Transcriptional Repression by a Conserved Intronic Sequence in the Nicotinic Receptor α3 Subunit Gene*

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The genes encoding the nicotinic acetylcholine receptor α3, α5, and β4 subunits are genomically clustered. These genes are co-expressed in a variety of cells in the peripheral and central nervous systems. Their gene products assemble in a number of stoichiometries to generate several nicotinic receptor subtypes that have distinct pharmacological and physiological properties. Signaling through these receptors is critical for a variety of fundamental biological processes. Despite their importance, the transcriptional mechanisms underlying their coordinated expression remain to be completely elucidated. By using a bioinformatics approach, we identified a highly conserved intronic sequence within the fifth intron of the α3 subunit gene. Reporter gene analysis demonstrated that this sequence, termed “α3 intron 5,” inhibits the transcriptional activities of the α3 and β4 subunit gene promoters. This repressive activity is position- and orientation-independent. Importantly, repression occurs in a cell type-specific manner, being present in cells that do not express the receptor genes or expresses them at very low levels. Electrophoretic mobility shift assays demonstrate that nuclear proteins specifically interact with α3 intron 5 at two distinct sites. We propose that this intronic repressor element is important for the restricted expression patterns of the nicotinic receptor α3 and β4 subunit genes.

Neuronal differentiation is a consequence of extrinsic and intrinsic regulatory cascades that ultimately act to regulate, both positively and negatively, gene expression. This process yields mature neurons that express a limited set of genes encoding proteins that perform specific functions (1–9). Part of this repertoire of genes is that of encoding proteins required for neuronal signaling, including neurotransmitter biosynthetic enzymes and their cognate neurotransmitter receptors. Acetylcholine is an excitatory neurotransmitter that interacts with both ionotropic and G-protein-coupled receptors. Signaling through ionotropic nicotinic acetylcholine (nACh)2 receptors is involved in a variety of behaviors ranging from muscle contraction to memory formation (10–13). In addition, acetylcholine and its receptors play important roles in neural development (14–17). Not surprisingly, compromised signaling through nACh receptors is implicated in a number of neurological disorders (18–35). Thus, understanding the molecular details underlying nACh receptor expression will shed light on various aspects of neural development and function as well as contribute to our understanding of several neuropsychological conditions.

In the nervous system, nACh receptors are encoded by a conserved family of genes consisting of at least 12 members, α2–α10 and β2–β4 (10, 11, 36–39). A significant effort has been put forth to elucidate transcriptional mechanisms controlling expression of the nACh receptor genes (40, 41). We and others have focused on the regulation of a genomic cluster of receptor genes, those encoding the α3, α5, and β4 subunits (42). As these three subunits are co-expressed in many neurons throughout the nervous system (43–48), the clustering of their genes raises interesting possibilities regarding regulation of their expression. For example, it is possible that they are coordinately expressed via a common regulatory mechanism. However, although the genes exhibit overlapping expression patterns, these patterns are not identical either temporally or spatially suggesting that in addition to a possible common regulatory mechanism, these genes are most likely subject to independent regulation as well. In this regard, it is important to note that a growing body of evidence suggests that the α3 and β4 subunit genes may be coordinately expressed, whereas the α5 gene may be independently regulated (47, 49–51). A number of laboratories, including our own, have identified several cis-acting transcriptional regulatory elements (52–66) and trans-acting regulatory factors (59, 65, 67–77) that are involved in the expression of the clustered nACh receptor subunit genes. The majority of the regulatory elements thus far described are involved in activating transcription and are located upstream of the nACh receptor genes that they regulate. We report here the identification and initial characterization of a highly conserved sequence in the fifth intron of the α3 subunit gene (referred to hereafter as “α3 intron 5”). We show that this intronic sequence is capable of repressing the transcriptional activities of the α3 and β4 subunit gene promoters and does so in a cell type-specific manner. In addition, we demonstrate that this repressive activity occurs independently of its position relative to the promoter and is also orientation-independent. Finally, we show that the intronic sequence directly interacts with nuclear proteins.
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**MATERIALS AND METHODS**

