The minimal promoter of the human α3 nicotinic receptor subunit gene has been mapped to a region of 60 base pairs and found to contain two Sp1 sites, one of which is essential for promoter activity. DNase footprinting has revealed the presence of another region of interaction with nuclear factors (named F2) immediately downstream of the Sp1 sites. This region has been found to be functional since it is capable of stimulating the minimal promoter. The F2 protection is completely found to be functional since it is capable of stimulating interaction with nuclear factors (named F2) immediately downstream of the Sp1 sites.

By means of computer-assisted analysis, multiple putative transcriptional effects may account for at least part of the neuro-specific expression profile of the α3 gene.

The identification of the genetic mechanisms underlying the expression of neural genes is rapidly becoming an invaluable approach for developing our understanding of the generation and maintenance of distinct neural phenotypes. Neuronal cholinergic-nicotinic receptors consist of subunits whose anatomical distribution and developmental regulation represent an attractive model for addressing such crucial questions (1–3). These molecules form a family of acetylcholine-gated cation channels, expressed in the autonomic and sensory ganglia, the adrenal medulla, and in distinct areas of the central nervous system, with a quaternary structure consisting of five transmembrane subunits assembled around a central channel. Eleven distinct neuronal nicotinic subunits have been cloned so far and classified into two subfamilies of eight (α2–α9) and three β subunits (β2–β4) (1, 4). In heterologous expression systems, α7, α9, and α9 are the subunits that can form homomeric receptors, whereas α2, α3, and α4 always need to be coexpressed with β2, β3, or β4 in order to generate functional channels. The α3 subunit is abundantly expressed in the post-ganglionic neurons of the autonomic nervous system (5, 6) where it participates as a ligand-binding subunit in the formation of the ganglionic type nicotinic receptor, whose activation generates the fast excitatory post-synaptic potential (7).

In a previous characterization of the 5′-regulatory sequences of the human α3 nicotinic receptor subunit gene, we identified a 350-bp region immediately upstream of the start codon that had the structural features of a multiple start site promoter and consistent transcriptional activity in neuronal cell lines (8). By means of computer-assisted analysis, multiple putative Sp1-binding sites were localized upstream of the cluster of transcription initiation sites, in a very GC-rich region that did not contain any TATA or CAAT box (Fig. 1).

A growing body of information on promoter structures has strengthened the idea that these features are common to several genes expressed in the nervous system (9–11). In the field of nicotinic receptors, all of the promoters characterized so far (12–14) conform to these rules, with the exception of the chicken β3 (15).

It is generally believed that in TATA-less promoters and in the absence of functionally equivalent structures such as the initiator, the recruitment of the TATA box-binding protein and consequent formation of the preinitiation complex may be mediated by Sp1 (16). However, the specific functional role and contribution of a given Sp1 site in this kind of promoter is often unpredictable a priori. For instance, in the bovine α5 nicotinic receptor subunit gene promoter, multiple functionally equivalent Sp1 sites cooperate to ensure basal functions (17), whereas the rat α3 promoter contains a unique Sp1 site whose mutation does not abolish the transcriptional activity of the regulatory region (18).

One crucial aspect of neuronal gene promoter functions concerns the mechanisms of neuro-specific and regional specific expression. The identification of REST/NRSF silencing factor supported the hypothesis that the repression of transcription in non-neuronal tissues is an essential step in the first global restriction of gene expression to neuronal tissues (19). However REST/NRSF could be proved to regulate or potentially regulate only a subset of neuronal genes (20), including the β2 subunit of the nicotinic receptor (21). In transgenic mice, the mutation of the REST/NRSF cognate cis-acting element in the context of the β2 promoter modifies the expression profile of this gene in

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The abbreviations used are: EMSA, electrophoretic mobility shift assay; kb, kilobase pair; hp, base pair; RSV, Rous sarcoma virus; DTT, dithiothreitol; PBS, phosphate-buffered saline.
the central nervous system but does not switch on its transcription in non-neuronal tissues (22), pointing out the role of positive transcriptional mechanisms specifically operating in neuronal cells.

In the present study, we define the human α3 minimal promoter, and we demonstrate the essential role of the members of the Sp family in its activity and the contribution of AP2 units. 100 μg/ml penicillin, 100 glucose, and grown in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 100 mg/ml streptomycin, and 2 mm l-glutamine. The HeLa cells were grown in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 100 μg/ml streptomycin, 2 mm l-glutamine, and 10 mm sodium pyruvate.

