Ets-2 Repressor Factor Silences Extrasynaptic Utrophin by N-Box-mediated Repression in Skeletal Muscle

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Utrophin is the autosomal homologue of dystrophin, the protein product of the Duchenne's muscular dystrophy (DMD) locus. Utrophin expression is temporally and spatially regulated being developmentally down-regulated perinatally and enriched at neuromuscular junctions (NMJs) in adult muscle. Synaptic localization of utrophin occurs in part by heregulin-mediated extracellular signal-regulated kinase (ERK)-phosphorylation, leading to binding of GABP α/β to the N-box/EBS and activation of the major utrophin promoter-A expressed in myofibers. However, molecular mechanisms contributing to concurrent extrasynaptic silencing that must occur to achieve NMJ localization are unknown. We demonstrate that the Ets-2 repressor factor (ERF) represses extrasynaptic utrophin-A in muscle. Gel shift and chromatin immunoprecipitation studies demonstrated physical association of ERF with the utrophin-A promoter N-box/EBS site. ERF overexpression repressed utrophin-A promoter activity; conversely, small interfering RNA-mediated ERF knock-down enhanced promoter activity as well as endogenous utrophin mRNA levels in cultured muscle cells *in vitro*. Laser-capture microscopy of tibialis anterior NMJ and extrasynaptic transcriptomes and gene transfer studies provide spatial and direct evidence, respectively, for ERF-mediated utrophin repression *in vivo*. Together, these studies suggest "repressing repressors" as a potential strategy for achieving utrophin up-regulation in DMD, and they provide a model for utrophin-A regulation in muscle.

INTRODUCTION

Duchenne's muscular dystrophy (DMD) is a fatal neuromuscular disease caused by gene mutations leading to qualitative or quantitative disturbances in dystrophin expression (Hoffman *et al.*, 1987). Dystrophin-related protein or utrophin is considered the autosomal homologue of dystrophin, because it shares extensive sequence homology as well as its size and abundance in muscle (Love *et al.*, 1989; Khurana *et al.*, 1990; Tinsley *et al.*, 1992). Utrophin and dystrophin also share functional properties such as the ability to associate with the dystroglycan complex and bind F-actin (Matsumura *et al.*, 1992; Winder and Kendrick, 1995; Rybakova *et al.*, 2002). Direct evidence for the ability of utrophin to functionally substitute comes from experiments demonstrating that utrophin overexpression driven either by transgenic means (Tinsley *et al.*, 1996; Rafael *et al.*, 1998; Tinsley *et al.*, 1998; Fisher *et al.*, 2001) or viral vectors (Gilbert *et al.*, 1999; Wakefield *et al.*, 2000; Cerletti *et al.*, 2003) can rescue dystrophin-deficient muscle. Despite these functional similarities, dystrophin and utrophin exhibit distinct localization in normal adult tissues.

Perinatally, utrophin is expressed throughout the sarcolemma, however, its expression declines as that of dystrophin increases (Khurana *et al.*, 1991; Clerk *et al.*, 1993), leading to its spatially restricted expression at neuromuscular and myotendinous junctions (NMJs and MTJs, respectively) of adult myofibers. These features and relevance toward a therapy for DMD provide a powerful impetus for better understanding transcriptional control of utrophin expression in myofibers (Jasmin *et al.*, 2002; Khurana and Davies, 2003; Nowak and Davies, 2004).

Although full-length utrophin protein can be generated using independently regulated A (Dennis *et al.*, 1996) and B (Burton *et al.*, 1999) promoters, myofiber utrophin expression is chiefly controlled via the utrophin-A promoter (Weir *et al.*, 2002; Chakkalakal *et al.*, 2003), and it is thus the subject of this investigation. Spatial and temporal expression of utrophin-A at the NMJ parallels that of nicotinic acetylcholine receptor (nAChR) subunits. Indeed, these genes share some features of transcriptional control. For example, the nAChR ϵ subunit and utrophin-A promoter N-box play a critical role in regulating expression levels and more importantly in restricting expression to the NMJ (Schaeffer *et al.*, 2001). We and others (Gramolini *et al.*, 1999a; Khurana *et al.*, 1999) have shown that the nerve-derived growth factor heregulin (HRG) causes a N-box-mediated increase in utrophin-A promoter activity via extracellular signal-regulated kinase-dependent activation of the Ets-related transcription factor complex GABP α/β , in a manner similar to that noted in the nAChR δ and nAChR ϵ subunit promoters (Falls *et al.*, 1993; Koike *et al.*, 1995; Sapru *et al.*, 1998; Schaeffer *et al.*,

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1998). Additionally, SP1 (Galvagni *et al.*, 2001; Gyrd-Hansen *et al.*, 2002), NFATc1/calcineurin (Chakkalakal *et al.*, 2003; Chakkalakal *et al.*, 2004), and myogenic factors (Gramolini and Jasmin, 1999; Perkins *et al.*, 2001) can also activate the utrophin-A promoter. Recently, the 5'-untranslated region of utrophin-A promoter has been shown to be important for regulation of utrophin protein levels during regeneration as well (Miura *et al.*, 2005). Indeed, several of these molecules or mechanisms are currently being investigated for development of utrophin up-regulation-based therapeutics for DMD (Chaubourt *et al.*, 1999; Krag *et al.*, 2004; St-Pierre *et al.*, 2004; Segalat *et al.*, 2005).

