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Negative regulatory elements upstream of a novel exon of the neuronal nicotinic acetylcholine receptor α2 subunit gene

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ABSTRACT

The expression of the nicotinic acetylcholine receptor α2 subunit gene is highly restricted to the *Spiriform lateralis* nucleus of the Chick diencephalon. As a first step toward understanding the molecular mechanism underlying this regulation, we have investigated the structural and regulatory properties of the 5′ sequence of this gene. A strategy based on the ligation of an oligonucleotide to the first strand of the cDNA (SLIC) followed by PCR amplification was used. A new exon was found ≈3kb upstream from the first coding exon, and multiple transcription start sites of the gene were mapped. Analysis of the flanking region shows many consensus sequences for the binding of nuclear proteins, suggesting that the 1 kb flanking region contains at least a portion of the promoter of the gene. We have analysed the negative regulatory elements present within this region and found that a silencer region located between nucleotide −144 and +76 is active in fibroblasts as well as in neurons. This silencer is composed of six tandem repeat Oct-like motifs (CCCCATGCAAT), but does not bind any member of the Oct family. Moreover these motifs were found to act as a silencer only when they were tandemly repeated. When two, four or five motifs were deleted, the silencer activity of the motifs unexpectedly became an enhancer activity in all cells we have tested.

INTRODUCTION

The nervous system is composed of a large variety of cell types embedded in an intricate structure of glial cells and organized in highly connected, yet functionally distinct layers and nuclei. Relative to other organs, the nervous system displays a much greater diversity of cell types distributed according to well defined patterns. Hence, genetic regulatory mechanisms in the nervous system provide more than simple tissue specificity: they control the three dimensional cellular patterns of gene expression. Mechanisms more sophisticated than the conventional regulation of gene expression by cell-specific enhancers thus occur in the central nervous system (1, 2), and one way to achieve such fine control is to regulate transcription by both positive and negative mechanisms. Several studies have revealed the existence of regulatory elements in neuronal genes that enhance or inhibit transcription in a cell-specific manner (3–11). However, these studies concern genes broadly expressed in the central nervous system, and the implications of these two mechanisms of regulation in the specification of groups of neurons or brain compartments cannot be inferred from these studies. We therefore decided to investigate the regulation of a particular neuronal gene exhibiting a highly restricted pattern of expression.

The nicotinic acetylcholine receptor (nAchR) plays a crucial role in the transmission of information within the central nervous system and each member of this family shows a distinct pattern of expression. At least eight members of the nAchR gene family have been characterized in vertebrates (12, 13) and brain regions expressing defined pattern of particular nAchR subunits have been mapped (14–16). These studies revealed that the gene encoding the α2 subunit (14) has the most restricted expression in both rodents and chicken. In the latter species, this gene is exclusively transcribed in the *Spiriformis Lateralis nucleus* (SpL) of the diencephalon (15, 16).

We therefore decided to investigate the genetic mechanisms governing the expression of the gene encoding the α2 subunit in the chick, aware of the possibility that the mechanisms that restrict the transcription of this gene to a highly localized diencephalic nucleus may have general applications in the specification of neuronal groups in the brain. In this paper, we describe a new non coding exon located 3kb upstream from the first coding exon. We also show that many transcription initiation sites for this gene are concentrated within a 300 bp region, and identify a silencer element located in the extreme 5′ flanking region of the gene. We show that this silencer is paradoxically

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composed of six enhancer elements and demonstrate that these elements are active in neuronal as well as in non-neuronal cell lines.

MATERIAL AND METHODS

Analysis of the 5' end

We performed the SLIC reaction exactly as described by Dumas-Milnes-Edwards *et al.* (19). Briefly, first strand synthesis of cDNA was performed using 6 μg of total RNA isolated from new-born chicken diencephalon. 6 pmol of primer CDNA was used to prime the reaction. In order to increase the relative abundance of the α2 mRNA, total RNA from new-born chick diencephalon was prepared and used the same day, to prevent degradation.

To ensure that the cDNA carried all the α2 transcribed sequences, we synthesised the first strand of cDNA using two different conditions. Common components of the two elongation buffer were: BSA 0,1 μg/μl, RNasin (Promega) 1u/μl, 10 mM Tris pH = 8,3, 1,6 mM $MgCl_2$, 8 mM KCl, 0,5 mM dNTP, 4 mM Sodium Pyrophosphate. In addition, we added either 70 mM β-Mercaptoethanol and 15 units of reverse transcriptase or 2 mM CH_3Hg, 28 mM β-Mercaptoethanol and 45 units of AMV reverse transcriptase (Promega). The reaction mixture was incubated for 50 min at 42°C, and stopped by heating for 10 min at 70°C after addition of 30 mM EDTA. After elimination of the primer and alkaline hydrolysis of the RNA, a modified oligonucleotide (A5') was ligated to the 3' end of the cDNA. The first PCR amplification (45 cycles) was performed using one half of the ligation mixture in a volume of 100 μl with 0,1 μM primers (A5'-1/PCR1 or A5'-1/PCR4) and 1 unit of Taq Polymerase in the buffer recommended by the supplier (GIBCO). A second amplification (35 cycles) was then carry out on 1/10 of the amplified products under the same conditions, using A5'-2/PCR2 or A5'2/PCR5 as primers. The parameters for both amplifications were: 92°C for 1 min, 57°C for 30 sec, 72°C for 45 sec. PCR products were analysed on a 2% agarose gel and then transferred to a nylon membrane (Hybond N). Filters were hybridized with ^{32}-P labelled oligonucleotide PCR3 or PCR6 in 6×SSC, 0,1%SDS, 1×Denhardt's solution at 42°C and washed at 42°C in 6×SSC, 0,1%SDS.

Cell culture and transfection

NIH3T6 (32) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal calf serum, 1% glutamine, 100U/ml Penicillin and 100μg/ml streptomycin.

PC12 (33) cells were grown in DMEM supplemented with 10% Horse serum, 5% Foetal calf serum, 1% glutamine, 100U/ml Penicillin and 100μg/ml streptomycin.

Undifferentiated SVLT cells were grown in DMEM supplemented with 10% Foetal calf serum, 1% glutamine, 100U/ml Penicillin and 100μg/ml streptomycin. SVLT cells were differentiated in DMEM supplemented with 2% Ultroser G (IBF) or in N2 medium after plating at appropriate concentration (see (34)).

