotide +1 in Fig. 1). The lowest band is a result of probe secondary structure (data not shown).

No canonical TATA or CAAT boxes were found upstream of the start site of transcription, although the start site is centered at an 8 nucleotide stretch that is identical to the 8 central nucleotides of the murine terminal deoxynucleotidyltransferase (TdT) genes initiator sequence (Smale and Baltimore, 1989). This latter sequence has been implicated to play a role in basal promotion and proper initiation of transcription.

Comparison of the rat  $\delta$ -subunit genes 5' flanking

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-1087 CACCCTCTCT TTCCAAGTGC TGGGAATTAA AGGTGTTGTG ATGGTTTGCA CATGCTTGGC CCAGGGAGTG GCACTATTAG
-1007 GAGGTGTGGC CTTGTTGGAG AAAGTGTGTC ACTGTGGTGG TGGGCTTGGG GACCCCTCTC CTAGCTGCCT AAGGATGCTC
-927 AGTCTGTTCC TGGCTTCCTT CAGGTGAAGA TGTAGAAGTC AGCTCCTCCT GCACCATGCC GGCCTAGATG CTGTCATGCT
-847 CCCACCTGAA CCACTGAACC TCTGAACCTG TAAGCCAGCC CCAATTAAAT GTTGTCTTTT ATAAACTTA CATTGGTCAT
-767 GGTGTCAGTT CACAGCAATA AGACCTGACT AAGGCAGGTG TGCCTGCCA CTCCTGGGTT AAGTGAATA TTTTAAAGA
-687 CGGGGGAATA TACATAAGTA CAGGTACAGC ACCAGCAAGA TGGTGAATA AGTAACAGTT GCCTGCTGTG ACAAGCCTGG
-607 GCTCACCTCA GAACCTCTG TAGAGAGAGA GAAAAACAAC TGTCACACAC ACACACACAC ACACACACAC ACACCAATAT
-527 TAAATAGAAT AGAAATGAAA AAATGATAAA ATCGTCGAAC AATAGACTGT TTGATTCCA CAGAACCACC TACAACAGA
-447 CACACATGTA CACACTCCAT ACACATATAT ACACACTCCG TACACACATG TACACACTCC ATACACATAT ACCACATGCA
-367 TAAAGAAGC TGGACGCAGG GCAGCTGCTA ACATGTCTTT CACCTCAGTC CTTTAAGGAC TGAAGTGGCA GTAAGTACC
-287 TATGTGTCAC CACCCTATCC ACAGCTACCA GCATGCCACA CAGACACACA GAGACACATA CACACACACC ATTGTCCCC
-207 CACCCTCTGC CACCCTACCC TACCAGGCC CACCCTACCA ACTAGCAGTG GCAGTCCCTG CCTGGGATCT TTGCATTCTG
-127 CCCTTAGGCT CCTGCCCTAG CTGGCAAACC CCACCCTCTC ATCACCAGCC TTCAAGTATC AGATTGCGTT TCCGGCCTCT
-47 TCTTTCCAAA CCCCTAAGCC GCCAGCACCT GTCCCCTTGC TTGCTCATT CCACAGCCAA CAGGCTGAAG GGGAGACATA
+34 AACCCAGTC AGTCGAGGT GGGCATGGCA GGGCCTGTGC CCACACTGGG GCTGCTGGCT GCCCTTGTG
+104 TGTGTGgtaaggggtggaatgactcccttcctgggggccttctatgccacctgggcccctcaccgcattggt
+176 gcccaccctcacatcatgcctaattcccactcccactcgccctttctaaccta

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**Fig. 1.** DNA sequence of the 5' end of the rat nAChR  $\delta$ -subunit gene. At the 3' end of the sequence is intron 1 typed in small case lettering. Preceding intron 1 is exon 1 extending 5' to nucleotide +1. Within this exon beginning with the initiator ATG codon, in bold type, and extending to nucleotide +109 is the translated sequence typed in larger lettering. The transcriptional start sites (+1 and +2) are indicated by asterisks(\*\*). The oligonucleotide sequence (+48 to +72) used in primer extension experiments is indicated with a thick underline, putative myogenic regulatory consensus sequences are double underlined and putative AP-2 consensus sequences are marked with a single thin underline. The SHUE box (–139 to –162) is marked with an overhead bracket. A palindromic sequence centered around the T residue at position –314 is indicated by a dashed underline. Various deletions used in this study are marked with arrows above the sequence with corresponding size next to the arrow.