However, equally important mechanisms controlling concurrent extrasynaptic down-regulation or repression of utrophin-A in myofibers that must occur to achieve expression at the NMJ rather than generalized expression throughout the sarcolemma remain unaddressed. Mechanistic clues arise from studies illustrating that restriction of neuronal sodium channel expression to the nervous system is partly due to RE-1 silencing transcription factor/neuronal-restricted silencing factor binding to a repressor element (Andres *et al.*, 1999; Ballas *et al.*, 2001; Lunyak *et al.*, 2002). Additionally an N-box-mediated silencing mechanism has been suggested for nAChR subunit expression in extrasynaptic regions of muscle (Koike *et al.*, 1995). In silico analysis of the human and murine utrophin-A promoter sequences suggest Ets-2 repressor factor (ERF) as a candidate molecule for N-box-mediated extrasynaptic repression of the utrophin-A promoter (Sgouras *et al.*, 1995). ERF is a novel member of the *ets* family and encodes a ubiquitously expressed 548-amino acid phospho-protein identified through its ability to repress the Ets-2 promoter via EBS binding (Sgouras *et al.*, 1995; Le Gallic *et al.*, 1999). ERF activity is regulated in part, by ERK-dependent changes in its phosphorylation status and subcellular localization due to nuclear shuttling, with CRM1 suggested to have a role in ERF export (Le Gallic *et al.*, 2004). Here, we use an array of in vitro and in vivo methodologies to delineate the role of ERF in the regulation of the utrophin-A promoter.

MATERIALS AND METHODS

Constructs

The pPUBF utrophin promoter luciferase reporter construct contains 1.3-kb of human utrophin promoter-A sequence (Dennis *et al.*, 1996), and it was a kind gift from Prof. Kay Davies (Oxford University). The pPUBFΔN-box is a pPUBF derivative with a deleted N-box/EBS sequence, and it has been previously described by our laboratory (Khurana *et al.*, 1999). Expression vectors containing human GABPα and -β1 subunits used were GABPα and GABPβ (Rosmarin *et al.*, 1995), and they were kindly gifted by Prof. Alan Rosmarin (Brown University). Expression vectors containing human ERF cDNA used were pSG5-ERF and GFP-ERF (Sgouras *et al.*, 1995; Le Gallic *et al.*, 1999), and they were kindly gifted by Prof. George Mavrothalassitis (IMBB, Greece).

Gel Shift Assay

Double-stranded probes encompassing the EBS and N-box of the human utrophin-A promoter (5'-GCTGATCTTCCGGAACAAAGTTGCT-3'; N-box underlined; EBS bold) or probes containing a mutated N-box/EBS site (5'-GCTGATCTTCCATCTACAAGTTGCT-3') were end labeled with [γ -³²P]ATP by using polynucleotide kinase. Unlabeled probes (10- and 100-fold excess) were used in cold competitor assays as outlined in Figure 1B. Recombinant human ERF (rhERF) was produced using a coupled in vitro transcription/translation system (Promega, Madison, WI), by using 0.5 μg (GFP)-ERF construct and unlabeled methionine. Additionally, we used nuclear extracts from C2C12 cell line (Active Motif, Carlsbad, CA). Gel shift analysis was performed using the Gel Shift Assay system (Promega). For supershifts, 5 μl of ERF-specific rabbit polyclonal antibody (S17C), and as controls, a nonspecific polyclonal antibody against the transcription factor Twist (Santa Cruz Biotechnology, Santa Cruz, CA), were added to the reaction mix for 1 h at room temperature before probe addition. Protein-DNA complexes were resolved on 4% polyacrylamide gels by using 0.25× Tris-glycine buffer at 200 V

for 2 h. Gels were dried and autoradiographed on a Typhoon 8600 Imager (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed on RNA obtained from ~100 mg of freshly dissected dystrophin-deficient (*mdx*) hindlimb muscle from different developmental stages, as described previously (Khurana *et al.*, 1999). Murine ERF and GABPα primers were designed to amplify a 490-base pair fragment of ERF spanning exons 1–4 (ERF1F, 5'-GATTGGCTACAAACCGGAGTCATCC-3') and ERF2R, 5'-GTCGGCAACCACAGGAGAGAAGAG-3') and a 409-bp fragment of GABPα spanning exons 8–10 (GABPα2F, 5'-CAGCTAAAGTGCAACGGTCCCCAAG-3' and GABPα1R, 5'-CCGTGCCAGTTCTTCTGTTCACACTC-3'). Primers used for MyoD were M_MyoD_1483F (5'-CTCCACATCCTTTGTTGT-3') and M_MyoD_1825R (5'-AGCGTCTTTATTTCCAACAC-3') and for Myogenin were M_Myogenin_924F (5'-CAATACACAAAGCACTGGAA-3') and M_Myogenin_1222R (5'-TCTGAGGAGAGAAAGATGGA-3'). Utrophin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were as described previously (Khurana *et al.*, 1999). PCR products were resolved on 4% acrylamide gels, dried, and radioactively incorporated products quantified using a Typhoon 8600 Imager and ImageQuant IQ Tools version 2.2 (GE Healthcare).

