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Targeting Transcriptional and Translational Mechanisms to Enhance Utrophin A Expression as a Therapy for Duchenne Muscular Dystrophy

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Targeting transcriptional and translational mechanisms to enhance utrophin A expression as a therapy for Duchenne Muscular Dystrophy

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ABSTRACT

Duchenne Muscular Dystrophy (DMD) is a fatal, neuromuscular disorder caused by mutations/deletions in the dystrophin gene. In skeletal muscle, dystrophin is expressed along the sarcolemma, providing a mechanical link between the cytoskeleton and the extracellular matrix; loss of dystrophin results in disrupted sarcolemmal integrity and progressive muscle wasting. Utrophin is the autosomal homologue of dystrophin and is present in skeletal muscles of DMD patients, although its expression is primarily restricted to the neuromuscular junction of mature skeletal muscle fibers. Enhancing expression of utrophin in skeletal muscles of DMD animal models prevents the dystrophic pathology; thus, it is of considerable interest to identify the mechanisms controlling utrophin expression in skeletal muscle, with the ultimate goal of identifying drugs that can stimulate utrophin levels in DMD patients.

Utrophin A expression is regulated by transcriptional mechanisms that are associated with the slow myogenic program. Here we uncovered that expression of utrophin A can be transcriptionally enhanced via its promoter by GW501516-mediated activation of the nuclear receptor PPARβ/δ, a known regulator of the slow myogenic program. Treatment of dystrophin-deficient *mdx* mice with GW501516 stimulated utrophin A expression at the sarcolemma and mitigated the dystrophic phenotype, as evidenced by increased sarcolemmal stability and decreased susceptibility of skeletal muscles to lengthening contractions.

Under multiple physiologically relevant conditions, enhancement of utrophin A protein levels in skeletal muscle are not accompanied by similar increases in mRNA

levels, suggesting a role for post-transcriptional control. We identified that utrophin A expression is enhanced via Internal Ribosome Entry Site (IRES)- mediated translation in response to skeletal-muscle regeneration and glucocorticoid treatment. Using transgenic reporter mice, we found that IRES-mediated translation of utrophin A occurs exclusively in skeletal muscle fibers and no other tissues. Finally, we identified that a *trans*-acting factor, eEF1A2, interacts with the utrophin A 5'-UTR and is involved in regulating IRES-mediated translation of utrophin A.

This work has identified novel transcriptional and translational mechanisms controlling expression of utrophin A in skeletal muscle. Our results suggest that GW501516 treatment might constitute a viable pharmacological therapy for DMD patients and IRES-mediated translational of utrophin A could serve as a new therapeutic target.

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List of Abbreviations

5'-UTR- 5'-Untranslated Region

AO-antisense oligonucleotide

βGAL- β-galactosidase

BiP- human immunoglobulin heavy chain binding protein

BMD- Becker muscular dystrophy

CaM- calmodulin

CaMK- Ca^{2+}/CaM-dependent protein kinase

CAT- chloramphenicol acetyltransferase

CTX-cardiotoxin

DAPC- dystrophin-associated protein complex

DMD- Duchenne muscular dystrophy

EBD- Evans blue dye

ECC- eccentric contraction

ECM-extracellular matrix

EDL-extensor digitorum longus

eEF1A2- eukaryotic elongation factor 1A2

FGF-II- fibroblast growth factor 2

GABP- GA binding protein

GRMD- golden retriever muscular dystrophy

hnRNP A1- heterogeneous nuclear ribonucleoprotein A1

IGF-I- insulin growth factor I

IRES- Internal ribosome entry site

ITAF- IRES-*trans* activating factors

MyHC- myosin heavy chain

NF-κB- Nuclear Factor kappa B

NMJ- neuromuscular junction

nNOS- neuronal nitric oxide synthase

PDN-6α–methylprednisolone-21 sodium succinate

PGC-1α- peroxisome proliferator-activated receptor γ coactivator 1α

PPAR- peroxisome proliferator-activated receptor

PPRE- PPAR response element

PTB- polypyrimidine-tract binding protein

RNA-BP- RNA-binding protein

RT-PCR- reverse transcription- polymerase chain reaction

TA- tibialis anterior

UCP2- uncoupling protein 2

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CHAPTER 1

1. GENERAL INTRODUCTION

I. Duchenne Muscular Dystrophy (DMD)

The muscular dystrophies are a group of hereditary neuromuscular disorders that are characterized by the progressive loss of skeletal muscle. The most common of these is Duchenne muscular dystrophy (DMD), a fatal disorder affecting approximately 1 in every 3500 boys (Emery, 1991). Diagnosis is made usually by 6 years of age, although the onset of symptoms begins around at 3 years of age, with the child having difficulty running, climbing stairs and often displaying a waddling gait due to weak proximal pelvic girdle muscles. Other clinical presentations include pseudohypertrophy of the calf muscles and proximal lower limb muscle weakness, which can be present as Gowers' maneuver (Gowers, 1879). Progressive lower limb muscle weakness causes wheelchair confinement, usually by age 12 (Emery, 1993). Death ensues usually by the mid-twenties usually due to cardiac or respiratory failure (Jennekens et al., 1991). Cardiomyopathy is present in >90% of patients, with heart failure thought to account for ~25% of deaths (Eagle et al., 2007). Loss of diaphragm muscle function is the major cause of respiratory failure, which accounts for ~75% of DMD deaths (Finsterer and Stollberger, 2003). DMD patients also display degrees of cognitive impairments that do not correlate with the progression or severity of the muscle disease (Anderson et al., 2002).

I.A. Dystrophin and the dystrophin associated protein complex (DAPC)

DMD is a monogenic disorder caused by mutations in the dystrophin gene (*DMD*); the first disease gene identified by positional cloning (Ray et al., 1985; Monaco

et al., 1986; Koenig et al., 1987). The *DMD* gene is located on Xp21.1 and is the largest in the human genome, being comprised of 79 exons that span ~2.5 Mb of genomic sequence (~0.1% of the human genome) (Blake et al., 2002). *DMD* mutations are inherited in an X-linked recessive fashion. However, due to the extremely large size of the gene, new mutations account for ~1/3 of DMD cases (Barbujani et al., 1990; Ahn and Kunkel, 1993). Most DMD cases are caused by deletions of the dystrophin gene (60%), while the remaining cases are attributed to point mutations (32%) or duplications (8%) (Muntoni et al., 2003).

The *DMD* gene encodes a full-length cytoskeletal protein of 427-kDa that is a member of the β-spectrin/α-actinin family (Koenig et al., 1988; Ahn and Kunkel, 1993). Alternative splicing and usage of alternative promoters gives rise to multiple shorter dystrophin isoforms that have contrasting expression patterns (Muntoni et al., 2003). The ~14 kb mRNA encoding full-length dystrophin is expressed in skeletal muscle, cardiac muscle and brain (Bies et al., 1992). In skeletal muscle, dystrophin is expressed at the sarcolemma (Watkins et al., 1988), where it plays an important structural role by linking the actin cytoskeleton to the extracellular matrix (ECM). Dystrophin is comprised of 4 major domains based on sequence homology and protein binding partners (**Fig. 1A**). The NH_2-terminal domain contains calponin homology modules that interact with actin filaments. This is followed by a large central rod domain that consists of spectrin-like repeats separated by hinge domains (Koenig et al., 1988). The presence of these repeats in the central rod domain are thought to make the protein more flexible and elastic, thus helping to protect the muscle cell from contraction induced damage (Grum et al., 1999). The third domain is a cysteine rich domain that includes two EF hand-like domains,

along with WW and ZZ modules (Koenig et al., 1988; Ponting et al., 1996). The final domain is the COOH-terminal domain. This domain, together with the cysteine rich domain interacts with the integral membrane protein β-dystroglycan (Ervasti and Campbell, 1991; Suzuki et al., 1992). β-dystroglycan interacts with α-dystroglycan at the extracellular side of the membrane, where it associates with extracellular matrix proteins such as laminin-2 (Ervasti and Campbell, 1991). The interactions of dystrophin with these and other proteins, including cytoplasmic, transmembrane and extracellular proteins, forms a complex referred to as the Dystrophin-Associated Protein Complex (DAPC) [also referred to as Dystrophin-Associated Glycoprotein Complex (DGC)]. Dystrophin and the DAPC thus provide a strong mechanical link between the inside of the muscle cell and the ECM that is essential to maintain the integrity of muscle fibers **(Fig. 1B)**.

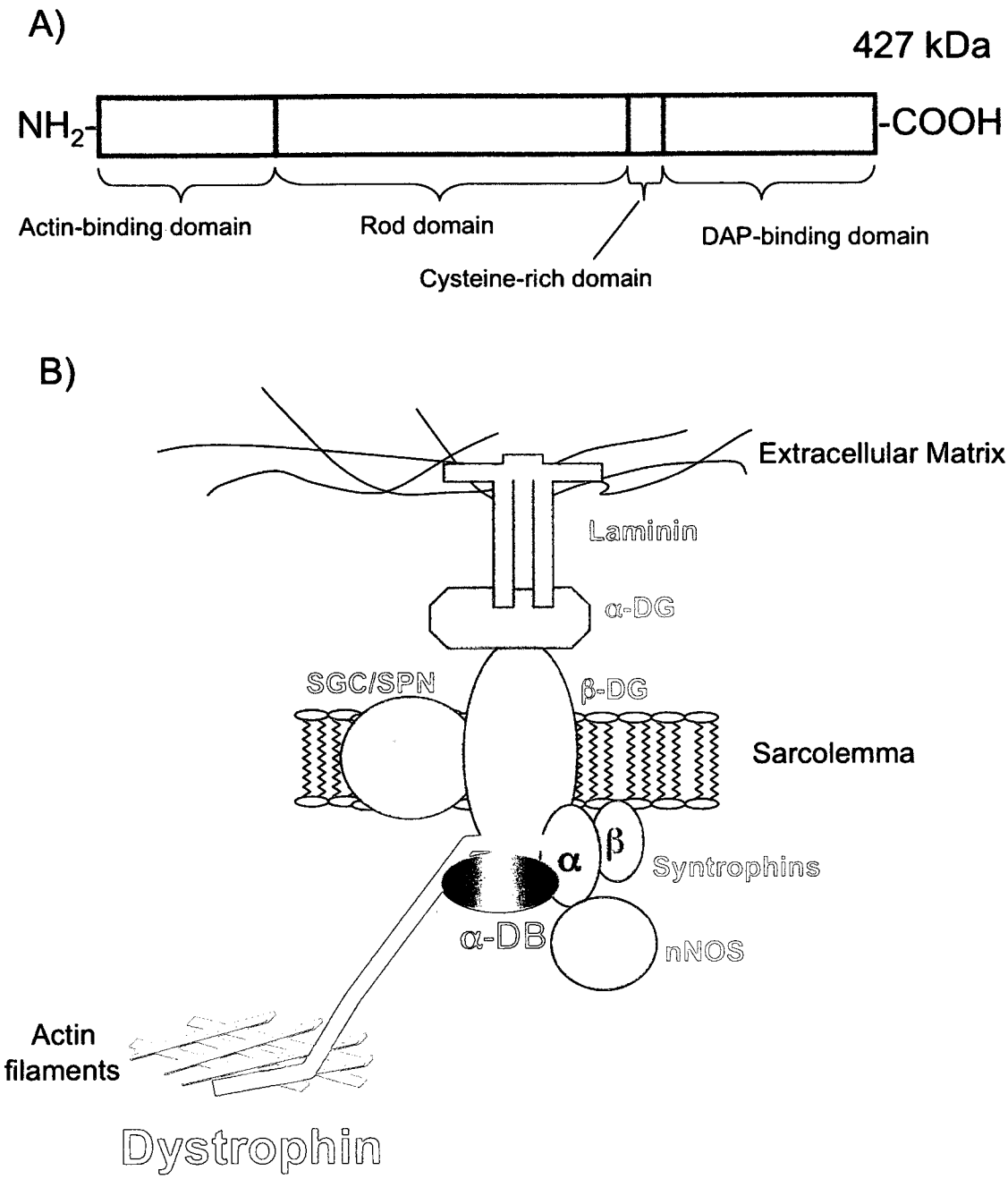
I.B. Skeletal muscle pathophysiology of DMD

It has been demonstrated that the absence of dystrophin expression in DMD patients results in a great reduction in DAPC members at the sarcolemma (Ervasti et al., 1990; Ibraghimov-Beskrovnaya et al., 1992). The absence of dystrophin and disruption of the DAPC causes instability of the sarcolemma, making DMD muscle fibers susceptible to damage and necrosis (Weller et al., 1990; Menke and Jockusch, 1991). In response to muscle wasting, muscle progenitor cells are activated to repair the degenerated muscle; however, as the disease progresses, muscle regenerative capacity diminishes, resulting in severe necrosis with lost muscle being replaced with fibrotic tissue.

Muscle inflammation is prominent in dystrophic muscles, and it appears that this inflammation is part of a specific immune response that also contributes to wasting

Fig. 1. Dystrophin associates with members of the DAPC at the sarcolemma

A) Organization of full-length dystrophin protein. B) Through binding actin filaments at its NH_2-terminus, and β-dystroglycan at its COOH-terminus, dystrophin provides a link between the ECM and the intracellular cytoskeleton. For simplicity, only some members of the DAPC are shown. β-DG, β -dystroglycan; α-DG, α -dystroglycan; nNOS, neuronal nitric oxide synthase; sarcoglycan complex (SGC); sarcospan (SPN); α-dystrobrevin (α-Db).


A)
427 kDa
NH2-
-COOH
Actin-binding domain
Rod domain
DAP-binding domain
Cysteine-rich domain
B)
Extracellular Matrix
Laminin
α-DG
SGC/SPN
β-DG
Sarcolemma
α
β
Syntrophins
α-DB
nNOS
Actin
filaments
Dystrophin

(Brunelli and Rovere-Querini, 2008). The susceptibility of the dystrophic muscle fibers to lesions causes leaking of cytoplasmic proteins such as creatine kinase into the serum (Florence et al., 1985) and an increase in intracellular Ca^{2+} levels (Bodensteiner and Engel, 1978). The increased Ca^{2+} levels in muscle cells alters Ca^{2+} -mediated signaling pathways, causing de-regulation of Ca^{2+} permeable channel activity and Ca^{2+} -mediated proteolysis (Alderton and Steinhardt, 2000; Ruegg et al., 2002; Vandebrouck et al., 2002).

It is currently thought that the pathology of DMD is not only caused by loss of sarcolemmal structural integrity and pertubation of calcium homeostasis, but is also a result of altered signaling pathways that are normally mediated by dystrophin at the DAPC. The DAPC is a multifunctional protein complex, having important roles linked to signal transduction cascades (Rando, 2001). For example, dystrophin interacts with neuronal nitric oxide synthase (nNOS) (Brenman et al., 1995) and several DAPC proteins bind Grb2, an adaptor protein involved in signal transduction (Yang et al., 1995; Oak et al., 2001). Signaling roles have been also been attributed to α-dystrobrevin and syntrophins because these proteins are required for muscle function in a manner that does not depend on the mechanical role of the DAPC at the sarcolemma (Crawford et al., 2000; Davies and Nowak, 2006). The lack of dystrophin in skeletal muscles also causes disruptions in JNK, p38 MAPK and GTPase signaling pathways (Batchelor and Winder, 2006). The massive dystrophin molecule likely plays other as of yet undeciphered roles in skeletal muscle, cardiac muscle and the brain. Further investigation into these roles should provide additional insights into how the lack of dystrophin results in muscle cell death.

I.C. Other muscular dystrophies

Defects in the dystrophin gene that result in the expression of truncated and partially functional dystrophin at the sarcolemma are the cause of Becker muscular dystrophy (BMD). BMD is less common than DMD, and the phenotype is more mild, with later onset and longer survival (Emery, 2002). Mutations in other genes encoding proteins that are involved in the function or composition of other DAPC protein members can result in other types of muscular dystrophies (Cohn and Campbell, 2000; Dalkilic and Kunkel, 2003). For example, mutations in the laminin α2 chain causes congential muscular dystrophy 1A (Helbling-Leclerc et al., 1995), and disruption of the genes encoding the sarcoglycan proteins causes several types of limb girdle muscular dystrophy (LGMD) (Vainzof et al., 1996). Mutations in dystroglycans have not been observed in human patients, but mutations in glycosyltransferases that post-translationally modify dystroglycans are known to cause different forms of muscular dystrophy (Dalkilic and Kunkel, 2003).

The importance of the DAPC in maintaining a link between the cytoskeleton and the ECM for proper muscle function is underscored by the aforementioned muscular dystrophies; however, other muscular dystrophies that display similar muscle pathologies result from defects in a wide range of proteins that are expressed in various cellular compartments within the muscle cell including the nuclear membrane, sarcomere and sarcoplasmic reticulum. These proteins include not only structural proteins, but also enzymes, glycosyltransferases and signaling molecules (Davies and Nowak, 2006). Thus, defects in the integrity of the sarcolemma do not account for all forms of muscular dystrophy, despite, in many cases, similar symptoms of muscle wasting.

I.D. The *mdx* model of DMD

The most commonly studied model of DMD is the *mdx* mouse. This mouse is null for dystrophin as a result of a spontaneous point mutation (Bulfield et al., 1984). The onset of the dystrophic pathology in this mouse is at 3 weeks of age, and is followed by continued necrosis and regeneration until ~8 weeks of age (McGeachie et al., 1993; Muntoni et al., 1993). After this peak phase, the muscle pathology progresses slowly, with a more severe phenotype not re-appearing before 1 year of age. Extensive fibrous connective tissue is present after 15 months (Lefaucheur et al., 1995). Despite the phenotype of the *mdx* being mild in comparison to the human disease, multiple parameters of the muscle pathology can be reliably assessed in the *mdx* mouse making it is an excellent model to test potential therapeutics. These techniques include, for example: histochemical stains to assess necrosis, regeneration and fibrosis; *in vivo* and *ex vivo* measurements of muscle force production after lengthening (eccentric) contractions; and assessment of the leakiness of muscle fibers (Grounds et al., 2008). Another DMD model is the golden retriever muscular dystrophy dog (GRMD). This animal displays a disease progression and more severe pathology that more closely resembles the human condition, making it useful for evaluating the benefits and, because of its large size, the toxicity of experimental therapies (Willmann et al., 2009).

II. Treating DMD

II.A. Current supportive therapy

Despite the identification of the molecular defect responsible for DMD being identified in 1987, there is still no curative treatment. Current therapy is only supportive, and includes physiotherapy, psychotherapy, nutritional support and cardiopulmonary support. Since most DMD patients die from muscular respiratory failure, mechanical support for ventilation and to aid in coughing is essential. Such interventions are estimated to prolong lifespan by 5 -10 years (Toussaint et al., 2007). Supportive pharmacological therapy for DMD includes treatment with the corticosteroids prednisone and deflazacort (Mendell et al., 1989; Biggar et al., 2001). These treatments have been shown to delay loss of ambulation and improve pulmonary function, but are associated with detrimental side effects such as weight gain (DeSilva et al., 1987; Fenichel et al., 1991). DMD patients are closely monitored for the development of cardiac problems, contractures and scoliosis. For DMD patients with heart failure, ACE inhibitors and beta-blockers can have beneficial effects (Ogata et al., 2009). Surgical correction for contractures and scoliosis is recommended in certain situations (Strober, 2006).

II.B. Experimental therapies for DMD

There are a wide range of novel therapeutic strategies under investigation to treat DMD, including gene replacement therapies, cellular therapies and more traditional pharmacological therapies.

II.B.i. Gene-therapy

One gene therapy approach aims to introduce a functional dystrophin protein in DMD patient muscles by viral-mediated gene transfer of dystrophin. Therapeutic viral vectors are limited in their carrying capacity, making delivery of full-length dystrophin difficult or unfeasible. The observation of a set of BMD patients exhibiting only a very mild dystrophy when 46% of dystrophin was deleted first suggested that truncated versions of dystrophin could be sufficient to restore the mechanical function of the DAPC (England et al., 1990). Indeed, transgenic introduction of mini-dystrophin genes that lack large portions of the central rod domain was later shown to prevent dystrophic symptoms in *mdx* mice (Phelps et al., 1995; Wells et al., 1995). These results further affirmed the importance of the dystrophin terminal domains in linking the actin cytoskeleton and the ECM along the sarcolemma. Early attempts using recombinant adenovirus vectors to deliver such mini-dystrophin molecules were met with some success in that some dystrophin expression could be restored to the sarcolemma and muscle function could be partially restored. However, one of the limitations of this approach was that the delivery of the therapeutic dystrophin molecule was confined to particular injected muscles (Ragot et al., 1993; Vincent et al., 1993; Deconinck et al., 1996). Since these pioneering studies, there has been steady progress in the design and implementation of viral vector based DMD therapeutics; including, improved delivery, immune system tolerance, and functional benefits (Hartigan-O'Connor and Chamberlain, 2000; Gregorevic et al., 2004b; Foster et al., 2006).

The most recent and promising iteration of this gene therapy strategy involves introducing truncated dystrophin molecules using recombinant adeno-associated virus

(rAAV) (Schultz and Chamberlain, 2008). Systemic delivery of the therapeutic agent can be achieved using this approach in both young (Gregorevic et al., 2004c) and old *mdx* mice (Gregorevic et al., 2008). While there has been impressive progress in the development of rAAV vectors over recent years (Trollet et al., 2009), one of the current hurdles is achieving systemic and sustained delivery to all striated muscles in large animals such as dogs and primates (Rodino-Klapac et al., 2007; Yue et al., 2008). In addition, although rAAV vectors can be delivered to mature, post-mitotic striated muscle, they are not integrated into the host genome and are not sustained in dividing cells. Lentiviral vectors, on the other hand, integrate into chromosomes and have been found to efficiently transduce both mature muscle fibers and satellite cells (Kimura et al., 2010); thus, they continue to be developed as potential DMD therapeutic agents.

Other gene therapy strategies aim to restore dystrophin expression by altering endogenous mRNA transcripts. Some DMD patients have nonsense *DMD* mutations that result in premature-termination codons. This causes the translational machinery to produce a truncated, non-functional dystrophin molecule that is degraded. When the mutations are found in exons that are non-essential to the function of dystrophin (such as in the central rod domain) these exons could be bypassed, thus restoring the reading frame and producing a near fully functional, but truncated dystrophin protein (Wilton and Fletcher, 2008). To induce exon-skipping in the *DMD* gene of such patients, a variety of antisense oligonucleotide (AO) strategies are under development (Trollet et al., 2009). Several of these approaches have been successful in alleviating the *mdx* mouse pathology using various delivery methods, including for example, delivery of modified snRNAs by AAV (Goyenvalle et al., 2004), administration of morpholino AOs (Fletcher et al., 2006)

and conjugation of AOs to cell-penetrating peptides (Yin et al., 2008). AO morpholinos were recently used to treat GRMD dogs (Yokota et al., 2009), and clinical trials of exon 51 skipping are in their early phases (van Deutekom et al., 2007; Kinali et al., 2009). Although exon 51 skipping would be applicable to only 13% of all DMD patients (Wilton and Fletcher, 2008), mutation specific single and double exon skipping strategies could theoretically be applicable to 83% of DMD patients (Aartsma-Rus et al., 2009).

II.B.ii. Cell-based therapy

In experimental cell-based DMD therapies, normal or corrected cells are delivered to the dystrophic muscle, in hopes that these cells will fuse with existing multinucleated muscle fibers and correct the dystrophic pathology. The first attempt of a cell-based therapy for DMD was myoblast transfer therapy. After the demonstration that transplanted wild type mouse myoblasts can fuse with dystrophic muscle fibers of the *mdx* mouse (Partridge et al., 1989), this therapy rapidly advanced to clinical trials (Law et al., 1993). The approach proved ultimately unsuccessful, in part due to limited survival of cells, inability of cells to migrate into the circulation and problems associated with immuosuppression (Farini et al., 2009). Since these initial attempts, various types of muscle precursor cells and stem cells have been evaluated for therapeutic potential (Peault et al., 2007; Darabi et al., 2008a).

In contrast to transplanted myoblasts, stem cells can migrate throughout the circulation; an important advantage considering the extensive mass of damaged muscles that would need to be corrected for an effective DMD therapy. Several types of stem cells have been found to have direct therapeutic relevance for the treatment of DMD. One recent example is embryonic stem cells, which were demonstrated to improve *mdx*

muscle regeneration and function after systemic transplantation (Darabi et al., 2008b). Some other promising stem cell therapy candidates include $CD133^+$ progenitor cells and mesoangioblasts because these can efficiently migrate through the blood vessels of dystrophic muscle and help reconstitute damaged fibers. Injection of immunocompromised *mdx* mice (*scid/mdx*) with $CD133^+$ progenitor cells (also known as $AC133^+$) has been found to ameliorate aspects of the dystrophic phenotype (Torrente et al., 2004), and these cells have been evaluated in clinical trials for safety (Torrente et al., 2007). Mesoangioblasts have been shown to cause recovery of dystrophin expression and restore proper muscle function in GRMD dogs when delivered intra-arterially (Sampaolesi et al., 2006). In addition to these types of cells, a distinct skeletal muscle precursor (SMP) population purified on the basis of several cell surface markers was recently shown to robustly engraft skeletal muscle, restoring dystrophin expression and causing improvements in muscle contractile function (Cerletti et al., 2008).

Many current stem cell based experimental therapies combine with gene-therapy approaches by employing *ex* vivo gene manipulation of autologous cells followed by transplantation. For instance, the aforementioned study using mesoangioblast transplantation in GRMD dogs also tested transplantation of autologous mesoangioblasts transduced with a lentivirus harboring a microdystrophin. Several of the treated animals displayed microdystrophin expression and improved morphology (Sampaolesi et al., 2006). In addition, a group was able to isolate $CD133^+$ from blood and muscle of DMD patients, and then successfully induce exon 51 skipping *ex vivo* using lentiviral vectors expressing AOs. Transplantation of these corrected cells into *scid/mdx* mice led to dystrophin expression and improved muscle function (Benchaouir et al., 2007).

This strategy of *ex vivo* manipulation of a DMD patient's stem cells followed by intra-arterial injection of the cells back into the same patient circumvents problems associated with immune rejection of transplanted cells. Moreover, *ex vivo* gene transfer avoids the possible problem of integration of the vector into the patient's genome near oncogenes since the cells can be tested for tumorigenicity prior to transplantation (Quenneville et al., 2007; Farini et al., 2009).

II.B.iii. Pharmacological therapy

Cell- and gene-based therapies hold great promise for DMD patients, but continue to be hampered by issues of safety, cost, delivery, sustained expression/survival, the potential for immune rejection, and other technical limitations. Conventional drug-based therapy avoids many of these problems, and thus continues to be a major focus for treatment.

The aforementioned therapeutic approaches all aim to introduce a functional dystrophin molecule in diseased muscles in order to provide a link between the intracellular cytoskeleton and the ECM. Other strategies attempt to counteract defects that are secondary to dystrophin deficiency through pharmacological means. One of these secondary defects stems from an immune response that exacerbates the dystrophic pathology. In this regard, treatment with immunosuppressants (Wehling-Henricks et al., 2004), modulating IKK/NF-kappaB signaling (Acharyya et al., 2007), and reducing levels of cytokines such as tumor necrosis factor (TNF) (Pierno et al., 2007) and osteopontin (Vetrone et al., 2009) have all been shown to ameliorate dystrophic features of the *mdx* mouse. Indeed, the only current pharmacological therapy for DMD is glucocorticoids, that exert their effects at least partially from their anti-inflammatoy

properties (Mendell et al., 1989; Biggar et al., 2001). Modulation of signaling by nitric oxide (NO), a potent signaling molecule that has anti-inflammatory properties, has been envisioned as a potential therapy. As previously mentioned, nNOS, the enzyme that catalyzes the production of NO, is associated with the DAPC; expressing an nNOS transgene (Wehling et al., 2001) or modulating nNOS activity (Barton et al., 2005; Voisin et al., 2005) reduces the extent of the dystrophic pathology in *mdx* mice. Oxidative stress is another secondary mechanism thought to contribute in the progression of DMD pathophysiology (Rando, 2002). Past clinic trials of using antioxidants for DMD patients were unsuccessful, but newer compounds evaluated in the *mdx* mouse show promise (Messina et al., 2006; Whitehead et al., 2008).

A major consequence of the dystrophic pathology is loss of muscle mass. Promoting muscle repair and increasing the growth and/or regeneration of dystrophic muscle by overexpressing Insulin Growth Factor (IGF)-1 (Barton et al., 2002) and blocking myostatin (Bogdanovich et al., 2002) are thus under consideration as potential therapies. Although this approach might be considered somewhat paradoxical because it aims to make more defective (dystrophin-negative) muscle, it nonetheless can cause functional improvement in DMD animal models. For instance, compensating for the loss of muscle mass using IGF-1 (Gregorevic et al., 2004a) and anabolic agents (Harcourt et al., 2007; Lynch et al., 2008) improves skeletal muscle function of *mdx* mice, and a neutralizing antibody to myostatin, MYO-029 has undergone phase I/II trials in DMD patients (Wagner et al., 2008).

In some cases, drugs approved for other diseases have been evaluated in the *mdx* mouse, and were found to improve the dystrophic phenotype. Such is the case for

imatinib, a drug approved for treating chronic myelogenous leukemia that attenuates muscular dystrophy in the *mdx* mouse apparently by modifying signaling pathways upstream of cytokine and fibrotic gene regulatory elements (Huang et al., 2009). The search for new therapeutically relevant molecules can involve high-throughput screens for regulation of proposed target genes. There is, however, no adequate *in vitro* model of the disease, making random screens difficult. *C. elegans* lacking a dystrophin-like gene has been proposed as a one model to test compounds. This approach led to the recent identification of carbonic anhydrase inhibitors as potentially useful drugs for treating DMD (Giacomotto et al., 2009).

Prior to the development of AOs as exon skipping gene-based DMD therapeutics, a pharmacological strategy using aminoglycoside antibiotics such as gentamicin to modify defective dystrophin transcripts was under investigation. Administration of gentamicin to *mdx* mice was found to restore dystrophin expression and protect against muscle damage (Barton-Davis et al., 1999). Gentamicin proved to be not a clinically useful drug, so high-throughput screening to identify compounds with similar properties was undertaken. This search identified a drug that could promote read-through of nonsense mutations that cause premature translation termination in the *DMD* gene and could provide functional benefits to *mdx* mice (Welch et al., 2007). This drug, ataluren (PTC-124), is undergoing clinical trials for treatment of the subset of DMD patients that would benefit from suppression of premature stop codons.

Other pharmacological strategies aim to overexpress certain proteins that can partially compensate for the structural defect at the sarcolemma caused by the loss of dystrophin. The predominant laminin binding integrin in skeletal muscle is $\alpha 7\ \beta 1$

integrin. Transgenic overexpression of α7-integrin ameliorates the *mdx* muscle phenotype (Burkin et al., 2001), whereas loss of its expression enhances the muscle pathology (Guo et al., 2006). Laminin is thought to regulate α7 β1 expression; in this regard, treatment using ECM proteins might benefit DMD patients since systemic administration of laminin-111 leads to improvements in *mdx* muscle function (Rooney et al., 2009). Using drugs to enhance the expression of other genes that can contribute to structural stability has also been explored. These include, for instance targeting ADAM12 (Moghadaszadeh et al., 2003a; Jorgensen et al., 2007) and GalNAc transferase (Nguyen et al., 2002; Martin et al., 2009). Finally, the most promising and well-studied target to improve the stability of the dystrophin deficient sarcolemma is utrophin, the autosomal homologue of dystrophin.

III- Utrophin

III.A. Utrophin is a suitable surrogate for dystrophin

Upregulating levels of utrophin is envisioned as a potential therapy to re-establish the integrity of dystrophic muscle fibers. Utrophin is the autosomal homologue of dystrophin, and in addition to the high degree of sequence identity between the two proteins, there is convincing evidence that utrophin can substitute for the lack of dystrophin if introduced at augmented levels at the sarcolemma. This was first demonstrated by the group of Kay Davies, who showed that introducing a truncated utrophin transgene based on the BMD-like mini-dystrophin transgene could reduce the dystrophic pathology of the *mdx* mouse (Tinsley et al., 1996). A more complete recovery was later demonstrated using a full-length utrophin transgene; interestingly, only a modest amount of utrophin overexpression at the sarcolemma led to dramatic functional improvements (Tinsley et al., 1998).

