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PROMOTER ELEMENTS CONFERRING NEURON-SPECIFIC EXPRESSION OF THE β2-SUBUNIT OF THE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR STUDIED *IN VITRO* AND IN TRANSGENIC MICE

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Abstract—Several genes encoding subunits of the neuronal nicotinic acetylcholine receptors have been cloned and regulatory elements involved in the transcription of the α2 and α7-subunit genes have been described. Yet, the detailed mechanisms governing the neuron-specific transcription and the spatio-temporal expression pattern of these genes remain largely uninvestigated. The β2-subunit is the most widely expressed neuronal nicotinic receptor subunit in the nervous system. We have studied the structural and regulatory properties of the 5′ sequence of this gene. A fragment of 1163 bp of upstream sequence is sufficient to drive the cell-specific transcription of a reporter gene in both transient transfection assays and in transgenic mice. Deletion analysis and site-directed mutagenesis of this promoter reveal two negative elements and one positive element. The positively-acting sequence includes one functional E-box. One of the repressor elements is located in the transcribed region and is the NRSE/RE1 sequence already described in promoters of neuronal genes.

In this paper, we describe the neuron-specific promoter of the gene encoding the neuronal nicotinic acetylcholine receptor β2-subunit.

Key words: transcription factors, silencer, E-box.

In situ hybridization[27,57,62] and immunohistochemistry[10,27,54] demonstrate that all subunits of the neuronal nicotinic receptors (nAchRs) cloned to date display a strict neuron-specific distribution. Yet, the transcription of some subunits appears restricted to a small set of neurons. For example, the α2-subunit transcripts are only detected in the spiriformis lateralis nucleus in the chick diencephalon[12,43] or the interpeduncularis nucleus in the rat.[58] Also the β3, β4 and α3-subunit transcripts are only detected in a small set of structures in vertebrate brain.[8,14,16,20,62] On the other hand, the distribution of the α4, α5, α7, and β2-subunit gene transcripts is much wider.[8,53,59,62] For example, the β2-subunit transcripts are found in the majority of neurons in the CNS, and all the peripheral neurons that express the nAchR.[22,27,50] As a consequence, a wide diversity of nAchR species occurs with defined patterns of expression involving diverse categories of neurons. For example, the neurons from medial habenula and those from the interpeduncularis nucleus are interconnected but express distinct sets of nAchR subunits (see Ref. 50 for review) with different physiological and pharmacological profiles.[44] It is therefore worthwhile to investigate the genetic mechanisms involved in the acquisition of these specific patterns of expression.

Only limited information is available, to date, about the genetic mechanisms which account for regulation of nAchR gene transcription in neurons. The promoter of the chick α7-subunit gene was analysed *in vitro*, but the DNA elements responsible for transcriptional regulation were not characterized.[38] In another study, the promoter of the α2-subunit gene was partially characterized and a silencer described and sequenced.[7]

The β2-subunit is expressed in the majority of the neurons in the brain.[27,54] Moreover, the timing of appearance of the β2-transcripts closely parallels that of neuronal differentiation.[62] We thus decided to study the genetic mechanisms which regulate its transcription.

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Abbreviations: β-gal, β-galactosidase; DMEM, Dulbecco's modified Eagle's medium; E, embryonic day; EDTA, ethylene diamine tetra acetate; FCS, fetal calf serum; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; nAchR, nicotinic acetylcholine receptor; NRSE, neural restrictive silencer element; P, postnatal day; PCR, polymerase chain reaction; PNS, peripheral nervous system; RACE, rapid amplification of cDNA ends; RE1, restrictive element; SLIC, single-strand ligation of cDNA.

EXPERIMENTAL PROCEDURES

Screening of the genomic library

The PCX49 plasmid[15] containing the entire rat cDNA (kindly provided by Drs J. Boulter and S. Heinemann, The Salk Institute, San Diego, Ca) was cut with EcoRI, the ≈2.2 kb fragment was isolated and used as a probe to screen an EMBL3 bacteriophage library of mouse DBA2 genomic DNA. One unique clone was obtained spanning ≈15 kb of DNA upstream and ≈5 kb downstream from the first exon. Figure 1 shows the nucleotide sequence of 1.2 kb upstream from the initiator ATG.

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number: X82655.

Mapping of the transcription initiation site

For the mRNA mapping, we used different batches of total RNA extracted from DBA2 embryos at stage embroynic day (E)13 or E15. The RNA samples were first digested with DNase I to avoid DNA contamination.

RNase protection. An XbaI/PstI fragment containing part of intron 1 was inserted into Bluescript SK (Stratagene). The plasmid was then linearized by BglII, and an RNA probe was synthesized using the T7 promoter. The protection experiments were then performed as described in Ref. 3.

RACE–PCR.[24] The mRNA was hybridized 5 min at 80°C with 10 pmol of primer. The synthesis of the cDNA was performed using 400 U MMLV (Gibco) for 45 min at 37°C in the buffer recommended by the supplier. After a phenol/chloroform extraction, the cDNA was ethanol precipitated. The terminal transferase reaction was performed in 0.2 M potassium cacodylate; 25 mM Tris–HCl pH 6.6; 25 mg/ml BSA; 1.5 mM $CoCl_2$; 50 nM dATP and 50 u terminal transferase (Boehringer) for 30 min at 37°C. After phenol/chloroform extraction and ethanol precipitation, one-tenth of the terminal transferase reaction was amplified using Promega's Taq DNA polymerase (30 cycles, 1 min at: 94°C; 55°C; 72°C). The amplified fragment was then loaded on an agarose gel. The gel was blotted and hybridized to oligonucleotide p2. We used pEx2 as a primer for cDNA synthesis, and p0/BEpT for polymerase chain reaction (PCR) to map mRNA from brain. OLUCI3 (synthesis of cDNA) and OLUCI2/BEpT (PCR) were used to map mRNA from transfected cells.

SLiL. The cDNA was first synthesized from 5 μg total RNA using pEx3 (6 pmol) as a primer in 50 mM Tris–HCl pH 8.3; 8 mM KCl; 1.6 mM $MgCl_2$; 5 mM spermidine; 0.5 mM dNTP; 1 u/μl RNasin; 0.1 mg/ml BSA; 70 mM β-mercaptoethanol; 80 u AMV (Promega) at 42°C for 45 min. The RNA was subsequently degraded in NaOH. The first strand of the cDNA was then ligated with the oligonucleotide A5′. The resulting single stranded cDNA was then submitted to two rounds of PCR amplification with oligonucleotides A5′-1/p0 and A5′-2/p1 (35 cycles: 94°C 1 min; 60°C 30 s; 72°C 45 s).

The sequence of the oligonucleotides were the following:

A5′: 5′-CTGCATCTATCTAATGCTCCTCTCGCTACC TGCTCACTCTGCGTGACATC
A5′-1: 5′-GATGTCACGCAGAGTGAGCAGGTAG
A5′-2: 5′-AGAGTGAGCAGGTAGCGAGAGGAG
p0: 5′-CCAAAGCTGAACAGCAGCGCCATAG
p1: 5′-AGCAGCGCCATAGAGTTGGAGCACC
p2: 5′-AGGCGGCTGCGCGGCTTCAGCACCACGGAC
pEx2: 5′-GCCGCTCCTCTGTGTCAGTACCCAAAAC CC
pEx3: 5′-ACATTGGTGGTCATGATCTG
BEpT: 5′-GCGGGATCCGAATTC $(T)_{21}$ A/C/G
OLUCI3: 5′-CGAAGTATTCCGCGTACGTGATG
OLUCI2: 5′-ACCAGGGCGTATCTCTTCATAGC

Plasmids

KS-Luci. The HindIII/KpnI restriction fragment of the pSVOAL plasmid[13] was subcloned in the corresponding site of Bluescript KS. The most 5′ EcoRI/BsmI (45 bp) fragment of the Luciferase gene was then deleted according to Ref. 13 and replaced by a *Sal*I site. The 342 bp PvuII/HindIII restriction fragment of SV40 containing the polyadenylation sites was subsequently subcloned into the EagI sites using adaptors.

*EE*1.2-*Luci.* The 1.2 kbp EcoRI/Eco47II fragment of the λβ2 phage was inserted in the EagI/SalI sites of KS-Luci using an adaptor. The 5′ end deletions of the promoter were obtained using Bal3.1 exonuclease.

