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An intronic enhancer containing an N-box motif is required for synapse- and tissue-specific expression of the acetylcholinesterase gene in skeletal muscle fibers

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ABSTRACT **mRNAs encoding acetylcholinesterase (AChE; EC 3.1.1.7) are highly concentrated within the postsynaptic sarcoplasm of adult skeletal muscle fibers, where their expression is markedly influenced by nerve-evoked electrical activity and trophic factors. To determine whether transcriptional regulatory mechanisms account for the synaptic accumulation of AChE transcripts at the mammalian neuromuscular synapse, we cloned a 5.3-kb DNA fragment that contained the 5′ regulatory region of the rat AChE gene and generated several constructs in which AChE promoter fragments were placed upstream of the reporter gene *lacZ* and a nuclear localization signal (nls). Using a recently described transient expression assay system in intact skeletal muscle, we show that this AChE promoter fragment directs the synapse-specific expression of the reporter gene. Deletion analysis revealed that a 499-bp fragment located in the first intron of the AChE gene is essential for expression in muscle fibers. Further analysis showed that sequences contained within this intronic fragment were (*i*) functionally independent of position and orientation and (*ii*) inactive in hematopoietic cells. Disruption of an N-box motif located within this DNA fragment reduced by more than 80% the expression of the reporter gene in muscle fibers. In contrast, mutation of an adjacent CArG element had no effect on nlsLacZ expression. Taken together, these results indicate that a muscle-specific enhancer is present within the first intron of the AChE gene and that an intronic N-box is essential for the regulation of AChE along skeletal muscle fibers.**

Acetylcholinesterase (AChE; EC 3.1.1.7) is an essential synaptic component in the nervous system because it is responsible for the rapid hydrolysis of acetylcholine released from nerve terminals. Although a single gene encodes AChE, alternative splicing and distinct processing of the catalytic subunits account for the multiplicity of molecular forms expressed at specific subcellular locations in muscle, neuronal, and hematopoietic cells (1, 2). In skeletal muscle, AChE accumulates at the neuromuscular synapse, where its expression is known to be markedly influenced by the levels of superimposed neuronal activation (refs. 3 and 4 and references therein).

Despite the wealth of information available on the plasticity of AChE confronted with altered levels of neuromuscular activation, our knowledge of the cellular and molecular mechanisms involved in the localization and activity-linked regulation of AChE in muscle is still rudimentary. Several recent studies have begun to explore the molecular basis underlying the accumulation of AChE at both avian and mammalian neuromuscular synapses. Results of these studies have shown that AChE mRNAs are ≈10-fold more abundant in synaptic versus extrasynaptic regions of muscle cells (5–7), yet the molecular events responsible for this compartmentalized expression of AChE transcripts remain to be elucidated. In this context, several levels of regulation, including transcriptional as well as post-transcriptional, may be considered. For instance, several recent studies have shown that post-transcriptional mechanisms operating at the level of transcript stability play a significant role in the regulation of AChE in differentiating myogenic (8), neuronal (9), and hematopoietic (10) cells maintained in culture. Alternatively, results from several laboratories have shown that the synaptic accumulation of mRNAs encoding the various acetylcholine receptor (AChR) subunits within the postsynaptic sarcoplasm results primarily from localized gene transcription occurring in junctional myonuclei (see refs. 11–13).

In the present study, we have thus examined whether local transcriptional activation of the AChE gene contributes to the synaptic accumulation of AChE transcripts within the postsynaptic sarcoplasm. Specifically, we determined the transcriptional activity and pattern of expression of several rat AChE promoter–reporter gene constructs along multinucleated muscle fibers *in vivo*. To this end, we employed a transient expression assay system in intact skeletal muscle that has recently proven useful to study the activity of several AChR promoters in synaptic versus extrasynaptic compartments of muscle cells (see refs. 14–19).

MATERIALS AND METHODS

Screening of a Rat Genomic Library. Genomic DNA from rat kidneys was partially digested with *Mbo*I and size-fractionated by ultracentrifugation on a continuous 10–40% sucrose gradient. DNA fragments between 9 and 23 kb were ligated to Lambda DASH II/*Bam*HI vector and packaged by using a Lambda DASH II/Gigapack II Cloning kit (Stratagene). Approximately 1.6×10^6 plaques were then screened with a ^{32}P-labeled rat AChE cDNA corresponding to the common coding region (base pairs 879–1722) (20). A positive clone containing an ≈9-kb insert was further purified. *Pst*I digestion of this clone yielded a 950-bp fragment that hybridized to the 5′ end of the rat AChE cDNA. This clone was then sequenced and designated as the rat AChE promoter (RAP)