All cells were transfected using Transfectam (IBF) according to the recommended protocol: cells (4−5 10^4 for SV-LT; 10^5 for PC12; 4.10^5 for 3T6) were plated in 60 mm Petri dishes. The next day, cells were transfected for 5 h in 1ml of 50% DMEM/50% Transfectam−DNA solution. We used 1.5 to 3 μg DNA and 6 to 12 μl Transfectam for each 60 mm petri dish.

For cotransfection, we used 0,5 μg of plasmid and 0,5 μg of oligonucleotide. The SV-Luci and α2.6Sil plasmid were cotransfected with either oligonucleotide SIL, OCT SP1, or with a non specific oligonucleotide (see below, NS). The absolute activity of the plasmids were normalized to the control SV-Luci plasmid cotransfected with the oligonucleotides. The activity of the SV-Luci plasmid cotransfected with SP1 is expressed as the percentage of the activity of SV-Luci cotransfected with NS. The activities of the α2.6Sil plasmid cotransfected with different oligonucleotides are expressed as the percentage of the activity of SV-Luci cotransfected with the same oligonucleotides.

Luciferase activity was measured 48 h later using a Berthold Lumat LB9501 luminometer.

All transfections were done in duplicate (PC12 cells and fibroblasts) or in triplicate (SVLT). All transfections were performed at least three times with at least two different plasmid preparations exept for cotransfection for which only one plasmid preparation was used.

Plasmid DNA

KS-Luci: The 1900 bp *Hind*III/*Kpn*I fragment of pO-Luc (64) was inserted in the corresponding restriction sites of Bluescript KS+ plasmid (stratagene).

SV40-Luci: An adapator containing sites for *Hind*III and *Sal*I was linked to the *Pvu*II restriction site of the ≈300 bp *Pvu*II/*Hind*III fragment of SV40. The resulting fragment was inserted in *Hind*III restriction site of KS-Luci.

α2BK7.0/SV-Luci: This plasmid contains 7 kb of genomic sequence upstream from exon 1, obtained from phage λ46.2 (15). This phage contains 15 kb of genomic DNA subcloned into the B*amHI* sitesof λ2001 (Stratagene), including the 6 α2 coding exon, 7kb upstream from exon 1 and 3 kb downstream from exon 6. The 5.5 kb *Xho*I/*Eco*RI fragment of the phage λ46.2 containing the extreme 5' region, was subcloned into the corresponding restriction sites of KS (α2BE5.5). The 1.4 kb *Eco*RI/*Kpn*I fragment of λ46.2 was then subcloned using adaptors into the *Eco*RI/*Spe*I restriction sites of α2BE5.5 (KS-BK7.0). Finally, the *Hind*III/*Kpn*I 1.9 kb fragment of KS-Luci was subcloned in the *Xba*I/*Not*I restriction sites of α2BK7 using adaptors.

α2.6Sil: The 891 *Pst*I/*Pst*I fragment of the α2BK7/SV-Luci was subcloned in both orientation into the *Pst*I restriction site of SV40-Luci. All experiments have been done with both kind of plasmids except the cotransfection experiments for which we have only used the 'sense' orientation.

Deletion mutants: The α2.6Sil plasmid was cut with *Sal*I and digested for various times with Bal-31 exonuclease. The DNA was then cut with *Hind*III to delete the remaining SV40 sequences. The overhanging ends were filled using the klenow fragment of DNA polymerase I and the plasmids were self ligated. The mutants obtained started at position −810, and ended at position: −144, −118, −80 and −41. The ≈700bp *Xba*I fragments of these plasmids were subcloned into the *Spe*I restriction site of SV40-Luci. The corresponding plasmids were: α2.0Sil, α2.1Sil, α2.2Sil and α2.4Sil.

The sequence shown in figure 2a has been submitted to the EMBL data library, and the accession number is X69511.

DNA mobility shift assays

Oligonucleotides were labeled either with γ^{32}P-ATP and T4 polynucleotide kinase, or with α^{32}P-dCTP and Klenow enzyme. Nuclear extracts were prepared from ≈10^7 cells as described

by (65). For binding, 1nmol of labeled oligonucleotide was mixed with 5μg of protein extract in 10 mM Hepes pH 8, 10% glycerol, 0,1 mM EDTA, 0,1 M NaCl, 2 mM DTT, 0,1 mg/ml BSA, 4 mM $MgCl_2$, 4 mM spermidine, 1mM PMSF, 3μg polydIdC in 20μl. The reaction was incubated for 10min on ice. The DNA-protein complexes were then analyzed on a 7% polyacrylamide gel run in 0,25× TBE.

The sequences of all oligonucleotides are described below.

Oligonucleotides

A5': 5'-CTGCATCTATCTAATGCTCCTCTCGCTACC TGCTCACTCTGCGTGACATC
A5'-1: 5'-GATGTCACGCAGAGTGAGCAGGTAG
A5'-2: 5'-AGAGTGAGCAGGTAGCGAGAGGAG
PCR1: 5'-ACCACACGAGGAGGGGTATGATG
PCR2: 5'-GGCACAGCGACGGGCTGAGTAGG
PCR3: 5'-CACAGGACACCCTGCTGGAGATGCCT
PCR4: 5'-GAGCCTCCTCGGCACAGCAT
PCR5: 5'-ACATCCTCCACTGGAGCCCA
PCR6: 5'-ACCCTCCTCCCTCCTCC
CDNA: 5'-TGGGGCTGTTTCTGCTCCCGGGTGG
Sil1: 5'-TCGAAGCCCCCATGCAATGTT
Sil2: 5'-TCGAAACATTGCATGGGGGCT
SIL = Sil1 hybridized to Sil2
OCT: 5'-CATATAAGATATGCAAATGAGTGGGAAGG (Double stranded)
SP1: 5'-TCGACTAACTCCGCCCAGTTC/5'-TCGAGAA CTGGGCGGAGTTAG (Double stranded)
Non Specific (NS): 5'-TTGCCTTACACCACTTATTCC AGGCATGCA (Double stranded)
Oligonucleotide A5' is modified as described in (19).