The mutations were introduced using the Sculptor kit (Amersham). In the neural restrictive silencer element/restrictive element (NRSE/RE1) sequence, the mutated sequence was: +24 ACCACTTACA instead of ACCACGGACA, as this mutation was shown to reduce the activity of the NRSE element.[41] In the E-box sequence, the mutated sequence was: −120 TCCTCAGG instead of TCCACTTG. Figure 6 shows that a nuclear protein is able to bind to the wild-type sequence, but not any more to the mutated sequence.

Cell lines and transfection

Neuroblastomas N1E115, SK-N-Be, HeLa and 3T6 fibroblasts, 293 Human kidney cells and SVLT striatal cells[21] were grown in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum (FCS) supplemented with 1% glutamine and 1% streptomycin. PC12 cells were grown in DMEM + 10% horse serum + 5% FCS supplemented with 1% glutamine and 1% streptomycin.

Cells were plated at 10^5 to 4×10^5 cells/60 mm^2 plates. The next day cells were transfected in 750 μl of DMEM + 2% Penicillin/Streptomycin for five to 12 h with 1 μg DNA mixed with 2.5 μl of Transfectam (IBF/Sepracor) in 150 mM NaCl. The Luciferase activity was measured 48 h later. DNA was prepared using Qiagen or Wizard prep (Promega) kits. When plasmid activities were compared, all plasmids were prepared the same day. At least two different DNA preparations were tested for each plasmid. All transfections were done in duplicate and repeated at least three times.

Transgenic mice

The Luciferase gene from EE1.2-Luci was excised and replaced by the nlsLacZ gene.[30] The β2-promoter/nlsLacZ fragment was electroeluted from a TAE agarose gel then further purified by ethanol precipitation, and finally resuspended in Tris–HCl 10 mM pH 7.5; EDTA 0.1 mM. The DNA solution (3 ng/ml) was injected into fertilized oocytes of C57BL6xSJL hybrids. Staining of tissues was performed as previously described.[40]

In situ *hybridization*

In situ hybridization was performed as described in Ref. 61. Temperature of hybridization was 37°C. The oligonucleotide used was:

5′-TGACAAAGCAGGTACATGGGTCAGCCGCAGG ACCTTCACGGAAGA

Gel shift assay

Oligonucleotides were labelled either with γ[^{32}P]ATP and T4 polynucleotide kinase, or with α[^{32}P]CTP and Klenow enzyme. Nuclear extracts were prepared from ≈10^7 cells as previously described.[7] For binding, 1 nmol of labelled oligonucleotide was mixed with 0.5 μg of protein extract in 10 mM HEPES, pH 8; 10% glycerol; 0.1 mM EDTA; 0.1 M NaCl; 2 mM diothiothreiotol; 0.1 mg/ml bovine serum albumin; 4 mM $MgCl_2$; 4 mM spermidine; 1 mM phenyl methyl sulphonyl fluoride; 1 μg polydIdC in 20 μl. The reaction was

incubated for 10 min on ice. The DNA-protein complexes were then analysed on a 7% polyacrylamide.

The oligonucleotides used in this experiment were double-stranded with the following sequences (the underlined nucleotides are changed between the mutated and the wild-type oligonucleotides, bold nucleotides highlight the putative binding sites):

E–D: 5′-TCCTCCCCTAGTAGTTCCATTGTGTTCCCTAG

Mut–E: 5′-CCTCCCCTAGTAGTTCCTCAGGTGTTCCCTAGA

S–E: 5′-CTAGCTCC**GGGGCGG**AGACTCCTCCCCTAGTAGTTCC**ACTTG**TGTTCCCTAG

RESULTS

Characterization of the 5′ flanking sequences of the gene encoding the β2-subunit

A λ phage containing the gene encoding the β2-subunit was cloned and a region surrounding the initiator ATG was sequenced (Fig. 1). The transcription initiation site was first mapped by RNase protection (Fig. 2A). This method allowed us to detect at least three initiation sites. However, minor additional start sites might not have been detected in these experiments. The size of the main protected band was estimated at about 150 nucleotides. To confirm and locate the initiation sites more precisely, we performed both rapid amplification of cDNA ends (RACE)-PCR[24] and single strand ligation of cDNA (SLIC)[18] which consist in the amplification of the primer extension product (Fig. 2B). Both techniques allowed us to subclone and sequence the same fragments corresponding to the four initiation sites described in Fig. 1. It is probable that the -13 start site is very rare and was not detected by RNase mapping.

Analysis of the sequence of the flanking region (Fig. 1) revealed several consensus DNA binding elements: an Sp1 site (-146), a cAMP responsive element binding[26,51] site (-287), a nuclear receptor response element[46] (-344 to -356), a GATA-3 site[32] (-1073), a weakly degenerate Octamer motif (-522). Moreover, an E-box (-118) contained in a dyad symmetrical element could be recognized. The proximal region (-245 to $+82$) also has an unusually high GC content (67%) and a high number of dinucleotide CpG that may have some regulatory significance.[2] Finally, a 20 bp sequence identical to the NRSE[41] or RE1[34] sequence was found in the 3′ end of the 1.2 kbp fragment ($+18$ to $+38$).

A 1.2 kbp fragment of flanking sequence of the β2-subunit gene promotes a neuron-specific expression in vitro

A construct was generated containing the 1163 bp EcoRI/Eco47III fragment (from -1125 to $+38$) of the β2-subunit 5′ flanking region fused to the Luciferase gene[13] (plasmid EE1.2-Luci). The polyadenylation sites of SV40 were inserted upstream from the β2-subunit sequences to avoid readthrough. The transcriptional activity of the plasmid EE1.2-Luci was then tested by transient transfection into pheochromocytoma (PC12) cells, neuroblastomas cell lines NIE 115 and SK-N-Be, SVLT a striatial cell line,[21] NIH3T6 or HeLa fibroblasts and human kidney cell line 293.

Using reverse transcription (RT)-PCR, we verified that the neuroblastomas and the PC12 cells normally express the β2 subunit mRNA but not the striatal SVLT cell lines or the 3T6 fibroblasts (data not shown). Table 1 shows that in PC12 cells and neuroblastomas, the 1.2 kbp fragment is 20- to 180-fold more active in mediating transcription of the reporter gene than in the other cell lines. In fibroblasts, 293 cells and SVLT cells, the transcriptional activity of the 1.2 kbp fragment is not significantly higher than that of the promoterless vector (Table 1). Therefore, the β2-subunit promoter is not active in these cell lines. These *in vitro* transfection experiments demonstrate that the 1163 bp fragment mimics the expression pattern of the endogenous β2-subunit gene, and thus contains a cell-specific promoter.

The 1163 bp promoter in transgenic mice

To test *in vivo* the 1163 bp promoter, the EcoRI/Eco47III fragment was linked upstream from the nls-β-galactosidase reporter gene.[30] The polyadenylation signals from SV40 were ligated downstream of the coding sequences. The resulting 4.7 kb fragment was subsequently microinjected into the male pronuclei of fertilized eggs from F1 hybrid mice (C57B16 × SJL). DNA extracted from the tails of the offspring was analysed for the presence of the β-galactosidase gene by PCR. Three independent founders were obtained and analysed for expression.

Two lines (13 and 26) had expression in neurons and the third line did not express at all. This shows that the 1163 pb promoter contains sufficient regulatory elements to drive neuron-specific expression *in vivo*. In the peripheral nervous system (PNS), both lines expressed in the same structure. In contrast, in CNS the labelling pattern of line 26 is a subset of that of line 13; we will only describe line 13 in detail. As expected, most peripheral β2-expressing ganglia expressed β-galactosidase (β-gal), whereas in the CNS only a subset of β2-positive regions expressed the β-gal. For instance, Fig. 3C shows that the vast majority of the neurons of the lumbosacral spinal cord express the β2-subunit transcripts, whereas only a subset of neurons in the ventral and dorsal horns display β-gal activity.