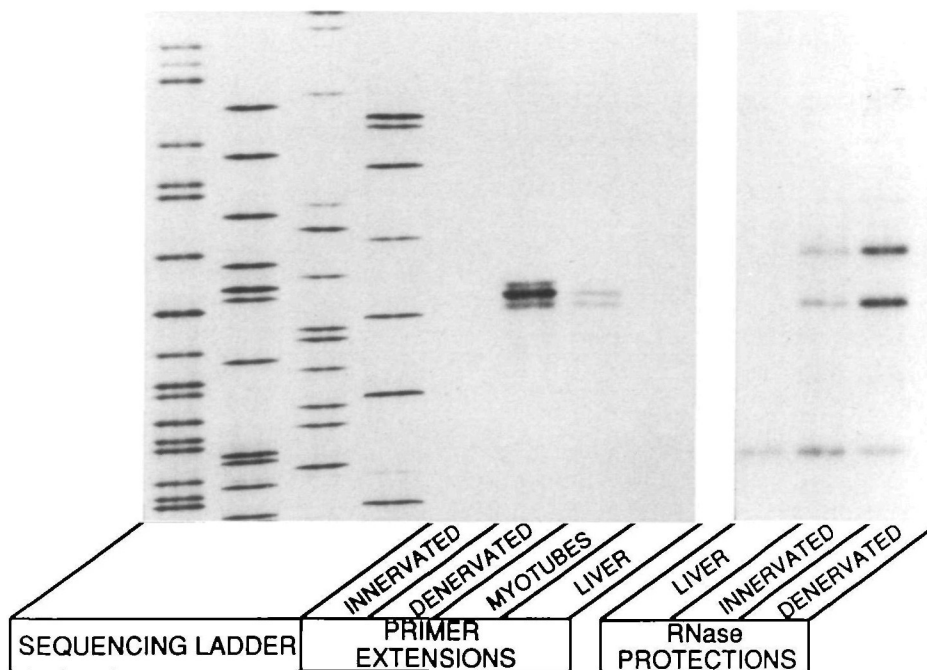
sequence with that of the published mouse and chick  $\delta$ -subunit sequences (Baldwin and Burden, 1988; Wang et al., 1990) showed about 90% identity with the mouse sequence for the 246 nucleotides upstream of the start site of transcription. In contrast, the chick sequence showed little homology with the rat sequence except for two regions: one centered around positions –22 of the rat (relative to transcriptional start site) and –110 of the chick (relative to translation initiator ATG), which contains a consensus sequence for binding myogenic regulators and shows a 12/14 bp match; and the second centered around positions –147 of the rat and –192 of the chick, which contains part of the SHUE box (Crowder and Merlie, 1988) and is identical for 8 nucleotides.

Upstream of the transcriptional start site, centered

around nucleotides –550 and –223, are two regions of DNA that contain 15 and 12 CA repeats, respectively. CA repeats have also been found in the mouse  $\delta$ - and  $\gamma$ -subunit genes. Finally, there is a 27 bp palindromic region centered around base pair –314 (dashed underline in Fig. 1).

#### *Identification of 102 bp sequence containing an element conferring regulation by muscle electrical activity*

We have found that muscle activity appropriately regulates nAChR gene expression in rat primary muscle cells. This effect is most pronounced when one compares cells stimulated with extracellular electrodes to those treated with the sodium channel blocker, TTX. Specifically, the  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -subunits of the nAChR



**Fig. 2.** Identification of the  $\delta$ -subunit genes transcriptional start site. Primer extension and RNase protections were used to identify the transcriptional start site of the rat nAChR  $\delta$ -subunit gene. Sizes of bands were determined by comparing with a DNA sequencing ladder. The extended products from denervated RNA correspond to nucleotides  $-1$ ,  $+1$  and  $+2$  (Fig. 1), while those from primary culture myotubes correspond to residues at positions  $+1$  and  $+2$  (Fig. 1). The lowest band in the RNase protections is a result of probe secondary structure (data not shown). The next middle band is consistent with transcription initiating at nucleotides  $+1$  and/or  $+2$  (Fig. 1). The upper band is consistent with transcripts initiating at nucleotide  $-1$ .