RESULTS

Structure of the 5' non translated sequence

Isolation and characterisation of the gene coding for the nicotinic receptor α2 subunit gene has been previously described (15, 17). As a first step toward identification of the regulatory region governing α2 gene transcription, we attempted to characterise the transcription initiation site of this gene. Due to the very low abundance of the mRNA in chick brain, we failed to detect any signal using Northern blots, S1 mapping, primer extension, RNase protection or RACE-PCR (18). To circumvent these difficulties, we used a new strategy [referred to as SLIC, (19)] based on the ligation of an oligonucleotide to the 3' end of the first strand of the cDNA using T4 RNA Ligase. The ligation and the following PCR strategies used in the first SLIC reaction are illustrated in figure 1a.

In order to increase the relative abundance of the α2 mRNA, total RNA from new-born chick diencephalon was prepared and used the same day, to prevent degradation. Prior to synthesis of the first strand of cDNA, RNA was denatured by heating or by treatment with methylmercuric hydroxide (20) to avoid problems with secondary structure. Synthesis was primed with an oligonucleotide located within exon 2 (17) to avoid genomic DNA contamination. A fifty nt long modified oligonucleotide (A5', see material and methods and figure 1a) was then ligated to the first strand of the cDNA. A first PCR amplification was performed using oligonucleotides A5'-1 and PCR1. The amplified fragment was then submitted to a second round of PCR using oligonucleotides A5'-2 and PCR2 (see figures 1a and 2a). The specific amplified products were subsequently characterised by Southern blot, using oligonucleotide PCR3 as an internal probe (figure 1b). This method allowed us to subclone and sequence the 5' end of the α2 cDNA. Based on this new sequence data, three novel oligonucleotides were designed (PCR4, PCR5, and PCR6, see figure 2a). A second SLIC reaction was then performed to ensure that the 5' ends were not truncations of the cDNA due to incomplete reverse transcription. Both SLIC reactions detected the same extremities, suggesting that the amplified fragments represent real 5' mRNA ends. The nucleotide sequence of this 5' region sequence is shown in figure 2a (numbered +1 to +536). Nine specific amplified products have been characterised (see figure 2a, arrows), revealing either different transcription initiation sites, arrest of the reverse transcriptase close to the true 5' end or very rapid degradation of mRNA. Rapid degradation might explain the relative inefficiency of the other methods. Interestingly, the size of the amplified products were approximately equal when the RNA was denatured by heat or by methylmercury hydroxide, suggesting that the reverse transcriptase had not been prematurely stopped by the secondary structure of the mRNA.

In order to elucidate the genomic organisation of the relatively long 5' end, we sequenced 4kb upstream from exon 1. Comparison of the sequences of the cDNAs with the genomic DNA revealed the presence of a new exon located 2.8 kb upstream from exon 1. The novel intron is bordered by consensus donor and acceptor sites (21). Thus the first coding exon of the

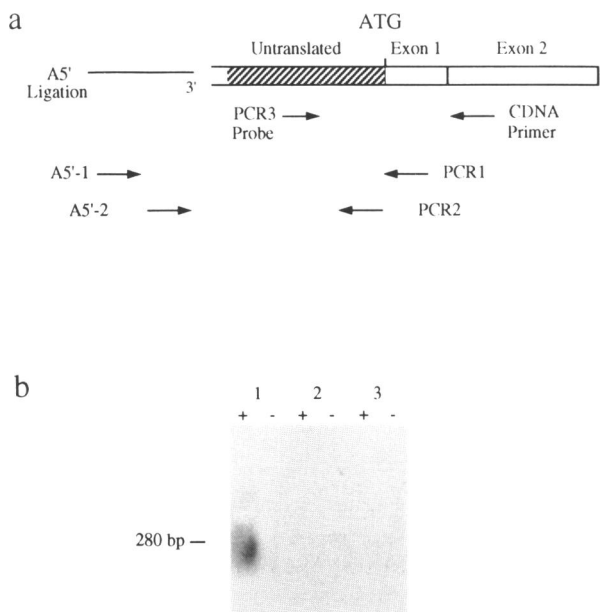

Figure 1. Identification of the α2 transcription start sites. **a.** Strategy used to characterise the 5' end of the α2 coding gene (SLIC). Oligonucleotides used for PCR are schematized by arrows. We verified that PCR3 was included within the transcribed region of the gene by RT-PCR (not shown). We first performed one SLIC using A5'-1 and A5'-2 together with PCR1 and PCR2, we then used PCR4 and PCR5 (see figure 2a) in a second SLIC, to confirm the previous result. **b.** Southern blot of the amplified SLIC product probed with PCR3. The amplification was performed using A5'-2/PCR2 and loaded onto a 1.2% agarose gel. Lane 1: 6 μg of total diencephalon RNA, Lane 2 and 3, control without Reverse transcriptase (lane 2) or without RNA. Minus: the single stranded cDNA and the A5' oligonucleotide were not ligated.