The expression of the transgene could be detected in the peripheral ganglia in E10.5–E11 embryos. The labelling was examined in E13 total embryos (Fig. 3A) and in brains at later ages (E17, postnatal day (P)0 and adulthood). At E13, labelling was prominent in PNS: strong labelling was observed in the dorsal root ganglia (DR, Figs 3 and 5C, D), some ganglia associated with the cranial nerves (the trigeminal, see Fig. 4A), geniculate, glossopharyngeal and vagal ganglia), the ganglia of the sympathetic chain (Fig. 4C, D), the ganglionic cells of the retina

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-1125  GGAATTCCTG AAAACACTCA AGTTTAAGTA AAAGGTAGGT AGGGGCACTG GGGTGATAAA
       EcoRI                                                      GATA-3

-1065  AGAGCTGGAG GGAACTACAT GTTTAAAAGA CCGAGGGCTA GGAGGGGTTA AATAGTCAAG
                                           1006E|→
-1005  GATCTTAAAG ACGTCGTCAA TAGCTAGAAT GTGGAGCTGA GACAGGCATT GACGAGATGA

-945   AGTCCGAAGC CTTTTGTCTG CTAAGTCTGC TTCAGACAGA AATCTTTTTG GTTGAAAGTG

-885   ACCACTGATC CACTAAGAAA AAAAAAGAGG TCCTTTTTGG GCTCAGTAGC TAAAACGGCA
        |→ 862E
-825   GGGCTTTCAA GATCAAACAT GTCATTGAGT TTTGACACCT CTCTCATCTT TGCTCTCTTT

-765   GTGTTAGCTT CATTCTTTCT GTGAAATGGT CCCCTGATCT CCCCAGAACA CAGCGTGGAA

-705   GGAACCATTG ATATTGGTTG CTTATGCAGA TCTCAGAACT TTCAAGGCCA CCTTCTTTTC

-645   AGGAGGTCTA GACCTATCTA GCTTAGATTC CCCAGGAGAA TGGCAAGATC TTGGCCTTGT

-585   CTGAGCTTAT GGAAGCAGAG AAGGGGGCAG GTGCAAAAGA CTCTCTTCCA GAACTCCGGA

-525   GAAATTTGCT TTTCAAAACT AGACAGCACC CTGCTGCCTA CTAAAGAAGT AGGTCCAAGG

-465   TCCTAATGTG CATATTCTCC GCTATACTCT TAGCTTTCCA GAAAACTAGA ATCATCAGTT

-405   TGGGTAAGAA CATAGAGGAA AACAGAAACG CCCCCCAACC TACCCCATGT CCAGAGAGCC

-345   TTGACCTACT TGTCTCCCTC CCACTCTCAA CCCTCCCAGT CTTGCTTCAA ACCTCTCCAC

                                                 283E|→
-285   GTCATGCCCC AACTTCGGAG CATTTGAACT CTGAGCAGTG GGGTCGCTTT CGCCTCAAGC
       CRE

-225   ACACCCCACC TCGGCAGGCC CAGTCAAAGG TCCCTCACAG GGACACCTTT TTTTCCCTGG

-165   GATCCCGCGC TTCGCCTCCG GGGCGGAGAC TCCTCCCCTA GTAGTTCCAC TTGTGTTCCC
                             Spl                              E-Box
       ↓r         133E|→            ↓h
-105   TAGAAGAGCA GCCGGGACGG CAAGAAGCCG GGACCTCCCC CTTCGTTCCA GGAACTGCCG

                                        ▽      ▽       ▽          ▽
-45    CGCAGTGGGC ACTTCAGCCC TGGAGGCCGC GAGCCCCACC CGGGTGAAGG CGGCTGCGCG
                                                      +1

+16    GCTTCAGCAC CACGGACAGC GCTCCCGTCC GCAGCCCTTG TGTCAGCGAG CGTCCGCGCT
           NRSE/RE1      Eco47III

+76    CGCGCTATGC AGGCGCATGG CCCGGTGCTC CAACTCTATG GCGCTGCTGT TCAGCTTTGG


+136   CCTCCTTTGG CTGTGTTCAG gtaagaatt
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Fig. 1. Nucleotide sequence of the region surrounding the initiator ATG of the β2-subunit gene. The four vertical arrowheads show the four extremities found using RACE-PCR and SLIC, corresponding to the transcription start sites. The vertical arrows indicate the position corresponding to the 5′ end of the longest rat (r) and human (h) β2-subunit cDNA clones.[15] The endpoints of the deletions used in the experiments described in Fig. 5 are indicated above the sequence. Nucleotides located in the intron are typed in lower case.

(Fig. 4A) and putative parasympathetic ganglia in the cardiac wall (Fig. 4B). At E13, clusters of positive cells were also present at several levels of the neuraxis, in both the brainstem and the prosencephalon. Clusters of stained neurons were also observed in the ventral and lateral spinal cord.

Later in development (E17), positive neurons were ound clustered in several basal telencephalic nuclei whereas dispersed cells were stained in the caudate-putamen. At the diencephalic level, positive clusters were present in the zona incerta and reticular thalamic nucleus, and in many hypothalamic nuclei. In the brainstem, most motor nuclei of cranial nerves (with the exception of the dorsal motor nucleus of the

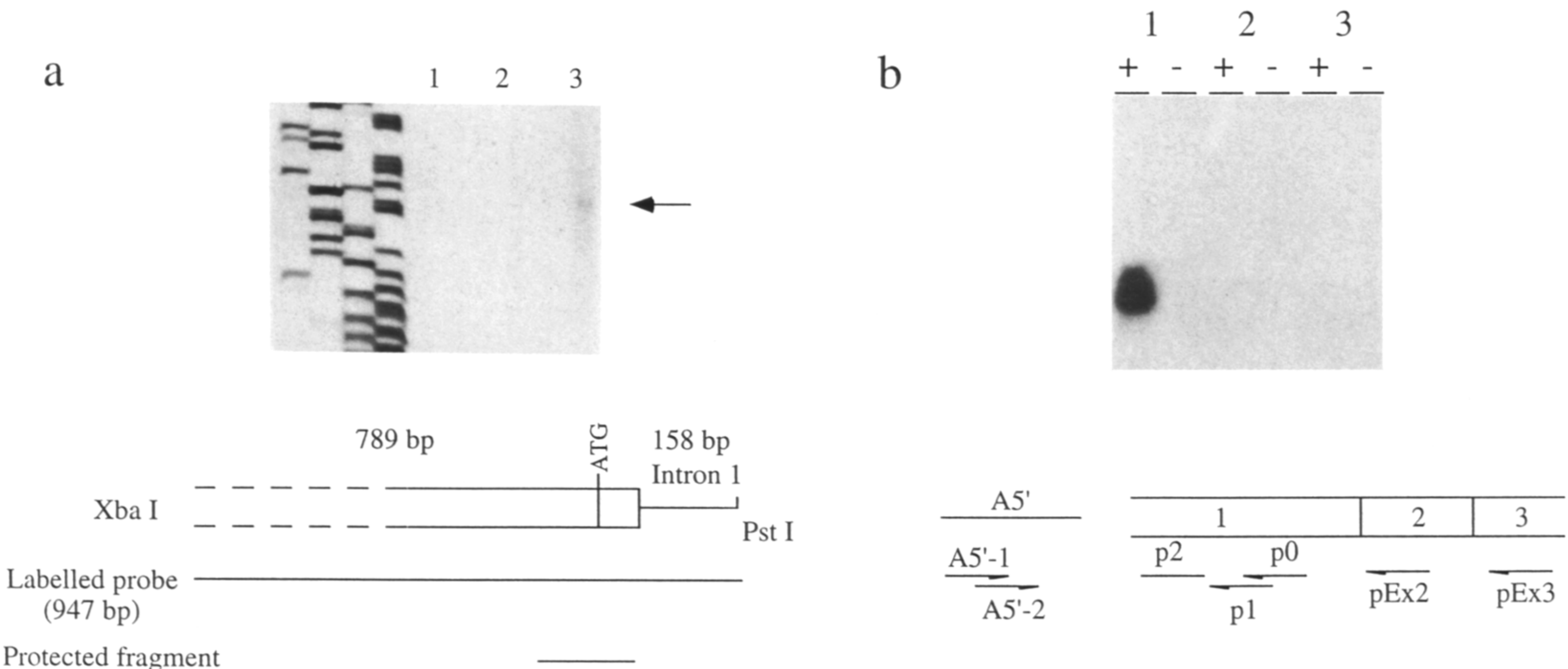

Fig. 2. Mapping of the 5′ end of the β2-subunit mRNA. (A) RNase protection experiments. Total RNA from DBA2 mouse brain (5 and 15 μg, lane 2 and 3 respectively) and yeast tRNA (15 μg, lane 1) were hybridized to a ^{32}P-labelled RNA probe containing 158 nucleotides of intron 1 (sequence data not shown), and 789 nucleotides of upstream sequences (−634/+155). The size of the protected bands were estimated according to the lower mobility in acrylamide of RNA as compared to DNA[3] and by comparison with the sequence of M13mp18 primed with the universal primer. The arrow on the right part of the gel points to the major protected band. (B) Identification of the transcription start site using SLIC. The lower part of the figure shows the strategy and describes the oligonucleotides used for the SLIC or the RACE-PCR. In the SLIC experiment, a primer extension was performed using oligonucleotide pEx3. The first strand of the cDNA was subsequently ligated to oligonucleotide A5′, and the resulting fragment was amplified using oligonucleotides A5′-1/p0 then A5′-2/p1. The amplified fragments were then loaded onto a 1.2% agarose gel. The gel was blotted and hybridized to oligonucleotide p2. Lane 1: 5 μg of total DBA2 mouse brain RNA. Lane 2–3: controls respectively without reverse transcriptase and without RNA. Minus: the T4 RNA polymerase was omitted. Same result was obtained using RACE-PCR.