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Abbreviations: AChE, acetylcholinesterase; AChR, acetylcholine receptor; β-gal, β-galactosidase; nls, nuclear localization signal; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assays; GABP, GA-binding protein; Inr, initiator element; MEL, murine erythroleukemia; TA, tibialis anterior; TK, thymidine kinase; RAP, rat AChE promoter; GRAP, NRAP, and FRAP, giant, N-box-containing, and functional RAP.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF134349).
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on the basis of its homology to both mouse and human AChE promoters. RAP was subsequently used as a probe to identify a 5.3-kb fragment, designated as the giant rat AChE promoter (GRAP), resulting from *Nco*I–*Ssp*I digestion of the initial ≈9-kb genomic clone. Sequences were obtained by using both the dideoxynucleotide chain termination method with Sequenase Version 2.0 (United States Biochemical) and the Dye-Deoxy Termination cycle sequencing (Applied Biosystems).

Reporter Constructs and *in Vivo* Analyses of Promoter–Reporter Gene Expression. Promoter fragments were subcloned into a LacZ reporter vector containing a nuclear localization signal (nls) (21). Mutagenesis was performed by using the Altered Sites II *in vitro* mutagenesis system (Promega). To prepare the thymidine kinase (TK)-LacZ constructs containing an intronic region from the rat AChE gene, a fragment from the first intron was excised with *Apa*I and *Sac*I and subcloned into pBluescript SK (Stratagene). This DNA fragment was subsequently excised with *Kpn*I and subcloned into pGEM 7Z. The orientation of the fragment was assessed by using *Eco*RI and *Fsp*I, and the intronic fragment was subcloned in either orientation upstream of the basic pTK-LacZ vector (CLONTECH).

Plasmid DNA was prepared by the Qiagen mega-prep procedure, and final pellets were resuspended in sterile PBS to a final concentration of 2–4 μg/μl. *In vivo* gene transfer into tibialis anterior (TA) muscles of mice was performed as described previously (14–16, 22, 23). Seven to 14 days later, injected muscles were excised and frozen in melting isopentane. Cryostat sections were processed for the simultaneous demonstration of β-galactosidase (β-gal) and AChE. The position of blue myonuclei indicative of AChE promoter activity was determined and compared with the presence of neuromuscular junctions by using a procedure established recently (15, 22, 23).

To determine biochemically the activity of various promoter–reporter gene constructs *in vivo*, 20 μl of a DNA mixture containing the appropriate AChE or TK construct and a plasmid encoding chloramphenicol acetyltransferase (CAT) driven by the constitutive simian virus 40 promoter were injected into TA muscles. Muscles were excised 7 days later and frozen in liquid nitrogen. Whole muscles were subsequently homogenized in a reporter lysis buffer (Promega), and the activities of β-gal and CAT were determined by luminescence (CLONTECH) and biochemical (Promega) assays, respectively, using kits. The β-gal values obtained with the promoter constructs were first corrected by subtracting the basal activity derived from a promoterless nlsLacZ plasmid, which gave values similar to those seen with noninjected muscles. The resulting β-gal activity was then normalized to CAT levels.

Transfection of Promoter–Reporter Gene Constructs in Cultured Cells. Friend murine erythroleukemia (MEL) cells were grown as described (10) and were transfected by using Superfect reagents (Qiagen). Forty-eight hours later, expression of β-gal driven by AChE promoter fragments was determined and compared with a constitutively expressed CAT plasmid. C2 muscle cells were grown and transfected as described (23).

Nuclear Extract and Electrophoretic Mobility-Shift Assays (EMSAs). Muscle nuclei were collected as described (24) with the exception that an additional purification step was performed by resuspending the nuclei in 27% Percoll and centrifugation at 29,000 × *g* for 15 min at 4°C (25). Nuclear proteins were extracted for 45 min on ice in a high-salt buffer containing 20 mM Hepes–KOH (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (vol/vol) glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM spermidine, 0.15 mM spermine, and 5 μg/ml aprotinin, leupeptin, and pepstatin (26). After centrifugation, the supernatant was diluted to reduce NaCl concentration to 150 mM and stored at −80°C.

