

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

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ABSTRACT At the neuromuscular synapse, innervation induces endplate-specific expression of adult-type nicotinic acetylcholine receptors by selective expression of their subunit-encoding genes ($\alpha_2\beta\epsilon\delta$) in endplate-associated myonuclei. These genes are specifically regulated by protein-tyrosine phosphatase (PTPase) activity. In addition, neuregulin/acetylcholine-receptor-inducing activity, a nerve-derived factor that stimulates nicotinic acetylcholine receptor synthesis, induces adult-type specific ϵ subunit gene expression via activation of a Ras/mitogen-activated protein kinase pathway. However, the DNA regulatory elements and the binding proteins that mediate PTPase and neuregulin-dependent gene expression remain unknown. Herein we report that PTPase, neuregulin, and Ras-dependent regulation of the ϵ subunit gene map to a 15-bp promoter sequence. Interestingly, this same 15-bp sequence appears to be necessary for low ϵ subunit gene expression in extrajunctional regions of the muscle fiber. Site-directed mutagenesis of a putative Ets binding site located within this 15-bp sequence, reduced PTPase, neuregulin, and Ras-dependent regulation. Overexpression of the rat muscle Ets-2 transcription factor resulted in a sequence-specific induction of ϵ subunit promoter activity. Further, a dominant negative mutant of Ets-2 abolished neuregulin-dependent induction of ϵ subunit gene expression. Thus, these results indicate a crucial role for the 15-bp element in determining synapse-specific and neuregulin-mediated motor neuron control of ϵ subunit gene expression and suggest the participation of Ets transcription factor(s) in this control.

Synapse formation and subsequent maturation involves complex interactions between the presynaptic cell and its postsynaptic target. As a model synapse, the neuromuscular junction provides a relatively simple system to study molecular mechanisms mediating some of these interactions. At this synapse, synaptic transmission between the motor neuron and its target muscle fiber is mediated by the muscle nicotinic acetylcholine receptor (nAChR), a multisubunit ligand-gated ion channel (1).

During the development of the neuromuscular synapse, nerve-evoked muscle activity suppresses expression of embryonic-type nAChRs ($\alpha_2\beta\gamma\delta$) throughout the muscle fiber (1, 2), whereas muscle innervation induces expression of adult-type nAChRs ($\alpha_2\beta\epsilon\delta$) at the endplate. This spatially restricted expression of adult-type nAChRs is largely a result of selective induction of their subunit encoding genes in endplate-associated myonuclei (1). The transcriptional mechanisms by which the motor neuron regulates gene expression in these subsynaptic nuclei are not well understood.

Motor-neuron-derived trophic factors named neuregulins and their receptors have been colocalized at the neuromuscular endplate (3–5). Neuregulins include neu differentiation factor, heregulin, glial growth factor, and acetylcholine-receptor-inducing activity, which are alternatively spliced products of a single gene (6). Several lines of evidence point to the crucial role of neuregulins in directing synapse-specific expression of the nAChRs at the neuromuscular junction (3–11). However, the underlying mechanism of this regulation is unclear.

Previously, we showed that protein-tyrosine phosphatase (PTPase) activity selectively suppresses muscle adult-type nAChR genes and proposed that changes in protein-tyrosine phosphorylation contributed to synapse-specific gene expression (12). Interestingly, neuregulins function as ligands for epidermal growth factor receptor-related (erbB) tyrosine kinases and stimulate adult-type nAChR synthesis (3–10). Whether the neuregulin signaling pathway differs from that perturbed by PTPase overexpression is not known. Further, synaptic induction of nAChR genes by neuregulin has been shown to require activation of a Ras/mitogen-activated protein (MAP) kinase pathway (13, 14). This raises the possibility that MAP-kinase-responsive transcription factors such as the Ets family of proteins (15–18) may be involved, at least in part, in mediating this regulation.

Because the ϵ subunit gene is unique to adult-type nAChRs and is locally expressed at the endplate, it serves as a marker for regulatory mechanisms involved in synapse-specific expression. In addition, this gene is most sensitive to neuregulin induction (9). However, there is no clear consensus regarding the element that mediates this induction. For example, one study identified a 6-bp element, referred to as the N-box (CCGGAA), that is crucial for synapse-specific expression of this gene (19), and a subsequent study concluded that this element is not required for acetylcholine-receptor-inducing activity (neuregulin)-dependent induction (20). Further, the transcription factors mediating this neuregulin-dependent and synapse-specific expression have not been identified.

In the present study, we show that a 15-bp ϵ promoter sequence plays a crucial role in PTPase, neuregulin, and Ras-dependent regulation of the rat nAChR ϵ subunit gene. *In vivo* expression suggest that this same DNA sequence participates in suppressing ϵ gene expression in extrajunctional regions of the muscle fiber. Further, overexpression of Ets-2 and a dominant negative Ets-2 mutant suggest the participation of Ets transcription factor(s) in neuregulin-mediated motor neuron control of ϵ subunit gene expression.

