The \(\beta_4\) subunit is a component of the neuronal nicotinic acetylcholine receptors which control catecholamine secretion in bovine adrenomedullary chromaffin cells. The promoter of the gene coding for this subunit was characterized. A proximal region (from \(-99\) to \(-64\)) was responsible for the transcriptional activity observed in chromaffin, C2C12, and COS cells. Within this region two cis-acting elements that bind transcription factors \(\mathrm{Sp1}\) and NF-Y were identified. Mutagenesis of the two elements indicated that they cooperate for the basal transcription activity of the promoter. The human \(\beta_4\) promoter, that was also characterized, shared structural and functional homologies with the bovine promoter. Thus, two adjacent binding elements for \(\mathrm{Sp1}\) and NF-Y were detected. Whereas the \(\mathrm{Sp1}\) site was an important determinant of the promoter activity, the NF-Y site may have cell-specific effects. Given that these promoters showed no structural or functional homology with the bovine promoter. Thus, two adjacent binding elements for \(\mathrm{Sp1}\) and NF-Y were detected. Whereas the \(\mathrm{Sp1}\) site was an important determinant of the promoter activity, the NF-Y site may have cell-specific effects. Given that these promoters showed no structural or functional homology with the previously characterized rat \(\beta_4\) subunit promoter (Bigger, C. B., Casanova, E. A., and Gardner, P. D. (1996) J. Biol. Chem. 271, 32842–32848) except for the involvement of an \(\mathrm{Sp1}\) binding element, we propose that constitutive expression of the \(\beta_4\) subunit gene in these three close species may be controlled by the general transcription factor \(\mathrm{Sp1}\). Nevertheless, other components could determine species-specific \(\beta_4\) subunit expression.

Cloning of nicotinic acetylcholine receptor (nAChRs)\(^1\) subunit cDNAs has revealed that the molecular heterogeneity of the gene families encoding the different receptor subunits is responsible for the pharmacological and functional diversity of nAChRs in the peripheral and central nervous systems (1, 2). The varied tissue-, region-, and development-specific distribution of nAChR subunits (3) has suggested that complex transcriptional mechanisms direct nAChR expression. Moreover, potential changes in subunit transcription in response to modulation of synaptic function, might have important consequences on the signals transduced by nAChRs (4, 5). For these reasons considerable effort has been dedicated to the elucidation of the molecular basis for the transcriptional regulation of neuronal nAChRs, and thus several cis- and trans-acting elements in the promoter of the different nAChRs subunits have been identified (6).

In our laboratory we have previously isolated and characterized the promoters of the bovine \(\alpha_5\) (7) and \(\alpha_7\) (8) subunits. These subunits are expressed in the chromaffin cells of the adrenal gland composing two different receptor subtypes, one of them formed by \(\alpha_7\) subunits (9) and the other by \(\alpha_3\), \(\beta_4\), and \(\alpha_5\) subunits (10). Interestingly, the genes of the latter subunits are clustered in the vertebrate genome (11, 12) and may have common patterns of regulation (13). We have analyzed here the bovine and human \(\beta_4\) promoters, finding that they are highly homologous in their proximal regions as well as in the cis-elements governing basal transcriptional activity. Although their sequences differ from the one of the rat \(\beta_4\) promoter (14, 15), the three promoters have in common their regulation by the ubiquitous transcription factor \(\mathrm{Sp1}\). Binding motifs for \(\mathrm{Sp1}\) have been also located in close proximity in the promoters of rat \(\alpha_3\) (16) and bovine (7) and human (17) \(\alpha_5\) subunits, suggesting that this transcription factor plays a fundamental role in the expression of several nAChRs subunit genes.

**EXPERIMENTAL PROCEDURES**

*Isolation and Analysis of the 5′-Flanking Sequence of the \(\beta_4\) Subunit*—For the bovine promoter a cDNA probe corresponding to 218 bp at the beginning of the coding sequence and the contiguous 38 bp of 5′-untranslated region (10) was used to screen a genomic library. For the human promoter, a cDNA probe corresponding to 67 bp at the beginning of the coding sequence and the contiguous 99 bp of 5′-untranslated region (15) was used to screen a genomic library. Both libraries were constructed in EMBL-3 SP6/T7 (CLONTECH, Heidelberg, Germany) and tested as previously described (9). In both cases several overlapping bacteriophage clones were purified and characterized.