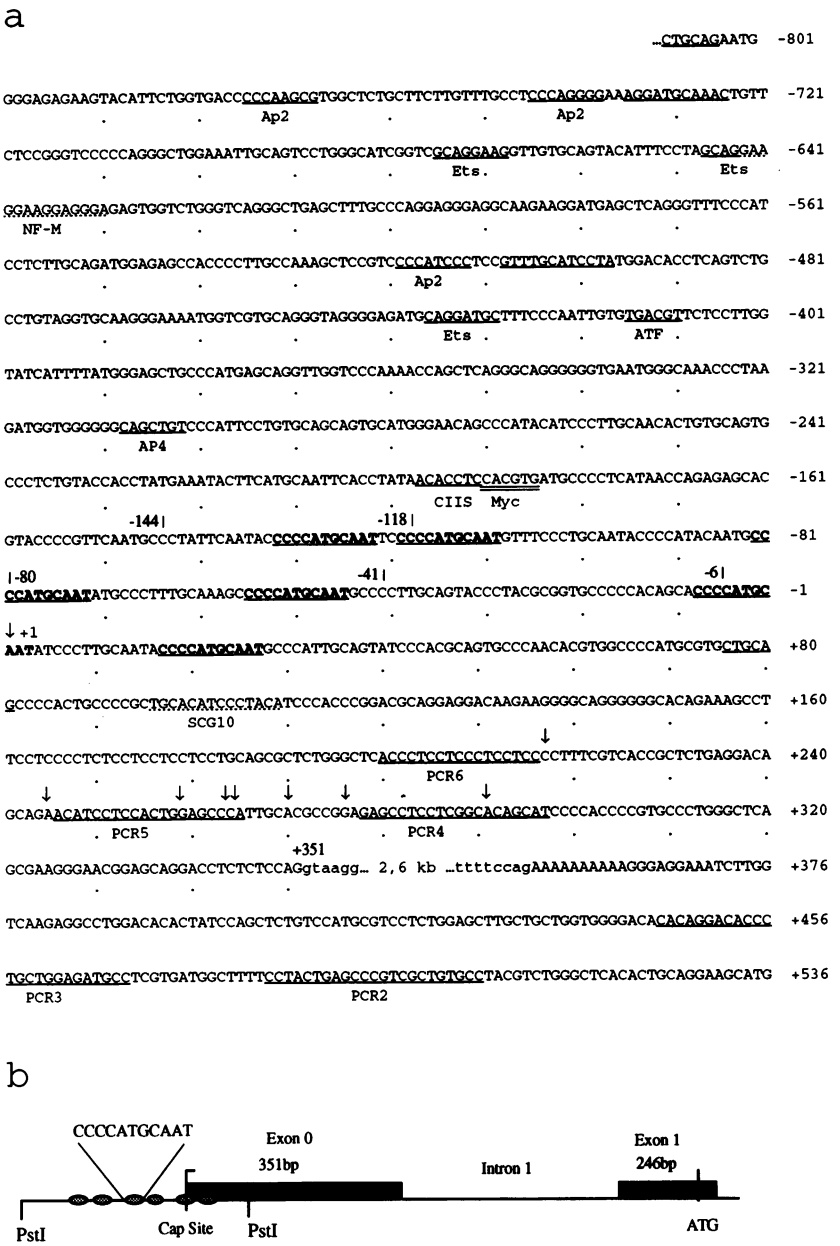

Figure 2. Nucleotide sequence and genomic structure of the 5′ region of the α2 coding gene. **a.** Nucleotide sequence of the 5′ flanking region. Transcription iniation sites are indicated by arrows. The most 5′ start site is designated +1. The sequence of the oligonucleotides used for the first PCR amplification, and for Southern blotting are underlined. Sequences conserved in NF-M or in SCG10 are underlined with a dashed line. The repeated Oct-like 11 nucleotide sequence is underlined and typed in bold. Intronic sequences are typed in lower case. Sequences with high homology to known protein-binding domains are underlined. An Oct-like inversely repeated sequence is also underlined (nt−519 and −736). The ATG starting at +534 is trhe initiator codon as determined by Nef *et al.* (17).

chick α2 subunit is 182 bp longer than previously described (17) (see figure 2b). In addition, although the genomic structure of several vertebrate nAchR subunits has been described, none of these genes has been shown, to our knowledge, to contain an untranslated exon [see however (22), and (23)]. The sequence of 1kb 5′ flanking sequence is shown in figure 2a. Examination of the 5′ upstream sequence (Figure 1a) reveals the presence of several consensus DNA-binding elements: Ap2 (3−8, 24), three Ets binding sites [nt −675, nt −647 and nt −436; (24−27)], an ATF binding site [nt −415; (28)], an Ap4 binding site [nt −308; (29)], a Myc binding site [nt −200; (26, 27, 30)], a CIIS element [nt −207; (31)] as well as six weakly degenerated octamer sequences (see below). No TATA or CAAT boxes were found. The presence of numerous DNA-binding sequences lends support to the hypothesis that at least part of the α2 gene promoter is contained within this sequence.

Silencing activity in the flanking region

Analysis of the expression pattern of the various neuronal nAchR subunit genes reveals that the α2 subunit gene displays the most restricted expression pattern (15, 16). From the onset, it appeared unlikely that such a stringent regulation would be conferred only

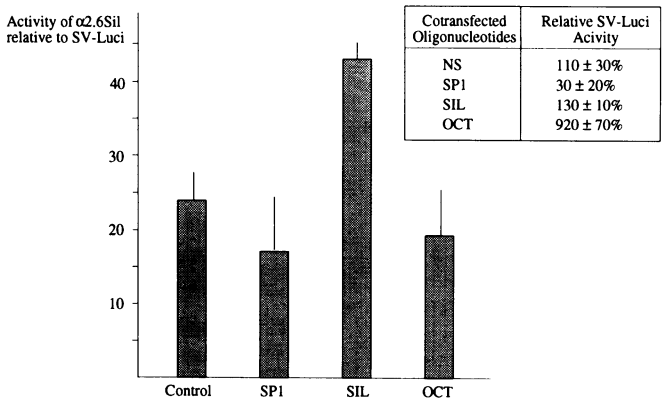

Figure 3. Relative activity of α2.6sil cotransfected with different oligonucleotides. In these experiments, both SV-Luci and αsil plasmids where transfected with the same oligonucleotides. The oligonucleotides used for each cotransfection are indicated beneath the graph. The units represents the activity of α2.6sil as a percentage of the activity of SVLuci. Control: both α2.6sil and SV-Luci plasmid were cotransfected with the oligonucleotide NS. Inset, activity of SV-Luci cotransfected with the oligonucleotides.

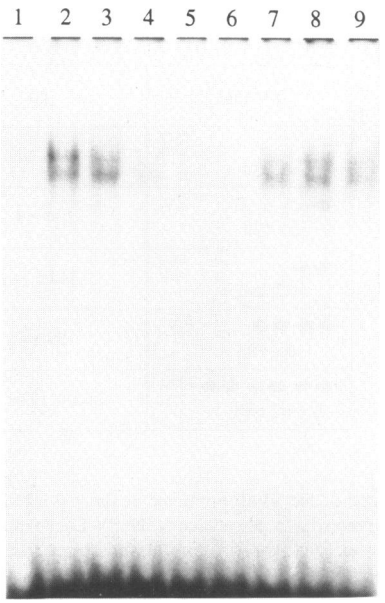

Figure 4. Characterization of the SIL binding protein and competition experiments. Autoradiogram of the mobility shift assay. Lane 1: no extract. Lane 2: no competitor. Lane 3–6. oligonucleotide SIL was added in 2, 10, 50 and 100 fold molar excess respectively. Lane 7–9: oligonucleotide OCT, SP1 and NS were used as competitor in 100 fold molar excess.

by positive regulatory mechanisms. We thus searched for negative cis-acting elements within the 5′ region of the α2 gene. Fibroblasts NIH3T6 (32), pheochromocytoma PC12 (33) and SVLT (34) cell lines were used as models of non-neuronal, peripheral and central neuronal cells respectively. Using the polymerase chain reaction with α2-specific primers, we confirmed that none of these cell lines expressed α2 mRNA (data not shown). This was expected as the nAchR α2 subunit gene is expressed only in a few brain regions (15, 35) and these cell lines have been isolated from regions that do not express α2.