Abbreviations: nAChR, nicotinic acetylcholine receptor; PTPase, protein tyrosine phosphatase; CAT, chloramphenicol acetyltransferase; MAP kinase, mitogen-activated protein kinase; CMV, cytomegalovirus.

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EXPERIMENTAL PROCEDURES

Expression Vectors. 5' and 3' ϵ promoter deletions were created with exonuclease III. Deletion endpoints were determined by DNA sequencing and appropriate deletions subcloned into the pXP (21) vector for expression studies (Fig. 1). ϵ -69 was subcloned upstream of the minimal enkephalin (MEK) promoter in pXP2 (22) because it showed little promoter activity on its own. The MEKpXP2 construct is not regulated by PTPase, neuregulin, or Ras. The internal deletion $\epsilon\Delta(-56/-67)$, was generated by first subcloning a *NcoI/KpnI* (blunted) fragment from ϵ 3' Δ 65, residing in BSSK (Stratagene), into the *NcoI/BspEI* (blunted) sites of ϵ -2000 BSSK (23). DNA sequencing confirmed that the sequence from positions -56 to -67 was deleted, and then we subcloned a *SmaI-XhoI* fragment into pXP2 for expression studies. ϵ subunit promoter mutants, ϵ MUT1 and ϵ MUT2, were created by PCR amplification of ϵ -2000 BSSK. Because the basal expression, as well as PTPase, neuregulin and Ets-2 responsiveness were very similar between the ϵ promoter constructs ϵ -5000 and ϵ -2000, we generated the internal deletion $\epsilon\Delta(-56/-67)$ and the mutants EMUT1 and EMUT2 in the 2-kb ϵ promoter construct. Further, because nucleotides -1 through -54 are dispensable for PTPase, neuregulin, and Ras-dependent expression (Fig. 2), we designed MUT1 and MUT2 oligonucleotide primers that were complementary to ϵ subunit DNA so that the amplified DNA's 3' end would reside at position -54. We used the M13 reverse sequencing primer that would hybridize upstream of the insert and result in an amplified product with a 5' end at position -2000. The MUT1 oligonucleotide sequence is 3'-CCTAATCCACTGTCAGG-GATTTGGATCAAATTTTCGAATAT-5' and the MUT2 sequence is 3'-CCTAATCCACTGTCAGGGATTTGGGCT-GGGCCTTCGAATAT-5'. These nucleotides span positions -54 to -87 of the ϵ subunit promoter and contain a *HindIII* site at their 3' end to facilitate subcloning. Mutants were confirmed by DNA sequencing and then subcloned into pXP2 for expression studies. The activated Ras expression construct harbors a constitutively active (G12V) T24 H-ras-1 gene in the

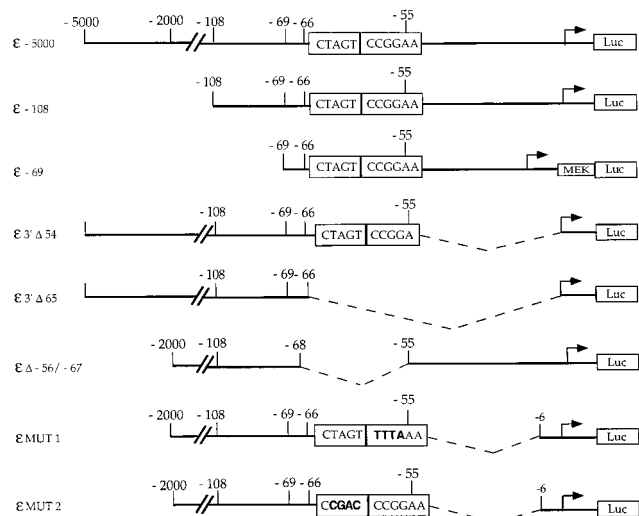


FIG. 1. Rat ϵ subunit promoter expression constructs. Schematic representation of expression constructs harboring the luciferase (Luc) structural gene driven either by a wild-type ϵ subunit promoter (ϵ -5000) or its various 5' (ϵ -108 and ϵ -69) or 3' (ϵ 3' Δ 54 and ϵ 3' Δ 65) deletion mutants. Dotted lines indicate the portion of DNA deleted to generate deletions. Mutations 1 and 2, respectively, represent the site-directed mutagenesis (shown in boldface type) of nucleotides CCGG (positions -59 and -56) to TTTA (ϵ MUT1) and nucleotides TAGT (positions -63 and -60) to CGAC (ϵ MUT2). The position of the transcriptional start site is indicated by an arrow.

pSVgpt vector (24). The dual specificity PTPase PTP CL100 (12, 25, 26) was used as a prototype PTPase for overexpression studies. Cytomegalovirus (CMV) chloramphenicol acetyltransferase (CAT) has been described (12). The γ -722 CAT construct harbors a fragment of γ subunit gene spanning nucleotides -754 to -33 (relative to the translation initiation site) driving CAT expression (27). Expression constructs used in *in vivo* studies harbor either a wild-type (ϵ -5000 or ϵ -2000) or a mutant (ϵ 3' Δ 65 or ϵ MUT1) ϵ subunit promoter driving nuclear-localized LacZ (nLacZ) expression. The pSV2CAT vector has been described (28).

