Multiple Functional Sp1 Domains in the Minimal Promoter Region of the Neuronal Nicotinic Receptor α5 Subunit Gene*

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The α5 subunit is a component of the neuronal nicotinic acetylcholine receptors, which are probably involved in the activation step of the catecholamine secretion process in bovine adrenomedullary chromaffin cells. The promoter of the gene coding for this subunit was isolated, and its proximal region was characterized, revealing several GC boxes located close to the site of transcription initiation (from −111 to −40). Deletion analysis and transient transfections showed that a 268-bp base pair region (−111 to +155) gave rise to −77 and 100% of the maximal transcriptional activity observed in chromaffin and SH-SY-5Y neuroblastoma cells, respectively. Site-directed mutagenesis of five different GC motifs indicated that all of them contribute to the activity of the α5 gene, but in a different way, depending on the type of transfected cell. Thus, in SH-SYS-5Y cells, alteration of the most promoter-proximal of the GC boxes decreased α5 promoter activity by −50%, whereas single mutations of the other GC boxes had no effect. In chromaffin cells, by contrast, modification of any of the GC boxes produced a similar decrease in promoter activity (50–69%). In both cell types, however, activity was almost abolished when four GC boxes were suppressed simultaneously. Electrophoretic mobility shift assays using nuclear extracts from either chromaffin or SH-SYS-5Y cells showed the specific binding of Sp1 protein to fragment −111 to −27. Binding of Sp1 to the GC boxes was also demonstrated by DNase I footprint analysis. This study suggests that the general transcription factor Sp1 plays a dominant role in α5 subunit expression, as has also been demonstrated previously for α3 and β4 subunits. Since these three subunits have their genes tightly clustered and are expressed in chromaffin cells, probably as components of the same receptor subtype, we propose that Sp1 constitutes the key factor of a regulatory mechanism common to the three subunits.

Nicotinic acetylcholine receptors (nAChRs) are members of the gene superfamily of neurotransmitter-gated ion channels.

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** This abbreviations used are: nAChRs, nicotinic acetylcholine receptors; bp, base pair(s); EMSA, electrophoretic mobility shift assay.

1 These multimeric receptors are heteromers or, in some cases, homomers of subunits (α2–α9 and β2–β4) that exhibit well defined and restricted expression patterns in vivo (1). The diversity of neuronal nAChRs arises, at least in part, from the different combinations of subunits able to form functional nAChRs (3). Thus, it is clear that their differential expression affects the electrophysiological and pharmacological properties of the resultant receptors (4). Moreover, potential changes in subunit expression in response to modulation of synaptic function might have important consequences on the signals transduced by nAChRs (5).

To understand how the regional and developmental expression of nAChR subunits is controlled, we have started to analyze the transcriptional mechanisms that regulate expression of the nAChR subunits expressed in chromaffin cells of the bovine adrenal gland. This cell type represents a relevant and accessible model in which to study a particular neuronal nAChR subtype with a well defined function. Previously, we cloned the bovine α3 (6), α5 and β4 (7), and α7 (8) subunits of neuronal nAChRs, which are expressed in chromaffin cells as components of the two nAChR subtypes typically present at the peripheral nervous system (9). We have also shown that nAChRs formed by α7 subunits are differentially expressed in adrenergic cells (10), probably as the result of transcriptional regulation, whereas α3, α5, and β4 subunits have a less restricted distribution in adrenergic and noradrenergic cells (7). Interestingly, the α3, α5, and β4 subunit genes have been found tightly clustered in the avian (11) and mammalian (12) genomes, with the α3 and α5 genes contiguous and having opposed transcription polarity. A number of studies have concentrated on the transcriptional regulation of the α3 and β4 subunits. Deneris and co-workers (13, 14) have shown that the POU domain factor SCIP/Tst-1 is able to activate the α3 subunit promoter, probably as a consequence of protein-protein interactions at the level of the basal transcriptional machinery. Furthermore, an enhancer located in the 3′-untranslated exon of the β4 subunit (15, 16), at the β4/α3 intergenic region, activates transcription from the α3 and β4 subunit promoters in a cell type-specific manner, possibly via a novel ETS domain factor, Pet-1, whose expression is almost restricted to the adrenal medulla (17). Considerable effort has also been dedicated to the transcriptional regulation of the β4 subunit. Thus, Gard-
ner and co-workers (18) have shown that Purα interacts with a 19-bp element in the β4 promoter. In addition, Sp1 (19) and Sp3 (20) transactivate the β4 promoter in a synergistic way, an effect possibly mediated by heterogenous nuclear ribonucleo-
that at least five positive regulatory elements exist in the α5 promoter proximal region. These elements, all of them GC boxes, were shown to interact with Sp1. Since the α3 promoter is also the target of Sp1 (22), we suggest the possible involvement of this transcription factor in a regulatory mechanism common to the α3, β4, and α5 subunits.