For EMSAs, synthetic oligonucleotides were 5′-CCTCGGGGTTCCGGAATTTCCAC-3′ (sense) and 5′-GTGGAAATTCCGGAACCCCGAGG-3′ (antisense) for the promoter N-box; 5′-CTGGAGAAGCCGGAACTACAG-CAG-3′ (sense) and 5′-CTGCTGTAGTTCCGGCTTCTC-CAG-3′ (antisense) for the intronic N-box at position +755; 5′-CCGGAGCTCCCGGAACACAGACGTC-3′ (sense) and 5′-GACGTCTGTGTTCCGGGAGCTCCGG-3′ (antisense) for the intronic N-box at position +823; and 5′-CTGCGAC-CCTAATTAGGGTCCCTA-3′ (sense) and 5′-TAGGGAC-CCTAATTAGGGTCGCAG-3′ (antisense) for the CArG-box. Annealed oligonucleotide probes were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The binding reaction mixture included 0.2 ng of labeled probes, 1.5–5 μg of poly(dI)·poly(dC), and 4 μg of nuclear extract. Polyacrylamide gel electrophoresis and exposure to Kodak XAR-5 films were performed as described (27). For competition assays, a 250-fold molar excess of unlabeled oligonucleotides was added to the binding reaction mixture prior to the addition of the labeled probes. Finally, antibodies to GA-binding protein (GABP) α and β (provided by S. McKnight, Univ. of Texas Southwestern Medical Center, Dallas) were used in supershift assays.

RESULTS

Sequence of the 5′ Regulatory Region of the Rat AChE Gene. We isolated a 5.3-kb DNA fragment from a rat genomic library by using the 5′ end of the rat AChE cDNA. Sequence

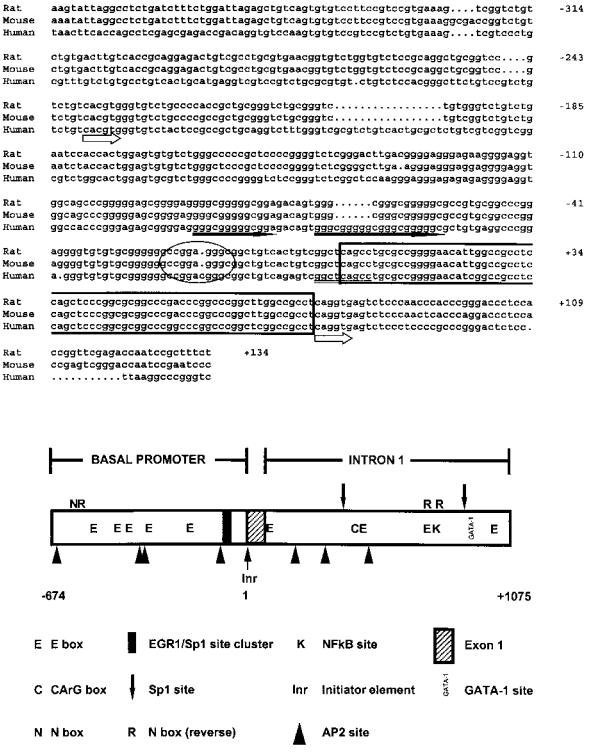

FIG. 1. 5′ regulatory region of the rat AChE gene. (*Upper*) Nucleotide sequence of the rat AChE promoter aligned with corresponding regions from the mouse and human genes. The first nucleotide in the initiator element (Inr; underlined) is designated as +1. Note that the sequence in the rectangular box (+5 to +75) exhibits more than 97% identity with exon 1 in the mouse and human AChE genes. Black arrows indicate EGR-1/Sp1 clusters; oval shows an AP2 site; and white arrows indicate E-boxes. (*Lower*) Schematic representation of the Inr and other consensus sites for DNA-binding proteins present in a larger promoter fragment as well as in the first intron of the gene.

analyses of this DNA fragment revealed that it is extremely G+C-rich and TATA-less. As previously reported for mouse and human AChE promoters (28, 29), this 5′ DNA fragment contains an initiator element (Inr) (Fig. 1). Because the transcriptional activation of TATA-less promoters is effected through the binding of transcription factor TFIID at the Inr (30, 31), thereby providing the primary transcriptional start site in this type of promoter (28, 32), we assigned the first nucleotide of the Inr in this DNA fragment as +1 (Fig. 1). Alignment of the region from −384 to +134 with the corresponding regions of the mouse (28) and human (29) promoters showed an overall identity of 99.5% and 69.5%, respectively (Fig. 1 *Upper*). On this basis, we designated this DNA fragment as GRAP (giant RAP).