*Amplification of α3 Intron 5 from Genomic DNA*—The PCR was used to amplify α3 intron 5 from genomic DNA. Two primers were designed for this purpose. The sequence of the upper primer was 5’-CGCCTGACACAGGGATAAGGGTCAT-3’, and the sequence for the lower primer was 5’-GGCGGGATCCCAGTTTAGGTAGGGCTCA-3’. The underlined sequences indicate XhoI (upper) and KpnI (lower) restriction endonuclease sites added to the primers to facilitate cloning of the amplified product. The following PCR protocol was used: 5 min at 94 °C (1 cycle), 1 min at 94 °C, 45 s at 53 °C, and 70 s at 72 °C (1 cycle); then 30 s at 94 °C, 30 s at 58 °C, and 70 s at 72 °C (30 cycles); and finally, 7 min at 72 °C. The expected and observed size for the amplified product was 1,078 bp.

*Plasmids*—The 1,078-bp α3 intron 5 PCR product was digested with XhoI and KpnI and cloned into XhoI/KpnI-digested pBluescript II SK(+) (Stratagene, La Jolla, CA) resulting in the construct pBSA3I5XK.

The α3 and β4 promoter regions were subcloned into the pGL3-Basic luciferase vector (Promega, Madison, WI). The α3 promoter sequence (nucleotides −2,036 to +64, relative to the major α3 transcriptional start site) was isolated from pX1A3HS (74) as follows: pX1A3HS was linearized with HindIII generating a 3’-overhang that was subsequently blunt-ended using the Klenow fragment of DNA polymerase I. The promoter sequence was liberated from the vector by digestion with XhoI and then subcloned into XhoI-digested pG3B4A3IU. The Klenow fragment of DNA polymerase I was used to amplify intron 5 from genomic DNA. Two primers were designed for this purpose. The sequence of the upper primer was 5’-CGCGGATCCCACAGGGATAAGGGTCAT-3’, and the sequence for the lower primer was 5’-GGCGGGATCCCAGTTTAGGTAGGGCTCA-3’. The underlined sequences indicate XhoI (upper) and KpnI (lower) restriction endonuclease sites added to the primers to facilitate cloning of the amplified product. The following PCR protocol was used: 5 min at 94 °C (1 cycle), 1 min at 94 °C, 45 s at 53 °C, and 70 s at 72 °C (1 cycle); then 30 s at 94 °C, 30 s at 58 °C, and 70 s at 72 °C (30 cycles); and finally, 7 min at 72 °C. The expected and observed size for the amplified product was 1,078 bp.

**TABLE 1**

Sequences of primers used for creating deletions of α3 intron 5

| Primer name | Sequence | Paired primer for PCR | Deletion created |
|-------------|----------|-----------------------|-----------------|
| U10A3IB     | 5’-CGCGGATCCCACAGGGATAAGGGTCAT-3’ | U10A3IB | pG3B4A |
| L36A3IB     | 5’-CGCGGATCCCACAGGGATAAGGGTCAT-3’ | L36A3IB | pG3B4B |
| L105A3IB    | 5’-CGCGGATCCCACAGGGATAAGGGTCAT-3’ | L105A3IB | pG3B4C |
| L725A3IB    | 5’-CGCGGATCCCACAGGGATAAGGGTCAT-3’ | L725A3IB | pG3B4D |
| L105A3IB    | 5’-CGCGGATCCCACAGGGATAAGGGTCAT-3’ | L105A3IB | pG3B4C |
| L725A3IB    | 5’-CGCGGATCCCACAGGGATAAGGGTCAT-3’ | L725A3IB | pG3B4D |

The α3 intron 5 sequence was liberated from the vector by digestion with XhoI and then subcloned into XhoI-digested pG3A3ID yielding the construct pG3A3ID. This was followed by subcloning the α3 and β4 promoters into pG3A3ID as follows: pX1A3HS was linearized with HindIII generating a 3’ promoter sequence was isolated from pBSKA31XK by first linearizing with KpnI and then using the exonuclease activity of T4 DNA polymerase to create a blunt end. The α3 intron 5 sequence was released from the vector by digestion with XbaI. This product was subcloned into pG3A3HX, which was first linearized with MluI, treated with Klenow to blunt-end the 3’-overhang, and then digested with NheI. The new construct was named pG3A3A3IU. To subclone the intronic sequence upstream with respect to the β4 promoter, α3 intron 5 was isolated from pBSKA31XK by first linearizing with KpnI and then using the exonuclease activity of T4 DNA polymerase to create a blunt end. The α3 intron 5 sequence was released from the vector by digestion with XhoI. The blunt-end/XhoI intronic sequence was subcloned into Smal/XhoI-digested pG3B4FH generating the construct pG3B4A3IU.