**EXPERIMENTAL PROCEDURES**

**Isolation and Analysis of the 5′-Flanking Sequence of the α5 Subunit—**A cDNA probe containing 152 bp of the 5′-end of exon 2 was used to screen a bovine genomic library in EMBL-3 SP6/T7 (Clontech, Heidelberg, Germany) as described by the manufacturer. Overlapped bacteriophage clones were purified and characterized. 

**RNase Protection—**Poly(A)^+^ RNA was directly selected from a lysate of bovine adrenal medulla by oligo(dT)-Dynabeads (Dynal, Oslo, Norway) and used in the RNase protection experiments. Probes were generated with SP6 and T7 polymerases (Boehringer Mannheim) as indicated by the manufacturer. Protected protection experiments were performed using an RNase protection kit with several other labeled RNAs of known size, which were also synthesized (8). Several overlapping bacteriophage clones were obtained with the pBR328-5LUC (see Fig. 3) or p111a5LUC (see Fig. 6) plasmid in the same cell type. When comparing α5, α7, and β4 promoters (see Fig. 4), representative constructs for each of the subunits, giving the maximal promoter activity, were chosen. They are indicated in the corresponding figure legends.

**Electrophoretic Mobility Shift Assay—**Crude nuclear extracts were prepared from chromaffin and SH-SYS-Y cells as described by Schreiber et al. (26). The DNA fragment corresponding to region −111 to −27 was obtained by digesting pBluescript subclones with EcoRI-KpnI and end-labeled by Klenow filling with [α-^32^P]dATP. The DNA-protein binding reaction volumes were 20 μl containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM diithiothreitol, 10% glycerol, 5 μg of bovine serum albumin, 2 μg of poly(dI-dC)-dAT (Pharmacia, Uppsala, Sweden), Biocytin, 2 μg of nuclear extract protein, and 20,000 cpm of [α-^32^P]pLabeled probe. Reactions were incubated for 10 min at room temperature; the labeled probe was added; and the incubation was continued for an additional 20-min period. For competition studies, the nuclear extract was incubated with the competing probe prior to the labeled probe during 20 min. Supershift assays were performed by preincubating nuclear extracts with 2 μl of antibodies against different transcription factors (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit IgG (Sigma) for 3 h on ice before probe addition.

**Western Blotting—**Ten μg of nuclear proteins/lane was separated by 10% SDS-polyacrylamide gel electrophoresis. Western blotting was carried out as described by Towbin et al. (27). After the transfer, nitrocellulose membranes were blocked overnight at 4 °C with 5% dry milk in Tris-buffered saline and incubated in the same way with the anti-Sp1 antibody (1:500) in phosphate-buffered saline and 5% dry milk. After incubation with the secondary antibody at room temperature for 2 h, the bands were visualized by a chromogenic reaction (Sigma Fast, nitro blue tetrazolium, Sigma).

**DNase I Footprinting—**The sense strand corresponding to region −111 to −37 of the α5 promoter was end-labeled by Klenow filling with [α-^32^P]dATP. Assays were performed with the Sure Track Footprinting kit (Pharmacia) according to the manufacturer's instructions. Recombinant Sp1 was incubated with the radiolabeled double-stranded fragment (~25,000 cpm) using the binding reaction conditions described above in the EMSA experiments (except for the absence of EDTA and the presence of 2.5 mM MgCl2). Immediately following the 30-min incubation at room temperature, the DNA and 1 μl of the anti-Sp1 antibody (1:500) in phosphate-buffered saline and 5% dry milk. After incubation with the secondary antibody at room temperature for 2 h, the bands were visualized by a chromogenic reaction (Sigma Fast, nitro blue tetrazolium, Sigma).

