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Identification of a DNA element determining synaptic expression of the mouse acetylcholine receptor δ-subunit gene

(neuromuscular junction/compartmentalized expression/enhancer/silencer)

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ABSTRACT **mRNAs for acetylcholine receptor genes are highly concentrated in the endplate region of adult skeletal muscle largely as a result of a transcription restricted to the subneural nuclei. To identify the regulatory elements involved, we employed a DNA injection of a plasmid containing a fragment of the acetylcholine receptor δ-subunit gene promoter (positions −839 to +45) linked to the reporter gene *lacZ* with a nuclear localization signal. Injection of the wild-type construct into mouse leg muscles yielded preferential expression of the reporter gene in the synaptic region. Analysis of various mutant promoters resulted in the identification of a DNA element (positions −60 to −49), referred to as the N box, that plays a critical role in subneural expression. Disruption of this 12-bp element in the context of a mouse δ-subunit promoter from positions −839 to +45 gives widespread expression of the reporter gene throughout the entire muscle fiber, indicating that this element is a silencer that represses δ-subunit gene transcription in extrajunctional areas. On the other hand, this element inserted upstream of a heterologous basal promoter preferentially enhances expression in the endplate region. This element therefore regulates the restricted expression of the δ-subunit gene both as an enhancer at the endplate level and as a silencer in extrajunctional areas. Furthermore, gel-shift experiments with mouse muscle extracts reveal an activity that specifically binds the 6-bp sequence TTCCGG of this element, suggesting that a transcription factor(s) controls the expression of the δ-subunit gene via this element.**

The muscle nicotinic acetylcholine receptor (AChR) is an integral membrane protein made up of four subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$ (fetal type) or $\alpha_2\beta\varepsilon\delta$ (adult type) that accumulates in the postsynaptic domain of the neuromuscular junction where it mediates efficient chemoelectrical transduction (for review, see ref. 1). Northern blot hybridization (2), *in situ* hybridization (3–6), and promoter studies (33) with transgenic animals (7–10) have demonstrated that the restricted distribution of the AChR protein at the endplate results, at least in part, from differential transcriptional regulation of AChR subunit genes in synaptic vs. extrasynaptic regions (for review, see ref. 11). Current models assume that in these different domains AChR genes are regulated by at least two distinct mechanisms. Factors of neural origin, such as calcitonin gene-related peptide (12, 13) or AChR-inducing activity/heregulin (14), enhance transcription and, thus, have been postulated to mediate local activation of transcription in endplate nuclei. It is also known that evoked and spontaneous electrical activity down-regulates AChR gene expression in adult and developing muscles (ref. 15; for review, see ref. 11).

In vivo studies using the DNA injection method (16), adenoviral vectors (17), and transgenic mice (18) have revealed that topologically distinct cis-acting elements contribute to junctional vs. extrajunctional transcription. For instance, fragments of mouse ε-subunit (16) and δ-subunit (18) promoters confer synaptic expression; moreover, in chicken α subunit (17) as in mouse δ-subunit promoters (18), E-box elements contribute to the repression by electrical activity in extrajunctional areas. Yet, to our knowledge, the precise determination of a DNA element that confers synapse-specific expression has not been reported.

To identify the DNA element(s) engaged in synaptic expression, we have used the mouse δ-subunit promoter sequence (19) and taken advantage of a fast semiquantitative technique of intramuscular injection of naked plasmid DNA (16). Here we report the identification of a 12-bp cis-acting element from the AChR δ-subunit gene that determines preferential transcription at the endplate.