We first showed that neither 6,8 kb upstream from exon 1 nor a shorter 891 bp *Pst*I/*Pst*I fragment (nt −810 to nt +81, see figure 2) was able to drive the expression of a reporter gene in these three cell lines (not shown). We next analysed the ability of α2 flanking sequences to repress transcription of a heterologous promoter. A plasmid (α2.6Sil) was constructed containing the 891bp (figure 2b) in both orientations upstream of the SV40 promoter using Luciferase as a reporter gene. The results presented in figure 5 were obtained with the α2 gene fragment in the sense orientation, similar results were obtained with α2 sequences in the antisense orientation (figure 5, result between parenthesis). Figure 5 shows that the expression of the α2.6Sil construct in 3T6 fibroblasts was approximately 4 times lower than the expression of a control plasmid containing the SV40 promoter upstream of the luciferase gene (SV-Luci). An equivalent decrease in luciferase activity was observed when a 7kb *BamHI*/*Kpn*I α2 fragment ending ≈60 bp downstream of exon 1 (17) was inserted upstream of the SV40 early promoter (not shown). From these experiments we conclude that (i) *cis*-acting DNA elements located within the 891bp *Pst*I/*Pst*I fragment are the only negative elements present within the 7 kb upstream of exon 1 (other regulatory elements might be present within this fragment but might not be active in our system), and (ii) these elements repress transcription irrespective of orientation or position (in the 7kb fragment, the 891 bp fragment is located ≈3kb upstream from the SV40 promoter). The 891bp *Pst*I/*Pst*I fragment thus by definition contains a silencer. These results cannot be due to an occlusion promoter effect (36, 37) since the α2 fragment is not active by itself in these cells.

An Oct-like tandem repeat of 11 nucleotide is involved in the silencing activity

If a trans-acting protein interacts with the α2 promoter to produce the silencing activity, we should be able to enhance the SV40 promoter activity by titrating out this protein. Cotransfection of the plasmid with oligonucleotide carrying the binding site for the putative repressor protein were performed for this purpose. To test the ability of double stranded oligonucleotides to affect transcription, we first cotransfected SV-Luci along with oligonucleotide containing the SP1 binding site, the Octamer sequence, the Oct-like 11-mer sequence, or a nonspecific sequence (described in Materials and Methods as oligonucleotide NS). In the nucleus, these oligonucleotides should specifically titrate the proteins, and thus act on the SV40 activity only if a corresponding binding site is present in the SV40 promoter. The boxed data in Figure 3 represent the ability of cotransfected oligonucleotide to interfere with the SV-Luci activity. As expected, transcription is significantly reduced (≈3 times) by the SP1 oligonucleotide (38), enhanced by the octamer sequence (39, 40), and not significantly affected by either SIL or NS oligonucleotide. However, although SP1 sites are essential for SV40 promoter activity (38), competition did not abolish the promoter activity entirely. This could be explained by the fact that we measured the average expression of the luciferase gene. Some transfected cells may have only been transfected by plasmids and no oligonucleotides. Another explanation is that the SP1 proteins may have not been completely titrated out by the oligonucleotides.

Analysis of the 5′ non-coding sequence reveals an 11 nucleotide motif (CCCCATGCAAT, underlined and typed in bold in figure 2a) that is repeated six times in the −130/+30 region of the gene. As mentioned, this 11-mer motif is homologous to the Oct binding site. The octamer motif (ATGCAAAT) has been identified in the regulatory region of many genes and often behaves as a

	Fibroblasts	PC12	SVLT undifferentiated	SVLT differentiated
SV-Luci	100	100	100	100
— SV α.0Sil	94 ± 12	ND	ND	ND
SV α2.1Sil	134 ± 8	262 ± 46	118 ± 31	101 ± 4
SV α2.2Sil	344 ± 14	273 + 61	168 ± 9	148 ± 21
SV α2.4Sil	199 ±40	218 ± 46	121 ± 9	69 ± 8
SV α2.6Sil	25 ± 4 (23 ± 5)	33 ± 2	80 ± 2	59 ± 2

Figure 5. Repression of the SV40 promoter by deleted α2 sequences. In the α2 sequences, the circles represent the sequence CCCCATGCAAT. The activity of the constructs is expressed as a percentage of the SV-Luci expression in that cell type. Shown are the mean and standard error of the mean for at least three independent experiments performed with two or more plasmid preparations. ND: not done.

transcriptional activator (41, 42). Some data show, however, that octamer-related sequences act as repressors of immediate-early HSV gene transcription (3). Furthermore, a neuronal form of Oct-2 can repress the activity of an octamer-containing promoter (9, 43). We therefore set out to examine the role of these elements by cotransfection experiments. Activity of SV-Luci and α2.6Sil were compared after cotransfection with oligonucleotides containing either an SP1 site, an octamer sequence (OCT) or the 11 nucleotide motif (SIL).

Cotransfection of the α2.6Sil and SV/Luci plasmid with oligonucleotides revealed interesting properties of the Oct-like sequence (figure 3). Indeed, the activity of α2.6Sil was significantly derepressed when it was transfected with oligonucleotide SIL. This demonstrates that the 11 nucleotide motifs located in the 5′ region are responsible for at least part of the silencing activity. The activity of the α2.6Sil plasmid did not significantly change upon cotransfection with either the SP1 or OCT oligonucleotide. This further confirms that the competition between SIL and α2.6Sil plasmid is specific.

Analysis of nuclear SIL binding proteins

Gel shift assays were performed on nuclear extracts from fibroblasts cells using the SIL oligonucleotides as a probe (figure 4) in order to characterize the nuclear proteins that interact with the 11 nucleotide sequence. Two distinct bands were detected when no competitor oligonucleotide was added (figure 4 lane 2), revealing two complexes between nuclear proteins and the oligonucleotide SIL. The slowly migrating complex was entirely competed by a 50 fold molar excess of unlabeled oligonucleotide (lane 5) whereas the fast migrating band was still slightly visible when the cold oligonucleotide was added in a 100 fold molar excess (lane 6). When binding competition was performed with 100 fold molar excess of either unrelated oligonucleotides (SP1, NS, Figure 4, lane 8 and 9) or control oligonucleotide containing octamer motif (lane 7), no decrease in specific signal was observed. Hence DNA-binding proteins detected in fibroblasts have a sequence specificity distinct from the ubiquitous Oct. Conversely, excess 11-mer motif did not compete for binding with Oct-1 to the control octamer sequence (not shown).