*RNase Protection*—Poly(A)\(^{+}\) RNA was directly selected from lysates of several bovine adrenal medullas by oligo(dT)-Dynabeads (Dynal, Oslo, Norway) and used in the RNase protection experiments. Probes were generated with SP6 and T7 polymerases (Roche Molecular Biochemicals, Barcelona, Spain), [\(\alpha^32\)P]cTP (Amersham Biosciences, Inc., Madrid, Spain) and the corresponding linearized templates (in the pSPT18 vector, Roche Molecular Biochemicals). A 462-bp Rsal-PstI fragment of the bovine \(\beta_4\) gene that included 286 bp 5′ to the beginning of the signal peptide sequence and 176 bp corresponding to the rest of the first exon and part of the second one was subcloned into the Smal and PstI sites of pSPT18. After linearization of the plasmid with EcoRI, an antisense probe of 496 nucleotides was synthesized with SP6 RNA
amplification of p99

GCGTCTTCC-3

-CTTTATGTTTTG-

oligonucleotides and an antisense primer (5′-end-oligo(dT)-Dynabeads or purchased from CLONTECH (human brain and adrenal tissues).

Plasmid Constructions—All β4 promoter-luciferase gene fusions were made in the pGL2-Basic vector (Promega, Madison, WI), introducing in its polynuker, upstream of the luciferase gene, the suitable β4 promoter fragments. These fragments were generated 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 express the luciferase gene under the regulation of the pBluescript and then transferred to pGL2-Basic or subcloned first in pBluescript, sequenced, and transferred to the appropriate construct from the corresponding figure legends.

RESULTS

Structure of the 5′-Flanking Region of the Bovine β4 Subunit Gene—A bovine genomic library was screened and several overlapping clones were isolated. Clone Cboββ4–11 contained ~13 kb of bovine genomic sequence including exon 1 and ~1.2 kb of 5′-flanking region. This region was further subcloned and sequenced (Fig. 1) revealing the lack of a TATA box. The 5′-end of β4 mRNA was mapped by RNase protection analyses (Fig. 2). A 496-residue antisense riboprobe (Fig. 2, Probe 1) yielded a protected fragment of ~309 bases that mapped transcription initiation to a thymine located 125 bp upstream of the initial ATG (arrowhead at position +1, in Fig. 1). Other protected fragments of smaller size and similar intensity were also observed, suggesting that alternative initiation sites exist. They are also indicated in Fig. 1 (arrowheads and small squares). To improve precision in the determination of the transcription initiation sites, a second overlapping probe was used (Fig. 2, Probe 2). In this case several protected fragments of 156, 155, 134, and 133 bp were observed and mapped transcription initiation to the same sites that the larger probe.

Functional Analysis of the Bovine β4 Subunit Promoter—A series of constructs was generated to determine the regions of the bovine β4 subunit promoter that contributed to its maximal activity (Fig. 3). These constructs were introduced into chromaffin, C2C12, and COS cells. Constructs containing 81 bp (p81β4LUC) or more (up to 1256 bp) of β4 promoter sequence plus 66 bp of 5′-noncoding region showed similar activity. Two shorter constructs (p83LUC and p99LUC) with ~60% reduction in promoter activity and further 5′ deletion (p46LUC) was even less active. The pattern of promoter activity was similar in the three cell types mentioned above, indicating that the tested constructs lacked elements able to confer cell-specific transcription. The only exception was construct p78LUC, which showed a ~20–30% decrease in activity in C2C12 and COS cells but not in chromaffin cells.
Therefore, in C2C12 and COS but not chromaffin cells, elements predominantly located between −353 and −787 with respect to the transcription initiation site may have a negative effect on β4 promoter activity. However, the largest construct tested (p1256LUC) also contains this region and exhibited increased activity (∼100–120%) in the three cell types.

