Role of Intronic E- and N-box Motifs in the Transcriptional Induction of the Acetylcholinesterase Gene during Myogenic Differentiation*

In this study, we examined whether an intronic N-box motif is involved in the expression of acetylcholinesterase (AChE) during myogenesis. We determined that AChE transcripts are barely detectable in cultured myoblasts and that their levels increase dramatically in myotubes. Nuclear run-on assays revealed that this increase was accompanied by a parallel induction in the transcriptional activity of the AChE gene. These changes in transcription were also observed in transfection experiments using AChE promoter-reporter gene constructs. Mutation of the intronic N-box at position +755 base pairs (bp) reduced by more than 70% expression of the reporter gene in myotubes. Disruption of an adjacent E-box, at position +767 bp, also reduced expression of the reporter gene following myogenic differentiation. Co-transfection experiments using AChE promoter-reporter gene constructs and a myogenin expression vector showed that expression of this regulatory factor increased expression of the reporter gene in myotubes. Although the AChE promoter contains multiple E-boxes, mutation of this intronic one was sufficient to prevent the myogenin-induced increase in reporter gene expression. Together, these results indicate that changes in AChE gene transcription occur during myogenesis and highlight the contribution of the intronic N- and E-box motifs in the developmental regulation of the AChE gene in skeletal muscle.

Acetylcholinesterase (AChE) is widely recognized as an essential component of cholinergic synapses. In both the central and peripheral nervous systems, it is responsible for the hydrolysis of acetylcholine released from nerve terminals thereby ensuring the efficiency of synaptic transmission. Although a single gene encodes AChE, several molecular forms can be generated as a result of alternative splicing and distinct post-translational processing (for reviews, see Refs. 1–3). It has been previously suggested that this polymorphism allows for the expression of AChE catalytic subunits in different cell types and at various subcellular locations where specific molecular forms may perform site-specific functions (4).

In skeletal muscle, AChE expression is known to be markedly influenced by the state of differentiation and innervation of the muscle fibers. Numerous studies have indeed reported dramatic changes in the expression of the enzyme during myogenic differentiation and synaptic differentiation (5, 9). Moreover, activity-induced changes in AChE transcript levels have been observed in both cultured myotubes as well as in skeletal muscle in vivo (10–14).

Despite these recent advances in our understanding of some of the biosynthetic events regulating AChE expression in skeletal muscle, our knowledge of the specific molecular mechanisms that account for these changes in mRNA expression remains largely unknown. Nonetheless, alterations in the stability of pre-synthesized AChE transcripts have been suggested to account for at least a portion of the changes in mRNA levels seen during myogenic differentiation (5, 9) and following muscle denervation (13). On the other hand, the contribution of transcriptional regulatory mechanisms has also been documented in several studies that examined expression of the AChE gene by nuclear run-on assays (13, 15) and promoter analyses (16–18). Together, these studies indicate therefore, that both transcriptional control mechanisms as well as post-transcriptional events contribute to the regulation of AChE mRNA levels in skeletal muscle cells.

In this context, we have recently begun to examine the mechanisms underlying the preferential accumulation of AChE transcripts within the postsynaptic sarcoplasm of muscle fibers (see Refs. 11, 17, 19). In these studies, we showed that the synaptic accumulation of AChE transcripts results, at least partially, from the local transcriptional activation of the AChE gene (17). By mutation/deletion analysis, we further demonstrated the key role of the first intron in regulating both the muscle-specific expression of the AChE gene as well as its preferential synaptic expression. In particular, our studies have shown the contribution of an intronic N-box motif and of the ets-related transcription factor GABP (see Refs. 20–24) in the synaptic regulation of the AChE gene in muscle fibers (17).
Given the key role of this intronic N-box in the regulation of AChE in adult skeletal muscle, we sought in the present study to determine whether this DNA element also participates in the control of the AChE gene during myogenic differentiation. In addition, we also examined whether other sites, located within the first intron, are also important in controlling AChE expression by focusing on an adjacent E-box motif.

**Experimental Procedures**

**Cell Culture**—Mouse C2C12 cells (ATCC, Manassas, VA) were cultured on Matrigel (Collaborative Biomedical Products, Bedford, MA)-coated 60-mm-diameter dishes in Dulbecco's modified Eagle's medium (Life Sciences/Life Technologies, Inc., Burlington, Ontario) supplemented with 10% fetal bovine serum, 292 ng/ml 1-glutamine, and 100 units/ml penicillin-streptomycin, in a humidified chamber at 37 °C containing 5% CO2. Confluent myoblasts were induced to differentiate and fuse into myotubes by replacing the growth media with differentiation media that contained low serum (5% horse serum). Culture media were changed every 48 h.