**Plasmid Constructions—**All α5 promoter-luciferase gene fusions were made in the pGL2-Basic vector (Promega, Madison, WI), introducing in its polylinker, upstream of the luciferase gene, the suitable α5 promoter fragment or fragments with restriction enzymes and directly cloned into pGL2-Basic or subcloned first in pBluescript and then transferred to pGL2-Basic. The vector pGL2-Control, which expresses the luciferase gene under the regulation of the SV40 promoter and enhancer sequences, was used to check luciferase activity. Deletion analysis of the most promoter-proximal region was performed by generating polymerase chain reaction fragments with suitable sense oligonucleotides and an antisense primer (5′-CTTTAT-GTTTGGGCGTCTCC-3′) that anneals to the pGL2-Basic vector downstream of the site of transcription initiation. The basic strategy for site-directed mutagenesis of the different elements in region −111 to −40 of the α5 promoter (see Fig. 6) consisted of the following steps. (a) We performed polymerase chain reaction (25 cycles at 94 °C for 10 s, 62 °C for 30 s, and 72 °C for 30 s) amplification of p111a5LUC (or its single or double mutants when double or quadruple mutants were desired, respectively) with appropriate mutagenic primers in the sense orientation, which generated restriction sites useful for further mutagenic constructions and to confirm mutagenesis. We used the same oligonucleotide mentioned above as antisense primer. The introduced mutations are indicated in lowercase letters in Fig. 6. (b) The corresponding linearized templates (in the pSPT18 vector, Boehringer Mannheim). A 431-bp BglII-PvuII fragment of the α5 gene that included 163 bp 5′ to exon 1 and 249 bp downstream in the same exon was subcloned into the BamHI and HindIII sites of pSPT18. After linearization of the plasmid with EcoRI, a probe of 479 nucleotides was synthesized with SP6 polymerase and, with a cRNA sense fragment of 366 nucleotides was synthesized by linearizing the template with HindII and using T7 polymerase. This cRNA protected a fragment of 358 nucleotides upon RNase treatment. Parallel experiments were carried out with a smaller probe that overlapped the first one. For this purpose, a 341-bp fragment of the α5 gene that included 319 bp of the 5′-end of the first probe (downstream of the HindII site mentioned above) was also subcloned into pSPT18. After linearization of the plasmid with EcoRI, a probe of 403 nucleotides was synthesized with SP6 polymerase. The same control sense cRNA used above then produced a protected fragment of 328 nucleotides when used instead of bovine adrenal RNA (see Fig. 2 for further explanations). RNase protection experiments were performed using an RNase protection kit (Boehringer Mannheim) as indicated by the manufacturer. Protected fragments were separated on a 7.5% urea and 8% acrylamide gel along with several other labeled RNAs of known size, which were also synthesized and used for calibration.

**RESULTS**

**Structure of the 5′-Flanking Region of the α5 Subunit Gene—**To examine the requirements for α5 subunit transcription, its promoter region was isolated and analyzed. A bovine genomic library was screened, and several overlapping clones were isolated. Clone λA5–21 contained ~16 kilobases of bovine genomic sequence including exons 1 and 2 and ~1.6 kilobases of 5′-flanking region. This region was further subcloned and sequenced (Fig. 1A). Comparison of this sequence to a database and sequencing of known transcription factors revealed the major features of the neuronal Preganglionic region of the α5 gene: the lack of a TATA box and the presence of several GC boxes, all of them concentrated into ~110 bases located 5′ to the transcription initiation site. Two perfect Sp1 consensus sites (5′-G/TGGGCGG/GGGC) were present within this GC-rich region. Additional sites with one mismatch, resembling perfect
consensus sequences, were also observed for Egr-1, Ap-1, Oct-1, and Ap-2. It is interesting to note the presence of a contiguous direct repeat of two 52- and 42-bp monomers (Fig. 1B), each of them containing one of the perfect Sp1 sites and two other elements to which this transcription factor could potentially bind.

The 5'-end of a5 mRNA was mapped by RNase protection analyses (Fig. 2). A 479-residue antisense riboprobe (Fig. 2, Probe 1) yielded two main protected fragments of 250 and 249 bases. The major one mapped transcription initiation to an adenosine present in a group of three (position 1, black arrow in Fig. 1) and located 40 bp downstream of the GC-rich repeats. To improve precision in the determination of the transcription initiation site, a second overlapping probe was used (Fig. 2, Probe 2). In this case, two protected fragments were also observed, which were 157 and 156 bp long and mapped transcription initiation to the same place. Therefore, these were tentatively considered the main transcription initiation sites. Other initiation sites may exist, as this is a typical feature of promoters without TATA boxes, and in fact, minor protected fragments of smaller size and intensity were also observed.