Muscle Ets-2 and Dominant Negative Mutant. A rat muscle Ets-2 clone was isolated by screening a denervated rat skeletal muscle cDNA library (5×10^5 phage) with a mouse Ets-2 DNA binding domain probe. Eight positive hybridizing phages were purified and analyzed by Southern blotting and DNA sequencing. All eight clones were Ets-2. Both strands of the largest clone (3.6 kb) were sequenced. This clone contained the complete Ets-2 coding sequence along with 5' and 3' untranslated sequence. The rat Ets-2 deduced amino acid sequence is 96% identical to the mouse Ets-2 sequence (29). For expression studies we subcloned the rat Ets-2 cDNA into the pBK-CMV expression vector (Stratagene). This vector was digested with *NheI* and *SpeI* to remove the vector's bacterial ATG codon that resides between the CMV promoter and the cDNA insert. A dominant negative construct was created that contained rat Ets-2 sequences encoding amino acids 280-443 (29). This sequence spans the conserved Ets DNA binding domain and lacks the transactivation domain (30).

Cell Culture. Primary rat muscle cultures were prepared as described (31). For experiments involving cotransfection with activated Ras, myotube cultures were used on day 5 after plating. For cotransfections with PTP CL100, fully differentiated myotube cultures on day 6 after plating were used. L6 cells were grown in DMEM supplemented with 10% fetal calf serum. All cells were grown in 60-mm dishes.

Neuregulin. Recombinant neuregulin (GGF2) was kindly provided by Cambridge Neuroscience (Cambridge, MA) and used at a final concentration of 5 nM.

Transfections. Cells were transfected as described (12). Briefly, for PTPase overexpression studies, cells were transfected with 5 μ g of test plasmid, 5-10 μ g of PTP CL100, and 15-20 μ g of CMV CAT. After a 1.5-h incubation with DNA precipitate, the cells were glycerol-shocked for 90 sec and placed in primary culture medium containing tetrodotoxin (2 μ g/ml) and cytosine arabinoside (2.8 μ g/ml). CMV CAT is not regulated by PTPase overexpression. For Ras expression studies, cells were transfected with 5-10 μ g of activated Ras, 20 μ g of γ -722 CAT, and 5 μ g of test plasmid. At 12 h after transfection, cells were placed in DMEM/0.5% fetal calf serum for 48 h before harvesting for luciferase and CAT assays. To study neuregulin-dependent regulation, L6 cells were transfected with 10 μ g of test plasmid and 20 μ g of γ -722 CAT. At 12 h after transfection, the cells were washed with PBS and placed in differentiation medium (DMEM/2% horse serum). Twenty-four hours after induction of differentiation, the cells were treated with neuregulin (5 nM) for 60 h before harvesting for luciferase and CAT assays. γ -722 CAT is not regulated by Ras or neuregulin. An identical procedure was followed for Ets-2 dominant negative experiments, except that the test plasmid and γ -722 CAT were supplemented either with 50 ng of Ets-2 dominant negative mutant expression vector or BSSK and transfected into L6 cells. For Ets-2 transactivation studies, L6 cells were transfected with 5 μ g of test plasmid, 20 μ g of pSV2 CAT, and 5 μ g of Ets-2 expression vector. At 12 h after transfection, cells were placed in DMEM/2% horse serum for 48 h before harvesting for luciferase and CAT assays.

In Vivo Expression Assays. The *in vivo* expression assay involving direct injection of DNA into muscle has been described (19, 22, 32, 33). Briefly, DNAs for direct injections