**RNA Extraction and Reverse-transcription and Polymerase Chain Reaction (RT-PCR)**—Total RNA was isolated from myoblasts and myotubes by using 1.0 ml of the TriPure Isolation Reagent (Life Sciences/Life Technologies, Inc.) per 60-mm culture dish according to the manufacturer's instructions. Briefly, following cell lysis with the TriPure reagent, chloroform was added and the solution was vortexed vigorously prior to centrifugation at 12,000 × g for 15 min at 4 °C. The RNA contained in the resulting aqueous layer was precipitated with isopropanol, and the pellet was washed several times with 70% ethanol. The RNA was then resuspended in RNase-free water and stored at −80 °C.

Quantitation of the amount of total RNA in each sample was performed using the Amersham Pharmacia Biotech Gene Quant II RNA/DNA spectrophotometer. Each sample was adjusted to a final concentration of 80 ng/μl. Semi-quantitative RT-PCR was carried out as previously described in detail elsewhere (11, 13, 19, 25). Reverse transcription, using random hexamers, was performed for 45 min at 42 °C, followed by a 5-min incubation at 99 °C. PCR was then used to amplify cDNAs corresponding to AChE and 12 S rRNA. Primers that amplify AChE T transcripts were synthesized according to a previous report (8). cDNAs corresponding to AChE and 12 S rRNA were standardized relative to the corresponding level of cDNA used in our studies (mNmE-NRAP). Each mutant promoter fragment was inserted into the luciferase reporter vector. Finally, a third mutant, in which the E- and N-box motifs were both mutated, was also used in our studies (mNmE-NRAP).

Plasmid DNA was prepared using the Qiagen (Chatsworth, CA) mega- or midi-prep procedure. Pellets were resuspended in sterile phosphate-buffered saline (PBS). Transfection of cultured myogenic cells was performed using the LipofectAMINE reagent kit (Life Sciences/Life Technologies, Inc.). Myoblasts, at 50–60% confluence, were transfected with the appropriate promoter-reporter gene construct. In the next cycle of freeze-thaw. The solution was then centrifuged at 15,000 × g, and the resulting supernatant was assayed for luciferase activity as described elsewhere (28).

Direct gene transfer into mouse skeletal muscle was performed as previously described (13, 17, 29). 25 μl of the appropriate promoter-reporter constructs, diluted at a final concentration of 2 μg/ml, were directly injected into adult mouse tibialis anterior (TA) muscles. Sest day, muscles were excised, quickly frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis. For detection of luciferase activity, muscles were homogenized in Reporter lysis buffer (Promega) following two cycles of freeze-thaw. The solution was then centrifuged at 15,000 × g, and the resulting supernatant was assayed for luciferase activity.

For both direct injection and transfection experiments, a chloramphenicol acetyltransferase (CAT) plasmid driven by the SV40 promoter, carrying two cycles of freeze-thaw. The solution was then centrifuged at 15,000 × g, and the resulting supernatant was assayed for luciferase activity.

**Nuclear Protein Extraction**—Nuclear proteins were extracted from myoblasts and myotubes as previously described (30). Briefly, myoblasts and myotubes were scraped into ice-cold PBS and collected by centrifugation at 200 × g for 5 min. The cells were resuspended and washed once in 1 ml of ice-cold PBS and centrifuged as above. The cells were resuspended in 1 ml of buffer A (10 mm Hepes, pH 7.9; 10 mm KCl; 1.5 mm MgCl2; 1.5 mm DTT; and 0.5 mm phenylmethylsulfonyl fluoride (PMSF)) and centrifuged at 200 × g for 5 min at 4 °C. The cells were then lysed in 300 μl of buffer B containing 0.1% Nonidet P-40 for 20 min on ice. The homogenate was spun at 15,000 × g for 10 min at 4 °C. The supernatant was discarded, and the pellet containing the nuclei was resuspended in 35 μl of buffer B (20 mm Hepes, pH 7.9, 420 mm NaCl, 1.5 mm MgCl2, 0.2 mm EDTA, 25% glycerol, 0.5 mm DTT, 0.5 mm PMSF, and the protease inhibitors spermidine, spermine, aprotinin, leupeptin, and pepstatin) for 45 min on ice to extract nuclear proteins. The nuclear extract was then obtained following centrifugation at 15,000 × g for 15 min. Equal volumes of the nuclear extract (20 μl) were loaded onto the gel for electrophoresis. The gel was stained with Coomassie blue, and the bands corresponding to the SV40 promoter, was used to control for the efficiency of transduction/transfection. The luciferase activity obtained in each sample was therefore normalized to CAT activity.