**Transient Transfections**—Both cell lines were transiently transfected by means of lipofection using the DMRIE-C reagent (Life Technologies, Eckhard). To blunt-end, and inserted in the appropriate orientation in the blunt-ended, and religation. The Firefly luciferase reporter gene is under the control of the firefly luciferase reporter gene is under the control of the BMV promoter.

**Preparation of Nuclear Extracts**—The nuclear extracts were prepared as described (25). Briefly, subconfluent cells were washed with PBS and detached with PBS + 1 mM EDTA, pelleted, washed once with PBS, resuspended in 5 packed cell volumes of hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM NaCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), and incubated for 10 min on ice. After centrifugation at 4 °C, the cells were resuspended in 3 packed cell volumes of the same buffer containing 0.5% Nonidet P-40 and homogenized in Dounce. After homogenization, cell lysis was checked in a microscope. The nuclei were centrifuged, washed once with the same buffer without Nonidet P-40, and resuspended in an appropriate volume of high salt buffer (5 mM Hepes, pH 7.9, 26% glycerol, 1.5 mM DTT) and run at a constant voltage of 150 V. Compete experiments and supershifts were carried out by preincubating the reactions with the appropriate amounts of cold oligonucleotide and antibody (anti-α3P) and purified on G-25 Sephadex column (Roche Molecular Biochemicals). All of the probes were 100% labeled. Reactions containing 5% poly(dI-dC) (Amersham Pharmacia Bio- tech), 100 mM NaCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), incubated at 4 °C for 30–60 min, and centrifuged at 21,000 × g at 4 °C for 20 min. The supernatant was aliquoted and stored at −80 °C. The protein content was measured using the Bradford assay.

**Gel Mobility Shift Assays**—The oligonucleotides used in the gel mobility shift assays were labeled by filling-in the protruding 5′ extremities of the double-stranded oligonucleotides with 3000 Ci/mmol end-labeling of the bottom strand, filled in with 10 mM MgCl2, 0.2 mM EDTA, 400 mM NaCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), incubated at 4 °C for 30–60 min, and centrifuged at 21,000 × g at 4 °C for 20 min. The supernatant was aliquoted and stored at −80 °C. The protein content was measured using the Bradford assay.

**In Vitro DNase Footprinting Assays**—To prepare the 376-bp probes, containing the Sac1-NcoI region of the α3 promoter, the 0.3-kb Sac1-NcoI/polybluescript plasmid was digested with either NcoI (to label the top strand) or HindIII (to label the bottom strand) and the oligonucleotides were labeled with α-[32P]dCTP and purified on G-25 Sephadex column (Roche Molecular Biochemicals). All of the probes were 100% labeled. Reactions containing 5% poly(dI-dC) (Amersham Pharmacia Bio- tech), 100 mM NaCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), incubated at 4 °C for 30–60 min, and centrifuged at 21,000 × g at 4 °C for 20 min. The supernatant was aliquoted and stored at −80 °C. The protein content was measured using the Bradford assay.

**In Vivo Footprinting Assays**—To prepare the 376-bp probes, containing the Sac1-NcoI region of the α3 promoter, the 0.3-kb Sac1-NcoI/polybluescript plasmid was digested with either NcoI (to label the top strand) or HindIII (to label the bottom strand) and the oligonucleotides were labeled with α-[32P]dCTP and purified on G-25 Sephadex column (Roche Molecular Biochemicals). All of the probes were 100% labeled. Reactions containing 5% poly(dI-dC) (Amersham Pharmacia Bio- tech), 100 mM NaCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), incubated at 4 °C for 30–60 min, and centrifuged at 21,000 × g at 4 °C for 20 min. The supernatant was aliquoted and stored at −80 °C. The protein content was measured using the Bradford assay.