To subclone β4 intron 5 into pG3A3HX upstream with respect to the α3 promoter, the intronic sequence was isolated from pBSKA31XK by first linearizing with KpnI and then using the exonuclease activity of T4 DNA polymerase to create a blunt end. The β4 intron 5 sequence was released from the vector by digestion with XbaI. This product was subcloned into pG3A3HX, which was first linearized with MluI, treated with Klenow to blunt-end the 3’-overhang, and then digested with NheI. The new construct was named pG3A3A3IU. To subclone the intronic sequence upstream with respect to the β4 promoter, α3 intron 5 was isolated from pBSKA31XK by first linearizing with KpnI and then using the exonuclease activity of T4 DNA polymerase to create a blunt end. The α3 intron 5 sequence was released from the vector by digestion with XhoI.

PCR was used to generate a set of 5’ and 3’ deletions of α3 intron 5 using pBSA3I5XK as the parental construct. Six primers were designed for this purpose (see Fig. 7 for a schematic representation of the deletions). The primer sequences and combinations are shown in Table 1. To facilitate subcloning, a BamHI recognition sequence was added to the 5’-end of the primers along with three additional nucleotides. The following PCR protocol was performed using a MyCycler thermal cycler (Bio-Rad): 5 min at 94 °C (1 cycle), 30 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C (30 cycles), followed by 7 min at 72 °C. The PCR products were subcloned into BamHI-digested pG3B4FH yielding the constructs pG3B4Ad, pG3B4Bd, pG3B4Cd, and pG3B4Dd. All constructs were verified by DNA sequencing.

**Cell Culture and Reporter Gene Assays**—Cell lines were maintained and transfected as described previously (74).
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Briefly, SN17 (78) and NIH3T3 cells (79) were transfected using a calcium phosphate procedure (Invitrogen), whereas PC12 cells (80) were transfected using a liposome-mediated approach (Lipofectamine, Invitrogen). Each cell line was transfected with the indicated test plasmid and a β-galactosidase expression vector, pRSVβGal. PC12 cells were differentiated with 100 ng/ml nerve growth factor (NGF; Upstate Biotechnologies, Inc., Lake Placid, NY) for 7 days after transfection. Cells were harvested either 48 h (SN17 and NIH3T3) or 7 days (PC12) following transfection and were assayed for luciferase (Luciferase Assay System, Promega) and β-galactosidase (Galacto-Star, Applied Biosystems, Foster City, CA) activities in a Lumimark microplate luminometer (Bio–Rad). To correct for differences in transfection efficiencies between dishes, the luciferase activity was normalized to β-galactosidase activity in that same sample. Statistical analysis was done using the Student’s t test.

**Real Time Quantitative PCR**—Real time reverse transcriptase (RT)-PCR was done using total RNA isolated from NGF-treated PC12, SN17, and NIH3T3 cells using a commercially available kit (RNasey midi kit, Qiagen, Inc., Valencia, CA). cDNA was synthesized from equal amounts of RNA (Retroscript, Ambion, Austin, TX) using primers specific for either the rodent α3 or β4 nACh receptor subunit genes. In addition to the nACh receptor genes, PCR was done with primers specific for cyclophilin for use as an internal control. The primer pairs used were as follows: α3, 5’-CGCCTGTTCAGTACCT-3’ and 5’-CTTCAGGACAGGTTT3’; β4, 5’-GAaGACAGGAATCACACTTGCC-3’ and 5’-CAGCCCGAGTGACCTT-3’; cyclophilin, 5’-AGGATACTGGTCTGGATCC-3’ and 5’-TTCCACTCTCCAAAAGCCAC-3’.

