GC- and E-box Motifs as Regulatory Elements in the Proximal Promoter Region of the Neuronal Nicotinic Receptor α7 Subunit Gene*

(Received for publication, April 9, 1998, and in revised form, May 26, 1998)

Carmen Carrasco-Serrano‡‡§§, Antonio Campos-Caro¶¶§§, Salvador Viniegra§§, Juan J. Ballesta***, and Manuel Criado‡‡‡‡

From the Departments of §Neurochemistry and **Pharmacology, and §§Instituto de Neurociencias, Universidad Miguel Hernández, 03550 San Juan, Alicante, Spain

The α7 subunit is a component of α-bungarotoxin-sensitive nicotinic acetylcholine receptors expressed in bovine adrenomedullary chromaffin cells. The proximal promoter of the gene coding for this subunit contains several GC-boxes and one E-box. Deletion analysis and transient transfections showed that a 120-base pair region (−77 to +43) including all of these elements gave rise to ~70 and 95% of the maximal transcriptional activity observed in chromaffin and SH-SY-5Y neuroblastoma cells, respectively. Site-directed mutagenesis of the different elements indicated that both GC and E motifs contribute to the activity of the α7 gene in a very prominent way. Using electrophoretic mobility shift assays, the upstream stimulatory factor (USF) was shown to be a component of the complexes that interacted with the E-box when nuclear extracts from chromaffin and SH-SY-5Y cells were used. Binding of the early growth response gene transcription factor (Egr-1) to three different GC-boxes was also demonstrated by shift assays and DNase I footprint analysis. Likewise, α7 promoter activity increased by up to 5-fold when α7 constructs and an Egr-1 expression vector were cotransfected into chromaffin cell cultures. Mutagenesis of individual GC-boxes had little effect on Egr-1 activation. By contrast, pairwise suppression of GC-boxes abolished activation, especially when the most promoter-proximal of the Egr-1 sites was removed. Taken together, these studies indicate that the α7 gene is likely to be a target for multiple signaling pathways, in which various regulatory elements are involved.

Nicotinic acetylcholine receptors (nAChRs) are members of a supergene family of neurotransmitter-gated ion channels (1, 2). Neurons in the central and peripheral nervous systems express a diversity of nAChRs with different electrophysiological and pharmacological properties (3, 4). This variety arises, at least in part, from the different combinations of subunits that can form functional nAChRs (5). Since each of the subunits exhibits well defined and restricted expression patterns in vivo (1), it is of interest to elucidate the mechanisms controlling the expression of nAChR subunit genes. At present, both positive and negative transcriptional regulatory elements have been described in several nAChR subunits (6–17).

The chromaffin cells of the adrenal medulla constitute a good model system in which to study the function and regulation of neuronal nAChRs. These paraneurons express the two main types of nAChRs present in the peripheral nervous system; the one sensitive to α-bungarotoxin is formed by α7 subunits (18), whereas the insensitive one is probably composed of α3, α5, and β4 subunits (19, 20). Acetylcholine triggers catecholamine secretion through a mechanism that apparently involves the latter nAChR subtype (21).

The function of the α-bungarotoxin-sensitive nAChRs in chromaffin cells remains obscure. The high Ca2+ permeability of these receptors (22) suggests their involvement in the control of intracellular Ca2+ levels. Previously, we have shown that they are differentially expressed in adrenergic cells (23), presumably providing these cells with an additional way of epinephrine release. Accordingly, α7 subunit expression is also restricted to adrenergic cells by a mechanism that appears to be mediated by the immediate early gene transcription factor Egr-1. This protein was found to be expressed exclusively in adrenergic cells and shown to bind to two sites within the proximal promoter of the α7 gene (23).

The present study was undertaken to further characterize the elements acting at this promoter. We report here that, in addition to the two Egr-1 sites previously described, at least two other positive regulatory elements exist. One of these elements, a GC-box, was shown to interact with Egr-1 (and also Sp1), whereas the other, an E-box, is recognized by transcription factors USF1 and USF2.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The isolation and characterization of the 5′-flanking sequence of the bovine α7 subunit gene has been previously described (23). All α7 promoter-LUC gene fusions were made in the pGL2-Basic vector (Promega, Madison, WI), introducing in its polylinker, upstream of the luciferase gene, the suitable α7 promoter fragments. Deletion analysis was performed by partially digesting an ApaI–HindIII fragment (from −339 to +43 in the α7 sequence) with BstUI, which leaves blunt ends at GCGC sequences, and subcloning the partial digests into pBluescript (Stratagene, Heidelberg, Germany) vector cut with HincII–HindIII. Sequence analysis allowed selection of the appropriate fragments, which were cloned into pGL2-Basic and further transfected.

