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Expression of the acetylcholine receptor δ-subunit gene in differentiating chick muscle cells is activated by an element that contains two 16 bp copies of a segment of the α-subunit enhancer

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The acetylcholine receptor is a multimeric membrane protein whose expression is activated during muscle differentiation and upon denervation of adult muscle. To gain insight into the coordinate expression of receptor subunits during myogenesis we have analyzed the chick muscle receptor δ-subunit gene upstream region. The δ-subunit gene lacks canonical promoter elements (CCAAT and TATA boxes). Nuclease protection and primer extension analysis revealed that transcription starts at six major and several minor sites between −110 and −30 upstream of the translational initiation site; two sites, at positions −77 and −66, give rise to ~50% of all transcripts. Using nested deletions of the proximal 960 bp of the 5′ flanking region of this gene we have identified a 62 bp sequence (−207 to −146) that activates transcription in a position independent manner. This enhancer-like element is activated during myotube formation; it contains two distinct functional moieties, each resembling the same 16 bp portion of the stage and tissue specific α-subunit gene enhancer which we have characterized previously [Wang *et al.* (1988) *Neuron,* 1, 527−534]. This common element, which also comprises several previously proposed skeletal muscle specific motifs [Buskin,J.N. and Hauschka,S.D. (1989) *Mol. Cell Biol.,* 9, 2627−2640; Mar,J.H. and Ordahl,C.P. (1988) *Proc. Natl. Acad. Sci. USA,* 85, 6404−6408], may account for the coordinate expression of the two subunits. The cell specificity of the δ-subunit gene 5′ flanking region is partly due to the enhancer, partly to an inhibitory element upstream of −207.

Key words: acetylcholine receptor/coordinate expression/ differentiation/enhancer/δ-subunit

Introduction

An investigation of the regulation of the expression of the acetylcholine receptor (AChR) in skeletal muscle is of interest for a number of reasons. Such an analysis will shed light on how the cholinoceptive phenotype is expressed during development, how denervation of adult muscle induces receptor expression, and how the synthesis of a multimeric protein, such as the AChR, is coordinated at the gene and post-transcriptional levels. Especially the latter objective requires the study of the expression of all constituent subunits of the AChR.

It has generally been assumed that the increase in receptor mRNA concentrations which accompanies the differentiation of myogenic cells, and which follows denervation of mature muscle fibers, is a result of transcriptional activation. Nuclear run-on analysis has confirmed this expectation for the α- and δ-subunits in differentiating cells (Buonanno and Merlie, 1986), and for the α-, δ- and γ-subunit genes after denervation (Tsay and Schmidt, 1989). A search for regulatory elements in receptor genes has therefore begun. Klarsfeld *et al.* (1987) observed that control of the chick receptor α-subunit gene during muscle differentiation was exercised by a 850 bp sequence upstream of the coding region. Wang *et al.* (1988) then discovered a potent stage and tissue specific enhancer close to the promoter of that gene. Multiple DNA−protein interactions in this region have been reported to occur in chick muscle during differentiation and after denervation (Piette *et al.*, 1989).

Similarly detailed information is also becoming available for other subunits. Crowder and Merlie (1986, 1988) investigated the vicinity of the mouse AChR δ- and γ-subunit genes (the '$\delta-\gamma$' locus) for DNase I hypersensitivity and found a single site specific for the differentiated muscle phenotype in the intergenic region, 1.7 kb upstream of the γ-subunit gene initiator codon. Two additional sites were found overlapping, or in close proximity to, the promoter of either gene; each site was defined as a region of ~300 bp. Baldwin and Burden (1988, 1989) analyzed the mouse δ-subunit gene upstream region in greater detail and found a myotube specific control element of 54 bp residing within the proximal 148 bp of the upstream flanking sequence.

The present paper presents an analysis of the 1 kb region immediately upstream of the coding region of the chicken δ-subunit gene. We show that there are both negative and positive elements present in the 5′ flanking region of this gene; from a comparison of an enhancer-like element with the α-subunit enhancer described previously we deduce a tentative consensus sequence for positive regulation of AChR subunits.

Results

Transcription start sites in the δ-subunit gene promoter

The upstream region of the δ-subunit gene (the proximal portion of which is shown in Figure 1) lacks typical TATA and CCAAT boxes in the promoter region. Such genes tend to have multiple start sites. Nuclease protection and primer extension analysis indeed revealed six major and several minor start sites, ranging in location from −110 to −30 (Figure 2); these observations were confirmed by analysis of $\delta\beta$G fusion gene transcripts (Figure 5). More than half of all transcripts are initiated at positions −77 and −66 upstream of the translation start codon, and sites at −110, −94, −90 and −62 account for 5−10% each of δ-subunit mRNA; in addition, minor start sites are located downstream of position −60, most of them clustering between −38 and −30.

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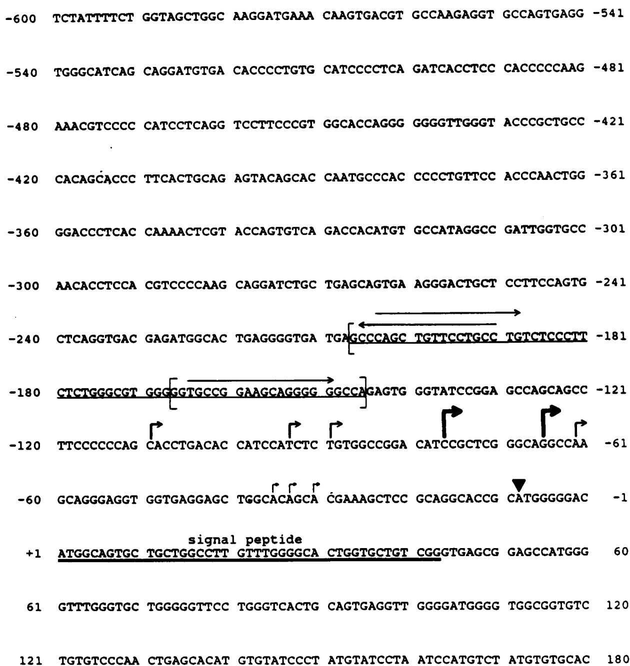