**Electrophoretic Mobility Shift Assay (EMSA)**—Equal amounts of nuclear proteins (5 μg) were incubated in a reaction mixture containing 17

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mM Hepes, 0.2 mM EDTA, 0.04 mM EGTA, 0.7 mM DTT, 140 mM NaCl, 0.5 mM MgCl₂, 36 mM KCl, 16% glycerol, and 5 μg of poly(dI-dC) for 10 min at room temperature. The reaction mixtures were then incubated with 0.2 ng of [³²P]ATP and [³²P]ATP (60 μCi) in a kinase buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5% glycerol, 0.2 mM EDTA, 0.04 mM EGTA, and 0.7 mM DTT) and incubated at 37 °C for 1 h. Labeled oligonucleotides were isolated by centrifugation through a Sephadex G-50 column (Amersham Pharmacia Biotech) at 3000 x g for 5 min.

RESULTS

Expression of the AChE Gene during Myogenic Differentiation—In a first series of experiments, we examined expression of the AChE gene during myogenic differentiation of mouse C2C12 cells grown in culture. Specifically, we first compared the relative abundance of AChE mRNAs in undifferentiated myoblasts (50% confluent), confluent myoblasts, and differentiated myotubes. As observed previously (see Refs. 5, 7), levels of AChE transcripts increased dramatically during myogenic differentiation. Fig. 1A shows that AChE mRNAs were barely detectable in undifferentiated myoblasts (50% confluent) and became more expressed, albeit still at low levels, in 100% confluent myoblasts. Upon differentiation and fusion of confluent myoblasts into multinucleated myotubes, AChE mRNA levels increased significantly (p < 0.05) and by 3- to 4-fold in myotubes exposed to differentiation media for 2 days (Fig. 1B). In 4-day-old myotubes, AChE mRNA levels were further increased reaching, in this case, more than 10-fold as compared with the levels seen in 100% confluent myoblasts.

We next examined whether this increase in AChE mRNA expression during myogenic differentiation could be attributed to changes in the transcriptional activity of the AChE gene. To this end, we performed nuclear run-on assays using myonuclei isolated from cultured C2C12 cells at various stages of myogenic differentiation (Fig. 2A). As expected (see for example Ref. 5), we observed an increase in the transcriptional activity of myogenin during myogenesis. Similarly, we also detected a gradual increase in the transcriptional rate of the AChE gene in muscle cells undergoing myogenic differentiation. This increase was transient, because in 4-day-old myotubes, the rate of transcription appeared to decrease toward levels seen in 100% confluent myoblasts. These results show, therefore, that the initial increase in AChE mRNA levels seen during myogenic differentiation was accompanied by an induction in the transcriptional activity of the AChE gene, thereby indicating that changes in transcription account for some of the increase in AChE expression.

The Transcriptional Induction of the AChE Gene during Myogenic Differentiation Depends on Intronic E- and N-boxes—In a subsequent series of experiments, we assessed the contribution of cis- and trans-acting elements in the transcriptional regulation of AChE during myogenic differentiation. Rat AChE promoter-reporter gene constructs were transfected into C2C12 myoblasts at −50 to −60% confluence (see “Experimental Procedures”). The cells were subsequently harvested when myoblasts became confluent or following myogenic differentiation. For these experiments, a rat AChE promoter fragment, termed NRAP (17), was linked to a luciferase reporter gene (Fig. 3). Additionally, several mutated NRAP fragments were also used in these studies (see Fig. 3, A and B), including one in which both the intronic E- and N-boxes had been mutated.

In myoblasts, NRAP appeared very active in differentiated myotubes, because the activity of luciferase was significantly (p < 0.05) higher (−6-fold). Furthermore, mutations of the intronic E- (mE-NRAP) and N- (mN-NRAP) boxes at position +767 and +755 bp (see Ref. 17), respectively, had no effect on expression of the reporter gene in these confluent myoblasts. By contrast, NRAP appeared very active in differentiated myotubes, because the activity of luciferase was significantly (p < 0.05) higher (−6-fold). Furthermore, mutations of the intronic E- and N-boxes markedly reduced expression of the reporter gene. Specifically, mutation of the N-box at position +755 bp led to a decrease of −75% (p < 0.05) in luciferase activity.

![Graph showing AChE mRNA levels during myogenic differentiation](https://www.jbc.org/)
Mutation of the adjacent intronic E-box essentially abolished the transcriptional activation of NRAP in differentiated myotubes, because the levels of luciferase were reduced by 90%. Due to this pronounced effect however, we were not able to demonstrate cooperativity between the adjacent E- and N-box motifs, because disruption of both DNA elements (mNmE-NRAP) reduced expression of the reporter gene to a comparable extent in differentiated myotubes.

