Transcriptional Regulation by Activation and Repression Elements Located at the 5′-Noncoding Region of the Human α9 Nicotinic Receptor Subunit Gene*

Received for publication, July 2, 2003, and in revised form, July 14, 2003
Published, JBC Papers in Press, July 14, 2003, DOI 10.1074/jbc.M307043200

Luis M. Valor‡, Mar Castillo§, José A. Ortiz¶, and Manuel Criado†
From the Department of Biochemistry and Molecular Biology and the Instituto de Neurociencias, Universidad Miguel Hernández-Consejo Superior de Investigaciones Científicas, 03550 San Juan, Alicante, Spain

The α9 subunit is a component of the neuronal nicotinic acetylcholine receptor gene superfamily that is expressed in very restricted locations. The promoter of the human gene has been analyzed in the human neuroblastoma SH-SY5Y, where α9 subunit expression was detected, and in C2C12 cells that do not express α9. A proximal promoter region (from −322 to +113) showed maximal transcriptional activity in SH-SY5Y cells, whereas its activity in C1C12 cells was much lower. Two elements unusually located at the 5′-noncoding region exhibited opposite roles. A negative element located between +15 and +48 appears to be cell-specific because it was effective in C2C12 but not in SH-SY5Y cells, where it was counterbalanced by the presence of the promoter region 5′ to the initiation site. An activating element located between +66 and +79 and formed by two adjacent Sox boxes increased the activity of the α9 promoter about 4-fold and was even able to activate other promoters. This element interacts with Sox proteins, probably through a cooperative mechanism in which the two Sox boxes are necessary. We propose that the Sox complex provides an initial scaffold that facilitates the recruiting of the transcriptional machinery responsible for α9 subunit expression.

Neuronal nicotinic acetylcholine receptors (nAChRs) are members of a supergene family of ion channels gated by neurotransmitters (1). They are pentameric oligomers composed of related subunits, which are commonly classified as agonist-binding (designated α2–α10) and structural (β2–β4) subunits. Unlike some nAChR subunits that have a relatively broad expression, the α9 subunit has been found only in very restricted areas such as the pituitary pars tuberalis, the olfactory epithelium, and the cochlea (2, 3). This limited expression could be the consequence of tight mechanisms of transcriptional regulation, which might be of great interest in understanding how the regional and developmental expression of neuronal nAChRs is controlled at the transcriptional level (see Ref. 4 for a review). For this reason, here we have analyzed the human α9 promoter, finding that two cis-elements unusually located at the 5′-noncoding region of α9 transcripts control in opposite ways the basal transcriptional activity of the α9 subunit gene.

EXPERIMENTAL PROCEDURES

Isolation and Analysis of the 5′-Flanking Sequence of the α9 Subunit—The human α9 coding sequence was obtained by PCR from a human pituitary cDNA library (Clontech, Heidelberg, Germany) by using the information contained in the GenBank™ sequence AJ243342. A fragment from the 5′-end was used to screen a human genomic library constructed in EMBL3 SP6/T7 (Clontech) and tested as described previously (5). A bacteriophage clone was purified and characterized. It contained ~4,600 bp of 5′-flanking region and at least the first exon.

5′-RACE Analysis of 5′ mRNA Ends—The 5′-end of α9 mRNA was mapped by 5′-RACE, as primer extension and RNase protection methods did not yield satisfactory results. For this purpose the Marathon Ready cDNA system from Clontech was applied to the previously mentioned cDNA library from human pituitary as indicated by the manufacturer. Two antisense oligonucleotides at the first and second exons (see Fig. 1) were used in parallel assays of DNA amplification. Their sequences were: from position +201 to +180, 5′-CTCAGTCTGGAGG-CAGCAAAG-3′; +602 to +578, 5′-CTAATCTGGAGG-GTCACT-TCAGG-3′. The resulting products were cloned and sequenced.