Fig. 1. Partial sequence of the 5′ flanking region of the chicken muscle AChR δ-subunit gene. Positions are numbered from the translation start site; this convention is adhered to throughout the article. Multiple transcription start sites are indicated by arrows whose size reflects extent of utilization in denervated chick muscle. Brackets indicate the boundaries of the enhancer element; note the internal 5′ end at position −167. The sites and orientations of regions resembling the α-subunit gene enhancer are indicated by horizontal arrows. The arrowhead marks the fusion with the CAT gene. The coding region is doubly underlined.

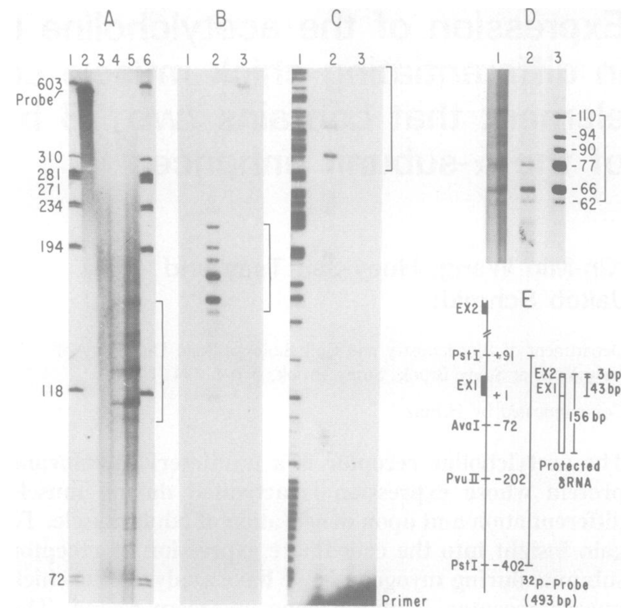

Fig. 2. Transcription initiation site of the δ-subunit gene. Transcription start sites were determined by three different techniques. **(A)** S1 protection analysis. A single stranded uniformly labeled probe, extending from −402 to +91 of the δ-subunit gene and containing all of exon 1 (43 bp) as well as the first three nucleotides of exon I was prepared and used as described in (E) and Materials and methods. **Lanes 1** and **6**; ΦX174RF DNA cut with *Hae*II; **lane 2**, probe; **lane 3**, probe hybridized with 90 μg yeast tRNA; **lane 4**, probe hybridized with 30 μg of total RNA extracted from chicken leg muscle (2 days after denervation); **lane 5**, probe hybridized with 90 μg of total RNA extracted from chicken leg muscle. The numbers on the left indicate fragment size in base pairs in the ΦX174 digest. **(B)** RNase protection analysis. A uniformly ^{32}P-labeled riboprobe, extending from −402 to +91 was hybridized to various RNA preparations, and, after RNase treatment and electrophoresis, visualized by autoradiography. **Lane 1**, 3 μg of poly(A)$^+$ RNA from chick brain; **lane 2**, 3 μg of poly(A)$^+$ RNA from denervated chick muscle; **lane 3**, probe only. **(C)** Primer extension analysis. A ^{32}P-end-labeled 28-mer oligodeoxynucleotide corresponding to positions +18 to +46 of the chick δ-subunit gene coding region (Nef *et al.*, 1984) was hybridized to various RNA preparations and extended with reverse transcriptase, following the protocol described in Materials and methods. **Lane 1**, length markers (one of four sequence ladders of an unrelated clone to determine transcript size); **lane 2**, primer hybridized to 30 μg poly(A)$^+$ selected RNA from chick muscle (2 days after denervation); **lane 3**, primer hybridized to 30 μg poly(A)$^+$ selected RNA from chick brain. Multiple low mol. wt bands are attributable to early termination. **(D)** Synopsis. **Lanes 1–3** represent aligned segments (indicated by brackets in panels A–D) of lanes A4/5, C2 and B2 respectively. **Lane 2** represents a longer exposure than C2 to improve visibility of faint transcripts. The numbers on the right represent distances from the ATG (+1) site. **(E)** Structure of probe for S1 nuclease and RNase protection analysis. On left, gene structure with restriction sites. Extent of protection (on right) is shown for the longest transcript (start site at −110). Ex, exon. Note that the protected RNA extends 3 bases downstream of exon I, since the 5′ trinucleotide sequences of intron I and exon II are identical (GTG).

Investigation of 5′ deletions

Constructs containing 5′ deletions of the δ-subunit upstream sequence fused to the chloramphenicol acetyltransferase (CAT) reporter gene ('δCAT'; see Figure 3 and Materials and methods) were transfected into several muscle cells and into 3T3 fibroblasts. Characteristic and reproducible differences in CAT expression were observed. In differentiated mouse C2C12 cells, removal of sequences to −429 affected expression very little, but deletion to −251 and further to −207 gave rise to a 2- to 3-fold activation. Further deletions then resulted in a decrease in activity, with the steepest reductions observed between −207 and −188, and −167 and −151. Comparable results were obtained in primary chick myotubes, except that the rise between −251 and −207, and the drop-off between −207 and −167 was steeper (Figure 4). The activity of the 5′-68 construct did not exceed 1% of that of the 5′-207 construct in either type of myotube, suggesting that the intervening sequence activates expression by at least two orders of magnitude. In L6 cells, a rat muscle cell line that expresses AChR at very low levels, and in the murine fibroblast line 3T3, weak activity appeared only after elimination of sequences upstream of −207. Low levels of activity in C2C12 myoblasts could be further reduced by culturing the cells in bromodeoxyuridine (BrdU), suggesting that they were caused by contamination with early differentiating cells.