Since these pioneering studies, introducing exogenous utrophin in muscle fibers of the *mdx* mouse has been shown to restore sarcolemmal expression of DAPC members and alleviate the dystrophic pathology using a variety of approaches. These include germline transfer of utrophin (Tinsley et al., 1996; Tinsley et al., 1998), somatic gene transfer of utrophin (Gilbert et al., 1999; Deol et al., 2007), and most recently, transfer of utrophin conjugated to the HIV cell-penetrating TAT domain (Sonnemann et al., 2009). Several studies have shown that recombinant utrophin and dystrophin are equivalent with regard to their ability to prevent the dystrophic pathology when introduced by viral vectors (Ebihara et al., 2000; Odom et al., 2008). For a DMD therapy, introducing a

utrophin transgene has a significant advantage over dystrophin transgenes since exogenously delivered dystrophin has the potential to initiate a cellular immune response against muscle fibers transduced with the vector, whereas, on the other hand, utrophin is expressed in many tissues, and thus the immune system of DMD patients are more likely to tolerate its overexpression.

Although utrophin upregulation via viral vectors is a viable therapeutic approach, there are many problems hindering the realization of viral-mediated gene transfer in the clinic (see *II.B.i*). The best opportunity for a utrophin-based DMD therapy involves increasing endogenous utrophin expression in DMD muscle fibers using pharmacological interventions. Such a drug therapy could be administered to patients systemically as utrophin over-expression in tissues other than muscle does not appear to cause detrimental effects (Fisher et al., 2001). Unlike AO- and suppression of premature termination codon- based strategies, a utrophin therapy should be effective for all DMD patients, regardless of the specific genetic defect. In order to unlock the therapeutic potential of utrophin as a drug target, a thorough understanding of the mechanisms regulating its expression is necessary.

III.B. Structure of utrophin gene and proteins

A cDNA fragment that was similar to dystrophin was first identified shortly after the discovery of dystrophin, and was found to correspond to a ~13 kb transcript that maps to chromosome 6q24 (Love et al., 1989). This transcript encodes a protein of 395 kDa, that was first called dystrophin-related protein (Tinsley et al., 1992), and later named utrophin due to its more ubiquitous tissue expression pattern compared to dystrophin (Khurana et al., 1990; Helliwell et al., 1992). Similarities in gene structure imply that the

existence of the two genes is a result of an ancient duplication event (Pearce et al., 1993). The utrophin gene, found on human chromosome 6, is ~900 kb in length and is composed of 74 exons (Pearce et al., 1993; Singh and Padgett, 2009). Several short isoforms of utrophin have been characterized. Most of these are transcribed from distinct promoter elements, with some displaying restricted expression patterns outside of muscle (Blake et al., 1995; Jimenez-Mallebrera et al., 2003).

The COOH-terminal and NH_2-terminal regions of full-length dystrophin and utrophin share a high degree of sequence identity (Tinsley et al., 1992; Blake et al., 2002). Both proteins interact with the DAPC at their cysteine rich and COOH-terminal domains, and both interact with actin filaments at their NH_2 domains (Matsumura et al., 1992; Moores and Kendrick-Jones, 2000) (**Fig. 2**). There is also a contribution of the central rod domain to the binding of actin filaments, although this appears to involve distinct modes of contact for the two proteins (Amann et al., 1999; Rybakova et al., 2006). Despite the similarities in the primary structure of the COOH-terminal domains, utrophin might interact differently with DAPC members. For instance, colocalization of utrophin with α-syntrophin at the NMJ is lost in α-syntrophin null mice, whereas dystrophin localization is unaffected (Adams et al., 2000).

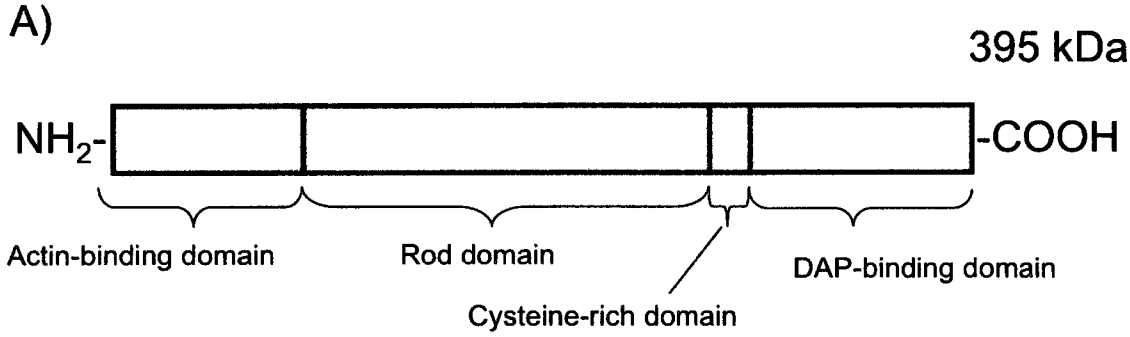
III.C. Expression pattern of utrophin

One of the main differences between full-length utrophin and dystrophin lies in their contrasting expression patterns. Early work established that in mature skeletal muscle fibers dystrophin is expressed at high levels along the entire length of the sarcolemma, whereas utrophin expression is confined to the myotendinous and neuromuscular junctions (NMJ) (Khurana et al., 1991; Ohlendieck et al., 1991). A

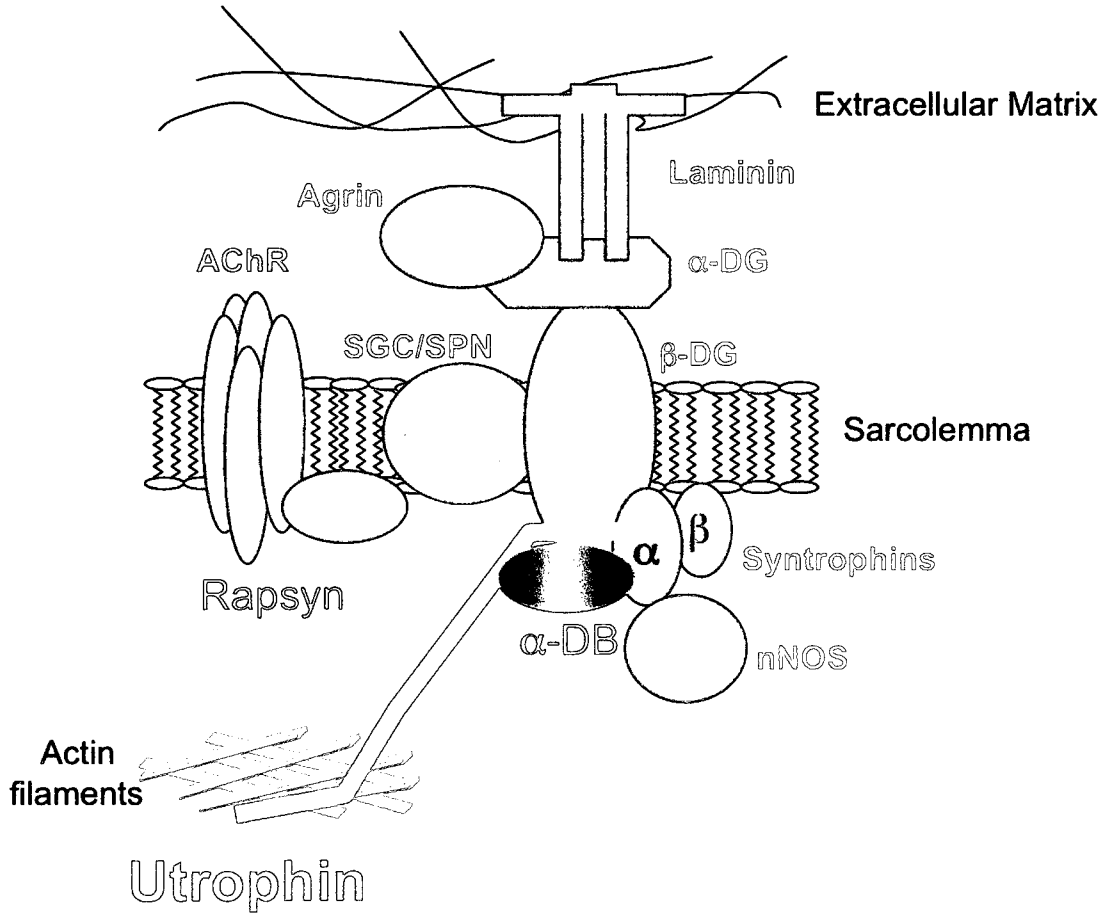
Fig. 2. Utrophin A Associates with the DAPC at the postsynaptic sarcolemma

A) Organization of utrophin A protein. B) Utrophin A expression in mature muscle fibers is primarily restricted to the neuromuscular junctions (NMJ) at the membrane in close proximity to acetylcholine receptors (AChR). Like dystrophin, utrophin A links the ECM with the intracellular cytoskeleton by associating with actin filaments at its NH_2-terminus and the DAPC at its COOH- terminus. For simplicity, only some members of the DAPC are shown. β-DG, β -dystroglycan; α-DG, α -dystroglycan; nNOS, neuronal nitric oxide synthase; sarcoglycan complex (SGC); sarcospan (SPN); α-dystrobrevin (α-Db).

A)


395 kDa
NH2-
-COOH
Actin-binding domain
Rod domain
DAP-binding domain
Cysteine-rich domain

B)


Extracellular Matrix
Laminin
Agrin
α-DG
AChR
SGC/SPN
β-DG
Sarcolemma
β
α
Syntrophins
Rapsyn
α-DB
nNOS
Actin
filaments
Utrophin

challenge for a utrophin-based drug therapy, therefore, is to stimulate expression of utrophin at appropriate levels along the entire sarcolemma of dystrophic muscle fibers including both synaptic and extrasynaptic compartments.

The expression and localization of utrophin when dystrophin expression is low suggests that endogenous utrophin can compensate for dystrophin. In contrast to the adult expression pattern, utrophin is expressed at high levels at the sarcolemma during development, and precedes expression of dystrophin (Clerk et al., 1993; Lin and Burgunder, 2000). This implies that utrophin performs the function of dystrophin in fetal and developing muscle fibers. Drug-based utrophin therapy can thus be thought of as an attempt to re-activate sarcolemmal expression of the fetal form of dystrophin. Such a strategy is not without precedent, as re-activation of fetal hemoglobin by drugs that induce its synthesis is a treatment for various β-hemoglobinopathies (Testa, 2009).

Other evidence points to a protective role for utrophin at the sarcolemma when dystrophin is absent. Mice that lack both utrophin and dystrophin (*dko*) exhibit a much more dramatic muscle phenotype compared to when utrophin or dystrophin are individually absent (Deconinck et al., 1997; Grady et al., 1997). The decline of skeletal muscle utrophin expression at ~15 days coincides with the onset of muscle necrosis in *mdx* mice (Roma et al., 2004), again suggesting a protective role. Utrophin levels are upregulated in striated muscles of the *mdx* mouse and in DMD patients, perhaps as a compensation for the lack of dystrophin (Karpati et al., 1993; Pons et al., 1994; Gramolini et al., 1999a). Recent studies have also shown that in DMD patients, utrophin

expression positively correlates with the length of time before a patient becomes wheelchair bound (Kleopa et al., 2006).

II.D. Utrophin A and utrophin B isoforms

Two full-length isoforms of utrophin have been identified and are called utrophin A and utrophin B. These two isoforms show tissue, cellular- and subcellular- specific expression patterns. The utrophin B isoform was identified after the A isoform (Burton et al., 1999); therefore, earlier descriptions showing near ubiquitous expression of utrophin, including striated muscle, smooth muscle, brain, peripheral nerves and kidney (Blake et al., 2002), did not differentiate between the two.

In skeletal muscle fibers, utrophin A is the major utrophin isoform, with its expression in mature fibers primarily confined to the myotendinous and neuromuscular junctions (Ohlendieck et al., 1991; Weir et al., 2002). However, sarcolemmal expression of utrophin A can be found under several conditions, including muscle regeneration, in *mdx* versus wild-type mice, and in slow-oxidative versus fast-glycotlytic muscle fibers (Gramolini et al., 1999a; Gramolini et al., 2001b; Weir et al., 2002). In contrast, utrophin B is not expressed within the muscle fiber, but is confined in striated muscles to the vascular endothelium (Weir et al., 2002; Baby et al., 2009).

Utrophin A and B display differential expression patterns in the brain, with utrophin A expression higher in neurons and astrocytes (Baby et al., 2009). Utrophin A expression is greater than utrophin B in the skeletal muscle, kidney and the choroid plexus, whereas their relative expression in lysates from lung and heart are more similar (Weir et al., 2002; Baby et al., 2009).

The two full-length isoforms are transcribed from distinct promoters and have distinct 5'-untranslated regions (5'-UTRs) (Dennis et al., 1996; Burton et al., 1999). The utrophin B promoter is found downstream from the translational start site of utrophin A, within the second intron of the utrophin gene. The two transcripts exhibit a common splicing pattern for exons 3 through 74. The resultant proteins are thus identical, except for N-terminal regions of 26 amino acids encoded by exon 2A for utrophin A, and 31 amino acids encoded by exon 1B for utrophin B (Burton et al., 1999) (**Fig. 3**).

III.E. Transcriptional regulation of utrophin A

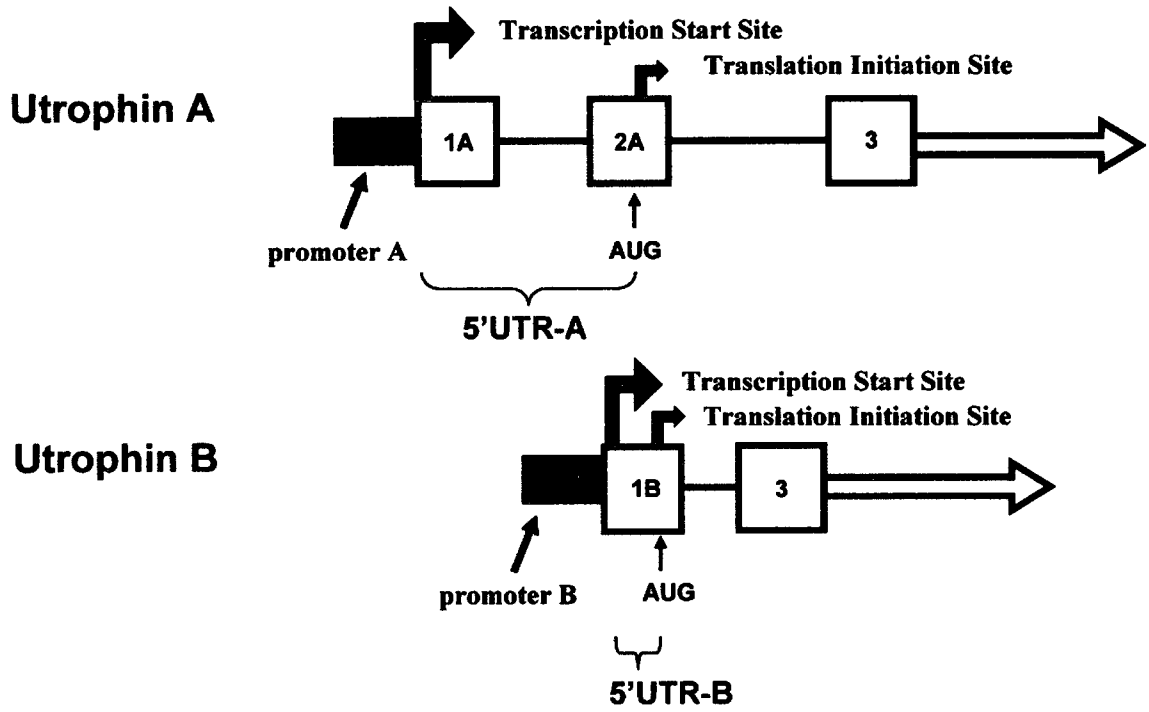
Given the similarities between dystrophin and utrophin proteins, it was hypothesized that differences in transcriptional regulation could account for the restricted expression pattern of utrophin in skeletal muscle. This lead to intensive investigation into the regulatory elements located within the utrophin A promoter (**Fig. 4**). The utrophin A promoter lacks the conventional TATA and CAAT motifs found in most eukaryotic promoters and is CpG rich (Dennis et al., 1996). The promoter region contains an N-box motif, which is an element known to be critical for directing the transcription of genes within the postsynaptic sarcoplasm (Koike et al., 1995). This element was shown to be important for the synaptic expression of utrophin A (Gramolini et al., 1997; Gramolini et al., 1998). The promoter A N-box motif is targeted by the ets-related transcription factor referred to as growth-associated binding protein (GABP) α/β (Gramolini et al., 1999b; Khurana et al., 1999). To induce utrophin transcription, GABPα/β is activated by an extracellular signal-related kinase (erk) pathway via the release of the nerve-derived trophic factor heregulin/neuregulin (NRG-1) and its interaction with ErbB tyrosine kinase receptors (Khurana et al., 1999). Heregulin also exerts epigenetic regulation of the

utrophin A promoter through the histone H3 kinase MSK1/2 (Basu et al., 2007). In this way, signals from the nerve terminal can direct the expression of utrophin within the postsynaptic sarcoplasm. Interestingly, the N-box motif appears to also be a target of repressive factors that work to keep utrophin A expression low at the sarcolemma. The Ets-2 repressor factor (ERF) has been shown to bind the N-box and repress utrophin A promoter activity, in particular at extrasynaptic regions of the sarcolemma (Perkins et al., 2007). The importance of the utrophin A promoter in driving transcription at the NMJ has been validated *in vivo*, using transgenic mice expressing a utrophin A promoter-reporter (Stocksley et al., 2005).

In addition to the N-box motif, the promoter region of utrophin A contains G/C elements that are targeted by Sp1 and Sp3 zinc finger-containing transcription factors that cooperate with GABP to activate transcription (Galvagni et al., 2001; Gyrd-Hansen et al., 2002). The phosphatase inhibitor okadaic acid can regulate activation of the utrophin A promoter through Sp1 (Rodova et al., 2004). Myogenic differentiation leads to the up-regulation of several synaptic proteins through the binding of the MyoD family of transcription factors to a DNA element called an E-box motif (Weintraub, 1993). Myogenic differentiation causes a ~2 fold increase in utrophin mRNA levels. Myogenic regulatory factors (MRFs) MyoD, myogenin and MRF4 all activate the utrophin A promoter, with this activation being abolished by mutation of an E-box in the promoter (Gramolini and Jasmin, 1999; Perkins et al., 2001). In addition to elements located in the utrophin A promoter, there is an intronic enhancer within the second intron of utrophin that regulates transcription of utrophin (Galvagni et al., 2002), and this element was found to be involved in driving activity of a reporter in skeletal muscles of transgenic

Fig. 3. Promoters and exons of utrophin A and utrophin B transcripts

The utrophin A transcript is driven by a CpG island-containing promoter. The 508 bp 5'-UTR expands through the first exon (termed 1A), into the second exon (2A). The translation initiation site is located within exon 2A. The utrophin B transcript is driven by a separate TATA- Inr+ promoter that is located within the large second intron of the utrophin gene. The translational start site is located in the first exon, termed 1B, and is preceded by a 5'-UTR of 74 nucleotides that is different from the utrophin A 5'-UTR. Exon 1B splices to exon 3 which is shared in common between the A and B transcripts.


Utrophin A
Transcription Start Site
Translation Initiation Site
1A
2A
3
promoter A
AUG
5'UTR-A
Utrophin B
Transcription Start Site
Translation Initiation Site
1B
3
promoter B
AUG
5'UTR-B

mice (Tanihata et al., 2008) (See **Fig. 4** for summary of utrophin A promoter elements).

III.F. Role of the slow myogenic program in regulating utrophin A expression

Transcriptional events known to be important for the regulation of skeletal muscle fiber type also play a role in regulating utrophin expression. Muscle fiber type can be classified based on the composition of the four different myosin heavy chain (MyHC) isogenes. Slow twitch muscle fibers express type I myosin heavy chain (MyHC), use primarily oxidative metabolism and thus are more resistant to fatigue. Type II MyHC containing fibers are classified into IIa fibers (also classified as slow twitch), which are both glycolytic and oxidative, and type IIx and IIb fibers which are primarily glycolytic (fast twitch) (Rivero et al., 1999). Fiber type specific gene expression is influenced by signals from the motor nerve, including regulation of intracellular Ca^{2+} levels (Sreter et al., 1987). Ca^{2+} dependent signaling mechanisms regulate expression of slow MyHCs and genes associated with oxidative metabolism (Meissner et al., 2000; Goldspink, 2003). Several Ca^{2+} responsive enzymes and transcription factors have been identified that can regulate fiber type, and these fall under two transcriptional signaling pathways, the calcineurin/NFAT (nuclear factor of activated T cells) and the CaMKII (Ca^{2+}/CaM-dependent kinase II) (Michel et al., 2007).

It has been known for some time that fast fibers of DMD patients are subject to greater damage that their slow counterparts (Webster et al., 1988). Moreover, a differential susceptibility to damage between these two muscle types has also been observed in the *mdx* mouse (Moens et al., 1993). A possible explanation for the reduced damage in the slower, more oxidative phenotype comes from the observation that utrophin is expressed in extrasynaptic regions of these fibers, and thus may mediate a

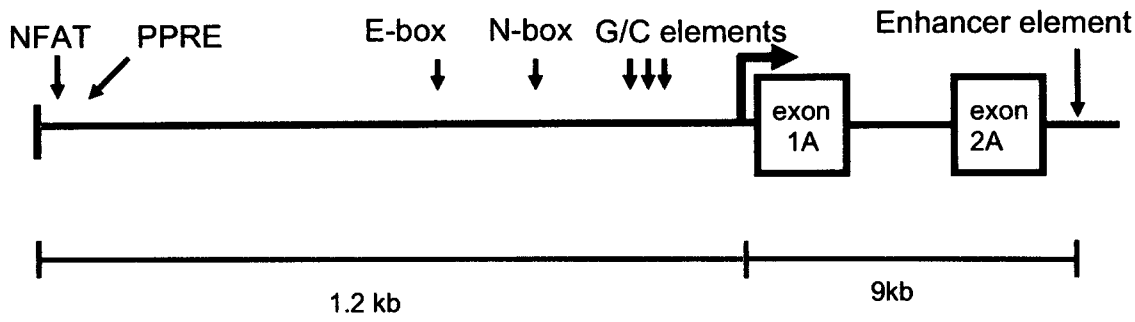


Fig. 4. Utrophin A promoter *cis*-regulatory elements.

Shown is a schematic representation of transcriptional *cis*-elements in the human utrophin A promoter region. Arrow denotes transcriptional start site. Diagram is not drawn to scale. The enhancer element is located in intron 2 of the utrophin gene. PPRE-PPAR response element half site.

protective effect against muscle damage (Gramolini et al., 2001b). In further support of a role for the slow myogenic program in regulating utrophin levels, it has been shown that stimulation of the slow muscle phenotype by functional overload results in greater expression of utrophin A (Chakkalakal et al., 2003a). These results suggested that regulatory mechanisms that determine the oxidative and contractile properties of skeletal muscle fibers contribute to the regulation of utrophin A expression.

III.F.i. Calcineurin/NFAT and Ca^{2+}/calmodulin signaling

Since the Ca^{2+} and calmodulin-dependent serine/threonine phosphatase calcineurin is known to be an important regulator of the slow myogenic program (Olson and Williams, 2000), it was hypothesized, based on the above findings, that calcineurin signaling could play a major role in regulating utrophin expression. Indeed, calcineurin activation can increase utrophin A mRNA levels in slow mouse muscle, whereas inhibition of calcineurin activity with cyclosporine reduces levels of utrophin A transcripts (Chakkalakal et al., 2003a). Calcineurin exerts at least part of its effects through activation of the transcription factor Nuclear Factor of Activated T cells (NFAT). Indeed, the utrophin A promoter region contains an NFAT binding site, and over-expression of NFAT induces transcription from the utrophin A promoter in muscle cell cultures (Chakkalakal et al., 2003a). Additionally, mutation of this NFAT binding site abolishes this effect (Angus et al., 2005). Together, these data suggested that stimulation of the slow myogenic program and increasing utrophin levels through activation of calcineurin/NFAT might be appropriate as a therapy to protect against degeneration of dystrophic muscle fibers.

III.F.ii. PGC-1α

Peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α) is a transcriptional co-factor that is important in driving the formation of slow-twitch muscle fibers. Overexpression of PGC-1α in skeletal muscle fibers by transgenic means causes a shift to MyHC type I and type IIa muscle fibers (Lin et al., 2002), whereas a skeletal muscle specific knockout of PGC-1α causes a shift from slow I and IIa fibers, to fast IIb and IIx fibers (Handschin et al., 2007b). PGC-1α was demonstrated to cooperate with GABPα to activate transcription of utrophin A (Angus et al., 2005). Following up on this discovery, it was found that PGC-1α in cooperation with GABP appears to play a role in regulating the expression of a broad range of muscle proteins that are expressed at the NMJ, including, for example, AChR subunits, ErbB2 and utrophin (Handschin et al., 2007a). Moreover, it was shown that heregulin stimulates phosphorylation of PGC-1α, and stimulation of NMJ gene expression by heregulin requires PGC-1α. Thus heregulin and PGC-1α work together in the same pathway to stimulate utrophin A promoter activity through GABP.

III.G. Compensating for dystrophin-deficiency by stimulating endogenous utrophin A expression

The mechanisms of utrophin A transcriptional regulation discussed above provide multiple targets for pharmacological interventions. Enabled by current understanding of the utrophin A promoter, several groups have been searching for small molecules or peptides that can stimulate transcription of utrophin. Utrophin transcription in muscle fibers of DMD patients could be activated directly through administration of transcription

factors/co-factors, or indirectly through either administration of growth factors or activation of upstream signaling pathways.

It is important to note again that only a modest enhancement of utrophin A levels at the sarcolemma is sufficient to counteract the progression of DMD in the *mdx* mouse. It has been shown that transgenic overexpression of full-length utrophin by only ~2 fold can favorably alter the dystrophic pathology in skeletal muscles (Tinsley et al., 1998). The timing of utrophin A enhancement is also important, given the age of pathology onset in the *mdx* mouse. Expression of a utrophin transgene induced post-natally is less beneficial than when expression is induced in utero (Tinsley et al., 1998).

III.G.i. Enhancing calcineurin/NFAT signalling and PGC-1α mitigates the dystrophic pathology

Since calcineurin/NFAT signaling plays a role in controlling expression of utrophin A, it is possible that manipulation of this pathway could be used as a therapeutic strategy to achieve increased expression of utrophin in dystrophic muscle. Proof of concept for such an approach came recently from experiments which showed that introduction of a transgene containing an activated form of calcineurin into *mdx* mouse muscles caused a ~ 2-fold increase in utrophin as well as significant improvements in several markers of the dystrophic pathology (Chakkalakal et al., 2004). In particular, dystrophin-deficient muscle fibers expressing the activated form of calcineurin showed an attenuation in muscle fiber damage as determined by a reduction in both fiber size variability and central nucleation. In addition, these mice exhibited decreased inflammation and a restoration of sarcolemmal integrity. Later experiments found that activating calcineurin also protects *mdx* muscles against contraction-induced injury

(Stupka et al., 2006) and enhances aspects of muscle regeneration (Stupka et al., 2008). Thus, activating calcineurin, or its downstream effector NFAT, is a potential therapy for DMD.

Further support for modulation of Ca^{2+} signaling as a therapeutic target for treating DMD comes from a study where *mdx* mice were crossed with mice expressing a CaM-binding peptide (CaMBP). CaMBP inhibits downstream signaling of calcineurin and CaMKs. Loss of function of these downstream pathways caused a downregulation of utrophin A expression and an exacerbation of the dystrophic pathology (Chakkalakal et al., 2006).

As previously mentioned, PGC-1α has been shown to be a key factor in regulating transcription of utrophin A (Angus et al., 2005). To test the suitability of PGC-1α as a therapeutic target for the treatment of DMD, *mdx* mice were crossed with PGC-1α expressing transgenic mice. A mitigation of several dystrophic symptoms was observed, including lower CK levels, reduced muscle damage and improved muscle function (Handschin et al., 2007a). Unfortunately, therapeutically useful molecules that act as activators of the slow myogenic program through calcineurin/NFAT signaling, Ca^{2+}/calmodulin signaling, and PGC-1α have not been identified. However, clinically relevant agonists to another factor that can mediate a fibre composition shift to more slow fibers have been developed. This factor is the nuclear receptor, Peroxisome proliferator-activated receptor (PPAR) *β/δ*.

III.G.ii. Possible role for PPARβ/δ

PPARs are part of the nuclear receptor superfamily of ligand-activated transcription factors. They are known to influence the transcription of genes involved in lipid metabolism and oxidative respiration (Luquet et al., 2005; Barish et al., 2006). These receptors form heterodimers with the retinoic X receptor (RXR) and bind to PPAR response elements (PPRE) of target promoters to stimulate transcription. All three PPAR isoforms α, γ and δ are present in skeletal muscle but the expression of PPARδ (also known as PPARβ, hereafter referred to as PPARβ/δ) in this tissue is 10- to 50-fold higher than the other isoforms (Muoio et al., 2002; Wang et al., 2004). An interaction between PGC-1α and PPARβ/δ has been demonstrated, and this interaction can be promoted by PPARβ/δ agonist treatment (Wang et al., 2003). Transgenic expression of activated PPARβ/δ causes a fiber composition shift to more slow, oxidative fibers, and enhanced endurance capacity (Luquet et al., 2003; Wang et al., 2003). Synthetic ligands to various PPARs are clinically useful drugs for the treatment of lipid disorders. Importantly, an experimental synthetic agonist of PPARβ/δ, GW501516, has been evaluated in clinical trials for the treatment of diabetes and dyslipidemia; thus, should GW501516-treatment show promise as a therapeutic in dystrophic animal models, its application as a therapy for DMD patients could be streamlined.

III.G.iii. Additional approaches to enhance endogenous utrophin A levels

An approach to upregulate endogenous utrophin A levels that could be developed into a DMD therapy involves heregulin treatment to stimulate utrophin A promoter activity. It has been shown that administration of a small peptide containing the epidermal growth factor-like region of heregulin ectodomain to *mdx* mice can increase

endogenous utrophin expression by ~3-fold (Krag et al., 2004). This caused amelioration of the dystrophic phenotype, as evidenced by a reduction in muscle degeneration and inflammation, and a decreased susceptibility of muscles to damage induced by eccentric contractions. The improvement in muscle function was deemed to specifically result from the upregulation of utrophin because treatment of mice deficient in both utrophin and dystrophin expression (dko mice) failed to reduce the muscle pathology.

Corticosteroid treatment, the only current drug therapy for DMD patients, has been shown to improve the dystrophic pathology of *mdx* mice (Anderson et al., 1996; Fisher et al., 2005; Keeling et al., 2007). In the case of treatment with the synthetic glucocorticoid deflazacort, this improvement coincides with enhanced utrophin A expression (St Pierre et al., 2004). Based on the finding that overexpression of nNOS can reduce the dystrophic pathology of the *mdx* mouse, administration of L-arginine, a substrate for nNOS, has been tested for potential therapeutic benefits. L-arginine has been reported to increase utrophin expression by as much as 2 to 3-fold in *mdx* mice, and indeed can cause improvements in muscle necrosis, contraction induced damage and inflammation (Chaubourt et al., 1999; Barton et al., 2005; Voisin et al., 2005; Hnia et al., 2008). Treatment with L-arginine, or other modulators of nNOS activity such as molsidomine (Voisin et al., 2005), could thus be potentially used as therapeutics for DMD. Additional drug treatments have been shown to improve the *mdx* mice pathology and enhance utrophin levels without direct evidence for utrophin acting as the mediating factor. For example, treatment with epigallocatechin-3-gallate and N-Acetylcysteine both improve the dystrophic phenotype and are associated with enhanced utrophin levels (Nakae et al., 2008; Whitehead et al., 2008).

Another approach to stimulate endogenous utrophin A expression in dystrophic muscle involves introducing artificial zinc finger-based transcription factors (ZF ATFs) that target the utrophin A promoter. One of these ZF ATFs, named Jazz, binds to a 9 bp element in the utrophin A promoter (Corbi et al., 2000). When Jazz is expressed in transgenic mice crossed with *mdx* mice, utrophin A expression is augmented by 1.8-fold and the dystrophic pathology is prevented (Di Certo et al., 2009). Although ZF ATFs are small, active at low concentrations, and have low immunogenicity, the factors would have to be delivered to DMD muscles using gene-therapy strategies or perhaps in the form of fusion proteins conjugated to cell penetrating peptides (Sonnemann et al., 2009). The limitations and impediments to realizing a therapy based on ZF ATFs are thus similar to those that involve introducing recombinant utrophin by gene-based approaches.