Further analyses of a smaller DNA fragment, termed NRAP (N-box-containing RAP), revealed the presence of several consensus sequences for DNA-binding proteins (Fig. 1). In addition to an EGR-1/Sp1 cluster, there are multiple E-boxes as well as a CArG element. Moreover, there are also binding sites for AP2, NF-κB, and GATA-1. In view of the recent evidence showing the crucial role of an N-box motif (TTCCGG) in the synapse-specific expression of AChR subunit (15–17) and utrophin (23) genes, it is noteworthy that there are four N-boxes present in NRAP with three being in a reverse orientation. Interestingly, several of the putative sites for DNA-binding proteins, including two N-boxes, are located in intron 1 of the rat AChE gene.

Expression of AChE Promoter–Reporter Gene Constructs in Muscle. To investigate the functional competence of the cloned DNA fragment in driving transcription *in vivo*, we first transduced mouse TA muscle fibers with plasmids containing various AChE promoter fragments linked to nlsLacZ (see Fig. 3*A*) by using a procedure recently developed (14–19, 22, 23). Histochemical analysis for detection of β-gal activity in transduced muscle fibers showed the presence of distinct blue myonuclei (Fig. 2). To assess whether GRAP conferred synapse-specific expression to the reporter gene in muscle fibers, the position of blue myonuclei was determined and compared with the presence of neuromuscular junctions identified by AChE histochemistry. Quantitative analysis revealed that GRAP was capable of directing the preferential expression of the reporter gene in synaptic compartments of muscle fibers, since approximately 40% of all the events were synaptic (Fig. 3). This percentage of synaptic events fits nicely with those recently obtained for the AChR δ and ε subunit promoters (15, 16), thereby suggesting that local activation of the AChE gene contributes to the enrichment of AChE transcripts within the postsynaptic sarcoplasm of muscle fibers. By contrast, expression of β-gal was more homogeneous along muscle fibers after direct injection of a plasmid containing nlsLacZ driven by the constitutive cytomegalovirus (CMV) promoter since, as expected (see refs. 15, 16, 22, and 23), only ≈12% of all events were synaptic.

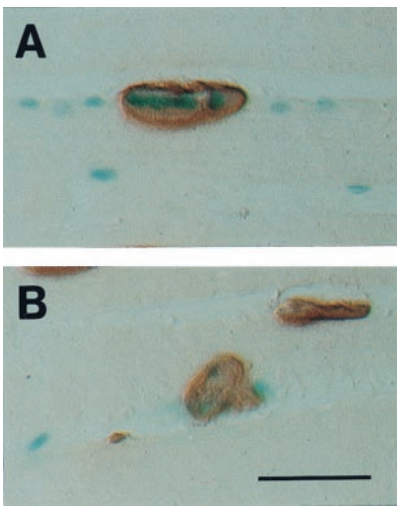

FIG. 2. Expression of AChE promoter–reporter gene constructs in synaptic compartments of mouse TA muscle fibers. (*A* and *B*) Cryostat sections stained histochemically for the simultaneous demonstration of β-gal (blue staining) and AChE (brown staining) activity. Note that the presence of blue nuclei coincides with the occurrence of neuromuscular junctions, reflecting AChE promoter activity within junctional myonuclei. (Bar = 75 μm.)

Intron 1 Is Essential for AChE Gene Expression in Muscle. To delineate DNA elements responsible for AChE expression in muscle, we deleted the bulk of the 5′ and 3′ ends in GRAP up to the region containing the N-boxes in the promoter and intronic regions (NRAP in Fig. 3), and injected the NRAP-nlsLacZ construct into TA muscles. Histochemical staining showed that the pattern of β-gal expression in transduced fibers was similar to that seen after injections of plasmids containing GRAP-nlsLacZ, indicating that NRAP is sufficient to drive the preferential synaptic expression of the reporter gene in muscle. To further characterize the DNA regulatory elements that confer synaptic expression, we deleted additional regions in NRAP. As shown in Fig. 3, removal of 133 nt (base pairs −807 to −674) and 258 nt (base pairs +817 to +1075) encompassing, respectively, the two N-boxes in the promoter region and one N-box in the first intron (see FRAP; functional RAP in Fig. 3), thereby leaving only one N-box intact in the intronic region, did not markedly affect the pattern of expression of the reporter gene (Fig. 3). However, further deletion from +318 to +817 in intron 1, abolished β-gal expression in muscle fibers (RAP in Fig. 3) suggesting that this intronic region is essential for expression of the AChE gene in muscle.

