The Journal of Neuroscience, April 1, 1997, 17(7):2273–2283

β43′: An Enhancer Displaying Neural-Restricted Activity Is Located in the 3′-Untranslated Exon of the Rat Nicotinic Acetylcholine Receptor β4 Gene

Jennifer McDonough and Evan Deneris

Department of Neurosciences, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Members of a neuronal nicotinic acetylcholine receptor subunit gene cluster ordered β4, α3, α5 in the vertebrate genome are expressed in highly restricted patterns in the PNS and CNS. Nothing is known, however, about the regulatory elements that control transcription of these genes in selected neuronal cell populations. We report here a novel enhancer, designated β43′, that is positioned in the β4 3′-untranslated exon. It is composed of two nearly identical 37 bp direct repeats that are separated by 6 bp. Multimerization of the enhancer upstream of the α3 minimal promoter results in synergistic activation. Analysis in different cell types, including three neural lines and primary keratinocytes, shows that β43′ is preferentially active in the neural line PC12, which expresses all members of the cluster. Mobility shift assays reveal a cell-type-specific complex, which forms with the first repeat of the enhancer and PC12 extracts. Complexes co-migrating with the PC12 cell complex are not detected with extracts from other lines, which suggests that PC12 cells contain a differentially expressed factor that may be important for the restricted activity of β43′. The cell-type-specific activity of the β43′ enhancer suggests that it is important for regulating restricted expression patterns of one or more clustered neuronal acetylcholine receptor genes. Its location within the β4 gene may be a selective pressure for maintaining tight linkage of clustered neuronal nAchR genes.

Key words: neuronal nicotinic receptors; cis-acting elements; enhancer; cell-type specific; gene transcription; 3′-untranslated exon

The tremendous diversity of vertebrate neural cell phenotypes implies an underlying complexity of cis regulatory element needed to control thousands of genes in the appropriate spatial and temporal patterns (He and Rosenfeld, 1991; Mandel and McKinnon, 1993). A key transcriptional element essential for neuron-specific expression of some genes is the neuron-restrictive silencer element (Mori et al., 1992) or repressor element 1 (Kraner et al., 1992). The neuron-restrictive silencer element/repressor element 1 silences transcription by binding a repressor factor REST (Chong et al., 1995) or NRSF (Schoenherr and Anderson, 1995), which is ubiquitously expressed in non-neuronal cells. Transgenic mice studies have shown, however, that positive modulation of transcription resulting from the binding of factors to cell- or region-specific enhancers is also required for controlling the varied patterns of transcription in the nervous system (Tuggle et al., 1990; Whiting et al., 1991; Zimmerman et al., 1994). This is also evident from studies with transgene constructs in which the deletion of relatively large fragments of DNA flanking promoter regions results in loss of transgene expression in specific neural cell populations (Vandaele et al., 1991; Min et al., 1994; Carroll et al., 1995). An example of an enhancer that directs expression to particular populations of neurons is the gonadotropin-releasing hormone gene enhancer (Whyte et al., 1995). This multicomponent enhancer is active specifically in a clonal line derived from hypothalamic neurosecretory neurons that secrete gonadotropin-releasing hormone. For the vast majority of genes expressed in the vertebrate nervous system, however, we know nothing about the organization of regulatory regions and transcription factors responsible for directing expression to specific neuronal cell types.

A cluster of nicotinic acetylcholine receptor (nAchR) genes ordered β4, α3, α5 in mammals and birds (Boulter et al., 1990; Couturier et al., 1990; Raimondi et al., 1992) is expressed in peripheral and central neurons. Neuronal expression patterns of these genes in the CNS are highly restricted (Duvoisin et al., 1989; Wada et al., 1989, 1990; Dineley-Miller and Patrick, 1992), which suggests the presence of enhancers with narrow cell specificities. PC12 cells express all members of the cluster (Boulter et al., 1990) as well as many other markers of peripheral and central neuronal phenotype and, therefore, these cells are an attractive system for investigating mechanisms of neural transcription (Kraner et al., 1992; Mori et al., 1992; Yoon and Chikaraishi, 1992).

We and others have begun to analyze transcriptional control of the nAchR α3 gene (Duvoisin and Heinemann, 1993; Boyd, 1994, 1996; Yang et al., 1994, 1995). We identified and characterized the promoter region of the rat nAchR α3 gene (Yang et al., 1994, 1995) and showed that it initiates transcription at multiple sites in PC12 cells and sympathetic neurons (Yang et al., 1994; Fyodorov and Deneris, 1996). The significant activity of this promoter in various cell lines, however, suggests that it lacks cell-type-specific information. To search for cis elements mediating cell-restricted activity, we have analyzed sequences that extend upstream of the α3 gene and that include part of the juxtaposed β4 gene. Reported here is a novel cell-type-specific enhancer, which is located in the β4 3′-untranslated exon.

Received Aug. 20, 1996; revised Jan. 14, 1997; accepted Jan. 15, 1997.

This work was supported by National Institutes of Health Grant RO1 NS29123 from the National Institute of Neurological Disorders and Stroke. We thank Eric B. Banks in Dr. Richard Eckert's laboratory in the School of Medicine for performing keratinocyte transfections. We also thank Nicole Francis and Dr. David Setzer for their helpful comments on this manuscript and Tim Miller for preparation of some nuclear extracts.

Correspondence should be addressed to Dr. Evan Deneris, Case Western Reserve University, School of Medicine, Department of Neuroscience, 2109 Adelbert Road, Cleveland, OH 44106-4975.

MATERIALS AND METHODS

Luciferase reporters

Reporters described here contain all or part of a 2.8 kb rat genomic *Sac*I fragment, α3(−2732/+47), with coordinates designated relative to the major α3 transcription start site. This fragment extends 1.3 kb into the upstream β4 gene (Yang et al., 1997). A 187 bp β4 3′-untranslated exon fragment at coordinates −2732/−2546 and exhibiting enhancer activity (Fig. 3) was used to prepare a series of SV40, α3, or β4 promoter constructs in the pGL2 series of luciferase vectors (Promega, Madison, WI). The relative activities of the α3 and β4 core promoters are approximately equal to each other and are 20–30% of SV40 promoter activity in PC12 cells. For reporter designations described below, numbers in brackets indicate the sequences beginning on the β4 side of the 2.8 kb *Sac*I fragment that are present in a particular construct, unless stated otherwise.

SV40 promoter reporters. A single copy of the 187 bp enhancer fragment was placed immediately upstream of and in reverse orientation relative to the SV40 promoter by subcloning a 1.1 kb *Sac*I/*Kpn*I fragment of α3(−2732/+47) into the polylinker of pGL2-promoter (Promega) to generate [1–1100]SV-luc. This intermediate was then cut with *Spe*I, treated with Klenow to blunt end, and cut with *Sma*I, and the gel-purified vector fragment religated to generate [1–187]SV-luc.

α3 Promoter reporters. Deletions from the 5′ end of α3(−2732/+47)-luc were made using convenient restriction sites. α3(−238/+47)-Luc, constitutes our α3 minimal promoter construct (Yang et al., 1995) and was used to prepare reporters in which all or part of the 187 bp enhancer fragment was subcloned in one or the other orientation either immediately upstream of the minimal promoter or downstream of the luciferase gene. To prepare a reporter, [1–187]α3-luc, in which the 187 bp fragment was positioned immediately upstream of the minimal promoter, α3(−2732/+47)-luc was cut with *Spe*I, treated with Klenow to blunt end, digested with PmlI, and then the gel-purified vector fragment was religated. A second reporter, [1–187]ΔPNα3-luc, in which enhancer fragment sequences 95–161 bp that lie downstream of the palindrome were removed, was prepared by digestion of [1–187]α3-luc with *Pst*I and NsiI, followed by religation of these compatible ends. To place the enhancer downstream of the luciferase gene and in the correct orientation relative to the α3 promoter, a 1.1 kb *Xho*I/*Sma*I fragment of [1–1100]SV-luc was subcloned into the *Sal*I and blunt-ended *Bam*HI sites located in the vector backbone of α3(−238/+47)-luc. The resulting construct was then cut with *Kpn*I and *Pst*I and treated with T4 DNA polymerase in the presence of dNTPs to blunt end, and the vector fragment was religated to generate [1–161]α3[dc]-luc. To place the enhancer downstream of the luciferase gene and in the reverse orientation relative to the α3 promoter, a 1.1 kb *Bgl*II/*Sma*I fragment of [1–1100]SV-luc was subcloned into the *Bam*HI and blunted-ended *Sal*I sites located in the vector backbone of α3(−238/+47)-luc. The resulting construct was then cut with *Kpn*I and *Pst*I and treated with T4 DNA polymerase in the presence of dNTPs to blunt end, and the vector fragment was religated to generate [1–161]α3[do]-luc. To prepare a reporter, [86–107]α3-luc, in which a single copy of the palindrome was placed upstream of the α3 promoter, complementary oligonucleotides were synthesized to generate *Kpn*I and blunt compatible ends, annealed, and subcloned into α3(−1607/+47)-luc vector that was cut with *Kpn*I and PmlI. To prepare a reporter, [1–107]α3-luc, in which both repeats of the enhancer fragment were placed upstream of the palindrome, the *Kpn*I and Ppu10I cut [86–107]α3-luc vector fragment was gel-purified. This linearized DNA was then used in a shotgun ligation with *Kpn*I, Ppu10I, ScaI, and *Bam*HI cut α3(−2732/+47)-luc DNA.

To place two copies of the enhancer fragment upstream of the α3 minimal promoter, the 1055 bp *Sma*I/*Eco*RI fragment of [1–107]α3-luc was ligated to a [1–107]α3-luc vector fragment that was cut with Ppu10I, blunt-ended with Klenow, and then cut with *Eco*RI. Three copies of the enhancer fragment were placed upstream of the α3 minimal promoter by repetition of the above process with the 2× enhancer construct and the 1055 bp *Sma*I/*Eco*RI fragment of [1–107]α3-luc.