RT-PCR Analysis—Poly (A) RNA was directly selected from SH-SY5Y cell lysates by oligo(dT)-Dynabeads (Dynal, Oslo, Norway) according to the manufacturer’s instructions. α9 subunit transcripts, as well as α7 and β4 transcripts as positive controls, were detected by an RT-PCR assay (Access RT-PCR System from Promega, Madison, WI). Briefly, samples of mRNA (200 ng) or in vitro synthesized cRNA (30 ng) in a final volume of 20 μl were reverse transcribed with 2 units of avian myeloblastosis virus (AMV) reverse transcriptase (45 min at 48 °C), and PCR was further performed for 30 cycles (30 s at 94 °C, 45 s at 60 °C, and 1 min at 68 °C) with 2 units of Tf1 DNA polymerase. The amplified DNA fragments extended over at least 1 intron or more, to rule out the possibility of amplifying a potential contamination of genomic DNA. Anyway, RT-PCR performed in the absence of AMV reverse transcriptase or RNA did not yield any DNA fragment. A similar analysis was performed with mRNA from C2C12 cells and 35 PCR cycles, and α7 but not α9 transcripts were detected.

Plasmid Construction—All α9 promoter-luciferase gene fusion genes were made in the pGL2-Basic vector (Promega), introducing in its polylinker upstream of the luciferase gene the suitable α9 promoter fragments. These fragments were generated with restriction enzymes and cloned directly into pGL2-Basic or subcloned first in pBluescript and then transferred to pGL2-Basic. Deletion analysis of the most promoter-proximal region was performed by generating either appropriate restriction enzyme fragments or PCR fragments with suitable sense oligonucleotides and an antisense primer (5′-CTTATGTTTTC-
FIG. 1. The 5′-region of the human α9 subunit gene. The nucleotide sequence of a fragment of genomic clone humα9—41 carrying exon 1 (with the protein sequence indicated underneath in italic), the 5’-region of intron 1 (indicated in lowercase letters), and ~1460 bp of 5′-flanking sequence are indicated. The positions of the oligonucleotides used in the RACE experiments are indicated as black arrowheads (arrows below the RACE experiments are indicated as positions of the oligonucleotides used in the 5′-RACE experiments). The 5′-ends of the oligonucleotides used in the transfection experiments are also indicated (X). The position of exon 2 (+478) has been deduced from GenBank™ sequence AY123244.

GGCGTCCCTCC-3′ that anneals to the pGL2-Basic vector downstream of the site of transcription initiation.

Cell Culture and Reporter Assays—SH-SYSY human neuroblastoma cells were grown in 90% Eagle’s minimal essential medium with Glutamax-1 (Invitrogen) and 10% fetal calf serum. C2C12 cells were grown in 85% Dulbecco’s modified Eagle medium and 15% fetal calf serum. Plasmids were purified by Concert columns (Invitrogen, manufacture discontinued). Both cell types were transfected by the calcium phosphate procedure (6). SH-SYSY (105 cells/well) or C2C12 cells (104 cells/well) on 24-well plates were incubated with 1.5 μg of the site of transcription initiation.

Electrophoretic Mobility Shift Assay—Crude nuclear extracts were prepared from cultured cells as described (7). Probes were obtained by annealing complementary oligonucleotides corresponding to region +66 to +80 and end-labeled by Klenow filling with [α-32P]dCTP (Amersham Biosciences). The DNA-protein binding reaction volumes were 20 μl containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 5 μg of bovine serum albumin, 2 μg of poly(dG-dC)•poly(dG-dC) (Amersham Biosciences), 2 μg of nuclear extract protein, and 20,000 cpm of 32P-labeled probe. Reactions were incubated for 10 min at room temperature, the labeled probe was added, and the incubation was continued for an additional 20 min. For competition studies, the nuclear extract was incubated with the competing oligonucleotide prior to the labeled probe for 20 min. In some experiments purified proteins were used instead of nuclear extracts. The high mobility group (HMG) domain of Sox10 (nucleotides 886–1135) fused to glutathione S-transferase (GST) was kindly provided by Dr. M. Wegner (Erlangen University, Germany). The protein was expressed in bacteria and purified as described (8). Recombinant Sox9 was obtained by applying the TNT Quick Coupled Transcription/Translation System (Promega) to the pcDNA3-HA-Sox9 plasmid, kindly provided by Dr. V. Harley (Prince Henry’s Institute, Clayton, Australia). A double-stranded oligonucleotide containing a consensus sequence for Sox proteins was used in some experiments. Its sequence was 5′-GGGAATTC-GGCGTTTGTTCCTCCCA-3′.