MATERIALS AND METHODS

Construction of the Mouse δ-Promoter–Reporter Genes. We cloned the region of positions −839 to +45 of the mouse AChR δ-subunit promoter by PCR by using a λ phage clone for the δ-subunit gene isolated from a mouse genomic library (DBA/2J strain, Clontech) as a template. The amplified fragment was cloned in the *Bam*HI and *Sal* I sites of KSnlsLacZ (16). The plasmid obtained was designated as mδ(−839/+45)nlsLacZ. The 5′ deletion fragments were also generated by PCR by using mδ(−839/+45)nlsLacZ as a template and cloned into the *Xba* I and *Sal* I sites of KSnlsLacZ. M3nlsLacZ was constructed by inserting a 65-bp basal promoter sequence, designated as M3, from the chicken α-subunit gene, positions −60 to +5, into the *Xba* I and *Sal* I sites of KSnlsLacZ. The 3′ deletion fragments were generated by PCR and cloned into the *Xba* I site of M3nlsLacZ. Site-directed mutagenesis was performed by the method of Maruta *et al.* (20) using sets of primers containing the mutated sequences.

DNA Injection Assay. The DNA injection assay was performed as described (16) except that DNA at 1 mg/ml was used. Injection of the δ-subunit promoter–nlsLacZ constructs gave clusters of blue nuclei, called "events," that coincide (synaptic) or not (extrasynaptic) with the motor endplate (see figure 1 of ref. 16) in some fibers. The absolute number of synaptic events, which represents the relative promoter activity in the synaptic region, and the ratio of synaptic events over total number of events are taken as an index of endplate-specific expression of the promoter. Six to 12 mice were used for each experiment and the experiments were repeated at least three times.

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Abbreviations: AChR, acetylcholine receptor; MCK, muscle creatine kinase.

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Gel Shift Assays. Frozen mouse leg muscles (1 g) were homogenized in 5 ml of 20 mM Hepes, pH 8.0/4 mM $MgCl_2$/1 mM EDTA/420 mM NaCl/0.5 mM phenylmethylsulfonyl fluoride/0.5 mM dithiothreitol by using a Polytron homogenizer. The homogenate was centrifuged for 30 min at 15,000 rpm in a SW41 rotor (Beckman). The proteins were ammonium sulfate-precipitated and resuspended in 1 ml of 20 mM Hepes, pH 8.0/100 mM KCl/0.2 mM EDTA/20% (vol/vol) glycerol. All the buffers were supplemented with chymostatin (2 μg/ml)/aprotinin (4 μg/ml)/0.1 mM *p*-aminobenzidine/pepstatin (1.5 μg/ml)/leupeptin (1 μg/ml)/antipain (2 μg/ml). The probe containing the N box was obtained by annealing the oligonucleotides 5′-GGCCGCGTTTCCGGCCTCT-3′ and 5′-CTAGAGAGGCCGGAAACGC-3′. Gel retardation assays were performed as described (17).

RESULTS

An 839-bp Mouse δ-Subunit Promoter Confers Preferential Synaptic Expression. Mapping of the δ-subunit promoter was investigated *in vivo* by DNA injection (16). In muscles injected with a muscle-specific plasmid MCKnlsLacZ (16), only 6% of the total number of clusters of blue nuclei were found at the endplate, indicating a random distribution of the events along the muscle fibers. On the other hand, in muscles injected with mδ(−839/+45)nlsLacZ, about 50% of the events were synaptic (Fig. 1 *B* and *C*). Since a longer promoter fragment (positions about −2400 to +45) did not yield an increase in synaptic events (data not shown), the promoter at positions −839 to +45 carries DNA elements sufficient for preferential synaptic expression.

A 5′ and 3′ Deletion Analysis of the δ-Subunit Promoter *in Vivo*. To identify these elements, we constructed a series of 5′ deletion mutants that are presented in Fig. 1*A*. The number of synaptic and extrasynaptic events and the ratios of synaptic/total events obtained from the muscles injected with mδ(−839/+45)nlsLacZ, mδ(−399/+45)nlsLacZ, mδ(−201/+45)nlsLacZ, and mδ(−90/+45)nlsLacZ were relatively constant (Fig. 1 *B* and *C*). The absolute number of synaptic events and the ratio of synaptic/total events slightly decreased in muscles injected with mδ(−60/+45)nlsLacZ but were still characteristic of a synaptic expression compared to muscle creatine kinase (MCK). The muscles injected with the mδ(−40/+45)nlsLacZ construct exhibited very few synaptic events. This indicates that the promoter region spanning the region from positions −60 to +45 contains the minimum information necessary to confer preferential synaptic expression and that a positive element is present between positions −60 and −40.