Tissue Culture and Transfection

Murine C2C12 muscle cells and *Drosophila* S2 embryonic cells were maintained and transfected as described previously (Gyrd-Hansen *et al.*, 2002). For cotransfection of human GABPα, GABPβ, and pSG5-ERF expression vectors, 1 μg of vector(s) and 1 ng of *Renilla* control plasmid (pRL-TK; Promega) were used. The mitogen-activated protein kinase kinase (MEK) inhibitor UO126 (10 μM; Promega) was added concurrent with transfection to block *Drosophila* (Gobert Gosse *et al.*, 2005) and mammalian MEK activity. Cells were allowed to express fusion genes for 12–24 h before analysis of cell extracts for luciferase activity by using the Dual Luciferase Assay kit (Promega) with a 20/20 luminometer (Turner Designs, Sunnyvale, CA). All assays were performed in triplicate with the transfection/assay process repeated using separate cultures for particular experiments as outlined in the figure legends.

MEK-dependent translocation of ERF from nucleus to cytoplasm was studied using C2C12 muscle cells that were incubated with MEK inhibitor UO126 for different times and analyzed using immunofluorescence of cells and immunoblotting of subcellular fractions as described previously for Ref1 cells (Le Gallic *et al.*, 1999, 2004). For immunofluorescent analysis, cells were fixed with ice-cold methanol for 10 min, incubated with anti-ERF antibodies (Santa Cruz Biotechnology), and detected using Alexa Fluor 546 donkey anti-goat secondary antibodies (Invitrogen, Carlsbad, CA). Images were acquired using a Radiance 2000 confocal laser imaging system (Bio-Rad, Hercules, CA). Nuclear and cytoplasmic fractions for immunoblotting were obtained by lysing the cells with 35 strokes in a glass Dounce homogenizer (pestle B) in buffer A (10 mM Tris-HCl, pH 7.5, 300 mM sucrose, and 1 mM EDTA). Nuclei were pelleted at 2000 × g for 5 min and washed with buffer B (10 mM HEPES, 10 mM NaCl, 1 mM KH₂PO₄, 5 mM NaHCO₃, 1 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% Nonidet P-40). All buffers were supplemented with 1 mM orthovanadate and protease inhibitor complete (Roche Diagnostics, Basel, Switzerland). To control for fractionation of nuclear and cytoplasmic compartments, aliquots of each of the fractions were monitored using fluorescent microscopy and DNA binding dyes. To control for loading equivalent amounts of proteins, the concentration of proteins was measured using a Bradford assay (Bio-Rad), and equal concentration (50 μg) of total protein from cytoplasmic and nuclear fractions were loaded and resolved using 3–8% Tris-acetate gradient SDS-polyacrylamide gel electrophoresis, electrophoresed onto polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA), and membranes were probed using anti-ERF antibodies (Santa Cruz Biotechnology). Blots were washed thoroughly, incubated with horseradish peroxidase-conjugated donkey anti-goat secondary antibodies (Promega), and enhanced chemiluminescence was performed as described by manufacturer (Pierce Chemical, Rockford, IL), by using X-Omat Blue XB-1 films (Eastman Kodak, Rochester, NY).

RNA Interference (Small Interfering RNA) Studies

Duplexed stealth RNA oligomers to the murine ERF sequence were designed using the BLOCK-iT RNA interference system (Invitrogen). Proliferating C2C12 cells (50% confluent) were transfected using LipofectAMINE 2000 (Invitrogen) with 25 pmol each of ERF-292 (5'-GGUUCACCUACAAG UUCAACUCAA-3'), ERF-326 (5'-GCUUGUCAAUACCCUUUCAUGCAU-3'), ERF-937 (5'-CCCACACCCAAAGCGUCUACAACUA-3'), and ERF-1268 (5'-GAUUAAGGUGAGGCCCA UCUCAGAA-3') or 100 pmol of an unrelated, scrambled control "egg" oligomer (5'-GCUUACUC AUCCAUGCAUCGGUAUG-3'). Transcript levels of utrophin, GAPDH, and ERF postoligomer addition were determined using semiquantitative RT-PCR. Analysis of ERF knockdown effects on utrophin promoter activity used 1 μg of pPUBF construct and transfection of oligomers after 24 h. Cells were incubated an additional 24 h before assaying for luciferase activity.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed with goat polyclonal anti-ERF antibodies (Santa Cruz Biotechnology) according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY). Briefly, cells from one 100-mm plate were treated with or without 2 nM heregulin for 15 min after overnight serum starvation, and they were cross-linked with 0.37% final concentration of formaldehyde. For U0126 treatment, cells were grown in presence of serum and treated with 10 μ M U0126 for 15 min. Cells were washed twice with ice-cold phosphate-buffered saline (PBS). Cell pellets were lysed in 200 μ l of lysis buffer and sonicated. Cell lysate was diluted 10-fold in ChIP dilution buffer and precleared with 120 μ l of protein A-agarose and 120 μ l of protein G-agarose. The precleared set was incubated with or without antibody at 4°C overnight with constant rotation. The antibody-chromatin complex was then collected with 60 μ l of protein A-agarose and 60 μ l of protein G-agarose, incubating 1 h at 4°C with constant rotation. The agarose beads were washed with wash buffers, and finally, chromatin was eluted with 500 μ l of elution buffer (0.1 M NaHCO₃ and 1% SDS) at room temperature. Beads were reverse cross-linked at 65°C overnight with 20 μ l of 5 M NaCl. One percent of input reserved before immunoprecipitation was reverse cross-linked in parallel. All solutions were supplemented with protease inhibitor complete (Roche Diagnostics), 1 mM Na₃VO₄ and 1 mM NaF. DNA was extracted with PCR purification kit (QIAGEN, Valencia, CA). Presence of utrophin-A promoter was detected in different sets by PCR in the presence of [α -³²P]dCTP with primers 5'-CCCAAACCTCAA-CAACCTCAGTAAAC-3' and 5'-CAAATTGTCCGAAAATGTGTGTC-3' designed to amplify 151 bp of utrophin-A promoter (NCBI accession no. X95524). Primers did not amplify products without appropriate templates. The products were separated using 12% acrylamide gel, and they were imaged using a Typhoon 8600 (GE Healthcare).