RESULTS

Structure of the 5′-Flanking Region of the Human α9 Subunit Gene—The 5′-end of α9 mRNA was mapped by 5′-RACE, as primer extension and RNase protection experiments produced inconsistent results. In 5′-RACE several DNA fragments were detected; their 5′-ends are indicated in Fig. 1 (black arrowheads). These fragments may represent true 5′-ends of the α9 RNA or may just be the result of arrests during reverse transcription. Because the largest fragment was obtained independently several times with two different oligonucleotides, we tentatively considered its 5′-end as the most probable site of transcription initiation.

To examine the requirements for α9 subunit transcription, its promoter region was isolated and analyzed. A human genomic library was screened, and mapping of a bacteriophage
sequences were also observed.

sites that showed mismatches in regard to perfect consensus /H11002 were reverse transcribed, and then PCR was performed as indicated under “Experimental Procedures” by using specific primers for a7 (lanes 1–3), a9 (lanes 4–6), and b4 (lanes 7–9) subunits. Samples without RNA were also used (denoted as –).

cloned showed the presence of the a9 5′-untranslated region (first exon) and further upstream sequences. A BamHI-HindIII fragment (Fig. 1) containing the first exon and part of the first intron was sequenced. Further comparison of this sequence to a data base of binding sequences of known transcription factors only revealed the lack of a TATA box and the presence of two adjacent Sox protein binding sites with opposite orientations and located 3′ to the transcription initiation site. Additional sites that showed mismatches in regard to perfect consensus sequences were also observed.

Functional Analysis of the Human a9 Subunit Promoter—A series of constructs was generated to determine the regions of the a9 subunit promoter that contributed to its maximal activity. We tried to study their transcriptional activity in cells that express a9 subunits, such as human keratinocytes (9) and the immortalized cell line UB/OC-2, which was developed from the organ of Corti of the transgenic H-2Kb-tsA58 mouse (10, 11).

However, no satisfactory results were obtained (see “Discussion”). Because the human neuroblastoma SH-SY5Y express a3, a5, a7, b2, b3, and b4 nAChR subunits (12), we decided to test for the presence of a9 transcripts by an RT-PCR assay. As shown in Fig. 2, a9 transcripts were detected in SH-SY5Y cells. As reaction controls, transcripts corresponding to a7 and b4 subunits were also amplified. Although this assay was not quantitative, it is apparent that a7 transcripts were more abundant than those corresponding to the b4 and a9 subunits.

These results suggest that the a9 gene is transcribed in SH-SY5Y and, therefore, that this cell line might be considered an adequate model in which to study the transcriptional activity of different a9 promoter constructs. In addition, mouse muscle C2C12 cells, which express the muscle-type nAChR (13) and the neuronal a5 and a7 nAChR subunit transcripts (14) but not a9 transcripts (not shown), were used for comparative purposes. Some representative constructs were also tested in pituitary derived GH3 cells, but the results did not differ from those found in C2C12 cells.

A construct containing 696 bp of a9 promoter sequence plus 32 bp of 5′-noncoding region was shown to be moderately active in both SH-SY5Y and C2C12 cells (Fig. 3, construct –696/+32), whereas a shorter construct with a 3′ deletion of the most proximal promoter region (construct –696/–317) showed a substantial decrease in promoter activity. By contrast, a construct containing the same 696 bp of promoter sequence plus 113 bp of 5′-noncoding region (construct –696/+113) exhibited a significant increase in luciferase activity of about 4- and 2.5-fold in SH-SY5Y and C2C12 cells, respectively. A further 5′ deletion (construct –322/+113) did not modify transcriptional activity. Interestingly, when the luciferase activity of constructs –696/+113 and –322/+113 was normalized for transfection efficiency (as determined by β-galactosidase activity) and compared in these two cell lines, it was about 3 times higher in SH-SY5Y cells than in C2C12 cells, which could account, at least in part, for the presence of a9 transcripts in SH-SY5Y but not in C2C12 cells. It is important to notice that the promoter activity of the different constructs was expressed as a percentage of the values obtained with construct [+66/+113], because the activity of the latter was similar in both cell lines, constituting a convenient comparative reference. Moreover, this construct contains elements important for transcription (see Fig. 5).