A series of 3′ deletion mutants was next constructed by cloning the 3′ deletion mutant promoters upstream of M3, a small heterologous basal promoter derived from the chicken α-subunit gene (Fig. 2*A*), which confers a low level of expression but no synaptic specificity to the reporter gene (Fig. 2 *B* and *C*). Although the level of expression of mδ(−839/−21)M3nlsLacZ, mδ(−839/−34)M3nlsLacZ, mδ(−839/−41)M3nlsLacZ, and mδ(−839/−49)M3nlsLacZ varied (Fig. 2*B*), the ratios of synaptic/total expression were as high as in mδ(−839/+45)nlsLacZ (Fig. 2*C*). Only mδ(−839/−61)M3nlsLacZ did not show preferential synaptic expression (Fig. 2*C*). Unexpectedly, the absolute number of the extrasynaptic events was found to be significantly higher than with any other 3′ deletion mutants. In other words, this construct gave events throughout the entire muscle (Fig. 2 *B* and *C*). Finally, mδ(−839/−150)M3nlsLacZ was not expressed in any fibers (Fig. 2 *B* and *C*). These results suggest the presence of two regulatory elements: (*i*) a nonspecific enhancer(s) in the region of positions −149 to −61 that nonspecifically enhances transcription throughout the muscle fiber and compares well with the activity in the region of positions −148 to −53 reported by Simon *et al.* (21) and (*ii*) a silencer located around positions −60 to −49 that specifically represses extrajunctional transcription and thus favors compartmentalization.

These 5′ and 3′ deletion studies thus demonstrate that the region from positions −60 to −49 overlaps a regulatory element important for specific synaptic expression, which we will refer to as the N box (for subneural), that possesses both enhancing and silencing activities depending on the context.

Identification of a 12-bp Element Involved in Synaptic Expression. To confirm this conclusion, we constructed a gap deletion mutant of the N box in the context of the promoter from positions −839 to +45, designated as mδ-839ΔNnlsLacZ (Fig. 3*A*). As shown in Fig. 3 *B* and *C*, the deletion of this element results in a dramatic change in the expression pattern of the reporter gene: a large number of extrasynaptic events were observed and both the increase in the number of extrasynaptic events and the decrease in the ratio of synaptic/total expression were significant ($P < 0.05$) by the Mann–Whitney test. This clearly confirms that the N box (*i*) determines synapse-specific expression and (*ii*) functions as a silencer in the extrasynaptic region.

The 5′ deletion experiment suggested the presence of an enhancer 3′ of position −60. We therefore examined the effect of small fragments from this region upon the heterologous basal promoter M3 (Fig. 3*D*). Since the 3′ boundary of the enhancer element was not known, we tested three fragments,

