The EMBO Journal Vol.17 No.11 pp.3078–3090, 1998

Implication of a multisubunit Ets-related transcription factor in synaptic expression of the nicotinic acetylcholine receptor

Laurent Schaeffer, Nathalie Duclert, Monique Huchet-Dymanus and Jean-Pierre Changeux[1]

CNRS UA D1284 'Neurobiologie Moléculaire', Institut Pasteur, 25 rue du Dr Roux, F-75724 Cedex 15, Paris, France

[1]Corresponding author
e-mail: changeux@pasteur.fr

In adult muscle, transcription of the nicotinic acetylcholine receptor (AChR) is restricted to the nuclei located at the neuromuscular junction. The N-box, a new promoter element, was identified recently and shown to contribute to this compartmentalized synaptic expression of the AChR δ- and ε-subunits. We demonstrate that the N-box mediates transcriptional activation in cultured myotubes and identify the transcription factor that binds to the N-box as a heterooligomer in myotubes and adult muscle. The GABP (GA-binding protein) α-subunit belongs to the Ets family of transcription factors, whereas the β-subunit shares homology with IκB and *Drosophila* Notch protein. GABP binding specificity to mutated N-box *in vitro* strictly parallels the sequence requirement for β-galactosidase targeting to the endplate *in vivo*. *In situ* hybridization studies reveal that the mRNAs of both GABP subunits are abundant in mouse diaphragm, with preferential expression of the α-subunit at motor endplates. In addition, heregulin increases GABPα protein levels and regulates phosphorylation of both subunits in cultured chick myotubes. Finally, dominant-negative mutants of either GABPα or GABPβ block heregulin-elicited transcriptional activation of the AChR δ and ε genes. These findings establish the expected connection with a presynaptic trophic factor whose release contributes to the accumulation of AChR subunit mRNAs at the motor endplate.

Keywords: Ets transcription factors/neuregulins/neuromuscular junction/nicotinic acetylcholine receptor/synaptic expression

Introduction

The neuromuscular junction constitutes a privileged system for investigation of the molecular mechanisms underlying the control exerted by a presynaptic nerve terminal on postsynaptic gene expression. In the adult muscle, the nicotinic acetylcholine receptor (AChR) is compartmentalized to the motor endplate (for reviews see Laufer and Changeux, 1989; Hall and Sanes, 1993; Duclert and Changeux, 1995). In fetal non-innervated muscle, the AChR is distributed evenly at the surface of the myotube. When an ingrowing motor nerve terminal contacts the myotube, the AChR molecules cluster under the motor nerve and the distribution of the receptor becomes restricted progressively to the postsynaptic domain (for a review see Hall and Sanes, 1993; Duclert and Changeux, 1995). The clustering of the receptor molecules accompanies the restriction of expression of AChR subunits (α, β, γ, δ, ε; for reviews see Galzi and Changeux, 1994; Karlin and Akabas, 1995) to the subjunctional nuclei (Merlie and Sanes, 1985; Fontaine *et al*., 1988; Goldman and Staple, 1989; Brenner *et al*., 1990; Klarsfeld *et al*., 1991; Sanes *et al*., 1991; Simon *et al*., 1992; Piette *et al*., 1993; Koike *et al*., 1995; Kues *et al*., 1995; Duclert *et al*., 1996). Such a compartmentalized transcription results from the combined action of two mechanisms. On one hand, nerve-evoked electrical activity in the muscle fiber represses transcription of the AChR genes in extrajunctional nuclei (Fontaine *et al*., 1988; Goldman *et al*., 1988; Fontaine and Changeux, 1989; Merlie and Kornhauser, 1989; Tsay and Schmidt, 1989). On the other hand, the neurotrophic factors calcitonin gene-related peptide (CGRP) (Fontaine *et al*., 1986; New and Mudge, 1986) and heregulin (Falls *et al*., 1993; Sandrock *et al*., 1997) stimulate the expression of the AChR genes and are, therefore, candidate first messengers responsible for enhanced transcription of AChR genes in the subsynaptic nuclei.

Until recently, the only consensus promoter element known to play a critical role in the transcriptional regulation of the genes of the AChR subunits was the E-box, the binding site for the myogenic factors (Piette *et al*., 1990; Jia *et al*., 1992; Prody and Merlie, 1992; Simon and Burden, 1993). Transgenesis and recombinant adenovirus technology demonstrated that the E-boxes are necessary, although not sufficient, to mediate the up-regulation of transcription of AChR genes following the silencing of electrical activity by denervation (Bessereau *et al*., 1994; Mendelzon *et al*., 1994; Tang *et al*., 1994). However, despite their contribution to the repression by electrical activity, E-boxes are not necessary to restrict the expression of AChR δ- and ε-subunit genes to the endplate (Duclert *et al*., 1993; Tang *et al*., 1994).

Using an *in vivo* DNA injection technique, a 6 bp promoter element, referred to as the N-box, was shown to restrict the transcription of the AChR δ- and ε-subunit genes to the synaptic region in adult mouse muscle (Koike *et al*., 1995; Duclert *et al*., 1996). In these promoters, the N-box specifically mediated transcriptional activation in the subsynaptic nuclei. Interestingly, within the context of a large version of the δ promoter, the N-box gained the additional ability to down-regulate transcription in extrajunctional areas (Koike *et al*., 1995). This supports the view that the upstream region of the δ promoter contains an element, absent from the ε promoter, which cooperates with the N-box to create a transcriptional repressing activity. The absence of this property in the

case of the AChR ε-subunit correlates well with the fact that the ε-subunit gene is never expressed in the extrajunctional regions of the muscle fiber (Brenner *et al*., 1990). Finally, N-boxes are present in the promoters of several other genes expressed at the level of the neuromuscular junction. For example, the AChR γ-subunit gene contains an N-box which binds the same protein as the N-boxes from the δ- and ε-subunits (unpublished results); in the utrophin gene promoter, an N-box lies in a critical region for the control of gene transcription (Dennis *et al*., 1996).

In this study, we demonstrate that the N-box is functional in cultured myotubes and behaves as a transcriptional activator. Taking advantage of this property, we identify the factor that activates transcription upon binding to the N-box. We show that the α-subunit of GA-binding protein (GABP) is expressed preferentially at the motor endplate. We further demonstrate that heregulin regulates phosphorylation of both GABP subunits, increases GABPα protein levels and activates transcription of AChR δ and ε genes in a GABP-dependent maner.

Results

The N-box mediates transcriptional activation in cultured myotubes

As a first step, we evaluated the contribution of the TTCCGG sequence of the N-box to transcriptional regulation in cultured myotubes. For this purpose, we constructed synthetic promoters containing three repeats of the N-box (N_3) placed upstream of a minimal promoter (M2) from the chick AChR α-subunit gene (Bessereau *et al*., 1993). Quantification of the luciferase activity in transfected C2C12 myotubes revealed a 50-fold higher expression of the reporter gene when the N_3M2 promoter was used instead of M2 (Figure 1A). The increase in luciferase activity did not occur when the N-box had been replaced by a mutated *in vivo* inactive version ($Nmut_3$) (Koike *et al*., 1995). Chick primary myotubes yielded comparable results (Figure 1A). These data demonstrate that the N-box behaves as a transcriptional activator in cultured myotubes.

The N-box binds a 58 kDa ubiquitous polypeptide

We next undertook the characterization of the N-box-binding activity previously identified in adult mouse muscle (Koike *et al*., 1995; Duclert *et al*., 1996). Gel retardation experiments using extracts from various tissues or cell types revealed the presence of an N-box-binding activity in adult muscle, chick primary myotubes, mouse C2C12 and Sol8 myotubes and non-differentiated C2C12 myoblasts. A similar activity was also detected in HeLa cells, 3T3 fibroblasts and neuroblastoma extracts, but was low in spleen extracts.

The molecular weight of the N-box-binding activity was determined by UV-induced covalent cross-linking to a bromodeoxyuridine-substituted N-box (Figure 2B). Autoradiography revealed a 68 kDa band. Since the molecular weight of the double-stranded oligonucleotide used for the UV cross-linking was 10 kDa, the cross-linked protein may plausibly correspond to either a single 58 kDa polypeptide or to several smaller polypeptides which would bind the N-box to form the observed 68 kDa complex.

A DNA affinity column specifically retains two polypeptides

In a subsequent step, attempts were made to identify the protein factor that binds the N-box. The screening of several muscle expression libraries did not lead to the identification of any N-box-binding protein. We then tried to purify this activity directly from nuclear extracts of C2C12 myoblasts using a DNA affinity chromatography strategy. After incubation of the extract with three repeats of the N-box immobilized on magnetic beads and several washes, the proteins were eluted by increasing KCl concentration. The various fractions were analyzed by SDS–PAGE and silver staining (Figure 3A). Gel retardation analysis revealed that an N-box-binding activity is retained on the beads and eluted with increasing KCl concentration (Figure 3B). The eluted fraction contained three major polypeptides of 67, 58 and 43 kDa. The 67 kDa polypeptide most likely corresponds to a fraction of bovine serum albumin (BSA) non-specifically adsorbed on the column. The presence of a 58 kDa band parallels the 58 kDa activity detected by UV cross-linking. However, the presence of a 43 kDa polypeptide in the purified fraction, undetected by the UV cross-linking technique, raised the possibility that the N-box binds a heterooligomeric factor, thus explaining the failure of the expression library screening approach.