are all suppressed by electrical activity, while the electrically insensitive  $\epsilon$ -subunit and muscle creatine kinase genes are unaffected (K. G. Chahine and D. Goldman, unpublished data). Fig. 3A, shows the  $\delta$ -subunit RNA is suppressed nearly 10-fold by electrical stimulation, while the creatine kinase gene (MCK) is slightly increased. Therefore, with a system in which to study muscle electrical activity established we isolated the  $\delta$ -subunit promoter and began to identify *cis*-acting elements that mediate this effect.

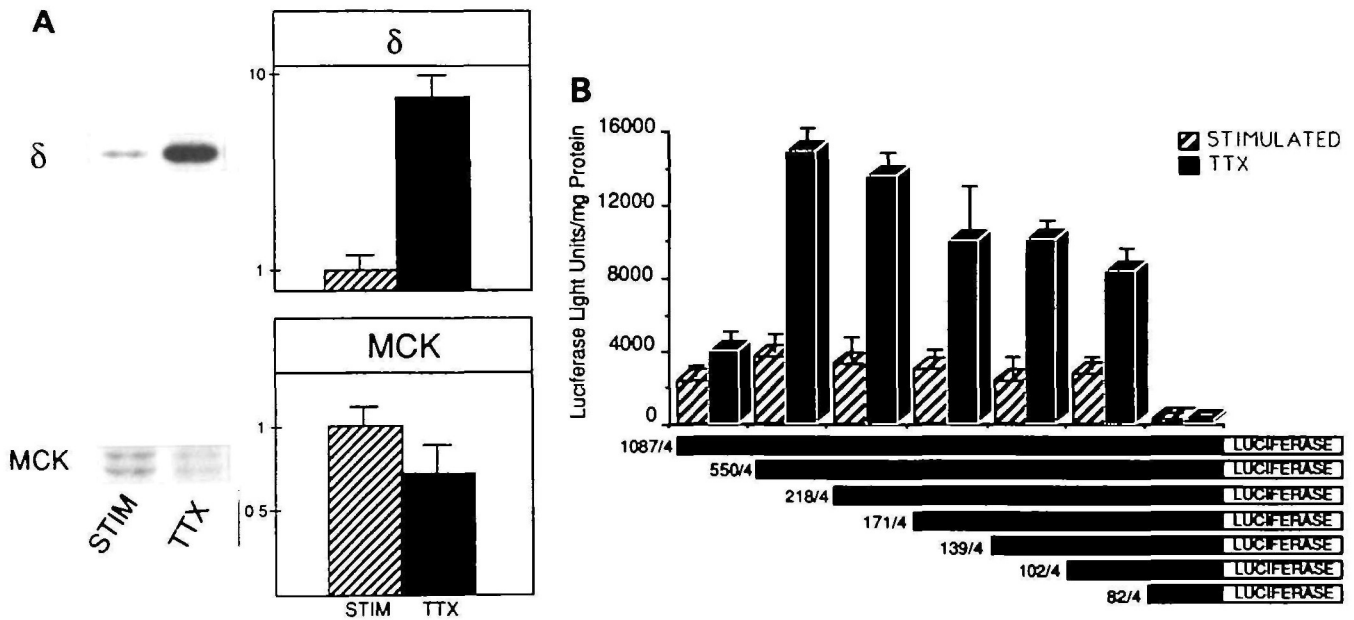
The first intron and most of exon 1, including the initiator ATG, were removed from the 3' end of BSSK(+) $\delta$ -1 by digestion with Bal 31 exonuclease. One of the clones generated from this digestion, BSSK(+) $\delta$ 1087/4, had its 3' end at nucleotide  $+11$  and extended 1098 nucleotides in the 5' direction as determined by DNA sequencing (Fig. 1). This clone served as the parent for all further deletions. Unidirectional deletions were created from the 5' end of this DNA using *ExoIII*. The precise 5' end of each of these deletions was determined by DNA sequencing. Clones were named according to the number of 5' nucleotides remaining upstream of the start site of transcription. These deleted promoters were subcloned into the pXP luciferase expression vector and transfected into rat primary myotube cultures that were either stimulated to contract with extracellular electrodes or kept inactive by addition of TTX for 3-5 days. Following transfection, the cells were returned to the stimulator or retreated with TTX and harvested 48-72 hours later. Transfection efficiency was normalized by co-transfecting cultures with MCKCAT. The MCK promoter like the endogenous gene is not suppressed by muscle electrical activity (unpublished data).