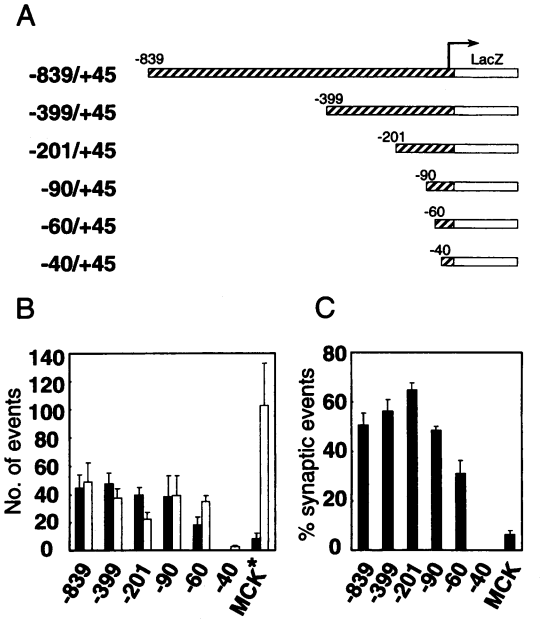

FIG. 1. Expression of 5′ deletion mutants in adult mouse muscle. (*A*) Structure of the 5′ deletion mutants. Hatched boxes, portion of the mouse δ-subunit gene promoter used; open boxes, *lacZ* reporter gene with a nuclear localization signal; arrow, position of the transcription start site in the δ-promoter according to refs. 19 and 24. (*B*) The number of synaptic and extrasynaptic events in the muscles injected with the 5′ deletion mutants. Solid and open bars, number of synaptic and extrasynaptic events, respectively. Data obtained with 5′ deletion mutants are presented as the mean number of events per 10 mice with SEM. *, The data for MCK are presented as those for one mouse. (*C*) Ratio of synaptic/total events in the 5′ deletion mutants. The mean values for synaptic per total events with SEM are shown. In *A*, "mδ" and "nlsLacZ" are omitted in the names of the constructs and are just indicated as −839/+45, etc. In *B* and *C*, only the positions of the nucleotides at the 5′ border of the mutants are shown at the bottom of the figures. Statistical analysis was performed by using a one-way analysis of variance (ANOVA) with overall significance $P < 0.01$ followed by Bonferroni's test. The ratios of synaptic/total events for constructs −839, −399, −201, −90, and −60 are significantly higher than that for MCK ($P < 0.05$) but that for construct −60 is significantly different from that for constructs −399 and −201 ($P < 0.05$) and from that for constructs −839 and −90 ($0.05 < P < 0.10$).

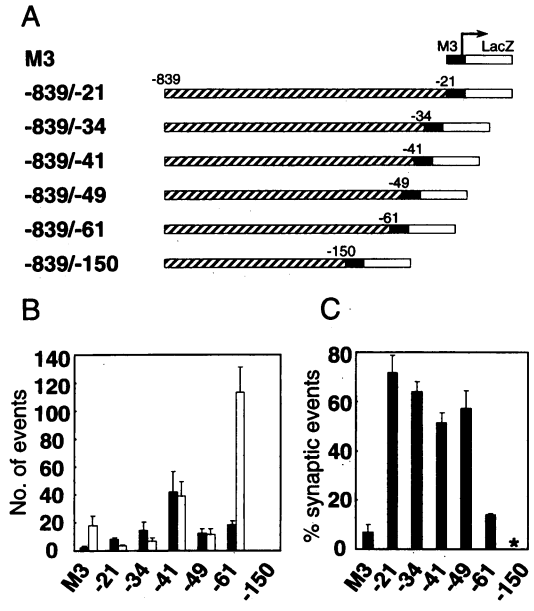

FIG. 2. Expression of 3′ deletion mutants in adult mouse muscle. Same as for Fig. 1 except that solid boxes indicate the portion of the basal promoter, M3, used. *, As no expression was ever observed for construct −150, the ratio could not be calculated. The number of extrasynaptic events for construct −61 is significantly different from that for all other constructs ($P < 0.05$). The ratio of synaptic/total events for construct −60 is significantly different from that for constructs −21, −34, −41, and −49 ($P < 0.05$).

positions −60 to −49 (N box), −60 to −41, and −60 to −34. The number of synaptic events and the ratio of synaptic/total events slightly, but significantly ($P < 0.05$), increased in muscles injected with N-M3nlsLacZ (about 30% of synaptic events) compared to those injected with M3nlsLacZ (Fig. 3 *E* and *F*). The other fragments, positions −60 to −41 and −60 to −34, yielded almost the same results (data not shown). In addition, a 6-bp substitution from positions −48 to −43 in the context of the promoter from positions −60 to +45 did not modify the synaptic expression pattern (data not shown). We therefore consider that the 3′ boundary of the enhancer does not extend downstream of position −49. Furthermore, the fragment from positions −60 to −41 also enhanced the expression of the basal promoter M3 when placed in the opposite orientation (data not shown). This element thus performs as an enhancer of synaptic expression. Yet, in the context of the promoter from positions −60 to +45, there were not as many events, and the ratio of the synaptic/total events was not as large, as was obtained by using the promoter from positions −839 to +45, so we may say that this enhancer contributes to, but is not sufficient, to fully explain the subneural transcription of the δ-subunit gene.