Deletions reveal an enhancer activity of the Oct-like sequences

Higher resolution mapping of the silencer elements was achieved by progressive deletion of the 3′ region of the α2.6Sil construct (from nt −144 to nt +76) (Figure 5). The region between nt −810 and nt −144 exhibited no silencing activity in fibroblasts (*cf.* activity of α2.0Sil, figure 5). This was not surprising since the α2.0Sil plasmid does not carry any of the 11 nucleotide sequences. Moreover, this rules out repression of transcription by any described DNA binding motif in the −810/−144 region. Thus, the active DNA elements are located in the flanking region of the gene (from nt −144 to nt +76) and most probably include only the previously described 11 nucleotide motifs. We next constructed deleted plasmids carrying 4 (α2.4Sil), 2 (α2.2Sil), or 1 (α2.1Sil) 11 nucleotide motifs (ending respectively at nt −41, −80, −118 and −144). These plasmids were then transfected into fibroblasts. Unexpectedly, progressive deletions of the repeated sequences revealed an increased expression of the SV40 promoter over control level (figure 5). Silencing or enhancing activity of the 11 nuclotide sequence seems to be dependant upon the total number of motifs present in the plasmid. A single motif has a significant enhancing activity (see the relative activity of α2.1Sil). Moreover, two 11 nucleotide elements promote a strong enhancement of the transcriptional activity of the SV40 promoter (relative activity of α2.2Sil). When two additional motifs are present on the α2 fragment (for a total of 4) a significant reduction in transcriptional activity is observed (compare relative activity of α2.2Sil and α2.4Sil). Yet, four motifs are still able to enhance transcriptional activity of the SV40 promoter (relative luciferase activity of α2.4Sil is still >100%). Thus, tandem repetition of enhancer sequences seems to be paradoxically responsible for the silencing activity of the α2 gene.

Eleven nucleotide sequences in neuronal cell lines

To further analyse the function of the α2 silencer in neurons, we transfected the α2.6Sil and the deleted constructs into two neuronal cell lines (SVLT, (34) and PC12(33)) and compared the luciferase activity of these constructs to the activity of the control SV-Luci plasmid. Results are presented in figure 5. In both cell lines, the α2.6Sil plasmid shows less luciferase activity

than the control plasmid. Thus, the silencer also represses the activity of an heterologous promoter in this neuronal context, but its activity seems to be variable from one cell line to another. Interestingly, α2.2Sil is still the most active plasmid suggesting that the 11 nucleotide sequence also has enhancer activity in neurons. This indicates that the 11 nucleotide motif has similar regulatory properties in neuronal and in non neuronal cells. We have also transfected a primary neuronal culture (cerebellar granular cells) and observed that the 11 nucleotide motif also behaves as an enhancer or silencer as a function of the number of motifs carried by the same DNA fragment (data not shown). Furthermore, transfection experiments using the SVLT cell lines show that the pattern of relative activity remains unchanged upon neuronal differentiation (see figure 5).

DISCUSSION

Structural analysis of the 5′ terminus of the chick α2 mRNA

Expression of the α2 gene is restricted in chick brain to the Spl nucleus. Even in this small diencephalic nucleus, the abundance of the α2 mRNA is low (see (15−17), data reported here). Transcribed α2 message cannot be detected in Northern blots either from mRNA extracted from total chick brain or from chick diencephalon. Since the expression of this mRNA seems to be highly restricted, we have set out to characterize transcriptional mechanisms that underlie cell-specific expression of α2 in the central nervous system. We have amplified primer extension products after ligation of an oligonucleotide to the 3′ end of the first strand of cDNA using T4 RNA Ligase. This approach allowed us to characterize a novel exon and to map the 5′ terminus of the α2 transcript to a site located ≈3kb upstream of the first coding exon.

More detailed sequence analysis of this region reveals several consensus motifs for DNA binding proteins, *viz.* Ap2 (24), Ets (24−27), ATF (29), CIIS (31), Myc (26, 27, 30) as well as a number of weakly degenerated motifs (Oct, Myc, Myd). Moreover, we have compared the α2 flanking sequence to other known neuronal flanking sequences. We found two sequences sharing high homology with the chicken middle-molecular weight neurofilament gene promoter (from nt −643 in the α2 gene and from nt −236 in NF-M (44), 92% homology shared by 13 nucleotides) or with the proximal regulatory region of the SCG10 gene (from nt +96 in α2 and from nt +73 in SCG10, (7), 94% homology shared by 17 nucleotides), see below