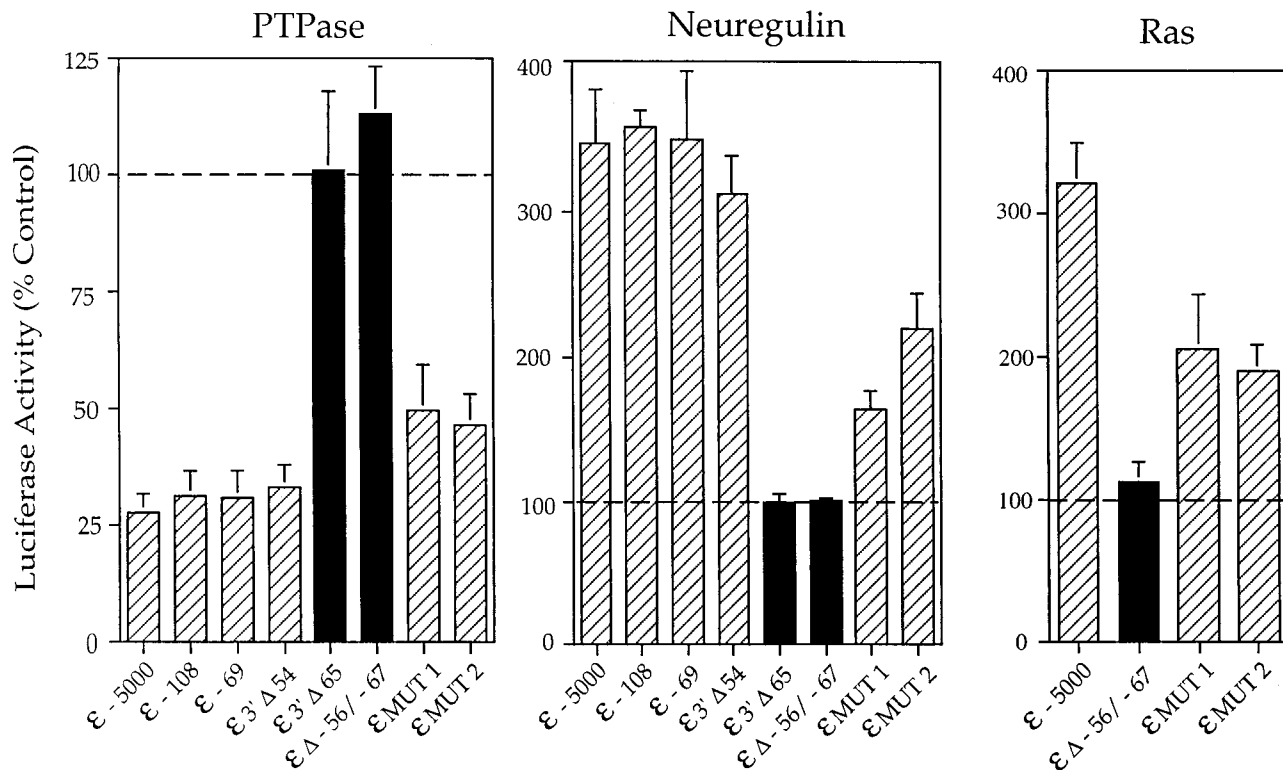


Fig. 2. Identification of a common 15-bp sequence crucial for PTPase, neuregulin, and Ras-dependent regulation of ϵ subunit promoter activity. (Left) Primary myotube cultures were cotransfected with a particular ϵ promoter/luciferase expression vector described in Fig. 1, CMV CAT (for normalization), with or without PTPase expression vector. Cells were harvested 24 h after transfection for luciferase and CAT assays. (Middle) L6 cells were cotransfected with a particular ϵ promoter/luciferase expression vector and γ -722 CAT (for normalization). Twenty-four hours after the induction of differentiation, cells were treated with buffer or recombinant neuregulin (5 nM) for 60 h before harvesting and assaying for luciferase and CAT activities. (Right) Primary myotube cultures were cotransfected with a particular ϵ promoter/luciferase expression vector, γ -722 CAT (for normalization), with or without activated-Ras expression vector. Cells were maintained in low serum medium (DMEM/0.5% fetal calf serum) for 48 h before harvesting for luciferase and CAT assays. Experiments were repeated a minimum of three times. Bar graphs represent the average of triplicate transfections normalized to CAT activity; error bars represent \pm SD. Dotted line indicates the control expression levels. Data are presented as a percent of control. Normalized luciferase activity for various constructs in the absence of cotransfection with a PTPase expression vector (control) was as follows: ϵ -5000, 207 (\pm 14.6); ϵ -108, 22 (\pm 3); ϵ -69, 477 (\pm 87); ϵ 3'Δ54, 949 (\pm 187); ϵ 3'Δ65, 1,165 (\pm 82); ϵ Δ(-56/-67), 1,090 (\pm 144); ϵ MUT1, 639 (\pm 68); ϵ MUT2, 1,042 (\pm 63). Normalized luciferase activity for various constructs in the absence of treatment with neuregulin (control) was as follows: ϵ -5000, 7,778 (\pm 1,070); ϵ -108, 481 (\pm 29); ϵ -69, 2,517 (\pm 162); ϵ 3'Δ54, 11,057 (\pm 961); ϵ 3'Δ65, 36,451 (\pm 6,110); ϵ Δ(-56/-67), 22,076 (\pm 1,557); ϵ MUT1, 16,472 (\pm 2,499); ϵ MUT2, 18,273 (\pm 3,677). Normalized luciferase activity for various promoter constructs in the absence of cotransfection with Ras expression vector (control) was as follows: ϵ -5000, 835 (\pm 76); ϵ Δ(-56/-67), 1,370 (\pm 205); ϵ 3'Δ65, 1,931 (\pm 314); ϵ MUT1, 1,368 (\pm 31); ϵ MUT2, 2,044 (\pm 115).