V. Post-transcriptional regulation of Gene Expression

One of the fundamental tenets in molecular biology is that genetic information is transmitted from DNA to mRNA to proteins. It was once assumed that the number of genes in eukaryotes would be proportional to biological complexity as a result of more genes encoding for more proteins. Whole-genome sequencing of organisms has revealed that this is certainly not the case, with rice having more protein coding genes than humans (Goff et al., 2002). Only a small proportion, ~2%, of the human genome is comprised of protein coding sequences (Venter et al., 2001). This sequencing data, along with decades of biological research, suggest that the regulation of gene expression, not the number of genes, is the core of biological complexity (Taft et al., 2007).

Regulation of gene expression was previously thought to occur almost exclusively through the interaction of transcription factors with *cis*-elements upstream of protein coding genes, as described in the lac operon model (Jacob and Monod, 1961). However, today RNA has emerged as much more than just a passive transmitter of information from DNA to proteins; instead RNA plays a central role in regulating gene expression. We now know that mRNA processing, alternative splicing, nuclear export, stability, transport and translation are all highly regulated processes. These post-transcriptional mechanisms can be regulated by proteins but also by non-coding RNA that operates through complementary pairing to target mRNAs, for instance in the form of short RNAs such as microRNAs (Bartel, 2009).

V.A. Evidence suggesting post-transcriptional control of utrophin expression

Several lines of evidence suggest that transcriptional mechanisms cannot account for the differential expression of utrophin under various conditions that have physiological and disease relevance. This first evidence for this came from a study that showed elevated expression of utrophin protein, but not its mRNA, in DMD patient skeletal muscles compared to those of healthy subjects (Gramolini et al., 1999a). This same study found that upregulation of utrophin protein during toxin-induced skeletal muscle regeneration was not accompanied by a similar induction in utrophin mRNA levels. Similar observations of upregulated utrophin A protein, but not mRNA have been made in *mdx* mice cardiac and skeletal muscles compared to wild-type mice (Weir et al., 2002). Changes in skeletal muscle utrophin expression during the lifespan of normal and *mdx* mice also appear to occur in a transcription-independent manner (Roma et al., 2004). In response to glucocorticoid treatment of muscle cells, utrophin protein, but not mRNA expression is enhanced (Pasquini et al., 1995; Courdier-Fruh et al., 2002). Other *in vivo* support for transcription independent control of utrophin come from *in vivo* studies showing upregulation of utrophin in response to sarcospan overexpression (Peter et al., 2008b), RhoA (Gauthier-Rouviere and Bonet-Kerrache, 2009) and myogenic Akt signaling (Peter et al., 2009). It is important to consider that an increase in utrophin protein independent of mRNA increase could be due to translational mechanisms or alternatively, post-translational mechanisms such as regulation of protein stability (Waheed et al., 2005).

V.B. Post-transcriptional regulation via utrophin A 3'-UTR

Regulation of mRNA stability, localization and translation occurs for multiple transcripts in skeletal muscle fibers, including subsynaptic regions of the sarcolemma (Chakkalakal and Jasmin, 2003). The 3'-UTR of mRNAs serves as a major target for post-transcriptional control. The murine utrophin 3'-UTR is ~2 kb in length, and contains multiple *cis*-elements that are targets for post-transcriptional control, including AU-rich elements (AREs), microRNA target sites and putative alternative polyadenylation signals. Early studies of post-transcriptional control found that the 3'-UTR of utrophin plays a role in mediating the stability and localization of the mRNA (Gramolini et al., 2001a). Post-transcriptional control was found to be an important contributor to the higher levels of utrophin A in slow versus fast muscles (Gramolini et al., 2001b) and later, an ARE in the utrophin 3'-UTR was found to be the *cis*-element responsible for the altered stability in these muscle types, apparently as a result of calcineurin signaling (Chakkalakal et al., 2008).

In recent years, particular attention has focused on regulation of mRNA stability and translation by microRNAs. MicroRNAs target complementary sequences generally found in the 3'-Untranslated Regions (3'-UTRs) of mRNAs to suppress their synthesis into proteins. There are several microRNAs that show muscle-specific expression patterns, and influence myogenic differentiation and disease processes (Chen et al., 2009; Williams et al., 2009). The expression pattern of microRNAs is altered in *mdx* and DMD patient skeletal muscles, suggesting that their mis-expression may contribute to the dystrophic pathology (Eisenberg et al., 2007; McCarthy et al., 2007; Greco et al., 2009). MicroRNAs also appear to regulate utrophin A expression, as evidenced by a report

claiming that the muscle-specific microRNAs miR-1 and miR-206 regulate utrophin A translation in muscle cells (Rosenberg et al., 2006).

V.C. Utrophin A 5'-UTR

The 5'-UTR of mRNAs can serve as targets for translational control mechanisms (see section V.I.B). The murine 5'-UTR of utrophin A is 508 nucleotides long and is comprised of 58% GC nucleotides. From the transcriptional start site, the 5'-UTR expands through the utrophin exon 1A to exon 2A where the translation initiation site for utrophin A is located (Dennis et al., 1996; Burton et al., 1999) (**Fig. 3**). The intron between exon 1A and exon 2A is 7015 bp in length (the splice site is located at nucleotide 417). The human utrophin A 5'-UTR is predicted to be 560 nucleotides in length. There is 77.5% sequence identity between the mouse and human 5'-UTRs in the first 298 nucleotides, and 63% identity overall.

VI- Translational Regulation

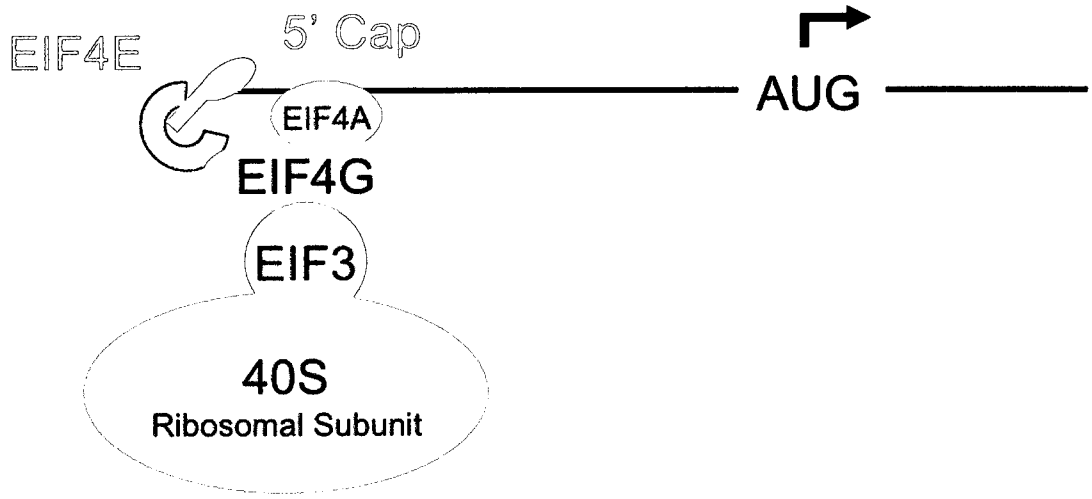
VI.A. Cap-dependent translation initiation in eukaryotes

Translational control is primarily regulated at the initiation of translation, as it is the rate-limiting step in protein translation. The majority of eukaryotic translation initiation proceeds via a scanning mechanism that is dependent on the presence of a 7-methyl guanosine cap, which is found at the 5'end of cellular mRNAs. The cap is recognized by the cap-binding factor eukaryotic initiation factor (eIF) 4E, which is one protein component of the eIF4F complex that also contains the RNA helicase eIF4A and the adaptor protein eIF4G (**Fig. 5**). The eIF4F complex recruits the 43S pre-initiation complex that consists of the 40S ribosomal subunit, the ternary complex eIF2-GTP-Met-tRNAi and eIFs 1, 1A, 3, and 5. Association of the 43S pre-initiation complex with the eIF4F cap-binding complex and the mRNA forms the 48S complex. The 48S complex scans along the 5'-UTR of the mRNA, reading successive triplets for complementarity to the Met-tRNAi anticodon. The AUG start codon, when encountered in a favorable context, matches with the anticodon of Met-tRNAi at the P (peptidyl) site of the ribosome. This causes scanning to be arrested, with eIF2-GDP and other eIFs being released. Subsequently, the large 60S ribosomal subunit joins to form the 80S initiation complex. The next appropriate aminoacyl-tRNA is accepted in the A (aminoacyl) site of the ribosome, and a peptide bond is formed, thus commencing the elongation phase of translation (Van Der Kelen et al., 2009).

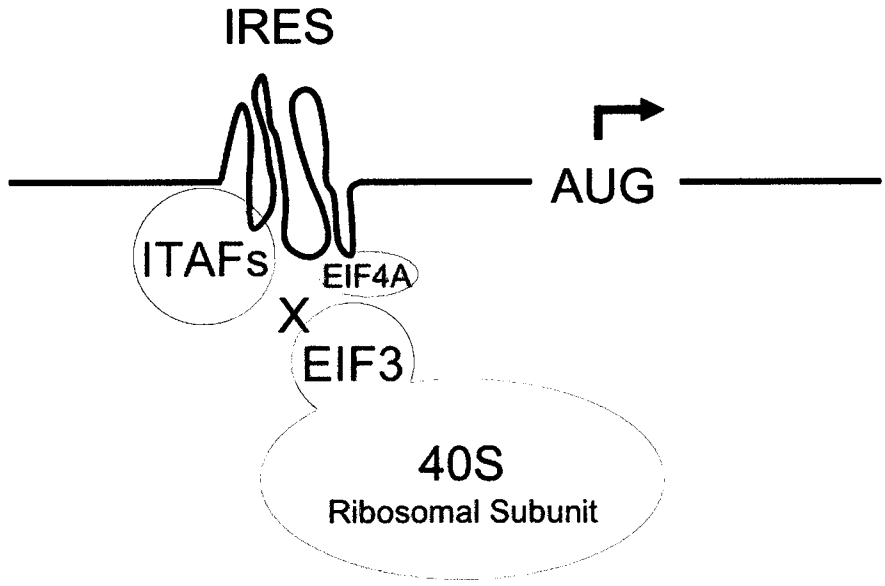
Fig. 5: Cap-dependent versus IRES-mediated translation initiation

A) eIF4E binds to the 5' 7-methyl guanosine cap of the mRNA as part of the eIF4F complex (eIF4E, eIF4A, eIF4G). eIF3 recruits the ribosome to this complex. Other eIFs and the ternary complex are not shown for simplicity. The 40S ribosomal subunit together with other factors (collectively, the 48S complex) scans along the mRNA until an AUG initiation codon is encountered. The 60S ribosomal subunit then joins, and translation elongation begins. **B)** Regions in the 5'-UTR that are composed of a sequence and structural motifs termed Internal Ribosome Entry Sites (IRES) can recruit the translational machinery independently of the 5' cap and eIF4E. Various IRES-trans-acting factors (ITAFs) mediate translation initiation through this mechanism. The involvement of other canonical translation initiation factors in IRES-mediated translation is not well understood, but in some cases includes proteolytically cleaved fragments of eIF4G (X). Figure adapted from (Holcik and Sonenberg, 2005).

A. Cap-dependent Translation initiation



B. IRES-mediated translation initiation



VI.B. Regulation of cap-dependent translation

The various steps of translation initiation provide many opportunities for regulation, including for example, AUG codon recognition, the joining of ribosomal subunits, and binding of the 43S pre-initation complex (Sonenberg and Hinnebusch, 2009). Phosphorylation events have particular importance in regulating translation initiation; for example, phosphorylation of eEF2α by stress-activated kinases (eg. GCN2, PKR, PERK) blocks ternary complex formation (Wek et al., 2006) and signaling through the mTOR pathway causes phosphorylation of multiple regulators of translation initiation, including the eIF4E-binding protein (4E-BP). In this regard, the PI3K/Akt/mTOR signaling pathway serves as a major regulator of translation initiation, responding to multiple signals including nutrient deprivation, stress and mitogens (Ma and Blenis, 2009).

It is possible that utrophin A is translationally regulated by cap-dependent mechanisms under the control of AKT signaling. Promoting myogenic AKT signaling in *mdx* mice ameliorates the dystrophic pathology, and causes a ~10-fold upregulation of utrophin protein levels. This induction is not accompanied by an increase in mRNA levels of utrophin A, suggesting that translational regulation is involved (Peter et al., 2009). Similar results have been obtained using inducible Akt transgenic mice (Blaauw et al., 2008).

VI.C. Translational regulation via the 5'-UTR of target mRNAs

Multiple mechanisms have been described to regulate translation via events targeting 5'-UTR regulatory regions. One example is regulation by upstream open

reading frames (ORFs) located in the 5'UTR of mRNAs (Sachs and Geballe, 2006). Upstream ORFs recruit the translational machinery, leading to the synthesis of short polypeptide chains and resulting in suppression of translation from the main open reading frame. Thirty percent of human genes are predicted to contain one or more upstream ORF (Suzuki et al., 2000). Another mechanism is the cap-dependent, scanning independent mechanism of initiation called ribosome shunting which is characterized by the ribosome skipping over highly structured regions of the 5'-UTR that contain hairpin loops that are inhibitory to ribosome scanning (Futterer et al., 1993). Targeting of microRNAs to the 5'-UTR provides another potential regulatory mechanism. Although microRNAs typically cause repression of gene expression via the 3'-UTR, several microRNAs have been shown to associate with the 5'-UTR of mRNAs, causing either inhibition and enhancement of translation (Lytle et al., 2007; Tsai et al., 2009).

VI.D. Cap-independent, IRES-mediated translation initiation

In some cases, translation initiation can occur independent of the 5' cap of the mRNA and independent of ribosome scanning. Highly structured regions in the 5'-UTR of picornavirus mRNAs (which are naturally uncapped) found to recruit the ribosome and components of the translational machinery provided the first examples on this alternative method of translation initiation (Jang et al., 1988; Pelletier and Sonenberg, 1988). The region meditating this type of initiation is called an Internal Ribosome Entry Site (IRES). Viral IRES elements sequester eukaryotic translation factors; thus, it was suggested that internal initiation might be a mechanism for regulation of some eukaryotic mRNAs (Pelletier and Sonenberg, 1988). The first cellular IRES-containing mRNA was discovered shortly thereafter by demonstrating that translation of BiP (human

immunoglobulin heavy chain binding protein) could occur in cells when cap-dependent translation was inhibited by poliovirus infection (Macejak and Sarnow, 1991). More than 70 cellular IRES elements have since been reported in the literature (Baird et al., 2006). Cellular mRNAs containing these elements are involved in a broad range of biological functions. Activation of several of these cellular IRES elements occurs under conditions where the availability of the cap-dependent translational machinery is compromised. Through this mechanism, the cell can maintain, or enhance protein synthesis for selected genes under stress conditions (Spriggs et al., 2008). The ability of IRES elements to drive translation under conditions where canonical translation factors are depleted suggests that alternative *trans*-acting factors are involved in IRES mediated translation.

VI.E. Mechanism of IRES-mediated translation

Association of the ribosome to viral IRES elements appears to depend mostly on the structure of the RNA. Study of viral IRES elements has led to a categorization of different IRES types based on common structures and requirement for interacting initiation factors and other RNA-binding proteins (RNA-BPs) (Hellen, 2009). The importance of structural determinants for driving IRES-mediated translation is highlighted by the observation that some viral IRESes can permit formation of active ribosomes without translation initiation factors (Wilson et al., 2000; Pestova and Hellen, 2003). Multiple mechanisms through which viral IRES-mediated translation occurs have been elucidated, such as modification of ribosome confirmation to permit mRNA entry, stabilization of the positioning of Met-tRNAi to the P-site of the ribosome, and regulation of the joining of ribosomal subunits (Hellen, 2009). In contrast, the *cis*-elements and structural requirements for cellular IRESes are less well defined (Baird et al., 2006).

Attempts to identify common structural motifs have been largely unsuccessful to date (Baird et al., 2007).

Instead of IRES elements being defined by general sequences and structures that permit cap-independent translation initiation, it appears that IRES-activity of cellular mRNAs depends on the interaction of the message with particular RNA-BPs along with a subset of eIFs (Lewis and Holcik, 2008; Fitzgerald and Semler, 2009) (**Fig. 5**). In keeping with this hypothesis, IRES-mediated translation for individual cellular mRNAs would seem to require a specific set of RNA-BPs tailored to regulate translation in a cell- and stress-specific manner. A range of RNA-BPs have been identified that are involved in cellular IRES-mediated translation (Fitzgerald and Semler, 2009). These RNA-BPs are called ITAFs (IRES *trans*-acting factors). Only a handful of ITAFs have been shown to regulate more than one IRES element; these include for example, polypyrimidine-tract binding protein (PTB), lupus autoantigen (La) and DAP5 (Spriggs et al., 2005). Multiple ITAFs can work in concert to enhance translation of IRES-containing mRNAs (Mitchell et al., 2001; Pickering et al., 2003), whereas other ITAFs have been identified as negative regulators of IRES activity (Lewis et al., 2007b). Several ITAFs are nucleo-cytoplasmic shuttling proteins. The stress-mediated localization of these proteins in the cell can regulate IRES activity of target mRNAs (Lewis and Holcik, 2008). To date, no universal ITAF has been identified, although PTB positively regulates several IRESes, apparently depending on the presence of a $(CCU)_n$ motif (Mitchell et al., 2005; Bushell et al., 2006a). Various mechanisms have been proposed on how ITAFs might generally regulate IRES-translation, such as by acting as chaperones to modify RNA structure and providing access to the ribosome (Spriggs et al., 2005).

2. STATEMENT OF PROBLEM, HYPOTHESES AND OBJECTIVES

Statement of problem and hypotheses: DMD is a fatal disorder for which there is no curative therapy. Utrophin can compensate for the lack of dystrophin in animal models of DMD, suggesting that upregulation of this protein in muscle fibers of DMD patients could prevent or mitigate the dystrophic pathology. A putative treatment for DMD involves using drug therapy to stimulate endogenous expression of utrophin. To realize this goal, a thorough understanding of the mechanisms regulating the expression and localization of utrophin A in skeletal muscle fibers is necessary.

Previous work has established that factors associated with the slow myogenic program regulate expression of utrophin A mRNA in skeletal muscle. PPARβ/δ is a nuclear receptor that plays a role in determining the slow-oxidative characteristics of muscle fibers, and a target site for PPARβ/δ is present in the utrophin A promoter. Synthetic ligands to PPARβ/δ are available. One of these agonists, GW501516, has been evaluated in clinical trials for the treatment of diabetes and dyslipidemia. **Hypothesis 1:** We hypothesize that treatment of *mdx* mice with GW501516 will activate PPARβ/δ and stimulate utrophin A expression leading to improvements in the dystrophic pathology.

Upregulation of utrophin A protein, but not mRNA, occurs in response to several physiologically relevant conditions in skeletal muscle, suggesting that regulatory mechanisms other than transcription are responsible for this expression pattern. **Hypothesis 2:** We hypothesize that the utrophin A expression can be translationally

regulated via events targeting the utrophin A 5'UTR, thus accounting for the upregulation of utrophin A protein, but not mRNA, in response to muscle regeneration and glucocorticoid treatment.

Objectives:

1- DETERMINE WHETHER ACTIVATION OF THE NUCLEAR RECEPTOR PPARβ/δ WITH GW501516 STIMULATES UTROPHIN A EXPRESSION AND PROVIDES PROTECTION TO DYSTROPHIC MUSCLE FIBERS (CHAPTER 2).

By monitoring mRNA levels and promoter activity of utrophin A, we will determine whether utrophin A expression can be enhanced by GW501516 treatment. Long-term treatment of *mdx* mice will be performed to assess whether PPARβ/δ activation can stimulate the slow myogenic program, restore stability to the sarcolemma, and confer functional improvements to dystrophic muscle fibers.

2- DETERMINE WHETHER UTROPHIN A IS TRANSLATIONALLY REGULATED VIA THE 5'-UTR DURING MUSCLE REGNERATION (CHAPTER 3).

The protein and mRNA expression pattern of utrophin is discordant in regenerating skeletal muscle, suggesting that translational control mechanisms might play a role. Using utrophin A 5'-UTR monocistronic reporter construct injections in control and regenerating muscles, we will determine whether the utrophin A 5'-UTR is a target of translational regulation under these conditions. We will test whether this translational

regulation occurs through Internal Ribosome Entry Site (IRES)-Mediated translation by performing bicistronic reporter experiments in skeletal muscles and C2C12 cells.

3- DETERMINE WHETHER UTROPHIN A IS REGULATED VIA IRES-MEDIATED TRANSLATION IN RESPONSE TO GLUCOCORTICOID TREATMENT (CHAPTER 4).

Corticosteroids are currently the only drugs used to treat DMD patients. Although it has been assumed that the benefit for patients comes from the anti-inflammatory effects of these drugs, the mechanism of action has not been determined. Utrophin protein is known to be upregulated in response to glucocorticoid treatment in cultured muscle cells. Using transient transfections and polysome profiling, we will determine whether translational regulation via the utrophin A IRES can account for this increase.

4- IDENTIFY TRANS-ACTING FACTORS THAT REGULATE THE UTROPHIN A IRES (CHAPTER 5).

IRES-mediated translation is controlled by the association of RNA-binding proteins known as IRES trans-acting factors (ITAFs). RNA-affinity chromatography will be employed to identify ITAFs that regulate IRES-mediated translation of utrophin A in skeletal muscle.

5- DETERMINE THE EXPRESSION PATTERN OF UTROPHIN A IRES ACTIVITY IN VIVO (CHAPTER 5).

IRES mediated translation of some cellular mRNAs occurs in a cell- and tissue-specific manner. Transgenic mice will be generated that harbor the utrophin A 5'UTR

bicistronic reporter construct. The pattern of IRES activity will be determined in various tissues of these mice, providing insight into the conditions and factors necessary to mediate translational regulation of utrophin A.

CHAPTER 2

Pharmacological activation of PPARβ/δ stimulates utrophin A expression in skeletal muscle fibers and restores sarcolemmal integrity in mature *mdx* mice

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Contributions of Authors: Conceived and designed the experiments: PM JVC JR BJJ. Performed the experiments: PM GB LB. Analyzed the data: PM LB JR BJJ. Provided Reagents: RH. Wrote the paper: PM BJJ.

Abstract

A therapeutic strategy to treat Duchenne muscular dystrophy (DMD) involves identifying compounds that can elevate utrophin A expression in muscle fibers of affected patients. The dystrophin homologue utrophin A can functionally substitute for dystrophin when its levels are enhanced in the *mdx* mouse model of DMD. Utrophin A expression in skeletal muscle is regulated by mechanisms that promote the slow myofiber program. Since activation of PPARβ/δ promotes the slow oxidative phenotype in skeletal muscle, we initiated studies to determine whether pharmacological activation of PPARβ/δ provides functional benefits to the *mdx* mouse. GW501516, a PPARβ/δ agonist, was found to stimulate utrophin A mRNA levels in C2C12 muscle cells through an element in the utrophin A promoter. Expression of PPARβ/δ was greater in skeletal muscles of *mdx* versus wild-type mice. We treated 5-7 week old *mdx* mice with GW501516 for 4 weeks. This treatment increased the percentage of muscle fibers expressing slower myosin heavy chain isoforms, and stimulated utrophin A mRNA levels leading to its increased expression at the sarcolemma. Expression of α1-syntrophin and β-dystroglycan was restored to the sarcolemma. Improvement of *mdx* sarcolemmal integrity was evidenced by decreased intracellular IgM staining and decreased *in vivo* Evans blue dye uptake. GW501516 treatment also conferred protection against eccentric contraction (ECC)-induced damage of *mdx* skeletal muscles, as shown by a decreased contraction-induced force drop and reduction of dye uptake during ECC. These results demonstrate that pharmacological activation of PPARβ/δ might provide functional benefits to DMD patients through enhancement of utrophin A expression.

Introduction

Duchenne Muscular Dystrophy (DMD) is a fatal muscle wasting disorder that results from mutations in the dystrophin gene (Anderson and Kunkel, 1992). A potential therapy for DMD involves stimulating expression of several target genes that have the ability to functionally compensate for the lack of dystrophin at the sarcolemma (Engvall and Wewer, 2003; Khurana and Davies, 2003). Utrophin is the autosomal homologue of dystrophin, and enhancing its expression by moderate amounts (2-fold) in animal models of DMD can compensate for the lack of dystrophin in skeletal muscle fibers and alleviate the muscle pathology(Tinsley et al., 1998; Chakkalakal et al., 2004; Krag et al., 2004; Miura and Jasmin, 2006; Peter et al., 2008a). Given the benefits that utrophin overexpression confers on dystrophic muscle fibers, it has been of considerable interest to elucidate the mechanisms that control its expression.

Although expression of utrophin A (the predominant isoform in skeletal muscle fibers) is primarily restricted to the post-synaptic membrane of the neuromuscular junction (NMJ) in mature muscle fibers, there are various conditions under which its expression can extend beyond the NMJ (Ohlendieck et al., 1991; Gramolini et al., 1997; Miura and Jasmin, 2006). Of particular interest is the extrasynaptic expression of utrophin A that is observed in slow, oxidative muscle fibers (Gramolini et al., 2001b; Chakkalakal et al., 2003b). Slow-twitch muscle fibers in both the dystrophin-negative *mdx* mouse model of muscular dystrophy and DMD patients show reduced damage in comparison to their fast-twitch counterparts (Webster et al., 1988; Moens et al., 1993).

This suggests that sarcolemmal expression of utrophin A in slow muscles protects against muscle damage.

In recent years, we have been interested in elucidating the mechanisms responsible for the increased sarcolemmal expression of utrophin A observed in slow oxidative skeletal muscle. In this context, we have shown that calcineurin, a phosphatase known to control the slow oxidative myofiber program (Olson and Williams, 2000), stimulates utrophin expression via binding of the transcription factor NFAT to the utrophin A promoter (Chakkalakal et al., 2003b; Angus et al., 2005). Importantly, stimulation of calcineurin/NFAT signaling in the *mdx* mouse increases sarcolemmal utrophin A expression, resulting in functional compensation for the lack of dystrophin (Chakkalakal et al., 2004; Stupka et al., 2006).

In addition to calcineurin/NFAT signaling, the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a key factor that can also drive the formation of slow oxidative muscle fibers (Lin et al., 2002). We found that PGC-1α cooperates with GABPα to stimulate utrophin A transcription in skeletal muscle cells (Angus et al., 2005), suggesting that PGC-1α could serve as a target for pharmacological interventions in the treatment of DMD. A recent study validated this strategy by showing that introduction of a muscle specific PGC-1α transgene into the *mdx* mouse stimulates expression of utrophin A and confers functional and structural improvement to dystrophic skeletal muscle fibers (Handschin et al., 2007a). Calcium/calmodulin (CaM) signaling is known to regulate the function of both PGC-1α and calcineurin/NFAT and to also promote the slow, high-oxidative myofiber program (Olson and Williams, 2000;

Handschin et al., 2003). We found that inhibition of CaM signaling in the *mdx* mouse causes a fiber-type shift to a faster phenotype, a reduction of utrophin A expression, and an exacerbation of the dystrophic pathology (Chakkalakal et al., 2006). Together, these studies suggest that activation of Ca^{2+}/Calmodulin (CaM) signaling pathways controlling calcineurin and PGC-1α might be beneficial to DMD patients.

Given the lack of an effective pharmacological therapy for DMD and based on the work described above, it is of considerable interest to identify small molecules that target signaling pathways controlling the slow myogenic program (Miura and Jasmin, 2006). Along these lines, an additional factor has recently been identified that controls the slow oxidative muscle phenotype. This factor is peroxisome proliferator-activated receptor (PPAR) β/δ, the first transcription factor identified that directly promotes the formation of slower, more oxidative muscle fibers (Luquet et al., 2003; Wang et al., 2004). PPAR nuclear receptors are ligand-activated transcription factors known to be important in regulating the transcription of genes involved in lipid metabolism and oxidative respiration (Luquet et al., 2005). Importantly, synthetic ligands to PPARs are in common clinical use and drugs that activate PPARβ/δ (the predominant isoform in skeletal muscle (Muoio et al., 2002)) are being clinically evaluated for the treatment of diabetes and dyslipidemia (Lopez-Soriano et al., 2006).

GW501516 is a PPARβ/δ specific agonist that has recently been shown in adult mice to increase the number of slow oxidative fibers and enhance running endurance when combined with exercise training (Narkar et al., 2008). Given the role of PPARβ/δ

in modulating slow fiber formation, we and others have previously proposed that treatment of *mdx* mice with PPARβ/δ agonists might improve muscle function by increasing expression of utrophin A (Chakkalakal et al., 2006; Miura and Jasmin, 2006; Davies and Khurana, 2007). Here, we evaluate whether PPARβ/δ activation with GW501516 directly stimulates utrophin A expression and indeed provides functional benefits to dystrophic *mdx* muscles.

Results

GW501516 treatment stimulates expression of PPARβ/δ transcriptional targets in C2C12 muscle cells. GW501516 is known to selectively activate PPARβ/δ and no other nuclear receptors (Oliver et al., 2001; Fredenrich and Grimaldi, 2004). We performed several positive controls to ensure that treatment of C2C12 cells with this drug increased expression of known PPARβ/δ transcriptional targets. PPAR nuclear receptors control target gene expression by interacting with promoter regions that contain a PPAR Response Element (PPRE). We thus transfected a luciferase reporter construct driven by a multimerized PPRE into C2C12 myoblasts, and then treated with GW501516 for 24 hrs at a concentration of 1 μM. This treatment induced luciferase expression from the reporter construct by ~3-fold ($P = 0.002$), demonstrating the effectiveness of the drug treatment in these cells (**Fig. 6a**). A reporter driven by the PGC-1α promoter, which is a known target of PPARβ/δ and contains a PPRE (Tanaka et al., 2003; Schuler et al., 2006), was enhanced by treatment with GW501516 by 1.7 fold ($P = 0.02$) (**Fig. 6a**). Levels of endogenous PGC-1α mRNA were increased by a similar magnitude of 1.7-fold ($P = 0.02$) (**Fig. 6b**). In complementary experiments, we treated C2C12 myoblasts and differentiated myotubes with GW501516, and found mRNA levels of uncoupling protein 2 (UCP2) a known target of PPARβ/δ (Dressel et al., 2003; Luquet et al., 2003), to be upregulated ~1.7-fold ($P = 0.005$) (**Fig. 6b**). Of relevance, these increases in expression of markers for the GW501516 effects are in line with data obtained by other groups (Luquet et al., 2003; Tanaka et al., 2003; Hondares et al., 2007).

PPARβ/δ activation by GW501516 stimulates utrophin A mRNA levels and promoter activity in C2C12 muscle cells. Having established the effectiveness of PPARβ/δ activation in C2C12 muscle cells, we next assessed whether utrophin A mRNA levels were stimulated in response to GW501516 treatment. Quantitative analysis revealed that GW501516 stimulated utrophin A mRNA levels in myoblasts by 1.7-fold ($P = 0.03$) (**Fig. 6b**). Similarly, treatment of differentiated C2C12 myotubes for 48 hrs at the same concentration of GW501516 increased utrophin A mRNA levels by 1.5-fold ($P = 0.02$) (**Fig. 6b**). It is important to note that the magnitude of utrophin A mRNA induction in response to GW501516 treatment observed here is similar to that observed for UCP2, PGC-1α and other PPARβ/δ-responsive genes (see above).

We next attempted to decipher whether the upregulation of utrophin A transcripts in response to GW501516 treatment resulted from direct transcriptional activation of the utrophin A promoter. To this end, a 1.3 kb human utrophin A promoter reporter construct (Dennis et al., 1996; Stocksley et al., 2005) (see **Fig. 6c**) was transfected into C2C12 myoblasts. The cells were induced to differentiate into myotubes, then treated for 24 hrs with GW501516. β-galactosidase RNA reporter levels driven by the utrophin A promoter were stimulated by 1.7-fold in response to GW501516 treatment ($P = 0.004$) (**Fig. 6e**, wild-type (WT) columns).