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rSp1 is an affinity-purified preparation from HeLa cells infected with vaccinia virus containing a full-length Sp1 cDNA. rAP2a is prepared from an Escherichia coli overexpressing clone by means of affinity purification. The reactions were performed in 80 μl, containing 40 μl of 2× binding buffer (see under "Gel Mobility Shift Assays"), 2 μg of double-stranded poly(dI-dC), 2 μg of bovine serum albumin, and 100 mm final salt concentration (NaCl + KCl) and were assembled and incubated on ice for 1 h. DNase I (DNase I-RNase free, Roche Molecular Biochemicals) was diluted in 1× binding buffer, 100 mM KCl, and 20 mM MgCl₂ and used at concentration of 0.1–0.4 units/μg DNA without extract and 1.4–2 units/μg DNA in the presence of extract. DNase I treatment was performed by adding 8 μl of DNase I to each sample, incubating the mixture for 150 s at 23 °C, and stopping the reactions by adding 40 μl of DNase I 2× stop buffer (1×, 100 mM NaCl, 10 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8, 0.5% SDS, 5 μg/ml proteinase K, 50 μg/ml glycogen). After 30 min of incubation at 50 °C, the reactions were precipitated by adding 100 μl of phenol/chloroform-extracted and ethanol-precipitated DNA with 0.3 μl sodium acetate, pH 7. The samples were resuspended in denaturing loading dye (0.1 M NaOH,formamide = 1:2; 0.01% bromphenol blue; 0.01% xylene cyanol) and loaded on a denaturing polyacrylamide-urea gel together with sequence reactions in order to evaluate the position of the bands.

RESULTS

Identification of the BsiWI-AccIII Sequence as an Essential Region of the α₃ Promoter—We investigated the functional role of the putative Sp1 and AP2 sites clustered immediately upstream of the transcription start sites of the human α₃ gene (Fig. 1). To do this, we deleted the 60-bp BsiWI-AccIII region in which most of them are contained from the BgIII-NcoI construct (0.35 BN in Refs. 8 and 24), and we measured the transcriptional activity of the resulting construct (BgIII-NcoIΔ60) in SKNBE and in HeLa cells. The BgIII-NcoIΔ60 construct showed a transcriptional activity that was reduced in SKNBE and in HeLa cells, respectively. We suggested that the BsiWI-AccIII region contained a basal promoter, whose activity is differently regulated by the adjacent regions in the two cell lines.

Footprinting Analysis of the 60-bp BsiWI-AccIII Region—In vitro DNase footprinting experiments were performed to characterize the DNA-protein interactions taking place in the 60-bp BsiWI-AccIII region. Fig. 3A shows the DNase protection patterns obtained by incubating a probe labeled on the top strand (containing the SacI-NcoI sequence of the α₃ promoter, −306/+1 in Fig. 1) with SY5Y and HeLa nuclear extracts or with the human Sp1 and AP2a recombinant proteins. Two distinct protected areas encompassing the BsiWI-AccIII region (F1 and F2) were observed with both extracts (Fig. 3A, lanes 3 and 4; F1 spans the −278/−229 region and includes the SacI-NcoI sequence of the α₃ promoter, −306/+1 in Fig. 1) with SY5Y and HeLa nuclear extracts or with the human Sp1 and AP2a recombinant proteins. Two distinct protected areas encompassing the BsiWI-AccIII region (F1 and F2) were observed with both extracts (Fig. 3A, lanes 3 and 4; F1 spans the −278/−229 region and includes the SacI-NcoI sequence of the α₃ promoter, −306/+1 in Fig. 1) with SY5Y and HeLa nuclear extracts or with the human Sp1 and AP2a recombinant proteins. Two distinct protected areas encompassing the BsiWI-AccIII region (F1 and F2) were observed with both extracts (Fig. 3A, lanes 3 and 4; F1 spans the −278/−229 region and includes the SacI-NcoI sequence of the α₃ promoter, −306/+1 in Fig. 1) with SY5Y and HeLa nuclear extracts or with the human Sp1 and AP2a recombinant proteins. Two distinct protected areas encompassing the BsiWI-AccIII region (F1 and F2) were observed with both extracts (Fig. 3A, lanes 3 and 4; F1 spans the −278/−229 region and includes the SacI-NcoI sequence of the α₃ promoter, −306/+1 in Fig. 1) with SY5Y and HeLa nuclear extracts or with the human Sp1 and AP2a recombinant proteins. Two distinct protected areas encompassing the BsiWI-AccIII region (F1 and F2) were observed with both extracts (Fig. 3A, lanes 3 and 4; F1 spans the −278/−229 region and includes the SacI-NcoI sequence of the α₃ promoter, −306/+1 in Fig. 1) with SY5Y and HeLa nuclear extracts or with the human Sp1 and AP2a recombinant proteins. Two distinct protected areas encompassing the BsiWI-AccIII region (F1 and F2) were observed with both extracts (Fig. 3A, lanes 3 and 4; F1 spans the −278/−229 region and includes the SacI-NcoI sequence of the α₃ promoter, −306/+1 in Fig. 1) with SY5Y and HeLa nuclear extracts or with the human Sp1 and AP2a recombinant proteins. Two distinct protected areas encompassing the BsiWI-AccIII region (F1 and F2) were observed with both extracts (Fig. 3A, lanes 3 and 4; F1 spans the −278/−229 region and includes the SacI-NcoI sequence of the α₃ promoter, −306/+1 in Fig. 1) with SY5Y and HeLa nuclear extracts or with the human Sp1 and AP2a recombinant proteins.
FIG. 2. Functional characterization of the 60-bp BsiWI-AccIII region of the human α3 gene promoter. The chimeric constructs (left) were transiently transfected in SY5Y and HeLa cells, together with the BSV-Renilla (SY5Y) and pTK-Renilla (HeLa) plasmids, as transfection efficiency controls (see "Experimental Procedures"). The bars on the right represent the transcriptional activity of the constructs given as fold increases over the activity of the promoter-less pGL3 basic vector. The data represent the means ± S.E. (error bars) of at least three independent experiments carried out in triplicate. On the left, A and B indicate the corresponding Sp1-binding sites, and the circle and the square indicate the mutations mutA and mutB, respectively, shown in detail in Fig. 5D. luc indicates the luciferase reporter gene. The dashed lines indicate the 5'-untranslated region of the luciferase reporter gene. SV40, SV40 minimal promoter.