Elements in the minimal promoter, between 81 and 63 bp upstream of the start site of transcription, appear to be critical for basal transcription of the β4 subunit gene in transient transfection assays, since: (a) their deletion produced ∼60% decrease in transcriptional activity, and (b) additional upstream sequences did not significantly increase activity. In this region the presence of inverted GC and CCAAT boxes was observed (Fig. 5A). Recombinant Sp1 produced a main retarded complex (lane 3, Fig. 5A, lane 3), coincident in position with one of those observed with nuclear extracts (arrowhead). By contrast, when the site 1 mutant was used as probe, neither the upper complex was formed with chromaffin extracts (Fig. 5A, lane 7) nor recombinant Sp1 retarded the probe (Fig. 5A, lane 8). This suggests that a protein from chromaffin extracts, which could be Sp1, is binding to the probe at site 1. When the site 2 mutant was used as probe the formation of the lower complex was abolished (Fig. 5A, lane 10) and Sp1 was able to form a complex (Fig. 5A, lane 13), coincident in position with one of those observed with nuclear extracts (arrowhead). Antibody supershift analysis was employed in an attempt to identify the proteins producing the retarded bands. The upper complex (arrowhead) observed with both the wild and the site 2 mutant probes was retarded by an anti-Sp1 antibody (Fig. 5A, lane 11), suggesting that a protein from chromaffin extracts, which is not Sp1, is binding to the probe at site 2. Antibody supershift analysis was employed in an attempt to identify the proteins producing the retarded bands. The upper complex (arrowhead) observed with both the wild and the site 2 mutant probes was retarded by an anti-Sp1 antibody (Fig. 5B, lanes 15 and 22, respectively), whereas no supershift was observed with antibodies against Sp3 (Fig. 5B, lane 16). The lower complex (circle) was shifted by an anti-NF-Yb antibody.
These results suggest that transcription factors Sp1 and NF-Y are binding to the probe. When using C2C12 and COS cell nuclear extracts a similar pattern of retarded bands was observed (Fig. 5C, lanes 24 and 27), although faster migrating complexes were also present with C2C12 extracts and the Sp1 upper band was less prominent. Again, the major band was supershifted with an anti-NF-Yb antibody (Fig. 5C, lanes 26 and 29).

Given that Sp1 and NF-Y bind to the GC and CCAAT boxes at sites 1 and 2, respectively (Fig. 5), and that the simultaneous alteration of these boxes produced a significant decrease of the transcriptional activity in luciferase reporter experiments (i.e. the activity of the double mutant p2-1/H92524LUC was 35, 52, and 42% of the one observed with the wild-type construct p99/H92524LUC in chromaffin, C2C12, and COS cells, respectively), we suggest that both, Sp1 and NF-Y, are involved in the transcriptional regulation of the bovine β4 promoter.

Structure of the 5' Flanking Region of the Human β4 Subunit Gene—Comparison of the β4 bovine promoter to its rat counterpart previously published (14, 15) did not show significant sequence homology. In an attempt to know whether this heterogeneity is extended to other species, we decided to isolate and characterize the human β4 promoter. A human genomic library was screened and several overlapping clones were isolated. Clone pH1101101 contained pH1101120 kb of human genomic sequence including exon 1 and probably exon 2 as well as pH110115 kb of 5' flanking region. This region was further subcloned and about 720 bp located 5' from the initial ATG were sequenced. This sequence was identical to the one deposited in the NCBI data base with accession number NT_010218 Region 389312–390032. A comparison of 1200 bp of bovine and human β4 promoter sequences adjacent to the transcription initiation site was performed with the Blast 2 program (22) and the result is shown in Fig. 6. Several homology regions were detected, rang-
significant homologies. The 5'-end of human with the rat and either the bovine or human promoters did not reveal homology flanked by stretches (ranging from 50 to 165 bp) accounting for more than half of the whole sequences. As indicated in the diagram of Fig. 6 (lower panel), the structure of both promoters is similar, revealing regions of high or moderate homology flanked by stretches (ranging from 50 to 165 bp) of dissimilar sequences. By contrast, comparisons performed describing this position as the initial ATG (indicated by initiation to sites located between 91 and 125 bp upstream of the initial ATG (indicated by arrowheads for major fragments and small squares for minor ones in Fig. 6). One of them was also predominant in experiments performed with human brain and adrenal mRNAs (Fig. 7) and for this reason we have numbered this position as +1 (also indicated as arrowhead at position +1 in Fig. 6). The multiple initiation sites for the human and bovine β4 subunit genes are approximately located in the same area (indicated as vertical boxes in lower panel of Fig. 6).