The unexpected increase observed upon adding the 5′-noncoding region, comprising +33 to +113, suggested that it might contain important elements for transcription of the a9 gene, inducing us to explore it in more detail. Thus, the luciferase activity of construct [+15/+113], where the basal promoter region has been removed, was lower (by about 2-fold in regard to construct [−696/+113]) but still significant, indicating that this region might facilitate RNA polymerase action by itself. Moreover, further 5′ deletions gave rise to activity increases in constructs [+34/+113], [+49/+113], and [+66/+113] to reach levels similar to construct [−696/+113]. This was especially dramatic in C2C12 cells, because removing the region comprising +15 to +48 yielded an activity equivalent to that found in SH-SY5Y cells. This fact suggested that some elements (denoted by a filled box in the scheme shown in Fig. 3) in the deleted region could play a negative role on transcription especially in C2C12 cells. Finally, larger deletions (constructs [−78/+113] and [−95/+113]) produced a dramatic decrease in activity, indicating that sequences in the deleted regions, located approximately 60–90 bp upstream of the main start site of transcription, may act as positive elements for transcription of the a9 subunit gene in transient transfection assays. In this region the presence of two Sox boxes (15, 16) (depicted as open boxes in the scheme shown in Fig. 3) in opposite orientations was observed between +66 and +79 (see Fig. 4A). When these two elements were inserted into pGL2-Basic (construct [+66/+80] in Fig. 3), substantial luciferase activity was detected, about 55 and 72% of the maximal activity observed in SH-SY5Y and C2C12, respectively. Therefore they appear to play an important role and were analyzed further (see Fig. 4).

Characterization of the Positive Element Present at the 5′-Noncoding Region—A functional analysis of the two Sox boxes was carried out by looking at the effects produced by their mutagenesis in the context of construct [+66/+113] (Fig. 4). Mutation of box 1 resulted in a 76 and 93% decrease in transcriptional activity in SH-SY5Y and C2C12 cells, respectively, close to the activity of constructs with the two boxes deleted (constructs [+76/+113] and [+95/+113] in Fig. 3). Mutation of box 2 produced less severe effects. Therefore, it appears that box 1 plays a more determining role in the transcriptional activity of the a9 subunit promoter than box 2, although both seem necessary for maximal activity and might integrate into a whole synergistic mechanism, as suggested by the electrophoretic mobility shift assay experiments to be described next.

DNA fragments carrying the wild-type +66 to +80 promoter region and the corresponding box 1 and box 2 mutants from the previous functional studies were labeled and incubated with increasing amounts of a fusion protein made of the HMG domain of Sox10 and GST. The HMG domain is a highly conserved DNA-binding region present in the Sox family of transcription factors (17). A retarded band was observed (Fig. 5A, lanes 1–4) when using the wild-type DNA fragment. Its intensity increased with increasing amounts of HMG-GST. By contrast, when the box 2 mutant was used as probe, the intensity of the retarded band was very reduced (Fig. 5A, lanes 5–8).
This reduction was even stronger when the box 1 mutant was used as probe (Fig. 5A, lanes 9–12). This finding suggests that the HMG domain of Sox10 is able to recognize the two boxes and that its affinity for them is higher if both are intact. Moreover, the single mutants results indicate that box 1 has higher affinity, in agreement with its predominant role in the transcriptional activity of the resultant constructs in C2C12 cells was compared with that shown by the corresponding parent constructs. As observed in Fig. 7, the insertion of the Sox element produced an increase in transcriptional activity in both promoters, which was larger (about 5-fold) with the bovine a7 subunit promoter than with the human a7 promoter. Therefore, it can be concluded that the Sox element present in the 5'-noncoding region of the a9 subunit gene can cooperate with other promoters acting as transcriptional activator.