The basic strategy for site-directed mutagenesis of the different elements at the −77 to −15 region of the α7 promoter (see Fig. 2) consisted of the following steps. (a) PCR (25 cycles of 94 °C for 10 s, 62 °C for 30 s, 68 °C for 45 s using the Expand kit from Boehringer...
Mannheim) amplification of p77α7LUC (or its single or double mutants when double or triple mutants were desired, respectively) was performed with suitable mutagenic primers in the sense orientation and an antisense primer (5′-CTTTATGTTTTTGGGCTTCTC-3′) that anneals to the pGL2-Basic vector downstream of the site of transcription initiation. The introduced mutations are indicated in lowercase letters in Fig. 2A and sometimes generated restriction enzyme sites that were useful for further mutant constructions and to confirm mutagenesis. The mutant sequences did not create any known binding site for transcription factors. Moreover, electrophoretic mobility shift assays (EMSAs), performed by using the mutant fragments as probes, never resulted in the formation of new retarded complexes. (b) PCR products were cloned into pBluescript, sequenced, and further transferred to the appropriate construct, which was also in pBluescript. (c) Constructs were cloned into the pGL2-Basic vector. A more direct cloning strategy from steps a to c was not possible, since a key restriction enzyme used in mutant construction and with a unique site in the α7 promoter fragment, PmaCI, has several sites in the pGL2-Basic vector.

**Cell Culture and Reporter Assays**—Chromaffin cells were isolated from bovine adrenal glands as described by Gandía et al. (24) and cultured in 90% Dulbecco’s modified Eagle medium (Sigma, Madrid, Spain), 10% fetal calf serum, with 10 mM cytosine arabinoside and 10 mM 5-fluoro-2′-deoxyuridine (Sigma) added to prevent fibroblast proliferation. SH-SY5Y human neuroblastoma cells were grown in 90% Eagle’s medium for 2 h, the bands were visualized by a chromogenic reaction following the 30-min incubation at room temperature, labeled probe was added, and the incubation continued for an additional 20-min period. For competition studies, the nuclear extract was incubated with the competing probe prior to the labeled probe during 20 min. Supershift assays were performed by preincubating nuclear extracts with 2 μg of antibodies against different transcription factors (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit IgG (Sigma) for 3 h on ice before the probe addition.

In some experiments, purified Egr-1 protein fused to glutathione S-transferase was used. The expression vector for the Egr-1 fusion protein was kindly provided by Dr. X. Cao (National University of Singapore), and the protein was expressed in bacteria and purified as described by Jain et al. (28). Recombinant Sp1 was obtained from Promega.

**Western Blots**—10 μg of nuclear proteins/lane were separated by 10% SDS-polyacrylamide gel electrophoresis. Western blots were carried out as described by Towbin et al. (29). After the transfer, nitrocellulose membranes were blocked overnight at 4 °C with 5% dry milk in phosphate-buffered saline, and incubated in the same way with the appropriate primary antibodies (1:500) in phosphate-buffered saline, 5% dry milk. After incubation with the secondary antibody at room temperature for 2 h, the bands were visualized by a chromogenic reaction (Sigma Fast, nitro blue tetrazolium, Sigma).

**DNase I Footprinting**—The antisense strand corresponding to region −77 to +43 of the α7 promoter was end-labeled by Klenow filling with [α-32P]dATP. Assays were performed with the Sure Track Footprinting kit from Amersham Pharmacia Biotech, according to the manufacturer’s instructions. Recombinant Egr-1 or Sp1 was incubated with the radiolabeled double-stranded fragment (25,000 cpm) using the binding reaction conditions described above in the EMSA experiments (except for the absence of EDTA and the presence of 2.5 mM MgCl2). Immediately following the 30-min incubation at room temperature, 0.5 mM CaCl2 and 1 mM MgCl2 were added to the reactions. This was followed by the addition of 1 unit of DNase I. The reactions were incubated at room temperature for 1 min and stopped with the addition of stop solution (SDS/EDTA). The DNA was prepared for loading onto an 8% polyacrylamide, 7 M urea sequencing gel (15,000 cpm/lane), and the Maxam-Gilbert A/G chemical sequencing reaction was included as reference ladder.
Deletion Analysis of the Promoter for the Bovine nAChR α7 Subunit—A series of constructs was generated to determine the regions of the α7 subunit proximal promoter (Fig. 1A) that contributed to its maximal activity. These constructs were introduced into SHSY-5Y and chromaffin cells, a neuroblastoma cell line and a primary cell culture, respectively, that express the α7 subunit endogenously (18, 30). In SHSY-5Y cells, the construct containing 77 bp of α7 promoter sequence plus 43 bp of 5′-noncoding region (p77α7LUC) showed the maximal activity (Fig. 1B). No significant changes in relative luciferase activity were observed when larger constructs were used (p125, p161, and p199α7LUC). In chromaffin cells, these constructs, particularly p199α7LUC, showed increased activity to yield ~145% of p77α7LUC. The activity of p77α7LUC in SHSY-5Y was about 90% reduced when 26 bp of the α7 promoter 5′-end were deleted further (p51α7LUC). In chromaffin cells, this deletion caused a 75% decrease in activity, with respect to p77α7LUC. Therefore, sequences in the minimal promoter, within 77 bp upstream of the α7 subunit gene expression.