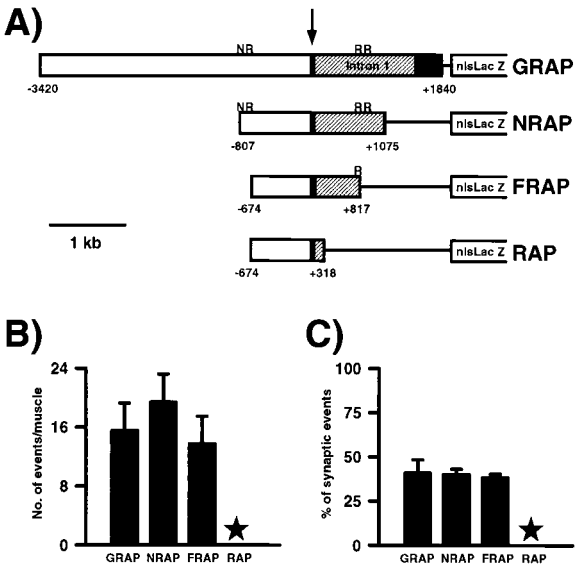

FIG. 3. (*A*) Schematic representation of several AChE promoter–reporter gene constructs used for the *in vivo* studies. Note that the four N-boxes (N) found in GRAP are retained in NRAP, whereas only the first intronic N-box at position +755 bp is still present in FRAP (R indicates those that are in reverse orientation). Arrow points to the first nucleotide of the Inr. (*B* and *C*) Total number of events (*B*) and percentage of synaptic events (*C*) seen in TA muscles after injection of the different constructs. Note that the pattern of expression of the reporter gene is unchanged despite large deletions of the 5′ and 3′ regions in the original 5.3-kb fragment (compare GRAP with NRAP and FRAP). However, note that deletion of an additional 499-bp intronic region abolished AChE promoter activity (compare FRAP with RAP). Star indicates no expression. Mean ± SE is shown; a minimum of 10 muscles were analyzed per construct.

To determine whether this intronic fragment is involved in enhancing specifically the expression of the AChE gene in muscle, we transfected hematopoietic and myogenic cells grown in culture. For these experiments, we compared the activity of FRAP- and RAP-nlsLacZ constructs in MEL versus C2 cells. In striking contrast to our findings obtained with muscle fibers *in vivo* (Fig. 3) and myotubes in culture (Fig. 4*A*), we observed that both constructs had a similar transcriptional activity in hematopoietic cells (Fig. 4*B*), indicating that the additional intronic region present in FRAP is distinctively involved in muscle-specific expression of the AChE gene.

In subsequent experiments, we examined whether the intronic fragment located between +318 and +817 could act as an enhancer of transcription. To this end, we engineered promoter–reporter gene constructs in which this DNA fragment was placed 5′ of the TK promoter fused to *lacZ*. Direct injections of these constructs into mouse muscle indicated that the basal TK promoter induced a low level of reporter gene expression in transduced fibers (Fig. 5). However, we noted a large and significant increase ($P < 0.001$) in β-gal activity when these constructs contained the AChE intronic region from +318 to +817 in either orientation.

Role of the Intronic N-Box in AChE Gene Expression. Since the expression studies indicated that important regulatory elements are contained within intron 1 of the AChE gene—i.e., between base pairs +318 and +817—we examined the role of specific DNA consensus elements known to be critical for expression of specific genes in skeletal muscle. Initially, we determined whether the CArG-box is functionally important for expression of the AChE gene by mutating its core consensus sequence (Fig. 6 *A* and *B*) and by comparing expression of the nlsLacZ reporter gene driven by either NRAP or its mutated counterpart mC-NRAP, after direct gene transfer

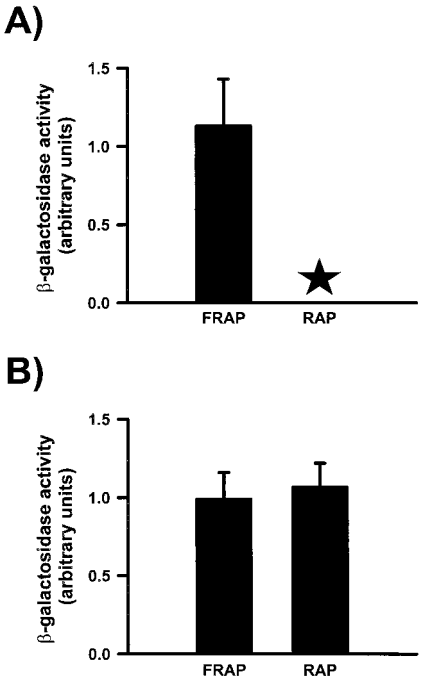

FIG. 4. Expression of AChE promoter–reporter gene constructs in hematopoietic and myogenic cells grown in culture. MEL and C2 cells were transfected with plasmids containing the reporter gene nlsLacZ and the AChE promoter fragment FRAP or RAP. Note that in contrast to myogenic cells (*A*; see also Fig. 3), both constructs were equally active in hematopoietic cells (*B*). In these assays (three independent experiments performed in triplicate), transfection efficiency was monitored by determining the expression of a constitutively expressed CAT plasmid. Star indicates no expression. Mean ± SE is shown.