The N-box binds two polypeptides corresponding to the α- and β-subunits of GABP

The N-box sequence contains the GGAA/T core sequence common to all known Ets-binding sites (Macleod *et al*., 1992). We thus tested the possibility that the N-box-binding activity corresponds to a member of the Ets transcription factor family. We first determined which members of the Ets family are present in muscle. We adopted an RT–PCR approach using adult mouse muscle poly(A) RNA and degenerate oligonucleotides matching the conserved sequences of the Ets DNA-binding motif. Cloning and sequencing of the amplified sequences revealed the presence of fragments corresponding to either Ets-2 or GABPα. Interestingly, GABP is the only known multimeric Ets factor. The apparent molecular weights of the GABP subunits are 58 and 43 kDa in the mouse (Brown and McKnight, 1992). All the other Ets factors bind DNA as monomers (Crépieux *et al*., 1994). GABP is composed of a large α-subunit, which contains the conserved DNA-binding Ets motif, and a smaller β-subunit. The β-subunit has no intrinsic DNA-binding activity but is required to obtain efficient DNA binding and nuclear localization of the α-subunit (LaMarco *et al*., 1991; Sawa *et al*., 1996). GABPβ binds to GABPα through a domain composed of repeats of a 33 amino acid ankyrin motif. This ankyrin motif is present in several developmentally important factors, including the *Drosophila* proteins Notch, IκB and lin12 (LaMarco *et al*., 1991). In mouse, GABPβ is encoded by two distinct but closely related genes, namely GABPβ1 and GABPβ2 (de la Brousse *et al*., 1994). Since both forms of GABPβ yield transcriptional activation (Sawa *et al*., 1996), the functional significance of two highly homologous forms

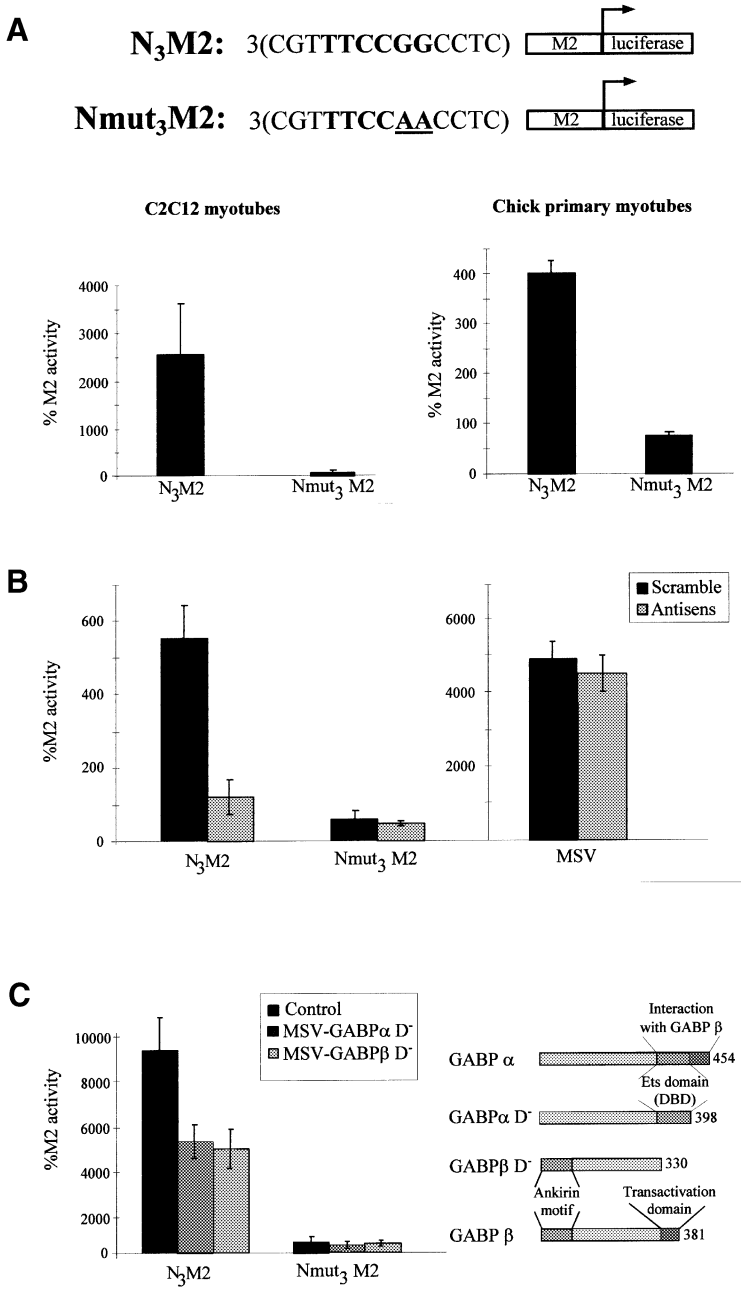

Fig. 1. The N-box and GABP activate transcription in cultured myotubes. (**A**) N-box-mediated transcription activation in C2C12 and chick primary myotubes. C2C12 and chick primary myotube cultures were transfected with either the N_3M2 or $Nmut_3M2$ constructs (1 μg/600 μl). Luciferase activity was measured after differentiation of the cells into myotubes. The results were normalized to the luciferase activity obtained with the M2 construct. (**B**) An antisense oligonucleotide directed against GABPβ blocks the N-box-mediated transcriptional activation in C2C12 myotubes. C2C12 myoblasts were co-transfected with either GABPβ antisense (light gray bars) or scramble (black bars) oligonucleotides (0.5 μM) and either the N_3M2 or $Nmut_3M2$ constructs (1 μg/600 μl). Luciferase activity was measured after differentiation of the cells into myotubes. The results were normalized to the activity of the M2 construct co-transfected with the appropriate oligonucleotide, and are presented as a percentage of this value. (**C**) Dominant-negative mutants of GABPα and GABPβ inhibit the N-box-mediated transcriptional activation in C2C12 myotubes. C2C12 myoblasts were co-transfected with either $GABP\alpha D^-$ or $GABP\beta D^-$ mutants, or control (1 μg/600 μl), and either the N_3M2 or $Nmut_3M2$ constructs (1 μg/600 μl). The mutants and control were placed downstream of the MSV promoter. Luciferase activity was measured after differentiation of the cells into myotubes. The results were normalized to the activity of the M2 construct. The results presented for the C2C12 myotubes are the average of at least three experiments in which each point was repeated in triplicate. For the chick primary myotubes, the experiments were repeated at least three times in triplicate, and the results of a typical experiment are presented. The error bars represent the standard deviation.

of GABPβ is still unknown. In this work, GABPβ refers to GABPβ1, since the antibodies, the antisense oligonucleotide and the recombinant proteins we used all correspond to GABPβ1. We have tested a rabbit serum directed against GABPβ2 in Western blot and in supershift experiments, and we could not detect GABPβ2 either in our purified GABP fraction or in the endogenous N-box-binding activity (data not shown). Moreover, the complete displacement of the N-box-binding activity by the anti-GABPβ1 antibody and the extent of the inhibition by the anti-GABPβ1 antisense oligonucleotide (described in the following results) suggest that GABPβ2 participates

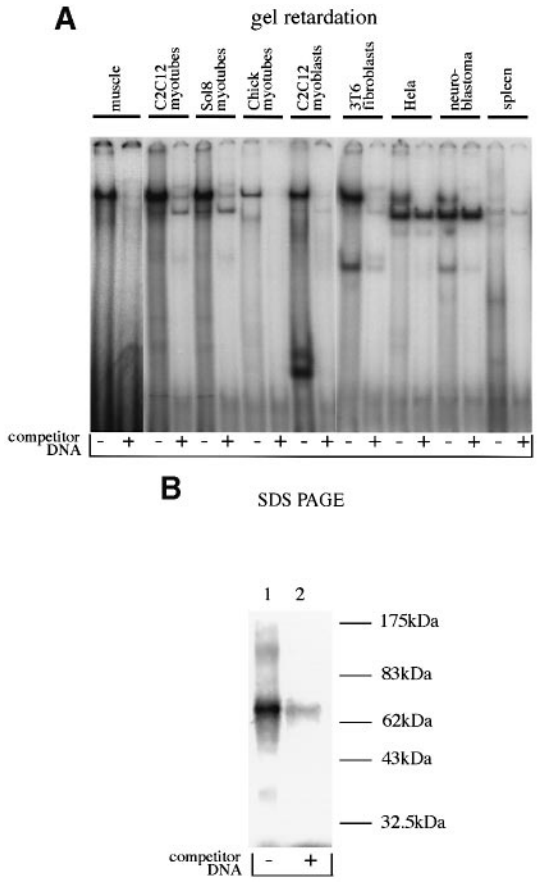

Fig. 2. The N-box binds a factor, present in various cell types, which contains a 58 kDa component. (**A**) Gel retardation experiments using the N-box sequence, either ^{32}P labeled as a probe (2 ng/lane), or unlabeled as a cold competitor. The protein extracts (10 μg/lane) used are indicated at the top of the gel. (**B**) Autoradiogram of denaturing SDS–polyacrylamide gel analysis of the UV cross-linked N-box-binding activity present in C2C12 myotubes. The same probe was used as for the gel retardation experiments except that the three central thymidine residues were substituted by bromodeoxyuridine. The presence or absence of competitor DNA (20-fold molar excess) is indicated at the bottom of each lane.

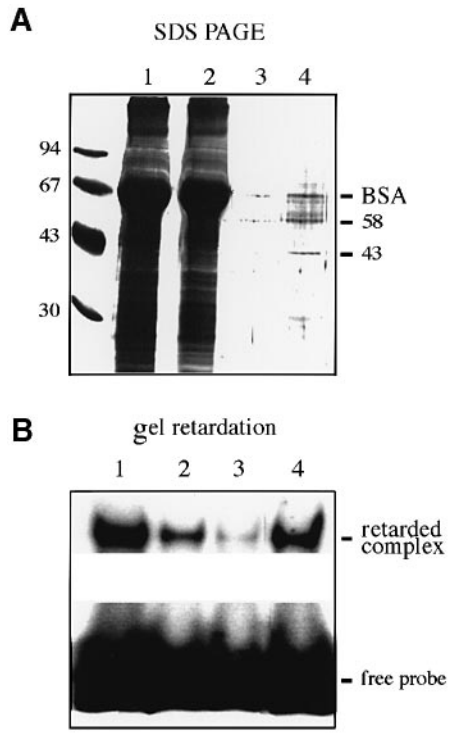

Fig. 3. Purification of the N-box-binding factor from C2C12 nuclear extracts on a DNA affinity column. The load (1), flowthrough (2), last wash (3) and eluted (4) fractions were analyzed by denaturing SDS–PAGE and protein silver staining (0.5, 2.5, 50 and 50 μl, respectively) (**A**), and gel retardation experiments using the N-box sequence as a probe (0.5, 2.5, 5 and 1 μl, respectively) (**B**). The sizes of the molecular weight markers and of the purified polypeptides are indicated in kDa on the left and right of (A), respectively; the band corresponding to BSA is also indicated. In (B), the retarded complex (top) and free probe (bottom) are indicated on the left.

neither in the endogenous N-box-binding activity, nor in the transcriptional activation by GABP.

Given that our low stringency PCR approach revealed the presence of GABP and that the apparent molecular weight of its subunits fits the size of the polypeptides we purified, it became legitimate to propose that the purified N-box-binding activity corresponds to GABP. To evaluate this possibility, we compared the mobilities obtained with the endogenous activity and with recombinant GABPα and β proteins in gel retardation experiments with the N-box (Figure 4B). The retarded complexes we obtained were indistinguishable. Moreover, the N-box-binding activity was competed by an oligonucleotide containing the consensus binding site for GABP (Watanabe *et al*., 1990), whereas an oligonucleotide containing an Ets-2 (Macleod *et al*., 1992) or Elf-1 (Wang *et al*., 1992) binding site had no significant effect (Figure 4A). In addition, an antibody directed against Ets-2 (Santa Cruz) did not affect the mobility of the N-box-containing complex in supershift experiments (Figure 4C). Altogether, these results strongly support the notion that the 58 and 43 kDa polypeptides retained by our affinity column correspond to GABPα and GABPβ, respectively.