Scanning Mutagenesis Experiments Identify a 6-bp Core Sequence in the N Box. To rule out the possibility that a nonspecific enhancer had been generated at the site of the gap deletion and to determine the core sequence of this enhancer/silencer, we introduced mutations into the N box in the context of the promoters at positions −839 to +45 and −60 to +45 (Fig. 4*A*). In the context of the promoter at positions −839 to +45, mutants N2, N3, and N4 showed expression patterns similar to that obtained with the gap deletion mutant (Fig. 4 *B* and *C*). On the other hand, mutations N1, N5, and N6 did not affect the expression pattern at all. These results suggest that the 6-bp region, positions −58 to −53, plays a critical role in the silencing activity. Since mutation N1 did not show the silencing activity, we consider that the 5′ boundary of the silencer does not extend upstream of position −60.

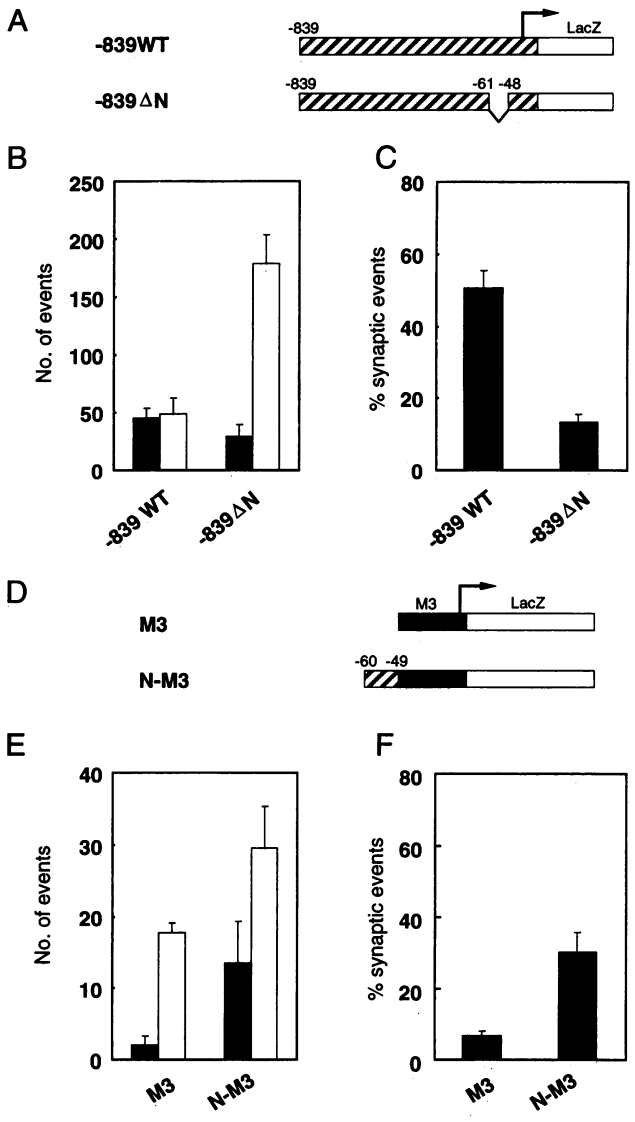

FIG. 3. Silencing and enhancing activities associated with the 12-bp element. (*A–C*) Silencing activity in the N box (same as for Fig. 1). (*D–F*) Enhancing activity in the N box (same as for Fig. 1).