Characterization of the Regulatory Elements Present at −77 to −15 of the α7 Promoter by Mutagenesis and Transient Transfections—A search for transcription factors that could interact with elements at the proximal promoter region of the α7 subunit revealed the existence of several GC-boxes (labeled 1, 2, and 4 in Fig. 2A) and an E-box (labeled 3 in Fig. 2A). In fact, our previous study on the bovine α7 promoter (23) characterized two of the GC-boxes (1 and 2, Fig. 2A) as binding sites for Egr-1 and demonstrated their involvement in activation of the α7 promoter by this transcription factor. However, construct p51α7LUC displayed low promoter activity despite containing these elements, which suggested the contribution of other elements between −51 and −77 to maximal promoter activity. Therefore, a systematic analysis of these putative regulatory elements was carried out, by looking at the functional effects produced by their mutagenesis in the context of p77α7LUC (Fig. 2B).

Single mutations of GC-boxes 1 and 2 had virtually no effect on functional activity relative to p77α7LUC. However, mutations of E-box 3 or GC-box 4 resulted in a 40–50% decrease in transcriptional activity, which may explain, at least partially, the large difference observed between p77α7LUC and p51α7LUC. Interestingly, when boxes 3 and 4 were left intact while mutating simultaneously boxes 1 and 2, a larger decrease (11 and 32% of activity relative to wild type in SHSY-5Y and chromaffin cells, respectively) was observed. Double mutations of GC-boxes 1 and 4 or GC-boxes 2 and 4 did not modify, however, the effect observed with the single mutation of box 4. By contrast, the decrease observed upon mutation of the E-box (37 and 50% of p77α7LUC activity in SHSY-5Y and chromaffin cells, respectively) was further enhanced when the GC-box 1 (but not GC-box 2) was mutated (11 and 24%). Finally, the triple mutations of the three GC-boxes or the first two plus the E-box did not modify the functional activity observed with the double mutant of boxes 1 and 2 (not shown). These results suggest that the four elements that were analyzed contribute to the transcriptional activity observed in both cell types, although in different and, in some cases, interrelated ways.

Characterization of the Regulatory Elements Present at −77 to +43 of the α7 Promoter by EMSA—DNA fragments carrying the wild-type −77/+43 promoter region and the corresponding E-box mutant (Mut 3) were labeled and incubated with nuclear extracts from chromaffin cells (Fig. 3) and SHSY-5Y (not shown). Several retarded bands, one of them very prominent
Antibody supershift analysis was employed to determine the identity of the proteins producing the retarded bands (Fig. 4). Since an oligonucleotide containing a CACGTG Myc-Max consensus sequence (Santa Cruz Biotechnology) was also able to compete the formation of bands related to the E-box (not shown), it was reasonable to assume that a transcription factor belonging to the basic helix-loop-helix family could bind to this element. Consequently, antibodies against Myc, Max, USF1, and USF2 were tested. In addition, antibodies against proteins that could bind to GC-boxes (Sp1, Egr-1, AP-2, and Sp3) were used in an attempt to identify other complexes. Part of the main complex observed in the presence of chromaffin and SHSY-5Y nuclear extracts was shifted by antibodies against USF1 and USF2 (lanes 4, 5, 20, and 21) but not with anti-Myc (not shown) or Max (lane 9) antibodies. Antibodies against Sp1 (lane 7), AP-2 (lane 8), and Sp3 (not shown) were unable to supershift the complexes, but an Egr-1 antibody produced a shift of part of the major band (lanes 6 and 22). Accordingly, this band represents a combination of different retarded complexes of similar size, contributed by USF1, USF2, and Egr-1. This was confirmed by using a mix of the three antibodies. In this case, a complete band supershift was observed (lane 23). Moreover, under conditions in which the E-box was disrupted (Mut 3 as probe) the main band appears less intense (lanes 11 and 15) and is totally shifted by an Egr-1 antibody (lanes 13 and 17).
cated that the E-box functions as a recognition element for both USF1 and USF2 or some other protein antigenically related to them, whereas the GC-rich elements appear to be the target of Egr-1 in a way that was further analyzed (see below).