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α2: −654    TTTCCTAGCAGGAAGGAAGGAGGGAG
NF-M: −247  GGGGAAAGGGTGAAGGAAGGAGTGTA
α2: +90     CCCCGCTGCACATCCCTACATCCCAC
SCG10: +67  CGAGCGTGCACATCCCTACAATGGCT
```

These two sequences may have important regulatory properties. These observations suggest that at least a portion of the α2 promoter is located in the −1028 flanking nucleotides described in figure 2a. The lack of TATA and CAAT boxes is not surprising as an increasing number of genes with such features are currently being found (5, 45, 46). Moreover, these promoters typically have several transcription start sites, as shown for the α2 subunit gene.

The presence of a non coding exon in a gene encoding an AchR subunit has never been described in vertebrates, but is known in Drosophila (22, 47). In vertebrates, the first four exons of the AchR subunits genes have the same boundaries in muscle or brain (13, 48), but the muscle nAchR genes have no exon upstream of exon 1. At this stage, it is not known whether this 'exon 0' is a general feature of the neuronal nAchR gene family or if it is limited to the α2 coding gene. Analysis of the flanking region of the α7 subunit gene, shows that this gene does not share such a structure, suggesting some specific role in the α2 subunit gene function (23).

Mechanism of silencing

As a first step toward identifying mechanisms underlying the restricted expression of the nAchR α2 coding gene in the brain, we have described negative regulatory elements present in the flanking region of the gene. We have shown that six repeated Oct-like motifs are distributed in the 5′ flanking region of the gene and are responsible for silencing activity. The repressing activity of the negative elements is likely to be due to cooperation between these six 11 nucleotide motifs. One of the repeated motifs is located in the transcribed region and this type of organization has been described for many genes (49, 50). Although each 11 nucleotide sequence is highly homologous to the octamer motif, our experiments show that the nuclear protein(s) involved in the silencing activity has a different binding specificity than that of the Oct proteins. This was confirmed by gel shift experiments and *in vitro* competition experiments. Moreover, transfection experiments with deleted plasmids carrying less than six 11 nucleotide motifs revealed that the silencing activity can be paradoxically reversed into an enhancing activity when four, two or one 11-nucleotide sequence are placed upstream of the control promoter. Several hypotheses can be proposed to account for such unusual behaviour of the deleted plasmids: 1. the flanking sequences surrounding the sequences can act to specify the physiological function of the motif, 2. the silencing activity observed after transfection is the functional consequence of the binding of a complex set of nuclear proteins to the DNA motifs; changing the spatial organisation of these DNA motifs could modify the binding properties and the cooperation of the nuclear proteins and thus their function, 3. the six 11 nucleotide motifs could titrate an activator protein present in limiting amounts and therefore inhibit its activity. The resulting activity of the whole DNA fragment could be a silencer activity. However this third hypothesis was not confirmed by cotransfection experiments since the cotransfected oligonucleotides actively titrate the nuclear proteins without increasing the silencing activity.

Are silencers involved in gene expression specific for different neuronal types?

In general, the information available on the genetic mechanisms governing neuronal gene expression is still rather limited (1, 2, 51). Growing numbers of *in vivo* studies on transgenic mice have revealed defined patterns of expression of transgenes controlled by various lengths of undefined flanking or intronic sequences (8, 52−57). *In vitro* studies have provided more precise characterisation of *cis*-acting elements involved in gene expression of different neuronal types (44, 45, 58−61). Increasing number of studies also described negative regulatory mechanisms governing expression of broadly expressed neuronal gene such as N-CAM (4), the HSV genes (3, 9), sodium channel (5, 11), the SCG10 (6, 7), the neurofilament (10), or the amyloid precursor (62). Experiments described here provide evidence for the negative regulation of expression of the gene encoding the α2 nAchR subunit. This gene contains tandem negative acting sequences able to repress transcriptional activity in fibroblasts

and neurons. At this stage, the activity of the silencer has not yet been tested in neurons from the SpL, because this nucleus cannot be easily dissected, and its cells are difficult to purify. However, an attractive hypothesis which still remains to be tested, is that the negative regulation of $\alpha 2$ gene transcription contributes to the highly restricted pattern of $\alpha 2$ gene expression. *In vivo* studies with mutated genes carrying no silencer should allow us to test for this possibility, and to gain new insights into the physiological role of this silencer. The approach used for the analysis of the compartmentalized expression of the nAchR genes in the developing innervated muscle (63) may thus validly be extended to the development of the more complex patterns of gene expression in the brain.

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REFERENCES

1. Struhl, K. (1991) *Neuron* **7**, 177–181.
2. He, X.and Rosenfeld, M. G. (1991) *Neuron* **7**, 183–196.
3. Kemp, L. M., Dent, C. L. and Latchman, D. S. (1990) *Neuron* **4**, 215–222.
4. Hirsh, M.-R., Gaugler, L., Deagostini-Bazin, H., Bally-Cuif, L. and Goridis, C. (1990) *Mol. Cell. Biol.* **10**, 1959–1968.
5. Maue, R. A., Kraner, S. D., Goodman, R. H. and Mandel, G. (1990) *Neuron* **4**, 223–231.
6. Mori, N., Schoenherr, C., Vandenbergh, D., J. and Anderson, D. J. (1992) *Neuron* **9**, 45–54.
7. Mori, N., Stein, R., Sigmund, O. and Anderson, D. J. (1990) *Neuron* **4**, 593–594.
8. Wuenschell, C. W., Mori, N. and Anderson, D. J. (1990) *Neuron* **4**, 595–602.