**Functional Analysis of the a5 Subunit Promoter**—A series of constructs was generated to determine the regions of the a5 subunit promoter that contributed to its maximal activity (Fig. 3). These constructs were introduced into SHSY-5Y and chromaffin cells, a neuroblastoma cell line and a primary cell culture, respectively, that express the a5 subunit endogenously (7, 28). In SHSY-5Y cells, the construct containing 111 bp of a5 promoter sequence plus 155 bp of 5'-noncoding region (p111a5LUC) showed the maximal activity. This construct covered all Sp1 sites already mentioned. A shorter construct (p65a5LUC) with only two Sp1 sites showed a 50% decrease in promoter activity, whereas further 5'-deletions that removed all the Sp1 sites (p17a5LUC, p156a5LUC) were inactive. In chromaffin cells, results were similar, suggesting that sequences in the minimal promoter, between 111 and 39 bp upstream of the start site of transcription, appear to be critical for basal transcription of the a5 subunit gene in transient transfection assays. Reporter constructs larger than p111a5LUC did not show significant changes in relative luciferase activity when expressed in chromaffin cells. However, in SHSY-5Y cells, a small but constant decrease was observed.
from one construct to the next larger one, being maximal with p752a5LUC, which showed a marked decrease in activity (36%). The largest construct tested (p1412a5LUC), however, exhibited increased activity (73%). Therefore, in SHSY-5Y but not chromaffin cells, elements predominantly located between –600 and –750 with respect to the transcription initiation site have a negative effect on α5 promoter activity.

We next compared the α5 promoter with the SV40 viral promoter, present in the vector pGL2-Control, and the nAChR α7 and β4 subunit promoters (Fig. 4). These subunits are also expressed in chromaffin and SHSY-5Y cells (7, 8, 28), probably forming part of the same (β4) or different (α7) nAChR subtype in which α5 is present. In addition, the β4 subunit gene is located in the same gene cluster as the α5 subunit gene (11, 12), as indicated previously. In chromaffin cells, the maximal nAChR subunit promoter activity corresponded to the α5 subunit, which accounted for about one-half of the activity shown by the SV40 promoter and was 10- and 2-fold higher that the activities of the β4 and α7 promoters, respectively. By contrast, in SHSY-5Y cells, the activities of the three subunit promoters were similar and about one-fifth the activity of the SV40 promoter. Therefore, it appears that some cell-specific differences exist among the three nAChR subunit promoters, which in any case are weaker than the SV40 viral promoter.

The α5 promoter constructs already tested in chromaffin and SHSY-5Y cells were also transfected into COS cells in an attempt to find elements that would confer cell specificity. Although this cell line does not express nAChRs, the pattern of promoter activity (Fig. 5) was similar to the one found in chromaffin cells. Thus, p111a5LUC showed the maximal activity. A shorter construct (p650a5LUC) with only two Sp1 sites showed a 44% decrease in promoter activity, whereas further 5′-deletions that removed all the Sp1 sites (p390a5LUC, p+705a5LUC, and p+560a5LUC) were inactive. Constructs larger than p111a5LUC did not show significant changes in luciferase activity relative to this construct. Interestingly, whereas in SHSY-5Y and chromaffin cells, the α5 promoter was clearly weaker than the SV40 promoter, present in the pGL2-Control vector, in COS cells, it was ~2-fold stronger than the viral promoter. Therefore, no cell-specific elements were found in the α5 promoter region analyzed.

Initially, four GC boxes present between –111 and –40, two of them representing perfect Sp1 consensus sites and another two with one mismatch, were chosen for mutagenic analysis. Single mutations of the four GC boxes indicated that all of them contribute to the activity of the α5 gene, but in a different way, depending on the type of transfected cell (Fig. 6). Thus, when the most promoter-proximal of the GC boxes was altered (site 1, Fig. 6A), α5 promoter activity in SHSY-5Y cells decreased to ~50% of that observed for the parent construct (p111a5LUC), whereas single mutations of the other three had no effect (Fig. 6B). The double mutant of sites 1 and 2 produced an activity close to the obtained for site 1, whereas the simultaneous mutation of sites 3 and 4 did not have any effect, further suggesting that sites 2–4, alone or in pairs, are not required for optimal promoter activity. Surprisingly, activity was almost abolished when all four GC boxes were altered simultaneously, which may indicate that sites 2–4, together with site 1, do integrate a whole synergistic mechanism required for basal promoter activity. By contrast, in chromaffin cells, mutation of any of the GC boxes produced a similar decrease in promoter activity (50–69%), and double mutants showed a further decline. Finally, as happened with SHSY-5Y cells, the simultaneous mutation of the four GC motifs produced the maximal decrease in activity.