β4 promoter reporters. A rat *Hin*dIII genomic fragment with one end in the β4 5′-untranslated exon and the other 2.8 kb upstream was subcloned into pGL2Basic (Promega). The resulting β4 promoter construct, β4(−2663/+137)-luc, has coordinates defined relative to the β4 transcription start site determined by Hu et al. (1994). To prepare a β4 minimal promoter construct, β4(−2663/+137)-luc was digested with *Bam*HI, Klenow-treated to blunt end, and then digested with *Eco*RI. The resulting 950 bp β4 promoter fragment was then subcloned into the vector fragment of α3(−2732/+47) that was obtained by digestion with *Sma*I and *Eco*RI to generate β4(−254/+137)-luc. To prepare a reporter, [1–187]β4-luc, in which a single copy of the enhancer fragment was placed immediately upstream of and in the correct orientation relative to the β4 minimal promoter sequences −254/+137, the reporter β4(−2663/+137)-luc was cut with *Bam*HI, treated with Klenow to blunt end, then digested with *Eco*RI. The resulting 950 bp promoter fragment was gel-purified and ligated to the vector fragment of α3(−2732/+47) that was digested with *Spe*I, Klenow-treated to blunt end, and then cut with *Eco*RI. To place a single copy of the enhancer fragment downstream of the luciferase gene, β4(−2663/+137)-luc and [1–161]α3[dc]-luc were cut with *Eco*RI and ScaI. The β4 promoter fragment was then ligated to the enhancer fragment of [1–161]α3[dc]-luc to generate [1–161]β4[dc]-luc.

Cell lines and transfections

Rat2 cells, a rat fibroblast line, and HeLa cells (American Type Culture Collection) were grown in DMEM supplemented with 10% (v/v) FBS (Hyclone Laboratories, Logan, UT). PC12 cells were grown in DMEM supplemented with 10% FBS and 5% heat-inactivated horse serum (Hyclone). ARIP cells, a rat pancreatic tumor line; Clone 9 cells, a rat liver line; mouse C1300 neuroblastoma, and rat C6 CNS glioma (ATCC) were grown in DMEM/Ham's F12K medium supplemented with 10% FBS. Penicillin G sodium (100 U/ml) and streptomycin sulfate (100 μg/ml) (Life Technologies, Gaithersburg, MD) were added to all media. Lines were maintained at 37°C and 7% CO_2.

Cell lines were electroporated as described previously (Yang et al., 1994) using a Bio-Rad (Hercules, CA) Gene Pulser. Semioptimal electroporation conditions were determined for different cell lines by electroporating Rous Sarcoma Virus (RSV)-luciferase at different voltages (200–350 V) and different capacitances (250–960 μF) followed by determination of luciferase activities in cell extracts 2 d later. Electroporations were performed at 290 V and 960 μF for PC12, Rat2, HeLa, C1300, and C6, and at 350 V and 960 μF for ARIP and Clone 9 cells. Approximately 10^6 cells in 0.4 ml electroporation buffer (0.1 M HEPES, pH 7.4, 137 mM NaCl, 6 mM dextrose, 7 mM Na_3PO_4) were transfected using equimolar amounts of Qiagen-purified (Qiagen, Hilden, Germany) luciferase reporter constructs. Human keratinocytes were transfected using lipofectin (Life Technologies) as described previously (Welter et al., 1996). RSV-βgal was co-transfected (5 μg/transfection) to control for transfection efficiency. Either the pGLC (Promega) luciferase reporter containing the SV40 promoter and enhancer or RSV-luciferase was used as positive controls. Luciferase activities were measured after either ~24 or ~48 hr for HeLa, Rat2, ARIP, and Clone 9 cells and after ~48 hr for PC12, C1300, and C6 lines and keratinocytes. Cell extracts were prepared with luciferase lysis buffer (Promega), and then extracts were used to measure luciferase activities with Promega reagents and β-galactosidase activities with a chemiluminescence substrate and reagents (Tropix, Bedford, MA).

Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared based on the method described by Schreiber et al. (1989). Approximately 10^7 cells were pelleted and resuspended in 800 μl of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 4 μg/ml leupeptin, 1 μg/ml aprotinin, 40 μg/ml bestatin), then swelled on ice for 15 min. A 10% solution of Nonidet NP-40 was added (50 μl), mixed and centrifuged 30 sec in a microfuge. The pellet was resuspended in 100 μl of cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 4 μg/ml leupeptin, 1 μg/ml aprotinin, 40 μg/ml bestatin, 20% glycerol), then centrifuged for 5 min in a microfuge, and the nuclear extract was frozen at −70°C until needed. An aliquot of each preparation was used to determine protein concentration (Bio-Rad). A variety of conditions were explored to optimize binding of proteins to probe. The [NaCl] in the binding buffer was varied from 0 to 50 mM. Binding reactions were performed at 4°C, 25°C, 30°C, or 37°C in the presence or absence of detergents and polyamines. For separation of complexes from probe, polyacrylamide concentrations in gels were varied from 4 to 8% with several different cross-linking ratios. Optimal conditions for binding were 2 μl of extract (~3 μg/μl protein) in buffer C, 6 μl of 2× binding buffer (40 mM Tris-Cl, pH 7.9, 20% glycerol, 2 mM DTT), 2 μg poly dI/dC, and ~70,000 cpm [^{32}P]-end-labeled double-stranded oligonucleotide in a total volume of 12 μl at room temperature for 30 min. The products were then run on a 6.5% polyacrylamide gel, 20:1 cross-link ratio, at room temperature in 1× Tris-glycine buffer (50 mM Tris base, 380 mM glycine, 2 mM EDTA). The probe used was a double-stranded 35mer including most of the first repeat 5′-CAA TGC CAC TTC CTT GTA TAA GCC TTC CCA TGA TC-3′ (Great American Gene Company, Ramona, CA).

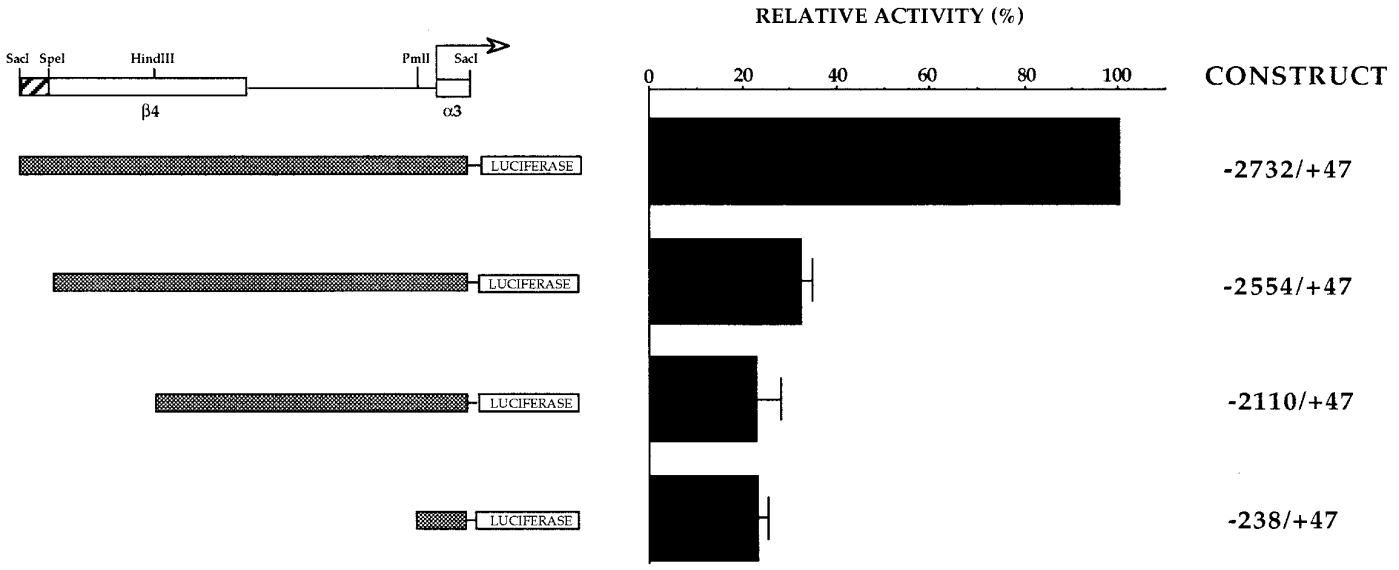

Figure 1. Identification of a positive transcriptional activity within the nAchR β4 gene 3′-untranslated exon. The schematic depicts a 2.8 kb *Sac*I fragment that contains the β4/α3 intergenic region as well as portions of β4 3′-untranslated and α3 5′-untranslated exon sequences (*rectangles*). The transcription start site region of the α3 promoter is shown by the *open arrow*, which is located ~1.4 kb downstream of the β4 gene. The *cross-hatched area* of the β4 3′-untranslated exon indicates the location of the 187 bp *Sac*I/*Spe*I fragment that activates transcription. Shown below the schematic are deletions of the 2.8 kb *Sac*I fragment that were used to localize the position of the positive transcriptional activity. Equimolar amounts of these reporters were transiently transfected into PC12 cells. Luciferase activity of each reporter construct was measured relative to that of the reporter containing the intact *Sac*I fragment (−2732/+47), which was set at 100%. Activities were obtained from at least three separate experiments in which duplicate transfections were performed and corrected for transfection efficiency with a co-transfected RSV-βgal plasmid. Error bars indicate mean ± SD.

EMSAs were repeated with several different batches of extract and probe preparations with similar results.