Laser Capture Microdissection (LCM) and Quantitative (q)PCR

Microscopy was performed using a PALM laser capture microscope with sections of adult rat tibialis anterior (TA) muscle (200–300 μ m² and 10 μ m in thickness) by using methods similar to those used for regional expression profiling of muscle (Budak *et al.*, 2004; Nazarian *et al.*, 2005). Extracted total RNA (yield typically between 50 and 150 ng) was used for one cycle linear amplification by using the Affymetrix two-cycle labeling kit (Affymetrix, Santa Clara, CA). Subsequently, amplified RNA (aRNA; yield typically 1000–5000 ng) was converted to cDNA by using an Invitrogen SuperScript RT kit and used as a template for qPCR. Taqman qPCR cholinergic receptor, nicotinic, α polypeptide 1 (Chrna1) (assay ID Rn00577938_m1), Utn (utrophin) (assay ID Rn00565137_m1, murine Ets-2 repressor factor (Erf) (mouse, assay ID Mm00468761_m1) and β actin primers (part 4352931E; Applied Biosystems, Foster City, CA). Rat v-ets erythroblastosis virus E26 oncogene homologue 1 (Ets1) (forward, 5'-TTGTGTGTGACCCGGAAGC-3' and reverse, 5'-GCTGATTATCCGGAAGGCC-3') primers were designed by primer expressed software version 2 for SYBR Green chemistry qPCR. An ABI 7500 qPCR machine (Applied Biosystems) was used for Taqman qPCR and ABI 7900HT qPCR machine (Applied Biosystems) was used for SYBR Green chemistry. cDNA (RNA) template (an aRNA equivalent of 3–5 ng/well cDNA used) of same animal was run in duplicate on the plate, and results were analyzed using the delta count method for relative gene expression analysis by using ABI SDS software version 1.3.1 (Applied Biosystems).

Direct Gene Transfer

All animal care and experimental procedures were performed in accordance with the guidelines established by the Canadian Council of Animal Care. These procedures were approved by the University of Ottawa Animal Care and Use Committee. Three- to 4-wk-old C57BL/6 mice were anesthetized, and anterior portions of both lower hindlimbs were shaved and disinfected. The underlying TA muscles were then directly injected with 25 μ l of DNA solution as described previously (Gramolini *et al.*, 1997; Chakkalakal *et al.*, 2003). The DNA solution contained equal amounts of the 1.3-kb human utrophin-A promoter-reporter (LacZ) construct (see Gramolini *et al.*, 1997; Chakkalakal *et al.*, 2003), the pPUBFAN-box construct (luciferase), a constitutively expressed chloramphenicol acetyl transferase (CAT) plasmid (to control for transduction efficiency), and either the ERF expression plasmid or its control (pSG5). Plasmid DNA was prepared using the Mega-Prep kit (QIAGEN, Mississauga, Ontario, Canada). DNA pellets were resuspended in sterile PBS at a final concentration of 1–2 μ g/ μ l. Five days postinjection, TA muscles were quickly excised, immediately frozen in liquid nitrogen, and stored at –80°C until processed for RT-PCR analysis. Total RNA was extracted by using TriPure (Roche Diagnostics, Indianapolis, IN) as recommended by the manufacturer. Quantitative RT-PCR was performed to determine the relative abundance of LacZ, luciferase, and CAT transcripts. Before RT-PCR, samples were digested using DNaseI to eliminate possible plasmid contamination (Gramolini *et al.*, 2001). RT-PCR assays were performed using previously described protocols and primers (Gramolini *et al.*, 1999b, 2001; Chakkalakal *et al.*, 2003). Cycle numbers varied depending on the primers used and they were all within the linear range of amplification. In all assays, negative controls consisted of RT mixtures in which total RNA was replaced with RNase-free water. PCR products were first visualized on 1.5% agarose gels containing ethidium bromide. Labeling intensity of the PCR products

that is linearly related to the abundance of cDNAs was then quantified using Digital Science 1D Image Analysis software (Eastman Kodak). Values were standardized relative to the amount of CAT present in the same sample. The Student's *t* tests were used to analyze the data.