Characterization of the Regulatory Elements Present at –111 to –27 of the α5 Promoter by EMSA—DNA fragments carrying wild-type promoter region –111 to –27 were labeled and incubated with nuclear extracts from chromaffin and SHSY-5Y cells (Fig. 7). Several retarded bands were observed in both cases (Fig. 7A, lanes 2 and 4). Some of them were common to both extracts (● and ○). Recombinant Sp1 produced a main retarded complex (lane 6), coincident in position with one of those observed with nuclear extracts (●). Larger complexes were also observed with recombinant Sp1, probably as the result of the simultaneous binding of two or more Sp1 mole-

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**FIG. 2. Determination of the α5 subunit gene transcription initiation site(s).** The 5′-end of the α5 subunit mRNA was mapped by RNase protection using two α5 probes whose structures are illustrated in the lower part of the figure. T, undigested probes; 479 (Probe 1, lane 1) and 403 (Probe 2, lane 5) bases; U, probes digested in the presence of yeast tRNA (lanes 2 and 6); C, probes digested in the presence of a sense cRNA control, yielding protected fragments of 358 (Probe 1, lane 3) and 328 (Probe 2, lane 7) bases; P, protected fragments using 7.5 μg of bovine adrenal medulla poly(A)+ mRNA (lanes 4 and 8). The sizes of several RNA fragments used for calibration of the gel are indicated to the left of each panel, whereas the sizes of the protected fragments are to the right.
Antibody supershift analysis was employed in an attempt to identify the proteins producing the retarded bands. One of the main complexes (●) was retarded by an anti-Sp1 antibody (arrowhead, lanes 3, 5, and 7), whereas no supershift was observed with antibodies against several transcription factors able (Sp3, Egr-1, and Ap-2) or not (Myc, Max, USF1, and USF2) to bind to GC boxes (data not shown). In addition to the main band, several minor ones were also displaced by the anti-Sp1 antibody when using chromaffin cell nuclear extracts (compare lanes 2 and 3). They are probably degradation products of Sp1 protein, which keep the capacity of binding to the DNA probe and being recognized by the antibody. The band labeled with an open circle (○) was more prominent in SHSY-5Y extracts (lanes 4 and 5), but we were not able to identify it. Competition experiments confirmed the specificity and identity of the complex formed by Sp1 (Fig. 7B).

Thus, an excess of unlabeled probe neutralized the formation of all complexes (lanes 10 and 13), whereas a synthetic oligonucleotide containing an Sp1 consensus sequence abolished the formation of the Sp1 complex (lanes 11 and 14). Western blot analysis of SHSY-5Y and chromaffin nuclear proteins indicated that Sp1 protein was indeed expressed in these cells (Fig. 7C). Two proteins bands (~95–100 kDa) were detected with anti-Sp1 antibodies in chromaffin (lane 15) and SHSY-5Y (lane 16) nuclear extracts, showing the same size as that previously described for Sp1 polypeptides (29). The amount of Sp1 protein detected in both extracts was approximately the same, and both bands had the same intensity. By contrast, recombinant Sp1 (lane 17) showed predominantly one species.

Interactions of Sp1 with the GC Boxes in the α5 Proximal Promoter—Recombinant Sp1 protein was used in DNase I foot-
Sp1 Regulatory Elements of Nicotinic Receptor Gene Expression

A

\[-111\text{Sp1GAGCCGG}
\]

\[\text{Sp1TGGGCCTAAGAGGTAAGTGGGCGGGG...}\]