Western blot analysis of SHSY-5Y and chromaffin nuclear proteins indicated that USF and Egr-1 proteins were indeed expressed in these cells (Fig. 5). The protein bands detected with anti-USF antibodies showed the same size than the previously described for USF1 (43 kDa) and USF2 (44 kDa) polypeptides (31). The band reacting with an Egr1 antiserum was somehow diffuse and had a size (∼82 kDa) close to the one described for this transcription factor (32). Since USF proteins bind to DNA as homo- or heterodimers (86–88 kDa) (33) and Egr-1 as monomers (82 kDa), the retardation of the complexes produced by both factors in EMSA would be expected to be very similar, as it actually occurred (Fig. 3).

Interactions of Egr-1 and Sp1 with the GC-boxes in the Proximal α7 Promoter—Recombinant Egr-1 and Sp1 proteins were used in EMSA and DNase I footprinting to document the preferences of these transcription factors for the three GC-rich elements previously characterized in transfection studies (Fig. 2). Sp1 was used despite EMSA experiments that had shown the lack of complex formation by this transcription factor in the conditions used in our assays. However, an interaction with this transcription factor cannot be ruled out, since it is expressed constitutively, and a very dynamic interplay between Egr-1 and Sp1 has been observed in a variety of promoters, depending on specific physiological conditions (34–36). In order to detect multiple interactions, two different amounts of protein were used in EMSA; the larger one was chosen to generate higher order complexes in the event that they could be formed. As shown in Fig. 6, both Egr-1 and Sp1 bound to the wild-type −77/+43 fragment (lanes 2 and 4, respectively). The use of larger amounts of proteins induced their binding to a second site (lanes 3 and 5, double dot). When a labeled fragment mutated in site 4 was used as probe, the action of Egr-1 was not significantly modified (lanes 7 and 8), except for a slight decrease in band intensity. By contrast, Sp1 could just produce a small amount of retarded complexes and only when used at high concentration (lane 10). Hence, it appears that the main binding site for Sp1 is located within box 4. Moreover, only if this site is intact, the binding of Sp1 to a second site seems to be facilitated (compare lanes 5 and 10). When the labeled probe had boxes 1 and 2 mutated, the two factors could bind to a unique site (lanes 12 and 14), since higher order complexes were not observed (lanes 13 and 15). This site is presumably box 4, because a probe with the three GC-boxes mutated was not retarded by any protein. Therefore, Egr-1 can bind to the three boxes, whereas Sp1 binds preferentially to box 4, which may facilitate the binding to a second site. These results were confirmed by DNase I footprinting (Fig. 7), using the same probe of the EMSA. Thus, Sp1 protected a domain that closely corresponds to box 4 (lane 2), whereas Egr-1 showed several protected regions (lane 3), which included the three GC-boxes, boxes 1, 2, and 4. Shown in Fig. 7 is the antisense strand, which was yielding the best resolution. The sense strand gave essentially the same footprint pattern.

Relationship between the GC-boxes and the Activation of the α7 Promoter by Egr-1—We have shown previously that an
Egr-1 expression plasmid can activate the \( \alpha_7 \) promoter when cotransfected with p38\( \alpha_7 \)LUC (23), a construct that contains only two of the three GC-boxes to which Egr-1 can bind (boxes 1 and 2). Given that the single mutation of box 4, an element that is absent in p38\( \alpha_7 \)LUC, or the double mutation of boxes 1 and 2 produced a marked reduction on basal promoter activity (Fig. 2B), it was of interest to study the functional effect of Egr-1 in the broader context of p77\( \alpha_7 \)LUC and its mutants with modified GC-boxes. As shown in Fig. 8, the activity of p77\( \alpha_7 \)LUC in chromaffin cells was increased by up to 5-fold when cotransfected with an Egr-1 expression plasmid. Single mutations of boxes 2 and 4 (pmut 2\( \alpha_7 \)LUC and pmut 4\( \alpha_7 \)LUC, respectively) did not modify this effect. A significant decrease in Egr-1 activation did occur with the single mutation of box 1 (pmut 1\( \alpha_7 \)LUC) or the simultaneous mutation of boxes 2 and 4 (pmut 4-2\( \alpha_7 \)LUC). However, the maximal reduction was observed when box 1 and any of the others were mutated at once. Thus, activation was barely observed for pmut 4-1\( \alpha_7 \)LUC, whereas pmut 2-1\( \alpha_7 \)LUC and pmut 4-2-1\( \alpha_7 \)LUC were insensitive to the action of Egr-1. These results suggested that GC-box number 1 is the main element for the induction of the \( \alpha_7 \) promoter by Egr-1, although the presence of any of the other GC-boxes is required for maximal activation.