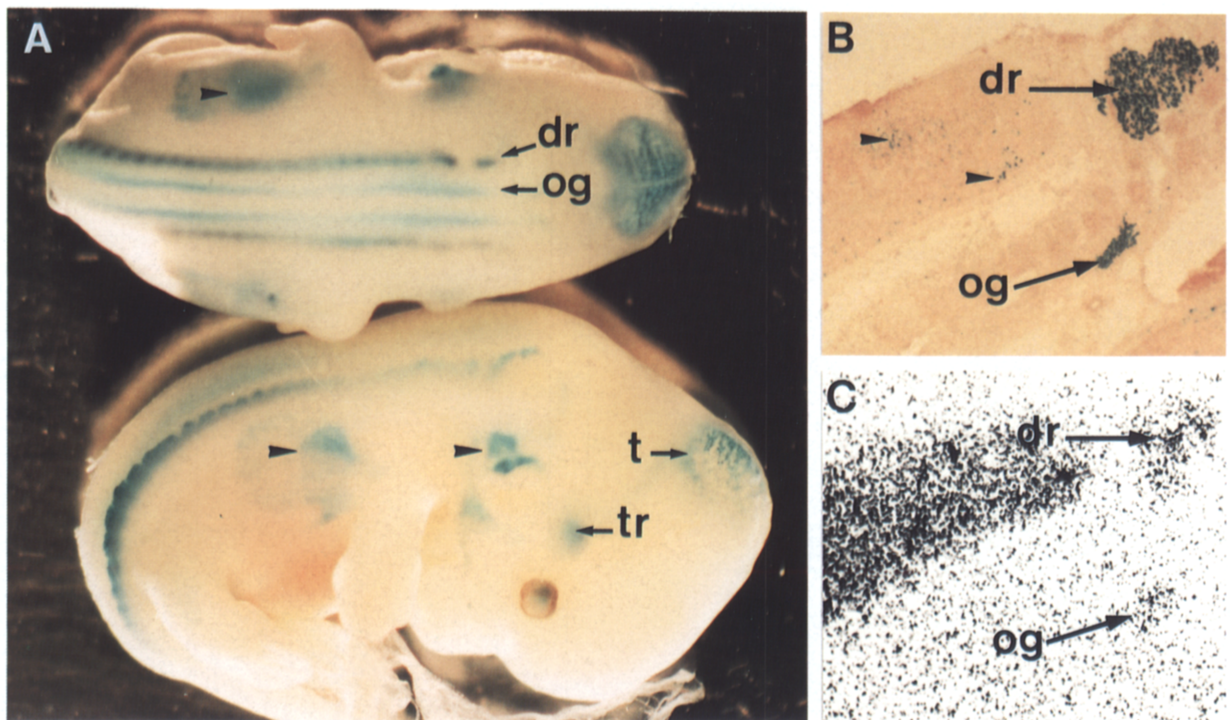

Fig. 3. Cell-specific expression of the β2-subunit promoter in transgenic mice. (A) Whole mount colouration of E13 embryos. The arrowheads point to ectopic expression in skin muscles. (B) Detection of the β-galactosidase activity in a parasagittal section of an E13 embryo at the lumbosacral level. Arrowheads indicate labelling in the ventral and dorsal horn of the spinal cord. (C) Detection of the β2-subunit transcripts in an adjacent section of the same embryo. dr, dorsal root ganglion; t, tectum; og, orthosympathetic ganglionic chain; tr, trigeminal ganglion.

vagus nerve) showed some to high labelling. In addition, the dispersed cells of the V mesencephalic nucleus appeared strongly stained, as well as the pontine nuclei, the prepositus hypoglossal nucleus and a few dispersed cells in the pontine tegmentum.

At P0 in line 13, the distribution of positive cells already appeared more restricted than at previous ages (for example labelling in basal telencephalon and oculomotor nuclei was clearly diminished). In the CNS of adult animals labelled cells were detected only in the hypothalamus. In line 13, some clusters of cells were stained in the mucosa of the gastrointestinal tract (stomach and duodenum) and in the pancreas. Ectopic labelling was detected in the genital tubercle and in several superficial muscles of line 13, but none of these tissues were stained in the line 26.

Identification of a minimal cell specific promoter

To investigate in more detail the regulatory elements involved in the promoter activity, we generated a series of plasmids containing 5′ deletions of the 1163 bp promoter. These plasmids were tested by transient transfection into fibroblasts and SK-N-Be cells. These two cell lines were chosen as they were the most easily transfected cell lines. Moreover, the neuroblastoma line was initially isolated from peripheral structures and is a convenient tool to study the regulatory elements carried by the 1163 bp promoter.

When 157 bp were deleted from the 5′ end of the 1163 bp promoter (plasmid 1006E-Luci, described in Fig. 1), the Luciferase activity did not significantly change in neuroblastomas but increased in fibroblasts (Fig. 5). When 144 bp were further deleted, the

Table 1. Cell-specific expression of the β2-subunit promotor *in vitro*

Cell type	PC12	SK-N-Be	N1E115	SVLT	3T6	293	Hela
Relative Luciferase activity	16.3 ± 2.3	156.5 ± 16.8	179.6 ± 5.4	0.8 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	1.1 ± 0.4

The Luciferase activity of the plasmids were normalized to the activity of the promoterless plasmid (KS-Luci, described in Experimental Procedures). RACE-PCR on mRNA extracted from SK-N-Be transfected with EE1.2-Luci, using Luciferase oligonucleotides (described in Experimental Procedures) showed that the amplified fragment had the expected size for the correct transcription initiation site (not shown).