The validity of the interpretation of the CAT results was confirmed by measurement of transcript synthesis. An analysis using β-globin constructs revealed not only that the correct start sites are used but also yielded the expected response pattern, i.e. intermediate activity with a 1 kb upstream region, strong activity after deletion to −207, and inactivation upon truncation to −151 (Figure 5). In myotubes (either C2C12 or primary chicken) truncation to −207 activated expression by about an order of magnitude; in fibroblasts, transcripts were at the limit of detectability even after elimination of the inhibitory upstream sequence.

The transcripts begin at the start sites except for the largest bands which may arise either from nonspecific read-through transcription originating inside the vector or—more likely

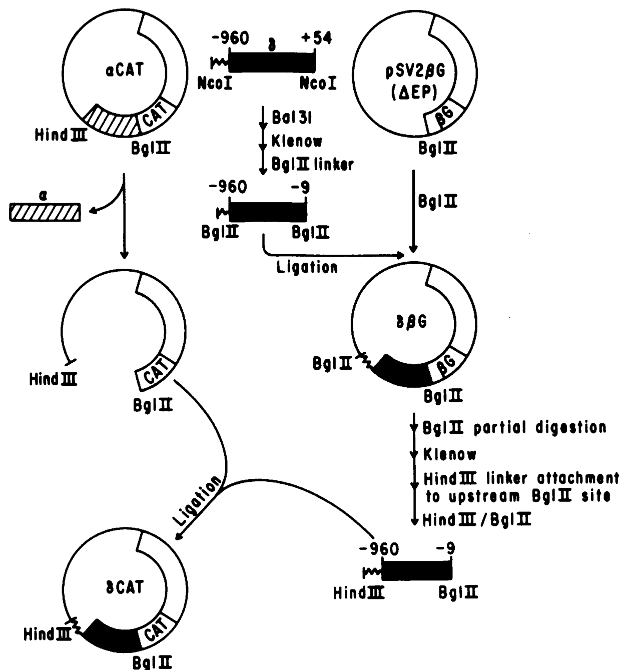

Fig. 3. Overview of plasmid construction. Details of the plasmid constructions outlined above are given in Materials and methods. The fragment derived from *Nco*I digestion of pL3 contains ~450 bp of ϕλL47 (zigzag line). δ-Subunit gene sequences are given in black, the 2 kb α-subunit gene upstream sequence is crosshatched.

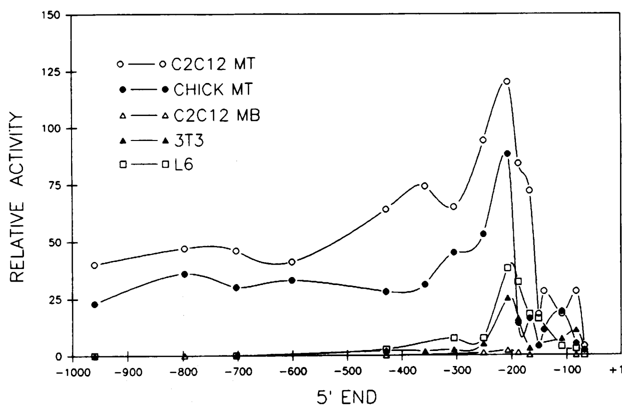

Fig. 4. Expression of 5′ deletions in muscle and non-muscle cells. Results of CAT assays with several deletion mutants. Numbers refer to the 5′ end of constructs produced by progressive deletion with *Bal*31 exonuclease (see Materials and methods). The plasmids pSV2CAT and pSV2CAT(ΔEP) (which possess or lack the SV40 promoter and enhancer respectively), serve as positive and negative controls. CAT activities are normalized to pSV2CAT.

in view of the variability of size and quantity of these bands—as artifacts of protection by residual plasmid DNA present in the RNA preparations used. Similarly small quantities of intact probe and of a band of ~495 nucleotides must be regarded as artifactual since their presence does not depend on protection by RNA. At any rate, no correlation exists between the presence of any of these bands and reporter gene expression; promoterless constructs such as −207(ΔP)CAT, in spite of massive 'readthrough transcription', express CAT only at background levels (see Table I).

5′ deletion fragments were also inserted, in both orientations downstream of pSV2CAT(ΔE) (a construct containing the SV40 promoter, but lacking an enhancer; Yang *et al.*, 1986) at the *Hpa*I site, ~1.5 kb from the SV40 promoter. These constructs displayed expression levels very similar to those of the 5′ deletions of δCAT (Figure 4), i.e. a stepwise reduction in activity upon truncation to −207, −167, and −83; no orientation dependence is apparent (Figure 6). It is noteworthy that regardless of location, partial activity (10−20%) survives deletion of sequences upstream of −146.

Experiments in which the 62 bp enhancer (see below) was inserted downstream of several truncated versions of the δ-promoter showed that δ (−207/−72)CAT (a construct which lacks the downstream promoter portion) and δ (−68)CAT (upstream portion deleted) still displayed transcriptional activity (data not shown).