FIG. 7. DNase I analysis of Egr-1 and Sp1 interactions within the proximal region of the \( \alpha_7 \) subunit promoter. The antisense strand of a 120-bp DNA fragment spanning nucleotides -77 to +43 of the bovine \( \alpha_7 \) subunit promoter was end-labeled by Klenow filling. The gel-purified fragment was incubated with 1 footprint unit of Sp1 (lane 2) or 10 \( \mu \)g of purified Egr-1 (lane 3) and treated with DNase I. A control reaction (lane 1) was performed in a similar manner, using bovine serum albumin as the protein component. Digestion with DNase I, fragments were resolved on an 8% denaturing polyacrylamide gel. A Maxam-Gilbert A/G chemical sequencing reaction was included as reference ladder (lane 4). To the right is a sequence summary of the protected domains, with numbers corresponding to the putative regulatory elements of Fig. 1.

Egr-1 expression plasmid can activate the \( \alpha_7 \) promoter when cotransfected with p38\( \alpha_7 \)LUC (23), a construct that contains only two of the three GC-boxes to which Egr-1 can bind (boxes 1 and 2). Given that the single mutation of box 4, an element that is absent in p38\( \alpha_7 \)LUC, or the double mutation of boxes 1 and 2 produced a marked reduction on basal promoter activity (Fig. 2B), it was of interest to study the functional effect of Egr-1 in the broader context of p77\( \alpha_7 \)LUC and its mutants with modified GC-boxes. As shown in Fig. 8, the activity of p77\( \alpha_7 \)LUC in chromaffin cells was increased by up to 5-fold when cotransfected with an Egr-1 expression plasmid. Single mutations of boxes 2 and 4 (pmut 2\( \alpha_7 \)LUC and pmut 4\( \alpha_7 \)LUC, respectively) did not modify this effect. A significant decrease in Egr-1 activation did occur with the single mutation of box 1 (pmut 1\( \alpha_7 \)LUC) or the simultaneous mutation of boxes 2 and 4 (pmut 4-2\( \alpha_7 \)LUC). However, the maximal reduction was observed when box 1 and any of the others were mutated at once. Thus, activation was barely observed for pmut 4-1\( \alpha_7 \)LUC, whereas pmut 2-1\( \alpha_7 \)LUC and pmut 4-2-1\( \alpha_7 \)LUC were insensitive to the action of Egr-1. These results suggested that GC-box number 1 is the main element for the induction of the \( \alpha_7 \) promoter by Egr-1, although the presence of any of the other GC-boxes is required for maximal activation.

DISCUSSION

Neuronal nAChRs play a significant role in the central and peripheral nervous systems, probably regulating transmitter release at many synapses (reviewed in Ref. 4). Their functional importance in these processes is evidenced just by taking into account the behavioral and cognitive effects of nicotine abuse (37) and the involvement of neuronal nAChRs in the pathogenesis of many neurological disorders (38). Several genes that encode nAChR subunits have been identified (1), and, depending on the subunit composition, their heterologous expression has shown clear differences in electrophysiological and pharmacological properties (5). For this reason, it is important to understand how the expression of the different subunits is regulated. The present study was aimed to characterize the cis-elements and trans-acting factors involved in the transcriptional regulation of the nAChR \( \alpha_7 \) subunit gene.