were twice banded in CsCl and resuspended in 0.15 M NaCl at 4 mg/ml. Male Sprague-Dawley rats, approximately 4 weeks old, were anesthetized with ether, and a small incision through the skin, covering the right and left tibialis anterior (TA) muscle, was made. Different syringes were used to inject 10 μ l of wild-type or mutant ϵ promoter expression vector into the left and right TA muscle, respectively. Seven days after injection, animals were sacrificed, and left and right TA muscles were removed, fixed, and stained for β -galactosidase activity (34). After staining individual muscle fibers were teased apart under a dissecting microscope to identify blue fibers (those containing nuclear localized β -galactosidase activity). Blue fibers were then stained for acetylcholinesterase (35) to identify endplates. The number of blue fibers was scored and those containing blue nuclei centered beneath an endplate were scored synaptic expression; all others were scored as extrasynaptic expression. Each experiment used four animals and was repeated at least four times.

RESULTS

PTPase, Neuregulin, and Ras-Dependent Regulation of ϵ Promoter Activity Map to a Common 15-bp Sequence. nAChR ϵ subunit gene expression can be suppressed by PTPase overexpression (12) or induced by neuregulin (8, 9). To

identify DNA sequences in the rat ϵ promoter that may mediate this regulation, we generated a series of 5' and 3' deletion mutants, some of which are schematically represented in Fig. 1. These deletion mutants identified a PTPase and neuregulin responsive element downstream of nucleotide -69 and upstream of nucleotide -54 (Fig. 2 Left and Middle). The internal deletion ϵ Δ(-56/-67) revealed that nucleotides -67 through -56 are essential for conferring this regulation (Fig. 2 Left and Middle). In addition, site-directed mutagenesis of nucleotides -59 through -56 (ϵ MUT1) or -63 through -60 (ϵ MUT2) caused a partial loss of PTPase and neuregulin-dependent regulation (Fig. 2). This is in stark contrast to deletion of 65 nucleotides from the 3' end of the promoter or deletion of nucleotides -67 through -56 that both resulted in complete loss of PTPase and neuregulin-dependent expression [ϵ 3'Δ65 and ϵ Δ(-56/-67) in Fig. 2]. Thus, these results point to a crucial role for the 15-bp DNA sequence (positions -69 and -55) in PTPase and neuregulin-dependent regulation of ϵ promoter activity.

Neuregulin has recently been shown to mediate its effects on ϵ gene expression via a Ras/MAP kinase pathway (13, 14). Because the deletions ϵ 3'Δ65 and ϵ Δ(-56/-67) are unresponsive to neuregulin (Fig. 2 Middle), we predicted these deletions would also be unresponsive to activation by Ras. Indeed, activated Ras had no effect on the deletions ϵ 3'Δ65

(data not shown) and $\epsilon\Delta(-56/-67)$, yet it caused a more than 300% increase in expression of ϵ -5000 (Fig. 2 *Right*). Ras-dependent regulation of deletion mutants, ϵ -69 and $\epsilon 3'\Delta 54$ were similar to ϵ -5000 (data not shown). In addition, as with neuregulin-dependent expression, the mutants ϵ MUT1 and ϵ MUT2 showed only a partial loss of Ras-responsiveness (Fig. 2).

Although 5' deletions ϵ -5000 and ϵ -2000 exhibited similar promoter activity (data not shown), ϵ -108 only retained approximately 10% of this activity, which is consistent with previous reports (23). In contrast, mutants $\epsilon\Delta(-56/-67)$, ϵ MUT1, and ϵ MUT2 and our 3' deletions exhibited an increase in promoter activity (see Fig. 2), which may reflect the removal of several negative elements.