The definitive proof concerning the identity of our endogenous N-box-binding activity came from supershift experiments. Incubation of the probe and nuclear extract of C2C12 myotubes along with a polyclonal rabbit antibody directed against either GABPα or GABPβ resulted in a decreased mobility of the N-box complex, whereas a pre-immune serum had no effect (Figure 4C). The anti-GABPα and anti-GABPβ antibodies also inhibited binding to the probe as shown by the decreased intensity of the supershifted complex compared with the signal obtained in the controls. This demonstrates that the endogenous N-box-binding activity contains both GABPα and GABPβ subunits. Since incubation with either the anti-GABPα or the anti-GABPβ antibody affected the totality of the N-box-bound complex (either supershifted or inhibited), we conclude that the endogenous N-box-binding activity we observe is composed primarily of GABPα and GABPβ.

Antisense oligonucleotides directed against GABPβ and dominant-negative mutants of GABPα or GABPβ block the N-box-mediated transcriptional activation in cultured myotubes

To demonstrate that, *in vivo*, the N-box is the target of GABP, we co-transfected C2C12 cells with the constructs described above and an antisense oligonucleotide directed against GABPβ (Figure 1B). This resulted in a 5-fold

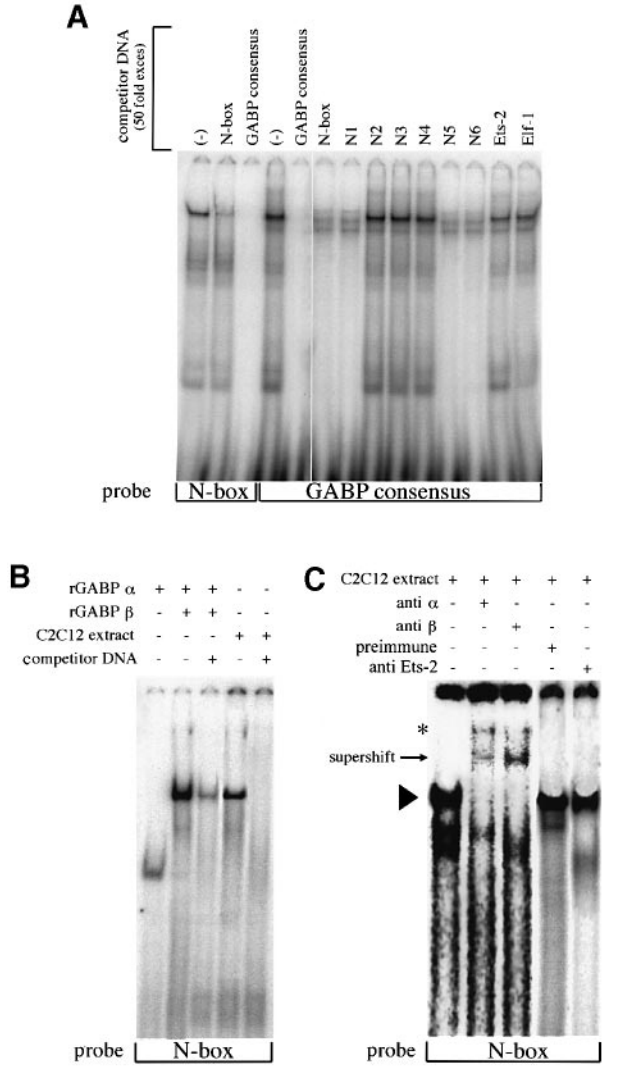

Fig. 4. The N-box-binding activity corresponds to the transcription factor GABP. (**A**) Gel retardation analysis of the DNA-binding specificity of the N-box-binding factor. C2C12 myotube nuclear extracts (10 μg/lane) were incubated with a ^{32}P-labeled probe (1 ng/lane) containing either the N-box sequence or the consensus binding sequence for GABP (the probe used in each lane is indicated at the bottom of the gel). Various competitor oligonucleotides (indicated at the top of the gel) were added to the reactions. (–), no competitor DNA; N1–N6, mutant N-boxes; Ets-2 and Elf-1, binding sites for Ets-2 and Elf-1, respectively. (**B**) Gel retardation comparison of the C2C12 N-box-binding activity and recombinant GABP migration profiles. The presence (+) or absence (–) of competitor DNA (20-fold molar excess of non-labeled probe) and the protein(s) used for each lane are indicated at the top of the gel. rGABPα and rGABPβ: baculovirus-produced recombinant mouse GABPα and GABPβ (100 ng/assay), respectively. C2C12 extract: 10 μg of C2C12 myotube nuclear extract. (**C**) The N-box-binding activity is supershifted by antibodies directed against GABPα or GABPβ. Gel retardation experiments were performed using C2C12 myotube nuclear extracts (10 μg/lane), ^{32}P-labeled N-box as a probe and in the presence of either an anti-GABPα (anti α), anti-GABPβ (anti β), pre-immune or anti-Ets 2 rabbit serum (0.5 μl/lane). The proteins added in each lane are indicated at the top of the gel. The supershifted bands are pointed out on the left and the bold arrow indicates the non-supershifted N-box-binding activity. The star indicates a non-specific band due to the antibodies.

inhibition of luciferase expression in C2C12 myotubes when using the N_3M2 promoter, whereas expression driven by $Nmut_3$M2 or M2 was only slightly affected. Under the same conditions, the control scrambled oligonucleotide caused only a 2-fold negative effect on luciferase levels. This 2-fold inhibition was most likely non-specific since most of the numerous phosphorothioate oligonucleotides we tested also yielded a small amount of inhibition of luciferase expression (data not shown). These results demonstrate that GABPβ, and thus GABPα which carries the DNA-binding motif of the complex, are necessary to activate transcription through the N-box in myotubes.

To specify further the contribution of GABP, we designed mutants of both GABPα and GABPβ. The mutant of GABPα (GABPαD^-) was obtained by deletion of the 56 C-terminal amino acids which are required for the formation of the GABPα–GABPβ heterodimer (Thompson *et al*., 1991; Sawa *et al.*, 1996). The mutant of GABPβ (GABPβD^-) was obtained by deletion of the 52 C-terminal amino acids which constitute the transactivation domain (Thompson *et al*., 1991; Sawa *et al.*, 1996). These mutants were placed under the control of a 400 bp fragment of the Moloney murine sarcoma virus promoter (MSV; van Beveren *et al*., 1982) and co-transfected in C2C12 myotubes together with the N_3M2 or $Nmut_3$M2 constructs. As found with the anti-GABPβ antisense oligonucleotide, we observe a blocking of the N-box-mediated transcriptional activation (Figure 1C).

Parallel effects of N-box mutations in vivo and in vitro on GABP binding

We next checked whether the sequence requirements for recombinant GABP binding fit those we determined previously for the *in vivo* activity of the N-box in subsynaptic compartmentalized expression (Koike *et al*., 1995; Duclert *et al*., 1996). We compared the effects of various mutations in the N-box on both β-galactosidase reporter gene expression at the neuromuscular junction using an *in vivo* DNA injection technique (Duclert *et al*., 1993) and the DNA-binding activity of recombinant GABP protein in gel retardation experiments (Figure 5). In the *in vivo* experiments, β-galactosidase was placed under the control of the AChR δ- or ε subunit gene promoter to test the N-box activity in its original context. The results show that each time a mutation in the N-box hindered the binding of GABP to its cognate site, the ability of the δ or ε promoter to restrict the expression of the reporter gene to the subsynaptic zone was altered. In addition, the parallel extends to the strength with which the mutations affect both GABP binding and synaptic expression: the more the DNA binding was affected, the less the expression was synaptic. This confirms that, *in vivo*, GABP binds the N-box and influences transcription of the δ- and ε-subunit genes, as it does in cultured myotubes.

Expression patterns of GABPα and GABPβ in muscle

We have shown that GABP activates transcription via the N-box and that, *in vivo*, the N-box specifically activates transcription in the subsynaptic nuclei. We thus examined GABPα and β mRNA distribution in mouse diaphragm muscles using *in situ* hybridization techniques (Figure 6). At embryonic day (ED) 18.5, the AChR δ-subunit transcripts are already compartmentalized under the motor endplates of the diaphragm. At this stage, both GABP subunits are expressed. The GABPβ mRNA level was found to be evenly distributed throughout the fiber. On