The 5′ deletion and the heterologous promoter analyses suggest that the promoter at positions −60 to +45 also contains an enhancer in the N box. Mutations in the core sequence of this enhancer should, therefore, reduce the overall expression level in the context of the promoter at positions −60 to +45. Indeed, mutations N2 and N3 yielded very low expression levels (both synaptic and extrasynaptic). With mutation N4 as well, few synaptic events were obtained, yet some extrasynaptic events could be observed (Fig. 4 *D* and *E*). These results suggest that the region spanning positions −58 to −53 is also important for activation of gene expression at synaptic sites.

We conclude therefore that mutations N2, N3, and N4, introduced in the contexts of positions −839 to +45 or −60 to +45, yield results consistent with the notion that this N box possesses both a synaptic enhancer and an extrasynaptic silencer function that map to the same domain at the resolution employed.

A Factor Binds to the N Box in Muscle Nuclear Extracts. By using a gel shift assay, the 12-bp element as a probe, and muscle extracts, we observed a retarded complex that disappeared in the presence of an excess of nonlabeled probe as a competitor (Fig. 5). Competition was also observed with DNA fragments

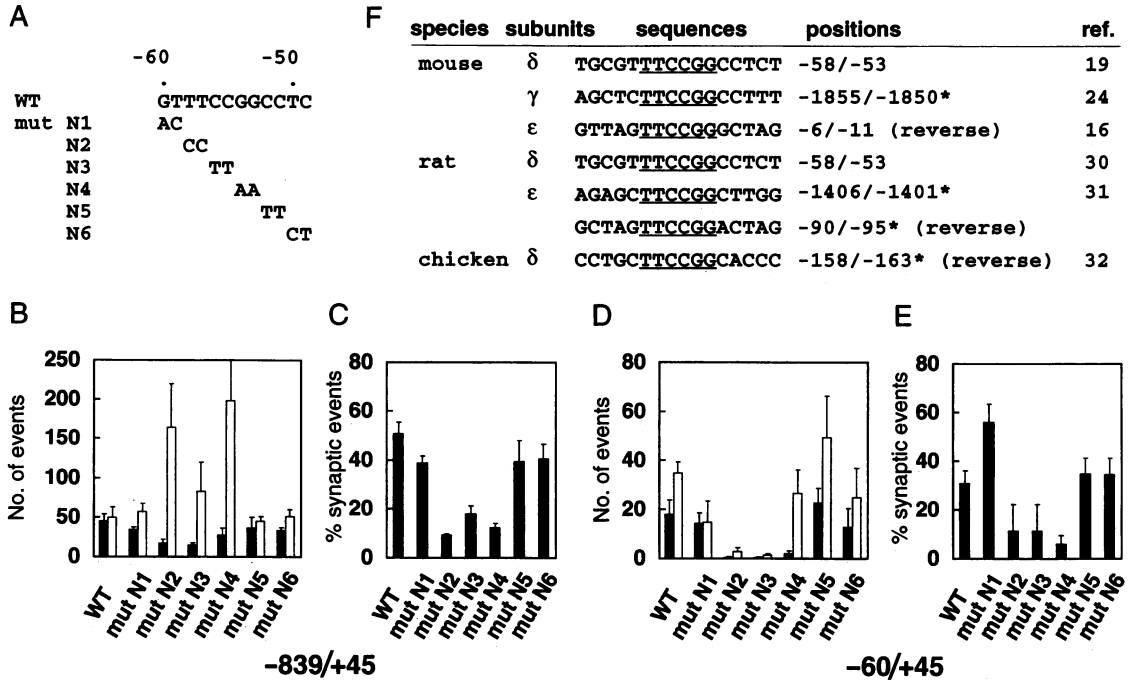

FIG. 4. Scanning mutagenesis experiments. (*A*) Mutations introduced in the N box, positions −60 to −49. The top line indicates the wild-type sequence; the lines below indicate the sequences of each mutant. (*B* and *C*) Same as for Fig. 1. Mutations were introduced in the context of the promoter from positions −839 to +45. The ratios of synaptic/total events for mut N2, N3, and N4 are different from that for wild type ($P < 0.05$). (*D* and *E*) Same as for Fig. 1. Mutations were introduced in the context of the promoter from positions −60 to +45. Due to the small number of events, it was not possible to apply a statistical test. (*F*) Sequences identical to the core sequence of the N box are present in other AChR subunit genes. Species and subunit of the genes and sequences are indicated; the nucleotide position of the identical sequence in each gene is indicated relative to either the transcriptional initiation site or the translation initiation site (marked by an asterisk).

carrying mutations N1, N5, and N6 but not with fragments containing mutations N2, N3, or N4. Thus, at least one factor binds to the probe in a sequence-specific manner and the sequence specificity of the binding activity correlates well with the sequence requirement determined in the functional assay described above.