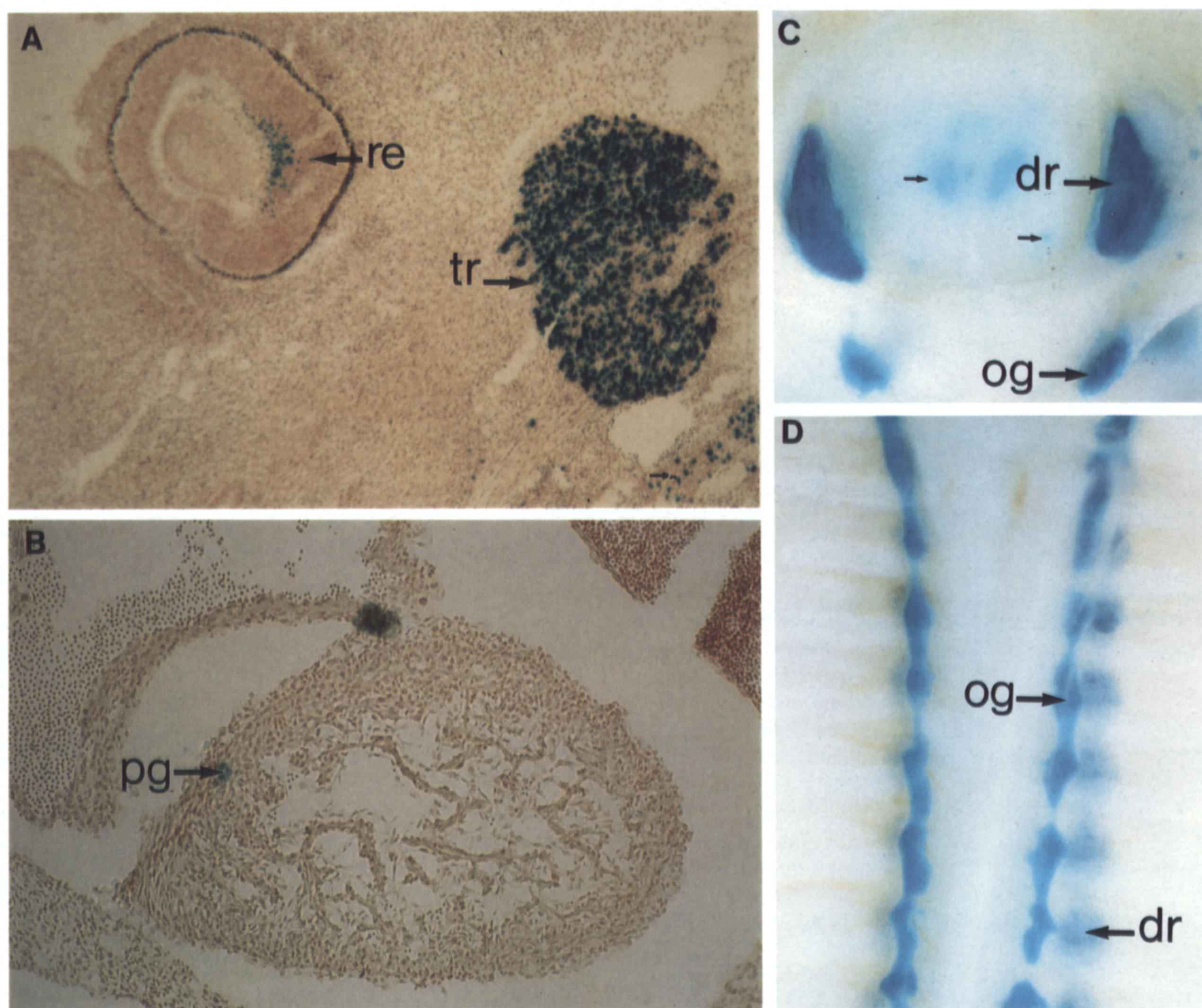

Fig. 4. Expression of β-galactosidae in transgenic mice. (A) Staining of the retina (re) and the trigeminal ganglia (tr) (E14.5). (B) Staining of cardiac parasympathetic ganglionic neutrons (pg) (E14.5). (C) Transverse section of the spinal cord (P1). dr, dorsal root ganglion; og, orthosympathetic ganglion. (D) Ventral view of the spinal cord (P1). The smaller arrows indicate neurons that have not been identified.

activity of the remaining promoter continued to increase in the fibroblasts but not in neuroblastomas (see plasmid 862E-Luci). Thus, the 157 and 144 bp deleted carry repressor elements which are only active in fibroblasts. However, the truncated 862 bp promoter still displayed a neuron-specific activity (Fig. 5, compare activity of 862E-Luci in both cell lines), showing that additional regulatory elements are carried by the 1.2 kbp promoter. Moreover, a repressor could be present between −824 and −245 (compare the activities of 862E and 283E-Luci in the neuroblastomas). This putative regulatory element was not further analysed. Indeed, a 283 bp promoter (plasmid 283E-Luci) is still ≈160 times more active in neuroblastomas than in fibroblasts, confirming the presence of another neuron-specific regulatory element in this proximal portion of the promoter.

When 150 bp were deleted from the 5′ end of the proximal 283 bp promoter, a very strong decrease of the transcriptional activity was detected in both fibroblasts and neuroblastomas (see activity of plasmid 133E-Luci). This shows that crucial positive regulatory elements have been deleted. These positive and negative elements were further investigated by deletion and mutation studies of the proximal portion of the promoter.

Negative and positive regulatory elements in the proximal region

The 3′ end of the β2-subunit promoter contains putative protein factor binding sites. To analyse the role of these elements in the β2-subunit gene

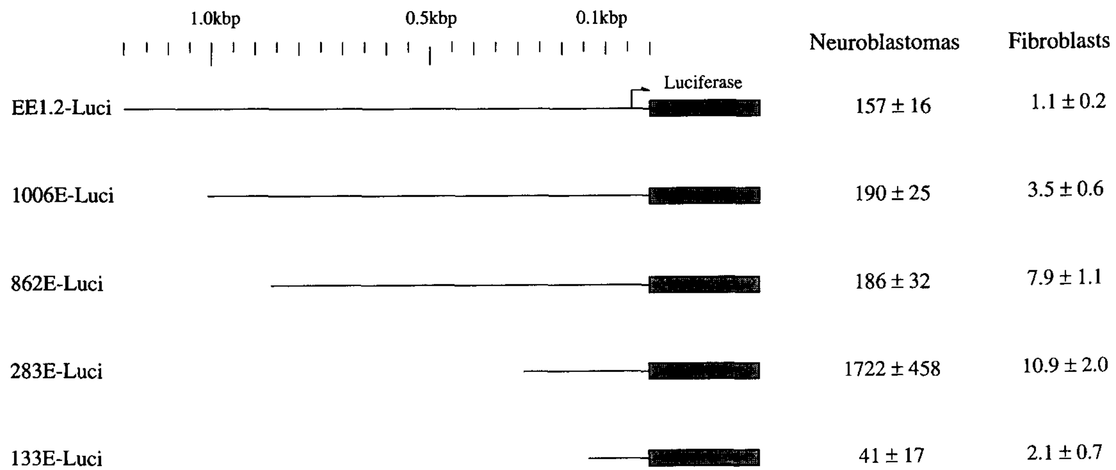

Fig. 5. Expression of the Luciferase fusion genes containing 5′ end deletions of the β2-subunit promoter. Plasmids are called nnnE-Luci, where nnn is the size in nucleotide of the insertion, and E is the 5′ end restriction site (*Eco*47III). The arrow indicates the transcription start site. The activities of EE1.2-Luci are from Table 1.

regulation, we generated plasmids containing mutations in these binding sites. Using deletion experiments, an activator was detected between −95 and −245 (see Fig. 3, the difference between 283E and 133E-Luci). As the E-box located at nt − 118 was a good candidate, we analysed the effect of mutations in this element on transcriptional activity. Table 2a shows a 40% reduction of the transcriptional activity of the mutated promoter compared to that of the wild-type promoter. The role of the E-box in non-neuronal tissues was more difficult to assess as the basal level of transcription was already low in fibroblasts.

To further understand the role of the E-box in the regulation of the promoter, we investigated the protein complexes able to interact with this sequence. Gel shift assays were performed using the 33 bp sequence (nt − 135 to −103, oligonucleotide E–D) as a probe. When the ^{32}P-labelled oligonucleotide was mixed with nuclear extracts from neuroblastomas or fibroblasts, three complexes were observed (see arrows, Fig. 6). All of them were fully displaced by an excess of the unlabelled oligonucleotide E–D. In contrast, no competition was observed when the competitor oligonucleotide was mutated within the E-box/Dyad (oligonucleotide Mut-E, see Fig. 6 lane "Mut-E"). This shows that the E-box/Dyad is the only element contained by the −135/−103 sequence able to bind nuclear protein. This sequence is likely to be involved in the activity of the β2-subunit promoter.

Table 2. Positive and negative regulatory elements in the proximal region of the 1163 bp promoter

	Fibroblasts (3T6)	Neuroblastomas (SK-N-Be)	
(a)			
EE1.2-Luci wild type	1.1	157	(100%)
EE1.2-Luci/NRSE/RE1	115.5 ± 13.8	502 ± 204	(320%)
EE1.2-Luci/E-Box	ND	94 ± 14	(60%)
(b)			
Mouse β2	TGCGCGGC . TTCAGCACCACGGACAGCGC . TCCCGTCC		
Sodium Channel (nt 29)	ATTGGGTT . TTCAGAACCACGGACAGCAC . CAGAGTCT		
SCG10 (nt 621)	AAAGCCAT . TTCAGCACCACGGAGAGTGC . CTCTGCTT		
Synapsin I (nt 2070)	CTGCCAGTC . TTCAGCACCGCGGACAGTGC . CTTCGCCC		
CAML1 (nt 1535)	TACAGGCC . TCCAGCACCACGGACAGCAG . ACCGTGAA		
Calbindin (nt 1093)	CCGAACGG . AGCAGCACCGCGGACAGCGC . CCCGCCGC		
Neurofilament (nt 383)	ATCGGGGT . TTCAGCACCACGGACAGCC . CCGCGGGG		
	TTCAGCACCACGGACAGCGC		

(a) Effect of mutations in the proximal part of the 1163 bp promoter. The activities of the wild-type or mutated promoters are normalized to the luciferase activity of the promotorless KS-Luci plasmid. The activities of EE1.2-Luci are from Table 1. ND, not done.