These experiments showed pXP $\delta$ 1087/4 contained all the necessary elements to confer electrical regulation

on promoter activity ( $-1087/4$  in Fig. 3B). The minimal *cis*-acting region that allowed promotion and still retained electrical regulation was 102 bp upstream of the transcriptional start site (Fig. 3B). Deletion of an additional 20 bp from the 5' end of pXP $\delta$ 102/4 resulted in loss of promoter activity. Within these 20 bp resides a putative AP-2 consensus sequence which may be required for promoter activity. These experiments also indicated that there is a negative element located between positions  $-1087$  and  $-550$ . Removal of this element increased the activity of the promoter about 4-fold in TTX-treated cells. Additional deletions have shown this element to reside between nucleotides  $-677$  and  $-550$  (data not shown). There was a gradual decrease in promoter activity down to deletion pXP $\delta$ 102/4. Further deletions resulted in a dramatic loss of promoter activity. Deletion pXP $\delta$ 102/4 retained approximately 65% of the activity observed in pXP $\delta$ 550/4.

*Elements conferring tissue and developmental stage-specific expression are contained within the same 102 bp fragment that confers regulation by muscle activity*

The muscle nAChR genes are specifically expressed in muscle cells after myoblasts fuse into multinucleated myotubes. Thus these genes are expressed in a tissue and developmental stage-specific manner. We assessed whether our various  $\delta$ -subunit promoter deletions retained tissue and developmental stage-specific expression by comparing their promoter activity in transfected C2 myoblasts and myotubes, and NIH 3T3 cells (Fig. 4). Transfection efficiency was normalized to a co-transfected SV40 $\beta$ -gal expression vector. These experiments revealed that all  $\delta$ -promoter deletions that contained a minimum of 82 bp upstream of the start site of transcription (pXP $\delta$ 82/4) had a significantly higher



**Fig. 3.** A 102 bp region of the  $\delta$ -subunit promoter is regulated by muscle electrical activity (A) RNase protection assay showing muscle electrical activity suppresses  $\delta$ -subunit, but not creatine kinase RNA levels in primary muscle cultures. Primary muscle cultures were either stimulated to contract with extracellular electrodes (STIM) or paralyzed with tetrodotoxin (TTX) for 5 days. Total RNA (10  $\mu$ g) was used in RNase protection assays as described in Materials and methods. The autoradiogram was exposed to X-ray film with an intensifying screen at  $-70^{\circ}\text{C}$  for 15 hours. Graphs show quantitation of these RNase protections. Values are arbitrary optical density units determined from scanning densitometry. Error bars are  $\pm$  standard deviation. (B) Graph showing promoter activity of various  $\delta$ -promoter deletions after transfection of expression vectors into stimulated or TTX-treated cells. Transfections were carried out with 15  $\mu$ g of the pXP $\delta$ -luciferase expression vector (various promoter deletions shown as horizontal bars below the graph) and 15  $\mu$ g of MCKCAT. Luciferase activity was normalized to protein, however normalization to CAT activity gave similar results. Bar graphs are the average of quadruplicate transfections normalized to total protein, error bars are  $\pm$  standard deviation.

level of expression in myotubes than in myoblasts or NIH3T3 cells (Fig. 4). However, this level of expression is significantly lower than constructs containing additional 5' DNA.