RESULTS

A 187 bp fragment of the β4 3′-untranslated exon activates transcription

Analysis of a 2.8 kb fragment extending upstream of the neuronal nAchR α3 transcriptional start site region revealed a subfragment that increases transcriptional activity of reporters in PC12 cells. This subfragment, which constitutes part of the β4 3′-untranslated exon, is likely to contain an enhancer and not an upstream α3 promoter, because PC12 cell-derived α3 exons are not detectable in this region of the cluster (Yang et al., 1997). To precisely localize the position of the putative enhancer, we prepared a set of α3 promoter 5′ deletion reporter constructs through the β4 3′-untranslated exon starting from −2732 relative to the major α3 transcription start site. The activity of each reporter was then quantitated after transfection into PC12 cells (Fig. 1). The results of this experiment showed that the activity of reporter α3(−2732/+47)-luc was approximately fivefold greater than that of α3(−238/+47)-luc, which is deleted down to the α3 minimal promoter (Yang et al., 1995). Deletion to −2554 resulted in a 70% decrease in activity, whereas additional deletion to −2110 produced only a small additional decrease to a level similar to the activity of the minimal promoter construct (Fig. 1). This analysis indicates that sequences responsible for the majority of the positive transcriptional activity reside within a 187 bp *Sac*I/*Spe*I fragment of the β4 3′untranslated exon, which is ~2.5 kb upstream of the α3 promoter.

Inspection of the 187 bp fragment revealed the presence of two motifs suggestive of enhancer elements (Fig. 2). First, two 37 bp direct repeats are present beginning at the β4 proximal end of the fragment and are separated from one another by 6 bp. There are only three mismatches between the repeats, and each mismatch is either a purine-to-purine substitution or a pyrimidine-to-pyrimidine substitution. Second, a nearly perfect 14 bp palindrome begins 7 bp downstream of the second repeat (Fig. 2).

The 187 bp fragment functions as an enhancer

To determine whether the 187 bp *Sac*I/*Spe*I fragment meets the criteria for a classical enhancer, we investigated the dependence of its activity on position and orientation relative to the α3 promoter. For this experiment, reporter constructs were made in which the 187 bp fragment was placed either immediately upstream of the α3 minimal promoter or downstream of the luciferase coding sequence. As shown in Figure 3, the 187 bp fragment stimulated the α3 minimal promoter severalfold. The magnitude of stimulation is similar to that obtained with the −2732/−2554 fragment (Fig. 1), thus confirming that most of the positive transcriptional activity of the −2732/−2554 region is located in the 187 bp fragment. As expected for an enhancer, we found that the 187 bp fragment produced comparable activations regardless of its position or orientation relative to the α3 promoter. (Fig. 3).

In the CNS, the expression patterns of the clustered β4 and α3 subunit genes only partially overlap, raising the possibility that there are cis elements that can activate particular members of the cluster but not others. On the other hand, because the β4 and α3 genes are co-expressed in numerous neuronal cell populations, especially in the PNS, they may be regulated by shared cell-type-specific cis elements. To determine whether the 187 bp *Sac*I/*Spe*I fragment is able to discriminate between the β4 and α3 promoters, we isolated the β4 promoter region from rat genomic cosmid clones (Yang et al., 1994). A *Hin*dIII fragment extending 2.8 kb upstream of the β4 5′-untranslated exon was isolated and subcloned into pGL2-Basic (Promega). The resulting reporter, β4(−2663/+137)-luc, and a truncated version, β4(−254/+137)-luc, with coordinates designated relative to the major β4 transcription start site reported by Hu et al. (1994) were used to

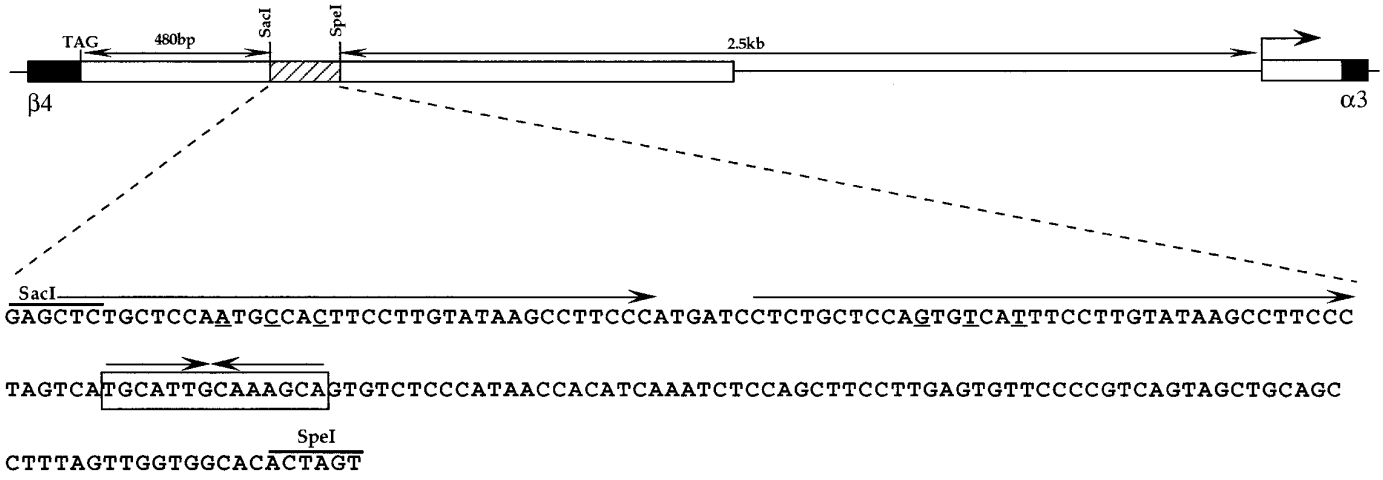

Figure 2. Genomic location and sequence features of the 187 bp *Sac*I/*Spe*I fragment. *Schematic*, The 187 bp fragment (*cross-hatched rectangle*) is part of the β4 3′-untranslated exon. It begins 480 bp downstream of the β4 translation stop codon (*TAG*) and is ~2.5 kb upstream of the α3 promoter. *Solid rectangles*, Protein coding sequences of α3 and β4; *open rectangles*, untranslated exon sequences; *thin lines*, intron and intergenic region sequences. *Sequence*, Within the *Sac*I/*Spe*I fragment are two 37 bp direct repeats. These repeats, shown by *long arrows* above the sequence, are separated by 6 bp and followed by a nearly perfect 14 bp palindrome (*boxed sequence*). Bases that differ between the two repeats are *underlined*.

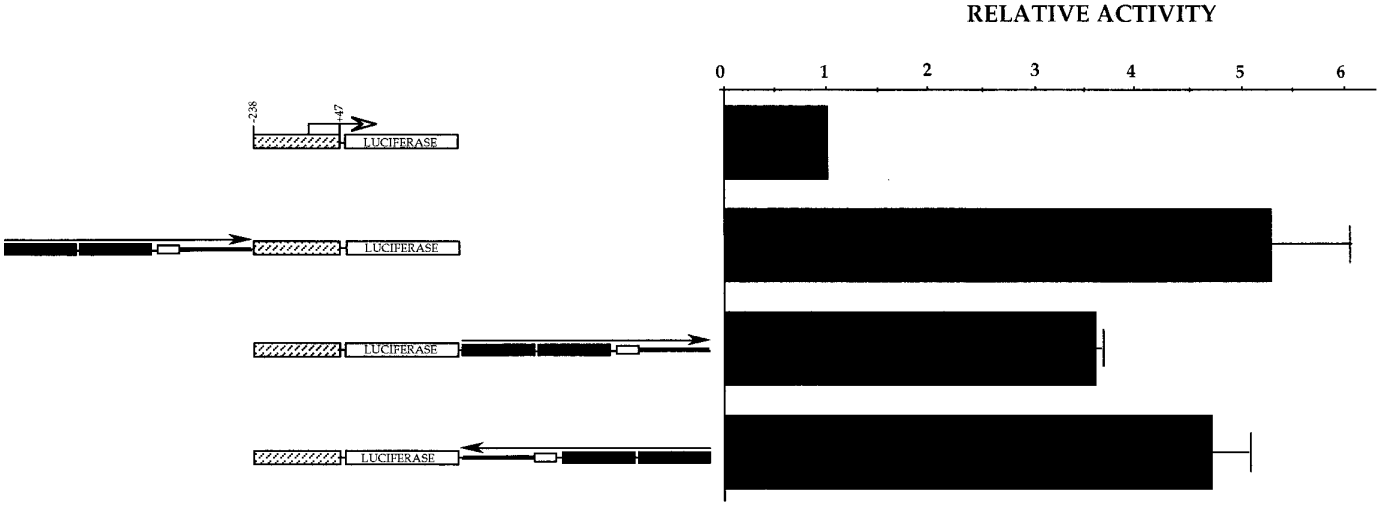

Figure 3. The 187 bp fragment displays characteristics of an enhancer: orientation and position independence. The 187 bp fragment was cloned either upstream of the α3 minimal promoter (*cross-hatched rectangle*) or downstream of the luciferase gene as indicated in the schematic. Sequence features of the fragment described in Figure 2 are represented by symbols (*tandem-filled rectangles*, 37 bp repeats; *open rectangle*, 14 bp palindrome; *thick line*, remainder of *Sac*I/*Spe*I fragment) below *dark arrow*, which indicates orientation of the element. The activity of reporter constructs containing the 187 bp fragment was measured relative to the α3 minimal promoter reporter α3(−238/+47)-luc in PC12 cells. Error bars indicate mean ± SD. Data are from at least two experiments in which duplicate transfections were performed and corrected for transfection efficiency by a co-transfected RSV-βgal plasmid.

prepare additional reporters in which the putative enhancer in the 187 bp fragment was placed either downstream or upstream of the β4 promoter, respectively. Transfection of these reporters into PC12 cells revealed that similar to the α3 promoter, equivalent activations were seen regardless of the position of the putative enhancer relative to the β4 promoter (Fig. 4*A*). Furthermore, the magnitude of activation was similar for both promoters. This result indicates that in cell culture, there is no fundamental selectivity of the putative enhancer for β4 and α3, which suggests that it may be capable of regulating both genes.