In Vivo Expression. The above experiments identified a 15-bp sequence crucial for PTPase, neuregulin, and Ras-dependent regulation of ϵ subunit promoter activity. Interestingly, within this DNA region resides a sequence, CCGGA, that was recently shown to be necessary for synapse-specific expression of the mouse ϵ subunit gene *in vivo* (19). However, our data suggests that this 15-bp sequence may have a more robust effect on synapse-specific expression than the CCGGA sequence alone [compare $\epsilon 3'\Delta 65$, $\epsilon\Delta(-56/-67)$, ϵ MUT1, and ϵ MUT2 in Fig. 2]. To test this hypothesis, we compared the *in vivo* expression of mutants $\epsilon 3'\Delta 65$ nLacZ and ϵ MUT1nLacZ with their corresponding wild-type constructs ϵ -5000nLacZ and ϵ -2000nLacZ, respectively. Although, the number of fibers showing synapse-specific expression were too low to obtain statistically significant results, we did observe a relatively robust extrasynaptic expression. Compared with their controls, mutants $\epsilon 3'\Delta 65$ nLacZ and ϵ MUT1nLacZ resulted in a 380% and 200% induction in extrasynaptic expression, respectively (Fig. 3). These results suggest that the 15-bp sequence essential for mediating PTPase, neuregulin, and Ras-dependent expression also contributes to synapse-specific expression of the ϵ -subunit gene *in vivo*, at least in part, by decreasing extrasynaptic expression.

Ets-2 Transactivates nAChR ϵ Subunit Promoter Activity. The 15-bp neuregulin response element identified above harbors at positions -60 to -55, the sequence TCCGGA that constitutes a putative Ets binding site. Interestingly, neuregu-

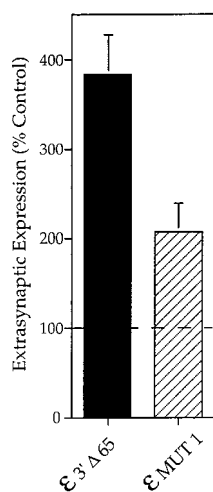


FIG. 3. *In vivo* expression analysis shows increased extrasynaptic expression for the 3' deletion mutant $\epsilon 3'\Delta 65$. Extrasynaptic expression of the mutant ϵ promoter constructs $\epsilon 3'\Delta 65$ and ϵ MUT1, relative to their wild-type constructs, ϵ -5000nLacZ and ϵ -2000nLacZ, respectively, was examined by using an *in vivo* expression assay. Data are presented as the mean values (percent of corresponding control) \pm SEM ($n = 4$). Dotted line indicates the control expression levels. Basal extrasynaptic expression for wild-type promoter expression vectors were 5.66 (± 0.86).

lin-dependent induction of the ϵ subunit gene requires activation of a Ras/MAP kinase signaling pathway that has the potential to activate Ets transcription factors (15–18). Therefore, we hypothesized that Ets proteins may be involved in mediating this neuregulin signaling. To test this hypothesis, we overexpressed Ets-2 with either ϵ -2000 or the deletion mutant $\epsilon\Delta(-56/-67)$ in L6 cells. We observed about a 4-fold induction of ϵ promoter activity by Ets-2 in a sequence-specific manner as indicated in Fig. 4.

An Ets-2 Dominant Negative Mutant Blocks Neuregulin-Dependent Induction of nAChR ϵ Subunit Promoter Activity.

To directly investigate whether an Ets transcription factor is involved in mediating neuregulin-dependent induction of ϵ promoter activity, we overexpressed an Ets-2 dominant negative mutant along with ϵ -5000 in L6 cells followed by treatment with either buffer or neuregulin. As shown in Fig. 5, overexpression of the Ets dominant negative mutant (50 ng per plate) completely abrogated neuregulin-dependent induction of ϵ promoter activity without affecting the basal expression of ϵ -5000.

DISCUSSION

We have identified a 15-bp DNA sequence (positions -69 and -55) in the rat nAChR ϵ subunit gene that contains sequences crucial for PTPase, neuregulin, and Ras-dependent regulation. Further, *in vivo* expression assays suggest this same sequence also participates in extrajunctional suppression of the ϵ subunit gene. Thus, our results point to a crucial role for the 15-bp element in determining synapse-specific and neuregulin-mediated motor neuron control of ϵ subunit gene expression. Finally, our data suggest that a functional Ets binding site resides within the ϵ promoter's 15-bp element and that an Ets transcription factor participates in mediating neuregulin-dependent control of ϵ promoter activity.

The finding that a common ϵ promoter DNA sequence mediates PTPase and neuregulin-dependent expression is not completely unexpected. Neuregulin has been shown to cause protein-tyrosine phosphorylation of its receptors (4, 6, 9), which may be targets of PTPase overexpression. In addition,

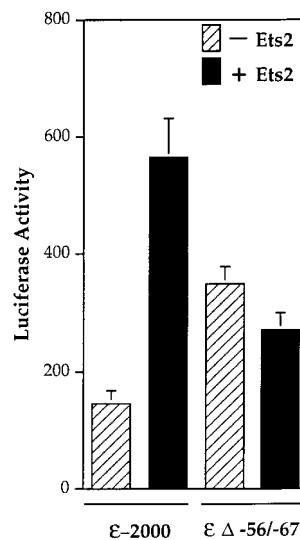


FIG. 4. Rat muscle Ets-2 induces nAChR ϵ subunit promoter activity. L6 cells were cotransfected with ϵ promoter/luciferase vector, ϵ -2000, or deletion mutant $\epsilon\Delta(-56/-67)$ (described in Fig. 1), SV2 CAT (for normalization), with or without the Ets-2 expression vector. Cells were maintained in differentiation medium (DMEM/2% horse serum) for 48 h before harvesting for luciferase and CAT assays. Experiments were repeated a minimum of three times. Bar graphs represent the average of triplicate transfections normalized to CAT activity; error bars represent \pm SD.