factors may be recruited to the F1 region in the absence of Sp1 factors. The competitor oligonucleotide did not affect the formation of the F2 region, thus indicating that Sp1 or Sp1-related factors were not involved directly (as also shown by using the Sp1 recombinant protein) or indirectly in this protection pattern.

Mapping and Functional Characterization of the Sp1-binding Sites in the 60-bp BsiWI-AccIII Region—The DNA-protein interactions in the BsiWI-AccIII region were further characterized by EMSA. Six retarded complexes were observed when a 60-bp probe corresponding to this region (Fig. 5D) was incubated with the SY5Y and HeLa nuclear extracts (Fig. 5A, lane 1, and Fig. 5B, lane 1); the formation of these complexes could be specifically competed by an excess of the same cold oligonucleotide (Fig. 5A, lanes 2 and 3). Competition experiments were also carried out using unlabeled oligonucleotides bearing canonical Sp1, AP2, or EGR sites (Fig. 5A, lanes 4–9); the Sp1 oligonucleotide prevented or greatly reduced the formation of all six bands (Fig. 5A, lanes 4 and 5), whereas AP2- and EGR-specific oligonucleotides were totally unable to compete for any of the observed bands (Fig. 5A, lanes 6–9). These data indicate that only the members of the Sp1 family efficiently bind the BsiWI-AccIII region, although two additional bands appeared when all of the Sp1 proteins were removed by competition (Fig. 5A, lanes 4 and 5), thus suggesting that other factors may bind the region under particular conditions. Supershift experiments carried out using antibodies against Sp1 (Fig. 5B, lanes 2 and 6), Sp3 (Fig. 5B, lanes 3 and 7), and Sp4 (Fig. 5B, lanes 4 and 8) showed that, in both the SY5Y and HeLa nuclear extracts, all three proteins interacted with the BsiWI-AccIII region participating in the generation of the four upper bands.

According to the predictions of the computer analysis, two putative Sp1-binding regions were identified in the BsiWI-AccIII sequence (Figs. 1 and 5D) as follows: one canonical site at the 5' end of the region (−249/−241, site A in Fig. 5D) and a cluster of three overlapping and partially degenerated sites immediately downstream (−233/−216, site B in Fig. 5D). In our footprinting experiments both regions were protected by members of the Sp1 family (Fig. 3), although the site B was only partially protected.