Given that both the intronic E- and N-box motifs appeared necessary for the transcriptional induction of the AChE gene during myogenic differentiation, we next investigated the contribution of trans-acting elements. To this end, we performed a series of EMSAs using radioactive oligonucleotides encompassing the AChE intronic N-box motif at position 1755 bp (17) and the intronic E-box motif at position 1767 bp. As expected (31, 32), we detected a relative increase in the binding activity to the E-box with nuclear proteins extracted from myotubes versus myoblasts (Fig. 4).

Analysis of the N-box binding activity revealed a single and specific band following incubation of the appropriate radiolabeled oligonucleotides with nuclear proteins isolated from C2C12 cells (Fig. 5A). This protein complex was effectively competed by the addition of a 250-fold excess of unlabeled wild-type probe, whereas an excess of unlabeled mutant probe, encompassing the same mutation as used to generate the mN-NRAP construct, failed to compete with this protein complex (Fig. 5A). In addition, this band was also supershifted when the extracts and oligonucleotides were incubated together with antibodies directed against GABPα and β (kindly provided by Dr. Laurent Schaeffer) thereby confirming the identity of the proteins present in this complex (Fig. 5B). The binding activity of GABP to the N-box motif appeared slightly elevated in nuclear extracts isolated from myotubes as compared with myoblasts (Fig. 5C). The slight increase in GABP expression in myotubes was confirmed by Western blot analysis (data not shown; see also Ref. 53).

Myogenin Increases Expression of AChE via the Intronic E-box Motif—In a separate series of studies, we further ascertained the role of myogenic factors and the intronic E-box at position 1755 bp in the regulation of the AChE gene during myogenic differentiation. To this end, we co-transfected into myoblasts a myogenin expression vector (33) along with a specific AChE promoter-reporter gene construct and harvested the cells after exposure to the differentiation media for 4 days. Fig. 6 shows that overexpression of myogenin increased by almost 2-fold (p < 0.05) the activity of luciferase in differentiated myotubes. As suggested by the experiments presented above, this increase in luciferase activity is dependent upon a single E-box motif, because co-transfection of the myogenin expression vector with the mE-NRAP construct, in which the single intronic E-box is mutated (at position +767 bp), totally pre-
transcriptional induction of the AChE gene in vivo—

Myogenic differentiation is a developmentally regulated process characterized by a series of coordinated biochemical and morphological changes accompanied by the fusion of mononucleated myoblasts into multinucleated myotubes. Together with cytoskeletal and contractile proteins, expression of several synaptic proteins, including AChR, N-CAM, utrophin, and AChE, is also enhanced to varying degrees during myogenesis. In this context, previous studies have shown that, in the case of AChR (34–37) and utrophin (38), the increased expression of these genes correlates well with the pattern of mRNA expression seen with co-transfection of the wild type NRAP construct (Fig. 6).

The Intronic E-box and N-box Regulate Expression of the AChE Gene in Vivo—In a final series of experiments, we assessed the contribution of these cis-acting elements in vivo by directly injecting into mouse TA muscles, the various promoter-reporter gene constructs and by monitoring 7 days later the activity of luciferase normalized to a co-injected constitutively expressed CAT plasmid. As previously observed (17), the NRAP fragment is very active in vivo and mutation of the intronic N-box at position +755 bp, reduced significantly \((p < 0.05)\) the expression of the reporter gene (Fig. 7). In agreement with our data obtained from cultured myogenic cells, functional disruption of the adjacent E-box (see Fig. 5B) in NRAP eliminated completely expression of the reporter gene further highlighting the crucial role of this DNA element in regulating expression of the AChE gene in muscle cells.

**DISCUSSION**

Together with the recent data obtained by Rossi et al. (39) and Rotundo,\(^2\) have also reported recently an increase in the expression of the AChE gene that parallels the induction in AChE mRNA at early stages of chick muscle cell development. Thus, as originally suggested by Merlie and Sanes (40) as well as by Klarsfeld (41), expression of genes encoding key synaptic proteins in muscle may indeed be coordinately regulated during myogenic differentiation.

In agreement with our findings, Rossi et al. (39) and R. L. Rotundo\(^2\) have also reported recently an increase in the expression of the AChE gene that parallels the induction in AChE mRNA at early stages of chick muscle cell development. Thus, as originally suggested by Merlie and Sanes (40) as well as by Klarsfeld (41), expression of genes encoding key synaptic proteins in muscle may indeed be coordinately regulated during myogenic differentiation.