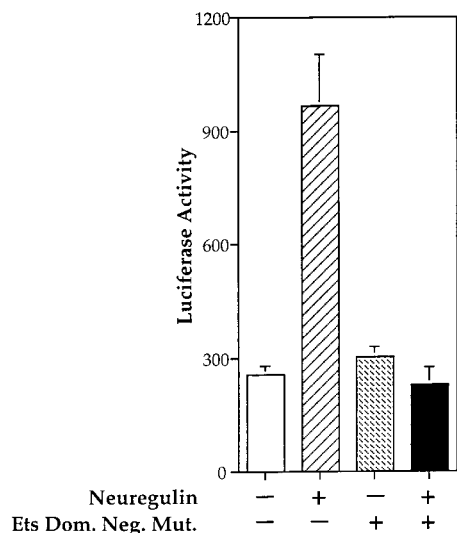


FIG. 5. Dominant negative mutant of rat muscle Ets-2 blocks neuregulin-dependent induction of nAChR ϵ subunit promoter activity. L6 cells were cotransfected with a wild-type ϵ promoter/luciferase vector, ϵ -5000 (described in Fig. 1), or γ -722 CAT (for normalization), with or without the dominant negative mutant of Ets-2. Twenty-four hours after the induction of differentiation, cells were treated with buffer or recombinant neuregulin (5 nM) for 60 h before harvesting and assaying for luciferase and CAT activities. Bar graphs represent the average of triplicate transfections normalized to CAT activity; error bars represent \pm SD.

neuregulin activates a Ras/MAP kinase signaling cascade (13, 14), and several MAP kinases have recently been shown to be specific targets for inactivation by members of the dual-specificity phosphatases of the CL100 family (36). If PTPase mediated its effect by decreasing the activity of neuregulin signaling components, one would predict a basal level of signaling in these cells in the absence of exogenous neuregulin. Indeed, differentiated muscle cell cultures express neuregulin (37), which may activate a neuregulin-dependent signaling cascade. Therefore, it is possible that PTPase overexpression mediates its effects on ϵ promoter activity by blocking this signaling cascade. Alternatively, PTPase overexpression may act on a neuregulin-independent signaling cascade that converges on the same DNA element that mediates neuregulin responsiveness. This latter scenario is in accord with the observation that the same DNA element mediates neuregulin-dependent induction (Fig. 2) and extrajunctional suppression of the ϵ gene (Fig. 3).

The fact that PTPase and neuregulin responsiveness are mediated by a common 15-bp DNA sequence in the ϵ subunit gene does not necessarily exclude the possibility that other PTPase-responsive elements will be unresponsive to neuregulin. In fact, our preliminary experiments suggest that a 104-bp fragment of the chicken α subunit promoter may harbor such an element (M.K.S. and D.G., unpublished observation).

Recently, DNA sequences participating in synapse-specific expression of the mouse δ and ϵ subunit genes have been identified (19, 32, 33). These *in vivo* expression studies suggest that an N-box (CCGGAA) is crucial for synapse-specific expression. Interestingly, this same sequence resides within the rat ϵ promoter's 15-bp regulatory sequence that we have identified as a PTPase and neuregulin response element (Fig. 2). Similar to our observation with the rat ϵ promoter, the N-box sequence in the mouse δ -subunit gene participated in both synaptic induction and extrasynaptic repression (32). However, the N-box identified in the mouse ϵ promoter was shown to only mediate synaptic induction, with little effect on extrasynaptic expression (19). This result is very different from

ours with the rat promoter. Although we do not know the reason for this difference, it may be attributable to the fact that we used a rat promoter containing 2-kb of 5' flanking DNA, whereas the mouse ϵ promoter constructs used for *in vivo* expression assays contained only 83-bp of 5' flanking DNA (19). We found that an N-box mutant created within a 154-bp rat ϵ promoter reduced expression below the limits of detection in our *in vivo* injection assays (data not shown), suggesting that 5' sequences upstream of nucleotide -154 participate in extrajunctional expression. Consistent with this possibility is the observation that the mouse 2.2-kb ϵ promoter exhibits more pronounced synapse-specific expression than a promoter construct containing only 83-bp of 5' flanking DNA (33). Similarly, DNA located immediately 5' of the N-box contributes to the synaptic expression of the rat ϵ promoter. This conclusion is supported by our *in vivo* expression data showing that ϵ MUT1 results in a 2-fold increase, whereas ϵ 3' Δ 65 results in a 4-fold increase in extrajunctional expression compared with their controls (Fig. 3). Inspection of the rat sequence from positions -69 to -55, which harbor sequences crucial for synapse-specific expression, identified two nucleotides that differ between rat and mouse (TAAACCTAGTCCGGA in rat and CAAACCTAGCCCGGA in mouse, where differences are underlined). Whether these subtle changes in sequence contribute to the observed regulatory differences between rat and mouse promoters is not known.