Relative quantification was determined using an ABI 7500 thermal cycler (Applied Biosystems) measuring real time SybrGreen (Bio–Rad) fluorescence. Calculations were done using the ΔΔCt method. Overall efficiencies of PCR were calculated from the slopes of the standard curves and were >95% for each primer pair. NGF-treated PC12 cells were used as a calibrator, and cyclophilin was used as an internal control. A cDNA clone for each gene was used as a positive control. Statistical analysis was done using the Student’s t test.

**Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extracts were prepared from SN17 cells using commercially available reagents (NE-PER extraction reagents, Pierce). For protein–DNA binding reactions, 0.5, 1, and 2 μg of nuclear extract were combined with binding buffer (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 5% glycerol, 2 mM dithiothreitol, 50 mM NaCl, 5 mM MgCl₂, 0.1 mM ZnCl₂, 2 μg of bovine serum albumin). The binding reactions were incubated at room temperature for 5 min and then combined with a DNA mixture containing 20 fmol of 32P-labeled DNA probe and 2 μg of poly(dI-dC). For competition experiments, specific competitors at 100-fold molar excess were added to the reaction. Following incubation at room temperature for 15 min, samples were loaded, without tracking dye, onto 4% non-denaturing acrylamide/bisacrylamide (40:1) gels in 1% TBE buffer. Electrophoresis was performed for 2–3 h at 185 V. Gels were dried under vacuum and subjected to autoradiography.

**RESULTS**

In an attempt to identify potentially important regulatory sequences within the β4/α3/α5 nACh receptor gene cluster (Fig. 1a), an alignment was done using the human and mouse genomic sequences. As shown in Fig. 1b, the expected high degree of conservation between exonic sequences was seen, particularly between the putative membrane-spanning regions. In addition, again as expected, there was relatively little conservation (<50%) between intronic sequences (Fig. 1b) with one notable exception: there was a striking degree of conservation (≈50%) between the human and mouse α3 subunit genes. The plot is an adaptation of a human–mouse comparison done using the VISTA program (98) with a window size of 100 bp and a minimal sequence identity of 75%. The 100-bp window was used as a smaller window as this optimizes alignments over regions of very high sequence identity. The x axis represents coordinates for the human sequence. Conservation of exonic sequences is indicated by the filled graph. The conservation of α3 intron 5 is shown by the open plot.

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**FIGURE 1. Conservation of an intronic sequence in the nACh receptor α3 subunit gene.** a, β4/α3/α5 nACh receptor gene cluster. The arrows indicate the directions of transcription. The expanded human and mouse α3 subunit genes are shown below the cluster. Black boxes indicate exons, and open boxes indicate intronic sequences. The gray box indicates the conserved 1,060-bp α3 intron 5 sequence. Not drawn to scale. b, alignment of the human and mouse nACh receptor α3 subunit genes. The plot is an adaptation of a human–mouse comparison done using the VISTA program (98) with a window size of 100 bp and a minimal sequence identity of 75%. The 100-bp window was used as a smaller window as this optimizes alignments over regions of very high sequence identity. The x axis represents coordinates for the human sequence. Conservation of exonic sequences is indicated by the filled graph. The conservation of α3 intron 5 is shown by the open plot.
As described below, these studies were extended to another cell line, SN17, a line derived from mouse septal cholinergic neurons (78). First, however, quantitative RT-PCR was carried out to determine the expression levels of the \( \alpha_3 \) and \( \beta_4 \) subunit genes in each of the three cell lines. As expected, NGF-treated PC12 cells express relatively high levels of both genes, whereas no expression of either gene was detected in NIH3T3 fibroblasts (Fig. 3). Interestingly, expression of the \( \alpha_3 \) and \( \beta_4 \) genes in SN17 cells was significantly lower than in NGF-treated PC12 cells (but still significantly higher than in NIH3T3 cells). Furthermore, the \( \alpha_3 \) subunit gene was expressed at relatively higher levels in SN17 cells than the \( \beta_4 \) gene (Fig. 3).