**DISCUSSION**

In this article we describe the characterization of the human nAChR a9 subunit promoter. An early attempt to perform functional assays in cells that express a9 subunits, such as human keratinocytes (9), failed because of the limited amount of available cells along with the difficulty in finding an efficient and reproducible transfection method. We also tested the immortalized cell line UB/OC-2, which expresses the a9 nAChR (10); it was efficiently transfected as indicated by expression of green fluorescent protein under the control of the human cytomegalovirus promoter. However, no luciferase activity was detected when different a9 promoter constructs were tested. The same happened with plasmids pGLControl and pCH110, which should express luciferase and β-galactosidase, respectively, under the control of the SV40 promoter. The reason for the lack of

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**Figure 3.** Deletion map analysis of human a9 gene promoter activity. SH-SY5Y and C2C12 cells were transfected with each of the constructs containing the different fragments of the a9 subunit promoter (indicated by the numbers corresponding to their 5'- and 3’-ends in regard to the major transcription initiation site) and the luciferase reporter as well as pCH110/β-galactosidase as a transfection efficiency control. The means ± S.E. (error bars) are given for at least two or three individual experiments carried out in triplicate. The closed and open boxes at the scheme represent the repression and activation domains, respectively.

**Figure 4.** Sox sites are functional elements required for a9 subunit gene expression. **A**, the Sox binding region of the a9 subunit promoter (nucleotides +66 to +80) is depicted with the two Sox boxes indicated by arrows pointing in opposite directions. Several nucleotides of each potential element were mutated, as indicated below the sequence to yield constructs analyzed in transfection experiments. **B**, the name of each mutant construct indicates the elements that have been altered. Plasmids were transfected into SH-SY5Y and C2C12 cells and their activity measured. Luciferase activity was normalized to the values obtained with construct +66/+113. Data are expressed as described in the legend for Fig. 3.

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**Figure 5.** A probe containing one consensus site for Sox proteins produced only the band of higher mobility (denoted as I in Fig. 6A), indicating that the bands of lower mobility obtained with the wild-type probe are probably the result of complex interactions involving the two Sox boxes and perhaps protein-protein interactions. Moreover, competition experiments (Fig. 6B) confirmed the specificity of the interactions, also showing that mutant probes (lanes 18–21 and 22–25) were less potent competitors than the wild-type probe (lanes 14–17). Therefore, these results suggest that the positive regulator located in the 5'-noncoding region of the a9 subunit gene is composed of two boxes to which proteins of the Sox family can bind. The identity of these proteins, which compose a family with about 25 members, remains to be elucidated.
activity of some promoters while others are active is unknown, but it may be related to the way this cell line was established and/or the particular cell culture conditions that are required (11). Therefore, we chose as a cell model the SH-SY5Y line, which also expresses a9 transcripts. Thus, the study of deletions of this promoter (Fig. 3) suggested the existence of three main regions: the typical basal promoter region located 5' to the transcription initiation site and two other domains, having negative and positive transcriptional effects, which are closely located at the 5'-noncoding region of the first exon.

The basal region was more active in SH-SY5Y than in C2C12 cells, and its boundaries could be roughly confined between -316 and -32 bp of the initiation site, because construct [-696/-317] was much less active than construct [-696/+32]. Moreover, constructs [-696/+113] and [-322/+113] produced equivalent results, confirming the relevance of the region close to the initiation site.

However, the most striking results were obtained when the

**Fig. 5.** Binding of Sox proteins to the Sox region of the a9 subunit promoter. A, the DNA fragment corresponding to region +66 to +80 was used as a gel mobility shift probe in the presence of increasing amounts (0.5, 1, 1.5, and 2 μg) of the HMG domain of Sox10 fused to GST, which were produced in bacteria and purified. Protein was incubated with the wild-type (+66/+80) DNA fragment (WT, lanes 1–4), and the corresponding DNA fragments mutated at either of the two Sox sites analyzed previously (lanes 5–8 and 9–12). B, a similar experiment was carried out with the complete Sox9 protein produced in vitro. In this case 0.5 (lanes 13 and 18), 1 (lanes 14, 16, and 19), and 1.5 μg (lanes 15, 17, and 20) of protein were incubated with the same DNA fragments as in described in A.