Because the *in vivo* synaptic expression of ϵ promoter constructs could not be measured reliably, we could not determine whether the 15-bp neuregulin responsive sequence suppresses ϵ subunit promoter expression throughout the muscle fiber or only in the extrajunctional compartment. Nevertheless because we have shown that this same sequence mediates neuregulin-dependent induction of the ϵ promoter and because neuregulin signaling is localized to the synapse, we propose that this element serves to suppress ϵ subunit expression extrajunctionally and induce it locally at the neuromuscular junction.

Although our results and that of others (19, 32, 33) suggest an important role for the N-box in mediating neuregulin-dependent and synapse-specific gene expression, there is one recent report that does not come to this conclusion (20). This latter study instead identified a promoter sequence, CCA-CAGCAGG, as an acetylcholine-receptor-inducing activity response element. This element corresponds to nucleotides -103 to -94 in the rat ϵ promoter. Surprisingly, our experiments using ϵ promoter constructs lacking these residues (ϵ -69 in Fig. 2) still exhibited neuregulin, PTPase, and Ras-dependent expression patterns. The reason for this difference is not clear but may reflect differences in the cell type used for expression assays. It is interesting to note that the one study reporting no role for the N-box in mediating neuregulin responsiveness used the C2C12 cell line, whereas our studies and others reporting an important role for this sequence used *in vivo* expression assays (19, 32, 33), primary rat muscle cultures and the L6 muscle cell line.

It is interesting that neuregulin responsiveness maps to a 15-bp sequence of the ϵ promoter that contains, at positions -60 to -55, the sequence TCCGGA. This sequence contains the core Ets binding site sequence, GGA (38, 39), as an inverted repeat. On the basis of the fact that neuregulin activates MAP kinase activity (13, 14) and that Ets proteins represent a family of MAP kinase responsive transcription factors (15-18), we hypothesized that the Ets transcription factors may participate in neuregulin-dependent induction of the ϵ subunit gene. Indeed Ets genes are expressed in skeletal muscle (as demonstrated by cloning Ets-2 from a rat skeletal muscle cDNA library) and Ets-2 overexpression transactivates ϵ promoter activity in a sequence-specific manner (Fig. 4). These results suggest a role for Ets transcription factors in regulation of nAChR ϵ subunit gene expression. A role for Ets

proteins in mediating neuregulin-dependent induction of ε promoter activity is further substantiated by the observation that an Ets dominant negative mutant blocks this induction (Fig. 5).

Our mutagenesis data suggest that Ets proteins may mediate their effect through the Ets binding site in the ε promoter's 15-bp neuregulin response element. However, we also demonstrated that sequences located just 5' of the Ets binding site are required for maximal neuregulin responsiveness (compare ε MUT1 and ε MUT2 in Fig. 2). This suggests that other proteins, in addition to Ets, participate in this regulation, which is consistent with the mechanism of action of Ets proteins (38). However, the observation that an Ets dominant negative mutant can completely abrogate neuregulin-dependent induction may suggest that Ets not only directly activates ε promoter activity through its Ets binding site but may indirectly activate it by inducing the expression of a protein that binds to sequences just upstream of the Ets binding site. Alternatively, it is possible that the Ets dominant negative mutant protein completely abrogates neuregulin signaling by assuming a conformation that sterically hinders interaction of transcription factors with these 5' flanking sequences in addition to its interference at the Ets binding site.

Finally, it is likely that differences in response to neuregulin, among the various nAChR subunit encoding genes, will be reflected in their neuregulin responsive elements. Except for the putative Ets binding site, there is little conservation of sequence between the ε subunit gene positions -69 to -55 and other nAChR subunit genes expressed at the synapse. This may reflect relative differences in activation of these genes by neuregulin, of which the ε gene appears to be most responsive (9). It is also interesting to note that simply containing a putative Ets-binding site sequence does not necessarily confer neuregulin or PTPase-dependent expression because ε 3' Δ 65 retains an upstream putative Ets binding site sequence 5'-CCGGAA-3' in reverse (centered around position -1368) yet is not regulated by neuregulin or PTPase overexpression (Fig. 2). These results support the idea that other sequences, in addition to the putative Ets binding site, participate in determining synapse-specific and neuregulin-mediated motor control of ε subunit gene expression.

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