Enhancement of utrophin A promoter activity resulting from GW501516 treatment is mediated by a PPRE half site. The consensus PPRE binding site consists of direct repeats of the hexameric sequence AGGTCA separated by 1 base. Sequence analysis of

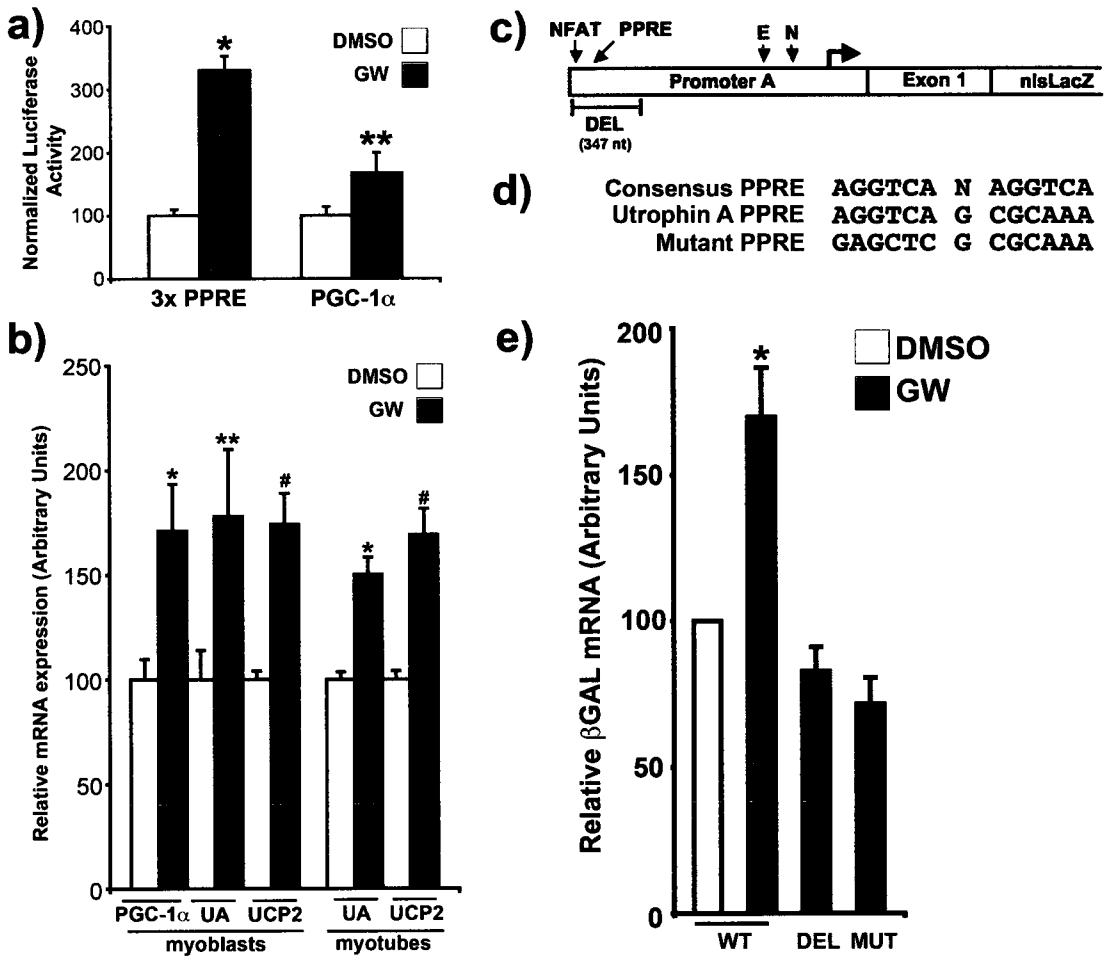
the utrophin A promoter revealed the presence of a conserved PPRE half site at the 5' end of the human utrophin A promoter (**Fig. 6c,d**). Although this is not a full consensus PPRE site, it has been previously shown that a half site can be sufficient to confer transcriptional activation by PPAR nuclear receptors (Barrero et al., 2003).

To assess the importance of this site, we removed the PPRE half site by creating a construct lacking the first 347 nucleotides of the utrophin A promoter region (DEL). GW501516 treatment of myotubes previously transfected with this reporter construct prior to differentiation caused no significant change in reporter levels ($P = 0.3$) (**Fig. 6e**). In order to demonstrate that the conserved PPRE half site is specifically responsible for mediating the effect of GW501516 treatment on utrophin A promoter activation, we mutated the PPRE half site (MUT) (See **Fig. 6d**). Reporter RNA levels driven from this promoter reporter construct were not significantly changed by treatment with GW501516 ($P = 0.09$) (**Fig. 6e**). Together, these results demonstrate that transcriptional activation through the PPRE half site located in the utrophin A promoter is responsible for the increase in utrophin A transcripts upon GW501516 treatment.

PPARβ/δ protein is more abundant in skeletal muscles of mdx mice compared to wild type mice. Since the objective of these experiments is to ultimately evaluate the potential usefulness of PPARβ/δ activation for the treatment of DMD, we set out to determine the expression pattern of PPARβ/δ in the *mdx* mouse. PPARβ/δ protein levels in skeletal muscles were compared between *mdx* mice and aged matched wild-type C57BL/10 mice. Using western blot analysis, we observed that the expression of PPARβ/δ was ~2- to 3

Fig. 6. Treatment of C2C12 cells with the PPARβ/δ agonist GW501516 induces utrophin A expression via a PPRE half site.

(**a**) C2C12 myoblasts transfected with a multimerized PPAR Response element (PPRE) luciferase reporter construct (PPRE X3-TK-luc) or a PGC-1α promoter reporter construct were treated for 24 hr with 1 μM GW501516 (GW) or a DMSO control. Promoter reporter activity was measured by renilla luciferase activity standardized to CAT reporter activity from a co-transfected pCAT plasmid. (DMSO vs GW: $*P = 0.002$, $**P = 0.02$). (**b**) GW501516 treatment of myoblasts and myotubes for 24 or 48 hr, respectively, increased PGC-1α, utrophin A (UA) and UCP2 mRNA levels as measured by quantitative RT-PCR. Values were standardized to actin mRNA levels. ($n = 3$-4, $*P = 0.02$, $**P = 0.03$, $\#P = 0.005$). Mean ± s.e.m. are shown. (**c**) Schematic of the 1.3 kb utrophin A promoter-reporter construct. Transcriptional regulatory elements are shown, including NFAT binding site (NFAT), PPAR Response Element (PPRE), E-box (E), and N-box (N). (**d**) The consensus PPRE sequence is shown compared to the utrophin A PPRE and mutant utrophin A PPRE. (**e**) C2C12 myoblasts were transfected with the 1.3 kb utrophin A promoter reporter construct (WT), or a reporter construct lacking the first 347 nucleotides of the promoter A sequence (DEL), or a construct harboring a mutated PPRE half site (MUT) and treated with GW for 24 hrs after 2 days in differentiation media. β-galactosidase RNA levels were measured using quantitative RT-PCR and standardized to CAT RNA expression from a co-transfected pCAT plasmid. ($n = 3$, $*P = 0.004$). Mean ± s.e.m. are shown.

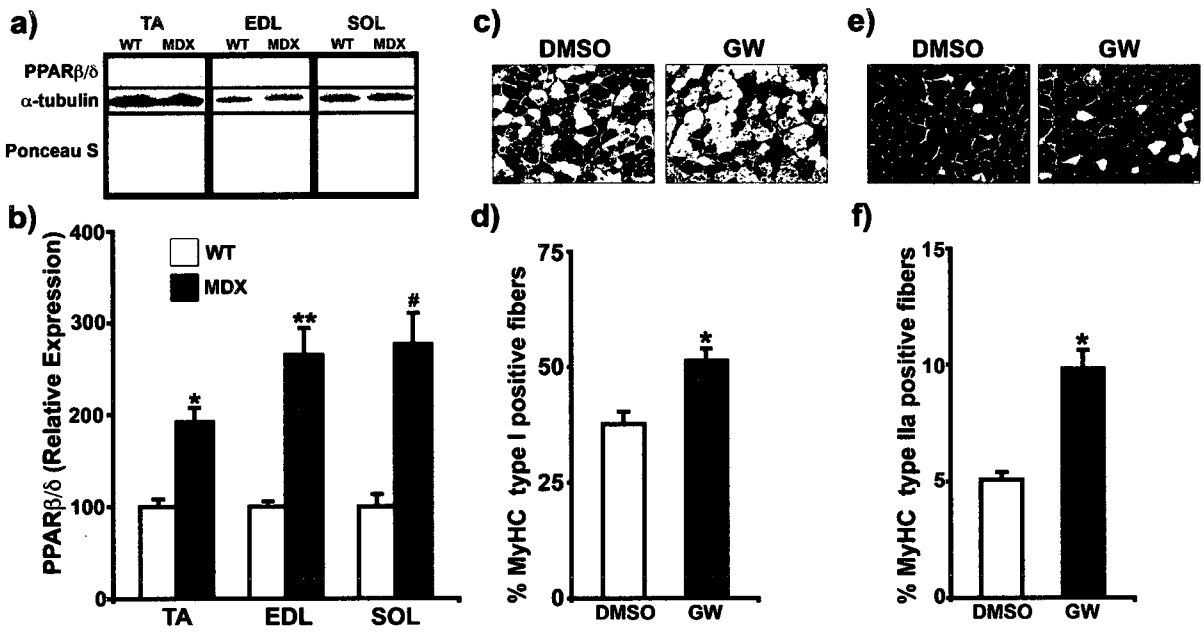

a)
Normalized Luciferase Activity
400
300
200
100
0
DMSO
GW
*
**
3x PPRE
PGC-1α
c)
NFAT
PPRE
E
N
Promoter A
Exon 1
nlsLacZ
DEL
(347 nt)
d)
Consensus PPRE AGGTCA N AGGTCA
Utrophin A PPRE AGGTCA G CGCAAA
Mutant PPRE GAGCTC G CGCAAA
b)
Relative mRNA expression (Arbitrary Units)
250
200
150
100
50
0
DMSO
GW
*
**
#
PGC-1α
UA
UCP2
myoblasts
myotubes
e)
Relative βGAL mRNA (Arbitrary Units)
200
150
100
50
0
DMSO
GW
WT
DEL
MUT

fold higher in *mdx* hindlimb muscles compared to wild type muscles (**Fig. 7a,b**, see figure legend for *P* values). We also examined heart tissue for PPARβ/δ expression in *mdx* vs WT mice, but were unable to detect a reliable signal (data not shown). We reasoned that the abundance of PPARβ/δ in dystrophic tissue could be advantageous in that increased amounts of PPARβ/δ would be available for activation by GW501516.

GW501516 treatment of mdx mice increases the percentage of slow, oxidative fibers and enhances utrophin A expression. In order to assess the effects of pharmacological activation of PPARβ/δ on dystrophic skeletal muscles, we treated *mdx* mice aged 5- to 7-weeks with GW501516 or vehicle control by gavage for 4 weeks. Treatment duration and delivery method was chosen based on previous studies (Wang et al., 2003; Narkar et al., 2008). To evaluate whether GW501516 promoted the slow myofiber program in *mdx* mice, we stained muscle sections with an antibody that detects myosin heavy chain (MyHC) type I positive fibers. Analysis of soleus muscles revealed a ~1.4-fold increase in the percentage of slow, oxidative MyHC type I fibers in the GW501516-treated *mdx* mice compared to vehicle treated mice ($P = 0.005$) (**Fig. 7c**). Very few MyHC type I positive fibers are normally found in mouse extensor digitorum longus (EDL) muscles and we found that GW501516 treatment did not increase the number of these fibers in EDL (data not shown). However, we did find a ~1.9-fold increase in the more oxidative type IIa positive fibers in EDL muscles of GW501516-treated *mdx* mice ($P = 0.003$). Skeletal muscles of *mdx* mice treated with GW501516 thus exhibited a slower, more oxidative phenotype.

Fig. 7. Increased PPARβ/δ expression in skeletal muscle of *mdx* mice permits stimulation of the slow myogenic program by GW501516.

(**a**) Western analysis performed on whole muscle lysates from 2 month old control C57BL/10 mice (WT) and *mdx* mice (MDX) to detect PPARβ/δ. Western blot for α-tubulin and Ponceau S staining is shown to demonstrate equal loading. (**b**) Densitometric analysis reveals that PPARβ/δ expression is greater in tibialis anterior (TA), extensor digitorum longus (EDL) and soleus (SOL) muscles of *mdx* mice compared to wild type controls. Note that similar results were obtained with 1 month old mice. Mean ± s.e.m. are shown. $n = 3$. (*mdx* vs WT: $^{*}P = 0.01$, $^{**}P = 0.03$, $^{\#}P = 0.02$). (**c**) Five to 7-week old *mdx* mice were treated daily with GW501516 (GW) or a DMSO vehicle control for 4 weeks. Cross sections from SOL muscles were stained for MyHC type I fibers (green) and laminin (red). (d) Quantification of the percentage of MyHC type I positive fibers reveals a 1.4- fold increase in the GW-treated *mdx* mice group compared to vehicle treated *mdx* mice. ($n = 5$, $^{*}P = 0.005$.) (e) Cross sections from EDL muscles were stained for MyHC type IIa positive fibers (green) and laminin (red). (f) Quantification of the percentage of MyHC type IIa positive fibers reveals a 1.9-fold increase in the GW-treated *mdx* mice group compared to vehicle treated *mdx* mice. (n=5, $^{*}P = 0.003$). Mean ± s.e.m. are shown.


a)
TA
EDL
SOL
WT MDX WT MDX WT MDX
PPARβ/δ
α-tubulin
Ponceau S
b)
PPARβ/δ (Relative Expression)
400
300
200
100
0
WT
MDX
*
**
#
TA
EDL
SOL
c)
DMSO
GW
d)
% MyHC type I positive fibers
75
50
25
0
*
DMSO
GW
e)
DMSO
GW
f)
% MyHC type IIa positive fibers
15
10
5
0
*
DMSO
GW

We next determined whether GW501516 treatment could stimulate utrophin A expression in *mdx* mice. Immunofluoresence analysis of cross sections from soleus muscles revealed that expression of utrophin A was increased in muscle fibers of agonist-treated *mdx* mice, including extrasynaptic regions of the sarcolemma (**Fig. 8a**). Quantitative analysis of the fluorescence intensity revealed a significant 1.5-fold increase ($P = 0.03$) in the GW501516 treated-*mdx* mice compared to vehicle-treated *mdx* mice (**Fig. 8b**). An increase in utrophin A expression was also observed at the mRNA level, as real time PCR analysis revealed that soleus muscles of agonist-treated mice contained 1.5-fold higher utrophin A mRNA levels ($P = 0.01$). Similar results were observed in the primarily fast-twitch EDL muscles (1.6-fold increase, $P = 0.04$) (**Fig. 8c**). As a positive control, UCP2 mRNA expression was measured and found to be upregulated by 7.5-fold in EDL ($P = 0.003$) and 2.5-fold in soleus ($P = 0.02$) of treated *mdx* mice (**Fig. 8c**). In line with these observations, a previous study found UCP2 levels to be increased by a similar magnitude in gastrocnemius muscles of PPARβ/δ transgenic mice, whereas other genes typical of slow oxidative fibers were modestly upregulated by less than 2-fold (Wang et al., 2004).

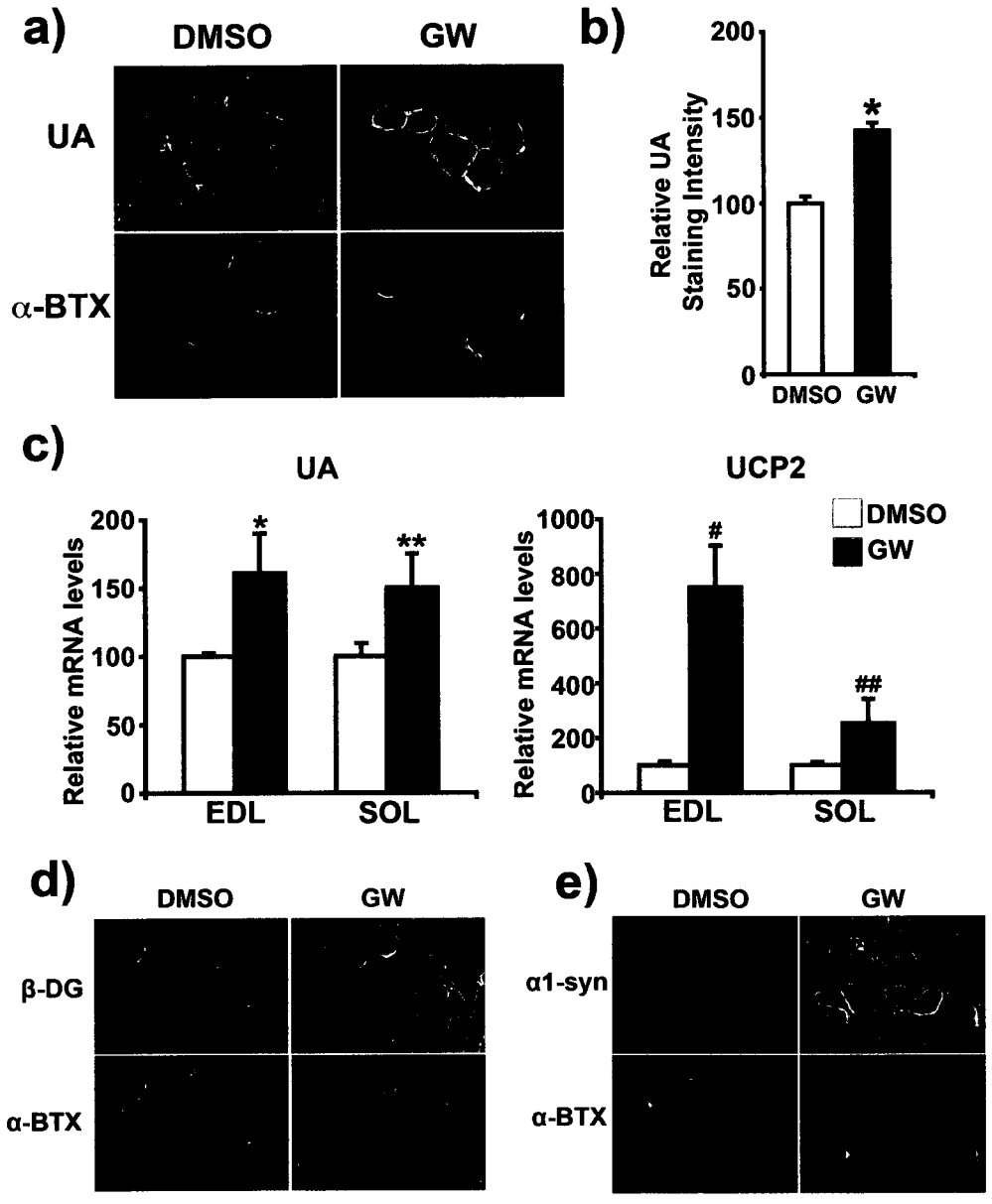
GW501516 treatment of mdx mice restores β-dystroglycan and α1-syntrophin localization to the sarcolemma. We next examined whether the increased sarcolemmal expression of utrophin A resulting from GW501516 treatment caused a reassembly of dystrophin-associated proteins at the sarcolemma. To this end, soleus cross-sections were stained with antibodies detecting members of the dystrophin associated protein complex (DAPC). In vehicle-treated *mdx* mice, β-dystroglycan expression was primarily restricted

to the neuromuscular junction (**Fig. 8d**). In contrast, *mdx* mice treated with the PPARβ/δ agonist were found to have enhanced β-dystroglycan expression along the sarcolemma (**Fig. 8d**). Expression of α1-syntrophin was similarly enhanced at the sarcolemma in GW501516-treated *mdx* mice compared to vehicle treated mice (**Fig. 8e**). It thus appears that upregulation of utrophin A resulting from PPARβ/δ activation reassembled the dystrophin-associated protein complex (DAPC) in *mdx* mice.

GW501516 treatment of mdx mice improves sarcolemmal stability. To determine whether reassembly of the DAPC resulting from GW501516 treatment improved the stability of the sarcolemma in *mdx* mice, soleus cross-sections of treated *mdx* mice were stained with an IgM antibody. Serum proteins such as IgM are normally extracellular, but disruption of the sarcolemma results in their intracellular presence (Straub et al., 1997; Chakkalakal et al., 2004). We observed a drastic reduction in the percentage of muscle fibers that had intracellular IgM staining, with vehicle-treated *mdx* mice showing 9 ± 4 % (mean ± s.e.m.) positive fibers versus 1 ± 1 % positive fibers for the GW501516 treated group ($P = 0.001$) (**Fig. 9a,b**). It thus appears that GW501516 treatment can prevent microtears of sufficient size to accommodate the transport of the rather large IgM across the sarcolemma of *mdx* mice. To further assess membrane stability using an *in vivo* approach and marker, we performed Evans blue dye (EBD) tail injections. In this commonly used method to assess muscle fiber damage, systemically delivered EBD diffuses into cells with leaky sarcolemma using albumin as a transporter (Straub et al., 1997; Bellinger et al., 2009). Quantitative analysis of positive EBD staining revealed that

Fig. 8. Treatment of *mdx* mice with GW501516 stimulates utrophin A expression and reassembles the dystrophin associated protein complex (DAPC) in skeletal muscle fibers.

Five to 7-week old *mdx* mice were treated daily with GW501516 (GW) or a DMSO vehicle control for 4 weeks. (**a**) Images from cross sections of soleus (SOL) muscle fibers co-stained using a utrophin A specific antibody (UA) and α-bungarotoxin (α–BTX). (**b**) Quantification of utrophin A staining reveals a significant increase in fluorescence intensity for the GW treated mice compared to vehicle treated controls. ($n = 4$, $^{*}P = 0.03$). (**c**) mRNA levels of utrophin A and UCP2 in SOL and EDL muscles of treated mice as measured by quantitative real-time RT-PCR and standardized to GAPDH levels ($n = 3$-4; GW vs DMSO: $^{*}P = 0.04$, $^{**}P = 0.01$, $^{\#}P = 0.003$, $^{\#\#}P = 0.02$). Mean ± s.e.m. are shown. (**d**) Representative images of *mdx* soleus cross sections stained with an antibody detecting β-dystroglycan (β-DG) or α-bungarotoxin (α-BTX). E) Representative images from cross sections of soleus muscles stained with an anti-α1-syntrophin antibody and α–BTX. Note the increase in β–DG and α1-syntrophin expression along the sarcolemma in the GW treated group, demonstrating re-assembly of the DAPC. $n = 3$.


a)
DMSO
GW
UA
α-BTX
b)
Relative UA Staining Intensity
200
150
100
50
0
*
DMSO
GW
c)
UA
Relative mRNA levels
200
150
100
50
0
*
**
EDL
SOL
UCP2
Relative mRNA levels
1000
800
600
400
200
0
#
##
DMSO
GW
EDL
SOL
d)
DMSO
GW
β-DG
α-BTX
e)
DMSO
GW
α1-syn
α-BTX

the percentage of damaged *mdx* myofibers in medial hamstring muscles was reduced by ~3-fold ($P = 0.002$) as a result of treatment with GW501516 (**Fig. 9c,d**).

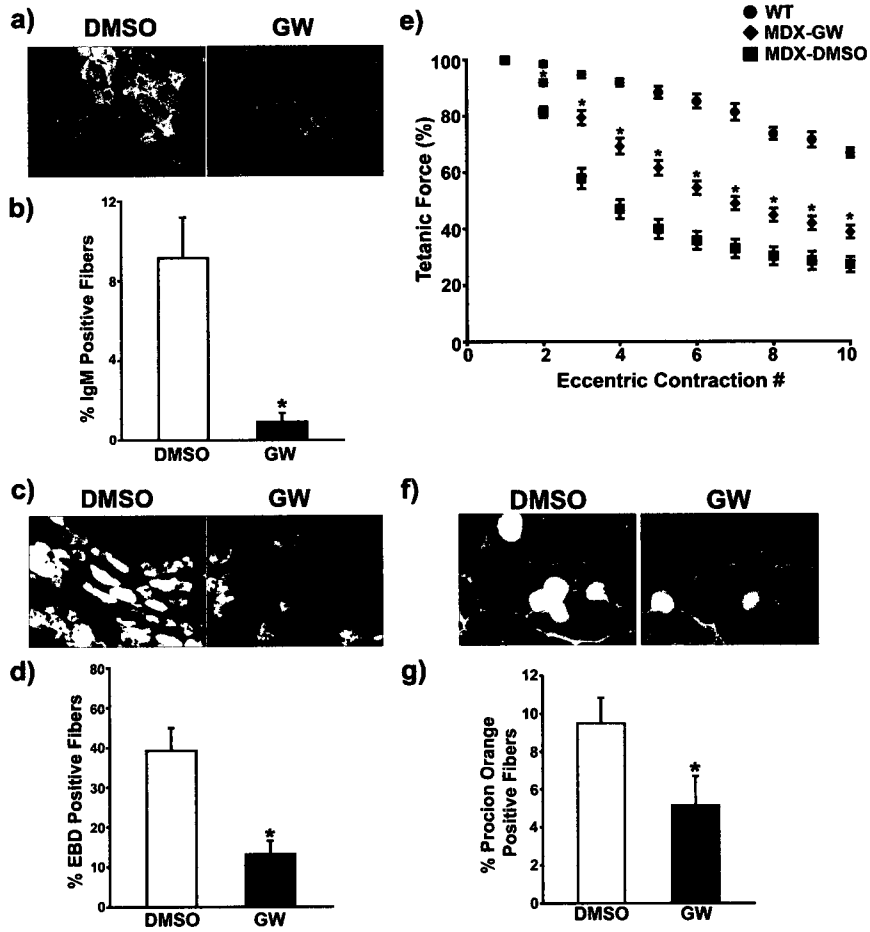
GW501516 treatment of mdx mice decreases force drop during eccentric contractions. To quantify the degree of functional improvement for *mdx* mice treated with GW501516, we also determined the extent of force drop during ten repetitive eccentric contractions (ECC). An excessive reduction in peak tetanic force following ECC is a specific sign of the susceptibility of dystrophin-deficient muscles to mechanical stress (Petrof et al., 1993; Tinsley et al., 1998; Blaauw et al., 2008). It is thus a well-accepted and quantitative method to assess the efficacy of potential DMD therapeutics.

Starting isometric force was found to be 344 +/- 20 mN for wildtype (WT) mice, 291 +/- 20 mN for vehicle-treated *mdx* mice (MDX-DMSO) and 298 +/- 10 mN for GW-treated *mdx* mice (MDX-GW). EDL muscles of *mdx* mice treated with GW501516 showed a significant reduction in post-ECC force drop throughout the protocol, i.e. from the 2^{nd} through 10^{th} eccentric contractions, compared to vehicle-treated *mdx* controls (ANOVA, $P < 0.001$) (**Fig. 9e**). In particular, there was a ~1.5-fold improvement from the 4^{th} to the 9^{th} eccentric contractions for the GW501516-treated *mdx* mice compared to vehicle-treated *mdx* mice. This force drop was not attributed to fatigue or a time effect since the changes in tetanic force of contralateral EDL muscles, simultaneously subjected to the same protocol without ECC, was less than 2% over the same time period.

During the ECC protocol, EDL muscles were bathed in 0.1% procion orange solution. This dye penetrates into the sarcoplasm of cells whose plasma membrane has been damaged, allowing for determination of the extent of cell membrane damage caused by the eccentric contractions. In the GW501516-treated group, ECC dye uptake was significantly decreased by almost 2-fold ($P = 0.001$) compared to the vehicle-treated group (**Fig. 9f,g**), providing further evidence that GW501516 treatment improves the sarcolemmal integrity of *mdx* mice.

Fig. 9. Treatment of *mdx* mice with GW501516 reduces the number of skeletal muscle fiber membrane lesions and decreases force drop due to eccentric contractions.

Five to 7-week old *mdx* mice were treated daily with GW501516 (GW) or a DMSO vehicle control for 4 weeks. (**a**) Cross sections of soleus muscles stained with anti-IgM antibody. (**b**) Quantification of the IgM staining revealed a 9-fold reduction in the percentage of intracellular stained fibers for the GW treated group ($n = 4$) compared to the DMSO group ($n = 3$) ($^*P = 0.001$). (**c**) Evans blue dye (EBD) was injected into the tail vein, and medial hamstring muscles were isolated 24 hours post-injection for analysis. Fibers permeable to EBD displayed intracellular red autofluorescence. (**d**) The percentage of permeable myofibers was significantly reduced by 3-fold in the GW group ($n = 5$, $^*P = 0.002$). (**e**) EDL muscles were subjected to ten eccentric contractions (ECCs) applied at 2 minute intervals at 25°C in physiological solution containing 0.1% procion orange. Force was normalized to tetanic force prior to the first ECC. The force drop was significantly less in the GW-treated *mdx* group (MDX-GW) (n=5) compared to vehicle-treated *mdx* group (MDX-DMSO) (n=6) from the 2^{nd} through 10^{th} ECC. *, significant difference between the MDX-DMSO and MDX-GW group (ANOVA, $^*P < 0.001$). ECC data from untreated wild-type C57Bl/10 mice (WT) is also shown (n=4). (**f**) Cross-sections of EDL after the ECC protocol. (**g**) Quantification of the number of procion orange positive fibers revealed that GW treatment significantly reduced sarcolemmal damage by almost 2-fold ($n = 5$, $^*P = 0.001$). Mean ± s.e.m. are shown.


a)
DMSO
GW
b)
% IgM Positive Fibers
12
8
4
0
DMSO
GW
c)
DMSO
GW
d)
% EBD Positive Fibers
80
60
40
30
20
10
0
DMSO
GW
e)
WT
MDX-GW
MDX-DMSO
Tetanic Force (%)
100
80
60
40
20
0
0
2
4
6
8
10
Eccentric Contraction #
f)
DMSO
GW
g)
% Procion Orange Positive Fibers
12
10
8
6
4
2
0
DMSO
GW

Discussion

Work by our group and others has recently demonstrated that interventions promoting the slow myogenic program, such as enhancing calcineurin/NFAT signaling and stimulating PGC-1α expression, upregulate utrophin A in muscle fibers and alleviate muscle damage in the *mdx* mouse (Chakkalakal et al., 2004; Stupka et al., 2006; Handschin et al., 2007a). Results presented here are the first to show that a similar outcome can be achieved using a therapeutically relevant molecule. We found that treatment of *mdx* mice with GW501516 caused an increase of utrophin A expression by ~1.5-fold, through a PPRE half site located in the utrophin A promoter. This increase of utrophin A expression was sufficient to improve sarcolemmal integrity and provide protection against eccentric contraction induced damage in *mdx* skeletal muscles. The functional benefits observed from this modest increase of utrophin A at the sarcolemma of dystrophic fibers is in accordance with multiple studies demonstrating that only a ~2-fold increase in utrophin A levels can fully correct for the dystrophic skeletal muscle phenotype of the *mdx* mouse (Tinsley et al., 1998; Chakkalakal et al., 2004).

We found that GW501516 treatment of *mdx* mice also resulted in a slower, more oxidative muscle phenotype as evidenced by an increase in the percentage of MyHC type I and type IIa positive fibers in the soleus and EDL, respectively. This finding is in agreement with a recent study that showed short-term pharmacological activation of PPARβ/δ in mice can increase oxidative fiber number (Gaudel et al., 2008). However, other studies have found that long-term treatment of sedentary wild-type mice with

PPARβ/δ agonist alone is ineffective at stimulating the slow myogenic program (Narkar et al., 2008; Kleiner et al., 2009). It is thus plausible that the increased expression of PPARβ/δ we detected in *mdx* skeletal muscles contributes to the appearance of more slow, oxidative muscle fibers and utrophin A upregulation after GW501516 treatment. This assertion is supported by the observation that mice expressing a skeletal muscle-specific activated PPARβ/δ transgene also show an increased amount of type I positive fibers (Wang et al., 2004).

In this study, we treated *mdx* mice beginning at 5-7 weeks of age. At this time-point, the first characteristic phase of muscle degeneration and necrosis in these mice has already developed (Whitehead et al., 2008). It is notable that agonist-treatment commenced even at this rather advanced stage of the dystrophic pathology successfully reassembled the DAPC, improved sarcolemmal integrity of existing fibers and mitigated force drop resulting from eccentric contractions. These data strongly suggest that GW501516 treatment might have important therapeutic benefits for DMD patients even after initial symptoms of muscle wasting arise.