**Fig. 6.** Binding of cell nuclear proteins to the Sox region of the a9 subunit promoter. A, the DNA fragment corresponding to the region +66 to +80 was used as a gel mobility shift probe in the presence of 2 μg of crude SH-SY5Y (S) (lanes 2, 5, 8, and 11) or C2C12 (C) (lanes 3, 6, 9, and 12) cell nuclear extracts. Protein was incubated with the wild-type probe (WT) (lanes 1–3) or with the corresponding probes mutated at either of the two Sox sites analyzed previously (lanes 4–6 and 7–9) as well as with a probe containing the consensus site for Sox proteins (lanes 10–12). The first lane in each series (–) is a probe run in the absence of protein extracts (lanes 1, 4, 7, and 10). Several complexes were observed (indicated by roman numerals at the left of the figure). B, the gel mobility assay was run using the wild-type DNA fragment as the labeled probe and nuclear extracts from SHSY-5Y cells. The first lane (lane 13) is without added competitor. Competitor DNA fragments wild-type (lanes 14–17) and mutants 1 (lanes 18–21) and 2 (lanes 22–25) were added in increasing amounts (10-, 30-, 100-, and 300-fold excess).
region between +15 and +113 was present, especially in SH-SY5Y cells, which we have shown to transcribe the α9 subunit gene. This region, which had transcriptional activity by itself (construct [+15/+113]), although lower than constructs containing the basal promoter region appears to contain two different elements. Thus, 5′ deletions between +15 and +48 produced an increase in transcriptional activity up to maximal levels, suggesting the existence of a negative element in the deleted area. This repression, although partial, was particularly evident in C2C12 cells, because repression was kept even with the larger constructs, which contain the basal promoter region. By contrast, this was not the case in SH-SY5Y cells, suggesting that complex interactions at the basal promoter region and the negative element would overcome the effect of the latter in these cells. Interestingly, the graded removal of the repressing effect (compare constructs [+15/+113], [+34/+113], and [+49/+113] in Fig. 3) suggests that the whole region between +15 and +48 is involved in repression.

Further 5′ deletions produced a strong decrease in activity, indicating that an activating element exists between +66 and +80. The most remarkable feature in this region was the presence of two sites for Sox proteins located in opposite orientations. These, in fact, are inverted repeats that confer a palindromic structure to the whole complex. Their alteration produced a significant decrease in luciferase activity, especially in site 1 (Fig. 4), which having the perfect consensus sequence for binding of Sox proteins might have higher affinity for them. Moreover, shift assays showed that these sites can bind the HMG domain of Sox10 as well as the complete protein Sox9 (Fig. 5), site 1 again being the one having more affinity for Sox proteins. However, binding is much higher when both sites are intact, suggesting that they constitute a whole symmetrical cooperative entity. This was confirmed with nuclear extracts, in particular the ones from SH-SY5Y cells (Fig. 6). They produced a complex pattern of retarded bands in the presence of the probe that has the two Sox boxes intact and a more simplified pattern with the mutants, which is nearly coincident with the one observed using a unique consensus Sox motif as probe. It is possible that the two Sox motifs provide a platform on which a protein complex can be built through protein-protein interactions. In this case the complex with the lower mobility would be the result of these interactions.

The identity of the Sox proteins involved in the transcriptional regulation of the α9 subunit gene remains to be elucidated. Some possible candidates could be Sox2/Dichaete, Sox5 and its isoform L-Sox5, Sox6, Sox9, or Sox10, because these proteins are expressed in embryonic structures (neural crest, otic vesicle, etc.; see Ref. 21 for a review) producing cell types that express α9 nAChRs later. In this context it is interesting to mention that Sox10 has been found to participate in the transcriptional regulation of the rat α3 and β4 subunit genes (22) and that mutations of the SOX10 gene leads to combined Waardenburg-Hirschsprung syndrome in humans (23). Classical features of the Waardenburg-Hirschsprung syndrome are deafness and pigmentation defects, and interestingly, α9 nAChRs are involved in hearing processes (24, 25) and are also expressed in keratinocytes (9). In any case, it might be difficult to identify which Sox protein is regulating α9 for two reasons: first, because the properties of DNA binding and structural modification (bending) of the HMG domains of different Sox proteins appear to be undistinguishable (26), and second because of the coexpression of several Sox proteins in the same cell type, which suggests a certain functional redundancy (27, 28). A possible strategy would be to search for proteins that determine the interaction specificity of Sox proteins (26), such as the ones with POU (Pit-1/Oct-1/Oct-2/unc-86) domains. Possible candidates could be Pit-1, involved in determination of the different cellular phenotypes of the anterior pituitary during organogenesis (29, 30), and Brn-3.1, essential for survival of cochlear ciliary cells (31).