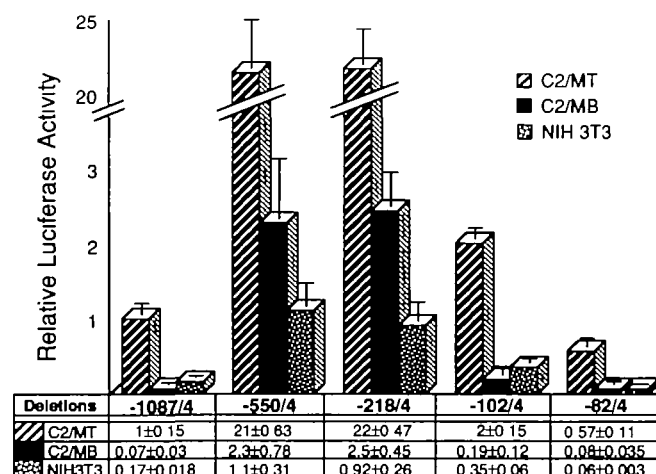
As was observed in transfected primary muscle cultures a negative element is located between nucleotides  $-1087$  and  $-550$ . This element is promiscuous in that it is active in all cell lines and primary cultures tested. Interestingly,  $\delta$ -promoter activity increased over 20-fold in C2 myoblasts and myotubes when the negative element was removed as compared to a 6-fold increase in NIH3T3 cells. These results are similar to those observed with the chick  $\delta$ -promoter (Wang et al., 1990).

Finally, we noticed two significant differences concerning  $\delta$ -promoter activity in the C2 muscle cell line compared to primary muscle cultures. First the negative element had a larger effect on  $\delta$ -promoter activity in the C2 cell line than it did in the primary rat muscle cultures. Therefore, its removal increased promoter activity over 20-fold in C2 cultures (Fig. 4), but only by about 3-fold in primary muscle cultures (Fig. 3). Second, the largest 5' deletion tested that still gave significant promoter activity in the C2 cells was pXP $\delta$ 82/4 (Fig. 4), while in primary cells this construct was at background levels of expression (Fig. 3). This latter result was not due to differences in transfection efficiency (data not shown).

## Discussion

We have presented data showing that rat primary muscle cultures can be used to study mechanisms by which muscle electrical activity regulates nAChR gene expression. In order to characterize the DNA sequences conferring activity-dependent regulation on these genes we cloned and sequenced the  $\delta$ -subunit genes 5' flanking DNA (Fig. 1). When  $\delta$ -promoter/luciferase expression vectors were introduced into rat primary muscle cultures that were either stimulated with extracellular electrodes or made inactive by TTX-treatment, the  $\delta$ -promoter conferred activity-dependent regulation on luciferase expression (Fig. 3). A series of deletions from the 5' end of the  $\delta$ -promoter identified a minimal 102 bp sequence that was sufficient for conferring this regulation on promoter activity. Further deletions resulted in loss of promoter activity, perhaps due to deletion of a putative AP2 site (Fig. 1). Expression from the MCKCAT vector used as an internal control, like the endogenous gene, was not suppressed by muscle electrical activity (data not shown).

One significant difference between the endogenous and transfected genes are the degree of regulation by muscle electrical activity. Generally, we can suppress the endogenous  $\delta$ -RNA about 10-fold by electrical stimulation, yet the transfected  $\delta$ -promoter is only



**Fig. 4.** An 82 bp region of the  $\delta$ -subunit promoter retains tissue and developmental stage-specific expression. C2 myoblasts (C2/MB), C2 myotubes (C2/MT) and NIH3T3 cells were transfected with expression vectors containing the luciferase structural gene driven by various  $\delta$ -promoter deletions. Transfections were carried out with 10  $\mu$ g of the pXP $\delta$ -luciferase construct (indicated on x-axis) and 10  $\mu$ g of SV40 $\beta$ -gal or CMVCAT. Values are reported as the activity relative to the full length pXP $\delta$ 1087/4 promoter construct expressed in myotubes. Data are the average of triplicate transfections normalized to SV40 $\beta$ -gal. Myoblast and NIH3T3 transfections were also normalized to CMVCAT or protein with similar results. Error bars are  $\pm$  standard deviation.

suppressed about 4-fold (Fig. 3). Although this difference may indicate additional post-transcriptional controls regulating  $\delta$ -RNA levels, we believe it may be accounted for by the observation that following calcium phosphate transfection the primary muscle cultures experienced a 12–16 hour period of inactivity. This would result in a higher level of promoter activity in stimulated cells causing a reduction in the observed effects of TTX. We have tried other transfection techniques, such as DEAE-dextran and lipofection, without success.