B

**FIG. 6. Sites 1–4 are functional elements required for α5 subunit gene expression.** A, the proximal region of the α5 subunit promoter (nucleotides –111 to –40) is depicted with the putative regulatory elements underlined. This region contains putative binding sites for transcription factor Sp1 (boxed). Several nucleotides of each potential element were mutated as indicated below the sequence to yield constructs analyzed in transfection experiments (B). B, the name of each mutant construct indicates the element(s) that have been altered (also crossed out in the scheme). Plasmids were transfected into SHSY-5Y and chromaffin cells, and activities were measured. Luciferase activity was normalized to values obtained with the p111α5LUC construct. Data are expressed as described in the legend to Fig. 3.

printing (Fig. 8) to document the preferences of this transcription factor for the four GC-rich elements previously characterized in transfection studies (Fig. 6). When using the same probe of the EMSA, Sp1 protected several domains (Fig. 8, lanes 3 and 4): the largest one corresponded to GC boxes 1 and 2 (large filled box on the left), whereas two additional domains were observed, especially at the highest Sp1 concentration (lane 4). One of them corresponded to GC box 4, and the other overlapped the 3′-half of GC box 3 and a downstream GC-rich element. This element was not as close to the Sp1 consensus binding sequence as the ones previously analyzed and, for this reason, was not included in our previous functional characterization (Fig. 6), but, given the DNase protection results, it was further characterized.

To confirm that an additional Sp1 site exists in the proximal region of the α5 subunit promoter, EMSA experiments were performed with recombinant Sp1, and the DNA fragment carrying wild-type promoter region –111 to –27 and the corresponding fragment mutated at the four original Sp1 sites were compared (Fig. 9). At low Sp1 concentration, a retarded band was observed with the wild-type probe (lane 3). The same band was observed in the case of the mutant probe, but it was very faint (lane 7). A higher Sp1 concentration increased the intensity of the retarded band with the wild-type probe and even induced the formation of complexes of larger size (lane 4), probably as the result of Sp1 binding to more than one site within a single DNA molecule. In the case of the quadruple mutant, the faint band was more intense (lane 8), but no larger complexes were observed, suggesting that this DNA fragment still has one intact Sp1-binding site. That this Sp1 site is the one immediately downstream of site 3 was demonstrated by mutating it (Fig. 10A) and performing EMSA experiments (Fig. 10, B and C). The combination of the same wild-type probe, already used, with nuclear extracts from chromaffin cells yielded the typical pattern in which a prominent band (Fig. 10B, lane 2, ●) was observed. The formation of this band, previously demonstrated to be antigenically related to Sp1 (Fig. 7B), was competed by the unlabeled probe (Fig. 10B, lanes 3 and 4), but not by the same probe mutated at the five Sp1 sites (lanes 5 and 6). Moreover, when the mutant fragment was used as the labeled probe, the prominent Sp1 band was not observed (lane 8). Finally, recombinant Sp1 was unable to produce retarded bands with the quintuple mutant (Fig. 10C, lanes 13 and 14), contrary to what occurred with the quadruple mutant (Fig. 9).

The functional significance of the fifth GC box was examined in chromaffin cells by transfecting constructs, made in the context of p111α5LUC (see Fig. 6), in which this element was mutated individually or in combination with the other four. In principle, this element seems to be unable to activate transcription by itself alone since the mutation of the other four, leaving this site intact, decreased activity to very low levels (Fig. 6). Thus, the contribution of this element to transcriptional activation, if any, should be expected in a cooperative manner, as it happens with the other four previously characterized. In fact, the single mutation had an effect on promoter activity similar to the one observed for the other single mutants (50% of the activity of p111α5LUC). The quintuple mutant had 14% of promoter activity (relative to p111α5LUC), close to the value obtained for the quadruple mutant (14%). Therefore, it seems that this element mediates the transcriptional activation of the α5 promoter in a collective way, combined with the other GC boxes.

**DISCUSSION**

Neuronal nAChRs mediate chemical synaptic transmission, probably regulating transmitter release at many synapses (reviewed in Ref. 5). A relatively large number of genes that encode nAChR subunits have been identified (1), having distinct, although overlapping patterns of expression in the central and peripheral nervous systems. This diversity constitutes, in large part, the molecular basis on which the variety of nAChR properties and neural responses to acetylcholine is established. The nAChR α5 subunit is widely expressed in the
peripheral nervous system (7, 30, 31) in combination with α3, β4, and, in some cases, β2 subunits (9, 32). Moreover, it has been demonstrated recently that the α5 subunit combines with α4, β2, α3, and β4 subunits (33, 34) in heterologous expression systems, modifying nAChR channel properties. Thus, some information has been obtained regarding α5 subunit function, but nothing was known until now about the cis-elements and trans-acting factors that regulate its expression.