Although not common, the presence of transcriptional regulatory elements at the 5′-noncoding region of genes is well documented, ranging from general elements such as the downstream promoter element (32) to others more specific (33–35) even within the nAChR family (36–38). These elements, however, are transcriptional enhancers that depend on their promoter region to exert their function. By contrast, in the case of the α9 subunit, the Sox complex can act as promoter by itself, at least in the conditions used in our transfection studies. This situation recalls the one found in the type I and II promoters of RNA polymerase III, where promoter elements are located within the transcribed region (for a review see Ref. 39). Here transcription factor TFIIIA serves as an adaptor, providing a platform that allows the multi-subunit complex called TFIIIC to be recruited. The primary function of the latter is to recruit TFIIIB, a heterotrimer able to bring the RNA polymerase III to the promoter and position it over the initiation region. In the case of the α9 subunit gene, Sox proteins interacting with the two Sox boxes would provide the initial scaffold that would facilitate further recruiting of the transcriptional machinery. The complex pattern observed in the shift assays performed with SH-SY5Y nuclear extracts would reflect the first steps of this process. Moreover, the activation of the α7 and β4 subunit promoters would indicate that the Sox elements could exert this facilitating function independently of the specific requirements of each promoter.

Acknowledgments—The excellent technical assistance of Susana Gerber is appreciated. We thank Drs. Vincent Harley and Michael Wegner for Sox9 and Sox10 clones, respectively, and Dr. Matthew C. Holley for the UB/OC-2 cochlear cells.
REFERENCES