The data presented above indicate that \( \alpha_3 \) intron 5 represses \( \alpha_3 \) and \( \beta_4 \) promoter activity in a cell line (NIH3T3) that does not express the two nACh receptor subunit genes but has no effect on promoter activity in a cell line (PC12) that does express the genes. As the \( \alpha_3 \) and \( \beta_4 \) mRNA levels in SN17 cells are significantly lower than in PC12 cells, we hypothesized that \( \alpha_3 \) intron 5 may repress promoter activity in SN17 cells. We tested this idea by transfecting SN17 cells with the reporter constructs. As shown in Fig. 4, \( \alpha_3 \) intron 5 does in fact repress promoter activity in SN17 cells. Interestingly, the repression seems to be greater for the \( \beta_4 \) promoter, consistent with the mRNA results (Fig. 3). In addition, the repressor activity of \( \alpha_3 \) intron 5 appears to be position-independent as the same level of repression was seen whether the intronic sequence was placed downstream or upstream relative to the two promoters (Fig. 4). We extended this approach to test the orientation dependence of \( \alpha_3 \) intron 5 repression. The data in Fig. 5 indicate that \( \alpha_3 \) intron 5 represses \( \beta_4 \) promoter activity independent of its orientation, being equally active in either orientation. Thus, the transcriptional activity of \( \alpha_3 \) intron 5 is both position- and orientation-independent, characteristics common to many transcriptional regulatory sequences.

Taken together, the data presented above suggest the existence of cell type-specific negative transcriptional regulatory sequences in \( \alpha_3 \) intron 5. As the intronic sequence spans approximately 1000 bp, we carried out a more in-depth sequence comparison with the goal of further localizing potential regulatory sequences. The comparison was done using the human, mouse, and rat genomic sequences spanning the \( \alpha_3 \) intron 5 region. We identified three subregions that exhibit at least 81% identity between the three genomes (Fig. 6). We refer to these subregions as Hot Points 1 (spanning 81 bp), 2 (73 bp) and 3 (38 bp). The experiments that follow focus on the func-
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FIGURE 4. Position-independent repression by α3 intron 5. The indicated constructs were transiently transfected into SN17 cells and assayed for luciferase activity 2 days after transfection. Fold activity was calculated relative to the normalized luciferase activity obtained by transfecting each of the intron-less promoter/luciferase reporter constructs. The results are from three independent experiments. The error bars represent standard errors. *, p = 0.000141; **, p = 0.0001.

FIGURE 5. α3 intron 5 repression of β4 promoter activity is orientation-independent. α3 intron 5 was subcloned in both orientations upstream of the β4 promoter in a luciferase reporter vector as shown on the left side of the figure. The filled boxes indicate the promoter sequences. Each construct was transiently transfected into SN17 cells and assayed for luciferase activity 2 days after transfection. Fold activity was calculated relative to the normalized luciferase activity obtained by transfecting each of the intron-less β4 promoter/luciferase reporter construct. The results are from three independent experiments. The error bars represent standard errors. *, p = 0.000981.

containing Hot Points 1 and 2. Transcription factor-binding site analysis of the nucleotide sequence encompassing this region revealed that numerous transcription factors (>30) could potentially bind to this region (not shown). We therefore sought to further delineate potential binding sites.

To test the ability of the intronic sequence to interact directly with nuclear proteins, as would be expected for a transcriptional regulatory region, we carried out EMSA. To facilitate this analysis, the 400-bp region containing Hot Points 1 and 2 was divided into halves with each half containing one of the Hot Points. These two 200-bp fragments were used as the probes in EMSA. We could not reliably detect specific protein-DNA interactions using the Hot Point 2 probe (not shown). However, when the fragment containing Hot Point 1 was used as the probe in EMSA with SN17 nuclear extracts, we detected three predominant specific protein-DNA complexes (Fig. 8, complexes 1–3). Importantly, the amount of each complex increased with increasing protein concentration, and the complexes were eliminated when the unlabeled probe fragment (referred to as G) was included as a competitor (Fig. 8). To further localize potential binding sites, four 50-bp double-stranded fragments (referred to as g1, g2, g3 and g4) spanning the 200-bp probe were used as competitors. As shown in Fig. 8, when g1 or g3 was used as a competitors, there was no effect on protein-DNA complex formation. However, when g2 was used as a competitor, the intensity of complex 3 was significantly reduced. Similarly, use of g4 as a competitor greatly reduced the intensity of complex 1 and essentially eliminated complex 2 (Fig. 8). These results indicate that this 200-bp region of α3 intron 5 specifically interacts with nuclear proteins isolated from a cell line in which the transcriptional repression is observed and, furthermore, that the binding occurs at two distinct sites, further narrowing the binding activities to two 50-bp regions. Finally, as many transcription factors require divalent cations to bind to DNA (84), we carried out EMSA in the presence of EDTA. Interestingly, complexes 2 and 3 were virtually eliminated in the presence of EDTA, whereas complex 1 was somewhat reduced (Fig. 8) suggesting that the protein(s) present in complexes 2 and 3 are most likely distinct from that present in complex 1.