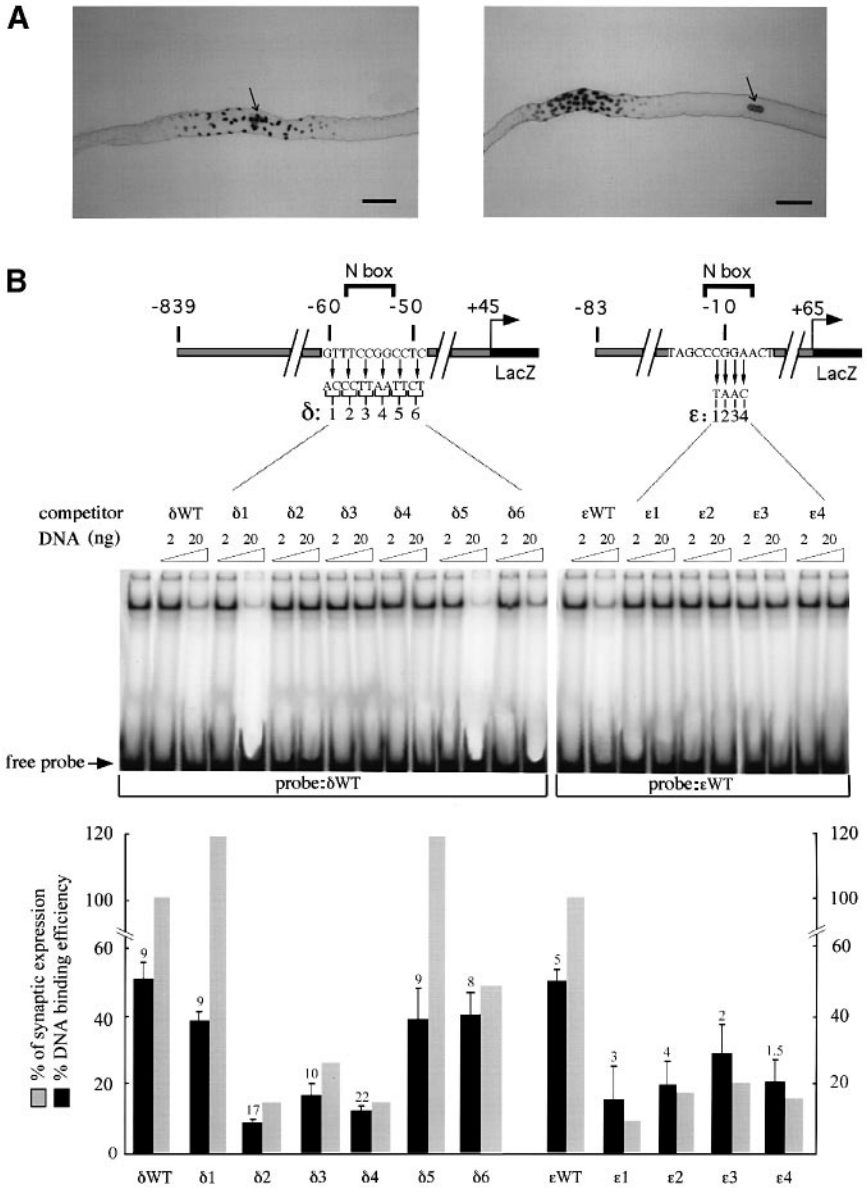

Fig. 5. Comparison of the effects of mutations in the N-box on the DNA binding of recombinant GABP and on the *in vivo* compartmentalization of reporter gene expression. (**A**) Muscle fibers expressing β-galactosidase after DNA injection. Left panel: synaptic expression. Right panel: extrasynaptic expression. The arrows indicate the acetylcholinesterase staining which marks the motor endplate. Bars: 100 μm. (**B**) Analysis of N-box mutation effects in gel retardation and *in vivo* using the muscle DNA injection technique. The various mutations introduced into the N-box of the AChR δ and ε promoters are indicated by the vertical arrows and named δ 1–6 and ε 1–4, respectively. For the gel retardation analysis, baculovirus-produced recombinant GABPα and β (100 ng/assay) were incubated with ^{32}P-labeled probe (2 ng/assay), either δ WT or ε WT as indicated at the bottom of the panel: δ WT, 5′-GGCCGCGTTTCCGGCCTCT-3′; ε WT, 5′-CTAGCCCGGAACT-3′. Binding to the probes was competed with non-labeled probe, either wild-type or containing the different mutations (δ 1–6 and ε 1–4). The type and amount (2 or 20 ng) of competitor DNA added to each reaction are indicated at the top of each lane. The results of a typical experiment were quantified (gray bars). They were expressed as the percentage competition yielded by the various mutants (which is proportional to their ability to bind GABP), competition by the wild-type probe being considered as 100%. For the *in vivo* analysis, the wild-type and mutant mouse AChR δ and ε promoters (from –839 to +45 and –83 to +65 for the AChR δ and ε promoter, respectively) were placed upstream of the β-galactosidase gene and a nuclear localization signal. The results of the injection of these constructs in the mouse tibialis anterior muscle are presented as the percentage of synaptic expression obtained with a given construct (black bars), and the average number of events per muscle obtained for each construct is indicated at the top of the corresponding bar. The values are derived from Koike *et al*. (1995) and Duclert *et al*. (1996). Each construct was injected at least three times in 12–24 muscles. The error bars represent the SEM.

the other hand, GABPα transcripts, although present in all the muscle fiber nuclei, showed a slight but significant compartmentalized expression in the subsynaptic nuclei. The same observations were made in the diaphragm of 3-day-old mice.

Heregulin enhances GABPα expression and the phosphorylation of both GABPα and GABPβ

We next investigated the possibility that GABP could be controlled by neural factors promoting the subjunctional transcription of the AChR genes, the best documented

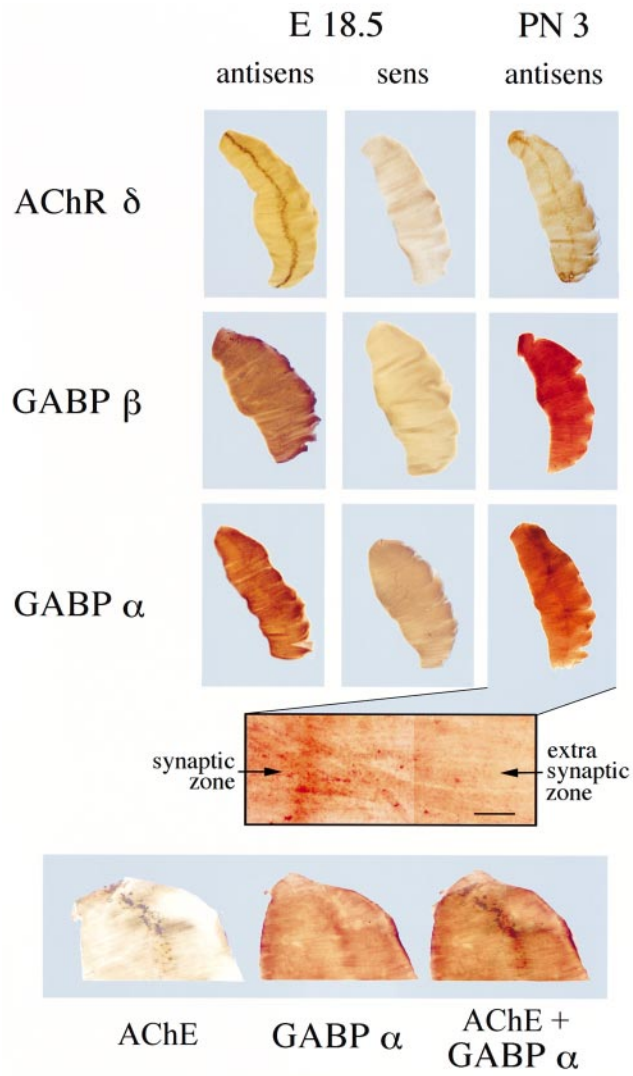

Fig. 6. Expression pattern of AChR δ-subunit, GABPα and GABPβ genes in mouse muscle. Diaphragm muscles from 18.5-day old mice embryos (E 18.5) and post-natal day 3 mice (PN 3) were hybridized with antisense and sense riboprobes to the AChR δ, GABPα and GABPβ mRNAs. The boxed picture shows a higher magnification of a PN 3 diaphragm hybridized with the GABPα antisense probe. The synaptic and extrasynaptic regions are indicated by arrows. The bar corresponds to 50 μm. The lower panel shows a portion of a diaphragm successively stained for acetylcholinesterase activity (AChE) and hybridized with the anti-GABPα probe (GABPα). On the right of the panel, both stainings were superimposed (AChE + GABPα).

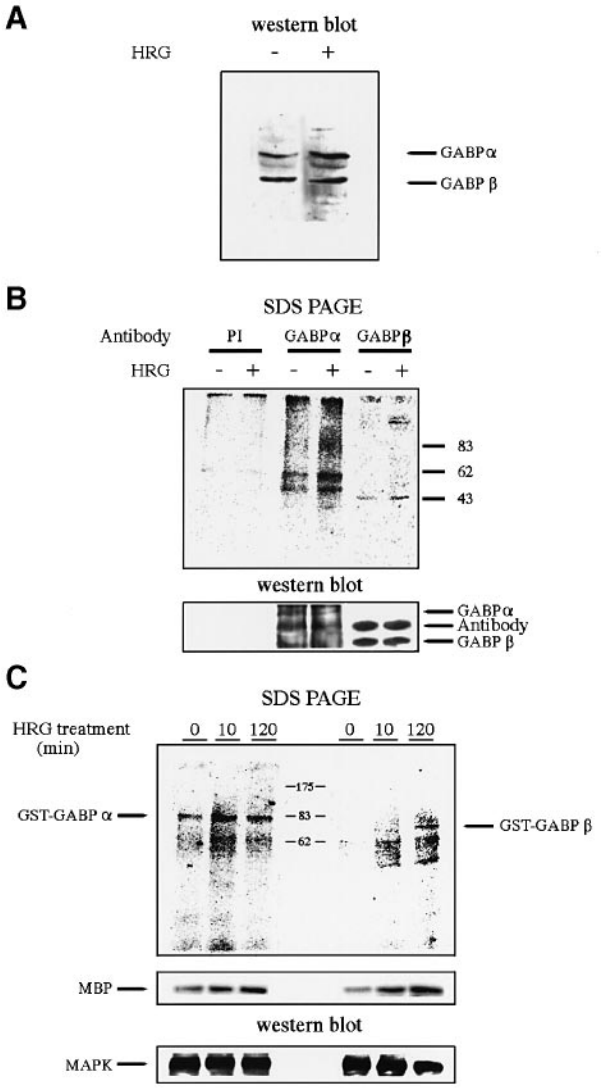

Fig. 7. Heregulin increases the GABPα protein level and modulates the phosphorylation of both GABPα and GABPβ. (**A**) Heregulin increases the GABPα but not the GABPβ protein level. Chick primary myotubes were either treated or not treated with 5 nM heregulin (HRG) for 24 h and analyzed in Western blot for the presence of GABPα and GABPβ (20 μg of total proteins per lane). The bands corresponding to GABPα and GABPβ are indicated on the left. (**B**) GABPα and GABPβ are phosphorylated in myotubes, and heregulin enhances their phosphorylation. Chick primary myotubes, treated or not with heregulin (HRG) in the presence of ^{32}P, were lysed and incubated with a rabbit pre-immune serum (PI), an anti-GABPα rabbit serum or an anti-GABPβ rabbit serum (GABPα and GABPβ). The immunoprecipitates were analyzed by SDS–PAGE and autoradiography or by Western blot using antibodies directed against GABPα and β. The molecular weights and the proteins visualized in the Western blot are indicated on the right. (**C**) MAP kinases phosphorylate GABPα and GABPβ and are stimulated by heregulin. MAP kinases were immunoprecipitated from non-treated and from 10 min or 2 h heregulin-treated (5 nM) chick primary myotubes. Immunoprecipitated MAP kinases were incubated in the presence of [γ-^{32}P]ATP (1 mCi/assay) with either GST–GABPα or GST–GABPβ fusion proteins (200 ng/assay, upper panel, left and right side, respectively) or MBP (middle panel). After electrophoresis through an SDS–polyacrylamide gel and transfer to nitrocellulose membrane, the reactions were analyzed by autoradiography. The membrane was also Western blotted for the presence of MAP kinases (MAPK, lower panel). The duration of the heregulin (HRG) treatment is indicated in minutes at the top (0, 10 and 120), and the molecular weights are given in kDa in the middle of the upper panel.

being heregulin (see Sandrock *et al.*, 1997, and references therein). To identify potential effects of heregulin on GABP, we first investigated its effects on GABPα and β protein levels in primary cultures of chick myotubes, an *in vitro* system reproducibly responsive to heregulin (Altiok *et al.*, 1995). Western blot analysis of heregulin-treated and non-treated cells revealed that heregulin treatment resulted in a 2-fold increase (average of six experiments, 25% variation between the experiments) in the GABPα protein level, while the GABPβ protein level was unaffected (Figure 7A).