A second criterion for enhancer activity is the ability to activate an heterologous promoter. To test for this property, the 187 bp fragment was placed immediately upstream of and in reverse orientation relative to the SV40 promoter. Transfection into PC12 cells revealed a sixfold stimulation of the SV40 promoter activity, which demonstrates that the putative enhancer can act equally well on an heterologous promoter (Fig. 4*B*). The inability to significantly stimulate a reporter carrying the SV40 enhancer, but no promoter, confirms that the 187 bp fragment itself does not behave as a promoter. Together, the results presented above show that the 187 bp fragment from the β4 3′-untranslated exon behaves as a classical enhancer. We have designated this enhancer β43′.

An intact repeat region is necessary and sufficient for β43′ activity

Having demonstrated that the 187 bp *Sac*I/*Spe*I fragment behaves as an enhancer, we next investigated which parts of it are neces-

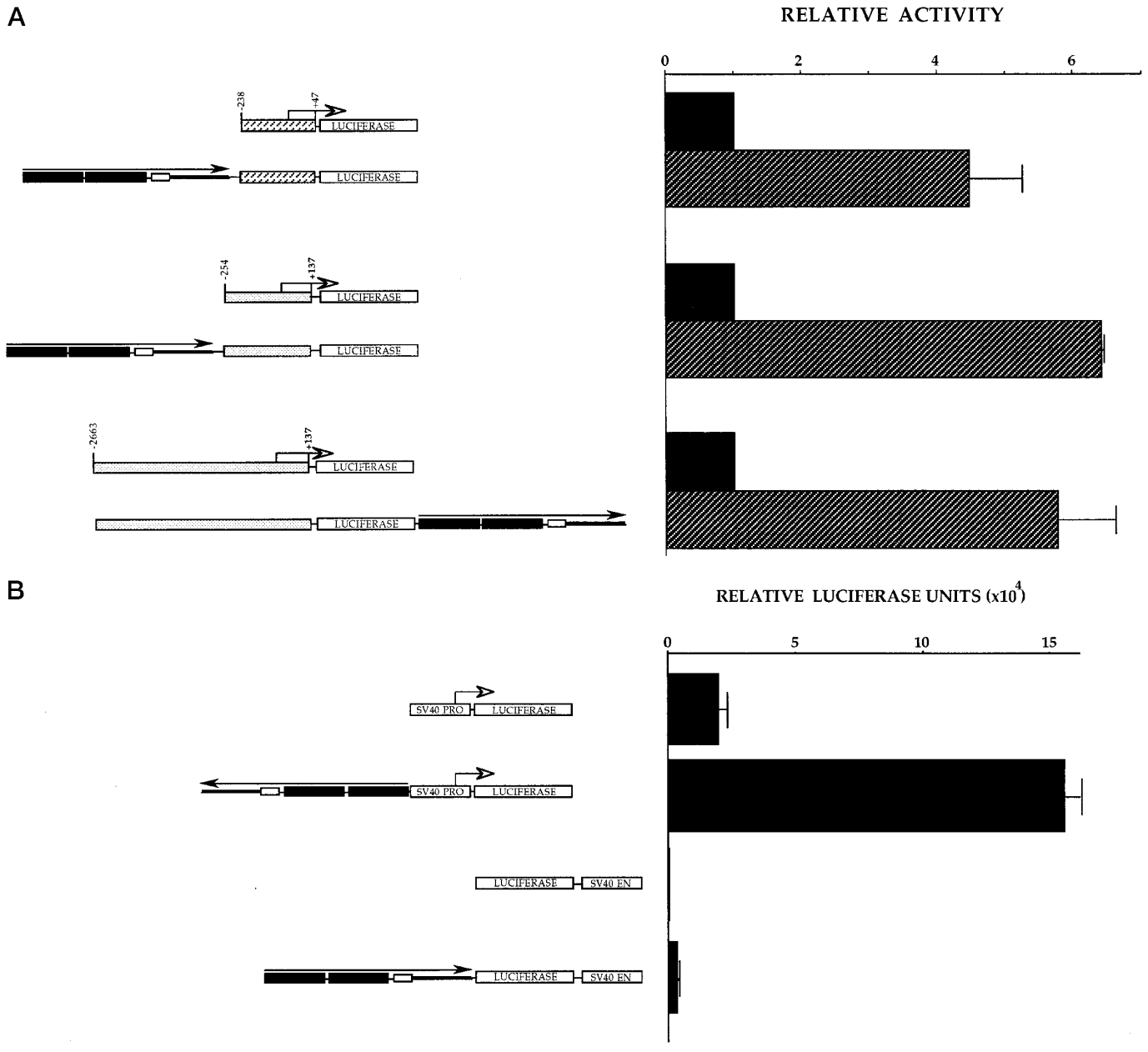

Figure 4. Equivalent activations of the β4, α3, and SV40 promoters by β43′. *A*, PC12 cells were transfected with constructs containing the 187 bp fragment either upstream or downstream of the β4 promoter (*stippled rectangles*) as shown in the schematic. The α3 minimal promoter constructs (*cross-hatched rectangles*) served as a positive control. The activity of reporter constructs containing the putative enhancer was measured relative to reporters containing the β4 or α3 promoter alone. Error bars indicate mean ± SD. Data are from at least two experiments in which duplicate transfections were performed and corrected for transfection efficiency by a co-transfected RSV-βgal plasmid. *B*, The 187 bp fragment was cloned in the indicated orientation upstream of either the SV40 promoter or SV40 enhancer, as shown in the schematic. Symbols representing sequence features of the fragment are as described in previous figures. Luciferase activities were calculated after correction for differences in transfection efficiency by a co-transfected RSV-βGal plasmid. Error bars indicate mean ± range for a typical experiment in which duplicate transfections were performed.

sary and sufficient for activity. For this experiment, we prepared reporters in which various segments of the fragment were placed immediately upstream of the α3 minimal promoter. The activities of each were then determined in PC12 cells and compared with the activity of the α3 minimal promoter in the presence or absence of an intact 187 bp fragment (Fig. 5, *lines 1* and *2*). We found that sequences downstream of the palindrome were dispensable for full activity, because deletion of these sequences did not decrease reporter activity (*line 3*). The palindrome was clearly not sufficient for activity, because an α3 reporter bearing a single copy of the palindrome was no more active than the minimal promoter (Fig. 5, compare *line 1* with *line 4*). Furthermore, an intact palindrome was not necessary for activity as shown by the retention of complete reporter activity when the palindrome and downstream sequences were deleted (*line 5*). These results point to the tandem repeat region as the essential segment of the β43′ enhancer. Enhancer elements often act synergistically when multimerized in cis to promoters (Sauer et al., 1995). To determine whether multiple copies of the tandem repeat region could act in this way, additional reporters were prepared in which two or three copies of

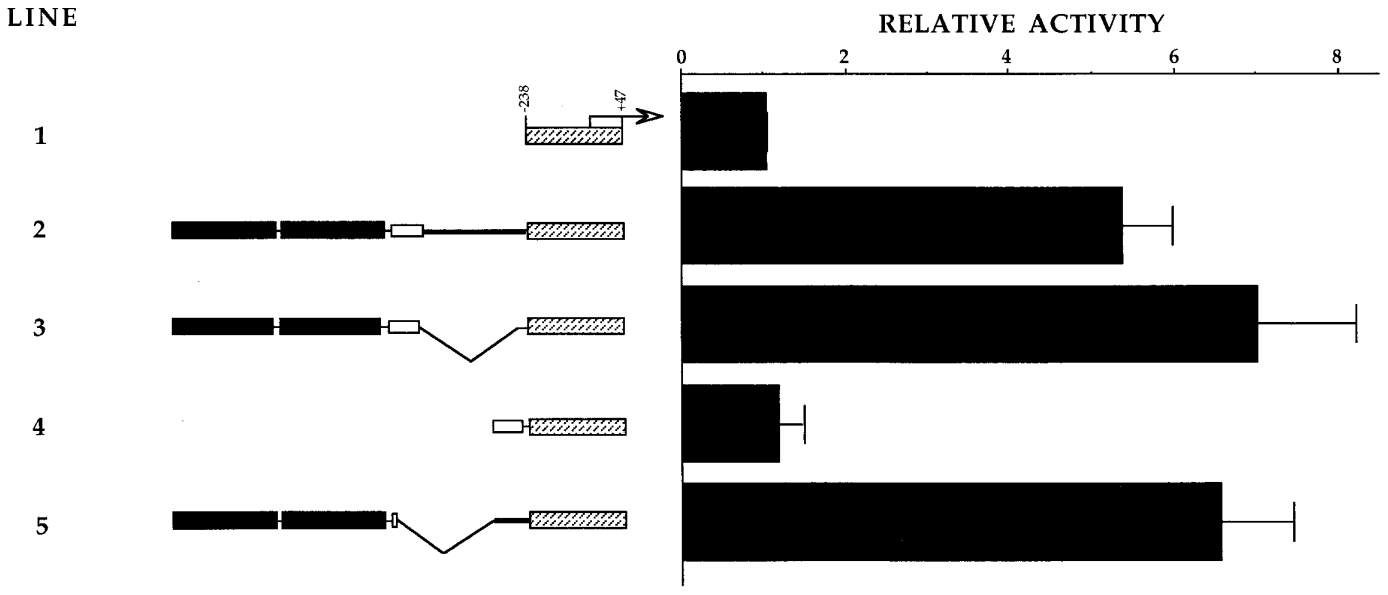

Figure 5. The repeat region is necessary and sufficient for β43′ activity. Comparison of α3 minimal promoter activities when linked in cis with different portions of the β43′ enhancer fragment. RSV-βgal-corrected activities are relative to the activity of the α3(−238/+47)-luc in PC12 cells. Error bars indicate mean ± SD, $n = 4$, except for *line 3*, in which error bars indicate mean ± range for two separate transfections. *Solid tandem rectangles*, 37 bp repeats; *open rectangle*, 14 bp palindrome; *thick line*, remainder of 187 bp *Sac*I/*Spe*I fragment; *cross-hatched rectangle*, α3 minimal promoter.