Other hallmarks of the *mdx* pathology including the presence of centrally located myonuclei and increased fiber size variability are already well in place in 5- to 7-week old *mdx* mice, and hence may be difficult to reverse (see (Huang et al., 2009)). We found that GW501516 treatment of mature *mdx* mice conferred no significant changes in these parameters, or in the amount of collagen infiltration (data not shown). Full prevention of the dystrophic pathology in *mdx* mice can typically only be achieved by transgene-

induced modifications initiated *in utero* or other non-pharmacological therapies commenced at an early age (Chakkalakal et al., 2004; Odom et al., 2007; Blaauw et al., 2008; Gregorevic et al., 2008; Sonnemann et al., 2009). In this regard, while conventional transgenic upregulation of utrophin by 2-fold can fully prevent the dystrophic pathology (Tinsley et al., 1998), post-natal overexpression of utrophin using a tetracycline-inducible transgenic system was found to cause more modest improvements (Squire et al., 2002). Treatment of DMD patients with relevant molecules such as GW501516 may thus significantly improve functional characteristics of existing and still partially functioning muscle fibers, but fail to completely reverse the extent of the pathology for symptoms linked to previous muscle fiber loss. Thus, a combination drug therapy aimed at enhancing sarcolemmal integrity of existing fibers while also promoting muscle fibers regeneration may prove ideal.

Our finding that GW501516 can stimulate utrophin A expression and improve sarcolemmal stability in mature *mdx* mice opens the possibility that it may be useful as a pharmacological treatment for DMD. This finding is particularly encouraging since several PPAR agonists are clinically approved drugs, and GW501516 has undergone phase II clinical trials for the treatment of dyslipidemia. Although there are multiple therapeutic avenues currently being investigated for the treatment of DMD, such as antisense-mediated exon skipping and cell replacement therapy (Cossu and Sampaolesi, 2007), conventional drug-based therapy has the advantages of being able to achieve systemic delivery and avoid complicating immune responses at a reasonable cost (Khurana and Davies, 2003).

Materials & Methods

GW501516 treatment. GW501516 (Alexis Biochemicals, Lausen, Switzerland) was dissolved in DMSO at 100 mM and diluted in 0.5% carboxymethylcellulose immediately prior to daily treatments. Five to 7-week old *mdx* mice (C57BL/10ScSc-DMD^{mdx}/J, Jackson Laboratories) were treated at 25 mg/kg/day with GW501516 or DMSO-only vehicle daily by gavage for 4 weeks. Mice were sacrificed and muscles were either frozen in liquid nitrogen and processed for RNA or protein analysis, or frozen in melting isopentane cooled with liquid nitrogen for histological and immunofluorescence analysis.

Plasmid transfections and RT-PCR analysis. All transient transfections were carried out using Lipofectamine 2000 (Invitrogen) while C2C12 myoblasts were 70-90% confluent. The PGC-1α promoter reporter construct was provided by Dr. M. Czubryt (University of Manitoba) (Czubryt et al., 2003). PPRE X3-TK-luc was obtained from Dr. B. Spiegelman (Dana Farber Cancer Institute) (Kim et al., 1998). For creation of the DEL construct, the first 347 nucleotides of the utrophin A promoter region (GenBank accession #X95523) was excised from the human utrophin A 1.3 kb promoter reporter (Dennis et al., 1996; Gramolini et al., 1997). Total RNA was isolated from cells or muscles using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNAse I (Fermentas, Burlington, Canada) to eliminate possible DNA contamination. Endogenous mRNAs and reporter RNA levels were measured by RT-PCR using previously described protocols and primer sets (Gramolini et al., 1999a; Lin et al., 2002; Chakkalakal et al., 2003b; Miura et al., 2005). Real time quantitative RT-PCR analysis was carried out on a

Stratagene MX3005p as previously described (Miura et al., 2008). The delta delta CT method was used to quantify expression of utrophin A and UCP2 relative to GAPDH.

Western Analysis, Immunofluorescence, and Assessment of Membrane Damage. Western analysis was performed essentially as previously described (Miura et al., 2005), with the exception that 60 μg of protein extract was separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. PPARβ/δ was detected using a polyclonal PPARδ antibody (Affinity Bioreagents, Golden, CO). α-tubulin protein levels were detected using the monoclonal antibody B512 (Abcam, Cambridge, MA). Quantification of bands by densitometry was performed on immunoblots using ImageJ (NIH) software. For immunofluorescence analysis of DAPC member proteins, images were acquired at 20x or 40x magnification on a Zeiss Axioshop-2 microscope. Muscle cryosections were stained with polyclonal utrophin A antibody (Miura et al., 2005), β-dystroglycan antibody (Dr. S. Carbonetto, McGill University) or α1-syntrophin antibody (Abcam) along with Alexa 488 conjugated α-bungarotoxin (Jackson ImmunoResearch, West Grove, PA). Quantification of the fluorescence intensity of utrophin A staining was performed on thresholded 40x magnified images using Northern Eclipse image analysis software. Immunostaining for these experiments was performed at the same time for all muscles in order to minimize variability in background fluorescence intensity. A standardized threshold was applied to grayscale images obtained at equal exposure times and the percentage of stained area per field of view was quantified. For each mouse, 2-4 random fields of view were analyzed from 3-4 cross-sections obtained from the midbelly of soleus muscles. Immunofluorescent fiber-type analysis was performed using undiluted

A4-840 antibody to detect MyHC I or undiluted SC-71 to detect MyHC IIa (Developmental Studies Hybridoma Bank, University of Iowa). Sections were co-stained with a polyclonal anti-laminin antibody at 1:1000 dilution (Sigma, St. Louis, MO) to allow detection of ECM surrounding the sarcolemma. Secondary detection was performed using appropriate FITC-conjugated anti-mouse antibodies and a TRITC-conjugated anti-rabbit antibody. Analysis of fiber type percentages was performed on 10x magnified images of 2-4 cross-sections from EDL and soleus muscles. For detection of sarcolemmal lesions, muscle cross-sections were stained with fluorescein-conjugated IgM anti-mouse secondary antibody (Sigma). For analysis, 3-6 random fields of view were examined on 4-6 cryosections for each muscle. To evaluate sarcolemmal lesions *in vivo*, a solution containing sterile 2% Evans blue due (EBD) (Sigma) in PBS was injected in the tail vein of mice at 0.1 ml/10 g body weight. After 24 hours, medial hamstring muscles were removed and cross-sections were prepared and analyzed as previously described (Chakkalakal et al., 2004). To quantify the percentage of EBD positive fibers, sections were also stained with an anti-mouse IgG-fluorescin antibody (Molecular Probes) to facilitate visualization of the sarcolemma.

Eccentric Contraction (ECC) Protocol. C57Bl/10 mice, DMSO- and GW-treated *mdx* mice were anesthetized as previously described (Cifelli et al., 2008), both EDL were excised and tendons were tied with surgical silk (#6). One tendon was attached to the force transducer while the other attached to fixed metal pin. Muscles were continuously immersed in physiological saline solution (Cifelli et al., 2008) and maintained at 25°C. Muscle length (Le) was adjusted to obtain maximum isometric tetanic force elicited every

5 min with 200 ms trains of supramaximal 10 V, 0.3 ms square pulses at 200 Hz. Force was monitored with a Cambridge ergometer (model 300, Aurora Scientific Inc., Aurora, ON, Canada) or a Kulite force transducer model BD100 (Kulite Semiconductor Products, Inc., Leonia, NJ, USA). Prior to the eccentric contractions, muscles were equilibrated for 30 min in physiological solution containing 0.1% procion orange dye (Sigma). Following equilibration, one EDL was subjected to the same isometric contractions while the other EDL underwent the ECC protocol consisting of 10 eccentric contractions applied at 2 min intervals. For the ECC, the stimulation train duration was prolonged to 700 ms and a 10% lengthening at a velocity of 0.5 Le/s was applied during the last 200 ms. Following the contraction protocol, cross-sections from the midbelly of EDL muscles were examined for intracellular green fluorescence. The periphery of the cross-sections was not included in the analysis due to auto-fluorescence.

Statistical Analysis. Significant differences between two means was determined using independent two-tailed *t* tests. For the ECC, a split-plot ANOVA design was used with the mice groups in the whole plot and the eccentric contractions in the split plot because all tetanic forces were obtained from the same muscle. Data is presented as mean ± s.e.m. throughout.

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CHAPTER 3

The Utrophin A 5'-UTR Confers IRES-Mediated Translational Control During Regeneration of Skeletal Muscle Fibers

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Contributions of Authors: Conceived and designed the experiments: PM JT MH BJJ. Performed the experiments: PM JT JVC. Provided Reagents: MH. Wrote the paper: PM BJJ.

Abstract

Utrophin up-regulation in muscle fibers of Duchenne muscular dystrophy (DMD) patients represents a potential therapeutic strategy. It is thus important to delineate the regulatory events presiding over utrophin in muscle in attempts to develop pharmacological interventions aimed at increasing utrophin expression. A number of studies have now shown that under several experimental conditions, the abundance of utrophin is increased without a corresponding elevation in its mRNA. Here, we examine whether utrophin expression is regulated at the translational level in regenerating muscle fibers. Treatment of mouse tibialis anterior (TA) muscles with cardiotoxin to induce muscle degeneration/regeneration, led to a large (~14-fold) increase in the levels of utrophin A with a modest change in expression of its transcript (40%). Isolation of the mouse utrophin A 5'-UTR revealed that it is relatively long with a predicted high degree of secondary structure. In control muscles, the 5'-UTR of utrophin A caused an inhibition on translation of a reporter protein. Strikingly, this inhibition was removed during regeneration indicating that expression of utrophin A in regenerating muscles is translationally regulated via its 5'-UTR. Using bicistronic reporter vectors, we observed that this translational effect involves an Internal Ribosome Entry Site (IRES) in the utrophin A 5'-UTR. Thus, IRES-mediated translation of utrophin A can, at least partially, account for the discordant expression of utrophin A protein and transcript in regenerating muscle. These findings provide a novel target for upregulating levels of utrophin A in DMD muscle fibers via pharmacological interventions.

Introduction

Duchenne muscular dystrophy (DMD) is the most prevalent inherited neuromuscular disorder since it affects one out every 3,500 male births (Emery, 1991). Although the genetic defect underlying DMD was identified 20 years ago (Anderson and Kunkel, 1992; Cohn and Campbell, 2000), there is still no effective treatment to alter the relentless progression of this debilitating neuromuscular disease (Chakkalakal et al., 2005). DMD is caused by deletions/mutations in the dystrophin gene which prevents production of full-length dystrophin molecules in skeletal muscle fibers. One therapeutic strategy to treat DMD consists in upregulating the endogenous levels of a protein in muscle fibers of affected patients which, once expressed at the appropriate location, could functionally compensate for the absence of dystrophin. An ideal candidate for such a role is utrophin (Jasmin et al., 2002; Khurana and Davies, 2003). Utrophin is a large cytoskeletal protein that displays a high degree of structural and functional similarity to dystrophin. In this context, overexpression of utrophin in muscle fibers of a DMD mouse model has been shown to alleviate the dystrophic pathology (Tinsley et al., 1992; Tinsley et al., 1996; Deconinck et al., 1997) thereby demonstrating that increased expression of utrophin could indeed yield important clinical benefits.

A major difference between utrophin and dystrophin relates to their pattern of expression in skeletal muscle fibers. Whereas dystrophin localizes along the cytoplasmic face of the entire muscle fiber, utrophin preferentially accumulates at the levels of the myotendinous and neuromuscular junctions (Ohlendieck et al., 1991; Bewick et al., 1992; Helliwell et al., 1992). To be valid as a therapeutic strategy for DMD, utrophin expression must therefore be extended in extrasynaptic compartments of dystrophic

muscle fibers. Thus, elucidating the nature of the molecular events controlling expression of utrophin in muscle fibers becomes important since it could lead to the rational design of pharmacological interventions aimed at upregulating utrophin in muscle fibers of DMD patients.

Two full-length isoforms of utrophin have now been identified and named utrophin A (Dennis et al., 1996) and utrophin B (Burton et al., 1999). These isoforms are encoded by mRNAs that are transcribed from two different promoters. These two mRNAs differ mostly in their 5' untranslated region (UTR) as well as in their initial coding portion resulting in protein products that slightly differ in their N-termini. Utrophin A is the major full-length isoform expressed in skeletal muscle fibers while expression of utrophin B is relatively higher in the vasculature (Weir et al., 2002). To date, most of the studies that have examined the mechanisms regulating utrophin A expression in muscle cells have focused on transcriptional regulation. For example, the synaptic accumulation of utrophin A has been shown to involve the local transcriptional activation of the utrophin A promoter through an N-box motif and the ets-related transcription factor GABP which is activated by heregulin stimulation (Gramolini et al., 1999b; Khurana et al., 1999). Recent studies have also shown that utrophin A is expressed at low levels in extrasynaptic compartments of slow muscle fibers and in this case, is subject to regulatory events involving the calcineurin/NFAT signalling pathway (Chakkalakal et al., 2003a; Chakkalakal et al., 2004).

In addition to transcriptional mechanisms, there is mounting evidence indicating that post-transcriptional mechanisms also regulate utrophin expression in muscle. This is coherent with the fact that post-transcriptional mechanisms are known to control

expression of several synaptic proteins in muscle cells (Chakkalakal and Jasmin, 2003). In particular, we have previously shown that distinct elements within the utrophin 3'-UTR are important for controlling the stability of utrophin transcripts in muscle cells and for targeting them to specific sub-cellular locations (Gramolini et al., 2001a; Gramolini et al., 2001b).

Converging lines of evidence also indicate that utrophin may be regulated at the translational and/or post-translational level. For example, we have previously demonstrated that utrophin levels are greatly upregulated during muscle regeneration without a corresponding increase in expression of its mRNA (Gramolini et al., 1999a). In the same study, we also noted that although utrophin levels are increased in muscle biopsy samples from DMD patients, levels of utrophin mRNA are similar to those seen in normal muscle samples (Chen et al., 2000; Gramolini et al., 2001b). Since these initial findings, several other laboratories have also reported discordant patterns of expression of utrophin protein and transcript. Specifically, Weir et al., detected an increase in the abundance of utrophin A in muscle from *mdx* mice with no parallel elevation in the levels of utrophin A transcript (Weir et al., 2002). Similarly, utrophin protein levels in muscle change significantly during the lifespan of *mdx* mice with little alterations in the expression of utrophin mRNAs (Roma et al., 2004). Finally, results from several other studies also support the idea that utrophin is regulated via translational mechanisms (Courdier-Fruh et al., 2002; Moghadaszadeh et al., 2003b). With this in mind, we have therefore initiated in the present study, a series of experiments to examine whether the regulation of utrophin A *in vivo* involves translational events.

Experimental Procedures

Generation of Reporter Constructs - To obtain the murine utrophin A 5'-UTR, reverse transcription and polymerase chain reaction (PCR) experiments using total RNA obtained from C2C12 myotubes was carried out. The 5' and 3' primers amplified a 508 base pair fragment (5' primer: 5'-GTTGTGGAGTCGCCCT-3' and 3' primer: 5'-CCCCATACTTGGCCAT-3') (Weir et al., 2002). This fragment was sequenced to confirm its identity. It was subsequently inserted into the multiple cloning site of pCMV SPORT-βGAL (Gibco/BRL, Burlington, ON) (called pβGAL here) to generate pUtrA/βGAL (see **Fig. 11** for all plasmids used). The utrophin A 5'-UTR was also inserted into the *Xho1* site of the bicistronic vector pβGAL/CAT (Holcik et al., 1999) to generate pβGAL/UtrA/CAT (see **Fig. 11**). The pβGAL/XIAP/CAT construct containing the ~1 kb XIAP 5'-UTR (Holcik et al., 1999) was used in our experiments as a control for IRES activity. The promoterless bicistronic constructs p(-cmv)βGAL/CAT and p(-cmv)βGAL/UtrA/CAT were created by removing the CMV promoter using *Nru1* and *HindIII* restriction sites. Excision of the β-galactosidase gene was accomplished by cutting with *Not1* and re-ligating to create the monocistronic pUtrA/CAT plasmid.

Cell Culture - C2C12 myoblasts were cultured in growth media in 35 mm plates as previously described in detail elsewhere (Gramolini et al., 1998). Cells were transfected when they reached 60-80% confluency. Two μg pβGAL and pUtrA/βGAL constructs were co-transfected with 2 μg of pCAT control vector (Promega, Mississauga, ON) using 7.5 μL of lipofectamine (2 mg/ml) (Invitrogen Life Technologies Inc., Burlington, ON) according to the manufacturer's instructions. Transfections using the bicistronic vectors

were performed using 4 μg of plasmid with 8 μL of lipofectamine (2mg/ml). Cells were lysed 24 hrs after transfection using 400 μL of 1 X reporter lysis buffer according to the manufacturer's instructions (Promega) and the reporter activity was determined as described below.

Direct Plasmid Injection and Cardiotoxin Treatment - For *in vivo* studies, 25 μL of cardiotoxin (Latoxan; Rosans, France) at 10^{-5} M was injected into the right tibialis anterior (TA) muscle of 4 week-old C57BL/10 mice (Charles River Laboratories, St. Constant, Quebec) to induce muscle degeneration and regeneration as previously described (Gramolini et al., 1999a). Seven days later, cardiotoxin-treated and control, untreated TA muscles were excised and frozen in liquid nitrogen. For immunofluorescence experiments (see below), some TA muscles were frozen in melting isopentane pre-cooled with liquid nitrogen.

To examine the expression of the reporter constructs during muscle regeneration, 25 μl of plasmid at 2 μg/μl in PBS was directly injected in both regenerating and control TA muscles three days after cardiotoxin treatment. Four days later, TA muscles were excised and frozen in liquid nitrogen. TA muscles injected with plasmids were homogenized using a Polytron in 1 ml of 1 X reporter lysis buffer (Promega), freeze-thawed twice and centrifuged for 20 min at 12,000g. Supernatants were collected and frozen until further analysis.

Reporter Assays - Assays for βGAL enzymatic activity were performed on homogenized TA muscles or C2C12 myoblast lysates using the β-Galactosidase Enzyme Assay system according to the manufacturer's instructions (Promega). CAT activity was measured by

analyzing the conversion of chloramphenicol to butyryl chloramphenicol by incorporation of [^{14}C]-butyryl coenzyme A using the CAT enzyme kit (Promega). Counting was performed in a liquid scintillation counter (Wallac 1414 Liquid Scintillation Counter with 1414 Winspectral Windows Software). Background levels for both reporter assays were determined by analyzing reporter activity in non-transduced TA muscle and non-transfected C2C12 cells.

RNA extraction and RT-PCR - Total RNA was isolated from C2C12 cells or TA muscle using TRIzol Reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. To quantitate the levels of endogenous transcripts in cardiotoxin-treated and untreated TA muscles, RT-PCR was performed to amplify utrophin A and S12 ribosomal RNA as previously described in detail elsewhere (Chakkalakal et al., 2003a). Negative controls consisted of RT mixtures in which total RNA was replaced by sterile, DEPC-treated water. For quantitation of endogenous utrophin A mRNA and S12 ribosomal RNA levels, PCR products were separated on vista green (Amersham Pharmacia Biotech, Piscataway, NJ) -containing agarose gels and the intensity of the signal was quantitated using ImageQuant version 5.1 (Molecular Dynamics/Amersham Biosciences, Oakville ON, Canada). The relative levels of utrophin A transcripts were standardized according to the amount of S12 ribosomal RNA levels present in the same samples.

For RT-PCR analysis of reporter expression in transfected C2C12 cells and transduced TA muscles, Trizol extracted RNA was first DNase I treated (Invitrogen) for one hour to eliminate plasmid contamination according to the manufacturer's instructions. For TA muscles injected with pβGAL and pUtrA/βGAL and co-transduced with pCAT, RT-PCR was carried out with primers to amplify a portion of CAT (5'-

TGGCAATGAAAGACGGTGAG-3'; 5'-GAAAACGGGGGCGAAGAAGT-3'), and a portion of βGAL (5'-GTGACGGCAGTTATCTGG-3'; 5' TTGGCAGTGCTCGTAGTA-3') mRNAs. Negative controls consisted of an RT mixture that had the reverse transcriptase replaced with DEPC-treated water. Denaturation was performed at 94°C for 45 s, followed by an annealing step of 55°C of 1 min and an extension step at 72 °C for 1 min for both βGAL and CAT PCRs. For βGAL amplification, 20-27 cycles were used for quantitative analysis, while 23-28 cycles were used for CAT. Cycling was followed by a final extension step at 72 °C for 10 min. It is important to note that for all RT-PCR experiments used to quantify the relative abundance of transcripts, cycle numbers were optimized to be within the linear range of amplification as previously described in detail (Jasmin et al., 1993; Michel et al., 1994; Gramolini et al., 2001b).

To control for the presence of an intact bicistronic reporter transcript following C2C12 transfections and direct plasmid injection into TA muscles with pβGAL/CAT and pβGAL/UtrA/CAT plasmids, RT-PCR using total RNA was performed using a 5' primer spanning 142 nucleotides of the LacZ gene and a 3' primer spanning 459 nucleotides of the CAT gene that flanked the utrophin A 5'-UTR (5' TTTTTCCCGATTTGGCTACA 3'; 5' TGAAACTCACCCAGGGATTG 3'). This RT-PCR strategy is summarized in **Fig. 15**. The RT-PCR products were subcloned using the TOPO TA cloning kit (Invitrogen) and sequenced to verified their identity.

For quantitative RT-qPCR, reverse transcription was carried out using the First-Strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ) with *Not* I- $d(T)_{18}$ primers. The quantitative real-time RT-PCR was performed using the QuantiTect SYBR

green RT-PCR kit (Qiagen) and analysed on a ABI Prism 7000 sequence detection system using the ABI Prism 7000 SDS Software. qPCR reactions were carried out to detect βGAL (5'ACTATCCCGACCGCCTTACT 3'; 5' CTGTAGCGGCTGATGTTGAA 3') and CAT (5' GCGTGTTACGGTGAAAACCT 3'; 5' GGGCGAAGAAGTTGTCCATA 3') for muscle samples transduced with the bicistronic vectors. In order to control for DNA contamination, qPCR reactions were performed directly on RNA samples.

Northern Blotting - For northern blot analysis, electrophoresis of total RNA was performed using a denaturing agarose/formaldehyde gel followed by transfer onto a hybond, positively- charged nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) for 2.5 hrs using downward alkaline transfer. The membranes were pre-incubated for 4 hrs at 42 °C in a shaking water bath with 10 ml of DIG easy hyb buffer (Roche, Mannheim, Germany). The membrane was then incubated overnight with a linearized cDNA probe for CAT (301 bp) or βGAL (333 bp) labelled with Redivue [α-^{32}P] dCTP (Amersham Biosciences) using the rediprime II random prime labelling system (Amersham Biosciences). Washes were carried out for 1 X 5 min and 1 X 10 min at room temperature with 2X SSC, 0.1% SDS, and then for 1 X 2 min at 65 °C with 0.1X SSC, 0.1% SDS. The blot was rinsed in 2X SSC and exposed to Kodak BioMax MR Film (Eastman Kodak Company, Rochester, NY).

Western Blotting - A polyclonal antibody directed against utrophin A was raised as previously described (Chakkalakal et al., 2003a). This utrophin A antibody was purified from production bleeds of antisera using the SulfoLink Kit (Pierce Biotechnology, Rockford, Il), according to the manufacturers instructions. For Western blots,

cardiotoxin-treated and untreated TA muscles from 4 week-old mice were homogenized in a previously described extraction buffer (Lu and Schneider, 2004) using a dounce homogenizer, then boiled and centrifuged for 10 min at 10,000 g. One hundred μg of the protein extracts were separated using a 5% SDS PAGE gel with a 4% stacking gel at 200 V for 5 hrs. Gels were transferred to Immobilion-P polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA) overnight at 4 °C. Membranes were incubated with Tris-buffered saline (TBST) with 0.1% Tween 20 and 5% dried skim milk for 1 hr, and then incubated for 1 hr with the primary antibody (1:10,000) diluted in 5% milk TBST buffer. The membranes were washed thoroughly in TBST, and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) secondary antibody (1:25,000) for 1 hr. Protein detection was carried out using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology) and exposed to Kodak BioMAX MR film (Eastman Kodak Company). The average intensity of the bands were quantitated using Kodak digital science 1D v. 3.0.2 image analysis software (Eastman Kodak Company). Equal loading of protein was confirmed with the SYPRO Ruby protein blot stain (Molecular Probes, Eugene, OR) as recommended by the manufacturer, and was further quantified using a monoclonal anti-Actin antibody (Sigma Aldrich, Oakville, ON).

Immunofluorescence - Immunofluorescence experiments were performed as previously described (Chakkalakal et al., 2003a). Briefly, the polyclonal anti-utrophin A antibody was used to identify utrophin A expression on longitudinal sections of untreated or cardiotoxin-treated TA muscles. Alexa 488-conjugated α-bungarotoxin 594 (Jackson

ImmunoResearch, West Grove, PA) was used to label acetylcholine receptors and hence, to identify the presence of neuromuscular junctions.

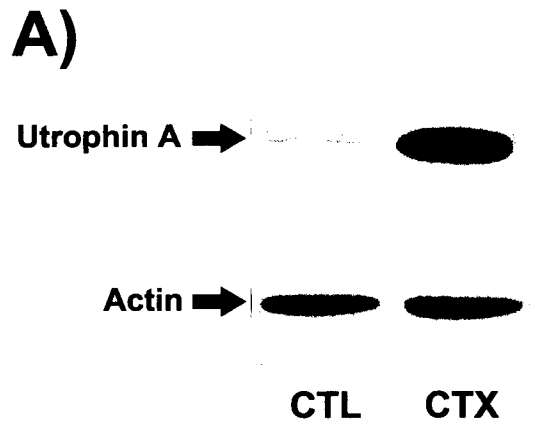
Statistical Analysis - The presence of significant differences between group means was determined using Student's t-test. The level of significance was set at $P < 0.05$. Means $\pm$ SEM are shown throughout.

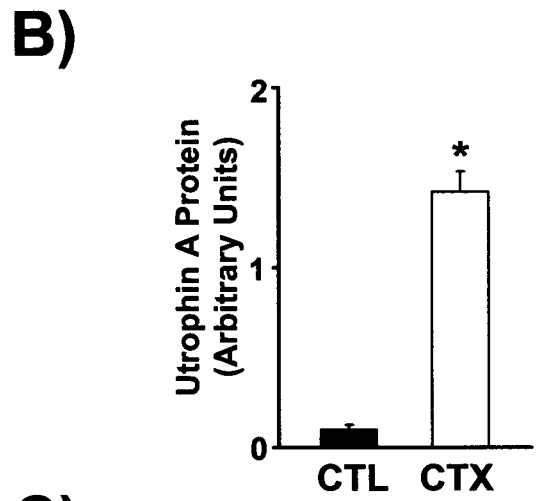
Results

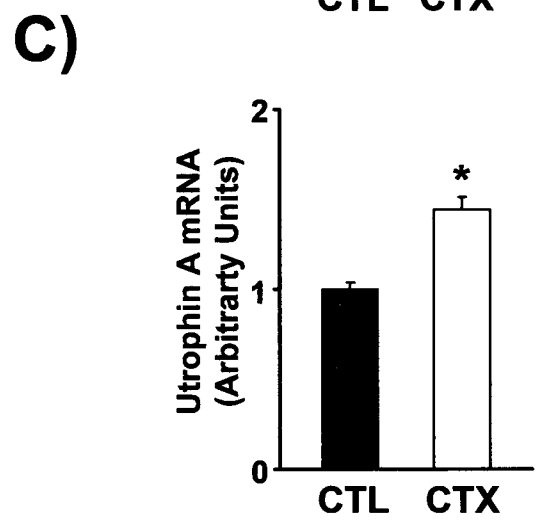
Discordant Expression of Utrophin A and its mRNA in Regenerating Muscle - Treatment of skeletal muscle with the snake venom cardiotoxin induces severe myonecrosis and subsequent muscle regeneration while leaving the innervating nerve intact. This is a well established model to study muscle regeneration (Yan et al., 2003; Charge and Rudnicki, 2004). Under these conditions, we have previously shown that the content of total utrophin (using an antibody that recognized both utrophin A and B isoforms) is significantly upregulated several days after cardiotoxin treatment (Gramolini et al., 1999a). Here, we examined the specific effect of cardiotoxin treatment on expression of the utrophin A isoform which is selectively expressed in skeletal muscle (Weir et al., 2002; Chakkalakal et al., 2003a). Western blot analysis of muscles treated with cardiotoxin for 3 to 10 days revealed that the greatest levels of utrophin A expression were obtained at 7 days after cardiotoxin treatment. At this time point, we found that utrophin A expression was increased ~14 fold ($P < 0.05$) compared to untreated control muscles (**Fig. 10A and B**). Immunofluorescence experiments revealed that while utrophin A expression in control muscles is confined to neuromuscular junctions (**Fig. 12A and B**) (Ohlendieck et al., 1991; Bewick et al., 1992; Helliwell et al., 1992), the increased expression of utrophin A in regenerating muscle fibers was observed along the entire length of the sarcolemma (**Fig. 12E and F**). However, in sharp contrast to this dramatic induction of protein, utrophin A mRNA levels were modestly increased in regenerating muscle (40% increase; **Fig. 10C**). This relatively small increase in the abundance of utrophin A mRNA is in stark contrast to the dramatic increase in protein

Fig. 10. Discordant expression of utrophin A and its mRNA in regenerating muscles.

A) A representative Western blot using a utrophin A specific antibody and proteins extracted from control (CTL) and cardiotoxin-treated (CTX) TA muscles. Also shown is a Western blot showing equal levels of actin in each sample (loading control). **B)** Quantitation of the signal intensity seen in the Western blot experiments. Note the large increase (~ 14-fold) in utrophin A. **C)** Levels of utrophin A mRNA standardized to S12 ribosomal RNA in control (CTL) and cardiotoxin-treated (CTX) muscles as measured by RT-PCR. Note the modest increase (~ 40%) in utrophin A mRNA. Values are in arbitrary units. Mean +/- SEM are shown. * denotes significant differences from control ($P < 0.05$).


A)
Utrophin A
Actin
CTL
CTX


B)
Utrophin A Protein
(Arbitrary Units)
2
1
0
*
CTL
CTX


C)
Utrophin A mRNA
(Arbitrarty Units)
2
1
0
*
CTL
CTX

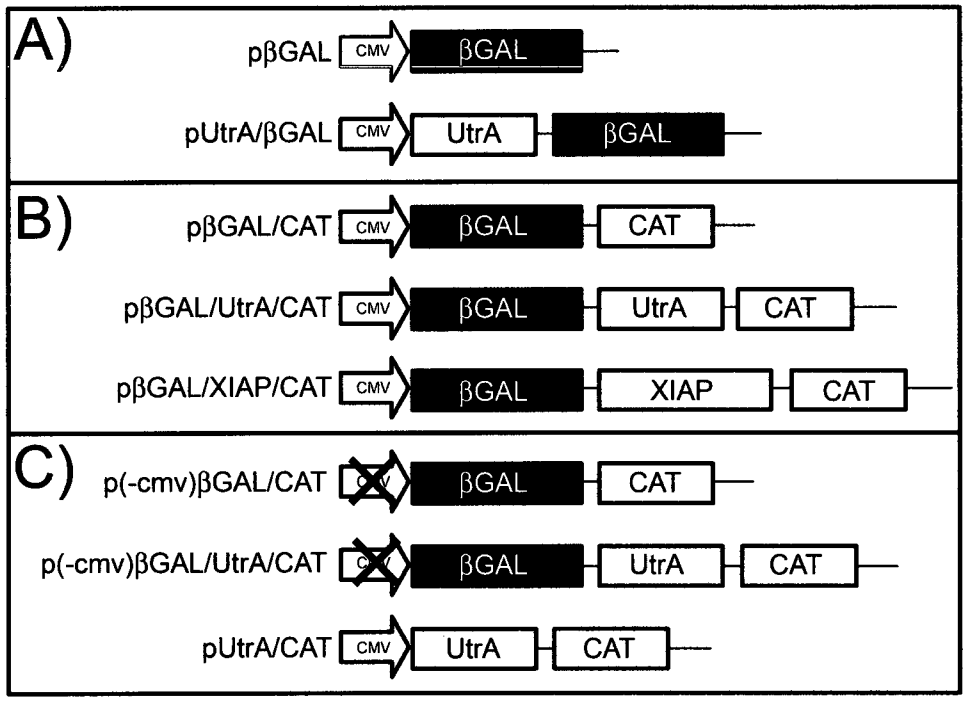
levels, thereby suggesting that changes in protein stability and/or in the translational efficiency of utrophin A mRNA could have an important regulatory function.