GABP subunits contain putative sites for phosphorylation by MAP kinases, and these kinases mediate the heregulin-elicited stimulation of transcription of AChR genes in cultured chick myotubes (Altiok *et al.*, 1997). In

addition, GABP phosphorylation by MAP kinases has already been reported in different cell systems (Flory *et al.*, 1996; Ouyang *et al.*, 1996). We thus investigated the phosphorylation of GABP subunits in chick primary myotubes. After a 2 h period of ^{32}P metabolic labeling, GABP α- and β-subunits were immunoprecipitated from the cell lysates, submitted to SDS–PAGE and transferred to nitrocellulose membranes. Autoradiography revealed that an anti-GABPα antibody, but not a pre-immune serum, immunoprecipitated a phosphorylated 58 kDa polypeptide. Moreover, a 2 h heregulin treatment of the cells increased phosphorylation of this polypeptide by 170% (average of four experiments, standard deviation = 20%) (Figure 7D). Thus, heregulin stimulated GABPα phosphorylation. Immunoprecipitations with an antibody directed against GABPβ revealed that phosphorylation of GABPβ was enhanced by 60% by heregulin treatment (average of four experiments, standard deviation = 10%) (Figure 7B).

To confirm this result, we immunoprecipitated MAP kinases from heregulin-treated (10 min and 2 h) and non-treated chick primary myotubes using an anti-MAP kinase antibody (anti-ERK2; Santa Cruz) (Figure 7C). The activity of the immunoprecipitated kinases was tested on either a purified GST–GABPα or GST–GABPβ fusion protein or with myelin basic protein (MBP). Autoradiography of the reactions performed with MBP and Western blotting showed that upon heregulin treatment, the activity of the MAP kinases increased while their protein level remained constant. The GST–GABPα fusion protein was also phosphorylated, and a 10 min heregulin treatment sufficed to increase its phosphorylation by 65% (average of six experiments, standard deviation = 15%). The phosphorylation level of GABPα in the 2 h heregulin-treated MAP kinases did not differ significantly from that in those treated for 10 min. Conversely, with the GST–GABPβ fusion protein, no phosphorylation could be detected with either the non-treated or 10 min heregulin-treated MAP kinases. However, when the 2 h heregulin-treated MAP kinases were used, GABPβ became phosphorylated. The same experiments were also performed using baculovirus-produced GABPα and GABPβ instead of the GST fusion proteins, and similar results were obtained (data not shown). These data demonstrate that both subunits of GABP are phosphorylated *in vivo* by MAP kinases and that heregulin enhances their phosphorylation, but with different kinetics.

Dominant-negative mutants of GABPα and GABPβ block heregulin-elicited transcriptional activation of the AChR δ and ε genes

Since a link could be established between heregulin and GABP, we next investigated whether GABP contributes to the heregulin-elicited transcriptional stimulation of the AChR genes observed in chick primary myotubes. Our antisense oligonucleotide was initially designed for mouse GABPβ and did not match chicken GABPβ. We thus turned to the dominant-negative mutants of GABPα and β we had constructed. First, the increase in AChR δ mRNA after heregulin treatment was measured by Northern blot in myotubes transfected with either one of the mutants. As a control, we used the same vector in which the inserts downstream of the MSV promoter were removed. Figure 8 shows that both mutants block the increase in AChR δ mRNA induced by heregulin. We next analyzed the effect of the mutants on the stimulation of AChR ε-subunit gene expression. In avians, no homolog of the mammalian AChR ε gene has ever been reported. We thus co-transfected the mutants together with the luciferase gene placed under the control of a 2200 bp fragment of the mouse AChR ε gene promoter (Duclert *et al.*, 1993). In this case also, the two mutants blocked the heregulin-dependent increase in luciferase expression.

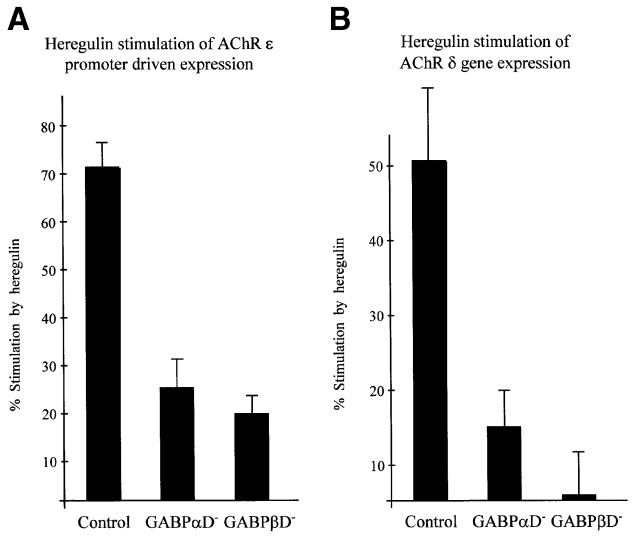

Fig. 8. (**A**) Dominant-negative mutants of GABP block heregulin stimulation of the AChR δ and ε promoters. Dominant-negative mutants of GABP block heregulin stimulation of luciferase expression controlled by the AChR ε gene promoter. Chick primary myotubes were co-transfected with either the mutants GABPαD⁻ or GABPβD⁻, or control (1 μg/600μl), and the luciferase gene controlled by a 2200 bp fragment of the mouse AChR ε promoter (1 μg/600 μl). The mutants and control were placed downstream of the MSV promoter. Luciferase activity was measured after differentiation of the cells into myotubes. The results are presented as the average ratio of the luciferase activity from non-treated and heregulin-treated cells. The experiments were done in quintuplicate and repeated at least three times. The error bars correspond to the SEM. Analysis of variance was performed using a one-way ANOVA ($P < 0.01$). (**B**) Dominant-negative mutants of GABP block heregulin stimulation of the endogenous AChR δ-subunit gene. Chick primary myotubes were transfected with either the dominant-negative mutants GABPαD⁻ or GABPβD⁻, or control (2 μg/600 μl). The mutants and control were placed downstream of the MSV promoter. The amount of AChR δ mRNA was measured in Northern blot and normalized to the amount of MCK mRNA. The results are presented as the ratio of the AChR δ mRNA in non-treated and heregulin-treated cells. The experiments were done in duplicate and repeated at least three times. The error bars correspond to the SEM ($P < 0.05$).

Discussion

In this report, we have demonstrated that the N-box, which we previously had shown to be a crucial element for the subsynaptic expression of the AChR δ- and ε-subunits, is also an activating element in cultured myotubes. Taking advantage of the *in vitro* activity of the N-box, we have further demonstrated that activation via the N-box is due

to the binding of the transcription factor GABP. In unraveling the mechanisms by which GABP promotes the compartmentalized expression of the AChR *in vivo*, we reveal a preferential expression of GABPα mRNA at the endplate, and the stimulation of GABPα expression by the neurotrophic factor heregulin.

After this manuscript was submitted, Sapru *et al.* (1998) reported studies with the AChR ε promoter from rat, which is highly homologous to its mouse counterpart. They showed that a 15 bp region, which contains the N-box, contributes to the heregulin-stimulated expression of a reporter gene regulated by the rat AChR ε promoter. They also showed that this effect is mediated by an unidentified Ets and involves the MAP kinase pathway. Here, we show that both subunits of GABP are phosphorylated by MAP kinases and that their phosphorylation increases upon heregulin treatment. Finally, using dominant-negative mutants of GABPα and GABPβ, we demonstrate that GABP is required for heregulin-stimulated transcription of the AChR δ- and ε-subunit genes.

GABP binds to the N-box and activates transcription in cultured myotubes

The 50-fold increase in luciferase expression observed upon grafting the three repeats of the N-box to a minimal promoter clearly demonstrates that the N-box behaves as an activator in cultured myotubes. This property is not specific to C2C12 mouse myotubes as the N-box is also functional in chick primary myotubes.

The low stringency PCR approach we have used to identify the various Ets genes expressed in muscle yielded a majority of sequences corresponding to Ets-2; others indicated the presence of GABPα, but no new Ets motif was ever amplified. Although not exhaustive, this approach has already been used successfully to identify new members of the Ets family (Brown and McKnight, 1992; Giovane *et al*., 1994; Lopez *et al*., 1994). Our results suggest that Ets-2 and GABPα are the best represented members of the Ets family in adult mouse muscle. Supershift experiments show that Ets-2 does not seem to be present in the N-box-binding activity. In addition, the mobility of GABP in gel retardation experiments differs greatly from that of the other Ets proteins (including Ets-2) which exhibit a much higher mobility (Jousset *et al*., 1997, and references therein). This is most likely due to the unique multimeric nature of GABP among the members of the Ets family. Two other Ets have a migration pattern that might correspond to GABP: Elf1 and ERF. However, Elf-1 and GABP preferentially bind to different sites (Brown and Mc Knight, 1992; Wang *et al*., 1992), and Figure 4A shows that competition by an oligonucleotide containing a strong binding site for Elf-1 is not efficient. In addition, Elf-1 and ERF have molecular weights (68 and 75 kDa, respectively) which are too different from GABPα to fit the results from the affinity purification or the UV cross-linking. Moreover, ERF is a repressor (Sgouras *et al.*, 1995), and we observe activation in myotubes. The combination of the site mutagenesis data, the migration profile of GABP, the composition of the affinity-purified fraction and the UV cross-linking experiment supports the conclusion that the N-box-binding activity we observe does not correspond to any known Ets other than GABP. Finally, the complete displacement of the N-box-binding complex by the anti-GABPα and β antibodies confirms that, at least in gel retardation experiments, GABP is the only muscle-derived protein which binds significantly to the N-box.

The inhibition of the N-box-dependent activation by the anti-GABPβ antisense oligonucleotide and the effect of the dominant-negative mutants demonstrate the implication of GABP in this process. However, it is still possible that other factors also contribute to the transactivation through the N-box in cultured myotubes. However, none could be detected using the gel retardation technique. Moreover, the antisense oligonucleotide decreased the expression of the N_3M2 construct to levels comparable with those obtained with M2 or $Nmut_3M2$, thus suggesting that GABP alone is responsible for the N-box-mediated transcriptional activation.

Possible mechanisms for GABP-mediated compartmentalized transcription of AChR genes at the neuromuscular junction

In previous studies, we have established the pivotal role of the N-box in restricting the transcription of the AChR δ- and ε-subunit genes at the neuromuscular junction in adult muscle (Koike *et al*., 1995; Duclert *et al*., 1996). We now demonstrate that GABP binding to the N-box enhances transcription in myotubes and that GABP is involved in the heregulin-dependent transcriptional activation of the AChR δ and ε genes.