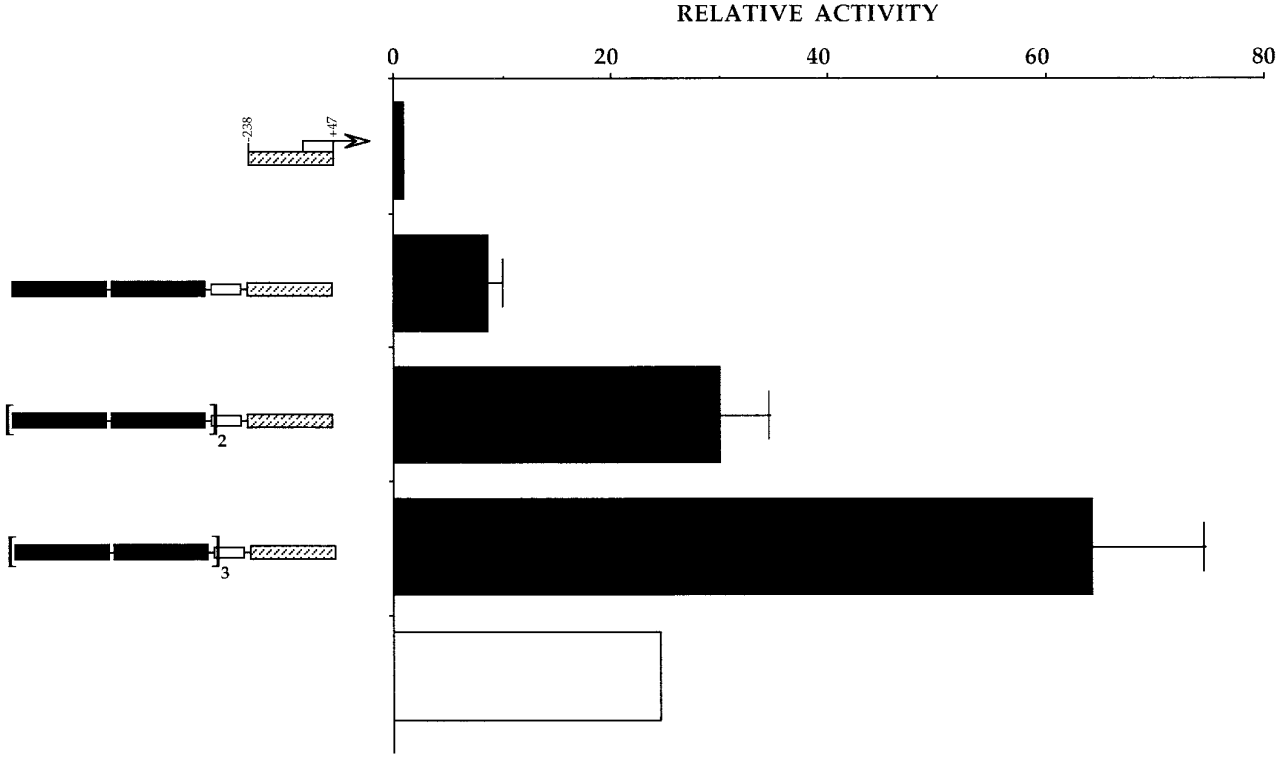

Figure 6. Multimerized repeat region activates synergistically. RSV-βGal-corrected luciferase activities were determined relative to the activity of the α3 minimal promoter construct α3(−238/+47)-luc for reporters bearing either one, two, or three copies of the β43′ repeat region cloned upstream of the α3 minimal promoter. Error bars indicate mean ± SD, $n = 3$. Schematic symbols are as described in previous legends. *Open data bar* represents predicted additive activity of the 3× enhancer reporter.

the repeat region were placed adjacent to one another and in front of the α3 promoter. The activities of these reporters were then compared with α3 minimal promoter activity in PC12 cells. As shown in Figure 6, the presence of additional copies of the repeat region resulted in a greater than additive activation of the α3 promoter, such that when three copies were present, a >60-fold stimulation was seen. These results show that multiple copies of the repeat region act synergistically to create a powerful cis

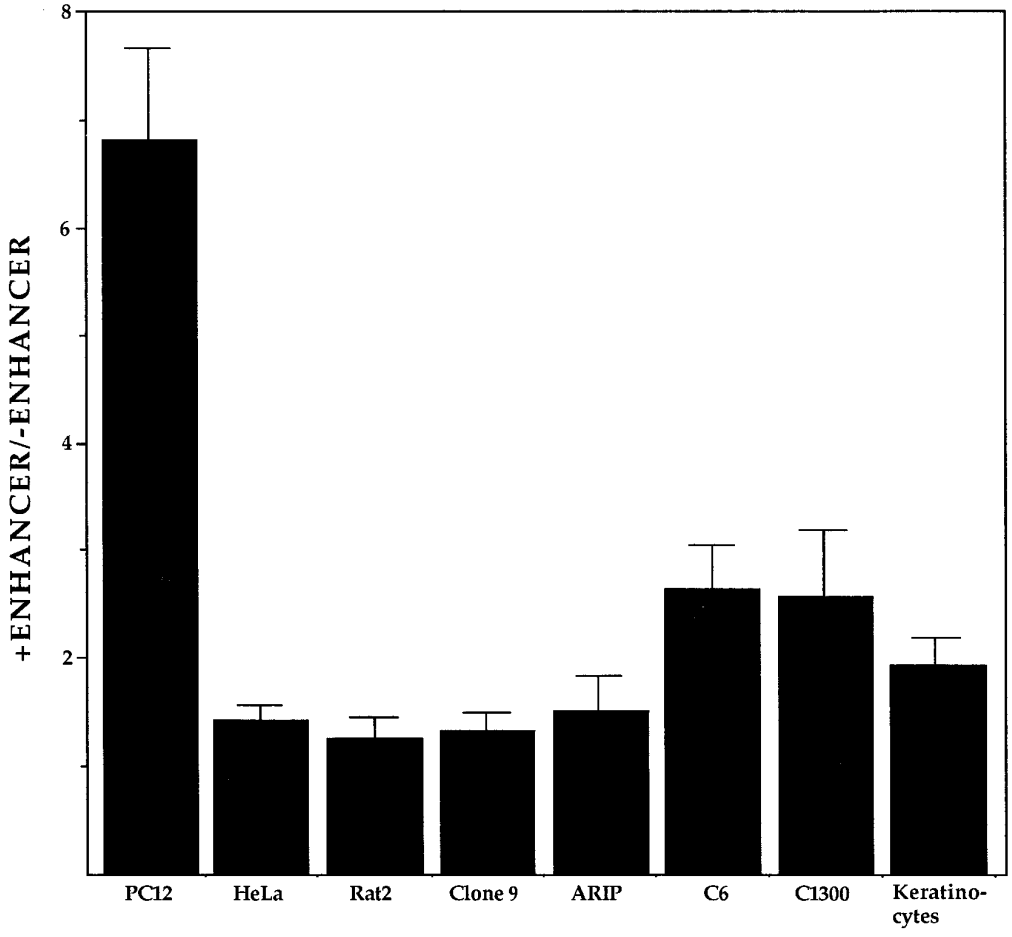

Figure 7. Cell-type-specific activity of β43′. Cell lines and primary keratinocytes were transfected with a luciferase reporter bearing a single copy of the 187 bp β43′ enhancer fragment placed immediately upstream of and in reverse orientation relative to the SV40 promoter. RSV-βgal-corrected data are presented as SV40 promoter activity in the presence of the enhancer divided by basal SV40 promoter activity. Error bars indicate mean ± SD, $n \geq 4$, except for keratinocytes, in which $n = 3$. *PC12*, Rat pheochromocytoma; *HeLa*, human adenocarcinoma; *Rat2*, rat fibroblast; *Clone 9*, rat liver; *ARIP*, rat pancreatic tumor; *C6*, rat CNS glioma; *C1300*, mouse neuroblastoma.

element and thus provide additional confirmation that the repeat region of the 187 bp *Sac*I/*Spe*I fragment is an enhancer.