1. Karlin, A. (2002) *Nat. Rev. Neurosci.* 3, 102–114
2. Elgoyhen, A. B., Johnson, D. S., Boulter, J., Vetter, D. E., and Heinemann, S. (1994) *Cell* 70, 705–715
3. Simmons, D. D., and Morley, B. J. (1998) *Brain Res. Mol. Brain Res.* 56, 287–292
4. Fornasari, K. I., Battaglioli, E., Terzano, S., and Clementi, F. (1998) in *Neuronal Nicotinic Receptors: Pharmacology and Therapeutic Opportunities* (Arneric, S. P., and Brioni, J. D., eds) pp 25–42, Wiley-Liss, New York
5. Garcia-Guzmán, M., Sala, F., Sala, S., Campos-Caro, A., Stühmer, W., Gutiérrez, L. M., and Criado, M. (1995) *Eur. J. Neurosci.* 7, 647–655
6. Graham, F. L., and van der Eb, A. J. (1973) *Virology* 52, 456–467
7. Schreiber, E., Matthias, P., Müller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* 17, 6419
8. Jain, N., Mahendran, R., Guy, R., Tan, Y. H., and Cao, X. (1996) *J. Biol. Chem.* 271, 13530–13536
9. Nguyen, V. T., Nduye, A., and Grando, S. A. (2000) *Am. J. Pathol.* 157, 1377–1391
10. Jagger, D. J., Griesinger, C. B., Rivolta, M. N., Holley, M. C., and Ashmore, J. F. (2000) *J. Physiol.* 527, 49–54
11. Rivolta, M. N., Grix, N., Lawler, P., Ashmore, J. F., Jagger, D. J., and Holley, M. C. (1998) *Proc. R. Soc. Lond. B Biol. Sci.* 265, 1595–1603
12. Groot Kormelink, P. J., and W. H. M. Luyten, W. H. M. L. (1997) *FEBS Lett.* 400, 309–314
13. Gardner, P. D., Heinemann, S., and Patrick, J. (1987) *Brain Res.* 427, 69–76
14. Campos-Caro, A., Carrasco-Serrano, C., Valor, L. M., Ballesta, J. J., and Criado, M. (2001) *DNA Cell Biol.* 20, 657–666
15. Harley, V. R., Lovell-Badge, R., and Goodfellow, P. N. (1994) *Nucleic Acids Res.* 22, 1500–1501
16. Peirano, R. L., Groch, D. E., Riehmacher, M., and Wegner, M. (2000) *Mol. Cell. Biol.* 20, 3198–3209
17. Kühbrecht, K., Herhabit, B., Sock, E., Hermans-Borgmeyer, I., and Wegner, M. (1998) *J. Neurosci.* 18, 237–250
18. Clarkson, M. J., and Harley, V. R. (2002) *Trends Endocrinol. Metab.* 13, 106–111
19. Carrasco-Serrano, C., Campos-Caro, A., Viniegra, S., Ballesta, J. J., and Criado, M. (1998) *J. Biol. Chem.* 273, 20021–20028
20. Valor, L. M., Campos-Caro, A., Carrasco-Serrano, C., Ortiz, J. A., Ballesta, J. J., and Criado, M. (2002) *J. Biol. Chem.* 277, 8866–8876
21. Wegner, M. (1999) *Nucleic Acids Res.* 27, 1409–1420
22. Liu, Q., Melnikova, I. N., Hu, M., and Gardner, P. D. (1999) *J. Neurosci.* 19, 9747–9755
23. Kühbrecht, K., Schmidt, C., Sock, E., Pingault, Y., Bondurand, N., Goossens, M., and Wegner, M. (1998) *J. Biol. Chem.* 273, 20033–20038
24. Fuchs, P. (2002) *Audiol. Neurootol.* 7, 40–44
25. Maison, S. F., Luebke, A. E., Liberman, M. C., and Zuo, J. (2002) *J. Neurosci.* 22, 10638–10646
26. Kamachi, Y., Cheah, K. S., and Kondoh, H. (1999) *Mol. Cell. Biol.* 19, 107–120
27. Collignon, J., Sockanathan, S., Hacker, A., Cohen-Tannoudji, M., Norris, D., Rastan, S., Stevanovic, M., Goodfellow, P. N., and Lovell-Badge, R. (1996) *Development* 122, 509–520
28. Lefebvre, V., Li P., and de Crombrugghe, B. (1998) *EMBO J.* 17, 5718–5733
29. Rosenfeld, M. G., Briata, P., Dasen, J., Gleiberman, A. S., Kioussi, C., Lin, C., O’Connell, S. M., Ryan, A., Szeto, D. P., and Treier, M. (2000) *Recent Prog. Horm. Res.* 55, 1–13
30. Andersson, B., and Rosenfeld, M. G. (2001) *Endoer. Rev.* 22, 2–35
31. Ryan, A. F. (2002) *Audiol. Neurootol.* 7, 138–140
32. Burke, T. W., and Kadonaga, J. T. (1997) *Genes Dev.* 11, 3020–3031
33. Yamamoto, K., Takeshima, H., Yamada, K., Nakao, M., Kino, T., Niishi, T., Kuchi, M., Kuratsu, J., Yoshimura, T., and Ushio, Y. (1999) *J. Biol. Chem.* 274, 4646–4654
34. Hernández-Torres, J., Yunta, M., and Lazo, P. A. (2001) *J. Biol. Chem.* 276, 35405–35413
35. van der Stoep, N., Quinten, E., and van den Elsen, P. J. (2002) *J. Immunol.* 169, 5061–5071
36. Besa, A., Salmons, A. M., Zoli, M., Le Novère, N., Picciotto, M., and Changeux, J. P. (1995) *Neurosci.* 108, 817–819
37. Fornasari, D., Battaglioli, E., Flora, A., Terzano, S., and Clementi, F. (1997) *Mol. Pharmacol.* 51, 250–261
38. Flora, A., Schulz, R., Battaglioli, E., Terzano, S., Clementi, F., and Fornasari, D. (2000) *Eur. J. Pharmacol.* 383, 85–95
39. Paule, M. L., and White, R. E. (2000) *Nucleic Acids Res.* 28, 1283–1298