Comparison of N-box mutation effects on the synaptic expression of a reporter gene in adult mouse muscle and on the binding of recombinant GABP shows that the GABP DNA-binding specificity strictly parallels the sequence requirements necessary to obtain an N-box-dependent synaptic expression pattern *in vivo*. Indeed, there is a robust correlation between the extent to which a mutation alters GABP binding to the probe and the relevant synaptic expression of the reporter gene. Moreover, GABP is involved in the transcriptional response of AChR genes to heregulin, which is concentrated at the neuromuscular junction (Jo *et al.*, 1995). In addition, GABPα mRNA shows preferential accumulation in the synaptic region *in vivo*. These observations support the conclusion that, in adult muscle, GABP is the factor which binds to the N-box and stimulates subsynaptic transcriptional activation.

The *in situ* hybridization experiments have further revealed that, although GABPα expression is higher in the subsynaptic nuclei, GABPβ mRNA is evenly distributed throughout the muscle fiber. This observation can be correlated with the fact that, in cultured myotubes, heregulin treatment specifically increases the GABPα protein levels. Since, *in vivo*, heregulin is concentrated at the neuromuscular junction (Jo *et al.*, 1995), a plausible hypothesis is that it accounts for the synaptic increase in GABPα expression. If the amount of GABPα was limiting for GABP activity, the compartmentalization of the GABPα subunit would suffice to enhance the GABP transcriptional activity in the subsynaptic nuclei.

Several alternative or complementary mechanisms may also contribute to the regulation of GABP activity. For instance, GABPα may bind different regulatory subunits according to its localization in the muscle fiber. Since GABPα dimerizes with proteins containing ankyrin

repeats, one has to consider the possibility that, in the extrasynaptic areas, GABPα binds to a subunit that sequesters it into the cytoplasm, as does the ankyrin repeat-containing protein IκB with the transactivator NF-κB (Blank *et al*., 1992). This possibility will be tested using suitable antibodies for immunofluorescence analysis. It is also plausible that different forms of GABPβ associate with GABPα: an active form at the endplate and an inactive form in the extrajunctional areas. Indeed, the GABPβ1 gene is alternatively spliced to produce two forms of the protein (de la Brousse *et al*., 1994). The smaller form has a shortened C-terminal end and seems to be transcriptionally inactive (Sawa *et al*., 1996). The probe we used for the *in situ* experiments does not discriminate between the two spliced forms, but the possibility of distinct distributions of the various spliced forms will be tested with suitable probes and antibodies.

Alternatively, the composition of GABP might remain unchanged throughout the fiber, with nerve-derived signals modulating its transcriptional activity. Heregulin, secreted by the nerve terminal or even by the muscle (Moscoso *et al*., 1995), accumulates in the basal lamina of the neuromuscular junction where it stimulates AChR expression (Jo *et al*., 1995; Sandrock *et al*., 1997). Our results demonstrate that, in addition to increasing GABPα protein levels, heregulin stimulates transcription of AChR δ and ε genes via GABP and influences the phosphorylation of both GABP subunits via the MAP kinase signaling pathway previously shown to be required for the stimulation of AChR expression (Tansey *et al*., 1996; Altiok *et al*., 1997). As heregulin is compartmentalized, the phosphorylation state of GABP most probably differs in the subsynaptic and extrasynaptic nuclei. Regulation of the transcriptional activity of GABP by heregulin-induced phosphorylation would be a means of obtaining synaptic specificity. This regulation could be achieved by affecting GABP stability, DNA-binding activity, heterodimerization, or its ability to regulate transcription by affecting its interactions with the basal transcriptional machinery or with transcriptional regulators. Interestingly, we have shown that GABPα and β display different phosphorylation kinetics. This suggests that although MAP kinases are implicated in both cases, the phosphorylation of GABPα and β may involve additional partners.

A possible negative regulatory role for GABP in extrasynaptic nuclei

In a previous study, we have demonstrated that, on one hand, in a truncated version of the AChR δ-subunit gene promoter, the N-box behaves as a subsynaptic activator, as in the AChR ε-subunit gene promoter. On the other hand, in a larger version of the δ promoter, the N-box gains the ability to function as an extrasynaptic silencer. This additional role of the N-box suggested the presence of an upstream element in the δ promoter, located between –839 and –60 and absent from the ε promoter, that cooperates with the N-box to inhibit transcription in the extrajunctional nuclei (Koike *et al.*, 1995; Duclert *et al.*, 1996). One possibility is that GABP confers such a dual function on the N-box. Indeed, in the case of the ribosomal protein genes, GABP serves both as an activator and as a repressor, depending on the promoter context in which its binding site is located (Genuario and Perry, 1996).

In both the AChR δ- and ε-subunit gene promoters, the N-box is located in the vicinity of an E-box and a CCCACCCC box (Baldwin and Burden, 1988; Duclert *et al*., 1993). The conservation of the immediate context of the N-box might be relevant and give additional cues concerning the modes of action of GABP in these promoters. Indeed, the Ets transcription factors are known to function most of the time in cooperation with other transcription factors (Crépieux *et al*., 1994). Moreover, a domain conserved among various Ets, including GABPα, has been proposed to form a basic helix–loop–helix (bHLH)-like motif (Seth and Papas, 1990) and thus to promote interactions with members of the bHLH family, among which are the E-box-binding myogenic factors. Synergism between Ets and bHLH proteins has been described in immunoglobulin gene expression (Rivera *et al*., 1993). Future studies will reveal if such interactions are relevant for the regulation of AChR expression.

Since the implication of the myogenic factors in AChR transcription regulation was shown (Piette *et al*., 1990), GABP has been the first transcription factor to be found that might play a pivotal role in AChR transcription compartmentalization to the subsynaptic nuclei. Moreover, GABP is regulated by the presynaptic trophic factor heregulin. These results will undoubtedly open new avenues of investigation towards the understanding of neural control of transcription. In addition, the various spliced forms of heregulin and their ErbB tyrosine kinase receptors are expressed in a wide variety of tissues in which they are actively involved in cell communication (for a review see Burden and Yarden, 1997). As GABP is most probably expressed in the various ErbB-containing tissues (LaMarco *et al*., 1991; de la Brousse *et al*., 1994), it might participate in the many aspects of the heregulin/ErbB signaling network.

Materials and methods

Cell cultures and transient transfection assays

Chick primary myotube cultures were obtained and cultured as previously described (Tansey *et al*., 1996). They were plated on 60 mm plates and transfected the next day for 3 h with 2 μg of DNA and 20 μl of lipofectamine (Gibco-BRL) in 2 ml of optiMEM (Gibco-BRL) according to the manufacturer's instructions. For the experiments of heregulin-stimulated transcription, the cells were treated with 5 nM recombinant heregulin 24 h before harvest. C2C12 cells (Genuario and Perry, 1996) (2×10^5) were plated on 35 mm dishes in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) containing 20% fetal calf serum (FCS; Gibco-BRL) and antibiotics. The myoblasts were transfected the next day for 3 h with 1 μg of DNA (or 0.5 mM double-stranded antisense oligonucleotides) and 12 μl of lipofectamine in 600 μl of optiMEM, according to the manufacturer's recommendations. The cells were next differentiated into myotubes for 2 days by replacing the FCS by 10% horse serum. For the experiments with the GABP dominant-negative mutants, no insert was placed between the MSV promoter and the SV40 poly(A) for the control.

Northern blot

RNA was purified according to Chomsky and Sacchi (1987), and 20 μg of total RNA was loaded in each lane. Probes were radiolabeled with [^{32}P]UTP (800 Ci/mmol, Amersham), using the Promega Riboprobe kit. The AChR δ and MCK probes were obtained from the pGEM3Z vector (Promega), in which 1.6 kb and 520 bp *Pst*I fragments of the chicken AChR δ and MCK genes, respectively, were cloned (Nef *et al*., 1984; Klarsfeld and Changeux, 1985)

Plasmid constructs and oligonucleotides

Constructs were made in KS-Bluescript vector (stratagene) in which the M2 minimal promoter (fragment –45 to +1 from the chicken AChR α gene promoter) and the luciferase gene (Bessereau *et al*., 1993) were introduced as previously described (Duclert *et al*., 1991). The three repeats of the wild-type (N_3) and mutated N-box ($Nmut_3$) were introduced upstream of M2 by cloning double-stranded synthetic oligonucleotides in the *Xba*I and *Sal*I sites: N_3, (sense) 5′-GGCCGCGTTTCCGGCCTCGTTTCCGGCCTCGTTTCCGGCCTCT-3′, (antisense) 5′-CTAGAGAGGCCGGAAACGAGGCCGGAAACGAGCCGGAAACGC-3′; $Nmut_3$, (sense) 5′-GGCCGCG TTTCCAACCTCGTTTCCAACCTCGTTTCCAACCTCT-3′, (antisense) 5′-CTAGAGAGGTTGGAAACGAGGTTGGAAACGAGGTTGGAAACGC-3′. All the oligonucleotides were from Genset (France). In the antisense oligonucleotides, the nucleotides were phosphorothioates.

Protein extracts

Extracts from tissues were obtained as previously described (Koike *et al*., 1995). Extracts from cultured cells were obtained using the technique described by Dignam (Changeux *et al*., 1990).

Mobility shift and supershift assays

Mobility shift assays were performed using 2 ng of ^{32}P-labeled probe in 20 µl at room temperature in 20 mM HEPES, pH 8.0, 50 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 4 mM spermidine, 0.1% NP-40, 15% glycerol, 1 µg of polydI–dC. After a 30 min incubation, the reactions were electrophoresed on 6% polyacrylamide gels in 0.5× TBE. The gel was then dried and autoradiographed on a phosphorimager screen (Molecular Dynamics). For the supershift experiments, 0.5 µl of polyclonal antiserum was added after the 30 min incubation at room temperature and the reaction was incubated for a further 20 min on ice prior to loading on gel.

The probes and competitor double-stranded oligonucleotide sequences (upper strand) were as follows: the N-box oligonucleotide sequence is derived from the mouse AChR δ-subunit gene promoter, 5′-GGCCGCGTTTCCGGCCTCT-3′; N1–N6 mutants were as previously described (Koike *et al*., 1995); GABP consensus, 5′-TTGGAAAACGGAAGTGACG-3′; Ets-2, 5′-TTGGTGGAGGAAGT-3′; Elf-1, 5′-TTGGTTTTTCCTCCTT-3′.

UV cross-linking

The binding to the probe and the electrophoresis of the resulting complex were performed as described for the mobility shift assay except that the three central T residues of the probe were substituted by three bromodeoxyuridine residues. After electrophoresis, the gel was UV irradiated at 320 nm (128 mW/cm^2 for 15 min) and autoradiographed. The gel slice containing the retarded complex subsequently was excised and the latter was eluted from the gel in 50 mM Tris pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 0.1% SDS and migrated through a denaturing SDS–polyacrylamide gel. Once dried, the gel was autoradiographed on a phosphorimager screen (Molecular Dynamics).