(b) Alignment of the proximal silencer of the β2-subunit promoter with other neuronal promoters. The sequences are taken from (Na channel,[39] accession number M31433), (SCG10,[42] M90489), (Synapsin I,[52] M55301), (CAML1 gene,[33] X63509), (Calbindin gene,[25] L11891), (Neurofilament gene,[63] X17102, reverse orientation). The numbering refers to the sequences in the GenBank/EMBL library.

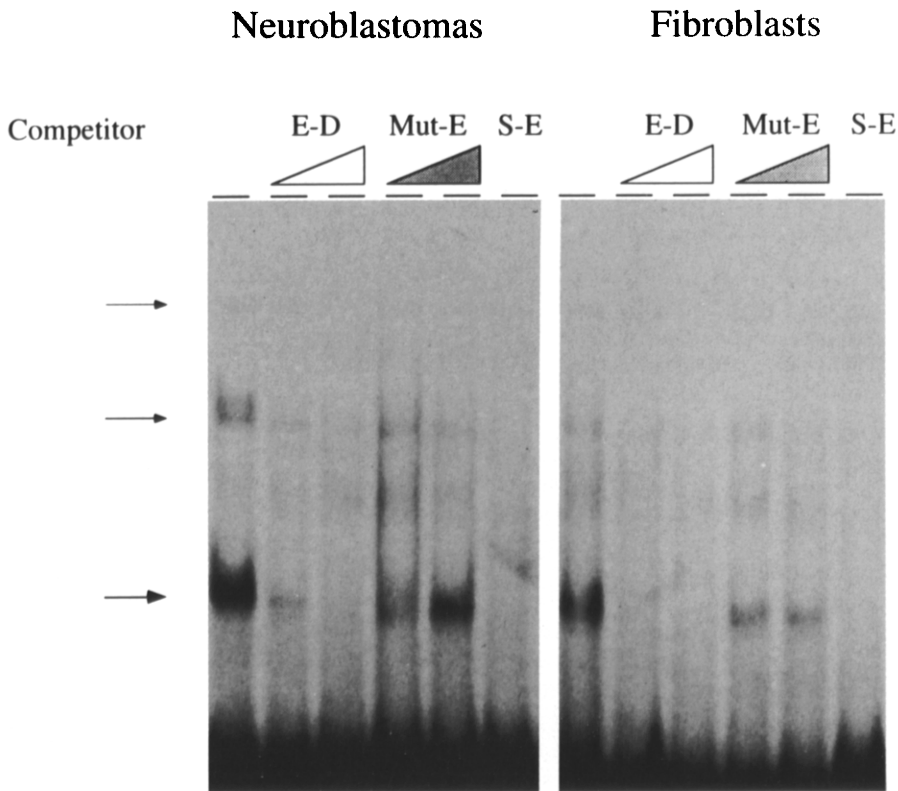

Fig. 6. Gel shift experiment. Autoradiogram of the mobility shift experiment. The probe used was a ^{32}P-labelled double-stranded E–D oligonucleotide. This oligonucleotide carries only the E-box/Dyad element whereas the oligonucleotide S–E carries the Sp1 binding site as well as the E-box/Dyad element. The competitor oligonucleotides were used in 10- and 100-fold molar excess, except for S–E that was used only in 100-fold molar excess.

An NRSE/RE1 sequence is also present in the proximal region and has been shown to act as a silencer in fibroblasts but not in PC12 cells or neuroblastomas.[34,36,41] Point mutation of this sequence in the context of the 1163 bp promoter resulted in a 115-fold increase of the transcriptional activity in fibroblasts, and only a three-fold increase in neuroblastomas (Table 2A). This sequence is thus responsible for at least part of the cell-specific expression of the β2 subunit gene.

DISCUSSION

Gene structure

We have cloned a genomic fragment containing the sequence encoding the mouse nAchR β2-subunit gene. Using RNase protection and amplification of primer extension products, we found one main and three minor transcription start sites (Fig. 1). The primer extension experiments were performed using two different reverse transcriptases, different batches of mRNA and different primers. These PCR-based techniques allowed us to amplify and subclone the same fragments, corresponding to transcription start sites rather than reverse transcriptase stops. The transcription start sites that we have characterized are located downstream from the position of the longest rat[15] and human[1] β2 cDNA 5′ end (see Fig. 1). This implies that in human and rat, another transcription start site is used. Such a discrepancy between species has already been demonstrated for the ϵ-subunit of the muscle nAchR.[17,19,55] In contrast with the α2-subunit gene,[7] no upstream exon could be detected.

Structural analysis of a 1.2 kbp flanking region disclosed many consensus motifs for nuclear protein binding including an Sp1 site and an E-box. Approximately 90 bp of the undeleted 1.2 kb promoter are transcribed and this region contains a NRSE/RE1 sequence.[34,41] Regulatory elements have already been described downstream of the transcription start site in different systems such as the Polyomavirus[9] or the *fos* gene.[35]

A 1163 bp promoter achieves cell-specific transcription

Transient transfection experiments showed that within the 1163 bp fragment is sufficient information to confer cell-specific expression of the nAchR β2-subunit gene. We showed that the same promoter directs a strict cell-specific transcription of the β-gal reporter gene. Moreover, the transgenic construct appears to be activated with the same timing as the

endogenous β2-subunit gene during the development of the early embryonic nervous system.[62] The 1163 bp promoter therefore carries regulatory sequences involved in the tissue and temporal specific transcription of the β2-subunit gene. At later stages of development, most of the peripheral β2 expressing neurons are still labelled (Fig. 3C, D). In CNS, the pattern of β-gal expression is different between the two lines and only a subset of the β2-expressing neurons expresses the transgene. This type of discrepancy between the expression of the transgene and the endogenous gene has already been described for the dopamine β-hydroxylase gene promoter[29,40] or for the *GAP*-43 gene.[56] Unexpected expression has been observed in transgenic line 13 in the genital tubercule and in skin muscles. This expression is likely to be due to the integration site of the transgene as these tissues are not stained in line 26. To our knowledge, most of the neuronal promoters studied by transgenesis display ectopic expression.[4,23,28,31,37,56]

By comparing β-gal-positive cell distribution with those of other known neuronal markers, it becomes apparent that a similarity exists with the distribution of choline acetyltransferase, TrkA (the high affinity nerve growth factor receptor) and p^{75} (the low affinity nerve growth factor receptor) expressing cells.[47–49,60] In particular, in developing rats, p^{75} is expressed in almost all the peripheral ganglia and central nuclei (with the exception of the zona incerta and hypothalamic nuclei) which express the transgene.[60] It is also interesting to note that p^{75} expression (like the expression of the β2-promoter transgene) is transient in many peripheral ganglia and brain nuclei, decreasing to undetectable levels at perinatal or early postnatal ages. It is therefore tempting to speculate that the β2-subunit promoter contains an element controlled by the activation of p^{75}, or that both the β2 transgene and p^{75} gene are controlled by a common regulator.

In conclusion, although the promoter seems to lack some regulatory elements active in the brain, the existing regulatory elements are sufficient to allow a cell- and development-specific expression of the β-gal in the PNS, in the spinal cord, and in several brain structures.

DNA regulatory elements

To further characterize the DNA elements involved in the transcription of the β2-subunit gene, we deleted or mutated the 1163 bp promoter and analysed the resulting constructs by transient transfection. A repressor element present in the distal 5′ end region is active in fibroblasts but not in neuroblastomas. This element thus accounts, at least in part, for the neuron-specific expression of the β2-subunit gene. Further analysis of the promoter shows that deleting 589 bp increases the activity in neuroblastomas but not in fibroblasts (Fig. 5, compare 862E and 283E-Luci). We have not yet characterized the mechanisms involved in this increase.

An NRSE/RE1 element is located at the 3′ extremity of the promoter. This element has already been shown to restrict the activity of promoters in neuronal cells.[34,36,41] In the 1163 bp promoter of the β2-subunit gene, point mutation of this sequence leads to a $\approx$100-fold increase of the transcriptional activity in fibroblasts implying that this sequence is involved in the neuron-specific expression of the β2-subunit gene. Moreover, sequence comparison shows that this sequence is highly conserved in rat and human β2-subunit cDNAs[1,15] (not shown) as well as in several promoters of genes expressed in the nervous system such as the middle-weight neurofilament gene, the CAM-L1 gene, the Calbindin gene or the cerebellar Ca-binding protein gene (see Table 2b).