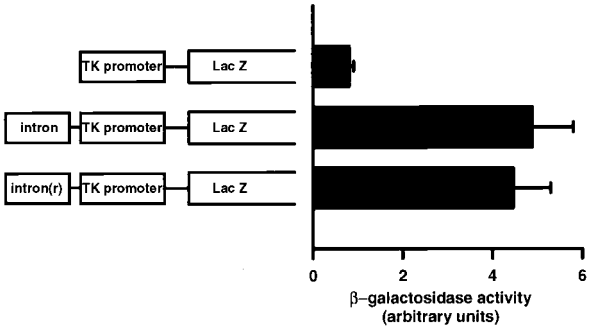

FIG. 5. Expression of β-gal in TA muscle fibers after injection of a plasmid containing the reporter gene *lacZ* with and without the intronic fragment placed in either orientation (r = reverse) upstream of the thymidine kinase (TK) promoter. Note the significant ($P < 0.001$; ANOVA) ≈6-fold induction in β-gal activity with the presence of the intronic DNA fragment in either orientation. Expression of β-gal was normalized to the activity of a coinjected CAT plasmid used to monitor transduction efficiency. Mean ± SE is shown; a minimum of 12 muscles were analyzed per construct.

experiments performed in TA muscles. Despite the loss of binding affinity for nuclear proteins, the mC-NRAP promoter fragment led to a level of β-gal expression comparable to that seen with the wild-type promoter ($P > 0.05$; Fig. 6*C*), suggesting that the CArG-box is dispensable for AChE gene expression in muscle.

Our sequencing data also showed that there are four N-boxes in the AChE gene: two are palindromically located at −694 and −692 bp from the Inr, whereas the others are located in intron 1 at positions +755 and +823 (Fig. 1*B*). To determine whether these DNA regulatory sites are capable of binding protein factors, EMSAs were performed with extracts of purified muscle nuclei. In experiments in which labeled oligo-

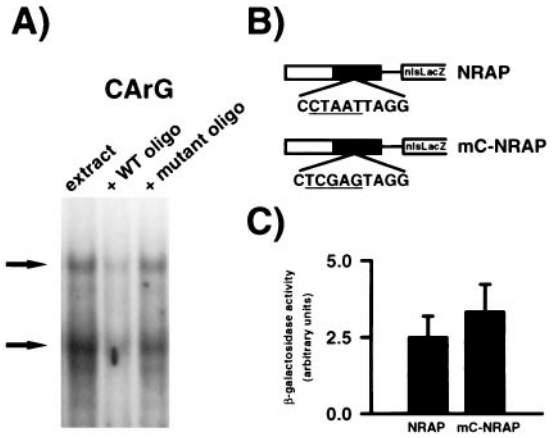

FIG. 6. Disruption of the CArG element does not affect expression of AChE promoter–reporter gene constructs in TA muscle. (*A*) EMSA using radiolabeled oligonucleotides containing the CArG-box. Note the presence of two major DNA–protein complexes (arrows) in muscle nuclear extracts whose formation was specifically blocked by competition with a 250-fold molar excess of the wild-type (WT) but not the mutant oligonucleotide. (*B*) Schematic representation of the nucleotides that were mutated (underlined) in the core region of the CArG element in NRAP to generate the mutant CArG-NRAP promoter fragment (mC-NRAP). As shown in *A*, this mutation resulted in a failure to compete for formation of specific DNA–protein complexes. (*C*) Expression of β-gal in TA muscles injected with reporter plasmids containing either NRAP or mC-NRAP. Note that disruption of this DNA regulatory element did not affect significantly ($P > 0.05$; Student's *t* test) expression of the reporter gene. Expression of β-gal was normalized to the activity of a coinjected CAT plasmid used to monitor transduction efficiency. Mean ± SE is shown; a minimum of 10 muscles were analyzed per construct.

nucleotides containing the N-box located at base pair +755 in the first intron were used, a single DNA–protein complex was observed (N int-1 in Fig. 7*A*). This N-box protein complex was specific, since its formation could be blocked by competition with a 250-fold molar excess of unlabeled oligonucleotides. In addition, mutation of the N-box consensus sequence as shown in Fig. 7*C* functionally abolished its protein-binding affinity as indicated by the inability of mutant oligonucleotides to compete effectively with the wild type for the formation of this protein complex (Fig. 7*A*). Interestingly, we detected considerably less protein binding when oligonucleotides containing the N-box at positions −694, −692, and +823 were used (compare N int-1 vs. N prom in Fig. 7*A*). These observations are in fact entirely consistent with our *in vivo* functional studies showing that the transcriptional activity of the various AChE promoter fragments is unaffected by deletions of these three N-boxes (see Fig. 3). In supershift assays, the binding activity to the N-box motif was shown to involve GABP α and β (Fig. 7*B*).