The Utrophin A 5'-UTR Confers Translational Regulation - On the basis of these findings, we next examined the possibility that utrophin is translationally regulated, and decided to specifically assess whether mechanisms involving the utrophin A 5'-UTR could account for the discrepancy between the levels of protein and transcript observed during muscle regeneration. To this end, we isolated the murine utrophin A 5'-UTR from total RNA obtained from C2C12 myogenic cells and found that it was relatively long (Weir et al., 2002). Using the mFOLD algorithm, this sequence was predicted to have a high degree of secondary structure (Zuker, 2003). Long 5'-UTRs often confer an inhibitory effect on cap-dependent translation. Therefore, we inserted this fragment into a reporter vector upstream of the β–Galactosidase reporter gene to examine whether the utrophin A 5'-UTR causes such translational inhibition (see schematics of constructs in **Fig. 11**).

The utrophin A 5'-UTR reporter construct (pUtrA/βGAL) or a parental control (pβGAL) was directly injected into control mouse TA muscles. An indication of the role that a 5'-UTR of interest plays in translational regulation can be obtained by measuring protein reporter activity and standardizing to the relative levels of reporter mRNA expressed from this plasmid (Bernstein et al., 1997; Shao and Ismail-Beigi, 2001). Results of these experiments are thus expressed as a ratio of reporter protein activity to reporter transcript levels. As shown in **Fig. 13A**, we observed in control TA muscles, a dramatic decrease in the ratio of protein to transcript for muscles transduced with the utrophin A 5'-UTR reporter construct (pUtrA/βGAL) compared to muscles transduced

Fig. 11. Schematic representation of reporter constructs.

A) Monocistronic constructs used for the direct gene transfer studies *in vivo*. Note the presence of the utrophin A 5'-UTR (UtrA) upstream of the reporter gene. The pCAT vector, which was used as a control for transduction efficiency, is not shown. **B)** Bicistronic constructs used to test for IRES activity. **C)** Promoterless and monocistronic versions of the bicistronic constructs used in control experiments.


A)
pβGAL
CMV
βGAL
pUtrA/βGAL
CMV
UtrA
βGAL
B)
pβGAL/CAT
CMV
βGAL
CAT
pβGAL/UtrA/CAT
CMV
βGAL
UtrA
CAT
pβGAL/XIAP/CAT
CMV
βGAL
XIAP
CAT
C)
p(-cmv)βGAL/CAT
βGAL
CAT
p(-cmv)βGAL/UtrA/CAT
βGAL
UtrA
CAT
pUtrA/CAT
CMV
UtrA
CAT

with the parental vector (pβGAL). This demonstrates that the 5'-UTR of utrophin A confers an overall inhibitory effect on translation in skeletal muscle fibers under control, steady-state conditions.

In order to study the effects of muscle regeneration on the ratio of reporter protein activity to transcript levels, we pre-injected TA muscles with cardiotoxin three days prior to direct plasmid injection of the reporter constructs. Muscles were excised seven days after the initial cardiotoxin injection. In these experiments, we reasoned that if the 5'-UTR of utrophin A plays a role in the discordant expression of endogenous utrophin A protein and transcript during muscle regeneration (see **Fig. 10** and **12**), then there should be a significant increase in the ratio of protein activity to transcript levels in regenerating muscles injected with pUtrA/βGAL. In accordance with our hypothesis, the relative ratio of protein to mRNA levels was dramatically increased in regenerating muscles injected with the construct containing the utrophin A 5'-UTR (**Fig. 13B**). Together, these results demonstrate that the inhibitory effect exerted by the utrophin A 5'-UTR on translation in control muscles was mitigated in cardiotoxin-treated TA muscles. These experiments further indicate that the increase in utrophin A protein expression observed in regenerating muscles (**Fig. 10** and **12**) involves a de-repression of translation inhibition conferred by the long and highly-structured utrophin A 5'-UTR. It should be noted that plasmid DNA uptake is significantly greater in regenerating muscle than normal muscle (Vitadello et al., 1994), thereby making it necessary to express the data in relative ratios of protein product activity to mRNA levels. Finally, it is important to note that removal of the CMV promoter completely abolished expression of the reporter gene in control and

Fig. 12. Utrophin A is expressed in extrasynaptic regions during muscle regeneration.

Immunofluorescence experiments performed with longitudinal sections of TA muscles using a utrophin A specific antibody. **A)** and **B)** show the localization of utrophin A in the synaptic regions of control muscle fibers. Arrows identify neuromuscular junction regions as identified by α-bungarotoxin staining of the acetylcholine receptor. **C)** and **D)** show the absence of utrophin A labelling in the extrajunctional regions of normal muscle fibers. **E)** and **F)** show the dramatic increase of utrophin A labelling at extrajunctional regions of regenerating muscle. Scale bar = 50 μM.

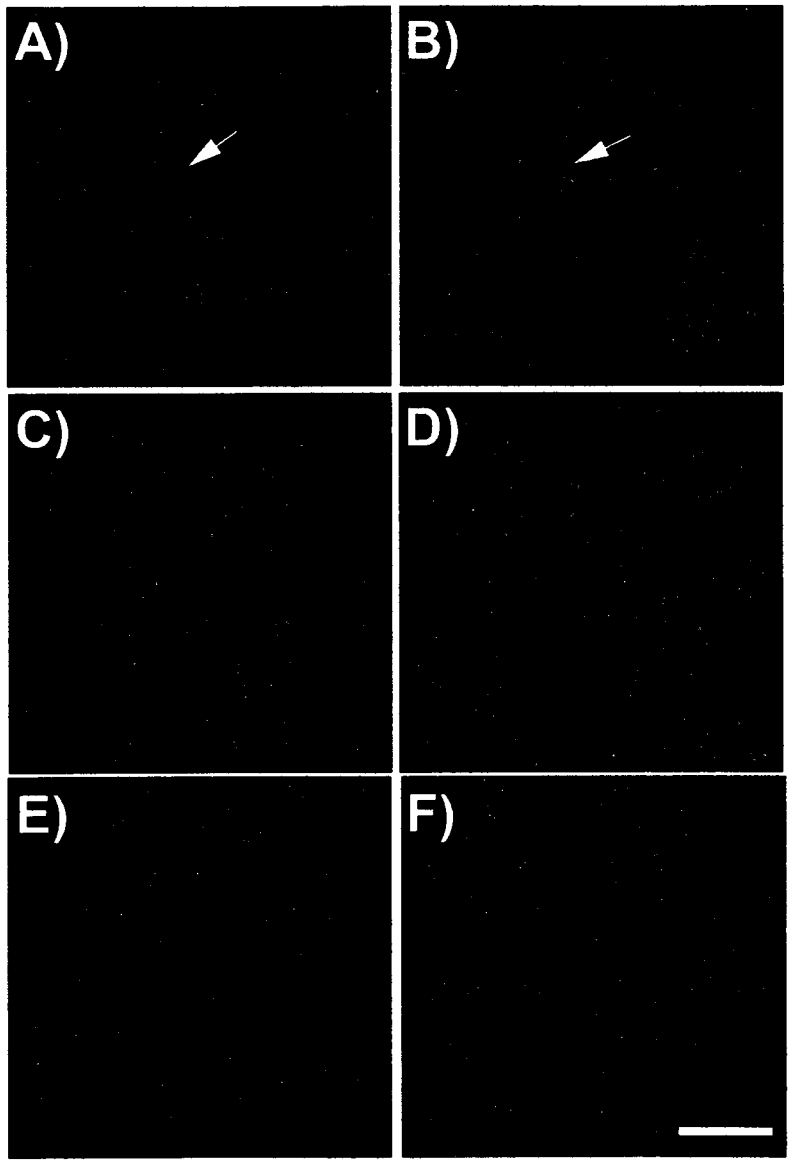
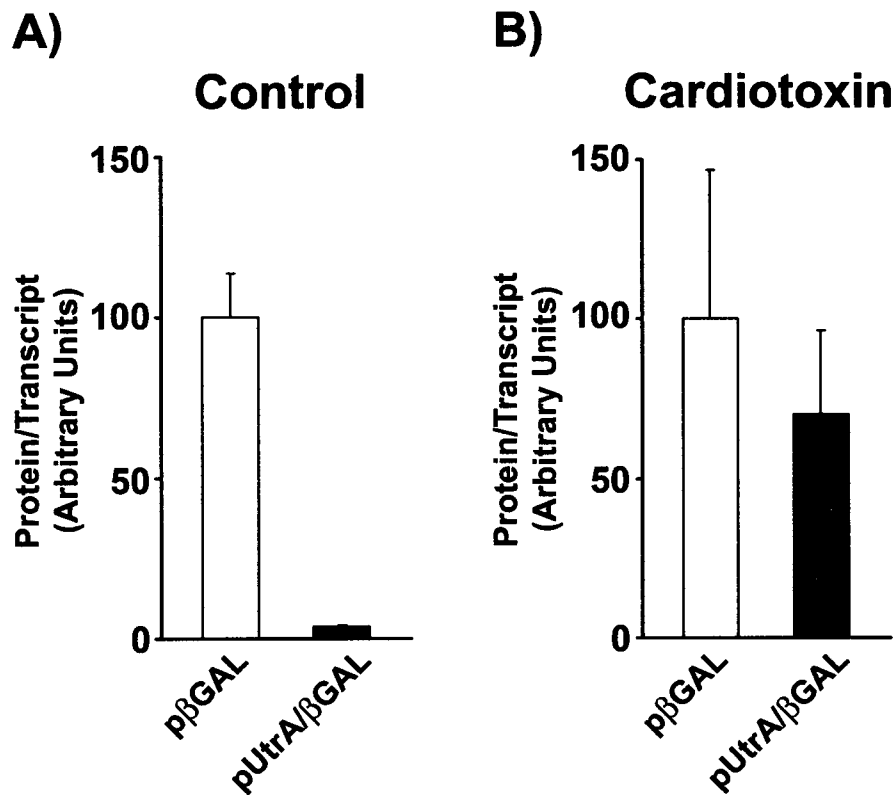

A)
B)
C)
D)
E)
F)

Fig. 13. The utrophin A 5'-UTR regulates expression of a reporter protein during muscle regeneration.

A) pβGAL or pUtrA/βGAL constructs (see Fig. 11) were directly injected into normal TA muscles along with the pCAT vector which was used to monitor the efficiency of gene transfer. Both βGAL reporter activity and βGAL mRNA levels were standardized to CAT reporter activity and CAT mRNA levels. Values are shown as a ratio of reporter protein levels, measured by β–galactosidase activity, to reporter mRNA levels, measured by RT-PCR, in order to obtain an indication of relative translational efficiency. The presence of the utrophin A 5'-UTR causes an inhibitory effect on translation of the reporter. **B)** Muscles were treated with cardiotoxin three days prior to plasmid injection to induce muscle regeneration. The regenerative state of the muscle causes the inhibitory effect of the 5'-UTR on translation to be partially removed, causing the reporter protein to be translated more efficiently. Mean +/- SEM are shown.


A)
Control
150
100
50
0
Protein/Transcript
(Arbitrary Units)
pβGAL
pUtrA/βGAL
B)
Cardiotoxin
150
100
50
0
Protein/Transcript
(Arbitrary Units)
pβGAL
pUtrA/βGAL

regenerating muscles indicating that the utrophin A 5'-UTR does not contain cryptic promoter activity (data not shown).

Identification of IRES Activity in the Utrophin A 5'-UTR in Regenerating Muscle - The above data are coherent with the idea that utrophin A is translationally regulated in regenerating muscles by mechanisms that target the 5'-UTR. Most commonly, translational regulation occurs at the point of translation initiation. One possible mechanism through which specific eukaryotic mRNAs are translated during periods of "cellular stress" involves translation initiation mediated by the presence of an Internal Ribosome Entry Site (IRES) (Holcik and Sonenberg, 2005). We therefore speculated that the utrophin A 5'-UTR contains an IRES that functions during the "stress" of cardiotoxin-induced muscle regeneration. A well established approach to assess IRES activity of 5'-UTRs consists in using bicistronic reporter constructs (Vagner et al., 2001; Merrick, 2004). Bicistronic vectors contain two reporter proteins, which enable the identification of IRES activity in a sequence of interest inserted between the two cistrons. The downstream cistron is only expressed if the sequence of interest contains an IRES. To test this hypothesis, we thus inserted the utrophin A 5'-UTR into a bicistronic vector (pβGAL/CAT see **Fig. 11**) previously used to identify IRESes (Holcik et al., 1999). The first cistron (βGAL) of this vector measures cap-dependent translation, while the second cistron (CAT) represents cap-independent translation.

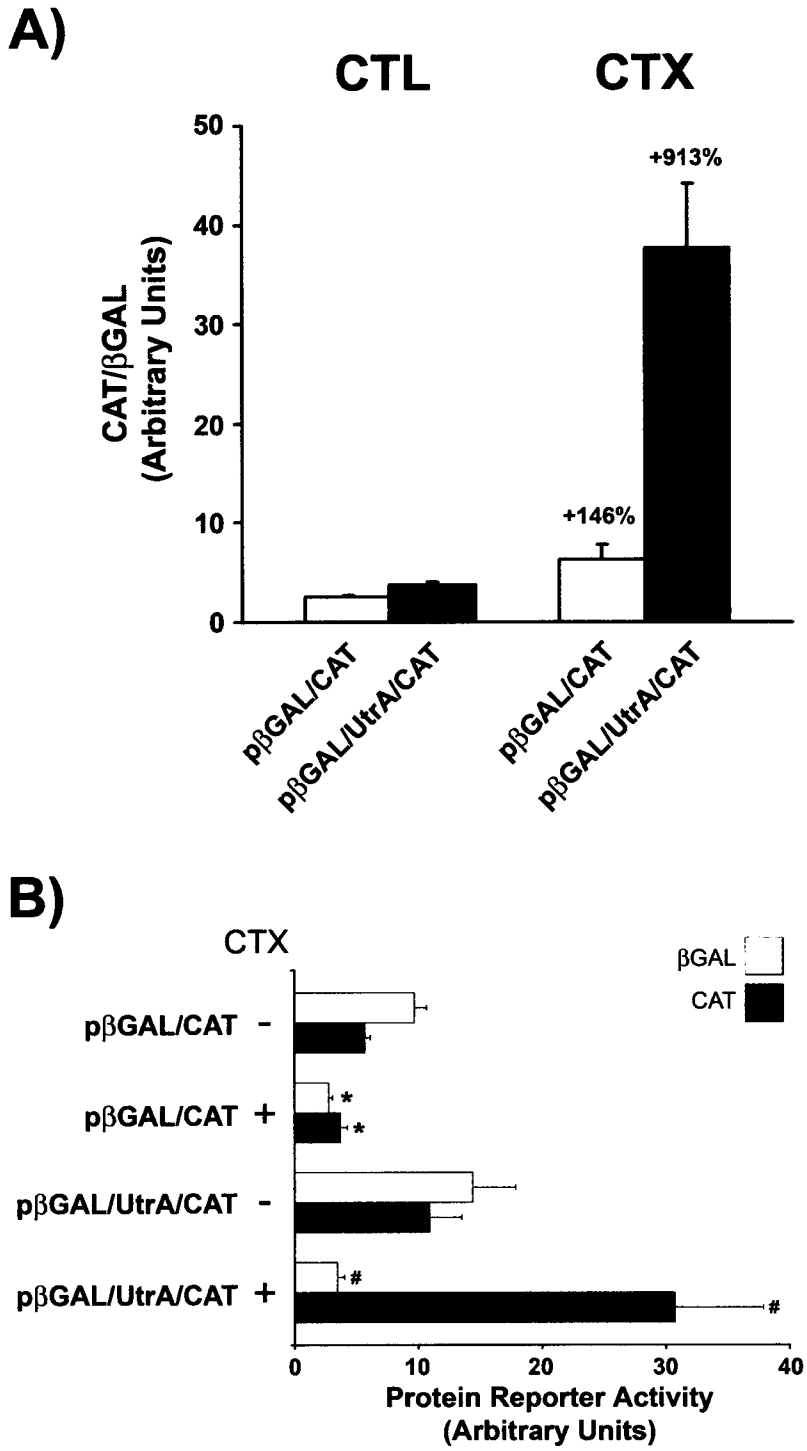
Cardiotoxin-treated and control TA muscles were injected with the bicistronic reporter constructs using the same time-frame as for injection with monocistronic reporter constructs (see above). As shown in **Fig. 14**, IRES activity is reported as a ratio of CAT/βGAL activity. A ratio significantly higher than that seen with the parental

vector is reflective of IRES activity. In control muscles transduced with the bicistronic constructs, no IRES activity above background was detected with the utrophin A 5'-UTR since similar CAT/βGAL ratios were found between pβGAL/CAT and pβGAL/UtrA/CAT-injected muscles. In cardiotoxin-treated muscles, there was a slight induction (~1.5-fold) in the ratio of CAT/βGAL for muscles injected with the parental plasmid (but, see below). However, there was a striking increase of more than 9-fold ($P < 0.05$) in the ratio of CAT/βGAL in regenerating muscles injected with the bicistronic vector containing the utrophin A 5'-UTR (**Fig. 14A**). The ratio of CAT/βGAL in pβGAL/UtrA/CAT-injected regenerating muscles was ~6-fold greater that that of pβGAL/CAT-injected regenerating muscles.

Further analysis of the data expressed as individual levels of CAT and βGAL revealed that the increased CAT/βGAL ratio seen for the parental vector in regenerating muscles was caused by a decrease in the level of the βGAL reporter, and not by an increase in CAT (**Fig. 14B**). Thus, the increased ratio seen for the parental vector in **Fig. 14A** is not reflective of spurious IRES activity but rather indicates a decrease in cap-dependent translation (determined by βGAL). In contrast, the increased ratio of CAT/βGAL mediated by the presence of the utrophin A 5'-UTR between the cistrons and seen in cardiotoxin-treated muscles, was clearly due to an increase in CAT activity (**Fig. 14B**). Similar to what was observed with the pβGAL/CAT constructs, we also detected a decrease in βGAL activity with the pβGAL/UtrA/CAT construct to an extent similar to that seen with the parental vector (**Fig. 14B**). It should be noted that the levels of βGAL for pβGAL/CAT and pβGAL/UtrA/CAT in the cardiotoxin treated muscle were not statistically different, while the levels of CAT were 8.3-fold greater in

Fig. 14. The utrophin A 5'-UTR displays IRES activity during muscle regeneration.

pβGAL/CAT or pβGAL/UtrA/CAT constructs were directly injected into control (CTL) and caridotoxin-treated (CTX) TA muscles. **A)** shows data reported as a ratio of CAT activity, measuring cap-independent translation, to βGAL activity, which measures cap-independent translation. A higher ratio of CAT/βGAL indicates IRES activity. The utrophin A 5'-UTR did not display IRES activity in control muscles. By contrast, we observed a significant induction (+ 913%) of utrophin A IRES activity in regenerating muscles. **B)** shows the individual levels of βGAL and CAT used to calculate the ratios shown in A). Note that the slight increase in apparent IRES activity seen with the ratios in regenerating muscles injected with the parental vector, is due to a decrease in βGAL activity and not because of an increase in CAT. In regenerating muscles injected with the constructs containing the utrophin A 5'-UTR, note also the decrease in βGAL activity, but in addition, the significant increase in CAT levels. (-) and (+) signs represent control or cardiotoxin-treated muscles, respectively. * denote significant differences between control and cardiotoxin-treated muscles injected with pβGAL/CAT. # denote significant differences between control and cardiotoxin-treated muscles injected with pβGAL/UtrA/CAT. Mean +/- SEM are shown, $n = 12$ per groups, $P < 0.05$.


A)
CTL
CTX
CAT/βGAL (Arbitrary Units)
50
40
30
20
10
0
+913%
+146%
pβGAL/CAT
pβGAL/UtrA/CAT
pβGAL/CAT
pβGAL/UtrA/CAT
B)
CTX
βGAL
CAT
pβGAL/CAT -
pβGAL/CAT +
pβGAL/UtrA/CAT -
pβGAL/UtrA/CAT +
*
*
#
#
0
10
20
30
40
Protein Reporter Activity
(Arbitrary Units)

pβGAL/UtrA/CAT-injected regenerating muscles compared to pβGAL/CAT- injected regenerating muscles. Therefore, the utrophin A 5'-UTR mediates cap-independent translation of the CAT reporter but only in cardiotoxin-treated muscle. This demonstrates that the utrophin A 5'-UTR contains an inducible IRES that becomes activated during muscle regeneration.

In parallel experiments, we also performed direct plasmid injection into TA muscles using a bicistronic vector containing the 5'-UTR of the X-chromosome linked inhibitor of apoptosis protein (XIAP), which is well characterized to contain an IRES element (pβGAL/XIAP/CAT) (Holcik et al., 1999; Holcik and Korneluk, 2000). Interestingly, XIAP IRES activity was dramatically lower in cardiotoxin-treated muscles compared to control muscles (data not shown), suggesting that the IRES-activating effect of muscle regeneration is gene specific.

To determine whether the IRES activity that we observed in regenerating muscles could be linked to aberrant splicing events, several control experiments were performed to ensure that CAT expression was due to genuine IRES-mediated translation of an intact bicistronic transcript. Due to the relatively low efficiency of transduction in mouse skeletal muscle, it is necessary to use a combination of PCR experiments to control for the possibility of splicing. First, RT-PCR was used to amplify the central region of the bicistronic RNA using primers flanking the utrophin A 5'-UTR sequence and portions of the βGAL and CAT reporter mRNAs (see **Fig. 15A**). As shown in **Fig. 15B**, we amplified RT-PCR products of the expected size using RNA extracted from cardiotoxin-treated muscles injected with pβGAL/UtrA/CAT. Sequencing of these PCR products confirmed their identity. Using this sensitive approach, we failed to detect smaller,

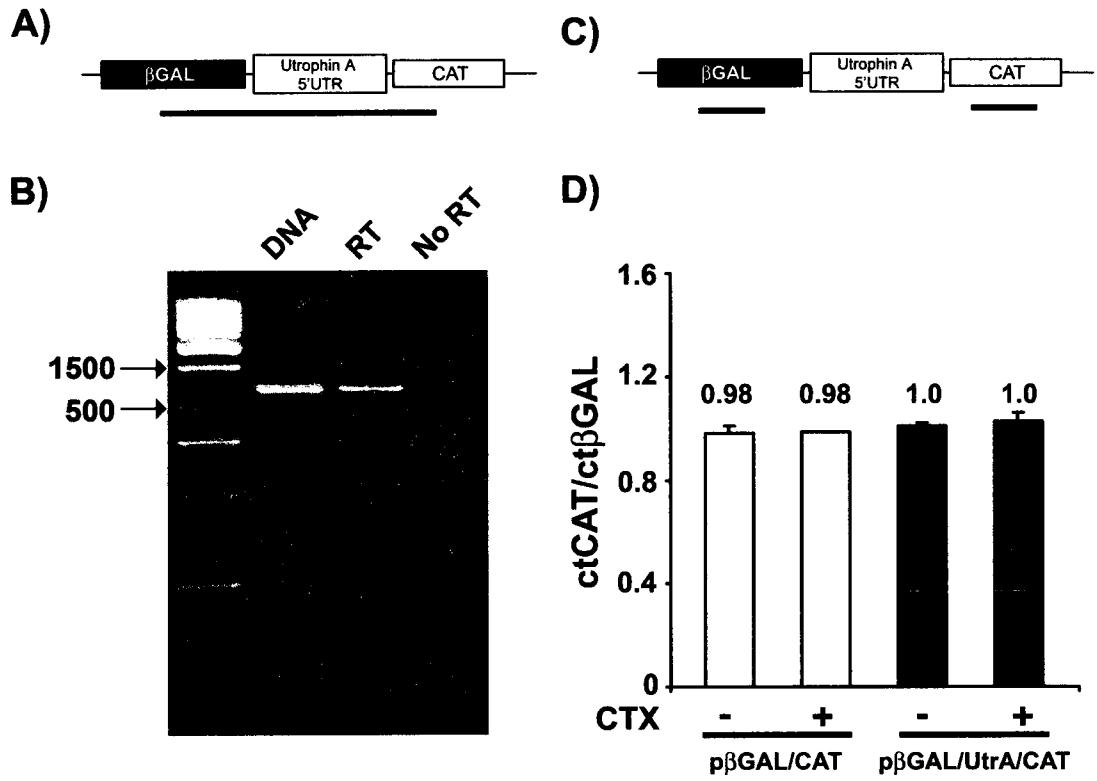
aberrantly-spliced mRNA further demonstrating that IRES-mediated translation of utrophin A occurs during muscle regeneration.

To further control for the possibility of aberrant splicing, additional experiments were performed. Specifically, we used qRT-PCR to amplify βGAL and CAT cistrons of the bi-cistronic mRNA (**Fig. 15C**). We reasoned that if the CAT activity from muscles transduced with the pβGAL/UtrA/CAT plasmid was due to IRES-mediated translation from intact, full-length bicistronic transcript then, βGAL and CAT cDNAs would be amplified by the qPCR reaction in a ratio of 1:1. Conversely, spurious splicing of the bicistronic mRNA would result in an increase of the CAT cistron RNA relative to βGAL cistron. This approach has in fact been used recently by others (Teshima-Kondo et al., 2004). We therefore processed RNA from control and cardiotoxin treated muscles transduced with the parental or 5'-UTR containing plasmids. For all samples, we found a ratio of 1:1 (**Fig. 15C**). This indicates that only full-length mRNAs were transcribed from both parental and 5'-UTR containing plasmids in both control and cardiotoxin treated TA muscles.

The Utrophin A IRES Is Also Active in Myoblasts - In separate experiments, we also assessed the activity of the utrophin A IRES in muscle cell culture. To this end, we transfected C2C12 myoblasts with the parental or utrophin A 5'-UTR-containing bicistronic vectors, harvested the cells 24 hrs later, and assessed IRES activity by determining reporter activity. We found that the presence of the utrophin A 5'-UTR conferred a ~9-fold ($P < 0.05$) induction in the ratio of CAT/βGAL compared to the ratio seen using the parental vector (**Fig. 16A**). This demonstrates that the utrophin A IRES element also mediates cap-independent translation in myoblasts. Interestingly, transfected

Fig. 15. RT-PCR analysis of bicistronic transcripts in injected muscles.

A) Schematic representation of the RT-PCR strategy. **B)** the "DNA" lane shows the PCR product amplified directly from pβGAL/UtrA/CAT plasmid. The "RT" lane shows the RT-PCR product amplified from RNA extracted from TA muscles injected with pβGAL/UtrA/CAT. Note that the transcripts are of the right molecular mass. Sequencing revealed that it contains the two portions of the reporter proteins as well as the intact utrophin A 5'-UTR suggesting the presence of full-length bicistronic transcripts. No smaller PCR fragments were observed indicating that no aberrant splicing occurred. "No RT" is an RT-PCR amplification of RNA extracted from TA muscle injected with pβGAL/UtrA/CAT with no reverse transcriptase in the RT reaction. This shows that amplification occurred from RNA and not from contaminating plasmid DNA which was eliminated by DNAse 1 treatment (see Methods). **C)** Schematic representation of the qRT-PCR strategy. **D)** qPCR using βGAL and CAT primers was performed on reverse transcriptase amplified RNA from TA muscles injected with the bicistronic reporter constructs. A ratio of amplified βGAL and CAT cDNA of 1:1 indicates the presence of an intact bicistronic transcript. Note that the ratio of cycle thresholds (ctCAT/ctβGAL) are basically equal to 1 and no ratios are statistically different from each other. Mean +/- SD are shown, $P > 0.05$.


A)
βGAL
Utrophin A 5'UTR
CAT
C)
βGAL
Utrophin A 5'UTR
CAT
B)
DNA
RT
No RT
1500
500
D)
ctCAT/ctβGAL
1.6
1.2
0.8
0.4
0
0.98
0.98
1.0
1.0
CTX
-
+
-
+
pβGAL/CAT
pβGAL/UtrA/CAT

cells that were induced to undergo myogenic differentiation for 4 days showed no IRES activity mediated by the utrophin A 5'-UTR (data not shown).

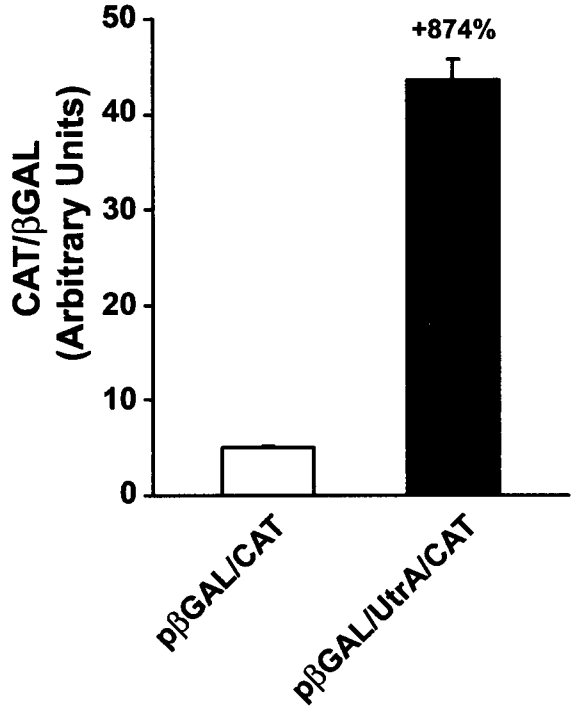
To ensure that expression of the downstream cistron was caused by genuine internal ribosome entry, we also examined the βGAL and CAT data individually. As shown in **Fig. 16B**, the increase ratio noted in cells transfected with the pβGAL/UtrA/CAT contruct was caused by a large increase ($P < 0.05$) in CAT activity. To further verify that this increased CAT activity was not due to the presence of a cryptic promoter in the utrophin A 5'-UTR, transfections of bicistronic reporter constructs in which the CMV promoter had been deleted, were performed. If the utrophin A 5'-UTR contains a cryptic promoter, CAT expression would occur in transfected cells despite the deletion of the viral promoter. Removal of the CMV promoter from the parental and utrophin A 5'-UTR vectors [p(-cmv)βGAL/CAT and p(-cmv)βGAL/UtrA/CAT, respectively] resulted in the loss of both βGAL and CAT reporter activity (data not shown). In agreement with these findings, no expression of an mRNA containing the CAT sequence could be detected by northern blots performed with total RNA extracted from cells transfected with the p(-cmv)βGAL/UtrA/CAT construct (data not shown). This further shows that the IRES activity of the utrophin A 5'-UTR is not due to cryptic promoter activity.

To control for the possibility of splicing events causing the expression of the CAT cistron from the pβGAL/UtrA/CAT vector in the transfected cells in culture, we performed RT-PCR and northern blot analyses to verify the presence of an intact

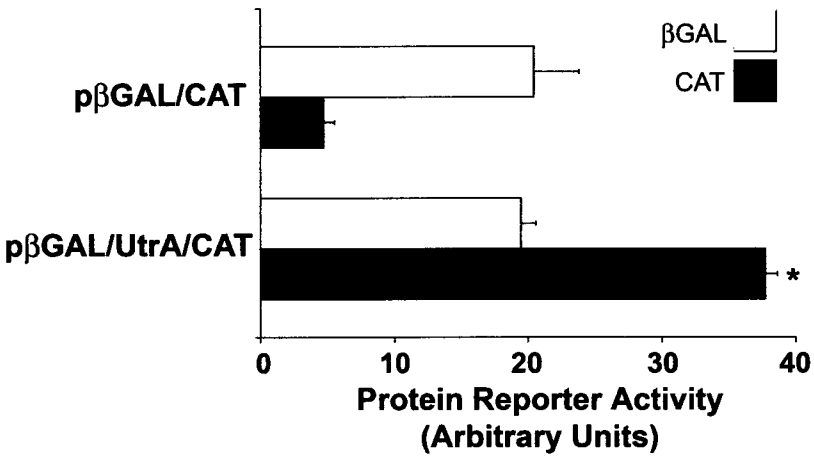
Fig. 16. The Utrophin A IRES is also active in C2C12 myoblasts.