**REFERENCES**

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tundo all observed a decrease in the transcriptional activity of the \( \text{AChE} \) gene in older myotubes, an observation entirely consistent with the results of Taylor and colleagues who also detected a slight reduction in the expression of the \( \text{AChE} \) gene in fully differentiated myotubes (5). Taken together, these results indicate that transcriptional mechanisms participate in the regulation of \( \text{AChE} \) mRNA expression during myogenic differentiation but only at the earliest stages.

As stated above, the increase in \( \text{AChE} \) mRNA expression that occurred in confluent myoblasts and 2-day-old myotubes can be solely explained by an increase in the transcriptional activity of the \( \text{AChE} \) gene. This increase in transcription appeared transient, because it returned toward control levels in older myotubes. At that stage however, \( \text{AChE} \) mRNA levels were further increased indicating, therefore, that post-transcriptional mechanisms likely account for this sustained increase. In this context, Taylor and colleagues have also implied an important contribution for post-transcriptional mechanisms during the later stages of muscle differentiation (5, 9). In agreement with this view, it is relevant to note that recent studies performed in our laboratory have also illustrated the key role of the intronic \( \text{AChE} \) promoter region during myogenic differentiation (5, 9).

Several recent studies have shown that the \( \text{ets} \)-related transcription factors GABP \( \alpha \) and \( \beta \) can bind to the N-box motif to transactivate genes encoding synaptic proteins (17, 22–24, 43). Therefore, we sought to determine whether expression of GABP was affected during myogenic differentiation. Electrophoretic mobility shift assays revealed that GABP-binding activity to the N-box was slightly increased in nuclear extracts from myotubes versus myoblasts, and this was confirmed by Western blot analysis. Although the increase appears rather modest, it is important to note that the transactivation potential of GABP is also influenced by its phosphorylation status with stronger transcriptional activation occurring in the ab-
The observed increase in DNA binding activity (22). These results, together with the promoter analysis, further highlight the importance of the trans-acting element GABP and its corresponding cis-acting element, i.e., the N-box motif, in the expression of AChE during myogenic differentiation.

The ets-related transcription factors often cooperate with other transcription factors (for reviews, see Refs. 44, 45), including AP-1 (46), or with cofactors such as the CREB-binding protein also known as CBP/p300 (47), to exert their effects. Because ets-related factors, including GABP, may also possess a conserved domain with homology to basic helix-loop-helix transcription factors such as myogenic factors (48), it appeared possible that GABP could in fact interact with myogenic factors to regulate expression of the AChE gene. This view appeared particularly attractive given the presence of an E-box in the immediate vicinity of the N-box motif at position +755 bp in the first intron of the AChE gene. Promoter analysis showed that indeed this E-box is crucial in regulating expression of the AChE gene during myogenic differentiation, because its mutation reduced by more than 90% expression of the reporter gene in transfected myotubes. Consistent with these findings, we further showed by co-transfection of AChE promoter-reporter gene constructs and a myogenin expression vector, that myogenin increases the expression of luciferase in myotubes. Remarkably, this effect was dependent upon a single E-box, because mutation of the intronic E-box prevented the myogenin-induced increase in reporter gene expression despite the presence of numerous E-boxes throughout the promoter region of the AChE gene (see Ref. 17). Our findings are therefore in agreement with the well-known effects of myogenic factors on expression of genes in differentiating muscle cells (for reviews, see Refs. 49–51). In addition, our direct plasmid injection in vivo (281–320) and normal mice (2250–2258) that indeed this E-box is crucial in regulating expression of the AChE gene. This view appeared particularly attractive given the presence of an E-box in the immediate vicinity of the N-box motif at position +755 bp in the first intron of the AChE gene. Promoter analysis showed that indeed this E-box is crucial in regulating expression of the AChE gene during myogenic differentiation, because its mutation reduced by more than 90% expression of the reporter gene in transfected myotubes. Consistent with these findings, we further showed by co-transfection of AChE promoter-reporter gene constructs and a myogenin expression vector, that myogenin increases the expression of luciferase in myotubes. Remarkably, this effect was dependent upon a single E-box, because mutation of the intronic E-box prevented the myogenin-induced increase in reporter gene expression despite the presence of numerous E-boxes throughout the promoter region of the AChE gene (see Ref. 17). Our findings are therefore in agreement with the well-known effects of myogenic factors on expression of genes in differentiating muscle cells (for reviews, see Refs. 49–51). In addition, our direct plasmid injection in vivo that indeed this E-box is crucial in regulating expression of the AChE gene.