From 5′-end deletion analysis (Fig. 1), we determined that the region located between nucleotides -77 and +43 was necessary for the basal promoter activity observed in SHSY-5Y cells. Additional positive elements, outside this region, may be operative in chromaffin cells, as demonstrated by the slight increase in the activity of larger constructs (Fig. 1 and also Ref. 23 for a comparison of larger fragments of the \( \alpha_7 \) promoter). However, the most striking fact was the large stepwise loss in promoter activity of p51\( \alpha_7 \)LUC with respect to a larger construct (p77\( \alpha_7 \)LUC). Since the region between -51 and -77 contains an E-box and a GC-rich sequence (labeled 3 and 4 in Fig. 1A, respectively), these elements appeared to be essential for promoter activity. Consequently, when any of these ele-
ments was mutated in the context of p77α7LUC, promoter activity was reduced to ~50% (Fig. 2). Nevertheless, these elements seem necessary but not sufficient for maximal promoter activity, since simultaneous mutation of other two downstream GC-boxes (labeled 1 and 2 in Fig. 1A), while keeping intact elements 3 and 4, produced a large decrease in activity (Fig. 2). This is also the case for other double mutant in which elements 1 and 3 had been removed. Therefore, a primary conclusion of this study is that the basal activity of the α7 subunit promoter is not defined by a predominant unique element; it rather seems to reflect the cumulative and concerted action of several transcription factors.

Element 3 corresponds to an E-box (Fig. 1). Several β2 E-box binding proteins have been identified and shown to bind the CACGTG consensus site (39). These include USF, which preferentially binds to this region when a thymidine residue precedes the consensus region (TCACGTG; see Ref. 39). Element 3 in the α7 promoter region has exactly this sequence. Our band shift assays identified USF1 and USF2 as the transcription factors that are binding to element 3. Although some minor complexes were only displaced by USF1 antibodies, the major band was supershifted by both anti-USF1 and USF2 antibodies, indicating that a heterodimer of both proteins constitutes the main E-box binding factor. Interestingly, nuclear extracts of human SHSY-5Y and bovine chromaffin cells yielded essentially the same band shift pattern, suggesting that regulation of the α7 promoter may follow similar pathways in both species. This assumption, however, will have to be confirmed once information about the human α7 promoter is available. The avian α7 promoter, by contrast, does not contain such an unambiguous USF binding site (6), although several CANNNTG sequences are present in the proximal promoter region. Their functionality, if any, has not been explored.

The other main elements in the core region of the α7 promoter are composed of GC-boxes. Our previous study indicated that the immediate early gene transcription factor Egr-1 was able to bind to elements 1 and 2 in the context of p83α7LUC (23). Here we have confirmed these results in a broader context (p77α7LUC) and shown that Egr-1 can also bind to another site (element 4). Moreover, the functionality of these elements is demonstrated when they are eliminated from p77α7LUC by site-directed mutagenesis; these three elements are needed for maximal basal activity, although they do not appear equivalent (Fig. 2). Thus, it is interesting that the individual mutation of site 4 produced a marked decrease in promoter activity, whereas its counterparts at sites 1 and 2 did not produce this effect, unless the mutations take place simultaneously. Therefore, a certain hierarchical order is suggested, in which site 4 appears critical for basal promoter activity, whereas at least one of the other two needs also to be operative. The co-transfection experiments with the Egr-1 expression plasmid (Fig. 8) confirmed that the three GC-boxes are involved in Egr-1 activation, but again in a nonequivalent manner. Thus, sites 2 and 4 can be individually removed without affecting Egr-1 activation, but site 1 needs to remain intact for maximal activation. In addition, when this site and any of the other two are simultaneously mutated, Egr-1 activation is abolished. Taken together, these results indicate that Egr-1 can bind to the three sites and perform its action by binding at least to two of them (preferentially the most promoter-proximal and -distant ones), probably through a synergistic mechanism.

In Fig. 9, we postulate a hypothesis on the basal transcriptional regulation of the α7 subunit gene, which takes into account our results and have been suggested by a recent model applied to the promoter of the nAChR β4 subunit gene (16). As happens with several nAChR subunits, the promoter of the α7 subunit is in a class of promoters that lack a canonical TATA box and, like many of them, it contains several GC-boxes (boxes 1, 2, and 4; Fig. 9) to which Egr-1 can bind. Upon binding to at least two elements, Egr-1 may be involved in interactions with other transcriptional co-activators and the basal transcription machinery to activate α7 subunit expression. The Egr-1 sites that have been detected are probably interrelated, perhaps by direct Egr-1 contacts (elements 1 and 2) or through other proteins (elements 1 and 2 with element 4). In addition, USF proteins are also critical in transcriptional activation upon interacting with the E-box of element 3. It is believed that USF1 affects transcription by interacting with the TFIID complex (40, 41) and, specifically, with the TFIID subunit TAFI55 (42). In the case of the α7 subunit promoter, the USF binding element is not closely adjacent to the transcription initiation site; hence, USF binding proteins may carry out their action through an additional protein, as it happens with PC5, a co-factor that has been demonstrated to mediate transcriptional activation by USF1 (43). Finally, a cooperative effect of Egr-1 (at site 1) and USF (at site 3) on transcriptional initiation appears plausible, if we consider that the double mutant of these elements (pmut 3–1 α7LUC) showed a decrease in promoter activity larger than the mere addition of the effects produced by the respective single mutants (pmut 1 α7LUC and pmut 3 α7LUC). For this reason, we have depicted a complex of co-activators whose action may depend on their simultaneous interactions with USF and Egr-1 proteins.