A) Bicistronic reporter constructs were transfected in C2C12 myoblasts and levels of reporter proteins assayed 24 hrs later. Note that in comparison to cells transfected with the parental construct, cells transfected with the construct containing the utrophin A 5'-UTR displays a large increase (+ 874%) in IRES activity. These data are reported as a ratio of CAT activity, measuring cap-independent translation, to βGAL activity, measuring cap-dependent translation. A higher ratio of CAT/βGAL is indicative of IRES activity. B) shows the individual levels of CAT and βGAL activity used to calculate the ratios presented in A). Note that the increased ratio is due to an increase in CAT activity. * denotes a significant difference between cells transfected with βGAL/CAT versus pβGAL/UtrA/CAT transfected cells. Mean +/- SEM are shown, $P < 0.05$.

A)


50
40
30
20
10
0
CAT/βGAL
(Arbitrary Units)
+874%
pβGAL/CAT
pβGAL/UtrA/CAT

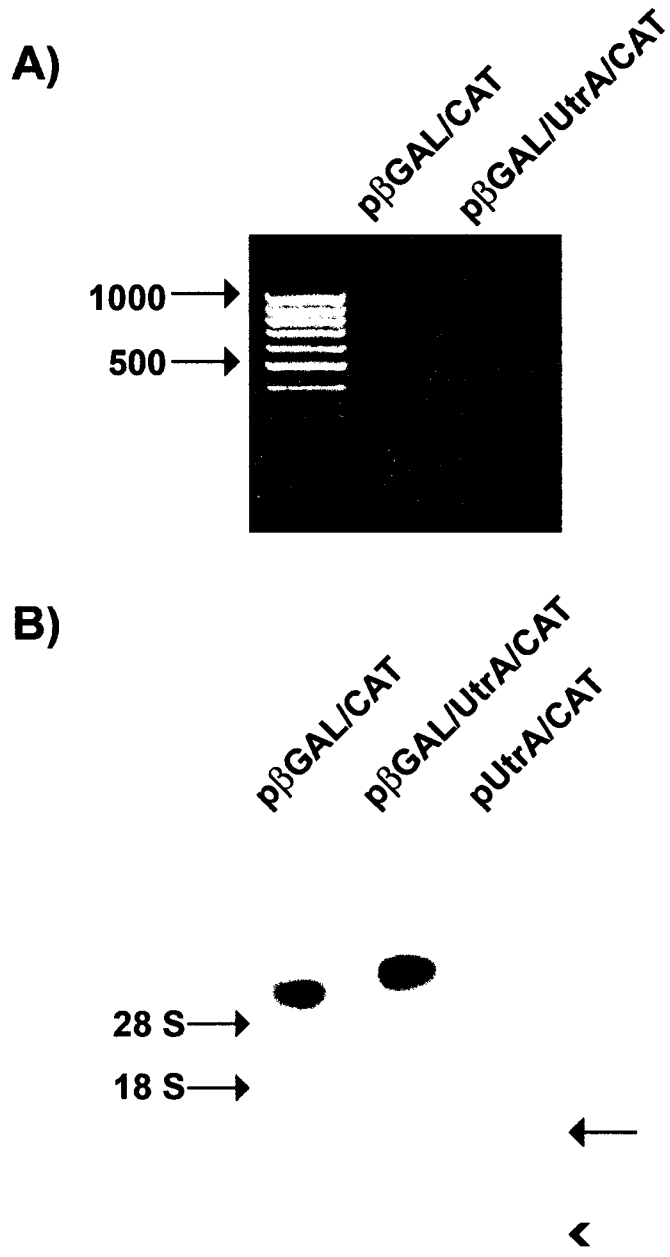
B)


βGAL
CAT
pβGAL/CAT
pβGAL/UtrA/CAT
*
0
10
20
30
40
Protein Reporter Activity
(Arbitrary Units)

bicistronic transcript. RT-PCR analysis of RNA from the pβGAL/CAT and the pβGAL/UtrA/CAT transfected C2C12 cells amplified single transcripts of the expected sizes (**Fig. 17A**). The larger PCR product seen in cells transfected with pβGAL/UtrA/CAT is due to the presence of the utrophin A 5'-UTR between the two cistrons. Northern blotting using a 301 bp probe for CAT was used to further confirm that the downstream cistron present in the bicistronic vector was being translated from a full-length, intact bicistronic transcript. As seen in **Fig. 17B** (lane 1), RNA species of the expected size were detected in cells transfected with the parental bicistronic vector (pβGAL/CAT). As expected, northern blot using RNA from cells transfected with the bicistronic vector containing the utrophin A 5'-UTR revealed the presence of a slightly larger RNA with no smaller products being detected (pβGAL/UtrA/CAT; lane 2). We also performed a northern blot using RNA from cells transfected with a vector containing the utrophin A 5'-UTR and CAT, but lacking βGAL, to show where a potential splice product should migrate (pUtrA/CAT; lane 3, see arrow). In addition, full-length transcripts from RNA extracted from cells transfected with the pβGAL/CAT or pβGAL/UtrA/CAT constructs were also detected by northern blot using a probe for βGAL (data not shown). Together, these results confirm that the IRES activity conferred by the utrophin A 5'-UTR is caused by genuine internal ribosome entry and not by aberrant splicing events.

Fig. 17. Analysis of bicistronic transcripts in transfected cells.

A) RT-PCR analysis performed on RNA extracted from cells transfected with pβGAL/CAT or pβGAL/UtrA/CAT using primers spanning a portion of βGAL and CAT (see Fig. 15A for RT-PCR strategy). The PCR products are of the expected sizes and sequencing confirmed their identity. Note the larger fragment seen in cells transfected with pβGAL/UtrA/CAT due to the presence of the utrophin A 5'-UTR. B) Northern blot analysis using a 301 bp probe that recognizes CAT was performed with RNA extracted from cells transfected with pβGAL/CAT or pβGAL/UtrA/CAT. Note the presence of single, intact bicistronic transcripts. Northern analysis of RNA extracted from cells transfected with pUtrA/CAT shows the migration of a lower molecular mass species (see arrow). This band corresponds to where a potential splice product from pβGAL/UtrA/CAT would migrate (see arrow). The arrowhead denotes where RNA from cells transfected with a monocistronic CAT vector would migrate (not containing the utrophin A 5'-UTR). The 28S marker corresponds to 4.7 kb, while the 18S marker corresponds to 1.9 kb.


A)
pβGAL/CAT
pβGAL/UtrA/CAT
1000
500
B)
pβGAL/CAT
pβGAL/UtrA/CAT
pUtrA/CAT
28 S
18 S

DISCUSSION

In recent years, several reports have implicated that utrophin is regulated via translational and/or post-translational mechanisms (see Introduction). Specifically, the discordant patterns of expression in the levels of utrophin and its mRNA in muscle cells placed under a number of experimental conditions indicate that utrophin expression is regulated by translational mechanisms and/or protein stability. The current study was designed to examine this issue by focusing on the possibility that utrophin expression, in addition to being controlled by transcriptional mechanisms, is also subjected to important translational events that ultimately modulate the overall abundance of utrophin in muscle fibers. Here, we show that the large increase in the expression of utrophin A during muscle regeneration is clearly not accompanied by a parallel increase in utrophin A transcripts. Additionally, we show that the utrophin A 5'-UTR has an inhibitory effect on translation of a reporter transcript in intact skeletal muscle fibers. Strikingly, a marked de-repression of this inhibition is observed in muscle fibers undergoing regeneration. Finally, using several complementary approaches, we show that the utrophin A 5'-UTR contains an IRES whose activity becomes highly stimulated in regenerating muscle.

IRES-mediated translation is an alternative to cap-dependent translation, the main mechanism of translation initiation in eukaryotes (Hellen and Sarnow, 2001). In cap-dependent translation, the eukaryotic initiation factor eIF4E binds to the ^{7}methyl guanosine-capped structure at the 5'end of an mRNA. Upon association of other initiation factors, Met-tRNA$_i^{Met}$ and the 40S ribosomal subunit, scanning is believed to occur in a 5' to 3' direction until a start codon in a favourable sequence context is encountered and protein synthesis begins (Preiss and Hentze, 2003). By contrast, in

IRES-mediated translation the 5' UTR harbours specific sequences that are thought to recruit ribosomes directly to the vicinity of initiation codon independently of the 5' capped structure (Holcik and Sonenberg, 2005). While cellular mRNAs are normally translated in a cap-dependent manner, there is a growing list of cellular mRNAs that are now known to be translated via cap-independent, IRES-mediated translation. Often, these mRNAs contain long and highly-structured 5'-UTRs.

Using bicistronic reporter vectors, we showed that the utrophin A 5'-UTR displayed, as expected, no IRES activity in intact muscles. However, in muscle induced to degenerate and regenerate by cardiotoxin treatment, the utrophin A 5'-UTR mediated a 9-fold increase in the expression of the downstream reporter. Several control experiments revealed that the bicistronic mRNA did not undergo aberrant splicing, while also showing that the utrophin A 5'-UTR does not contain cryptic promoter activity. Based on these findings, it appears therefore that the induction of the muscle regenerative program serves as an appropriate signal to create an environment causing activation of the utrophin A IRES. Thus, cap-independent translation via the utrophin A 5'-UTR IRES can at least partially account for the large discrepancy in protein and transcript levels observed in regenerating skeletal muscle.

Skeletal muscle regeneration that follows after injury and chemical insult, involves the activation of quiescent satellite cells and promotion of their entry into the mitotic phase. These cells proliferate, terminally differentiate, and fuse to form newly formed mutli-nucleated muscle fibers (Charge and Rudnicki, 2004). Interestingly, and in agreement with our *in vivo* findings, we observed in the present studies that the utrophin A 5'-UTR also displayed IRES activity in myoblasts growing in culture at 9.1-fold over

parental levels, (**Fig. 16**). In contrast, after transfected myoblasts were induced to differentiate to form multinucleated myotubes, we observed no significant IRES activity (data not shown). This is unlikely to be coincidental since before they fuse to form myotubes, C2C12 myoblasts are similar to satellite cells that are activated during muscle regeneration. Our results obtained with cultured cells as well as with muscle *in vivo* are strongly supported by the fact that cap-dependent translation is often down-regulated during cell cycle activation and differentiation, while IRES-mediated translation of specific genes persist under these conditions (Pozner et al., 2000; Sachs, 2000). Indeed, βgal reporter levels, that in our experimental system are indicative of cap-dependent translation, were reduced in cardiotoxin treated samples.

Many of the mammalian IRES elements found to date mediate cap-independent translation of proteins that are expressed during periods of "cellular stress" when cap-dependent translation may become less efficient (Johannes et al., 1999; Hellen and Sarnow, 2001; Holcik and Sonenberg, 2005). An interesting possibility therefore is that the "stress" of muscle regeneration causes the induction of IRES activity for a variety of mRNAs. In this context, several other proteins that are known to be involved in muscle regeneration have also been shown to be regulated via IRES-mediated translation. Specifically, it is known that injection of fibroblast growth factor II (FGF-2) in muscles of *mdx* mice improves regeneration (Lefaucheur and Sebille, 1995). Additionally, expression of insulin growth factor I (IGF-I) receptor is up-regulated during muscle regeneration (Levinovitz et al., 1992). It is therefore highly relevant to note that the FGF-2 5'-UTR contains an IRES (Creancier et al., 2000) and that the receptor for IGF-I is also subject to IRES-mediated translational control (Giraud et al., 2001). In addition, both the

leader 2 5'-UTR of IGF-II (Pedersen et al., 2002) and the A and C leaders of FGF-I (Martineau et al., 2004) can cause translation initiation via internal ribosome entry. Thus, muscle regeneration may indeed represent a type of "cellular stress" that promotes IRES-mediated translation of a subclass of mRNAs whose expression may be crucial to ensure the success of the regenerative response.

In recent years, there has been an emergence of studies identifying translational regulation at specific postsynaptic sites. The majority of such work has focused on translational events that occur in dendrites and which impact on synaptic plasticity (Eberwine et al., 2001; Kelleher et al., 2004; Klann and Dever, 2004). Such local regulation provides a mechanism for protein synthesis to occur rapidly in a distinct subcellular compartment under the influence of in-coming inputs from presynaptic nerve terminals. Map 2 and Arc are two mRNAs that are localized to dendrites and whose translation can be regulated by the presence of IRES elements in their 5'-UTRs (Pinkstaff et al., 2001). These findings are important and supportive of our current results since: i) utrophin A is an important component of the postsynaptic apparatus in muscle fibers; and ii) both Map 2 and Arc are also cytoskeletal proteins.

There is solid evidence now indicating that translational regulation does occur at the *Drosophila* neuromuscular junction (Sigrist et al., 2000; Menon et al., 2004). Furthermore, there are indirect observations implicating translational regulation as an important control step in the synthesis of synaptic proteins in mammalian muscle. For instance, it has been shown that expression of the acetylcholine receptor α–subunit is regulated at the translational level in rat primary muscle cells (Horovitz et al., 1989). Moreover, a decrease in translation has been shown to account for the reduction in

acetylcholinesterase expression in skeletal muscle following glucocorticoid treatment (Brank et al., 1998). Finally, we have recently demonstrated that staufen, a double stranded RNA-binding protein involved in mRNA targeting and translation, accumulates within the postsynaptic membrane domain of the neuromuscular junction (Belanger et al., 2003).

In summary, we have demonstrated that the utrophin A 5'-UTR can drive IRES mediated translation during muscle regeneration. This finding provides an explanation for the discrepancy between utrophin A protein and transcript levels observed in various studies. Although these findings do not exclude the possibility that post-translational mechanisms, such as alterations in protein stability, may also partially account for these discrepancies between protein and mRNA levels (Moghadaszadeh et al., 2003b; Roma et al., 2004; Waheed et al., 2005), our data add considerably to the growing literature highlighting the importance of translational control for the regulation of synaptic proteins in muscle and nerve. Additionally, our results provide an additional target from which pharmacological interventions may be rationally designed to stimulate utrophin A expression in muscle fibers from DMD patients in attempts to alter the progression of this neuromuscular disorder and provide functional benefits.

Footnotes

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CHAPTER 4

IRES-Mediated Translation of Utrophin A is Enhanced by Glucocorticoid Treatment in Skeletal Muscle Cells

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Contributions of Authors: Conceived and designed the experiments: BJ PM MH. Performed the experiments: PM MA. Analyzed the data: BJ PM MA MH. Wrote the paper: BJ PM MH.

ABSTRACT

Glucocorticoids are currently the only drug treatment recognized to benefit Duchenne muscular dystrophy (DMD) patients. The nature of the mechanisms underlying the beneficial effects remains incompletely understood but may involve an increase in the expression of utrophin. Here, we show that treatment of myotubes with 6α–methylprednisolone-21 sodium succinate (PDN) results in enhanced expression of utrophin A without concomitant increases in mRNA levels thereby suggesting that translational regulation contributes to the increase. In agreement with this, we show that PDN treatment of cells transfected with monocistronic reporter constructs harbouring the utrophin A 5'-UTR, causes an increase in reporter protein expression while leaving levels of reporter mRNAs unchanged. Using bicistronic reporter assays, we further demonstrate that PDN enhances activity of an Internal Ribosome Entry Site (IRES) located within the utrophin A 5'-UTR. Analysis of polysomes demonstrate that PDN causes an overall reduction in polysome-associated mRNAs indicating that global translation rates are depressed under these conditions. Importantly, PDN causes an increase in the polysome association of endogenous utrophin A mRNAs and reporter mRNAs harbouring the utrophin A 5'-UTR. Additional experiments identified a distinct region within the utrophin A 5'-UTR that contains the inducible IRES activity. Together, these studies demonstrate that a translational regulatory mechanism involving increased IRES activation mediates, at least partially, the enhanced expression of utrophin A in muscle cells treated with glucocorticoids. Targeting the utrophin A IRES may thus offer an important and novel therapeutic avenue for developing drugs appropriate for DMD patients.

INTRODUCTION

Glucocorticoid administration is currently the only drug treatment known to offer real clinical benefit to patients suffering from Duchenne muscular dystrophy (DMD). Glucocorticoids used to treat DMD include prednisone (Mendell et al., 1989) and its oxazoline derivative, deflazacort (Biggar et al., 2001). DMD patients treated with glucocorticoids exhibit delayed progression of muscle weakness (Fenichel et al., 1991) and remain ambulatory for a greater period of their lives (DeSilva et al., 1987). The mechanism by which patients benefit from glucocorticoid treatment is not fully understood, although it is thought that the clinical benefits arise in part from the anti-inflammatory and immunosuppressive effects of these drugs (Tidball and Wehling-Henricks, 2004). Previous work has shown that deflazacort treatment of the *mdx* mouse, a dystrophin deficient model of DMD, can alleviate symptoms of the dystrophic pathology and results in the stimulation of utrophin A expression in skeletal muscle fibers (St Pierre et al., 2004). This observation is important since one therapeutic strategy for the treatment of DMD involves the stimulation of endogenous utrophin levels in dystrophic skeletal muscle fibers (Khurana and Davies, 2003; Miura and Jasmin, 2006). In this context, utrophin upregulation represents an interesting therapeutic strategy for DMD since it is the autosomal homologue of dystrophin, the protein missing from DMD muscle fibers. Several previous studies have in fact shown the ability of utrophin to functionally compensate for the absence of dystrophin in various animal models of DMD (Tinsley et al., 1996; Gilbert et al., 1999; Cerletti et al., 2003). Since stimulation of utrophin expression may be one mechanism by which DMD patients benefit from glucocorticoid

treatment, it thus becomes important to define the molecular targets through which these drugs act to increase utrophin expression in muscle cells.

While utrophin expression can be enhanced at the transcriptional level in response to deflazacort treatment in *mdx* mice (St Pierre et al., 2004), several groups have also demonstrated that utrophin is regulated at the translational or post-translational level in response to glucocorticoid treatment. Indeed, it has been shown that treatment of cultured muscle cells with glucocorticoids causes an increase in utrophin protein expression without corresponding changes in utrophin transcript levels (Pasquini et al., 1995; Courdier-Fruh et al., 2002). This discrepancy between utrophin protein and mRNA levels occurs also under other conditions, as we first demonstrated in muscle fibers of DMD patients and regenerating mouse muscle fibers (Gramolini et al., 1999a). Similar observations have been made when examining utrophin expression in *mdx* skeletal muscle (Weir et al., 2002). Thus, utrophin appears to be regulated by translational and/or post-translational mechanisms under diverse conditions.

Recently, we provided further evidence that translational control can account for the large increase in utrophin protein expression seen during regeneration of mouse skeletal muscle in the absence of concomitant changes in utrophin transcript levels (Miura et al., 2005). By using direct injection of monocistronic and bicistronic reporter vectors harbouring the utrophin A 5' untranslated region (5'-UTR) into mouse skeletal muscle, we showed that the utrophin A 5'-UTR contains an Internal Ribosome Entry Site (IRES) that is quiescent in adult muscle fibers, but becomes preferentially activated upon

the stress of muscle regeneration (Miura et al., 2005). IRES-mediated translation is an alternative mechanism of translation initiation that is believed to occur independently of the methyl^7 guanosine cap structure at the 5'end of an mRNA. This cap-independent mechanism of translation initiation regulates translation of specific eukaryotic mRNAs in response to stressful conditions in cells where cap-dependent translation is compromised (Holcik and Sonenberg, 2005). Since glucocorticoid treatment of muscle cells results in an increase in utrophin expression without alterations in mRNA levels, we hypothesized that IRES-mediated translational control is in fact the mechanism responsible for enhanced utrophin expression under these conditions. Here, we tested this hypothesis and demonstrate that PDN treatment enhances the activity of the utrophin IRES that in turn allows for efficient recruitment of utrophin mRNA into polysomes despite the overall inhibition of protein synthesis that is caused by treatment with PDN.

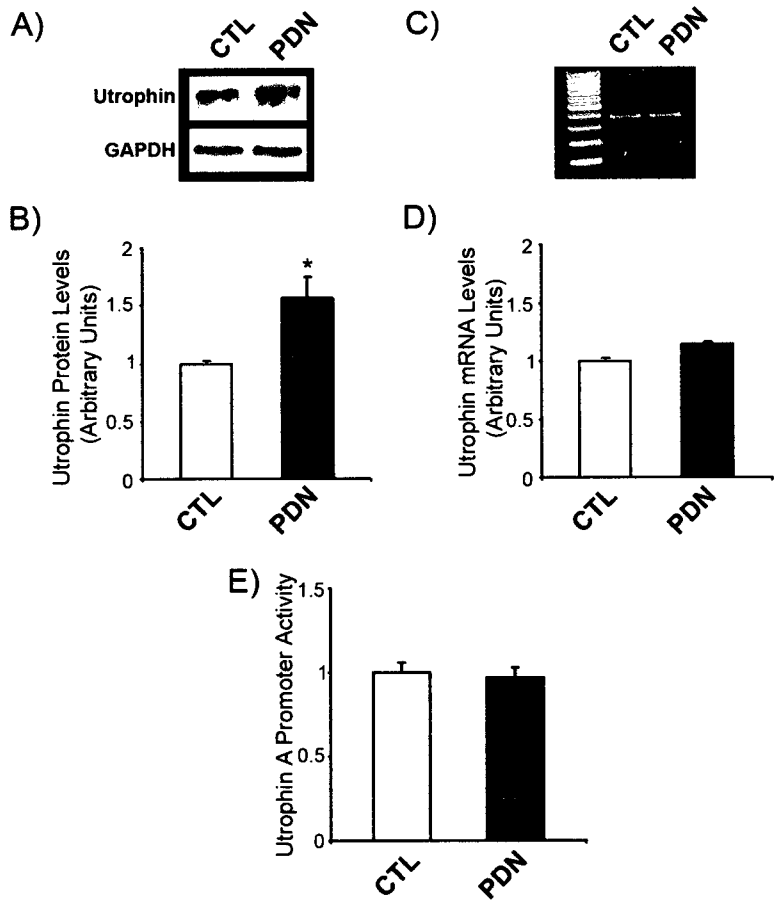
RESULTS

Glucocorticoid Treatment of C2C12 Myotubes Stimulates Utrophin Protein Expression Without Affecting mRNA Levels- Earlier studies have demonstrated that utrophin is stimulated at the protein, but not mRNA level in muscle cells treated with various glucocorticoids (Pasquini et al., 1995; Courdier-Fruh et al., 2002). We thus treated C2C12 myotubes for 3 days with the glucocorticoid 6α-methylprednisolone-21 sodium succinate (PDN) at a concentration previously shown to increase utrophin protein levels by ~ 40% (500 nM) (Courdier-Fruh et al., 2002). As expected, Western blot analysis showed that utrophin protein levels were ~1.5 fold greater ($p < 0.05$) in PDN- versus DMSO (control: CTL)- treated myotubes (**Fig. 18A, B**). In contrast to this induction upon PDN treatment, and in accordance with previous findings (Courdier-Fruh et al., 2002), we observed no increase in utrophin mRNA levels by both end point PCR (**Fig. 18C, D**) and quantitative PCR (**Fig. 21B**) ($p > 0.05$). Similarly, utrophin promoter activity was not induced by PDN treatment, as revealed by transient transfection experiments using a utrophin A promoter-reporter construct (**Fig. 18E**).

The Utrophin A 5'-UTR Regulates Expression of a Reporter Protein in Response to Glucocorticoid Treatment- We next performed a complementary set of experiments to test the hypothesis that PDN treatment enhances translation of utrophin A transcripts via events targeting its 5'-UTR. To this end, we transfected myoblasts with a monocistronic construct driven by the CMV promoter that contained the utrophin A 5'-UTR upstream of a CAT reporter (pUtrA/CAT) (**Fig. 19A**). After inducing myoblasts to differentiate, we

Fig. 18. Discordant expression of utrophin and its mRNA in glucocorticoid-treated C2C12 myotubes.

C2C12 myotubes were treated with either DMSO (CTL) or 6α-methylprednisolone-21 sodium succinate (PDN) at 500 nM for 3 days. Cells were harvested and protein or RNA was isolated for analysis. **A)** Western blot analysis for utrophin (drp2 antibody) and GAPDH. **B)** Quantification of the signal intensity seen in the Western blot experiments shows that utrophin levels are stimulated ~ 1.5 fold by PDN treatment. **C)** Representative ethidium bromide-stained agarose gels showing utrophin PCR product amplified from CTL- and PDN-treated cells. **D)** Quantification of the RT-PCR product bands shows that utrophin mRNA levels are unchanged by PDN treatment. Levels of utrophin mRNA are standardized to GAPDH mRNA. Values are in arbitrary units. **E)** Cells were transfected with a utrophin A promoter-reporter construct and treated as above. Quantification of β-galactosidase activity (standardized to a co-transfected pCAT plasmid) demonstrates that the utrophin A promoter is not activated by PDN treatment. Transcriptional mechanisms thus cannot explain the increased expression of utrophin protein following PDN treatment. Mean +/- S.E. are shown. Experiments were performed in triplicate, n=3. *, significant differences from control ($p < 0.05$).

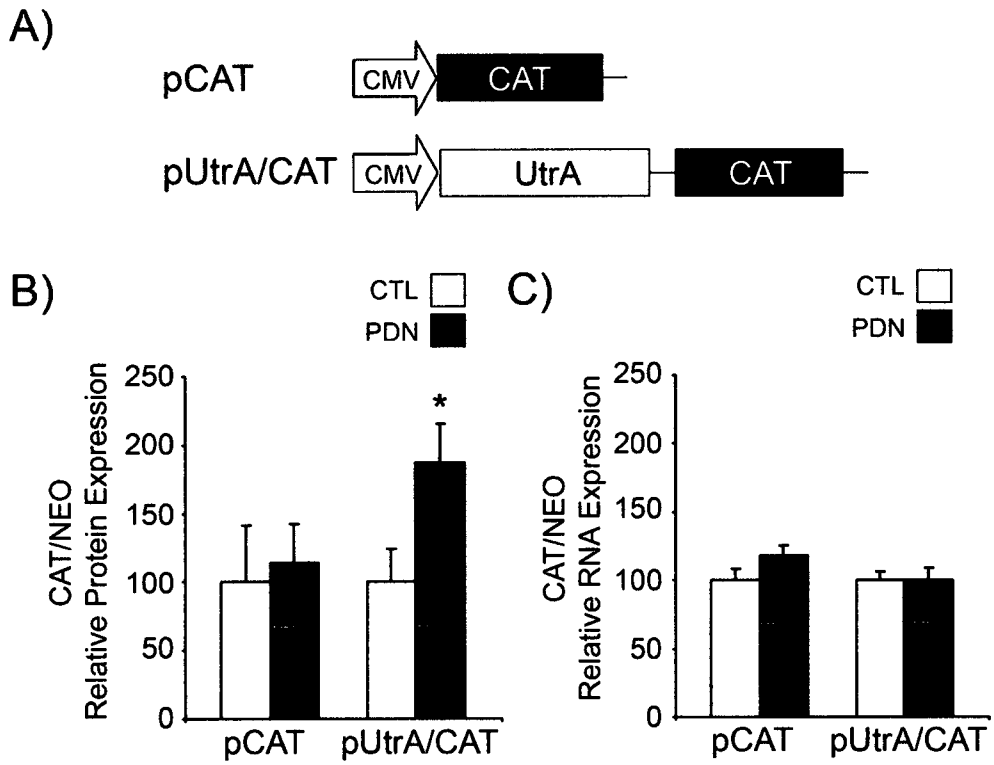

A)
CTL
PDN
Utrophin
GAPDH
C)
CTL
PDN
B)
Utrophin Protein Levels
(Arbitrary Units)
2
1.5
1
0.5
0
*
CTL
PDN
D)
Utrophin mRNA Levels
(Arbitrary Units)
2
1.5
1
0.5
0
CTL
PDN
E)
Utrophin A Promoter Activity
1.5
1
0.5
0
CTL
PDN

treated these cells with DMSO (CTL) or PDN for 2-3 days. Levels of CAT reporter protein were then measured and standardized to levels of neomycin protein, expressed from the same plasmid via a separate promoter. The results show that PDN treatment caused an increase in the relative expression of CAT protein by ~1.5-2 fold ($p < 0.05$) in cells transfected with the pUtrA/CAT plasmid. In contrast, CAT protein levels remained unaffected by PDN treatment in cells transfected with the parental pCAT construct (**Fig. 19B**). Quantitative analysis of the levels of CAT and neomycin mRNAs by RT-PCR revealed that relative CAT mRNA levels were not affected by PDN treatment in muscle cells transfected with either the pCAT or pUtrA/CAT construct (**Fig. 19C**). Thus, the observed increase in CAT protein expression without concomitant induction of CAT mRNA expression, strongly suggests that PDN treatment causes increased translation of the UtrA/CAT mRNA.

Glucocorticoid Treatment Enhances Utrophin A IRES Activity- In subsequent experiments, we determined whether enhanced translation mediated by the utrophin A 5'-UTR in response to PDN treatment results from induced IRES activity. For these studies, we used bicistronic reporter constructs and assays as described previously (Miura et al., 2005). C2C12 myoblasts were first transfected with either a bicistronic construct containing the utrophin A 5'-UTR inserted between the two cistrons (pβGAL/UtrA/CAT) (**Fig. 20A**) or an empty vector control (pβGAL/CAT). Increased expression of the downstream cistron (CAT), relative to the upstream cistron (βGAL) provides an indication of enhanced IRES activity. Assessment of utrophin A IRES activity in cells treated with PDN at concentrations ranging from 5 nM to 1 mM revealed that maximal

Fig. 19. The utrophin A 5'-UTR regulates translation of a reporter protein following PDN treatment of C2C12 myotubes.

A) Monocistronic reporter constructs used in these experiments. pCAT is driven by the CMV promoter, pUtrA/CAT contains the utrophin A full-length 5'-UTR (UtrA) subcloned upstream of the CAT reporter. C2C12 myoblasts were transfected with either pCAT or pUtrA/CAT constructs, induced to differentiate and then treated for 3 days with PDN (500 nM) or DMSO (CTL). **B)** Relative CAT protein reporter activity levels. Values are shown as a ratio of CAT reporter protein activity to neomycin (NEO) protein reporter activity. The neomycin reporter is expressed from a separate promoter on the pCAT construct and serves as an internal control. Note the increase in CAT reporter levels in response to PDN treatment when the utrophin 5'-UTR is present. **C)** Relative levels of CAT containing mRNA reporter transcripts, standardized to NEO reporter mRNA levels, as assessed by RT-PCR. Note that in contrast with was is seen with reporter protein levels, CAT reporter RNA levels are unchanged by PDN treatment independent of the presence of the utrophin A 5'-UTR. Values are in arbitrary units. Mean +/- S.E. are shown representing the average of three experiments performed in triplicate. *, significant difference from DMSO control (CTL) ($p < 0.05$).


A)
pCAT
CMV
CAT
pUtrA/CAT
CMV
UtrA
CAT
B)
CTL
PDN
CAT/NEO
Relative Protein Expression
250
200
150
100
50
0
*
pCAT
pUtrA/CAT
C)
CTL
PDN
CAT/NEO
Relative RNA Expression
250
200
150
100
50
0
pCAT
pUtrA/CAT

utrophin A IRES activity was induced at 500 nM (data not shown). As shown in **Fig. 20B**, 500 nM PDN treatment of muscle cells transfected with pβGAL/UtrA/CAT led to a significant ~ 1.5 fold increase ($p < 0.05$) in IRES activity as measured by an increased ratio of CAT to βGAL. This increase in relative IRES activity of the pβGAL/UtrA/CAT transfected cells is due to a stimulation in expression of the downstream cistron thus reflecting a genuine increase in IRES activity (**Table 1**). This level of induction in the activity of the utrophin A IRES is consistent with the observed increase in endogenous utrophin protein levels following treatment with PDN (See **Fig. 19A, 19B** and (Courdier-Fruh et al., 2002; Courdier-Fruh et al., 2003)). In contrast to the enhancement of utrophin IRES activity, the activity of the FGF-IA (Galy et al., 1999) and FGF-II (Martineau et al., 2004) IRES elements were not enhanced by PDN treatment ($p > 0.05$).