Deletion experiments described in Fig. 5 show that an essential activator element is present between nucleotides −245 and −95. An Sp1 binding site and an E-box could be detected in this region. Sp1 sites are ubiquitous factors whereas E-boxes have been involved in several genetic regulatory mechanisms in muscle (see Ref. 6 for the nAchR α1-subunit) as in neurons.[26] Dyad elements have also been reported in some neuronal promoters such as those of the tyrosine hydroxylase gene,[61] the SCG10 gene,[42] the GAP43 gene[45] or in the flanking region of the N-CAM gene.[11] Results shown in Table 2a demonstrate that in neuroblastomas, the 1163 bp promoter mutated in the E-box/Dyad is slightly but significantlyless active than the wild-type promoter. Moreover, a gel shift assay (Fig. 6) further demonstrates that the E-box/Dyad is able to bind specific complexes. This suggests that the E-box/Dyad is responsible for at least part of the activation of β2-subunit gene transcription. However, transactivation experiments of heterologous promoters suggest that the E-box may co-operate with the Sp1 site located 27 bp upstream to positively activate the transcription (data not shown). Such a co-operation of an E-box and an Sp1 binding site has already been demonstrated for the regulation of the muscle nAchR α1-subunit transcription.[5]

CONCLUSION

We have shown that the β2-subunit gene is primarily regulated by negatively acting elements and by one positive element which comprises an E-box. This double regulation seems to be a general feature shared by several neuronal genes and allows fine tuning of the transcription of neuronal genes. Moreover, our transgenic studies show that the 1163 bp promoter confers a tight neuron-specific expression, but lacks some developmental or CNS-specific regulatory elements. Further joint *in vivo* and *in vitro* studies will help to characterize the DNA sequences involved in the β2-subunit gene expression, and to study the role of each DNA element in the temporal and cell-specific transcription.

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REFERENCES

1. Anand R. and Lindstrom J. (1990) Nucleotide sequence of the human nicotinic acetylcholine receptor β2 subunit gene. *Nucl. Acids Res.* **18,** 4272.
2. Antequera F. and Bird A. (1993) Number of CpG islands and genes in human and mouse. *Proc. natn. Acad. Sci. U.S.A.* **90,** 11995–11999.
3. Ausubel F. M., Brent R., Kingston R. E., Moore D. D., Seidman J. G., Smith J. A. and Struhl K. (1994) *Current Protocols in Molecular Biology.* (ed. Janssen K.) John Wiley and Sons, Inc.
4. Banerjee S. A., Hoppe P., Brilliant M. and Chikaraishi D. M. (1992) 5′ flanking sequences of the rat tyrosine hydroxylase gene target accurate tissue-specific, developmental, and trans-synaptic expression in transgenic mice. *J. Neurosci.* **12,** 4460–4467.
5. Bessereau J.-L., Mendelzon D., LePoupon C., Fizman M., Changeux J.-P. and Piette J. (1993) Muscle-specific expression of the acetylcholine receptor a-subunit gene requires both positive and negative interactions between myogenic factors, Spl and GBF factors. *Eur. molec. Biol. Org. J.* **12,** 443–449.
6. Bessereau J.-L., Stratford-Perricaudet L., Piette J., Le Poupon C. and Changeux J.-P. (1994) *In vivo* and *in vitro* analysis of electrical activity-dependent expression of muscle acetylcholine receptor genes using adenovirus. *Proc. natn. Acad. Sci. U.S.A.* **91,** 1304–1308.
7. Bessis A., Savatier N., Devillers-Thiery A., Bejanin S. and Changeux J. P. (1993) Negative regulatory elements upstream of a novel exon of the neuronal nicotinic acetylcholine receptor alpha-2 subunit gene. *Nucl. Acids Res.* **21,** 2185–2192.
8. Boulter J., O'Shea-Greenfield A., Duvoisin R., Connolly J., Wada E., Jensen A., Gardner P., Ballivet M., Deneris E., McKinnon D., Heinemann S. and Patrick J. (1990) Alpha3, alpha5 and beta3: three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. *J. biol. Chem.* **265,** 4472–4482.
9. Bourachot B., Yaniv M. and Herbomel P. (1989) Control elements situated downstream of the major transcriptional start site are sufficient for highly efficient polyomavirus late transcription. *J. Virol.* **63,** 2567–2577.
10. Britto L., Keyser K., Lindstrom J. and Karten H. (1992) Immunohistochemical localization of nicotinic acetylcholine receptor subunits in the mesencephalon and diencephalon of the chick (*Gallus gallus*). *J. Comp. Neurol.* **317,** 325–340.
11. Chen A., Reyes A. and Akeson R. (1990) Transcription initiation sites and structural organization of the extreme 5′ region of the rat neural cell adhesion molecule gene. *Molec. Cell. Biol.* **10,** 3314–3324.
12. Daubas P., Devillers-Thiery A., Geoffroy B., Martinez S., Bessis A. and Changeux J.-P. (1990) Differential expression of the neuronal acetylcholine receptor α2 subunit gene during chick brain development. *Neuron* **5,** 49–60.
13. de Wet J., Wood K. V., DeLuca M., Helinski D. R. and Subramani S. (1987) Firefly luciferase gene: Structure and expression in mammalian cells. *Molec. Cell. Biol.* **7,** 725–737.
14. Deneris E. S., Boulter J., Swanson L. W., Patrick J. and Heinemann S. (1989) β3: A new member of nicotinic acetylcholine receptor gene family is expressed in brain. *J. biol. Chem.* **264,** 6268–6272.
15. Deneris E. S., Connolly J., Boulter J., Wada E., Wada K., Swanson L. W., Patrick J. and Heinemann S. (1988) Primary structure and expression of β2: a novel subunit of neuronal nicotinic acetylcholine receptors. *Neuron* **1,** 45–54.
16. Dineleymiller K. and Patrick J. (1992) Gene transcripts for the nicotinic acetylcholine receptor subunit, Beta4, are distributed in multiple areas of the rat central nervous system. *Molec. Brain Res.* **16,** 339–344.
17. Dong K., Yu K. and Roberts J. (1993) Identification of a major up-stream transcription start site for the human progonadotropin-releasing hormone gene used in reproductive tissues and cell lines. *Molec. Endocr.* **7,** 1654–1666.
18. Dumas Milnes Edwards J.-B., Delort J. and Mallet J. (1991) Oligodeoxiribonucleotide ligation to single stranded cDNA: a new tool for cloning 5′ ends of mRNAs and for constructing cDNAs libraries by *in vitro* amplification. *Nucl. Acids Res.* **19,** 5227–5232.
19. Dũrr I., Numberger M., Berberich C. and Witzemann V. (1994) Characterization of the functional role of E-box elements for the transcriptional activity of rat acetylcholine receptor ϵ-subunit and γ-subunit gene promoters in primary muscle cell cultures. *Eur. J. Biochem.* **224,** 353–364.
20. Duvoisin R., Deneris E., Patrick J. and Heinemann S. (1989) The functional diversity of the neuronal nicotinic acetylcholine receptors is increased by a novel subunit: beta 4. *Neuron* **3,** 487–496.
21. Evrard C., Borde I., Marin P., Galiana E., Prémont J., Gros F. and Rouget P. (1990) Immortalization of bipotential glio-neuronal precursor cells. *Proc. natn. Acad. Sci. U.S.A.* **87,** 3062–3066.
22. Flores C. M., Rogers S. W., Pabreza L. A., Wolfe B. B. and Kellar K. J. (1992) A subtype of nicotinic cholinergic receptor in rat brain is composed of alpha4-subunit and beta2-subunit and is up-regulated by chronic nicotine treatment. *Molec. Pharmac.* **41,** 31–37.
23. Forss-Petter S., Danielson P. E., Catsicas S., Battenberg E., Price J., Nerenberg M. and Sutcliffe J. G. (1990) Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. *Neuron* **5,** 187–197.
24. Frohman M. A., Dush M. K. and Martin G. R. (1988) Rapid production of full length cDNA from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. natn. Acad. Sci. U.S.A.* **85,** 8998–9002.
25. Gill R. K. and Christakos S. (1993) Identification of sequence elements in mouse calbindin D-28k gene that confer 1,25-dihydroxyvitamin D-3- and butyrate-inducible responses. *Proc. natn. Acad. Sci. U.S.A.* **90,** 2984–2988.