Although a number of neuronal genes appear to be regulated in a cell-type-specific manner by silencer elements (44, 45), we have not detected them in the α7 subunit promoter. If they exist, they might be acting at sites not included in our constructs. Another possibility of tissue- or cell-specific regulation could be through the antagonistic effect that other factors can exert by binding at the E- and GC-boxes. For instance, members of the Sp transcription factor family could bind to G/C-rich elements and block the activating effect of Egr-1. Although neither Sp1 nor Sp3 was shown to interact with the GC-boxes in the context of our gel retardation assays with nuclear extracts from chromaffin and SHSY-5Y cells, we have demonstrated that recombiant Sp1 can bind to element 4 in the basal promoter of the α7 subunit. Therefore, depending on the concentration, cell cycle-regulated expression, or phosphorylation state of Egr-1 and Sp factors, an interplay between them could be established, as has been demonstrated for other promoters (34, 35, 36, 46–48). A similar effect could take place upon binding of Myc family members to the E-box element 3. According to a previous study (39), the surrounding sequence of element 3 in the α7 subunit promoter is optimal for the binding of USF proteins, but certain physiological conditions could alter binding specificity or protein availability. Finally, if certain co-activators are needed for coupling to the RNA polymerase II complex, as we propose in Fig. 9, their concentration, function-
ality, etc. in a determined cellular type would also be significant in regulating a7 subunit expression.

Acknowledgments—We thank Y. Wang, V. P. Sukhatme, and X. Cao for Egr-1 plasmids. The excellent technical assistance of Eva Martı´nez is also appreciated.