DISCUSSION

The physiological diversity of nACh receptors stems from the incorporation of five subunits into either homo- or hetero-meric structures. As at least 12 genes encode nicotinic receptor subunits expressed in the nervous system, there is the potential for a substantial number of functionally distinct receptor subtypes. The molecular details of how particular receptor subtypes are expressed in a given neuron largely remain a mystery, but regulation at the level of transcription is obviously a critical factor. The work presented above focuses upon two tightly linked nACh receptor genes, those encoding the α3 and β4 subunits. The observation that these subunits are co-expressed and co-regulated in many cell types in the central nervous system and peripheral nervous system is consistent with the hypothesis that their expression is because of coordinated transcriptional regulation. Several laboratories are actively pursuing this hypothesis, and substantial work has revealed a number
of shared regulatory features between the two genes. First, by using in vitro approaches, it has been shown that the α3, α5, and β4 promoters can directly interact with and be trans-activated by the widely expressed transcription factors Sp1 and Sp3 (56, 59, 62, 65, 70). This observation extends to the third gene in the cluster, the α5 subunit gene, which also appears to be regulated by Sp1 (73). This is interesting in that neither the α3, α5, nor the β4 promoter region contains a TATA box, and it has been postulated that the Sp factors are linked to the basal transcriptional machinery via interactions with tethering factors in order to activate transcription from TATA-less promoters (85, 86). These tethering factors may be cell type-specific. Second, the α3 and β4 promoters can be trans-activated by the cell type-specific regulatory factors, Sox10 and SCIP/Tst-1/Oct-6 (53, 74). Third, in a recent elegant study by the Deneris laboratory (66), an enhancer, referred to as β4', located in the 3′-untranslated region of the β4 subunit gene, was shown to play a critical role in vivo in the expression of the clustered genes in the adrenal gland. In addition, this group demonstrated that a conserved noncoding region, CNR4, located 20 kb pairs upstream of the β4 coding sequence, plays a key role in directing expression of the clustered genes in the pineal gland and superior cervical ganglion (66). CNR4 may also be important for cell type-specific expression of the clustered genes in the central nervous system (66). Thus, as with other systems, a picture is emerging that the coordinated expression of the clustered nACh receptor genes results from interactions between ubiquitously expressed and cell type-specific transcription factors with cis-acting elements located within the cluster. This is consistent with the so-called transcription factor combinatorial control mode in which transcription factors act in combination to control neuronal cell type specification (87, 88). Despite this progress, however, many gaps remain, particularly with respect to the mechanisms regulating central nervous system expression of the clustered receptor genes.

Using bioinformatics in an attempt to identify structurally conserved, and thus potentially functionally conserved, non-coding sequences led to the identification of α3 intron 5. Given the striking degree of sequence identity between the human, mouse, and rat α3 intron 5 sequences, we tested the hypothesis that the intronic sequence is involved in regulating transcription from the α3 and β4 subunit gene promoters. The data strongly suggest that α3 intron 5 functions as a repressor of α3 and β4 promoter activity. In fibroblasts, α3 promoter activity is decreased 40% whereas β4 promoter activity is down by 70% when α3 intron 5 is located either upstream or downstream of

FIGURE 6. Sequence alignment of the α3 intron 5 regions of human, mouse, and rat. The BLAST program (NCBI) was used to align the three sequences. Three regions of high similarity were identified: Hot Point 1 (dashed box; 84% identity between the species), Hot Point 2 (solid box; 81% identity), and Hot Point 3 (dashed/dotted box; 86% identity). Numbering is relative to the first base pair of the mouse intronic sequence.