9. Lillycrop, K. A., Dent, C. L., Wheatley, S. C., Beech, M. N., Wood, N. N. and Latchman, D. S. (1991) *Neuron* **7**, 381–390.
10. Shneidman, P. S., Bruce, J., Schwartz, M. L. and Schlaepfer, W. W. (1992) *Molecular Brain Research* **13**, 127–138.
11. Kraner, S. D., Chong, J. A., Tsay, H.-J. and Mandel, G. (1992) *Neuron* **9**, 37–44.
12. Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S. and Patrick, J. (1986) *Nature* **319**, 368–374.
13. Couturier, S., Bertrand, D., Matter, J.-M., Hernandez, M.-C., Bertrand, S., Millar, N., Valera, S., Barkas, T. and Ballivet, M. (1990) *Neuron* **5**, 847–856.
14. Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E. S., Swanson, L. W., Heinemann, S. and Patrick, J. (1988) *Science* **240**, 330–334.
15. Daubas, P., Devillers-Thiery, A., Geoffroy, B., Martinez, S., Bessis, A. and Changeux, J.-P. (1990) *Neuron* **5**, 49–60.
16. Morris, B. J., Hicks, A. A., Wisden, W., Darlison, M. G., Hunt, S. P. and Barnard, E. A. (1990) *Molec. Brain Res.* **7**, 305–315.
17. Nef, P., Oneyser, C., Alliod, C., Couturier, S. and Ballivet, M. (1988) *EMBO J.* **7**, 595–601.
18. Frohman, M. A., Dush, M. K. and Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8998–9002.
19. Dumas Milnes Edwards, J.-B., Delort, J. and Mallet, J. (1991) *Nucl. Acids Res.* **19**, 5227–5232.
20. Bailey, J. M.and Davidson, N. (1976) *Anal. Biochem.* **70**, 35–46.
21. Jackson, I. J. (1991) *Nucleic Acids Research* **19**, 3795–3798.
22. Sawruk, E., Hermans-Borgmeyer, I., Betz, H. and Gundelfinger, E. (1988) *FEBS Lett.* **235**, 40–46.
23. Matter-Sadzinski, L., Hernandez, M.-C., Roztocil, T., Ballivet, M. and Matter, J.-M. (1992) *EMBO. J.* **11**, 4529–4538.
24. Faisst, S.and Meyer, S. (1992) *Nucl. Acids Res.* **20**, 3–26.
25. Wasylyk, B., Wasylyk, C., Flores, P., Begue, A., Leprincet, D. and Stehelin, D. (1990) *Nature* **250**, 814–817.
26. Gutman, A.and Wasylyk, B. (1991) *Trend Genet.* **7**, 49–54.
27. Ghysdeal, J.and Yaniv, M. (1991) *Curr. Op. Cell Biol.* **3**, 484–492.
28. Hurst, H. C., Totty, N. F. and Jones, N. C. (1990) *Nucl. Ac. Res.* **19**,.4601–4609.
29. Hu, Y., Luscher, B., Admon, A., Mermod, N. and Tjian, R. (1990) *Gene and Dev.* 1741–1752.
30. Låscher, B.and Eisenman, R. N. (1990) *Genes and Dev.* **4**, 2025–2035.
31. Savagner, P., Miyashita, T. and Yamada, Y. (1990) *J. Biol. Chem.* **265**, 6669–6674.
32. Todaro, G. J.and Green, H. (1963) *J. Cell. Biol.* **17**, 299–313.
33. Greene, L. A.and Tischler, A. S. (1982) *Adv. Cell Neurobiol.* **3**, 373–414.
34. Evrard, C., Borde, I., Marin, P., Galiana, E., Prémont, J., Gros, F. and Rouget, P. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3062–3066.
35. Wada, E., Wada, K., Boulter, J., Deneris, E., Heinemann, S., Patrick, J. and Swanson, L. W. (1989) *J. Comp. Neurol.* **284**, 314–335.
36. Kadesh, T.and Berg, P. (1986) *Mol. Cell Biol.* **6**, 2593–2601.
37. Proudfoot, N. J. (1986) *Nature* **322**, 562–565.
38. Barrera-Saldana, H., Takahashi, K., Vigneron, M., Wildeman, A., Davidson, I. and Chambon, P. (1985) *EMBO J.* **4**, 3839–3849.
39. Gruda, M. C.and Alwine, J. C. (1991) *J. Virol.* **65**, 3553–3558.
40. Ondek, B.and Herr, W. (1991) *J. Virol.* **65**, 1596–1599.
41. Schöler, H. R. (1991) *Trends Genet.* **7**, 323–329.
42. Kemler, I., Bucher, E., Seipel, K., Muller-Immergluck, M. M. and Schaffner, W. (1991) *Nucleic Acids Research* **19**, 237–242.
43. Dent, C. L., Lilycrop, K. A., Estride, J. K. and Latchman, D. S. (1991) *Molecular and cellular biology.* **11**, 3925–3930.
44. Zopf, D., Dineva, B., Betz, H. and Gundelfinger, E. D. (1990) *Nucl. Ac. Res.* **18**, 521–529.
45. Sehgal, A., Patil, N. and Chao, M. (1988) *Mol. Cell. Biol.* **8**, 3160–3167.
46. Sauerwald, A., Hoesche, C., Oschwald, R. and Kilimann, M. W. (1990) *J-Biol-Chem* **265**, 14932–14937.
47. Bossy, B., Ballivet, M. and Spierer, P. (1988) *EMBO J.* **7**, 611–618.
48. Buonanno, A., Mudd, J. and Merlie, J. P. (1989) *J. Biol. Chem.* **264**, 7611–7616.
49. Lamb, N. J. C., Fernandez, A., Tourkine, N., Jeanteur, P. and Blanchard, J.-M. (1990) *Cell* **61**, 485–496.
50. Tamura, T.and Mikoshiba, K. (1991) *FEBS Let.* 75–78.
51. Korner, M., Rattner, A., Mauxion, F., Sen, R. and Citri, Y. (1989) *Neuron* **3**, 563–572.
52. Forss-Petter, S., Danielson, P. E., Catsicas, S., Battenberg, E., Price, J., Nerenberg, M. and Sutcliffe, J. G. (1990) *Neuron* **5**, 187–197.
53. Vidal, M., Morris, R., Grosveld, F. and Spanopoulou, S. (1990) *The EMBO Journal* **9**, 833–840.
54. Kaneda, N., Sasaoka, T., Kobayashi, K., Kiuchi, K., Nagatsu, I., Kurosawa, Y., Fujita, K., Yokoyama, M., Nomura, T., Katsuki, M. and Nagatsu, T. (1991) *Neuron* **6**, 583–594.
55. Vandaele, S., Nordquist, D. T., Feddersen, R. M., Tretjakoff, I., Peterson, A. C. and Orr, H. T. (1991) *Gene and Dev.* **5**, 1136–1148.
56. Wirak, D. O., Bayney, R., Kundel, C. A., Lee, A., Scangos, G., Trapp, B. D. and Unterbeck, A. J. (1991) *EMBO J.* **10**, 289–296.
57. Miskimins, R., Knapp, L., Dewey, M. and Zhang, X. (1992) *Brain. Res. Dev. Brain. Res* **65**, 217–221.
58. Chelly, J., Hamard, G., Koulakoff, A., Kaplan, J.-C., A., K. and Berwald-Netter, Y. (1990) *Nature* **344**, 64–65.
59. Boyce, F. M., Beggs, A. H., Feener, C. and Kunkel, L. M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1276–1280.
60. Messing, A., Behringer, R., Hammang, J., Palmiter, R., Brinster, R. and Lemke, G. (1992) *Neuron* **8**, 507–520.
61. Yoon, S. O.and Chikaraishi, D. M. (1992) *Neuron* **9**, 55–67.
62. Izumi, R., Yamada, T., Yoshikai, S., Sasaki, H., Hattori, M. and Sakaki, Y. (1992) *Gene* **112**, 189–195.
63. Changeux, J. P., Babinet, C., Bessereau, J. L., Bessis, A., Cartaud, A., Cartaud, J., Daubas, P., Devillers-Thiery, A., Duclert, A., Hill, J. A., Jasmin, B., Klarsfeld, A., Laufer, R., Nghiem, H. O., Piette, J., Roa, M. and Salmon, A. M. (1990) *Cold Spring Harbor Symp Quant* **55**, 381–396.
64. de Wet, J., Wood, K. V., DeLuca, M., Helinski, D. R. and Subramani, S. (1987) *Mol. Cell. Biol.* **7**, 725–737.
65. Screiber, E., Matthias, P., Müller, M. M. and Schaffner, W. (1989) *Nucl. Acid Res.* **17**, 6419.