Deletion Analysis of the Promoter for the Human nAChR β4 Subunit—A series of constructs was generated to determine the regions of the β4 subunit proximal promoter (Fig. 8) that contributed to its maximal activity. These constructs were introduced into SHSY-5Y cells, a human neuroblastoma cell line that express the β4 subunit endogenously (23) as well as in mouse muscle C2C12 cells, that express the muscular-type nAChR (24, 25). In both cell lines, the construct containing 74 bp of β4 promoter sequence (considering the initiation site labeled as +1 in Fig. 6 as reference for numbering) plus 97 bp of 5'-noncoding region (p74h4LUC) showed the maximal activity. When the luciferase activity of this construct was normalized for transfection efficiency and compared in these two cell lines it was about 8 times higher in SHSY-5Y cells than in C2C12 cells. On the other hand, the activity of p74h4LUC was 29 and 35% of the activity shown by pGL2Control in C2C12 and SHSY-5Y cells, respectively. When larger constructs were used (p255 and 960h4LUC) the relative luciferase activity decreased up to 60–70% of the activity observed with p74h4LUC. The activity of p74h4LUC in SHSY-5Y and C2C12 cells was about 90% reduced when 46 bp of the β4 promoter 5’-end were deleted further (p28h4LUC) and was barely detectable upon the additional deletion of 37 bp (p19h4LUC). These results suggest that elements located between 74 and 28 bp of the transcription initiation site are essential for transcription.

Characterization of the Regulatory Elements Present at −741 to −44 of the Human β4 Promoter by Mutagenesis and Transient Transfections—A search for transcription factors which could interact with elements at the proximal promoter region of the

| LUCIFERASE ACTIVITY (% of p99β4LUC) |
|-------------------------------------|
| **Chromaffin** | **C2C12** | **COS** |
| p4β4LUC | ![Graph](http://example.com/graph1.png) | ![Graph](http://example.com/graph2.png) |
| p39β4LUC | ![Graph](http://example.com/graph3.png) | ![Graph](http://example.com/graph4.png) |
| p63p4LUC | ![Graph](http://example.com/graph5.png) | ![Graph](http://example.com/graph6.png) |
| p81p4LUC | ![Graph](http://example.com/graph7.png) | ![Graph](http://example.com/graph8.png) |
| p99β4LUC | ![Graph](http://example.com/graph9.png) | ![Graph](http://example.com/graph10.png) |
| p140β4LUC | ![Graph](http://example.com/graph11.png) | ![Graph](http://example.com/graph12.png) |
| p353β4LUC | ![Graph](http://example.com/graph13.png) | ![Graph](http://example.com/graph14.png) |
| p787β4LUC | ![Graph](http://example.com/graph15.png) | ![Graph](http://example.com/graph16.png) |
| p1256β4LUC | ![Graph](http://example.com/graph17.png) | ![Graph](http://example.com/graph18.png) |

Fig. 3. Deletion map analysis of bovine β4 gene promoter activity. Chromaffin, C2C12, and COS cells were transfected with each of the plasmids (named pβ4LUC with the number of promoter base pairs included in the construct) containing the luciferase reporter under the control of the different fragments of the β4 subunit promoter and pCH110β-galactosidase as a transfection efficiency control. Promoter activity was normalized to values obtained with the p81β4LUC construct because it contains elements important for transcription (see Fig. 4). Although construct p81β4LUC also contains these elements it was not chosen as reference because one of the elements is just at its 5’-end and it was not known how this would affect its activity. The mean ± S.E. (error bars) are given for at least two or three individual experiments, carried out in triplicate.