RESULTS

ERF Can Physically Associate with the Utrophin-A N-Box/EBS Site

To determine whether the utrophin-A promoter N-box/EBS sequence is recognized by ERF, a double-stranded probe spanning the region (Figure 1A) was incubated with recom-

Figure 1. ERF binds to the utrophin-A promoter N-box/EBS element. (A) Sequence of the human utrophin-A promoter probes containing the N-box/EBS motif used in gel shifts. The N-box is in bold italics; the EBS is in a white box. Mutant refers to the gel shift probe used that contains a mutant N-box/EBS site. (B) The N-box/EBS site in the human utrophin A promoter binds ERF. rhERF was in vitro synthesized and 0.5–10 μ l (0.5–10; lanes 2–5) was incubated with the labeled oligonucleotide probe. Five microliters each of C2C12 extract was used for analysis of endogenous ERF in lanes 17–22. Formation of a single specific DNA-protein complex (gray arrow) was observed with rhERF (lanes 2–5), which could be specifically competed away with 10- and 100-fold excess of wild-type cold competitor oligonucleotide (lanes 6 and 7) but not the mutant probe (lanes 8 and 9). rhERF did not bind a double-stranded probe of the same region with a mutated N-box/EBS site (lanes 10–12). The black arrow indicates ERF-DNA complex supershifted in the presence of 5 μ l of ERF-specific antibodies (lane 14) but not in the presence of a nonspecific antibody against the transcription factor Twist (lane 13). White arrows represent free radiolabeled probe. Wt, wild-type probe; mt, N-box/EBS mutant probe; [ERF], amount of rhERF protein in microliters; c, comp, unlabeled N-box/EBS oligonucleotide; α ERF, ERF S17C rabbit polyclonal antibody.

Figure 2. The human utrophin-A promoter is *trans*-repressed by ERF overexpression and MEK inhibition, which may be alleviated by GABP α / β . (A) Changes in subcellular fractions and subcellular localization of ERF protein in C2C12 cells caused by inhibition of MEK by using U0126. Nuclear shuttling of ERF noted over a 30-min treatment period as demonstrated by i) immunoblotting or ii) immunofluorescence by using anti-ERF antibodies, as described in *Materials and Methods*. Bar, 25 μ m. (B) The human utrophin-A promoter is *trans*-repressed by ERF overexpression and MEK inhibition, which may be alleviated by GABP α / β . (i) The 1.3-kb human utrophin-A promoter-luciferase construct pPUBF showing location of the N-box/EBS according to X95523. The pPUBFΔN-box construct is a deletion that removes the N-box/EBS. (ii) pPUBF or pPUBFΔN-box derived firefly luciferase activities were normalized to pRL-TK-derived *Renilla* luciferase activity and expressed as a percentage of normalized luciferase activity (black column). Utrophin promoter-reporter constructs and a transfection control pRL-TK

binant ERF (Sgouras *et al.*, 1995) for gel shift assays. The protein-DNA complex could be competed with excess unlabeled N-box/EBS probe and supershifted using ERF-specific antibodies (Figure 1B). The protein-DNA complex could not be competed using a probe containing mutations in the N-box/EBS. The complex was not supershifted using an irrelevant antibody. Additionally, a mutant probe was not retarded when incubated with ERF (Figure 1B). To demonstrate the existence of ERF and show its ability to form the complex in the context of muscle, we performed the gel shift and supershift by using nuclear extracts obtained from C2C12 muscle cell lines. As shown in Figure 1B, a protein-DNA complex was formed that was able to retard probe migration and able to be competed with excess unlabeled probe as well as supershifted using ERF-specific antibodies in muscle cell lines, suggesting the relevance of this mechanism for muscle *in vivo*.

Having demonstrated the ability of ERF to associate with the N-box/EBS site of the utrophin-A promoter using gel shift assays, we asked whether the ERK-dependent nuclear shuttling mechanisms previously characterized in Ref-1 cells (Le Gallic *et al.*, 2004) are functional in C2C12 muscle cell lines. To test this, we performed subcellular fractionation experiments to determine the levels of ERF in the nuclear and cytoplasmic fractions of C2C12 muscle cell lines after incubation with U0126, an inhibitor for MEK, the upstream kinase for ERK1/2 at different times. As shown in Figure 2Ai, incubation with U0126 led to increased ERF levels in the nuclear compared with cytoplasmic fractions. Next we used anti-ERF antibodies for indirect immunofluorescent confocal microscopy to independently validate the MEK-dependent nuclear shuttling by visualizing changes in subcellular localization of ERF in C2C12 cells when incubated with U0126. As shown in Figure 2Aii, incubation with U0126 caused ERF to preferentially localize in the nucleus. To demonstrate the effect of ERF on utrophin-A promoter activity, we used the pPUBF utrophin-A luciferase reporter construct containing the entire human utrophin promoter (Dennis *et al.*, 1996), as well as the pPUBFΔN-box reporter construct that contains a deletion mutation of the N-box region (Khurana *et al.*, 1999) (Figure 2Bi). *Drosophila* S2 cells were cotransfected with pPUBF and 1) expression constructs encoding GABP α / β (Rosmarin *et al.*, 1995) as a positive control or 2) ERF expression vector pSG5-ERF. In agreement with previous studies (Khurana *et al.*, 1999; Gyrd-Hansen *et al.*, 2002), GABP α / β *trans*-activated the utrophin A promoter fourfold ($407 \pm 39.2\%$) (Figure 2Bii). In contrast, ERF *trans*-repressed the

vector were cotransfected into S2 cells along with equimolar combinations of the following: GABP α / β expression vectors (GABP, gray columns), ERF expression vector pSG5-ERF (ERF, white columns), GABP α / β and ERF vectors (light gray column), or a 10 μ M final concentration of MEK inhibitor U0126 (white column), and luciferase activity was assayed after a 24-h incubation. GABP α / β *trans*-activated the utrophin promoter construct almost fourfold (gray column), whereas decreases to 12 and 33% of normalized promoter activity was noted with ERF overexpression and MEK inhibition, respectively. However, *trans*-repression by ERF was completely competed by GABP α / β *trans*-activation (light gray column). No difference in pPUBFΔN-box reporter activity was observed upon the addition of ERF (96%). Luciferase values are the means of triplicate wells in nine separate experiments ($n = 27$) for pPUBF and ERF and in three separate experiments ($n = 9$) for MEK inhibition and GABP α / β . Error bars are SEM. (iii) Immunoblot controls showing that transfection of GABP and ERF expression vectors causes an increase in protein levels in transfected C2C12 cells.