To investigate the specific contribution of these sites to Sp1 binding and promoter function, two point mutations (mutA and mutB) were introduced in the BsiWI-AccIII region (Fig. 5D). The mutation of the site A (mutA) severely affected the binding of the Sp1 factors (Fig. 5C, compare lanes 1 and 4), abolishing complexes I and II and drastically reducing complexes III and IV. The mutation of the site B (mutB) did not affect complexes III and IV but severely reduced the formation of complexes I and II (Fig. 5C, compare lanes 1 and 7). The presence of both mutations (mutAB) completely abolished the binding of the Sp1 factors (Fig. 5C, lanes 10 and 11). Supershift experiments using the mutated oligonucleotides as probes showed that the two mutations similarly affected the binding of Sp1, Sp3, and Sp4 (data not shown), thus indicating that neither site A nor site B were selective for a certain Sp factor in vitro. To study the functional role of these sites, the BglII-NcoI mutA and BglII-NcoI mutB constructs were generated, and their transcriptional activities were measured in SY5Y and HeLa cells (Fig. 2). MutA was found to reduce the transcriptional activity of the BglII-NcoI construct 16 times in the SY5Y and 7 times in the HeLa cells, whereas mutB did not have any significant effect. The effect of the deletion of the BsiWI-AccIII region was therefore largely recapitulated by removing a single Sp1-binding site essential for the α3 promoter activity.

Molecular Characterization of the F2-protected Region—DNase footprinting experiments with both SY5Y and HeLa nuclear extracts revealed the presence of an additional footprint (F2) that spanned the −219/−176 region and thus included the last 20 bp at the 3’ end of the BsiWI-AccIII fragment (Figs. 3 and 4). As the AP2α recombinant protein was found to interact with the −196/−130 fragment, including the 3’ half of the F2 region (Figs. 3 and 4), we used competitive footprinting to investigate the involvement of this transcription factor in F2 formation (Fig. 6). With both nuclear extracts, the oligonucleotide bearing a canonical AP2 site not only appeared to prevent the protection of the −196/−176 region but also seemed to abolish the formation of the entire F2 region (−219/−176; Fig. 6, lanes 5 and 8). As already shown in Fig. 4, an Sp1 canonical oligonucleotide did not impair F2 protection; moreover, the AP2-specific oligonucleotide did not affect the protection pattern of any other region of the probe, thus suggesting that its effects on F2 were specific and direct. To confirm the specificity of the AP2 oligonucleotide, EMSA and supershift experiments were carried out using SY5Y and HeLa nuclear extracts.
The top strand and cleotide was completely ineffective (Fig. 7, B, proteins. The formation of F2 protection, we analyzed the footprinting pattern of a probe spanning the the position of the SacI-NcoI region, labeled on the bottom strand were incubated with 45 μg of SY5Y (lanes 2 and 3) or HeLa (lane 4 and 5) nuclear extracts and 2 footprinting units of either rSp1 (lane 6) or rAP2α (lane 7)-purified proteins as described under “Experimental Procedures.” A 5000-fold molar excess of cold oligonucleotide containing one Sp1 canonical binding site was added as a competitor to the reactions in lanes 3 and 5. Lane 1, no extract. The asterisk indicates an artificial band due to probe folding. The protected regions are indicated on the right of the autoradiogram, where F1 and F2 indicate protections by nuclear extracts and Sp1 and AP2α are protected by recombinant proteins. The numbers on the left indicate the reference nucleotides on the probe.

(Fig. 7). Two retarded bands were observed with both cell lines, both of which were completely supershifted by an antibody directed against AP2α (Fig. 7, lanes 2 and 16), thus indicating that both complexes contained this transcription factor.

In order to define further the DNA sequences required for the formation of F2 protection, we analyzed the footprinting pattern of a probe spanning the −325/−176 region, whose 3′ coincided with the 3′ of the F2 region. Under these conditions, F2 protection still occurred, and competition experiments showed the same dependence on AP2α as that observed with the longer probe (Fig. 8, lanes 3–5), thus indicating that the tested sequence contained all of the elements required for F2 formation. However, unexpectedly, the AP-2α recombinant protein was unable to bind the −196/−176 region of this probe (Fig. 8, lane 6), which indicated that the sequences downstream of the SacII site were required for the binding of the recombinant protein to the 3′ of the F2 region. Consistently, a −226/−165 oligonucleotide (SacII in Fig. 7), which included the entire F2 region, was unable to bind the nuclear AP2α in EMSA (data not shown), but the same oligonucleotide appeared to prevent, albeit modestly, the binding of the nuclear AP2 to its canonical consensus site (Fig. 7, lanes 7 and 8 and 14 and 15). On the contrary, an unrelated GC-rich, EGR-specific oligonucleotide was completely ineffective (Fig. 7, lanes 5 and 6 and 12 and 13). These findings primarily indicated that the AP2-bind-
Promoter of Human $\alpha_3$ Nicotinic Receptor Subunit

should be due to the presence of the F2-protected region. We also observed that the BsiWI-SacII construct had 1.8 times more activity than the BsiWI-AccIII plasmid (Fig. 9). Taken together, these data indicate that the BsiWI-AccIII region can be independently stimulated by the upstream (BglII-BsiWI) and downstream (AccIII-SacII) regions and that their effects were fully additive (BglII-SacII construct in Fig. 9).