It is interesting that as we deleted DNA from the 5' end of the  $\delta$ -promoter its activity was consistently affected to a greater extent in TTX-treated cells than in stimulated cells (Fig. 3B). Perhaps the proteins that bind these upstream regulatory elements interact with a second protein that is expressed at low levels or not at all in active muscle. This would be consistent with the observation that ongoing protein synthesis is required to maintain the elevated level of nAChR expression seen in inactive muscle (Duclert et al., 1990).

The nAChR  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ -genes are all suppressed by muscle electrical activity. The sequences mediating this effect may be common among these different genes. We compared the electrically responsive 102 bp sequence of the rat  $\delta$ -promoter to the other nAChR gene sequences. Except for the mouse and chick  $\delta$ -promoters the other nAChR promoters showed little sequence similarity to this 102 bp region. However, it is important to note that within this 102 bp region at

position -22 is a consensus sequence binding site for myogenic regulators (Lassar et al., 1991). Both the chick  $\delta$ -(sense strand) and  $\alpha$ -promoter (antisense strand) sequences show a 12/14 and 10/14 match with the rat  $\delta$ -promoter sequence in this region. Myogenic regulatory sequences are found in all the nAChR promoters characterized to date (Baldwin and Burden, 1988; Gardner et al., 1987; Klarsfeld et al., 1987; Wang et al., 1990). Although these sequences play an important role in activating muscle specific genes during development (Wright et al., 1989) they may also participate in other regulatory mechanisms.

That this myogenic regulatory sequence at position -22 might participate in developmental regulation of nAChR  $\delta$ -subunit expression is supported by the observation that pXP $\delta$ 82/4 is expressed at significantly higher levels in C2 myotubes than myoblasts or NIH3T3 cells (Fig. 4). Albeit this level of expression is about 5-fold lower than pXP $\delta$ 102/4. The reason for this latter decrease in expression is likely due to removal of a putative AP2 consensus sequence located within the 20 bp deleted from pXP $\delta$ 102/4 (Fig. 1).

Since the amount of expression drops as we delete DNA from pXP $\delta$ 218/4 (Figs 3 and 4), it is likely that additional sequences involved in tissue and developmental stage-specific expression exist within this region of DNA. Consistent with this interpretation, one finds pXP $\delta$ 218/4 contains an additional myogenic regulatory sequence (position -218) and 2 additional AP2 sequences (positions -211 and -104) compared to pXP $\delta$ 82/4. The reason we do not detect pXP $\delta$ 82/4 expression in primary muscle cultures (Fig. 3) but do detect it in C2 myotubes may simply reflect a difference in the level of expression of *trans*-acting factors in these two sources of muscle cells.

Surprisingly, we find a low but above background level of expression of various  $\delta$ -promoter constructs in NIH3T3 cells (Fig. 4). The reason for this is not known. Clearly, the endogenous gene is not expressed in these cells. One possibility is that there are additional *cis*-acting sequences required for tissue specific expression. Alternatively, as has been found for the skeletal  $\alpha$ -actin promoter (Yisraeli et al., 1986), appropriate DNA methylation may be important for completely suppressing  $\delta$ -promoter activity in non-muscle cells.

There are many parallels between myogenesis and muscle inactivity: (1) they both cause induction of nAChR gene expression (Evans et al., 1987); (2) myogenic regulators are induced in a developmental stage-specific manner (Wright et al., 1989) and suppressed by muscle activity (Eftimie et al., 1991) as are the nAChR genes (Evans et al., 1987; Goldman et al., 1988), and (3) DNA elements conferring developmental stage-specific expression and regulation by electrical activity have been localized to the same 102 bp region of DNA that contains a consensus binding site for myogenic regulators (this report). Therefore, it is tempting to speculate that this latter sequence participates in both induction of muscle specific genes during myotube formation and responsiveness of the nAChR genes to muscle activity. Since the *trans*-acting pro-

tein(s) that binds this regulatory sequence may be one of many hetero-dimers (Lassar et al., 1991), one might be able to obtain differential effects (developmental versus electrical) depending on which dimer is bound to this site.

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