Analysis of 3′ deletions

A fragment with 5′ end at position −207 was digested with *Bal*31 to identify the 3′ boundaries of the positive regulatory element described above. The activity of a CAT construct containing the undigested (5′−207/3′−9) sequence in combination with the SV40 promoter equalled that of pSV2CAT (which contains the SV40 enhancer) and exceeds that of an enhancerless control [namely, pSV2CAT(ΔE)] by about two orders of magnitude (Table I, Figure 7). Truncation to position −72 (3′) was tolerated, regardless of the orientation of the transposed fragment. Further deletion led to a 5-fold reduction in activity of the fragment oriented in the opposite direction. When inserted in the correct orientation, the activity proved more resistant to 3′ deletions; such constructs with inserts deleted to between −89 and −146 were about two to three times more active than their opposite-orientation counterparts. Truncation to −165 resulted in weak (≤20%) residual activity in either orientation (Figure 7). Further deletions were impractical due to the small size of the resulting oligonucleotide. These experiments revealed that the enhancer activity resides in a 62 bp stretch (from −207 to −146; the '62 bp enhancer').

Tissue specificity

To assess tissue and stage specificity, both 5′ and 3′ deletion mutants were examined in myoblasts (undifferentiated C2C12 cells), myotubes (differentiated C2C12 cells and primary chick myotubes), and non-muscle cells. CAT constructs comprising the δ-promoter and the entire upstream sequence are expressed in both avian and murine myotubes at rates equalling 30−50% of the 5′ (−207) deletion mutant (Figure 4). In fibroblasts and myoblasts no activity is seen when the entire upstream sequence is present. Expression appears, however, in fibroblasts, upon progressive deletion of 5′ sequences, and reaches a peak corresponding to 25% of pSV2CAT when deletion advances to position −207. Similar results are seen with HeLa cells (at peak, 19% of the pSV2CAT activity). The same constructs remain silent in myoblasts (trace activity in myoblasts is attributable to incipient differentiation). To investigate how different cell types respond to the isolated enhancer element, constructs containing it downstream of the SV40 promoter were transfected into various muscle and non-muscle cells (Table I). Regardless of promoter origin the enhancer was found to be active in fibroblasts and differentiated muscle cells, but displayed little activity in myoblasts, even in the

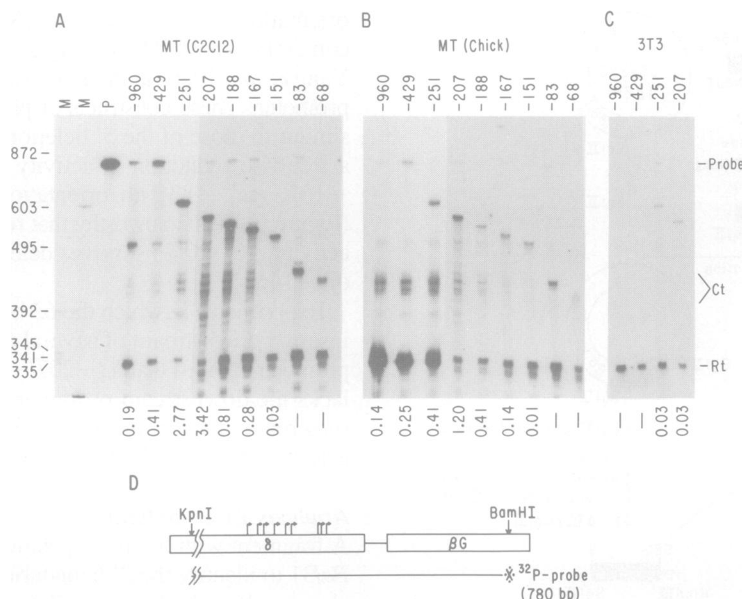

Fig. 5. S1 nuclease protection analysis of δβG. Nuclease protection analysis was performed as described in Materials and methods. The numbers on top are endpoints of 5′ deletions, the numbers on the bottom are the intensity ratios of test and reference transcripts (note that each test transcript 'Ct' is an ensemble; for quantitation the individual band intensities are summed). Abbreviations are: M, mol. wt marker (ΦX174/*Hae*III and ΦX174/*Hin*cII); P, probe; Ct, correct transcripts (multiple bands due to multiple start sites); Rt, reference transcript (pβCH41); –, undetectable. S1 mapping results are shown for C2C12 myotubes (**A**), chick primary myotubes (**B**) and 3T3 cells (**C**). Probes are shown in (**D**) multiple start sites are indicated by arrows. Numbers on top indicate the 5′ end; numbers at bottom represent activities normalized to the internal control pβHC41 and represent averages of two to three independent experiments. The band at 495 is an artifact that appears even in the absence of a protecting sequence.

Table I. Activity of the 62 bp enhancer in different cell types

Cell type	Combined with SV40 promoter				δ-subunit promoter	
	pSV2CAT	pSV2CAT(ΔE)	62+	62−	−207(ΔP)CAT	−207CAT
MT(chick)	100	5	41	20	ND	88
MT(C2C12)	100	1	68	25	7	93
MB(C2C12)	100	0	0	0	ND	3
3T3	100	6	30	14	1	25
HeLa	100	6	27	12	0	19
TE671	100	17	78	ND	10	80
RD	100	4	25	ND	2	54

Constructs were transfected into various cell types and analyzed as described in Materials and methods. Activities are normalized to that of an equal amount of cotransfected pSV2CAT. Abbreviations used: pSV2CAT(ΔE), pSV2CAT without enhancer; 62+, pSV2CAT(ΔE), with 62 bp enhancer inserted downstream in forward orientation; 62−, pSV2CAT(ΔE), with 62 bp enhancer inserted downstream in reverse orientation; −207(ΔP)CAT, −207CAT with a −145 to −9 (i.e. promoter) deletion; MB, myoblast; MT, myotube; ND, not determined; 3T3, HeLa, TE671, and RD, cell lines described in Materials and methods. Values represent averages of five (MTC2C12) or two experiments (MBC2C12; 3T3) or single observations (all others).

absence of the inhibitory upstream element; not surprisingly the enhancer also was active in cell lines expressing the receptor constitutively (RD, TE671).