The β43′ enhancer displays cell-type-specific activity

We next investigated whether the activity of β43′ was cell-type-restricted. For this experiment, the enhancer was tested upstream and in reverse orientation relative to the SV40 promoter in a variety of cell lines from diverse tissue origins. We chose to use the SV40 promoter for these assays because of its robust activity in a variety of cell lines; however, results similar to those presented below were obtained in the context of the α3 promoter (J. McDonough and E. Deneris, unpublished observations). For each cell line assay, positive-control luciferase reporters bearing the SV40 promoter and enhancer, the CMV promoter, or the RSV promoter were transfected in sister cultures to ensure the linearity of luciferase activity. In addition to PC12 cells, two other neural lines, C1300 and C6, were tested. The C6 line is derived from a CNS glioma and C1300 from a mouse neuroblastoma. The four non-neural lines were HeLa cells, which are derived from a human adenocarcinoma, Rat 2 cells, a rat fibroblast line, Clone 9 cells, a rat liver line, and ARIP cells, which are a rat pancreatic tumor line. Northern blot analyses showed that among these lines, only the PC12 line expresses the α3 gene (McDonough and Deneris, unpublished observations). In contrast to the six- to sevenfold activation of the SV40 promoter in PC12 cells, a less than twofold activation was seen in each of the non-neural lines, which shows that the enhancer does indeed exhibit cell-type-restricted activity (Fig. 7). Interestingly, although the enhancer showed some activity in C6 and C1300 cells, it was weak relative to its activity in PC12 cells. Because human keratinocytes have been shown to express the α3 and β4 genes (Grando et al., 1995), we also tested β43′ activity in the primary cultures of these cells. As shown in Figure 7, β43′ activity was virtually inactive in these cells. Together, these results suggest that the activity of β43′ is highly restricted even among different neural cell types, which makes it an attractive candidate for participating in the neuron-restricted expression of one or more of the clustered neuronal nAchR genes.

The β43′ repeat forms a unique complex with PC12 cell nuclear extracts

To begin to characterize the nuclear proteins that bind β43′ and to determine whether PC12 cells express unique β43′ binding factors that correlate to its cell-type-specific activity, EMSAs were performed with a radiolabeled oligonucleotide bearing the first repeat of β43′ and nuclear extracts prepared from cell lines described in Figure 7. With PC12 cell extracts, a complex RBP1 was detected (Fig. 8*A*). RBP1 represents specific binding to

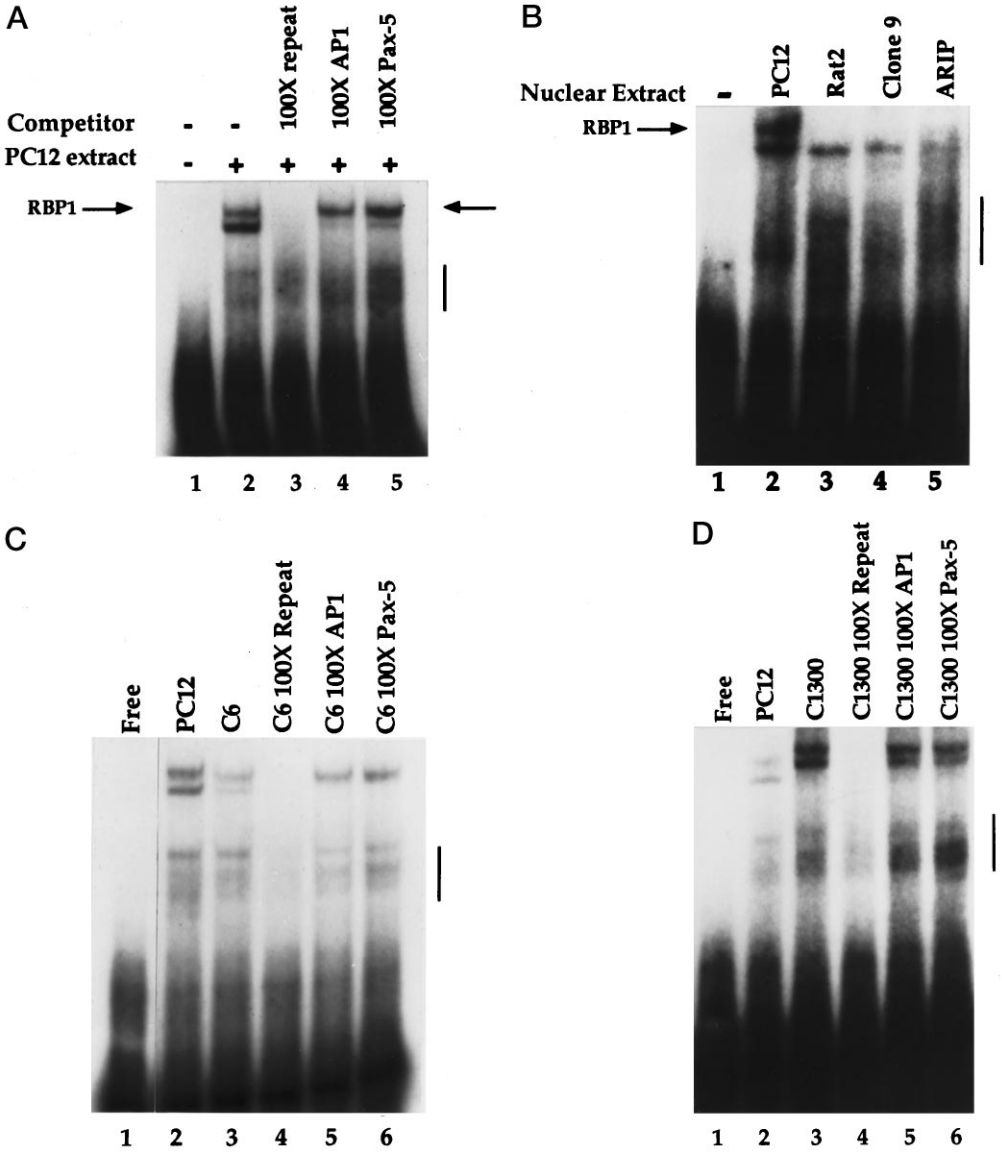

Figure 8. The β43′ repeat forms a unique complex with PC12 extracts. A duplex oligonucleotide probe bearing a single copy of the β43′ first repeat was used in mobility shift assays with nuclear extracts prepared from the indicated cell lines, as described in Methods and Materials. *A*, Analysis of the PC12 cell extract. *Lane 1*, Free probe; *lane 2*, PC12 extract; *lane 3*, PC12 extract incubated with 100-fold molar excess unlabeled first repeat-bearing oligonucleotide; *lane 4*, PC12 extract incubated with 100-fold molar excess AP1-bearing oligonucleotide; *lane 5*, PC12 extract incubated with 100-fold molar excess Pax-5-bearing oligonucleotide. *B*, Analysis of the indicated rat non-neural extracts. *Lane 1*, Free probe; *lane 2*, PC12 extract; *lane 3*, Rat 2 extract; *lane 4*, Clone 9 extract; *lane 5*, ARIP extract. *C*, Analysis of C6 nuclear extract. *Lane 1*, Free probe; *lane 2*, PC12 extract, slowest mobility complex is RBP1; *lane 3*, C6 extract; *lane 4*, C6 extract incubated with 100-fold molar excess unlabeled first repeat-bearing oligonucleotide; *lane 5*, C6 extract incubated with 100-fold molar excess AP1-bearing oligonucleotide; *lane 6*, C6 extract incubated with 100-fold molar excess Pax-5-bearing oligonucleotide. *D*, Analysis of C1300 nuclear extract. *Lane 1*, Free probe; *lane 2*, PC12 extract, slowest mobility complex is RBP1; *lane 3*, C1300 extract; *lane 4*, C1300 extract incubated with 100-fold molar excess unlabeled first repeat-bearing oligonucleotide; *lane 5*, C1300 extract incubated with 100-fold molar excess AP1-bearing oligonucleotide; *lane 6*, C1300 extract incubated with 100-fold molar excess Pax-5-bearing oligonucleotide. *Vertical line* adjacent to each of the autoradiograms marks a set of ubiquitous low-molecular-weight complexes detected with all extracts and, therefore, their distribution does not correlate with the cell-type-specific activity of β43′. *Arrows* point to the position of the PC12 cell-derived RBP1 complex.

probe, because a 100-fold molar excess of unlabeled repeat eliminated formation of this complex, but competition with an equivalent molar excess of oligonucleotides bearing either AP1 binding sites or Pax-5 binding sites failed to compete (Fig. 8*A*). Competition with unlabeled second repeat also inhibited RBP1 formation, which suggests (as expected) that the two repeats are able to bind the same protein or proteins (data not shown). A slightly faster migrating complex just below RBP1 was also detected, but this may represent nonspecific binding to probe, because as shown in Figure 8*A*, 100-fold molar excess of each competitor oligonucleotide eliminated nearly all of this complex. Oligonucleotides bearing octamer sites or AP2 sites also eliminate this complex, which further support the idea that this complex represents nonspecific binding to probe (data not shown). Interestingly, when extracts from the rat non-neural cell lines were tested, complexes co-migrating with RBP1 were not detected (Fig. 8*B*). One complex was detected in each of these extracts, which co-migrated with the PC12 nonspecific complex. These are likely to be non-

specific complexes as well, because similar to the PC12 nonspecific complex, competition with oligonucleotides bearing AP1 and Pax-5 bindings sites eliminated the Rat 2 complex (data not shown) and a similar C6-derived complex (see below).

Analysis of the C6 and C1300 extracts revealed more than one complex, some of which appeared to be similar in size to the PC12 cell-derived RBP1 complex. As shown in Figure 8*C*, extracts from C6 cells formed two complexes. The lower mobility complex represents specific binding to probe, because a 100-fold molar excess of unlabeled repeat eliminated formation of this complex, but competition with an equivalent molar excess of oligonucleotides bearing either AP1 binding sites or Pax-5 binding sites did not compete. However, this complex is not likely to be formed by the same PC12 cell protein forming RBP1, because the mobilities of these complexes are slightly different, suggesting that they represent novel complexes (Fig. 8*C*). The higher mobility complex formed with C6 extracts co-migrated with the nonspecific complex formed with PC12 extracts and, similar to PC12 extracts, this probably represents nonspecific binding to probe, because its formation was eliminated by competition with AP1- and Pax-5-bearing oligonucleotides. Two complexes were detected with C1300 extracts, and both of these represented specific binding, because although a 100-fold molar excess of unlabeled repeat completely eliminated these complexes, an equivalent excess of AP1 and Pax-5 competitors did not inhibit formation of these complexes. However, similar to the protein(s) in C6 cells that binds to the repeat, the repeat-binding proteins in C1300 appear to be different from the PC12 cell protein that forms RBP1. Together these results suggest that among the neural and non-neural cell lines investigated, PC12 cells contain a differentially expressed factor (or factors) that specifically binds the β43′ enhancer repeat, and this binding activity correlates well with the cell-type-specific activity of β43′.