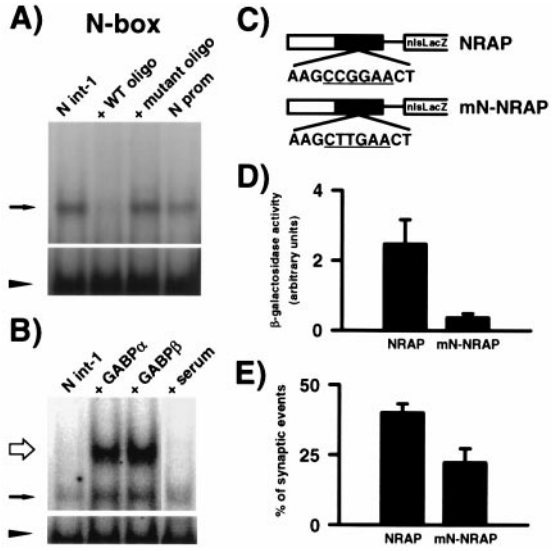

FIG. 7. Disruption of the N-box motif reduces drastically expression of AChE promoter–reporter gene constructs in TA muscle. (*A*) EMSA using radiolabeled oligonucleotides containing the N-box motif. Note that one specific DNA–protein complex (arrow) was formed when a 24-bp oligonucleotide encompassing the first intronic N-box at position +755 (N int-1) was used. Formation of this protein complex was blocked by competition with a 250-fold molar excess of unlabeled wild-type oligonucleotides (WT oligo). Mutation of the core sequence as shown in *C*, abolished its protein-binding capacity, as indicated by the inability of the mutant oligonucleotides to compete in formation of specific protein complex. Note also that oligonucleotides containing the two palindromic N-box motifs located in the promoter region (N prom) displayed a weaker affinity for specific protein complexes. Arrowhead indicates the amount of unbound radioactive oligonucleotides present in each sample. (*B*) The protein complex was supershifted (white arrow) by an additional incubation with antibodies against either GABP α or GABP β. (*C*) Schematic representation of the nucleotides that were mutated (underlined) in the core region of the first intronic N-box motif at position +755 in NRAP to generate the mutant N-box-NRAP promoter fragment (mN-NRAP). As shown in *A*, this mutation resulted in a failure to compete in formation of specific DNA–protein complexes. (*D*) Expression of β-gal in TA muscles injected with reporter plasmids containing either NRAP or mN-NRAP. Note that disruption of this DNA regulatory element essentially abolished ($P < 0.001$; Student's *t* test) expression of the reporter gene, indicating that the N-box is involved in enhancing expression of AChE in muscle. Expression of β-gal was normalized to the activity of a coinjected CAT plasmid used to monitor transduction efficiency. (*E*) The percentage of synaptic events was also significantly reduced ($P < 0.005$; Student's *t* test) in muscles injected with reporter plasmids containing mN-NRAP. Mean ± SE is shown; a minimum of 10 muscles were analyzed per construct.

On the basis of our promoter analysis and EMSA, we next examined whether this intronic N-box is essential for the regulation of the AChE gene in muscle. To this end, we directly injected into TA muscle a plasmid containing the reporter gene nlsLacZ driven by the NRAP promoter fragment mutated within the intronic N-box at position +755 (mN-NRAP in Fig. 7*C*). In comparison with the wild-type AChE promoter fragment NRAP, mN-NRAP lost more than 80% of its transcriptional activity ($P < 0.001$; Fig. 7*D*), indicating therefore that this intronic N-box plays a critical role in the expression of AChE in muscle. Along with this dramatic reduction in the level of expression, we also observed a significant decrease ($P < 0.005$) in the percentage of synaptic events (Fig. 7*E*). By contrast, mutation of this intronic N-box did not alter expression of the reporter gene in MEL cells.