To confirm enhancer action at the transcriptional level, we inserted the 62 bp fragment at the *Hpa*I site downstream of pSV2βGΔE, and transfected this construct into chick and mouse myotubes and into fibroblasts. In differentiated muscle cell results seem independent of the reporter gene used: strong activation of the SV40 promoter, with a significant orientation effect (i.e. the enhancer is about two to three times more active when inserted in the forward than in the opposite orientation). The 62 bp fragment in the correct orientation is about two to three times weaker than the α-subunit derived enhancer (Figure 8). In fibroblasts the α-construct and the δ-enhancer display low activity. This contrasts with the readily detected CAT activity in experiments when the δ-enhancer is under the control of the SV40 promoter or under its own promoter (see Figure 4 and Table I). The apparent discrepancy between results obtained with β-globin and CAT constructs may arise from differences in assay sensitivity or post-transcriptional effects such as CAT enzyme stability.

Discussion

Promoter structure

In contrast to the α-subunit, the δ-subunit gene promoter region lacks the canonical TATA and CCAAT elements; it is moderately abundant in GC; and transcription is initiated from several sites, giving rise to six major and several minor transcripts. This situation is reminiscent of 'house-keeping' genes, e.g. those coding for HMG CoA reductase (Reynolds *et al.*, 1984), dihydrofolate reductase (McGrogan *et al.*,

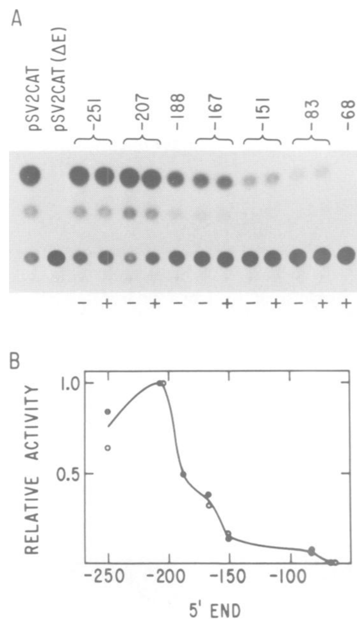

Fig. 6. Analysis of combinations of the SV40 promoter with δ 5′ deletions in C2C12 myotubes. (**A**) Various deletion fragments were inserted, in both orientations, at the *Hpa*I site of pSV2CAT(ΔE), 1.5 kb downstream of the SV40 promoter. Constructs were tested in C2C12 myotubes as described. The 3′ end of all fragments is at −9. 5′ ends are indicated by numbers; the sign refers to orientation (+, forward; −, opposite). (**B**) Graphic representation. Values are normalized to the most active fragment; averages of two experiments are shown. Open symbols, forward; filled symbols, reverse orientation.

1985), EGF receptor (Ishii *et al.*, 1985), and c-K-*ras* (Yamamoto and Perucho, 1988). Whether individual start sites play specific functional roles is not known at the present time. While chick muscle cells, both *in vivo* and *in vitro*, prefer the upstream sites, transfected C2C12 myotubes utilize proximal as well as distal sites (Figure 5). Perhaps this is related to the fact that the upstream portion of the enhancer is required for function in chicken cells, while more proximal elements suffice for at least partial activity in mouse myotubes (see below). The entire −140 to −9 region of the δ-subunit gene may be considered as an extended promoter or as a domain comprising several promoters and one (or more) weak enhancer(s). At least two non-overlapping fragments (upstream of −72, and downstream of −68) function independently as promoters.

The 62 bp enhancer

The δ upstream region stimulates transcription of the enhancerless pSV2CAT(ΔE) construct by about two orders of magnitude. This activity resides in a 62 bp sequence (−207 to −146), upstream of the first start site. The element functions independently of location and promoter provenance (SV40 or δ) and thus can be regarded as an enhancer. It also retains activity upon inversion but, for unknown reasons, constructs containing the element inserted in correct polarity were two to three times more active than their opposite-orientation counterparts; the sequence between −83 and −72 appears to be responsible for this effect.

Regarding enhancer structure we note interesting differences and similarities with the α-subunit gene. While the α-enhancer is fairly compact, the δ-enhancer contains at least two distinct elements whose 5′ ends are located

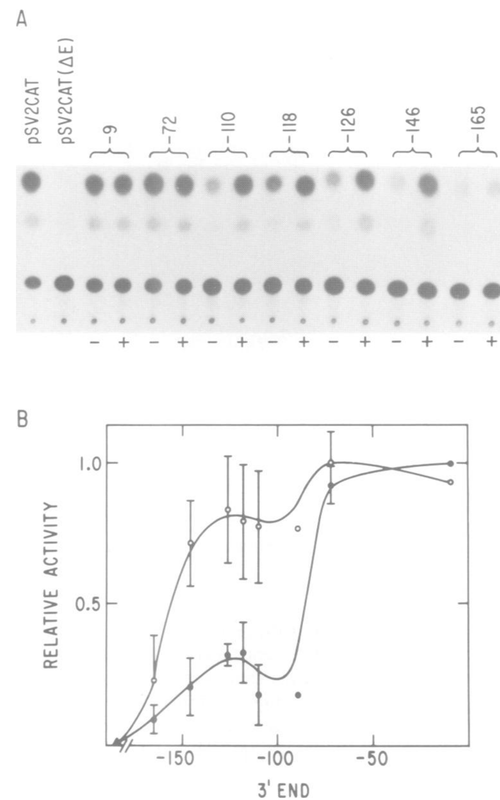

Fig. 7. Activity of 3′-deletions in C2C12 myotubes. (**A**) 3′ deletions were prepared as described in Materials and methods inserted, in both orientations, at the *Hpa*I site of pSV2CAT(ΔE), 1.5 kb downstream of the SV40 promoter. Constructs were tested in C2C12 myotubes as described. Numbers indicate the 3′ end; signs refer to orientation (+, correct; −, opposite). (**B**) Graphic representation as described in the legend to Figure 6. Most data points are results (mean and SEM) of two or more experiments.