A similar regulatory profile was also observed in HeLa cells, thus suggesting that the described effects were not specific to neuronal cells (Fig. 9).

We have previously reported the functional characterization of the AccIII-NcoI downstream region (8, 24), and we have shown that it works as a composite regulatory element stimulating the transcription of the $\alpha_3$ gene in a neuro-specific fashion. In order to evaluate whether this region can exert its neuro-specific action directly on the minimal promoter, we measured the activity of the BsiWI-AccII construct and found that the downstream region actually worked as a positive regulator of transcription by directly stimulating the minimal promoter. However, inconsistent with our previous results, this stimulation was not neuro-specific, as it occurred in both SY5Y and HeLa cells (Fig. 9). By comparing the activities of the BglII-AccIII, BsiWI-NcoI, and BglII-NcoI constructs in SY5Y, we found that the BglII-BsiWI and AccIII-NcoI regions together exerted an effect that was greater than the sum of the activities of each region alone, whereas the effects of the same regions were not even additive in HeLa cells. Thus the neuro-specific activation of the minimal promoter observed with the BglII-NcoI construct depends on synergistic interactions between the upstream and downstream regions, and not only on the downstream region.

In order to test the role of the F2 region in such interactions, we measured the activity of the BglII-NcoI F2 construct that lacked the AccIII-SacII sequence. In SY5Y, this construct showed a 60% reduction in activity in comparison with the BglII-NcoI construct, thus suggesting that the F2 region participates in the activation of the minimal promoter in the context of the BglII-NcoI region. The activity of this construct also indicated that the positive effect of the SacII-NcoI region on the minimal promoter (see BsiWI-NcoI F2 construct in Fig. 9) was only additive and not synergistic with that of the upstream region BglII-BsiWI. Therefore, in SY5Y cells, the synergistic interactions between the upstream and the downstream regions requires the presence of both the AccIII-SacII (F2) and SacII-NcoI downstream subregions.

In HeLa cells, the activity of the BglII-NcoI F2 construct was less than that of the BglII-AccIII and BsiWI-NcoI F2 constructs. In this cell line the positive effects independently exerted by the BglII-BsiWI and SacII-NcoI regions on the promoter were not only not additive but a negative interference between the two regions seemed to occur.

Taken together, these data suggest that the enhanced activity of the $\alpha_3$ promoter observed upon transfection in neuroblastoma cells, in comparison with non-neuronal cell lines, depends on the opposite effects on the minimal promoter produced by the combination of the upstream and downstream regions in neuronal and non-neuronal cells.

**DISCUSSION**

In this study we describe the characterization of a 60-bp sequence (BsiWI-AccIII) in the 5'-flanking region of the human $\alpha_3$ nicotinic receptor subunit gene, which has the features of a

**FIG. 5.** Mapping of the Sp1-binding sites in the BsiWI-AccIII region by EMSA. In each reaction, 1 fmol of probe (10,000–20,000 cpm) was incubated with 2 µg of nuclear extract, as described under “Experimental Procedures.” A, the 60 probe was incubated with the SY5Y nuclear extract. The competitors were carried out by adding 500 or 2500× molar excess of the indicated cold oligonucleotides (lanes 2 and 3; Sp1, lanes 4 and 5; EGR, lanes 6 and 7; AP2, lanes 8 and 9). B, the 60 probe was incubated with HeLa (lanes 1–7) and SY5Y (lanes 8–10) nuclear extracts. Supershift analyses were performed by adding the specific antibodies as follows: AbSp1 (lanes 2–6), AbSp2 (lanes 3–7), and AbSp4 (lanes 4–8) as indicated. C, the indicated oligonucleotides (mutA, lanes 1–3; 60mutA, lanes 4–6; 60mutB, lanes 7–9; 60mutAB, lanes 10 and 11) were incubated with the SY5Y nuclear extract, and × 100 or × 500 molar excess of the Sp1 oligonucleotide was added to the reactions loaded in lanes 2 and 3, 5 and 6, and 8 and 9 and 100× in lane 11. The asterisks indicate nonspecific bands. D, sequence of the 60 probe corresponding to the BsiWI-AccIII region. The putative Sp1-binding sites, mutA and mutB mutations, and sites A and B are shown.
The 376-bp probe labeled on the top strand was incubated with SY5Y (lanes 3–5) and HeLa (lanes 6–8) nuclear extracts and a Sp1–purified protein as described under “Experimental Procedures.” The competitions were performed by adding a 5000-fold molar excess of either Sp1 (lanes 4 and 7) or AP2 (lanes 5 and 8) canonical oligonucleotides. Lanes 1 and 2, no extract/protein. The numbers on the left indicate the reference nucleotides on the probe; the protected regions are indicated on the right (see also Fig. 3B).