promoter by $\sim 88\%$ ($12.3 \pm 2.2\%$). Use of the MEK inhibitor U0126 and the subsequent nuclear export of ERF also resulted in an approximately threefold decrease in promoter activity ($32.1 \pm 9.3\%$). Utrophin promoter reporter assays were also performed using cotransfection of ERF and GABP α/β expression constructs together in *Drosophila* S2 cells (Figure 2B). To determine whether the N-box was required for ERF-mediated repression, we transfected cells using the pUBF Δ N-box construct. As shown in Figure 2Bii, ERF was unable to repress the mutant utrophin promoter lacking the N-box motif. Additionally, immunoblotting by using anti-ERF and anti-GABP antibodies was performed on cells that had been transfected with the ERF and GABP constructs, respectively, to verify that the expression constructs were functional (Figure 2Biii). Together, these assays demonstrated that 1) ERF-mediated *trans*-repression could be successfully competed by GABP α/β -mediated promoter activation and 2) that the N-box motif is required for ERF-mediated *trans*-repression of the utrophin promoter.

ERF Knockdown Enhances Endogenous Utrophin mRNA Levels and Utrophin-A Promoter Activity

Because ERF is able to bind and repress promoter activity, we sought to delineate the effect of siRNA-mediated silencing of ERF on 1) endogenous utrophin mRNA levels and 2) utrophin-A promoter activity. To ensure selective knockdown, four siRNA oligomers corresponding to regions of ERF outside the conserved N-box/EBS binding domain with no homology to other known gene were transfected into C2C12 muscle cells, and semiquantitative RT-PCR was performed after 24 h. ERF siRNA decreased endogenous ERF levels to 2%, and it enhanced endogenous utrophin levels (1.4-fold) compared with an unrelated scrambled oligomer control (egg), after adjustment to GAPDH levels (Figure 3Ai). We postulated the increase in utrophin mRNA was due to increased promoter activity as a result of decreased ERF levels; therefore, pUBF was transfected 24 h postoligomer addition, and C2C12 cells were assayed for luciferase activity after an additional 24-h period (Figure 3Bii). In concordance with our hypothesis, a twofold elevation of promoter activity ($199.1 \pm 24\%$) was observed for ERF oligomers in comparison with the egg control. Additional transfection experiments were performed to address whether siRNA-mediated silencing of ERF altered the program of myogenic differentiation in the cultured myotubes and hence secondarily effected utrophin expression via altered expression of myogenic factors (Gramolini and Jasmin, 1999; Perkins *et al.*, 2001). As shown in Figure 3Aiii, no alterations in expression levels of the myogenic factors MyoD and myogenin were detected using semiquantitative RT-PCR after transfecting with ERF siRNA oligomers or scrambled egg control, making it highly unlikely that the alterations of utrophin promoter activity were due to changes in expression of myogenic factors. Rather, the increase in utrophin promoter activity noted in Figure 3A was indeed a direct result of decreased ERF levels due to a siRNA-mediated mechanism, as postulated.

ChIP Analysis Demonstrates ERF Association with the Utrophin-A Promoter

To independently validate the interaction between ERF and the utrophin-A promoter and the role of MEK and heregulin in modulating the promoter (Gramolini *et al.*, 1999a; Khurana *et al.*, 1999), we used anti-ERF antibodies to perform ChIP analysis of the utrophin-A promoter in C2C12 cells that had been treated with MEK inhibitor U0126 or heregulin for 15 min or left untreated. As shown in Figure

Figure 3. Inhibition of ERF gene expression enhances utrophin mRNA levels and utrophin-A promoter activity in C2C12 cells. (Ai) Semiquantitative RT-PCR of ERF, utrophin, and GAPDH transcript levels in murine C2C12 cells after treatment with either 100 pmol (left lane) of an unrelated, scrambled control egg oligomer, or 25 nmol each of four siRNA complementary to ERF. ERF siRNA oligomers caused a specific decrease in ERF transcript to $\sim 2\%$ and an ~ 1.4 -fold increase in utrophin mRNA after adjustment to GAPDH levels. (ii) ERF knockdown causes an increase in utrophin promoter activity. The pUBF utrophin promoter-luciferase construct was transfected into C2C12 cells 24 h after either egg or ERF siRNA oligomer transfection, and luciferase activity was assayed after an additional 24-h incubation. An approximate twofold elevation of utrophin promoter activity in C2C12 cells was observed with the ERF oligomer mix (1.99 ± 0.24) in comparison with the egg scrambled control. Luciferase values are the means of four separate experiments performed in triplicate ($n = 12$). Error bars are SEM. (iii) Semiquantitative RT-PCR of MyoD, myogenin, and GAPDH transcript levels in murine C2C12 cells after treatment with either 25 nmol each of four siRNA complementary to ERF (left lanes, 1–3) or 100 pmol (right lanes, 1–3) of an unrelated, scrambled control egg oligomer and assayed after 24 h. The – marked lane shows a negative control for RT-PCR where reaction was performed without template. No significant changes in MyoD or myogenin transcript levels were noted. Gels show results of three separate experiments. (B) Chromatin immunoprecipitation analysis of the utrophin-A promoter with ERF antibodies in C2C12 cells, demonstrating changes in ERF occupancy of the utrophin-A promoter upon treatment with MEK inhibitor U0126 and HRG treatment for 15 min. (i) RT-PCR of utrophin-A promoter from ERF-precipitated chromatin from C2C12 cells. (ii) Quantification of changes in ERF occupancy compared with the untreated ERF controls shows increased (1.63-fold) and decreased (0.64-fold) ERF levels with MEK inhibitor U0126 and HRG, respectively. Controls incubated without antibodies are shown below.