between −207 and −188, and −167 and −151 respectively. 3′ deletion experiments indicate that the 3′ end of the enhancer does not extend beyond position −146. Both moieties comprise a short sequence resembling a 16 bp motif (TGCCTGGAACAGGTGG) that occurs in the α-enhancer. The near element (located between −167 and −146) includes a 14/16 match, while the far element (downstream of −207) contains a 12/16 match in reverse orientation. The sequence of the far element, read in the forward orientation, also resembles the region further upstream (CAGCTGTCA-TGCCTG) of the α-enhancer (a 14/16 match); this is a consequence of the partially palindromic character of the −107 to −84 section of the α-enhancer (see Figures 1 and 9). Accordingly, one would expect an antiparallel fit between the downstream element and the α enhancer as well; however, the similarity is quite modest (8/16 match). The consensus sequence that can tentatively be deduced for AChR enhancers (see Figure 9) is composed of several modules that have been implicated previously in tissue specific expression. It contains two copies of the CANNTG motif present in the MyoD1 target sequence and characteristic of many muscle specific regulatory regions (Buskin and Hauschka, 1989; Lassar *et al.*, 1989); the CANNTG elements flank an 'M-CAT' similarity and a TGCCTGG sequence both of which have been proposed as muscle specific motifs (Mar and Ordahl, 1987; Baldwin and Burden, 1989).

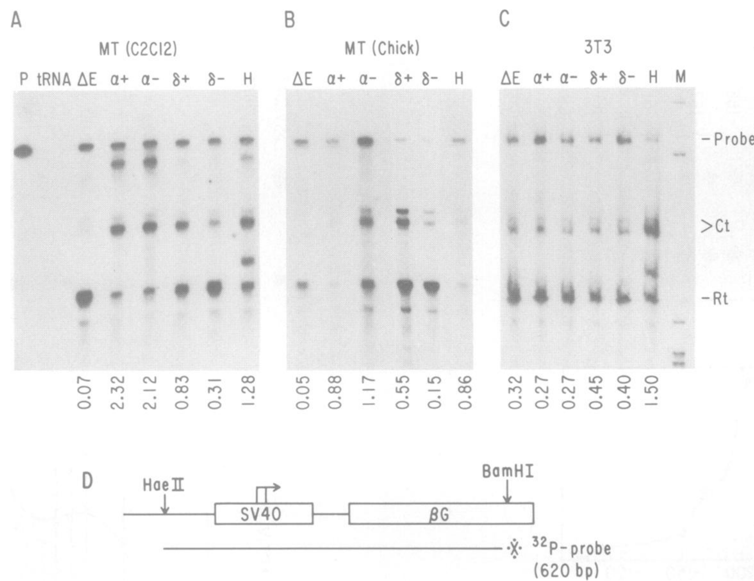

Fig. 8. S1 nuclease protection analysis of the 62 bp enhancer inserted downstream of SV40βG. The 62 bp enhancer (positions −207 to −146, see Figure 1) was inserted, in both orientations, at the *Hpa*I site of pSV2CAT(ΔE), 1.5 kb downstream of the SV40 promoter. Activities were compared with the α-enhancer inserted at the same site. Constructs were tested in C2C12 myotubes (**A**), chick primary myotubes (**B**) and 3T3 fibroblasts (**C**). (**D**) Structure of probe. Abbreviations: P, probe; ΔE, pSV2β(ΔE); α, α-enhancer (Wang *et al.*, 1988) inserted; δ, δ-enhancer (62 bp) inserted; H, human cytomegalovirus enhancer inserted; +, forward orientation; −, reverse orientation; M marker (ΦX174/*Hae*III). Numbers at bottom are intensity ratios of test and reference (pβCH41) transcripts.

It is tempting to speculate that the similarity in functional sequences permits the same *trans*-acting factor (or factors) to activate both the α- and δ-subunit gene regulatory regions and thereby ensure their coordinate expression.

A negative element

A notable difference between α- and δ-upstream regions is the presence, in the latter, of an inhibitory element. Such negative elements have been described for a number of genes including the histocompatibility complex gene Eα (Thanos *et al.*, 1988) and the genes coding for the retinol binding protein (Colantuoni *et al.*, 1987), α1-antitrypsin (Simone *et al.*, 1987), β-interferon (Goodbourn *et al.*, 1986), interleukin 2 (Nabel *et al.*, 1988), and the T cell receptor α-subunit (Winoto and Baltimore, 1989). In these genes, expression in nonspecific cells can be effected by elimination of the inhibitory sequence. The negative element of the δ-subunit gene is active in both nonspecific cells and myofibers. The narrow peak of activation seen in the 5′ deletion series (which is especially conspicuous in the βG/chicken myotube experiments; Figure 5) suggests that the inhibitory element is adjacent to the enhancer.

In transfection experiments the δ-enhancer, in combination with the SV40 promoter, displays about half the strength of the α-enhancer. This contrasts with the finding that levels of δ-specific messages in normal and denervated muscle (Shieh *et al.*, 1988) are ~15-fold lower than α-subunit mRNA concentrations. The relatively weak expression of the δ-subunit gene at the message level (unless caused by post-transcriptional effects, e.g. faster turnover) is at least in part accounted for by the negative element which is active not only in non-muscle cells but also in differentiated myotubes. In addition, the δ-subunit promoter appears to be weaker than the α-subunit promoter.