![Graph](http://example.com/graph19.png)

Fig. 4. Sites 1 and 2 are functional elements required for β4 subunit gene expression. A, the proximal region of the β4 subunit promoter (nucleotides −99 to −64) is depicted with the putative regulatory elements box. This region contains putative binding sites for transcription factors Sp1 and NF-Y (denoted by numbers 1 and 2, respectively). 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. Plasmids were transfected into chromaffin, C2C12, and COS cells and activities were measured. Luciferase activity was normalized to values obtained with the p99β4LUC construct. Data are expressed as described in the legend to Fig. 3.
FIG. 5. Binding of cell nuclear proteins to the proximal region of the β4 subunit promoter. A, the DNA fragment corresponding to region −99 to +66 (WT −99/+66) or the analogous mutants of sites 1 (Mut 1) and 2 (Mut 2) were used as gel mobility shift probes in the presence of 2 μg of crude chromaffin (CR, lanes 2, 4, 5, 7, 10) and cell nuclear extracts. Two prominent bands (filled dot and arrowhead) were observed with chromaffin nuclear extracts. Two of them (arrowhead) was also observed with recombinant Sp1. Competition with unlabeled probe added in 10-fold (lane 4) and 100-fold (lane 5) excess, decreased significantly the amount of retarded complexes. B, probes WT −99/+66, Mut 1, and Mut 2 were used with chromaffin nuclear extracts in a supershift assay. The band labeled with an arrowhead was supershifted by Sp1 antibodies (lanes 15 and 22) but not by Sp3 antibodies (lane 16). The band labeled with a filled dot was supershifted by NF-Y antibodies as deduced by the lower intensity of the NF-Y band in the presence of antibody (compare lanes 18 and 19). Lanes 17, 18, and 19 (F) are probes run in the absence of protein extracts C, the gel mobility assay was run using DNA fragment −99 to +66 as the labeled probe and nuclear extracts from C2C12 cells (lanes 24–26) and COS cells (lanes 27–29). The prominent band, indicated by a filled dot, was displaced with NF-Y antibodies (lanes 26 and 29).
Fig. 6. Comparison of the nucleotide sequence of the 5′-flanking regions of the bovine and human nAChR β4 subunit genes. Upper panel, the nucleotide sequences of the bovine (BOV) and human (HUM) proximal regions of the β4 subunit promoters are depicted, including the initial ATG (bold) and the signal peptide sequences. Comparisons were performed with the Blast 2 program (22) and the degree of identity is indicated by bars located above the sequences according to the following code: □, 60–75% identity; ▤, 75–85% identity; □□, >85% identity.
new band located slightly below the NF-Y complex appeared (square). In the case of the GC boxes, only disruption of GC-box 2 abolished the binding of Sp1 (lane 15) whereas the mutant of GC-1 (lane 17) produced a pattern similar to the wild probe. Therefore, NF-Y binds to the inverted CCAAT box and Sp1 to the adjacent GC box. In fact, when both boxes were simultaneously mutated none of these transcription factors were able to produce retarded complexes (lane 19). Similar results were obtained with nuclear extracts from C2C12 cells (not shown).

**DISCUSSION**

Two classes of nAChRs have been identified on bovine chromaffin cells. One class, probably formed by α3, α5, and β4 subunits, is present in all chromaffin cells of the adrenal medulla (10) and appears to be representative of many nAChRs present in the peripheral nervous system. The other binds α-bungarotoxin, is expressed only in adrenergic cells (26) and contains α7 subunits (9). In our laboratory we have previously isolated and characterized the bovine α7 (8, 26) and α5 (7) subunits promoters. In this article we describe the characterization of the β4 subunit promoter in the bovine and human species and compare them with its rat counterpart, that has been previously studied in great detail (14, 15, 27, 28).