26. Guillemot F., Lo L.-C., Johnson J. E., Auerbach A., Anderson D. J. and Joyner A. L. (1993) Mammalian *achaete-scute* homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**, 463–476.
27. Hill J. A., Zoli M., Bourgeois J. P. and Changeux J. P. (1993) Immunocytochemical localization of a neuronal nicotinic receptor—the beta-2-subunit. *J. Neurosci.* **13**, 1551–1568.
28. Hoesche C., Sauerwald A., Veh R. W., Krippl B. and Kilimann M. W. (1993) The 5′-Flanking region of the rat Synapsin-I gene directs neuron-specific and developmentally regulated reporter gene expression in transgenic mice. *J. biol. Chem.* **268**, 26494–26502.
29. Hoyle G. W., Mercer E. H., Palmiter R. D. and Brinster R. L. (1994) Cell-specific expression from the human dopamine beta-hydroxylase promoter in transgenic mice is controlled via a combination of positive and negative regulatory elements. *J. Neurosci.* **14**, 2455–2463.
30. Kalderon D., Roberts B. L., Richardson W. D. and Smith A. E. (1984) A short amino acid sequence able to specify nuclear location. *Cell* **39**, 499–509.
31. Kaneda N., Sasaoka T., Kobayashi K., Kiuchi K., Nagatsu I., Kurosawa Y., Fujita K., Yokoyama M., Nomura T., Katsuki M. and Nagatsu T. (1991) Tissue-specific and high-level expression of the human tyrosine hydroxylase gene in transgenic mice. *Neuron* **6**, 583–594.
32. Ko L. J. and Engel J. D. (1993) DNA-binding specificities of the GATA transcription factor family. *Molec. Cell Biol.* **13**, 4011–4022.
33. Kohl A., Giese K. P., Mohajeri M. H., Montag D., Moos M. and Schachner M. (1992) Analysis of promoter activity and 5′ genomic structure of neural cell adhesion molecule L1. *J. Neurosci. Res.* **32**, 167–177.
34. Kraner S. D., Chong J. A., Tsay H.-J. and Mandel G. (1992) Silencing the type-II sodium channel gene—a model for neural-specific gene regulation. *Neuron* **9**, 37–44.
35. Lamb N. J. C., Fernandez A., Tourkine N., Jeanteur P. and Blanchard J.-M. (1990) Demonstration in living cells of intragenic negative regulatory element within the rodent *c-fos* gene. *Cell* **61**, 485–496.
36. Li L. A., Suzuki T., Mori N. and Greengard P. (1993) Identification of a functional silencer element involved in neuron-specific expression of the synapsin-I gene. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1460–1464.
37. Logan C., Khoo W. K., Cado D. and Joyner A. L. (1993) Two enhancer regions in the mouse En-2 locus direct expression to the mid-hindbrain region and mandibular myoblasts. *Development* **117**, 905–916.
38. Matter-Sadzinski L., Hernandez M.-C., Roztocil T., Ballivet M. and Matter J.-M. (1992) Neuronal specificity of the α7 nicotinic acetylcholine receptor promoter develops during morphogenesis of the central nervous system. *Eur. molec. Biol. Org. J.* **11**, 4529–4538.
39. Maue R. A., Kraner S. D., Goodman R. H. and Mandel G. (1990) Neuron-specific expression of the rat brain type II sodium channel gene is directed by upstream regulatory elements. *Neuron* **4**, 223–231.
40. Mercer E., Hoyle G., Kapur R., Brinster R. and Palmiter R. (1991) The dopamine beta-hydroxylase gene promoter directs expression of E. coli lacZ to sympathetic and other neurons in adult transgenic mice. *Neuron* **7**, 703–716.
41. Mori N., Schoenherr C., Vandenbergh D. J. and Anderson D. J. (1992) A common silencer element in the SCG-10 and Type-II Na^+ channel genes binds a factor present in non-neuronal cells but not in neuronal cells. *Neuron* **9**, 45–54.
42. Mori N., Stein R., Sigmund O. and Anderson D. J. (1990) A cell type-preferred silencer element that controls the neural-specific expression of the SCG10 gene. *Neuron* **4**, 593–594.
43. Morris B. J., Hicks A. A., Wisden W., Darlison M. G., Hunt S. P. and Barnard E. A. (1990) Distinct regional expression of nicotinic acetylcholine receptor genes in chick brain. *Molec. Brain Res.* **7**, 305–315.
44. Mulle C., Vidal C., Benoit P. and Changeux J.-P. (1991) Existence of different subtypes of nicotinic acetylcholine receptors in rat habenulo-interpeduncucular. *J. Neurosci.* **11**, 2588–2597.
45. Nedivi E., Basi G., Akey I. and Skene J. H. P. (1992) A neural-specific GAP-43 core promoter located between unusual DNA elements that interact to regulate its activity. *J. Neurosci.* **12**, 691–704.
46. Parker M. G. (1993) Steroid and related receptors. *Curr. Opin. Cell Biol.* **5**, 499–504.
47. Pioro E. P. and Cuello A. C. (1990) Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and correlation with the cholinergic system—I. Forebrain. *Neuroscience* **34**, 57–87.
48. Pioro E. P. and Cuello A. C. (1990) Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and correlation with the cholinergic system—II. Brainstem, cerebellum and spinal cord. *Neuroscience* **34**, 89–110.
49. Ringstedt T., Lagercrantz H. and Persson H. (1993) Expression of members of the trk family in the developing postnatal rat brain. *Devl Brain Res.* **72**, 119–131.
50. Role L. W. (1992) Diversity in primary structure and function of neuronal acetylcholine receptor channels. *Curr. Opin. Neurobiol.* **2**, 254–262.
51. Sassone-Corsi P. (1988) Cyclic AMP induction of early adenovirus promoters involves sequences required for E1A trans-activation. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7192–7196.
52. Sauerwald A., Hoesche C., Oschwald R. and Kilimann M. W. (1990) The 5′-flanking region of the synapsin I gene. A G + C-rich, TATA- and CAAT-less, phylogenetically conserved sequence with cell type-specific promoter function. *J. biol. Chem.* **265**, 14932–14937.
53. Seguela P., Wadiche J., Dineleymiller K., Dani J. A. and Patrick J. W. (1993) Molecular cloning, functional properties, and distribution of rat brain-alpha7—A nicotinic cation channel highly permeable to calcium. *J. Neurosci.* **13**, 596–604.
54. Swanson L. W., Simmons D. M., Whiting P. J. and Lindstrom J. (1987) Immunohistochemical localization of neuronal nicotinic receptor in the rodent central nervous system. *J. Neurosci.* **7**, 3334–3342.
55. Toussaint C., Bousquet-Lemercier B., Garlatti M., Hanoune J. and Barouki R. (1994) Testis-specific transcription start site in the aspartate-aminotransferase housekeeping gene promoter. *J. biol. Chem.* **269**, 13318–13324.
56. Vanselow J., Grabczyk E., Ping J., Baetscher M., Teng S. and Fishman M. C. (1994) GAP-43 transgenic mice: dispersed genomic sequences confer a GAP-43-like expression pattern during development and regeneration. *J. Neurosci.* **14**, 499–510.
57. Wada E., Wada K., Boulter J., Deneris E., Heinemann S., Patrick J. and Swanson L. W. (1989) Distribution of alpha2, alpha3, alpha4, and beta2 neuronal nicotinic subunit mRNAs in the central nervous system: a hybridization histochemical study in rat. *J. comp. Neurol.* **284**, 314–335.

58. Wada K., Ballivet M., Boulter J., Connolly J., Wada E., Deneris E. S., Swanson L. W., Heinemann S. and Patrick J. (1988) Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor. *Science* **240,** 330–334.
59. Wada K., Dechesne C., Shimasaki S., King R., Kusano K., Buonnano A., Hampson D., Banner C., Wenthold R. and Nakatani Y. (1989) Sequence and expression of a frog brain complementary DNA encoding a kainate-binding protein. *Nature* **342,** 684–689.
60. Yan Q. and Johnson E. M. (1988) An immunohistochemical study of the nerve growth factor receptor in developing rats. *J. Neurosci.* **8,** 3481–3498.
61. Yoon S. O. and Chikaraishi D. M. (1994) Isolation of two E-box binding factors that interact with the rat tyrosine hydroxylase enhancer. *J. biol. Chem.* **269,** 18453–18462.
62. Zoli M., Le Novère N., Hill Jr J. A. and Changeux J.-P. (1994) Developmental regulation of nicotinic receptor subunit mRNAs in the rat central and peripheral nervous system. *J. Neurosci.* **15,** 1912–1939.
63. Zopf D., Dineva B., Betz H. and Gundelfinger E. D. (1990) Isolation of the chicken middle-molecular weight neurofilament (NF-M) gene and characterization of its promoter. *Nucleic Acids Res.* **18,** 521–529.

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