We have attempted to study the effect of the negative element on the activity of heterologous promoters. Constructs

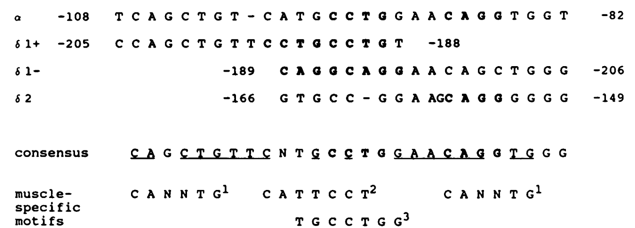

Fig. 9. The enhancer consensus sequence. α refers to the central portion of the a-subunit gene enhancer, δ1 to the 'far' or upstream enhancer element (5′ terminus between −207 and −188), δ2 to the 'near' or downstream element (5′ terminus between −167 and −151) and signs to orientation. A frequent tetranucleotide motif, which has also been reported in mouse β- and γ-subunit upstream regions (Crowder and Merlie, 1988), is printed in bold. Palindromic elements in the enhancer consensus sequence are underlined. [1]Proposed by Lassar *et al.* (1989). [2]'M-CAT' motif, proposed by Mar and Ordahl (1988). [3]Proposed by Baldwin and Burden (1989).

containing the negative element several hundreds of base pairs away from an SV40 promoter reveal no inhibitory effects (data not shown), suggesting that perhaps its action diminishes with distance. At present we do not know the 5′ extent of the negative element.

Tissue specificity

Two mechanisms contribute to the tissue specific expression of the δ-subunit gene. First, the positively acting element, i.e. enhancer plus promoter, is most active in myotubes. Although a direct comparison of enhancer strength in two different cell types is not possible, normalization to the activity of a nonspecifically acting promoter region, e.g. that of SV40, can be attempted. It turns out that while 5′ flanking sequences derived from SV40 and δ are comparable in activity in differentiated muscle cells, the δ sequences are only ~1/5 to 1/4 as active as the SV40 elements in non-muscle cells, e.g. 3T3 fibroblasts and HeLa cells; signifi-

cantly higher activity was observed in the human rhabdomyosarcoma lines RD and TE671, both of which express the muscle type AChR (Table I).

Equally important is the presence of the inhibitory element. While this element reduces receptor gene transcription by several-fold in myotubes (2- to 3-fold when measured in the CAT assay; ~10-fold when measured by transcript analysis), it virtually eliminates expression in non-muscle cells (put the other way around and more quantitatively, we estimate that in fibroblasts activity of the δ-gene promoter, upon elimination of the inhibitor, increases by two orders of magnitude). The regulatory situation thus appears similar to that of the rat insulin 1 gene where, according to a proposal by Nir *et al.* (1986), a negative *trans*-acting factor active in cells not expressing insulin is over-ridden by a dominant positive factor present in insulin expressing cells. The inhibitor sequence does not determine stage specificity, i.e. the suppression of the δ-subunit gene in myoblasts prior to fusion; instead, developmental expression is controlled by elements downstream of −207.

When assayed by transcript formation using β-globin constructs, the enhancer, even after complete removal of the inhibitory region, exhibits only trace activity in 3T3 fibroblasts. This observation was made repeatedly and seemingly disagrees with the CAT results. One might presume that this means that CAT expression in non-muscle cells is entirely nonspecific (arising from translation of read-through transcripts) and that, consequently, tissue specificity is determined by the enhancer alone. Alternatively it can be argued that the enhancer is weakly active in fibroblasts, giving rise to low, barely detectable levels of transcript which, due to much higher post-transcriptional expression efficiency in these cells and/or greater assay sensitivity, produce relatively strong CAT signals. We favor the latter possibility because −207(ΔP)CAT, a CAT construct without a promoter that nevertheless should permit read-through transcription, is silent in 3T3 cells (Table I); the situation in HeLa cells is similar.

Species specificity

The recent publication of the mouse δ-subunit gene upstream region (Baldwin and Burden, 1988; Crowder and Merlie, 1988) invites a structural as well as functional comparison with the chicken sequence. Functionally the two upstream regions are different in that the mouse gene harbors a unique start site with TATA- and CCAAT-like elements at the proper positions. The sequences of the chicken and mouse 5′ flanking regions also differ considerably, with the exception of a 15/18 bp match that overlaps the transcription start site in the mouse and centers on the 5′-most major cap site at position −110 of the chicken α-subunit gene. In the murine gene, information for stage and tissue specific expression is contained within a 54 bp region of the 5′ untranslated sequence (Baldwin and Burden, 1989). This element functions equally well in both orientations, but is inactivated upon placement 1.7 kb downstream of the δ-subunit promoter, in obvious contrast to the chicken δ-subunit element. The chicken 62 bp enhancer contains a TGCCTGT heptamer (−194/−188) which resembles the TGCCTGG motif found in several other muscle specific genes, including the one coding for the mouse δ-subunit (Baldwin and Burden, 1989). No other obvious similarity with the chicken enhancer consensus sequence is present in the 54 bp region of the mouse gene. The question then arises why the chicken δ-subunit gene enhancer is active in a murine host. One possible explanation is that the chicken δ-subunit gene is recognized by the α regulatory apparatus of the mouse cell. Though plausible, the tacit assumption here is that recognition of the α enhancer in the chicken by murine factors is the result of structural similarity of the regulatory regions of the α genes in the two species. Unfortunately, without structural information on the mouse α-subunit upstream region, this remains a speculation.

Materials and methods

Construction of chimeric genes

Plasmid pL3 which contains the entire genomic sequence of the δ-subunit (~4.5 kb; Nef *et al.*, 1984) in pUC8 was treated with *Nco*I to release a 1.4 kb fragment which includes about 450 bp of ϕλL47 DNA at the 5′ end. *Bal*31 digestion produced a 3′ end 8 bp upstream of the first ATG of the δ coding sequence. Upon addition of *Bgl*II linkers the fragment was inserted into pSV2βG(ΔEP) (which contains the rabbit β-globin gene, but lacks both SV40 promoter and enhancer) at the *Bgl*II site. A clone of the correct orientation was selected, and partial digestion followed by *Hin*dIII linker attachment to the 5′-end yielded the δβG construct. Insertion of the *Hin*dIII/*Bgl*II fragment from δβG into the modified αCAT produced the δCAT construct (see Figure 3); αCAT is a plasmid that contains 2 kb of upstream sequence of the α-subunit gene fused to the CAT coding region (X.-M.Wang, unpublished data). Deletion breakpoints and fusions were checked by DNA sequencing.