The core promoter regions of both the bovine and human β4 subunits do not contain TATA boxes, but do have G + C-rich domains. This characteristic is also found in the rat β4 promoter (14) as well as in the promoters of the α2 (29), α3 (30, 31), α5 (7, 17), and α7 (26, 32–34) subunits. From 5′-end deletion analysis (Fig. 3) of the bovine promoter, we determined that the region located between nucleotides −99 and +66 was necessary for the basal promoter activity detected in chromaffin, C2C12, and COS cells. A comparison of normalized (for transfection efficiency) luciferase activity values of p99 β4LUC in the three mentioned cell types, indicated that promoter activity was similar in chromaffin and COS cells whereas in C2C12 was about 40% lower. Since COS cells do not endogenously produce β4 subunits, it is possible that promoter elements needed for cell-specific expression are not included within the promoter fragments used in this study. Nevertheless, elements located between −353 and −787 appear responsible for a slight decline in activity in C2C12 and COS cells and could be involved in a silencing mechanism. However, this mechanism might not be totally effective or may need additional elements, since the largest construct p1256 β4LUC regains activity despite containing the mentioned sequence. A large loss in promoter ac-

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Non-homologous stretches are also indicated (= = =) with the number of bases they contain. The putative regulatory elements are in capital letters and underlined in the two promoter sequences. Numbering in both sequences considers the initial ATG site as +1. The multiple sites of transcription initiation in the human promoter are indicated below the sequence with arrowheads (main sites) and small squares (secondary sites). Lower panel, an schematic diagram of the two proximal promoter regions depicting the homologous fragments (same code as above), the non-related stretches (——), the approximate location of the regulatory elements identified in the bovine promoter (NF-Y/Sp1), and the location of the regions protected in the RNase protection analysis (vertical boxes). The accession number of the human sequence in GenBank™ is AF453877.
Regulatory Elements of Nicotinic Receptor Gene Expression

Activity was observed when 36 bp were deleted from the 5'-end (compare p63β4LUC with respect to the larger construct p99β4LUC, Fig. 3). The most remarkable feature in the deleted region, between −64 and −99, was the presence of sites for Sp1 and NF-Y (labeled 1 and 2, respectively, in Fig. 4A). These transcription factors, or other proteins closely related immunologically with them, were able to bind to the mentioned elements (Fig. 5). Therefore, these elements appeared to be suitable candidates for controlling promoter activity. Consequently, when they were simultaneously mutated in the context of p99β4LUC, promoter activity was strongly reduced (Fig. 4B). The sum of effects due to the single alteration of these sites was lower than the effect of mutating them simultaneously, suggesting that Sp1 and NF-Y act cooperatively to play a crucial role in the transcriptional regulation of the bovine β4 gene. Both, Sp1 and NF-Y, are ubiquitously expressed transcription factors that play a major role in the transcription of many genes (35–37). Moreover, there are abundant examples of promoters that require the concerted action of Sp1 and NF-Y (see Refs. 38–42 for recent studies), and in two cases a physical interaction between these factors has been demonstrated (43, 44). In addition, other positive elements, located between −63 and +66 may be required for optimal transcription, given that constructs p39β4LUC, p63β4LUC (Fig. 3), and p2–1β4LUC (Fig. 4) that do not contain the Sp1 and NF-Y sites, still exhibited about 30–40% of the maximal promoter activity. However, if these additional factors exist, they were not detected with the standard conditions used in our EMSA experiments.