DNA affinity purification of GABP

Biotinylated double-stranded N_3 oligonucleotide (200 pmol) was coupled to 1 mg of streptavidin magnetic beads (Dynal) according to the manufacturer's instructions. The beads were then incubated with 200 µg of nuclear extract of C2C12 myoblasts for 30 min at 4°C in 400 µl of gel retardation assay buffer supplemented with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mg/ml BSA (fraction V, Sigma). The beads subsequently were washed three times with 400 µl of the same buffer except that the KCl concentration was increased to 100 mM and the BSA was removed for the last two washes. The proteins retained on the beads were then eluted in 50 µl by increasing the KCl concentration of the buffer to 400 mM.

Low stringency PCR for the Ets gene family

The degenerate primers were designed in the most conserved regions of the Ets domain (Macleod *et al*., 1992) and their sequences were as follows: 5′-YTITGGSAITTYYTIYTISA-3′ and 5′-AIYTTITCRTARTTCAT-3′.

In situ hybridization studies and riboprobes

Diaphragm muscles were dissected from OF1 mice and hybridized with riboprobes as previously described (Piette *et al*., 1993). The riboprobes for the mouse AChR δ-subunit, GABPα and GABPβ1 were obtained using cDNA fragments spaning from nucleotides +4 to +420, +1 to +553 and +485 to +990, respectively. The presence of acetylcholinesterase was detected using the method described by Koelle and Fiedenwald (1949).

In vivo DNA injection

The detailed protocol is described in Duclert *et al.* (1993). Briefly, 30 µl of a 20% sucrose solution, containing 3 mg/ml or 1 mg/ml of plasmid, respectively, carrying the AChR promoters ε or δ, was injected into both tibialis anterior muscles of 3-week-old mice. One week after injection, the muscles were dissected and the fibers expressing β-galactosidase were stained for acetylcholinesterase to localize the synaptic zone (Koelle and Fiedenwald, 1949). Microscopic observation indicated whether or not the β-galactosidase-expressing nuclei were co-localized with the motor endplates. Synaptic events corresponded to those for which co-localization was observed, and the percentage of synaptic events is given by the ratio of the number of synaptic events to the total number of events.

Generation of a polyclonal GABPα antibody

A GABPα–GST fusion protein was generated by inserting the mouse GABPα cDNA into the *Nco*I–*Eco*RI sites of the pGEX3X vector (Pharmacia). The fusion protein was produced in *Escherichia coli* (strain XL-1 blue, Stratagene) and purified on a glutathione–Sepharose column (Pharmacia) according to the manufacturer's instructions. The purified protein was used to immunize rabbits following a standard protocol. In Western blot, a major band at 58 kDa was detected in C2C12 nuclear extracts. A band of the same size was also detected in extracts from cells infected with GABPα-expressing baculovirus, whereas no band was detected when the cells were infected with GABPβ-expressing baculovirus. For the immunoprecipitations, the anti-GABPα antibody was affinity purified on blots as previously described (Beall and Mitchell, 1986).

Metabolic labeling and immunoprecipitations

Chick primary myotube cultures on 60 mm plates were incubated for 2 h in phosphate-free MEM (ICN), the medium was then changed and 0.8 mCi/ml of ^{32}P (Amersham) were added. After 2 h, the cells were harvested in the immunoprecipitation buffer: 50 mM HEPES pH 8.0, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 10 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin for GABPα immunoprecipitations, and 50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 2 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 10 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin for GABPβ immunoprecipitation. The lysate supernatant was incubated with either 100 ng of purified GABPα antibody or 1 µl of anti-GABPβ serum, for 90 min at 4°C. Then 20 ml of protein A–agarose beads (Santa Cruz) were added and the incubation was continued for a further 60 min. The beads were then washed five times in the GABPα immunoprecipitation buffer and resuspended in denaturing polyacrylamide gel loading buffer (Laemmli, 1970). Half of the reactions were loaded on SDS gels and transferred to nitrocellulose membranes for autoradiography on a phosphoimager screen (Molecular Dynamics) and Western blotting using chemiluminescent detection (ECL, Amersham). To avoid the introduction of errors due to variations in GABPα or β levels from lane to lane, the level of phosphorylation of GABPα or GABPβ measured in each lane was normalized to the amount of the corresponding protein estimated by scanning the result of the Western blot.

The MAP kinase immunoprecipitations were performed as for GABPα, except that 1 µg of anti-ERK2 goat antibody (Santa Cruz) and protein G–agarose beads (Santa Cruz) were used and the two final washes were performed in the MAP kinase assay buffer.

MAP kinase assays

One-third of the immunoprecipitated MAP kinases was used in each assay. The reactions was performed for 30 min at 30°C in 50 µl of 25 mM HEPES pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 50 µM ATP and 10 µCi of [γ-^{32}P]ATP as previously described (Skolnik *et al*., 1993). MBP (0.5 mg) (Sigma) or 100 ng of purified GST–GABPα or GST–GABPβ fusion proteins were used as substrates. The reactions were stopped by addition of denaturing gel loading buffer (Laemmli, 1970); one half was loaded on denaturing polyacrylamide gels and transferred to nitrocellulose membranes for autoradiography on a phosphorimager screen (Molecular Dynamics) and Western blotting.

Acknowledgements

We are grateful to Jacques Ghysdael (Orsay, France) for his gift of mouse GABPα and GABPβ cDNAs and baculovirus-produced proteins. We thank Steven L.McKnight (Tularik Inc., San Francisco CA) who provided us with anti-GABPβ1 and anti-GABPβ2 antibodies. Heregulin was kindly supplied by Mark Sliwkowski (Genentech Inc., San Francisco, CA). We are also grateful to Henriette Nuret and Martine Soudant for preparation of primary cultures. We also thank Jean-Louis Bessereau, Alain Bessis, Elisabeth Brown, Aymeric Duclert, Alban de Kerchove and Satoshi Koike for fruitful discussions and critical reading of the manuscript. This work was supported by the Association Française contre les Myopathies, the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Collège de France, the Ministère de l'éducation nationale, de la recherche et de la technologie, the EEC (Biotech. and Biomed.) and the Direction des Recherches et Etudes Techniques.

References

Altiok,N., Altiok,S. and Changeux,J.P. (1997) Heregulin-stimulated acetylcholine receptor gene expression in muscle—requirement for MAP kinase and evidence for a parallel inhibitory pathway independent of electrical activity. *EMBO J.*, **16**, 717–725.

Baldwin,T.J. and Burden,S.J. (1988) Isolation and characterization of the mouse acetylcholine receptor delta subunit gene: identification of a 148-bp *cis*-acting region that confers myotube-specific expression. *J. Cell Biol.*, **107**, 2271–2279.

Beall,J.A. and Mitchell,G.F. (1986) Identification of a particular antigen from a parasite cDNA library using antibodies affinity purified from selected portions of Western blots. *J. Immunol. Methods*, **86**, 217–223.

Bessereau,J.L., Mendelzon,D., Le Poupon,C., Fiszman,M., Changeux,J.P. and Piette,J. (1993) Muscle specific expression of the acetylcholine receptor alpha subunit gene requires both positive and negative interaction between myogenic factors Sp1 and GBF factors. *EMBO J.*, **12**, 443–449.

Bessereau,J.L., Stratford-Perricaudet,L.D., Piette,J., Le Poupon,C. and Changeux,J.P. (1994) *In vivo* and *in vitro* analysis of electrical activity-dependent expression of muscle acetylcholine receptor genes using adenovirus. *Proc. Natl Acad. Sci. USA*, **91**, 1304–1308.

Blank,V., Kourilsky,P. and Israel,A. (1992) NF-kappa B and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. *Trends Biochem. Sci.*, **17**, 135–140.

Brenner,H.R., Witzemann,V. and Sakmann,B. (1990) Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses. *Nature*, **344**, 544–547.

Brown,T.A. and McKnight,S.L. (1992) Specificities of protein–protein and protein–DNA interaction of GABP alpha and two newly defined ets-related proteins. *Genes Dev.*, **6**, 2502–2512.

Burden,S. and Yarden,Y. (1997) Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron*, **18**, 847–855.

Changeux,J.P. *et al*. (1990) Compartmentalization of acetylcholine receptor genes expression during development of the neuromuscular junction. *Cold Spring Harbor Symp. Quant. Biol.*, **55**, 381–396.

Chomczynski,P. and Sacchi,N. (1987) Single step method for RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

Crepieux,P., Coll,J. and Stehelin,D. (1994) The Ets family of proteins: weak modulators of gene expression in quest for transcriptional partners. *Crit. Rev. Oncogen.*, **5**, 615–638.

de la Brousse,F, Birkenmeier,E.H., King,D.S., Rowe,L.B. and McKnight,S.L. (1994) Molecular and genetic characterization of GABP beta. *Genes Dev.*, **8**, 1853–1865.

Dennis,C.L., Tinsley,J.M., Deconinck,A.E. and Davies,K.E. (1996) Molecular and functional analysis of the utrophin promoter. *Nucleic Acids Res.*, **24**, 1646–1652.

Duclert,A. and Changeux,J.P. (1995) Acetylcholine receptor genes expression at the developing neuromuscular junction. *Physiol. Rev.*, **75**, 339–367.

Duclert,A., Piette,J. and Changeux,J.P. (1991) Influence of innervation on myogenic factors and acetylcholine receptor α-subunit mRNAs. *NeuroReport*, **2**, 25–28.

Duclert,A., Savatier,N. and Changeux,J.P. (1993) An 83 nucleotide promoter of the acetylcholine receptor epsilon-subunit gene confers preferential synaptic expression in mouse muscle. *Proc. Natl Acad. Sci. USA*, **90**, 3043–3047.

Duclert,A., Savatier,N., Schaeffer,L. and Changeux,J.P. (1996) Identification of an element crucial for the sub-synaptic expression of the acetylcholine receptor epsilon-subunit gene. *J. Biol Chem.*, **271**, 17433–17438.

Falls,D.L., Rosen,K.M., Corfas,G., Lane,W.S. and Fischbach,G.D. (1993) ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the Neu ligand family. *Cell*, **72**, 801–815.

Flory,E., Hoffmeyer,A., Smola,U., Rapp,U.R. and Bruder,J.T. (1996) Raf-1 kinase targets GA-binding protein in transcriptional regulation of the human immunodeficiency virus type 1 promoter. *J. Virol.*, **70**, 2260–2268.

Fontaine,B. and Changeux,J.P. (1989) Localization of nicotinic acetylcholine receptor alpha-subunit transcripts during myogenesis and motor endplate development in the chick. *J. Cell Biol.*, **108**, 1025–1037.