The core promoter region of the α5 subunit does not contain TATA and CAAT boxes, but it does have several GC-rich domains, a feature found in the promoters of the α2 (35), α3 (13), β4 (20), and α7 (10, 36) subunits. Moreover, the structure of the α5 subunit promoter appears highly organized with two direct repeats (Fig. 1B), each containing crucial elements for promoter activity. From 5′-end deletion analysis (Fig. 3), we determined that the region located between nucleotides –111 and +115 was necessary for the basal promoter activity detected in SHSY-5Y and chromaffin cells. A large loss in promoter activity was observed when 72 bp were deleted from the 5′-end (compare p39α5LUC with respect to the larger construct p111α7LUC) (Fig. 3). These constructs were also transfected into COS cells (Fig. 5), which do not endogenously produce α5 subunits, and were even more potent in luciferase activity

![Fig. 7. Binding of cell nuclear proteins to the proximal region of the α5 subunit promoter.](image)

**Fig. 7.** Binding of cell nuclear proteins to the proximal region of the α5 subunit promoter. A, the DNA fragment corresponding to region –111 to –27 was used as a gel mobility shift probe in the presence of 2 μg of crude chromaffin (CR; lanes 2 and 3) or SH-SY-5Y (SH; lanes 4 and 5) cell nuclear extracts or recombinant Sp1 (0.2 footprint units, 1 footprint unit/μl) (lanes 6 and 7). Lane 1 is probe run in the absence of protein extracts. Lanes 3, 5, and 7 represent complexes obtained upon adding anti-Sp1 antibodies (Ab). A prominent band (●) was supershifted by Sp1 antibodies (arrowhead). Other complexes were also observed (○ and □), but were not displaced by any of the antibodies tested (see “Results”). B, the gel mobility assay was run using DNA fragment –111 to –27 as the labeled probe and nuclear extracts from chromaffin (lanes 9–11) and SH-SY-5Y (lanes 12–14) cells. Lanes 9 and 12 were without added competitor (Comp.; –). Competitor DNA fragment –111 to –27 (PR; lanes 10 and 13) and an oligonucleotide with a consensus site for Sp1 (Sp1; lanes 11 and 14) were added in 100-fold excess. Lane 8 is probe run in the absence of protein extracts. C, nuclear proteins from chromaffin (lane 15) and SH-SY-5Y (lane 16) cells and recombinant Sp1 (lane 17) were separated by SDS-polyacrylamide gel electrophoresis on a 10% resolving gel. Following Western blotting with Sp1 antibodies, two molecular species of ~97 kDa were detected. N.P., nuclear protein.

![Fig. 8. DNase I analysis of Sp1 interactions within the proximal region of the α5 subunit promoter.](image)

**Fig. 8.** DNase I analysis of Sp1 interactions within the proximal region of the α5 subunit promoter. The sense strand of the same DNA fragment (–111 to –27) used in EMSA was end-labeled by Klenow filling. The gel-purified fragment was incubated with 0.4 (lane 3) or 1 (lane 4) footprint unit (fpu) of Sp1 and treated with DNase I. A control reaction (Cont.; lane 2) was performed in a similar manner, using bovine serum albumin as the protein component. Following digestion with DNase I, fragments were resolved on an 8% denaturing polyacrylamide gel. A Maxam-Gilbert A/G chemical sequencing reaction was included as a reference ladder (lane 1). To the left is a sequence summary of the protected domains, with numbers corresponding to the putative regulatory elements in Fig. 6.
when using a viral promoter as a reference. Therefore, it is possible that promoter elements needed for cell-specific expression are not included within the promoter fragments used in this study. Nevertheless, the action of negative elements seems evident in SHSY-5Y cells, as demonstrated by the slight but gradual decrease in the activity of larger constructs. Elements located between –660 and –752 appear responsible for the largest decline in activity and could be involved in a silencing mechanism. It is evident that this mechanism might not be totally effective in SHSY-5Y cells since the largest construct (p14090a5LUC) regains activity, and in fact, SHSY-5Y cells endogenously express a5 subunits. However, it could be of relevance in tissues where a5 subunit expression is repressed, although, at least in COS cells, such a mechanism does not seem to be operative.