Albeit the high sequence identity (84.4%) between the coding regions of the bovine and rat β4 subunits (10), their proximal promoter regions did not show significant sequence homology. In an attempt to clarify the significance of this heterogeneity we decided to isolate and characterize another β4 subunit promoter, the one from the human species. As shown in Fig. 6, the structures of the human and bovine promoters are highly homologous, containing regions of 60–95% identity separated by non-related stretches of 50–165 bp, and indicating that there may be common regulatory mechanisms leading to their expression. This was confirmed upon transfection and EMSA experiments that demonstrated the involvement of Sp1 and NF-Y in the regulation of the human β4 promoter. However, the contribution of these factors was different from the one exhibited in the bovine promoter for several reasons. First, the situation of the elements to which these factors bind is different in the two promoters and despite the existence of several regions of moderate to high homology, they are located in sequence stretches that are not homologous. Second, in the absence of the Sp1 site, the promoter activity that remains is clearly lower in the human promoter (Fig. 9) than in its bovine counterpart (Fig. 4), suggesting a different action mechanism for this transcription factor in the two promoters. And finally, the role of NF-Y also appears to be different in the two cases. Thus, in the bovine promoter NF-Y acts in a concerted manner with Sp1, whereas the role of NF-Y in the human promoter appears to depend on the cell context where promoter activity is being tested. Thus, in SHSY-5Y cells appears to be irrelevant, while in C2C12 and COS cells could be involved in a repressing mechanism as it is suggested by the increase of transcriptional activity observed upon its mutation (Fig. 9). A possible explanation for this effect would be that the absence of NF-Y facilitates the binding of an activating factor. In fact, EMSA experiments performed with a probe in which the NF-Y site had been altered (Fig. 10B, lane 15, small square) revealed the formation of a complex that was not observed with the wild probe. However, this shift pattern was observed not only with C2C12 extracts (not shown) but also with nuclear extracts from SHSY-5Y cells (Fig. 10), in which promoter activity was not modified by the NF-Y mutation. Alternatively, and given that there is a 3-bp overlap within the Sp1 and NF-Y sites, the absence of NF-Y would alleviate steric restrictions and increase the binding of Sp1, thus helping to enhance promoter activity. Such a situation, however, would be expected to occur in all the tested cell lines and one would anticipate the same effect of the NF-Y mutant in the three cell lines analyzed, what was not the case. Therefore, it is possible that more complex mechanisms are implicated in the differential effect of the NF-Y

**Fig. 8.** Deletion map analysis of human β4 gene promoter activity. SHSY-5Y and C2C12 cells were transfected with each of the plasmids, which were named pβ4LUC with the number of promoter base pairs included in the construct (numbering considers in this case as +1 one of the major protected fragments in the RNase experiments) and contained the luciferase reporter under the control of the different promoter regions. Data are expressed as in Fig. 3.

**Fig. 9.** Site 2 is the major determinant of the human β4 subunit promoter activity. A, the proximal region of the human β4 subunit promoter (nucleotides −74 to −38) is depicted with the putative regulatory elements boxed. This region contains putative binding sites for transcription factors Sp1 (boxes of solid lines, elements 1 and 2) and NF-Y (dashed box, element 3). Several nucleotides of each potential element were mutated as indicated below the sequence to yield constructs analyzed in transfection (panel B) experiments. B, the name of each mutant construct indicates the element(s) that have been altered. Plasmids were transfected into SHSY-5Y, C2C12, and COS cells, and their activities were measured. Luciferase activity was normalized to values obtained with the p60hβ4LUC construct. Data are expressed as in Fig. 3.
transcription factors were used to identify the proteins producing the lanes 7 and 11 protein extracts. In Sp1, respectively. The excess of competitor oligonucleotides with consensus sites for NF-Y and H11002 subunit promoter. The identification of Sp1 and NF-Y as proteins binding to the -elements 2 and 3 of the proximal region of the human nicotine receptor gene was used as gel mobility shift probe in the presence of crude SHSY-5Y cell nuclear extracts. When site 3 was altered, the formation of a new complex, lane 9, was observed. The band labeled with an arrowhead, whereas the band labeled with an asterisk, was observed (lanes 12, 14, 18, and 20) or absence (lanes 12, 14, 16, 18, and 20) of SHSY-5Y cell nuclear extracts. When site 3 was altered, the formation of a new complex, labeled with a square, was observed (lanes 15 and 21).

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Luis M. Valor, Antonio Campos-Caro, Carmen Carrasco-Serrano, José A. Ortiz, Juan J. Ballesta and Manuel Criado

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