Fontaine,B., Klarsfeld,A., Hökfelt,T. and Changeux,J.P. (1986) Calcitonin gene-related peptide, a peptide present in spinal cord motoneurons, increases the number of acetylcholine receptors in primary cultures of chick embryo myotubes. *Neurosci. Lett.*, **71**, 59–65.

Fontaine,B., Sassoon,D., Buckingham,M. and Changeux,J.P. (1988) Detection of the nicotinic acetylcholine receptor alpha-subunit mRNA by *in situ* hybridization at neuromuscular junctions of 15-day-old chick striated muscles. *EMBO J.*, **7**, 603–609.

Galzi,J.L. and Changeux,J.P. (1994) Neurotransmitter-gated ion channels as unconventional allosteric proteins. *Curr. Opin. Struct. Biol.*, **4**, 554–565.

Genuario,R.R. and Perry,R.P. (1996) The GA-binding protein can serve as both an activator and repressor of ribosomal protein gene transcription. *J. Biol. Chem.*, **271**, 4388–4395.

Giovane,A., Pintzas,A., Maira,S.M., Sobieszczuk,P. and Wasylyk,B. (1994) Net, a new ets transcription factor that is activated by Ras. *Genes Dev.*, **8**, 1502–1513.

Goldman,D. and Staple,J. (1989) Spatial and temporal expression of acetylcholine receptor RNAs in innervated and denervated rat soleus muscle. *Neuron*, **3**, 219–228.

Goldman,D., Brenner,H.R. and Heinemann,S. (1988) Acetylcholine receptor alpha-, beta-, gamma-, and delta-subunit mRNA levels are regulated by muscle activity. *Neuron*, **1**, 329–335.

Hall,Z.W. and Sanes,J.R. (1993) Synaptic structure and development: the neuromuscular junction. *Cell/Neuron*, **72**, 99–121.

Jia,H.T., Tsay,H.J. and Schmidt,J. (1992) Analysis of binding and activating functions of the chick muscle acetylcholine receptor gamma-subunit upstream sequence. *Cell. Mol. Neurobiol.*, **12**, 241–258.

Jo,S.A., Zhu,X., Marchionni,M.A. and Burden,S.J. (1995) Neuregulins are concentrated at nerve–muscle synapses and activate ACh-receptor gene expression. *Nature*, **373**, 158–161.

Jousset,C. *et al*. (1997) A domain of tel conserved in a subset of ets proteins defines a specific oligomerization interface essential to the mitogenic properties of the tel-pdgfr-beta oncoprotein. *EMBO J.*, **16**, 69–82.

Karlin,A. and Akabas,M.H. (1995) Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron*, **15**, 1231–1244.

Klarsfeld,A. and Changeux,J.P. (1985) Activity regulates the levels of acetylcholine receptor α-subunit mRNA in cultured chicken myotubes. *Proc. Natl Acad. Sci. USA*, **82**, 4558–4562.

Klarsfeld,A., Bessereau,J.L., Salmon,A.M., Triller,A., Babinet,C. and Changeux,J.P. (1991) An acetylcholine receptor alpha-subunit promoter conferring preferential synaptic expression in muscle of transgenic mice. *EMBO J.*, **10**, 625–632.

Koelle,G.B. and Fiedenwald,J.S. (1949) A histological method for localizing cholinesterase activity. *Proc. Soc. Biol. Med.*, **70**, 617–622.

Koike,S., Schaeffer,L. and Changeux,J.P. (1995) Identification of a DNA element determining synaptic expression of the mouse acetylcholine receptor delta-subunit gene. *Proc. Natl Acad. Sci. USA*, **92**, 10624–10628.

Kues,W.A., Sakmann,B. and Witzemann,V. (1995) Differential expression patterns of five acetylcholine receptor subunit genes in rat muscle during development. *Eur. J. Neurosci.*, **7**, 1376–1385.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

LaMarco,K., Thompson,C.C., Byers,B.P., Walton,E.M. and McKnight,S.L. (1991) Identification of Ets- and notch-related subunits in GA binding protein. *Science*, **253**, 789–792.

Laufer,R. and Changeux,J.P. (1989) Activity dependent regulation of gene expression in muscle and neuronal cells. *Mol. Neurobiol.*, **3**, 1–54.

Lopez,M., Oettgen,P., Akbarali,Y., Dendorfer,U. and Libermann,T.A. (1994) ERP, a new member of the ets transcription factor/oncoprotein family: cloning, characterization, and differential expression during B-lymphocyte development. *Mol. Cell. Biol.*, **14**, 3292–3309.

Macleod,K., Leprince,D. and Stehelin,D. (1992) The ets gene family. *Trends Biochem. Sci.*, **17**, 251–256.

Mendelzon,D., Changeux,J.P. and Nghiêm,H.O. (1994) Phosphorylation of myogenin in chick myotubes: regulation by electrical activity and by protein kinase C. Implications for acetylcholine receptor gene expression. *Biochemistry*, **33**, 2568–2575.

Merlie,J.P. and Kornhauser,J.M. (1989) Neural regulation of gene expression by an acetylcholine receptor promoter in muscle of transgenic mice. *Neuron*, **2**, 1295–1300.

Merlie,J. and Sanes,J.R. (1985) Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibers. *Nature*, **317**, 66–68.

Moscoso,L.M., Chu,G.C., Gautam,M., Noakes,P.G., Merlie,J.P. and Sanes,J.R. (1995) Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/heregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. *Dev. Biol.*, **172**, 158–169.

Nef,P., Mauron,A., Stalder,R., Alloid,C. and Ballivet,M. (1984) Structure linkage, and sequence of the two genes encoding the delta and gamma subunits of the nicotinic acetylcholine receptor. *Proc. Natl Acad. Sci. USA*, **81**, 7975–7979.

New,H.V. and Mudge,A.W. (1986) Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature*, **323**, 809–811.

Ouyang,L., Jacob,K.K. and Stanley,F.M. (1996) GABP mediates insulin-increased prolactin gene transcription. *J. Biol. Chem.*, **271**, 10425–10428.

Piette,J., Bessereau,J.L., Huchet,M. and Changeux,J.P. (1990) Two adjacent MyoD1-binding sites regulate the expression of the acetylcholine receptor α-subunit gene. *Nature*, **345**, 353–355.

Piette,J., Huchet,M., Houzelstein,D. and Changeux,J.P. (1993) Compartmentalized expression of the α and γ-subunits of the acetylcholine receptor in recently fused myotubes. *Dev. Biol.*, **157**, 205–213.

Prody,C.A. and Merlie,J.P. (1992) The 5′-flanking region of the mouse muscle nicotinic acetylcholine receptor beta-subunit gene promotes expression in cultured muscle cells and is activated by MRF4, myogenin and MyoD. *Nucleic Acids Res.*, **20**, 2367–2372.

Rivera,R.R., Stuiver,M.H., Steenbergen,R. and Murre,C. (1993) Ets proteins: new factors that regulate immunoglobulin heavy-chain gene expression. *Mol. Cell. Biol.*, **13**, 7163–7169.

Sandrock,A.J., Dryer,S.E., Rosen,K.M., Gozani,S.N., Kramer,R., Theill,L.E. and Fischbach,G.D. (1997) Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction *in vivo*. *Science*, **276**, 599–603.

Sanes,J.R., Johnson,Y.R., Kotzbauer,P.T., Mudd,J., Hanley,T., Martinou,J.C. and Merlie,J.P. (1991) Selective expression of an acetylcholine receptor–LacZ transgene in synaptic nuclei of adult muscle fibers. *Development*, **113**, 1181–1191.

Sapru,M.K., Florance,S.K., Kirk,C. and Glodman,D. (1998) Identification of a neuregulin and protein-tyrosine phosphatase response element in the nicotinic acetylcholine receptor ε subunit gene: regulatory role of an Ets transcription factor. *Proc. Natl Acad. Sci. USA*, **95**, 1289–1294.

Sawa,C., Goto,M., Suzuki,F., Watanabe,H., Sawada,J.I. and Handa,H. (1996) Functional domains of transcription factor hgabp-beta-1/e4tf1-53 required for nuclear localization and transcription activation. *Nucleic Acids Res.*, **24**, 4954–4961.

Seth,A. and Papas,T.S. (1990) The c-ets-1 proto-oncogene has oncogenic activity and is positively autoregulated. *Oncogene*, **5**, 1761–1767.

Sgouras,D.N., Athanasiou,M.A., Beal,G.J.,Jr, Fisher,R.J., Blair,D.G. and Mavrothalassitis,G.J. (1995) ERF: an Ets domain protein with strong transcriptional repressor activity, can suppress ets-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. *EMBO J.*, **14**, 4781–4793.

Simon,A.M. and Burden,S.J. (1993) An E box mediates activation and repression of the acetylcholine receptor δ-subunit gene during myogenesis. *Mol. Cell. Biol.*, **13**, 5133–5140.

Simon,A.M., Hoppe,P. and Burden,S.J. (1992) Spatial restriction of AChR gene expression to subsynaptic nuclei. *Development*, **114**, 545–553.

Skolnik,E.Y., Batzer,A., Li,N., Lee,C.H., Lowenstein,E., Mohammadi,M., Margolis,B. and Schlessinger,J. (1993) The function of GRB2 in linking the insulin receptor to Ras signaling pathways. *Science*, **260**, 1953–1955.

Tang,J., Jo,S.A. and Burden,S.J. (1994) Separate pathways for synapse-specific and electrical activity-dependent gene expression in skeletal muscle. *Development*, **120**, 1799–1804.

Tansey,M.G., Chu,G.C. and Merlie,J.P. (1996) Aria/hg regulates Achr epsilon subunit gene expression at the neuromuscular synapse via activation of phosphatidylinositol 3-kinase and ras/mapk pathway. *J. Cell Biol.*, **134**, 465–476.

Thompson,C.C., Brown,T.A. and McKnight,S.L. (1991) Convergence of Ets- and notch-related structural motifs in a heteromeric DNA binding complex. *Science*, **253**, 762–768.

Tsay,H.J. and Schmidt,J. (1989) Skeletal muscle denervation activates acetylcholine receptor genes. *J. Cell Biol.*, **108**, 1523–1526.

van Beveren,C.P., Rands,E., Chattopadhyay,S.K., Lowy,D.R. and Verma,I.M. (1982) Long terminal repeats of murine retroviral dans: sequence analysis, host-proviral junctions, and integration sites. *J. Virol*., **41**, 542–556.

Wang,C.Y., Petryniak,B., Ho,I.C., Thompson,C.B. and Leiden,J.M. (1992) Evolutionarily conserved Ets family members display distinct DNA binding specificities. *J. Exp. Med.*, **175**, 1391–1399.

Watanabe,H., Wada,T. and Handa,H. (1990) Transcription factor E4TF1 contains two subunits with different functions. *EMBO J.*, **9**, 841–847.

Received October 10, 1997; revised and accepted April 1, 1998