DISCUSSION

We report the isolation of a 5.3-kb DNA fragment located in the 5′ region of the rat AChE gene as well as its functional characterization in skeletal muscle. Alignment of this DNA fragment with available human and murine promoter elements (28, 29) revealed a significant degree of sequence identity as exemplified by the presence of highly conserved DNA binding sites for transcription factors. Additional *in vivo* experiments confirmed that this DNA fragment functions indeed as a promoter, since it induces expression of a reporter gene in muscle fibers. Although DNA fragments corresponding to the promoter region of the mouse and human AChE genes have been studied previously in cultured cells (28, 33–35), *Xenopus laevis* embryos (29), and transgenic mice (36), no previous data showed that an AChE promoter fragment confers transgene expression in muscle fibers *in vivo*.

Previous studies have shown that, similar to the transcripts encoding the various AChR subunits, AChE mRNAs are approximately 10-fold more abundant in synaptic versus extrasynaptic compartments of muscle fibers (5–7). However, in contrast to the progress made recently in our understanding of the mechanisms underlying the expression of AChR mRNAs at the neuromuscular synapse, there is currently no information concerning the molecular events responsible for maintaining a high concentration of AChE transcripts within the postsynaptic sarcoplasm of muscle fibers. On the basis of the compartmentalized transcriptional activation of AChR subunit genes within synaptic myonuclei (see refs. 11–13), it may be argued that enhanced transcription of the AChE gene within these nuclei also accounts for the accumulation of AChE transcripts within the postsynaptic sarcoplasm. Using a transient transfection assay system recently employed to study AChR subunit (14–19) and utrophin promoters (22, 23) in intact muscle fibers, we show here that a DNA fragment located in the 5′ region of the AChE gene leads to the preferential expression of a reporter gene in synaptic compartments of muscle fibers. Taken together, these data indicate, therefore, that local activation of genes is a general mechanism employed by muscle fibers to ensure sufficient quantities and appropriate location of postsynaptic membrane proteins along muscle fibers.

Our deletion studies have led to the identification of a region located in the first intron which appears critical for expression of the AChE gene in muscle cells. In agreement with our current data, Taylor and colleagues (37) have recently observed that, indeed, intron 1 is necessary for expression of AChE gene constructs in C2 cells. In our experiments, we further showed that this intronic DNA fragment failed to enhance expression of a reporter gene in hematopoietic cells. Moreover, it significantly increased in muscle, the expression of *lacZ* driven by the heterologous TK promoter when positioned in either orientation. Together with the observation that this element is functional at the 5′ end of the heterologous

promoter as well as in its native downstream location in the AChE gene, these results strongly suggest that this intronic DNA fragment acts as an enhancer in an orientation- and position-independent manner in addition to being tissue-specific.

Recent studies have identified a 6-bp sequence termed an N-box, which is critical for the synapse-specific expression of AChR subunit genes (15, 16, 18) as well as the utrophin gene (23). Interestingly, there are four N-boxes within the 5′ regulatory region of the rat AChE gene. However, our deletion and mutation analyses revealed that only the N-box located in the first intron at position +755 was essential for expression of the AChE gene in muscle fibers. These functional data are in fact entirely consistent with our EMSAs showing that, although all N-boxes in the AChE gene appear to bind specifically the same protein complex, the latter intronic N-box clearly displays the highest binding activity. Because all N-box oligonucleotides used in our EMSA have the same 6-bp core element, these results further indicate that protein binding affinity of the N-box depends on flanking sequences. Together, these data indicate not only that the N-box motif may regulate expression of several genes from an upstream position (15, 16, 23) but also that it can function as a muscle-specific enhancer in a downstream location.

On the basis of the various studies that have examined so far the role of the N-box motif in the regulation of genes encoding synaptic proteins, it is becoming apparent that this DNA regulatory element can in fact act as an enhancer and/or repressor in synaptic versus extrasynaptic compartments of muscle fibers. Transcription factors belonging to the Ets family (38, 39) are currently becoming recognized as important regulators of AChR gene expression, given their ability to bind the N-box motif. For example, in tissue culture experiments, GABP and Ets-2 were shown recently to transactivate AChR subunit promoters (18, 19). Additional studies have also highlighted the contribution of Erp and Sap1a in the repression of the ε subunit promoter (40), thereby indicating that the complement of Ets factors expressed along muscle fibers can in fact mediate the transcriptional activation or repression of genes encoding synaptic proteins in synaptic versus extrasynaptic regions of muscle fibers. Since the N-box motif is present in the promoter (see refs. 15 and 41) as well as intronic regions (present study) of several genes encoding synaptic proteins, it appears that Ets-related transcription factors represent key determinants mediating the development and maintenance of the postsynaptic apparatus because they may lead ultimately to the coordinate activation of this subset of genes within synaptic myonuclei.

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