REFERENCES

1. Sargent, P. B. (1993) Annu. Rev. Neurosci. 16, 403–443
2. Karlin, A. & Akassh, M. H. (1995) Neuron 15, 1231–1244
3. McGehee, D. S. & Role, L. W. (1995) Annu. Rev. Physiol. 57, 521–546
4. Role, L. W. & Berg, D. K. (1996) Neuron 16, 1077–1085
5. Papke, R. L. (1993) Proc. Neurobiol. 41, 599–531
6. Matter-Sadzinski, L., Hernández, M.-C., Roztocil, T., Ballivet, M. & Matter, J.-M. (1992) EMBO J. 11, 4529–4538
7. Bessis, A., Savatier, N., Devillers-Thiéry, A., Bejanin, S. & Changeux, J.-P. (1995) J. Biol. Chem. 270, 15143–15147
8. Yang, X., McDonough, J., Fyodorov, D., Morris, M., Wang, F. & Deneris, E. S. (1994) J. Biol. Chem. 269, 10252–10264
9. Boyd, R. T. (1994) J. Neurobiol. 25, 960–973
10. Hernández, M.-C., Erkman, L., Matter-Sadzinski, L., Roztocil, T., Ballivet, M. & Matter, J.-M. (1995) J. Biol. Chem. 270, 3224–3233
11. Milton, N. G. N., Bessis, A., Changeux, J.-P. & Latchman, D. S. (1995) J. Biol. Chem. 270, 15143–15147
12. Fyodorov, D. & Deneris, E. (1996) Mol. Cell. Biol. 16, 1077–1085
13. Boyd, R. T. (1996) Neurosci. Lett. 208, 73–76
14. Bigger, C. B., Casanova, E. A. & Gardner, P. D. (1996) J. Biol. Chem. 271, 32842–32848
15. Fyodorov, D., Nelson, T. & Deneris, E. (1996) Mol. Cell. Biol. 16, 5004–5014
16. Bigger, C. B., Melnikova, I. N. & Gardner, P. D. (1997) J. Biol. Chem. 272, 14990–14995
17. Bigger, C. B., Melnikova, I. N. & Gardner, P. D. (1997) J. Biol. Chem. 272, 25976–25982
18. Fyodorov, D., Nelson, T. & Deneris, E. (1998) J. Neurobiol. 34, 151–163
19. García-Guzmán, M., Sala, F., Sala, S., Campos-Caro, A., Stühmer, W., Gutierrez, L. M. & Criado, M. (1995) Eur. J. Neurosci. 7, 647–655
20. Criado, M., Alamo, L. & Navarro, A. (1992) Neurochem. Res. 17, 281–287
21. Campos-Caro, A., Smillie, F., Domínguez del Toro, E., Rovira, J. C., Vicente-Aguilera, P., Chapuli, J., Juiz, J. M., Sala, S., Sala, F., Ballesta, J. & Criado, M. (1997) J. Neurochem. 68, 488–497
22. Seguésa, P., Wadiche, J., Dineley-Miller, K., Dani, J. A. & Patrick, J. W. (1993) J. Neurosci. 13, 596–604
23. Criado, M., Domínguez del Toro, E., Carrasco-Serrano, C., Smillie, F. I., Juiz, J. M., Viniegra, S. & Ballesta, J. J. (1997) J. Neurosci. 17, 6554–6564
24. Gandía, L., Casado, L. F., López, M. G. & Garcia, A. G. (1991) Br. J. Pharmacol. 103, 1073–1078
25. Graham, F. L. & van der Eb, A. J. (1973) Virology 52, 456–467
26. Gupta, M. P., Gupta, M., Zak, R. & Sukhatme, V. P. (1991) J. Biol. Chem. 266, 12813–12816
27. Schreier, E., Matthias, P., Müller, M. M. & Schaftner, W. (1989) Nucleic Acids Res. 17, 6419
28. Jain, N., Mahendran, R., Philp, R., Guy, G. R., Tan, Y. H. & Cao, X. (1996) J. Biol. Chem. 271, 13530–13536
29. Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
30. Lukas, R., Norman, S. & Lucero, L. (1993) Mol. Cell. Neurosci. 4, 1–12
31. Sawadogo, M., Van Dyke, M. V., Gregor, P. D. & Roeder, R. G. (1988) J. Biol. Chem. 263, 11985–11993
32. Cao, X., Koski, A., Gasheer, A., McKinnern, M., Morris, C. G., Gaffney, R., Hay, R. & Sukhatme, V. P. (1990) Mol. Cell. Biol. 9, 1931–1939
33. Gregor, P. D., Sawadogo, M. & Roeder, R. G. (1990) Genes Dev. 4, 1730–1740
34. Skerka, C., Decker, E. L. & Zipfel, P. F. (1995) J. Biol. Chem. 270, 22500–22506
35. Cui, M.-Z., Parry, G. C. N., Oehl, P., Larson, H., Smith, M., Huang, E.-P., Adamson, E. D. & Mackman, N. (1996) J. Biol. Chem. 271, 2731–2739
36. Khachigian, L. M., Lindner, V., Williams, A. J. & Collins, T. (1996) Science 271, 1427–1431
37. Dani, J. A. & Heinemann, S. (1996) Neuron 16, 905–908
38. Lindstrom, J. (1997) Mol. Neurobiol. 15, 193–222
39. Bendall, A. J. & Molley, P. L. (1994) Nucleic Acid Res. 22, 2801–2810
40. Sawadogo, M. & Roeder, R. G. (1985) Cell 43, 165–175
41. Kokubo, T., Takada, R., Yamashita, S., Gong, D.-W., Roeder, R. G., Horikoshi, M. & Nakatani, Y. (1993) J. Biol. Chem. 268, 17554–17558
42. Ciang, C.-M. & Roeder, R. G. (1995) Science 267, 531–536
43. Halle, J. P., Stelzer, G., Goppelt, A. & Meisterernst, M. (1995) J. Biol. Chem. 272, 1755–1760
44. Chong, J. A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J. J., Zheng, Y., Boutros, M. C., Altschuller, Y. M., Frohman, M. A., Kramer, S. D. & Mandel, G. (1995) Cell 80, 949–957
45. Schoenherr, C. J., Paquette, A. J. & Anderson, D. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9881–9886
46. Ebert, S. N. & Wong, D. L. (1995) J. Biol. Chem. 270, 17299–17305
47. Khachigian, L. M., Williams, A. J. & Collins, T. (1996) J. Biol. Chem. 270, 27679–27686
48. Trejo, S. R., Fah, W. E. & Ratner, L. (1997) J. Biol. Chem. 272, 27411–27421