3B, ERF was associated with the utrophin-A promoter, and its levels were modulated by U0126 and heregulin. Consistent with the gel shift assays, subcellular localization and reporter assays (Figure 2) as well as previous literature on heregulin-mediated utrophin promoter activation (Gramolini *et al.*, 1999a; Khurana *et al.*, 1999), MEK inhibitor U0126 increased, and conversely heregulin decreased, the levels of ERF associated with the utrophin-A promoter, respectively.

Utrophin, GABP α , and ERF Expression during Development

To address the approximately eightfold perinatal reduction of utrophin levels that leads to relatively low levels (typically 0.01% of message) in the adult compared with embryonic muscle (Khurana *et al.*, 1990, 1991), we analyzed utrophin, GABP α , and ERF transcript levels by using semiquantitative RT-PCR at six times from embryonic day 16- (e16) to adult (1-yr-old *mdx* mouse hindlimb muscle (Figure 4). Utrophin and GABP α transcripts were most abundant during embryogenesis (e16), and they showed a decline of 2.5- and 1.7-fold, respectively, by peri-

natal day 2 (p2). Analysis of adult muscle indicated that transcripts had decreased to 11.6-fold for utrophin and 12.1 for GABP α from values obtained during embryogenesis (Figure 4B). Interestingly, ERF transcript levels remained high from late embryogenesis to adult muscle similar (cf. 4.9 and 5.1, respectively), with the highest level detected at p14 (5.74 arbitrary units standardized to GAPDH).

ERF Expression Is Localized Extrasynaptically in Adult Limb Muscle

To understand the mechanisms behind the spatially restricted expression of utrophin at NMJs in adult myofibers, we used LCM to selectively study gene expression levels in synaptic versus extrasynaptic regions in adult rat TA muscle (Figure 5). Demonstrating the sensitivity and specificity of this approach, we found evidence for highly enriched transcription at the synapse for the well-known synaptic markers α subunit of nAChR or *Chrna1* (163-fold), utrophin (7.9-fold), and *Ets1* (17.9-fold). Interestingly, ERF expression was not detected at the synapse; however, it was exclusively expressed extrasynaptically in TA muscle in vivo (Figure 5B).

Direct Gene Transfer Confirms N-Box-mediated ERF Repression of the Utrophin-A Promoter In Vivo

Finally, we confirmed the ability of ERF to repress the wild-type utrophin-A promoter in vivo by coinjecting the wild-type utrophin promoter-A reporter plasmids with a CAT control plasmid to monitor transduction efficiency into TA muscles of 4-wk-old control mice with control or ERF plasmids. Five days later, muscles were harvested, RNA was extracted, and RT-PCR analysis for LacZ and CAT was performed. In comparison with the control plasmid, promoter-A activation was significantly impaired by cotransfection with the ERF expression vector (31.5%; $p < 0.01$; Figure 6). To determine whether the N-box was required for ERF-mediated repression, we performed additional experiments by using the pUBF Δ N-box construct as a reporter plasmid. As shown in Figure 6, ERF was unable to repress the mutant utrophin promoter lacking the N-box motif in vivo. These results support and extend our findings from cultured muscle cells to skeletal muscle in vivo.

DISCUSSION

In this study, we used gel shift (Figure 1), utrophin-A promoter reporter assays and monitoring of ERF subcellular translocation studies (Figure 2), siRNA-mediated knock-down studies and ERF-utrophin promoter ChIP analysis (Figure 3), in vitro expression/developmental time course (Figure 4) qPCR expression/localization in muscle (Figure 5), and direct gene transfer analysis (Figure 6) to identify and characterize ERF-mediated repression as a novel mechanism for regulation of the utrophin-A promoter at a transcriptional level.

This study addresses a fundamental question in molecular and cellular biology—how to achieve selective localization of genes/gene products such as AChR or utrophin at synapses. In skeletal muscle, the problem is more extreme (than in neurons), because muscle is composed of elongated multinucleated cells, and the muscle synapse or NMJs occupy only an extremely small portion of the cell membrane; yet, synaptic localization is achieved with considerable fidelity. Although subsynaptic transcription plays an important role in enriching genes such as AChR and utrophin at the NMJ (Schaeffer *et al.*, 2001), we suggest that the converse mechanisms of extrasynaptic silencing may play an impor-

Figure 4. Transcript levels of GABP α , utrophin, and ERF in *mdx* mouse muscle. Semiquantitative RT-PCR of utrophin, GABP α , ERF, and control GAPDH transcript levels at various times from embryonic day 16 to adult (12-mo-old) muscle. Utrophin and GABP α transcripts are shown after a 48-h exposure, and GAPDH and ERF are shown after 24-h exposure. The graph illustrates arbitrary units of each PCR product measured by ImageQuant Tool software and expressed as a ratio to GAPDH at each time point after 24-h exposure. Left y-axis refers to ratios to GAPDH obtained for utrophin and GABP α , and right y-axis refers to ERF:GAPDH ratios. E, embryonic; p, perinatal.