5′ deletions. Using exonuclease *Bal*31, the 5′ flanking region was progressively deleted from either the *Nae*I site at −797 or the *Kpn*I site at −429; this was followed by attachment of *Hin*dIII linker and self-ligation with the most upstream *Hin*dIII site.

3′ deletions. δ(−207)CAT was cut at the *Bgl*II site and digested with *Bal*31, followed by Klenow fill-in, *Xho*I linker attachment and self-ligation.

*Hin*dIII−*Xho*I fragments, after Klenow fill-in, were isolated from a series of such deletions and inserted into pSV2CAT(ΔE) at the *Hpa*I site downstream of the SV40 promoter. The 62 bp enhancer fragment was also inserted into pSV2βG(ΔE) at the same *Hpa*I site. The plasmids pSV2CAT(ΔE) and pSV2CAT(ΔEP) were derived from pSV2CAT by deletion of either the enhancer or the enhancer and promoter combined, as described previously (Yang *et al.*, 1986). The preparation of pSV2βG and its mutants was described previously (Wang *et al.*, 1988).

Cell culture and transfections

The murine C2C12 cell line and 3T3 fibroblasts were grown and transfected as described previously (Wang *et al.*, 1988). When myoblasts were to be tested, BrdU was added to the medium (at 5 μg/ml) to suppress differentiation (Schimmel *et al.*, 1977), unless stated otherwise. Chick embryo myogenic cells were cultured as described (Pezzementi and Schmidt, 1981). The human cell lines RD and TE671 [the latter, originally thought to be of neuronal origin, has recently been recognized as a rhadomyosarcoma line. (Chen *et al.*, 1989; Stratton *et al.*, 1989)] were obtained from ATCC and were grown in DME containing 10% fetal calf serum. For CAT experiments, sample DNA (10 μg) and pCH110 (2 μg) as an internal control were co-transfected into cells, cultured in 100 mm dishes, using the calcium phosphate precipitation technique; for transcript analysis, 25 μg of β-globin construct and 0.5 or 1.0 μg of internal control (pβHC41; Boshart *et al.*, 1985) were used per 150 mm dish.

Nuclease protection assay

For start site determination, a uniformly ^{32}P-labeled single stranded probe was prepared from an M13mp10 subclone containing the *Pst*I fragment from −402 to +91 inserted in the right orientation (Yang *et al.*, 1985). The *Pst*I fragment was also subcloned into Bluescript SK^{+}, and a uniformly ^{32}P-labeled riboprobe was synthesized and used as described previously (Tsay and Schmidt, 1989). For the analysis of δβglobin or pSV2βG(ΔE) fusion constructs, the *Kpn*I−*Bam*HI fragment of δβG was end-labeled at the *Bam*HI site. Hybridization and S1 nuclease mapping were carried out as described by Wang *et al.* (1988); autoradiographic signals were quantitated by densitometry using a Joyce-Loebl Chromoscan III densitometer.

Primer extension analysis

An oligodeoxynucleotide representing a δ-subunit gene fragment from +18 to +46 was synthesized and end-labeled with [γ-^{32}P]ATP, using T4 kinase. Hybridization and reverse transcription followed the protocol described in Ausubel *et al.* (1987), except that hybridization (at 30°C) was allowed to proceed for 48 h.

Enzyme assays

Three days (in the case of myotubes) or 2 days (myoblasts, fibroblasts and HeLa cells) after transfection, cultures were washed twice with 5 ml phosphate buffered saline, incubated for 5 min at room temperature with 2 ml of 40 mM Tris, 1 mM EDTA, 0.15 M NaCl, and cells scraped off, collected by centrifugation and suspended in 100 µl of 0.25 M Tris−HCl, pH 7.8. An extract was obtained by several freeze−thaw cycles followed by centrifugation at 4°C for 5 min. β-Galactosidase was assayed colorimetrically according to Miller (1972). Briefly, 10 µl of cell extract were mixed with 310 µl of 'Z buffer' (0.1 M sodium phosphate, pH 7.0, 1 mM magnesium sulfate, 50 mM β-mercaptoethanol) and 80 µl of substrate solution (13 mM *o*-nitrophenyl-β-D-galactopyranoside in 100 mM sodium phosphate pH 7.0). The mixture was allowed to stand for 30 min at 37°C or until sufficient color was generated whereupon the reaction was stopped by the addition of 200 µl 1 M calcium carbonate, and absorbance at 420 nm determined. Chloramphenicol acetyltransferase (CAT) was assayed by the procedure of Davis *et al.* (1987) in volumes of cell extract adjusted to contain equal amounts of β-galactosidase. Briefly, 125 µl of substrate solution (a mixture of 70 µl 1 M Tris−HCl, pH 7.8, 20 µl 4 mM acetyl coenzyme A, 4 µl 0.5 mM [^{14}C]chloramphenicol; a total of ~300 000 c.p.m. and 31 µl water) were added to the extract, and the mixture incubated at 37°C for 1 h. The substrate and its acetylated derivatives were extracted with ethyl acetate and analyzed by TLC [silica plates; solvent: chloroform/methanol 19:1 (v/v)], followed by autoradiography. Quantitation was performed either by densitometry of developed X-ray film or by means of an imaging scanner (Ambis).

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