Sp1 oligo
AP2 oligo

FIG. 7. EMSA analysis of the AP2-specific oligonucleotide. 1 fmol of the AP2 probe was incubated with 4 μg of SY5Y (lanes 1–8) and HeLa (lanes 9–16) nuclear extracts. Supershift analysis was performed by adding an antibody specific for the AP2α factor to the reactions loaded in lanes 5 and 16. The competitions were carried out by adding 500 and 2500× molar excess of cold oligonucleotides (AP2, lanes 3 and 4 and 10 and 11; EGR, lanes 5 and 6 and 12 and 13; SacII, lanes 7 and 8 and 14 and 15). The SacII oligonucleotide spans the sequence −226/−165 and contains the entire F2 region.

minimal promoter. Its removal dramatically affected the transcriptional activity of a previously characterized 350-bp BgIII-NcoI construct, as well as that of a construct containing the entire β3–α3 intergenic region (data not shown), whereas it retained some transcriptional activity and could be independently stimulated by the upstream and downstream regions, thus indicating that it contains the basal elements required for transcription initiation.

DNase protection and EMSA analyses of the BsiWI-AccIII region led to the identification of two sites (A and B) that are both capable of binding Sp1 and Sp1-related factors, although with apparently different affinity. However, mutation analyses revealed that the site A was essential for the activity of the entire BgIII-NcoI region and probably accounted for the critical role of the 60-bp region in this context, whereas the mutation of the site B, which actually reduced the functional activity of the promoter, Site B therefore seems to be dispensable under the basal conditions under which we tested the effects of its mutation, but it may play a role under stimulated conditions, when other factors may be recruited at the promoter and interact with Sp1 or Sp1-related factors on site B.

The data obtained with site A confirmed that Sp1 plays a critical role in the transcription initiation of GC-rich TATA-less multistart site promoters such as the human α3 nicotinic receptor subunit gene. The regulation of these promoters is poorly understood. Sp1 is known to interact with multiple components of the transcriptional machinery, such as TATA box-binding protein (16), TAF55 (28), TAF110 (29–31), and TFIIIB (28, 32), and may therefore play a critical role in the formation/recruitment of the pre-initiation complex on these promoters. Sp1 is a ubiquitous transcription factor; its activity is generally constitutive, but it can be directly regulated by...
O-glycosylation (33) and phosphorylation (34) or by interactions with other factors. A number of transcription factors have been documented as acting in combination with Sp1 or promoting its displacement from an overlapping site (35–41). Among these, Sp3 and Sp4 belong to the same family as Sp1 and are closely related to it (42, 43). Sp1, Sp3, and Sp4 share a highly homologous DNA binding domain that recognizes the same sequences in vitro with identical affinities. Sp3 is ubiquitously expressed and generally acts as a repressor by competing with Sp1 binding to the same sequence (44–46). Sp4 is found in many cell lines, but in vivo, its mRNA is abundant in the brain and barely detectable in other organs. It generally acts as an activator, but it can also repress Sp1-activated transcription in the same way as Sp3 (47, 48). It has been suggested that the cell content of Sp1, Sp3, and Sp4 is a critical factor influencing the transcription of Sp1-dependent promoters, such as the housekeeping gene promoters (47). Both the A and B Sp1-binding sites identified in the a3 promoter also bind to Sp3 and Sp4 in vitro, and so these factors might participate in the regulation of the a3 promoter by competing or cooperating with Sp1.