DISCUSSION

Highly restricted expression patterns of clustered neuronal nAchR genes are likely to be established, in part, by cell-type-specific enhancers. Described here is an enhancer, β43′, which is positioned within the 3′-untranslated exon of the neuronal nAchR β4 gene ~2.5 kb upstream of the α3 gene. The cell-type-specific activity of this enhancer suggests that it is important for regulating patterns of one or more clustered neuronal nAchR genes.

Several lines of evidence support the conclusion that β43′ is an enhancer and not a second α3 promoter. First, its activity does not depend on its position or orientation relative to a test promoter. Second, it activates transcription when placed in reverse orientation to the SV40 promoter. Third, no significant activity is seen unless β43′ is linked in *cis* to a promoter. Fourth, multimerization of the element upstream of the α3 minimal promoter results in synergistic activation. Fifth, no PC12 cell-derived α3 exon sequences can be detected in the 3′-untranslated exon of β4 (Yang et al., 1997).

The enhancer was identified within a 187 bp fragment that we subdivided, based on sequence features, into three regions. The first region proximal to the β4 translation stop codon bears two nearly identical 37 bp repeats, which are separated by a 6 bp spacer segment. There are only three nucleotide differences between the two repeats, and these differences are clustered near one another. The second region located 6 bp downstream of the repeats consists of a nearly perfect 14 bp palindrome. The remainder of the 187 bp fragment constitutes the third region, which is 80 bp. We investigated the importance of each of these regions for positive transcriptional activity by testing deletions of the 187 bp fragment and found that the second and third regions were dispensable for full activity. Although the palindrome in the second region did not exhibit activity in our assays, it is still possible that this sequence motif has a function in other cell types. Our results, therefore, demonstrate that the activity of the enhancer in PC12 cells is mediated by sequences in the repeat region. Computer-assisted comparison with transcription factor-motif databases (Computational Biology and Informatics Laboratory, University of Pennsylvania, TESS database) revealed some weak similarities to DNA binding sites described previously. The majority of the repeat sequence, however, appears to be unrelated to consensus sequences previously described. This, together with the relatively long length of the β43′ repeats, suggest that the repeats may constitute a unique cis regulatory interface for multiple interacting DNA binding proteins, some of which are perhaps novel transcription factors.

The expression patterns of the clustered neuronal nAchR subunit genes are complex. In the CNS, members of the cluster are expressed in selected populations of neurons but not in glia. The three genes are co-expressed in peripheral ganglia neurons and, at least in ciliary ganglia neurons, the subunits encoded by these genes are assembled together into a neuronal nAchR subtype (Conroy and Berg, 1995). The expression of these genes in peripheral ganglia is regulated differentially during development and in response to cell–cell interactions, which arise from presynaptic innervation and synaptic connections with target tissues (Boyd et al., 1988; Devay et al., 1994; Mandelzys et al., 1994; Levey et al., 1995). It is not clear, however, whether differential regulation occurs at the level of gene transcription, a post-transcriptional step, or both. Outside of the nervous system, the only tissues reported to express members of the cluster are thymus, which expresses α3 (Mihovilovic and Roses, 1993), muscle, which expresses β4 and α5 (Corriveau et al., 1995), and human keratinocytes, which express α3 and β4 (Grando et al., 1995). This suggests that control of the three clustered genes is achieved, at least in part, through the activity of enhancers with narrow cell-type specificities. The β43′ enhancer is intriguing in this sense, because although it has strong activity in PC12 cells, it is nearly inactive in several non-neural lines and human keratinocytes. Moreover, it is only weakly active in two other neural lines, the mouse C1300 neuroblastoma line and rat C6 CNS glioma line. PC12 cells are the only cell line used here that express the endogenous α3 gene. Thus, the activity of β43′ is remarkably restricted and in a manner that correlates with major aspects of nAchR expression patterns. The narrow cell specificity of β43′ is correlated with the formation of a protein–DNA complex, RBP1, in PC12 cell nuclear extracts that was not detected in other cell line extracts. Our findings suggest, therefore, that the preferential activity of β43′ in PC12 cells results from interactions with at least one cell-type-specific regulatory protein.

As described above, the expression patterns of clustered neuronal nAchR genes overlap with one another, which is consistent with the heteromeric composition of nAchR subtypes (Duvoisin et al., 1989; Wada et al., 1989; Boulter et al., 1990; Dineley-Miller and Patrick, 1992; Corriveau and Berg, 1993; Mandelzys et al., 1994). One way in which co-expression among these genes might be controlled is through shared cis regulatory elements positioned to influence more than one member of the cluster. For example, regulatory elements located between members of the Hox complexes control Hox genes on either side of the elements (Gérard et al., 1996; van der Hoeven et al., 1996). The β43′ enhancer is,

perhaps, a shared cell-type-specific cis element, because it is located between the β4 and α3 coding regions, which may allow it to influence both the β4 and α3 promoters. It also has the ability to act equally well on both the β4 and α3 promoters irrespective of orientation and is preferentially active in a neural cell type that expresses both genes. The expression patterns of the clustered genes, however, are not identical, which suggests that gene-specific cis elements may play a role in establishing individual patterns of transcription. Although our transient assays indicate that there is no fundamental discrimination between β4 and α3 promoters by β43′, it is possible that additional mechanisms involving chromatin insulator sequences may isolate the endogenous β4 gene from the influence of the enhancer (Cai and Levine, 1995). Thus, an important future goal is to use transgenic methods to determine which of the three clustered genes is influenced by β43′ *in vivo* and whether β43′ is a shared cell-type-specific cis element.

Control of nAchR gene transcription in muscle is achieved, at least in part, through the interaction of myogenic factors with E boxes located upstream of nAchR subunit genes (Piette et al., 1990; Gilmour et al., 1991; Simon and Burden, 1993). In contrast to nAchR genes expressed in muscle, very limited information is available regarding the cis-acting elements that regulate neuronal nAchR gene transcription. Daubas et al. (1993) showed that an α2 transgene construct bearing the entire avian α2 gene coding region, as well as 7 kb upstream and 3 kb downstream, was expressed in a neuron-specific manner in selected CNS nuclei. In a separate study, these investigators identified a silencer near the transcription start site region of the α2 gene, which was active in both neural and non-neural cells (Bessis et al., 1993). At least part of the silencing activity was found to reside in six Oct-like repeats distributed over a 160 bp region both upstream and downstream of the most 5′ start site. Near the start site region of the α3 gene is a G-rich motif, which mediates Sp1 transactivation and binds Sp1-immunoreactive material in PC12 cells extracts. Mutation of this site nearly abolishes α3 promoter activity (Yang et al., 1995). A similar element is present near the β4 promoter start site and is thought to bind novel factors enriched in brain (Hu et al., 1995). It is not clear, however, what role, if any, these cis elements play in controlling restricted patterns of neuronal nAchR gene transcription.

Expression of the avian nAchR β3 gene is limited to inner nuclear and ganglion cells of the retina and sensory ganglia. Interestingly, Hernandez et al. showed, using transient transfection assays in freshly dissociated cultures of central neurons, that a 143 bp β3 promoter fragment was active in retinal neurons isolated at an early developmental stage but not at other stages or in non-neural cells (Hernandez et al., 1995). Thus, cis elements directing highly restricted expression of a reporter gene to the appropriate β3-positive cell types are present within the β3 promoter region. The results presented here suggest that the α3 gene may be regulated differently from β3 in that control of what is apparently a housekeeping-type promoter near the α3 coding region is regulated by distant cell-type-specific enhancers. This difference in regulation may arise, because although α3 expression is restricted, it is not as restricted as β3 and, therefore, more complex and diverse regulatory regions may be required to control α3 in a wider range of cell types. We propose that β43′ is one of the cis-acting regulatory interfaces required to restrict expression of one or more of the neuronal nAchR cluster genes to neurons. Its presence within another gene may be a selective pressure for maintaining the tight linkage of the β4 and α3 genes.

REFERENCES

Bessis A, Savatier N, Devillers-Thiery A, Bejanin S, Changeux JP (1993) Negative regulatory elements upstream of a novel exon of the neuronal nicotinic acetylcholine receptor alpha 2 subunit gene. Nucleic Acids Res 21:2185–2192.

Boulter J, O'Shea-Greenfield A, Duvoisin RM, Connolly G, Wada E, Jensen A, Gardner PD, Ballivet M, Deneris ES, McKinnon D, Heinemann S, Patrick J (1990) α3, α5 and β4: three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a cluster. J Biol Chem 265:4472–4482.

Boyd RT (1994) Sequencing and promoter analysis of the genomic region between the rat neuronal nicotinic acetylcholine receptor β4 and α3 genes. J Neurobiol 25:960–973.

Boyd RT (1996) Transcriptional regulation and cell specificity determinants of the rat nicotinic acetylcholine receptor α3 gene. Neurosci Lett 208:73–76.

Boyd RT, Jacob MH, Couturier S, Ballivet M, Berg DK (1988) Expression and regulation of neuronal acetylcholine receptor mRNA in chick ciliary ganglia. Neuron 1:495–502.

Cai H, Levine M (1995) Modulation of enhancer-promoter interactions in the *Drosophila* embryo. Nature 376:533–536.