The most remarkable feature in the deleted region, between –111 and –39, was the presence of at least four closely located Sp1 sites (labeled 1–4 in Fig. 6A). Therefore, these elements appeared to be suitable candidates for controlling promoter activity. Consequently, when these elements were simultaneously mutated in the context of p111a7LUC, promoter activity was almost suppressed, thus suggesting that they play a crucial role in the transcriptional regulation of the a5 gene. Moreover, DNase I footprinting with purified Sp1 protein revealed regions of extended protection, which contained the four Sp1-binding sites and also an additional GC-rich element, close to site 3 (Fig. 8). This site, in contrast to the others, which have no or one mismatch with respect to a perfect Sp1 consensus binding sequence, has two mismatches and, for this reason, was not considered in the initial analysis of promoter activity. However, EMSA (Fig. 10) and gene reporter experiments confirmed the footprinting data indicating that Sp1 can also bind to this site with a functional significance. We postulate that Sp1 contacts the DNA through multiple interactions to form a higher order complex that would accomplish transcription activation at a higher level (38). The individual contribution of each Sp1 element to the formation of this hypothetical higher order complex appears differently defined, depending on the cell context. Thus, in SHSY-5Y cells, the only critical site is the most promoter-proximal, perhaps because the possibility of
physically direct interactions between Sp1 at this site and the basal transcriptional machinery. The importance of the others only emerges if they are simultaneously altered, suggesting that Sp1 acts synergistically as a whole on the complete region. By contrast, in chromaffin cells, transcriptional activity seems to depend on each of these sites, as alteration of any of them produces a decrease in promoter function. If we assume that the formation of a higher level complex requires several Sp1 sites, then the possibility exists that in SHSY-5Y but not chromaffin cells, other elements can substitute for the Sp1 sites that were gradually altered by mutagenesis. This substitution would be not possible, however, when all of the Sp1 sites are modified. This assumption is supported by the fact that the double mutant of sites 3 and 4 shows only an insignificant decrease (11%) with respect to the activity of the parent construct p111a5LUC (Fig. 6B), whereas the deletion construct p65a5LUC, which lacks the region between –111 and –66, including sites 3–5, but also other sequences, shows a marked decrease (48%) in activity. Perhaps some additional element present in the deleted region becomes effective in SHSY-5Y cells when some but not all of the Sp1 sites are altered. Whatever the mechanism, the primary conclusion of this study is that the basal activity of the α5 subunit promoter is defined by an array of Sp1-binding elements to which this transcription factor can bind to activate transcription in a synergistic manner.

In addition to silencing mechanisms, the α5 subunit promoter, which appears strongly dependent on a ubiquitously transcription factor like Sp1, may be regulated in a tissue-specific manner by this factor in several ways. (a) Availability of Sp1, as levels of this protein have been shown to vary among different tissues (39). (b) Competition with other members of the Sp family that could bind to the same elements. For instance, Sp3 acts with Sp1 in a concerted way to transactivate the nAChR β4 subunit (21). However, we have not found binding of Sp3 to the α5 promoter in EMSA of chromaffin and SHSY-5Y nuclear extracts. In other cases, Sp3 has been shown to repress the action of Sp1 (40–42). (c) Formation of heteromeric complexes between Sp1 and other proteins. Protein p107, a member of the retinoblastoma family of proteins, binds Sp1 and represses Sp1-dependent transcription (43). Sp1 also interacts with the RelA subunit of transcription factor NF-κB (44) and the cellular protein YY1 (45). (d) Action of Sp1 as a physical link between proximal and distal promoter elements via DNA bending (46) or looping (47). Finally, we would like to emphasize the role that Sp1 could play in a potential common regulatory mechanism of the α3, β4, and α5 subunits. As has been already mentioned, these subunits compose the predominant nAChR subtype in the peripheral nervous system (7, 9, 32) and have their genes clustered in the genome (11, 12). Therefore, a concerted regulation of the expression of the three subunits is plausible. Given that Sp1 (and other members of the Sp family) seems to play a crucial role in the regulation of the α3 (22), β4 (20, 21), and α5 (this study) subunits, we propose that this transcription factor, either directly or through other proteins that can regulate its activity, may play the coupling role in the coordinated regulation of the three subunits. Other factors, or perhaps a differential regulation of Sp1, specific to a determined neuronal cell subset, would be responsible for the independent regulation of each subunit, which also must take place, given the dissimilar pattern of expression of these subunits in certain places (48, 49).