By using DNase protection assays, we identified another region of interaction with nuclear factors (F2), which is located immediately downstream of sites A and B. The F2-protected region directly activates the minimal promoter and also participates in its regulation in the context of the 0.35-bp F2 region. Competitive footprinting showed that the F2-protected region was entirely and specifically competed by an AP2 construct, Student's t test, p < 0.05.

These findings suggest a model in which AP2a recombination protein could not directly bind the F2 region in footprinting experiments, and no binding of nuclear AP2a to the F2 sequence was detected by EMSA.

AP2α was originally isolated as a sequence-specific transcription factor binding to the enhancer regions of SV40 and human metallothionein IIA genes (49). It is expressed in neural crest lineages, in which it plays fundamental functions during mammalian embryogenesis and, specifically, in the morphogenesis of the peripheral nervous system (50). Moreover, it is also known to be activated by treatment with phorbol esters and agents that increase cAMP levels, thus suggesting that external stimuli can influence its functions (51).

Functional AP2-binding sites have been described in many viral and cellular regulatory regions (52–54). However, recent studies have shown that AP2α can regulate gene expression independently from DNA binding by means of heteromerization with other transcription factors. These interactions can lead to the inhibition (55, 56) or activation (57) of the interacting factor, thus suggesting that AP2α is capable of regulating gene transcription either as a sequence-specific regulator or as a cofactor that controls the activity of other nuclear proteins.

It is interesting that the binding sites identified by the AP2α recombinant protein did not seem to interact with the endogenous nuclear AP2α factor. This may be due to competition with other sequence-specific factors recognizing overlapping elements; however, mechanisms involving the sequestering of endogenous AP2α by other nuclear proteins or post-translational modifications affecting its DNA binding specificity might also be considered, as already suggested in the literature (49, 51, 58).

In order to understand how the ubiquitous activity of the 60-bp minimal promoter is re-directed in such a way that the expression of the a3 gene is mainly confined to the neural phenotype, we studied the effects exerted by the upstream (100-bp BglII-BstIWI) and downstream regions (20-bp AccIII-SacI, essential for F2 protection, and 180-bp SacII-NcoI) on the activity of the BstIWI-AccIII region in SY5Y and HeLa cells.

In both cell lines, all three regions independently activated the minimal promoter, whereas different results were obtained when more than one region was present. In SY5Y cells, the combined stimulatory effects of the upstream and downstream regions were greater than the sum of the transcriptional activities conferred by the individual regions. This synergistic activation of the minimal promoter seemed to depend on the presence of all three regions, since each downstream region alone appeared to produce only additive effects in combination with the upstream sequence.

In HeLa cells, the activity exerted by the upstream and downstream regions together was less than the sum of their activities; this was apparently due to negative interference between the SacII-NcoI and the upstream region, since together their activity was even lower than that of each sequence alone. These findings indicate that opposite interactions occur...
between the regions upstream and downstream of the minimal promoter in SY5Y and HeLa cells. The synergistic interactions described in SY5Y have also been found in SK-N-BE, another neuroblastoma cell line, but not in any of the other cell lines analyzed so far: those not expressing the α3 endogenous gene, HeLa TE671 (8), K562 (24), DAOY,2 or non-neuronal cells expressing the α3 endogenous gene, such as the MOLT4 T-lymphocyte cell line (24). This may therefore be a specific mechanism for controlling α3 gene expression in neurons, which may rely on a neuro-specific TAF/cofactor that mediates the synergistic activation of the α3 minimal promoter by non-tissue-specific activators bound upstream and downstream of it. HeLa cells may simply lack this cofactor or may even contain a specific corepressor mediating a negative interference occurring between the two regulatory regions.

Sequence-specific transcription factors can make direct contacts with different components of the transcriptional machinery (in particular with specific TAFIIs) thus allowing the TFIIID complex to integrate multiple signals from different regulators (59, 60). TAFIIs are core promoter-selective and can be expressed in a tissue-specific fashion, so that they can account for tissue-specific gene regulation. Recent studies indicate that a sequence regulator can interact with both coactivator and corepressor complexes which in turn either stimulate or inhibit the formation or the activity of the pre-initiation complex (61, 62). The alternative binding of non-tissue-specific transcription factors to tissue-specific coactivators or corepressors may therefore provide a simple and flexible mechanism for restricting gene expression to specific cell types.

We propose as a working hypothesis that this kind of mechanism might be responsible for the specific expression of the human α3 nicotinic receptor subunit gene in neuronal cells.

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