FIGURE 7. Deletional analysis of α3 intron 5. The indicated 5' and 3' deletions were generated using PCR (see "Materials and Methods" for details) and then subcloned downstream of the β4 promoter. The resulting constructs were transiently transfected into SN17 cells. h1, h2, and h3 refer to Hot Points 1, 2, and 3, respectively. The results are from three independent experiments. The error bars represent standard errors. *, p = 0.00371; **, p = 0.0001.
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The top portion of the figure depicts the 203-bp Hot Point 1 region of α3 intron 5 used as the probe in EMSA. Below the probe are schematics of the various unlabeled competitors referred to as G, g1, g2, g3, and g4. Nuclear proteins were isolated from SN17 cells and used as the protein source for EMSA. The 1st lane is the probe without nuclear extract (N.E.). The next three lanes are EMSA using the indicated amounts of nuclear extract. The next five lanes are competition experiments using 100-fold molar excess of each unlabeled competitor and 1 μg of nuclear extract. The last lane is an EMSA done in the presence of EDTA and 1 μg of nuclear extract. Specific protein-DNA complexes are indicated by numbered arrows on the left of the gel.

The promoters. In addition, the repressive activity of α3 intron 5 is orientation-independent. These two features suggest that α3 intron 5 is a classical silencer element as has been described for numerous genes from a wide variety of organisms (89–91). Most importantly, the repressive activity is seen only in cell lines that do not express the α3 and β4 genes (NIH3T3) or express them at very low levels (SN17); the activity was not seen in α3- and β4-expressing cells (differentiated PC12 cells). Thus, α3 intron 5 appears to be a cell type-specific repression element regulating the activities of at least the α3 and β4 nicotinic receptor subunit gene promoters.

Negative transcriptional regulatory elements have been identified for other nicotinic receptor subunit genes, all of which are located upstream of their respective coding regions. For example, Ebihara et al. (92) identified an Alu repeat as well as other sequences that function as repressors of α6 promoter activity. It is not clear if their repressive activities are cell type-specific. Similarly, Bessis et al. (93) identified negative regulatory elements upstream of the α2 subunit gene. The cell type specificity of these elements is unclear, although their activity is variable from one cell line to another. This same group also identified a neuron-repressive silencer element in the promoter region of the β2 subunit gene (94). Interestingly, the neuron-repressive silencer element silences or enhances transcription depending on the cellular context within the nervous system, whereas in non-neuronal cells, it functions exclusively as a silencer. No neuron-repressive silencer element has been detected across the β4/α3/α5 gene cluster. More recently, Valor et al. (95) identified a cell type-specific 34-bp repression element in the 5′-noncoding region of the α9 subunit gene. We did not detect any significant homology between this element and α3 intron 5. Overall, these negative regulatory elements appear to be gene-specific, but all are thought to be involved in the restricted expression patterns of the nicotinic receptor subunit genes by mechanisms that remain to be elucidated.

How might α3 intron 5 exert its transcriptional regulatory effect? In light of the EMSA data, it seems likely that nuclear proteins interact with α3 intron 5 and function as transcriptional repressors. Such proteins are generally classified as passive or active repressor factors (96). The interactions of these factors with α3 intron 5 are presumably coupled in some way to the basal transcriptional machinery, possibly through further protein/nucleic acid or protein/protein interactions (97). For example, the α3 intron 5-interacting nuclear proteins may interact with general transcription factors during early stages of transcription complex assembly, thereby blocking further assembly. Another possibility is that the repressor proteins recruit repressive chromatin remodeling complexes that subsequently return the nucleosomal state of the promoter region to its pre-transcriptional form. Alternatively, the repressor may recruit a histone deacetylase to the nicotinic receptor subunit gene promoters. This could lead to decreased affinity of TFIID for the promoters and decrease the accessibility of DNA at the promoters. Finally, the orientation- and position-independent nature of α3 intron 5 repression suggests considerable structural flexibility with respect to the protein/DNA interactions and may provide a mechanism by which α3 intron 5 exerts long range effects. In any of these cases, cell type specificity must be imposed at some point given the cell type-specific activity of α3 intron 5. One way in which this may be achieved is if the proteins that interact with the intronic sequence are expressed in a spatially restricted manner. Testing this hypothesis depends on the identification of the proteins that interact with α3 intron 5, which is the subject of current investigation.

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