Carroll SL, Schweitzer JB, Holtzman DM, Miller ML, Sclar GM, Milbrandt J (1995) Elements in the 5′ flanking sequences of the mouse low-affinity NGF receptor gene direct appropriate CNS, but not PNS, expression in transgenic mice. J Neurosci 15:3342–3356.

Chong JA, Tapia-Ramirez J, Kim S, Toledo-Aral JJ, Zheng Y, Boutros MC, Altshuller YM, Frohman MA, Kraner SD, Mandel G (1995) REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell 80:949–957.

Conroy WG, Berg DK (1995) Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit combinations. J Biol Chem 270:4424–4431.

Corriveau RA, Berg DK (1993) Coexpression of multiple acetylcholine receptor genes in neurons: quantification of transcripts during development. J Neurosci 13:2662–2671.

Corriveau RA, Romano SJ, Conroy WG, Oliva L, Berg DK (1995) Expression of the neuronal acetylcholine receptor genes in vertebrate skeletal muscle during development. J Neurosci 15:1372–1383.

Couturier S, Erkman L, Valera S, Rungger D, Bertrand S, Boulter J, Ballivet M, Bertrand D (1990) α5, α3, and non-α3: three clustered avian genes encoding neuronal nicotinic acetylcholinereceptor-related subunits. J Biol Chem 265:17560–17567.

Daubas P, Salmon AM, Zoli M, Geoffroy B, Devillers-Thiery A, Bessis A, Medevielle F, Changeux JP (1993) Chicken neuronal acetylcholine receptor alpha 2- subunit gene exhibits neuron-specific expression in the brain and spinal cord of transgenic mice. Proc Natl Acad Sci USA 90:2237–2241.

Devay P, Qu X, Role L (1994) Regulation of nAchR subunit gene expression relative to the development of pre- and postsynaptic projections of embryonic chick sympathetic neurons. Dev Biol 162:56–70.

Dineley-Miller K, Patrick J (1992) Gene transcripts for the nicotinic acetylcholine receptor subunit, beta4, are distributed in multiple areas of the rat central nervous system. Mol Brain Res 16:339–344.

Duvoisin RM, Heinemann S (1993) Transcription control elements of the rat neuronal nicotinic acetylcholine receptor subunit α3. Braz J Med Biol Res 26:137–150.

Duvoisin RM, Deneris ES, Patrick J, Heinemann S (1989) The functional diversity of the neuronal nicotinic acetylcholine receptors is increased by a novel subunit: β4. Neuron 3:487–496.

Fyodorov D, Deneris E (1996) The POU domain of SCIP/Tst-1/Oct-6 is sufficient for activation of an acetylcholine receptor promoter. Mol Cell Biol 16:5004–5014.

Gérard M, Chen J-Y, Gronemeyer H, Chambon P, Duboule D, Zakany J (1996) *In vivo* targeted mutagenesis of a regulatory element required for positioning the *Hoxd-11* and *Hoxd-10* expression boundaries. Genes Dev 10:2326–2334.

Gilmour BP, Fanger GR, Newton C, Evans SM, Gardner PD (1991) Multiple binding sites for myogenic factors are required for expression of the acetylcholine receptor γ-subunit. J Biol Chem 266:19871–19874.

Grando SA, Horton RM, Pereira EF, Diethelm-Okita BM, George PM, Albuquerque EX, Conti-Fine BM (1995) A nicotinic acetylcholine receptor regulating cell adhesion and motility is expressed in human keratinocytes. J Invest Dermatol 105:774–781.

He X, Rosenfeld MG (1991) Mechanisms of complex transcriptional regulation: implications for brain development. Neuron 7:183–196.

Hernandez MC, Erkman L, Matter-Sadzinski L, Roztocil T, Ballivet M, Matter JM (1995) Characterization of the nicotinic acetylcholine receptor beta 3 gene. Its regulation within the avian nervous system is effected by a promoter 143 base pairs in length. J Biol Chem 270:3224–3233.

Hu M, Theobald Whiting N, Gardner PD (1994) Nerve growth factor increases the transcriptional activity of the rat neuronal nicotinic acetylcholine receptor β4 subunit promoter in transfected PC12 cells. J Neurochem 62:392–395.

Hu M, Bigger CB, Gardner PD (1995) A novel regulatory element of a nicotinic acetylcholine receptor gene interacts with a DNA binding activity enriched in rat brain. J Biol Chem 270:4497–4502.

Kraner SD, Chong JA, Tsay H-J, Mandel G (1992) Silencing the type II sodium channel gene: a model for neural-specific gene regulation. Neuron 9:37–44.

Levey MS, Brumwell CL, Dryer SE, Jacob MH (1995) Innervation and target tissue interactions differentially regulate acetylcholine receptor subunit mRNA levels in developing neurons *in situ*. Neuron 14:153–162.

Mandel G, McKinnon D (1993) Molecular basis of neural-specific gene expression. Annu Rev Neurosci 16:323–345.

Mandelzys A, Pié B, Deneris ES, Cooper E (1994) The developmental increase in ACh current densities on rat sympathetic neurons correlates with changes in nicotinic ACh receptor α-subunit gene expression and occurs independently of innervation. J Neurosci 14:2357–2364.

Mihovilovic M, Roses AD (1993) Expression of alpha-3, alpha-5, and beta-4 neuronal acetylcholine receptor subunit transcripts in normal and myasthenia gravis thymus. Identification of thymocytes expressing the alpha- 3 transcripts. J Immunol 151:6517–6524.

Min N, Joh TH, Kim KS, Peng C, Son JH (1994) 5′ upstream DNA sequence of the rat tyrosine hydroxylase gene directs high-level and tissue-specific expression to catecholamine neurons in the central nervous system of transgenic mice. Mol Brain Res 27:281–289.

Mori N, Schoenherr C, Vandenbergh DJ, Anderson DJ (1992) A common silencer element in the SCG10 and type II Na^+ channel gene binds a factor present in nonneuronal cells but not in neuronal cells. Neuron 9:45–54.

Piette J, Bessereau JL, Huchet M, Changeux JP (1990) Two adjacent MyoD1-binding sites regulate expression of the acetylcholine receptor α-subunit gene. Nature 345:353–355.

Raimondi E, Rubboli F, Moralli D, Chini B, Fornasari D, Tarroni P, De-Carli L, Clementi F (1992) Chromosomal localization and physical linkage of the genes encoding human alpha3, alpha5, and beta4 neuronal nicotinic receptor subunits. Genomics 12:849–850.

Sauer F, Hansen SK, Tjian R (1995) DNA templates and activator-coactivator requirements for transcriptional synergism by *Drosophila bicoid*. Science 270:1825–1828.

Schoenherr CJ, Anderson DJ (1995) The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. Science 267:1360–1363.

Schreiber E, Matthias P, Muller MM, Schaffer W (1989) Rapid detection of octamer binding proteins with "mini-extracts," prepared from a small number of cells. Nucleic Acids Res 17:6419.

Simon AM, Burden SJ (1993) An E-box mediates activation and repression of the AChR δ subunit gene during myogenesis. Mol Cell Biol 13:5133–5140.

Tuggle CK, Zakany J, Cianetti L, Peschle C, Nguyen-Hu MC (1990) Region-specific enhancers near two mammalian homeo box genes define adjacent rostrocaudal domains in the central nervous system. Genes Dev 4:180–189.

Vandaele S, Nordquist DY, Feddersen RM, Tretjakoff I, Peterson AC, Orr HT (1991) Purkinje cell protein-2 regulatory regions and transgene expression in cerebellar compartments. Genes Dev 5:1136–1148.

van der Hoeven F, Zakany J, Duboule D (1996) Gene transpositions in the HoxD complex reveal a hierarchy of regulatory controls. Cell 85:1025–1035.

Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW (1989) Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. J Comp Neurol 284:314–335.

Wada E, McKinnon D, Heinemann S, Patrick J, Swanson LW (1990) The distribution of RNA encoded by a new member of the neuronal nicotinic acetylcholine receptor gene family (α5) in the rat central nervous system. Brain Res 526:45–53.

Welter JF, Gali H, Crish JF, Eckert RL (1996) Regulation of human involucrin promoter activity by POU domain proteins. J Biol Chem 271:14727–14733.

Whiting J, Marshall H, Cook M, Krumlauf R, Rigby PWJ, Stott D, Allemann RK (1991) Multiple spatially specific enhancers are required to reconstruct the pattern of Hox-2.6 gene expression. Genes Dev 5:2048–2059.

Whyte DB, Lawson MA, Belsham DD, Eraly SA, Bond CT, Adelman JP, Mellon PL (1995) A neuron-specific enhancer targets expression of the gonadotropin-releasing hormone gene to hypothalamic neurosecretory neurons. Mol Endocrinol 9:467–477.

Yang X, McDonough J, Fyodorov D, Morris M, Wang F, Deneris ES (1994) Characterization of an acetylcholine receptor alpha 3 gene promoter and its activation by the POU domain factor SCIP/Tst-1. J. Biol Chem 269:10252–10264.

Yang X, Fyodorov D, Deneris ES (1995) Transcriptional analysis of acetylcholine receptor alpha 3 gene promoter motifs that bind Sp1 and AP2. J Biol Chem 270:8514–8520.

Yang X, Yang F, Fyodorov D, Wang F, McDonough J, Herrup K, Deneris E (1997) Elements between the protein coding regions of the adjacent β4 and α3 acetylcholine receptor genes direct neuron-specific expression in the CNS. J Neurobiol, in press.

Yoon SO, Chikaraishi DM (1992) Tissue-specific transcription of the rat tyrosine hydroxylase gene requires synergy between an AP-1 motif and an overlapping E box-containing dyad. Neuron 9:55–67.

Zimmerman L, Lendahl U, Cunningham M, McKay R, Parr B, Gavin B, Mann J, Vassileva G, McMahon A (1994) Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron 12:11–24.