DISCUSSION

Synaptic expression of the δ-subunit gene in adult mouse muscle is regulated positively and negatively by several elements present in the promoter region from positions −150 to +45. One of them (positions −60 to −49), which we refer to as the N box, plays a critical role in the subneural compartmentalization process. Indeed, mutation of TTCCGG within the N box in the context of the promoter at positions −839 to +45 dramatically converts the expression pattern of the reporter gene from a junctional to a nonsynaptic pattern. Moreover, the core sequence TTCCGG of the N box is present in several AChR subunit genes (Fig. 4*F*). The fragments of the chicken α-subunit 850-bp promoter (22), mouse α-subunit promoter from positions −750 to +32 (23), and mouse γ-subunit promoter from positions −758 to −5 (relative to the translation initiation site) (24), which did not contain this sequence, failed to confer synaptic expression by using this assay in the adult mouse (data not shown). On the other hand, an 83-bp mouse ε-subunit promoter that contains this element in the inverted orientation confers synapse preferential expression (16). It is therefore likely that the N box controls the concerted synaptic expression of at least some of the AChR genes in the adult.

Neural factors such as AChR-inducing activity and calcitonin gene-related peptide might enhance synaptic transcription via the N box. Interestingly, a 181-bp mouse δ-subunit promoter (25) and a 150-bp mouse ε-subunit promoter (26), both of which contain the 6-bp core sequence of the N box (Fig. 4*F*), confer a response to AChR-inducing activity.

Electrical activity represses transcription of the mouse δ-subunit gene at least in part by reducing activation by myogenic factors at the level of the E box (approximately position −20) (18, 27, 28). N-box-mediated repression clearly differs from this E-box-mediated repression of the δ-subunit, however. The N box actively represses constitutive activation in extrajunctional regions through a nonspecific enhancer at positions −149 to −61. Therefore, once the N box is disrupted, transcription is no longer repressed in the extrajunctional

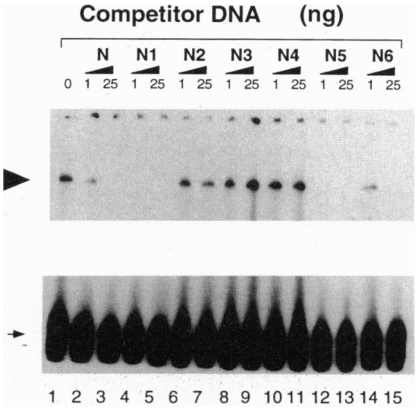

FIG. 5. Gel shift experiments identify an activity that specifically binds the N box in adult muscle extracts. The probe containing the N-box sequence was incubated with a mouse muscle extract (lane 1). In lanes 2–15, the reaction was performed in the presence of nonlabeled competitor DNA: N is the oligonucleotide used to prepare the probe (lanes 2 and 3) and N1–N6 correspond to the oligonucleotide N carrying the mutations N1–N6 described in Fig. 4, respectively (lanes 4–15). Arrow and arrowhead indicate the free probe and the retarded complex, respectively.

regions (Figs. 3 and 4). This observation is consonant with results for the γ-subunit that demonstrated a repression of extrajunctional AChR transcription by neural factors distinct from evoked electrical activity (29).

A decisive piece of information for the elucidation of the still enigmatic enhancer/silencer functions of the N box in the compartmentalized expression of the AChR subunit genes might result from the identification of the protein factor(s) that specifically interact with the N box.

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