Figure 5. ERF expression is restricted exclusively to extrasynaptic regions in muscle, suggesting a role in utrophin repression. (A) Microdissection steps of synaptic and extrasynaptic regions of rat TA muscle. Cryosections of 10 μ m (TA) are stained with Karnowsky and Root's method to show NMJ localization by using PEN membrane glass slides, and they are visualized using PALM light microscopy at 20 $\times$ power. (A and D) Visualization of TA tissue before LCM. (B and E) Same slide cut by laser for synaptic and nonsynaptic tissue parts intending to capture. (C and F) Section visualized post-LCM, showing removal of synaptic and nonsynaptic material by catapulting to the extraction buffer. Bars, 100 μ m. (B) Taqman qRT-PCR amplification plots of ChRNA1 (muscle), Utrn, Ets1, Erf, and β -actin are shown on the left side of Figure 5B. The x-axis shows PCR cycle numbers up to 40, and the y-axis illustrates the Δ of normalized reporter fluorescence dye (Δ Rn), which refers to new product formation in each cycle. Color codes used refer to β -actin in TA-synaptic (purple) and TA-nonsynaptic (green) and the gene of interest in TA-synaptic (red) and TA-nonsynaptic (blue) in PCR analysis. Relative gene expression of analysis data results for duplicate wells of each gene are shown as bar graphs with standard deviations, with a star indicating statistically significant differences. Because ERF (blue lines) did not reach threshold levels in this analysis, its expression level is illustrated as an electrophoretic agarose (1%) gel product at the end of 40 cycles. (C) Summary of the relative expression synaptic:nonsynaptic ratio for all genes is illustrated.

tant role as well. ERF-mediated repression provides a novel molecular mechanism contributing to concurrent extrasynaptic silencing that must occur to achieve NMJ localization of utrophin-A in muscle. Because ERF expression seems to be exclusively extrasynaptic in TA rat muscle (Figure 5), we postulate that within these regions (or in unstimulated muscle), low levels of utrophin transcription exist, in part, due to occupation of the N-box/EBS site by ERF that may serve as a constitutive damper of expression (Figure 7). However, the

repression is repressed at synaptic regions (or when stimulated by neurite-associated HRG). On binding of HRG to the HER 2,3,4 receptors at the NMJ, ERK is activated by MEK-dependent phosphorylation, leading to nuclear localization and facilitating two events, each of which favor increased utrophin transcription to occur: 1) multiple serine/threonine residue phosphorylation of ERF, causing MEK-dependent nuclear export and a loss of repressor activity, hence derepressing the promoter (or making it more likely to be acti-

Figure 6. Direct gene transfer demonstrates N-box-mediated utrophin-A promoter repression by ERF *in vivo*. Utrophin promoter-A constructs (wild type; with N-box) were coinjected with CAT (to monitor transduction efficiency) into TA muscles of 4-wk-old control mice with control (pSG5 vector) or ERF (pSG5-ERF) expression plasmid. A different cohort was injected using the mutant utrophin promoter constructs (Δ N-box; without N-box). Five days later, muscles were harvested, and then RNA extracted and qPCR analysis was performed. Values obtained for LacZ or luciferase are standardized relative to the amount of CAT present in the same sample, with the ERF-injected samples compared with their respective control (normalized to 100%). Student's *t* tests were used to analyze the data. The asterisk denotes the statistically significant ($p < 0.02$) decrease in promoter-A activation ($\sim 30\%$) observed with the ERF plasmid (black bar) versus the control injected plasmid (white bar). No significant differences were observed using the Δ N-box construct. Error bars represent SEM; sample size for wild type, $n = 5$; Δ N-box, $n = 10$ for both ERF and control experiments.

Figure 7. Model for transcriptional regulation of utrophin promoter-A via the N-box/EBS site in muscle. Transcriptional model of the utrophin-A promoter pre- and post-ERK nuclear localization by HRG stimulation at the NMJ. Multiple arrows represent increased transcription. P, phosphorylated protein; dotted and full arrowed lines represent potential and defined signaling cascades, respectively. For more information, please refer to the text.

vated); and 2) activation of GABP α/β , leading to greater N-box/EBS binding as well as synergistic activation of the utrophin promoter with Sp1. Consistent with this, ERK immunoprecipitated from HRG-treated C2C12 cells phosphorylated ERF in a MEK-dependent manner by using *in vitro* kinase assays (unpublished observations).

In conclusion, we have identified promoter repression as a novel mechanism that leads to utrophin down-regulation in extrasynaptic regions of skeletal muscles. We think that derepressing the promoter by blocking the activity of repressor molecules (such as ERF or ERF-like molecules) either alone or in concurrence with positively *trans*-activating ETS family members may be a functional means to synergistically enhance utrophin promoter activation as a therapeutic strategy for DMD. However, it is important to test these hypotheses *in vivo* before assigning these important roles to the ERF transcription factors.

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