We previously demonstrated that the utrophin A 5'-UTR contains a *bona fide* IRES element, and that the reported IRES activity as measured using bicistronic vectors was not due to the presence of an internal promoter or spurious splicing events (Miura et al., 2005). To ensure that the observed induction of utrophin A IRES activity seen here in response to PDN treatment was due to a genuine increase in cap-independent translation, we performed quantitative RT-PCR analysis on cells transfected with pβGAL/UtrA/CAT and treated with PDN or DMSO as control. Analysis of the amplification profiles of the βGAL and CAT cDNA products revealed no change in the proportion of βGAL to CAT cistron RNA in PDN- versus DMSO-treated cells (**Fig. 20C**). In addition, we transfected a utrophin bicistronic construct lacking the CMV promoter, p(-cmv)βGAL/UtrA/CAT (Miura et al., 2005), and found that CAT activity was not induced by PDN treatment

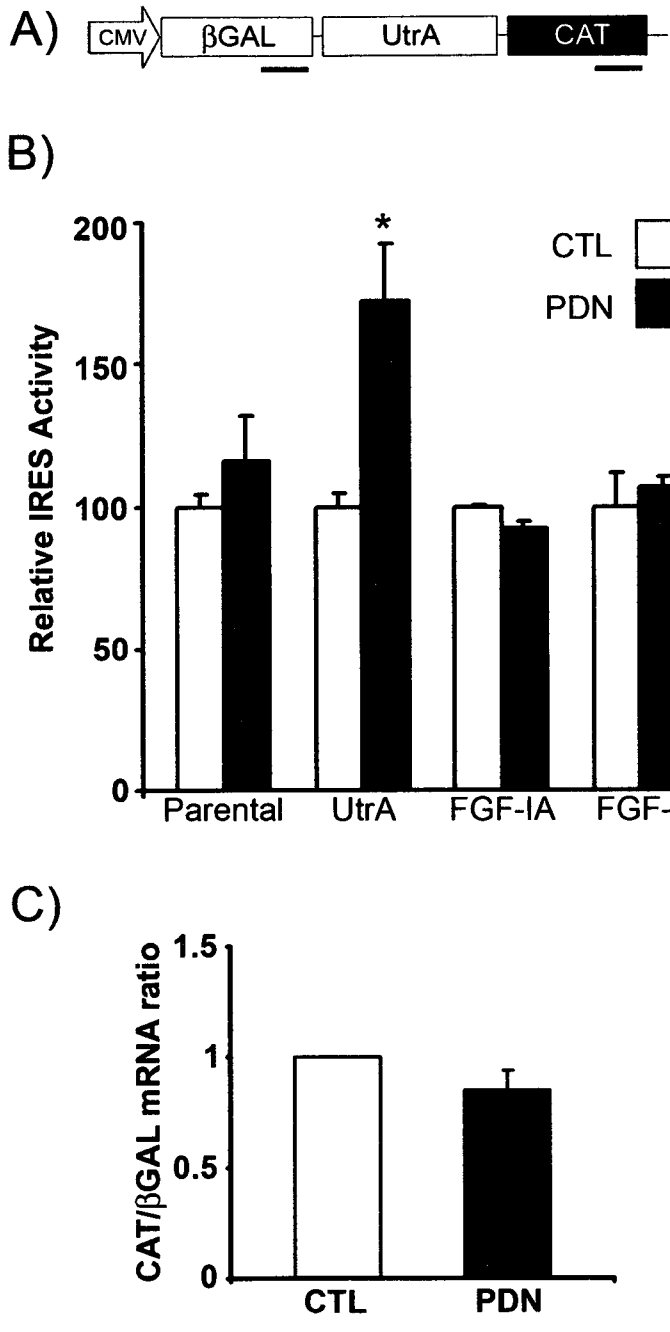
Construct	Treatment	βGAL	CAT
Parental	DMSO	100.0 +/- 2.0	100.0 +/- 0.8
Parental	PDN	103.7 +/- 5.2	117.4 +/- 15.5
UtrA	DMSO	100.0 +/- 3.9	100.0 +/- 6.2
UtrA	PDN	97.8 +/- 3.0	167.8 +/- 19.1

Table 1: Individual βGAL and CAT expression levels for cells transfected with parental or utrophin A 5'-UTR bicistronic constructs and treated with DMSO or PDN.

Values correspond to the relative levels of IRES activity shown in Fig. 20B for cells transfected with pβGAL/CAT (Parental) or pβGAL/UtrA/CAT (UtrA). Note the increase of CAT activity when the UtrA transfected cells are treated with PDN. Values are standardized to DMSO treated condition. Mean +/- S.E. is shown.

Fig. 20. Utrophin A IRES activity is stimulated by PDN treatment of C2C12 myotubes.

A) Schematic representation of bicistronic vector containing the utrophin A 5'-UTR (pβGAL/UtrA/CAT). Regions amplified by quantitative RT-PCR in control experiments (data presented in C) are indicated with black bars beneath the βGAL and CAT cistrons. **B)** C2C12 myoblasts were transfected with either an empty vector bicistronic reporter construct (parental), or bicistronic reporter constructs harbouring IRES elements from FGF-IA, FGF-II and utrophin A (UtrA). Cells were induced to differentiate and then treated for 3 days with PDN or DMSO (CTL). Relative IRES activity is reported as a ratio of CAT (measuring cap-independent translation), to βGAL (measuring cap-dependent translation). Data from PDN-treated samples are standardized to control, DMSO-treated samples. See Table 1 for individual expression levels of the βGAL and CAT cistrons. Mean +/- S.E. are shown representing the average of four experiments performed in triplicate. *, significant difference from DMSO control (CTL) ($p < 0.05$). Note that the effect of PDN is specific for the utrophin A 5'-UTR. **C)** Quantitative RT-PCR using βGAL and CAT primers was performed on RNA from treated C2C12 cells transfected with pβGAL/UtrA/CAT to exclude the possibility of spurious splicing or cryptic promoter activity of the utrophin A IRES. The lack of significant difference in the ratio of CAT to βGAL cDNA products between CTL- and PDN-treated cells indicates, as expected, the presence of an intact bicistronic transcript. The CAT/βGAL ratio is expressed as $2^{-[Ct(CAT)-Ct(bGAL)]}$ (see materials and methods for details). Values are in arbitrary units. Mean +/- S.D. are shown representing the average of three experiments performed in triplicate.


A)
CMV
βGAL
UtrA
CAT
B)
Relative IRES Activity
200
150
100
50
0
*
CTL
PDN
Parental
UtrA
FGF-IA
FGF-II
C)
CAT/βGAL mRNA ratio
1.5
1
0.5
0
CTL
PDN

($p > 0.05$) (data not shown). Together, these experiments indicate that the PDN-induced increase in IRES activity is not a result of aberrant splicing or cryptic promoter activity.

Analysis of Polysome-Bound Utrophin A Transcripts During Glucocorticoid Treatment- Previous studies have shown that glucocorticoids can cause inactivation of key members of the translational machinery in muscle cells (Shah et al., 2000c) and compromise protein synthesis in skeletal muscle (Shah et al., 2000b). In order to assess whether PDN treatment of C2C12 myotubes attenuates global translation we fractionated muscle cell lysates by sucrose density gradient centrifugation. In **Fig. 21A**, the optical density profiles of typical sucrose gradients are shown. The top of the gradient contains free mRNAs and ribosomal subunits (40S, 60S, 80S) while the bottom of the gradient contains mRNAs associated with polysomes. Analysis of the optical density profiles demonstrated a reduction in the relative amount of mRNAs associated with polysomal fractions in PDN-treated cells thereby indicating an overall attenuation of protein synthesis under these conditions (**Fig. 21A**).

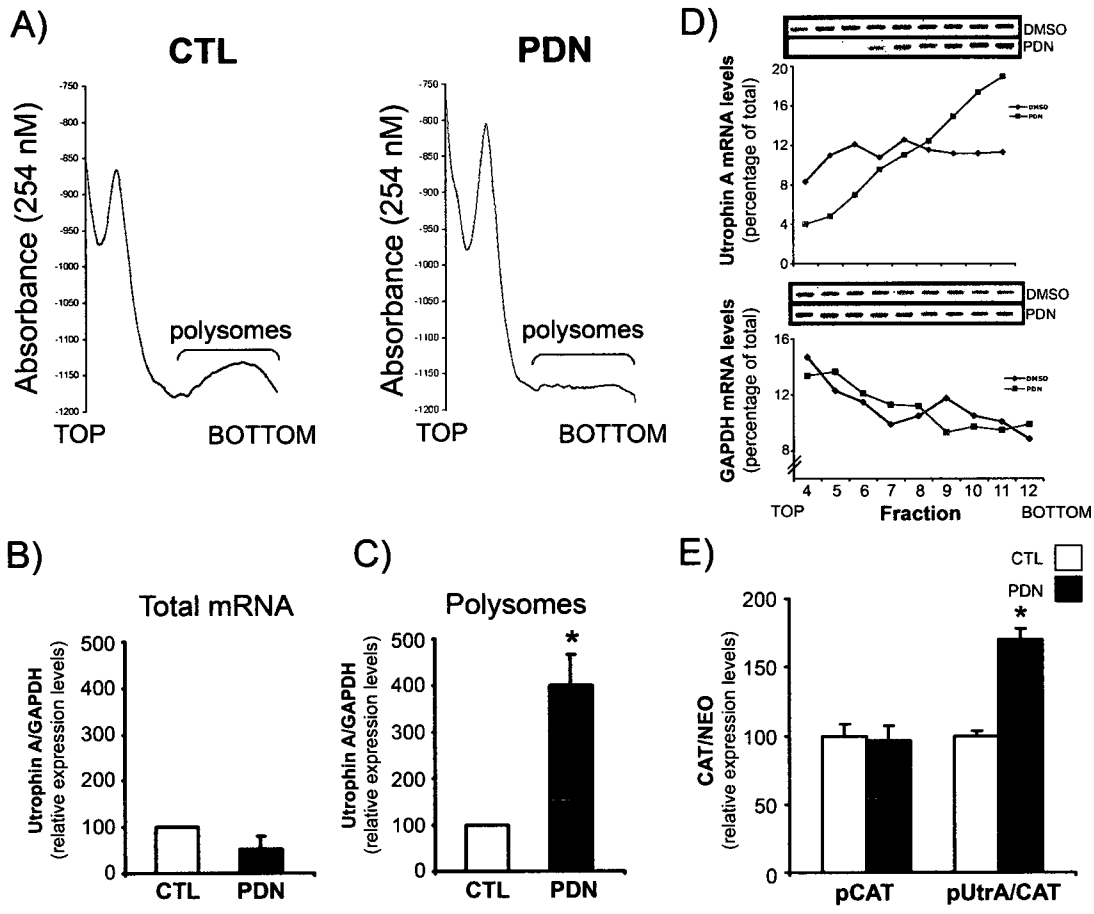
We next compared the expression profile of utrophin A mRNA in total cellular mRNA extracted prior to sucrose density ultracentrifugation to that of mRNA isolated from the pooled polysome fractions of both DMSO- (CTL) and PDN-treated myotubes. As expected (see **Fig. 18C** and **D**), quantitative RT-PCR analysis revealed that expression of utrophin A mRNA was not changed by PDN treatment in the total cellular mRNA samples (**Fig. 21B**). In contrast, we observed an ~4-fold enrichment in the levels of endogenous utrophin A transcripts relative to GAPDH in polysome fractions from PDN-

treated myotubes versus controls (**Fig. 21C**). This comparison between total levels of utrophin A transcripts and their association with polysomal fractions indicates that PDN promotes the recruitment of utrophin A mRNA to polysomes despite a general depression in protein synthesis. Such recruitment of utrophin A transcripts to polysomes is further indication that PDN treatment causes a translational induction of utrophin expression. In order to examine the distribution of utrophin A mRNA within the polysome fractions, we performed RT-PCR analysis on individual ~0.5 mL fractions from the polysome enriched regions of gradients obtained from control and PDN-treated cells (fractions 4-12). As shown in **Fig. 21D**, we found that utrophin A mRNA shifted toward the heavier polysome fractions in PDN treated cells. In contrast, the distribution of GAPDH mRNAs in polysomes were unaffected by PDN treatment (**Fig. 21D**) confirming the observation that GAPDH protein levels remain unaffected in cells treated with PDN (**Fig. 18A**). This shift in distribution to the heavier polysome fractions is further indication that utrophin A translation is enhanced by glucocorticoid treatment.

The Utrophin A 5'-UTR is Responsible for Recruitment of Utrophin A Transcript to Polysomes Upon Glucocorticoid Treatment- Based on these findings, we decided to examine whether the utrophin A 5'-UTR could promote the recruitment of reporter transcripts to polysomes upon PDN treatment. We thus transfected myoblasts with the pUtrA/CAT or pCAT monocistronic constructs (**Fig. 19A**), differentiated cells into myotubes and then treated them with DMSO or PDN. Polysome fractionation experiments were performed as described above, and relative levels of CAT- and

Fig. 21. Utrophin A mRNA becomes enriched in polysomes in response to PDN treatment.

A) Representative polysome profiles of C2C12 myotube cell lysates fractionated by sucrose density ultracentrifugation. C2C12 myotubes were treated with DMSO (CTL) or with PDN (500 nM) for two days. Cells were then treated with cycloheximide and lysed. Nuclei and cellular debris were removed by centrifugation. Cell lysates were layered on a continuous sucrose gradient.Absorbance of the gradients was measured at 254 nm from top to bottom via a peristaltic pump and RNA-containing sucrose gradients were fractioned for a total of 12 fractions. Note the decrease in polysomal RNA in PDN-treated cells, indicating decreased global protein translation. **B)** The overall change in endogenous utrophin A mRNA levels in response to PDN-treatment was measured by qRT-PCR from total mRNA samples and is shown relative to GAPDH mRNA, n=3. **C)** The change in endogenous utrophin A mRNA levels in the pooled polysome fractions from cells treated with DMSO or PDN relative to GAPDH mRNA. Note the increased presence of utrophin A transcript in the polysome fraction of PDN treated cells, n=3. **D)** RT-PCR was performed to detect utrophin A and GAPDH mRNA in individual polysome enriched fractions (fractions 4-12) of the sucrose gradient. Quantification of the amplified bands (See Materials and Methods) demonstrates a shift of utrophin A mRNA to the heavier polysome fractions of the gradient for the PDN treated cells. Data are representative of three independent experiments. Results were confirmed by qPCR. **E)** Cells were transfected with pCAT and pUtrA/CAT (see Fig. 19A) and treated as above. qRT-PCR analysis of CAT reporter transcript expression standardized to neomycin (NEO) reporter transcript expression was carried out on mRNA isolated from the pooled polysome fractions. Note the increase in polysome-associated CAT reporter transcript in response to PDN-treatment, n=3. All values are in arbitrary units. Mean +/- S.E. are shown. *, significant difference from control ($p < 0.05$).


A)
CTL
PDN
Absorbance (254 nM)
polysomes
TOP
BOTTOM
D)
DMSO
PDN
Utrophin A mRNA levels
(percentage of total)
GAPDH mRNA levels
(percentage of total)
Fraction
B)
Total mRNA
Utrophin A/GAPDH
(relative expression levels)
C)
Polysomes
E)
CTL
PDN
CAT/NEO
(relative expression levels)
pCAT
pUtrA/CAT

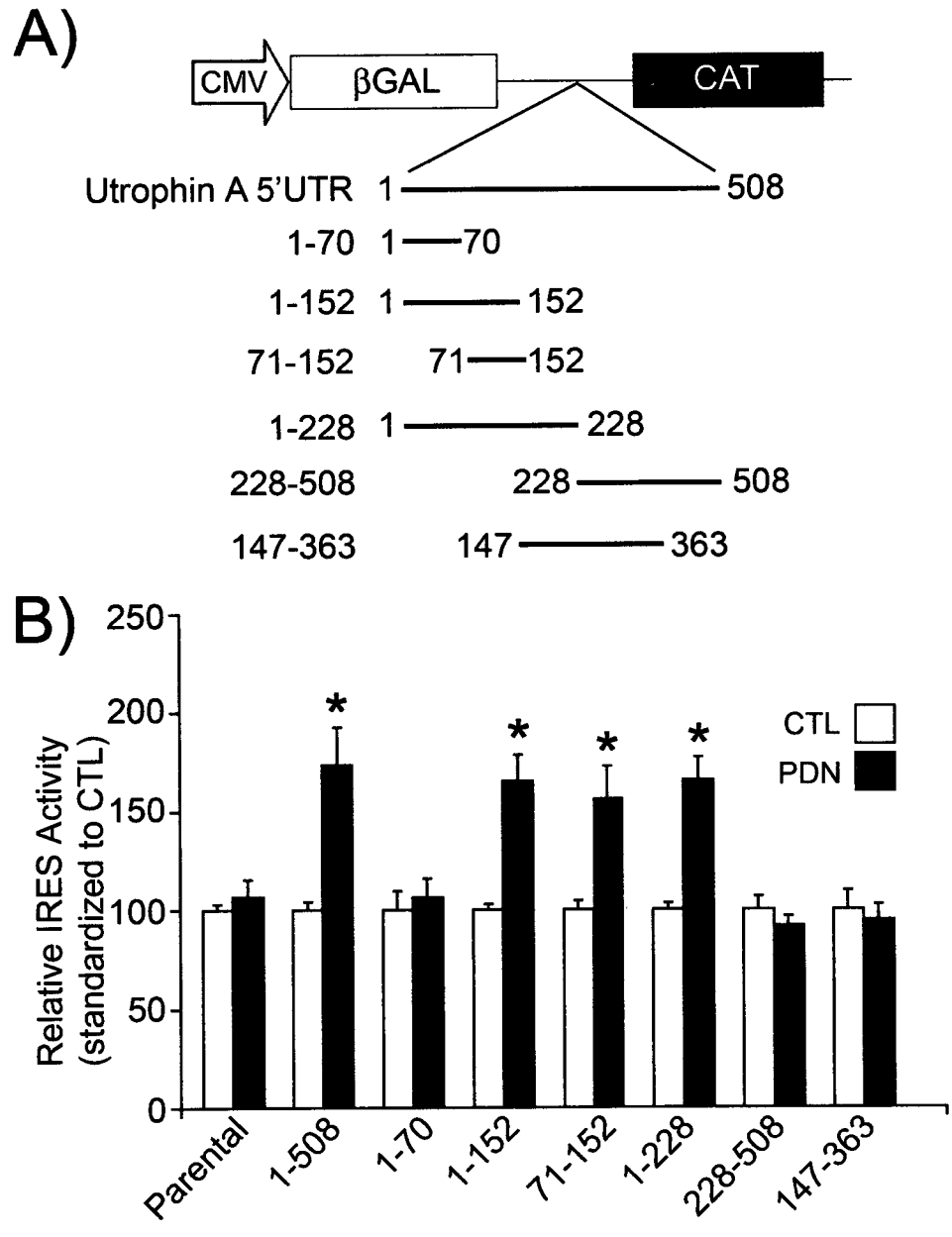
neomycin-containing mRNA were quantified by RT-PCR. This analysis revealed that PDN treatment caused a significant recruitment ($p < 0.05$) of reporter mRNA expressed from the pUtrA/CAT construct to polysomal fractions (**Fig. 21E**). In contrast, no change in the levels of polysome-associated reporter mRNAs was observed following PDN treatment of cells transfected with the parental pCAT plasmid that lacks the 5'-UTR (**Fig. 21E**). Thus, the utrophin A 5'-UTR plays a key role in recruiting endogenous utrophin A transcripts to polysomes upon PDN treatment.

Mapping of the Glucocorticoid-Responsive Utrophin A IRES Element- In order to delineate the region of the utrophin A 5'-UTR that confers PDN-induced IRES activity, several truncated variants of the utrophin A 5'-UTR were created and inserted into the bicistronic vector (**Fig. 22A**). Interestingly, PDN treatment was found to enhance IRES activity in muscle cells transfected with bicistronic constructs containing the first 152 or 228 nucleotides of the utrophin A 5'-UTR by ~1.5 fold ($p < 0.05$) (**Fig. 22B**). This level of induction is similar to that observed using the full-length 5'-UTR, i.e., construct containing nucleotides 1-508 in **Fig. 22B** (see also **Fig. 19B**). In contrast, the 147-363 region of the 5'-UTR, as well as the 228-508 region did not confer enhanced IRES activity in response to PDN. Further truncation of the 5'-UTR from its 3' end to the first 70 bases (1-70) also abolished the responsiveness to PDN. These experiments suggested that nucleotides 71-152 encompass the PDN-responsive IRES element. Indeed, transient transfection of a bicistronic construct harbouring this region demonstrated an ~1.5 fold induction in IRES activity upon PDN treatment ($p < 0.05$). Individual expression levels of the βGAL and CAT cistrons for each construct and treatment are shown in **Table 2**.

These data demonstrate that the 71-152 fragment can recapitulate the PDN-mediated induction in IRES activity seen with the full-length 5'-UTR.

Fig. 22. Elucidation of the minimal PDN-responsive utrophin A IRES element.

A) Schematic representation of bicistronic constructs harbouring truncated regions of the utrophin A 5'-UTR. **B)** Cells were transfected with the various bicistronic constructs, treated with DMSO (CTL) or PDN (500 nM) for 2 days and analyzed for IRES activity, reported as a ratio of CAT to βGAL protein reporter activity. IRES activity in PDN treated cells is standardized to IRES activity in CTL cells. See Table 2 for individual expression levels of the βGAL and CAT cistrons for each construct and treatment. Mean +/- S.E. are shown representing the average of 3-5 independent experiments, performed in triplicate. *, significant difference from control ($p < 0.05$).


A)
CMV
βGAL
CAT
Utrophin A 5'UTR 1 508
1-70 1 70
1-152 1 152
71-152 71 152
1-228 1 228
228-508 228 508
147-363 147 363
B)
Relative IRES Activity (standardized to CTL)
250
200
150
100
50
0
CTL
PDN
*
Parental
1-508
1-70
1-152
71-152
1-228
228-508
147-363

Construct	Treatment	βGAL	CAT
Parental	DMSO	100.0 +/- 1.9	100.0 +/- 2.9
Parental	PDN	102.0 +/-6.0	104.1 +/- 9.1
1-508	DMSO	100.0 +/- 3.3	100.0 +/- 4.8
1-508	PDN	89.5 +/- 8.6	137.5 +/- 14.3
1-70	DMSO	100.0 +/-6.6	100.0 +/- 6.7
1-70	PDN	94.6 +/- 4.5	102.7 +/- 8.5
1-152	DMSO	100.0 +/- 1.4	100.0 +/- 1.6
1-152	PDN	76.0 +/- 5.7	122.5 +/- 7.2
71-152	DMSO	100.0 +/- 5.0	100.0 +/- 1.6
71-152	PDN	110.2 +/- 8.0	169.0 +/- 9.1
1-228	DMSO	100.0 +/- 2.2	100.0 +/- 3.9
1-228	PDN	75.4 +/- 5.2	120.6 +/- 3.2
228-508	DMSO	100.0 +/- 3.9	100.0 +/- 4.3
228-508	PDN	84.8 +/- 4.4	78.3 +/- 4.4
147-363	DMSO	100.0 +/- 3.8	100.0 +/- 7.2
147-363	PDN	84.1 +/- 4.9	84.8 +/- 10.7

Table 2: Individual βGAL and CAT expression levels for cells transfected with bicistronic constructs harbouring truncated regions of the utrophin A 5'-UTR and treated with DMSO or PDN.

Values correspond to the levels of relative IRES activity for the various constructs shown in Fig. 22. Values are standardized to DMSO treated condition. Mean +/- S.E. is shown.

DISCUSSION

Several groups have previously observed a discordant expression pattern of utrophin protein and mRNA in response to glucocorticoid treatment. Initial studies showed that treatment of cultured muscle cells from DMD patients with the glucocorticoid dexamethasone resulted in increased utrophin protein expression while leaving the level of utrophin transcripts unaffected (Pasquini et al., 1995). Similarly, treatment of normal and dystrophin-deficient myotubes with PDN caused an increase of utrophin protein by approximately 40% in both cell types without altering mRNA levels (Courdier-Fruh et al., 2002). Furthermore, various traditional Chinese medicines that tested positive for glucocorticoid-like activity can also stimulate utrophin protein expression in muscle cells (Courdier-Fruh et al., 2003). Collectively, these studies suggest that gene regulation at the level of transcription cannot explain the stimulation of utrophin protein expression in response to glucocorticoids.

In the present study, we thus examined whether translational regulatory mechanisms could account for the increased expression of utrophin protein following glucocorticoid treatment. Using multiple and complementary approaches, we show that an enhancement of IRES-mediated translation of utrophin A can account, at least partially, for the increased expression of utrophin A protein in response to PDN treatment of C2C12 myotubes. These results are important particularly in the context of identifying small molecules that could upregulate the endogenous levels of utrophin A in muscle from DMD patients since they show that known drugs can indeed increase the activity of

the utrophin A IRES thereby promoting protein synthesis from a pool of already synthesized and translationally competent transcripts. Together with our previous findings obtained in regenerating muscle fibers (Miura et al., 2005), our current data further illustrate that the utrophin A IRES can be activated by physiologically relevant stimuli.

The enhancement of utrophin translation in response to PDN treatment, as assessed by both monocistronic (**Fig. 19**) and bicistronic (**Fig. 20**) reporter transfection experiments, was found to be similar to the enhancement of endogenous utrophin protein expression in C2C12 cells (**Fig. 18 A,B**). Nonetheless, the increase in utrophin protein levels might also be partially accounted for by an increase in utrophin protein stability. We thus attempted to assess the contribution of protein stability to the enhancement of utrophin expression in response to PDN treatment by treating cells with PDN and cycloheximide. Unfortunately, we found that 48-72 hrs of cycloheximide treatment (10 μg/mL) did not significantly reduce utrophin protein levels as assessed by western blot (see also (Bonet-Kerrache et al., 2005)), thus precluding a thorough assessment of the contribution of protein stability to the enhancement of utrophin expression by PDN treatment.

Several groups have observed that glucocorticoid treatment can cause a reduction in global translation rates. This has in fact been shown to occur in both cultured muscle cells as well as in skeletal muscle of live animals (Shah et al., 2000d; Shah et al., 2000c, a; Shah et al., 2002).The mechanisms by which translation rates are depressed by

glucocorticoids in muscle cells have been linked to multiple pathways including inhibition of ribosomal S6 protein kinase (P70 S6K) activity (Shah et al., 2000c; Shah et al., 2002) and dephosphorylation of eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (Shah et al., 2000d; Shah et al., 2000a).

Using sucrose gradient sedimentation, we also observed a reduction in the amount of RNA present within polysomal fractions suggesting a decrease in overall translation. In contrast, both endogenous utrophin A transcripts and reporter mRNAs containing the utrophin A 5'-UTR, became enriched within polysomes under these conditions. It is thus interesting to note that utrophin A translation is clearly enhanced by a treatment that simultaneously compromises global translation rates. Several mammalian mRNAs have been shown to exhibit enhanced IRES-mediated translation under conditions where global translation rates are depressed such as during viral infection, mitosis and ER stress (Sarnow, 1989; Qin and Sarnow, 2004; Warnakulasuriyarachchi et al., 2004). Our current findings showing an activation of the utrophin A IRES following PDN treatment, fit extremely well with these earlier observations. Together, these results suggest that IRES elements provide an alternative route by which specific mRNAs continue to be translated when the proper functioning of the cap-dependent translational machinery becomes compromised.

While glucocorticoid treatment downregulates global protein synthesis in skeletal muscle cells, several lines of evidence have shown that it can stimulate expression of several proteins at the translational level (Verdi and Campagnoni, 1990; Colson et al.,

1992; Sarantos et al., 1994; Ambuhl et al., 1999), including the Na^+/K^+-ATPase $\alpha 1$ subunit, which appears to be translationally regulated by glucocorticoids via its 5'-UTR (Devarajan and Benz, 2000; Cougnon et al., 2002; Nordsborg et al., 2005) (P.M., M.H., B.J.J., unpublished observations). Along these lines, it is important to note that another steroid hormone, testosterone, has been shown to alter IRES-mediated translation of the Fibroblast Growth Factor 2 (FGF-II) mRNA. In this case, it was found that treatment of an FGF-II IRES reporter mouse with testosterone activated the FGF-II IRES in a manner dependent upon the availability of androgen receptors (Gonzalez-Herrera et al., 2006). Interestingly, testosterone treatment also caused an increase in the binding and interaction of multiple proteins from testes to an FGF-II 5'-UTR probe (Gonzalez-Herrera et al., 2006).

The nature of the exact mechanisms causing an increase in the activity of the utrophin IRES following PDN treatment of muscle cells remains for the moment unclear. Previous work by others has shown that the increase in utrophin protein expression in response to PDN treatment can be inhibited by simultaneously treating cells with the glucocorticoid receptor antagonist RU486 (Courdier-Fruh et al., 2002; Courdier-Fruh et al., 2003) thereby indicating that this increase is dependent upon activation of the glucocorticoid receptor. It may thus be envisioned that activation of the glucocorticoid receptor stimulates signaling pathways that modulate the association of RNA-binding proteins to the utrophin 5'-UTR, thereby enhancing IRES-mediated translation.

A common set of RNA-binding proteins that are required for IRES-mediated translation to occur has not been identified, although several proteins, termed IRES-*trans* acting factors (ITAFs), have been shown to modulate the activity of different IRESes. These proteins include, for example, polypyrimidine-tract binding protein (PTB) (Kim et al., 2000; Bushell et al., 2006b), the caspase-cleaved form of DAP5/p97 (p86) (Henis-Korenblit et al., 2002; Lewis et al., 2008) and heterogeneous nuclear ribonucleoprotein (hnRNP) A1 (Bonnal et al., 2005; Lewis et al., 2007b). Future studies will aim to identify the ITAFs that mediate IRES-driven translation of utrophin during both glucocorticoid treatment and muscle regeneration.

Treatment of DMD patients with the corticosteroids prednisone and deflazacort slow the progression of the muscle disease. However, this effect is not sustained and the treatment is associated with many detrimental side effects such as weight gain, behavioural abnormalities and osteoporosis (Lo et al., 1995; Fisher et al., 2005). Thus, glucocorticoid treatment does not constitute a viable long-term treatment for DMD. Nonetheless, this treatment increases the length of time a patient remains ambulatory while increasing muscle mass (Rifai et al., 1995) and improving pulmonary function (Bonifati et al., 2000; Campbell and Jacob, 2003). Therefore, an increased understanding of the mechanisms by which glucocorticoids improve muscle function in DMD patients is critical in ultimately designing similar drugs with longer lasting benefits and fewer side effects.

In addition to utrophin upregulation, it is important to note that glucocorticoid treatment appears to benefit dystrophic muscle via anti-inflammatory actions, including reducing macrophage and T cell (CD2+ and CD8+) accumulation in skeletal muscle (Kissel et al., 1991; Wehling-Henricks et al., 2004). Therefore, the beneficial effect of prolonged glucocorticoid treatment for DMD patients likely stems from the combinatorial impact associated with utrophin upregulation and the anti-inflammatory response.

Given the role of the utrophin A 5'-UTR in mediating the translational response to PDN treatment demonstrated here, it may prove useful to begin screening for drugs that target and stimulate utrophin A IRES activity. Based on previous work assessing the therapeutic potential of utrophin to mitigate the dystrophic phenotype of the *mdx* mouse, such a drug would have to stimulate utrophin expression levels only by ~2-fold in order to show dramatic improvement in muscle function (Tinsley et al., 1998; Chakkalakal et al., 2004; Krag et al., 2004).

MATERIALS AND METHODS

C2C12 Cell Culture and Drug Treatments- C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were grown as previously described (Miura et al., 2005) on matrigel-coated plates (BD Biosciences, Bedford, MA) in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (Cansera, Toronto, ON, Canada) and 100 units/mL of ampicillin/streptomycin. Cells were kept at 37 °C in a water-saturated atmosphere containing 5% CO_2. Upon reaching 80-90% confluency, the growth media was replaced with differentiation media containing 2% Horse Serum (Invitrogen). Cells were maintained in differentiation medium for 2-3 days. For drug treatments, 6α-methylprednisolone-21 sodium succinate (referred to as PDN) (Sigma, St. Louis, MO) was dissolved in DMSO to make 500 μM stocks. DMSO (used as control; CTL) or PDN was mixed with differentiation media at concentration of 500 nM and added to the cells (Courdier-Fruh et al., 2002). Fresh media and drug were added each day for 2-3 days of treatment before cell harvesting. In order to monitor the effectiveness of the drug treatments, transient transfections of luciferase reporter constructs harbouring the hormonal response region of the mouse mammary tumor virus promoter (Ponta et al., 1985) were performed.

Western Blot Analysis- Proteins were isolated from myotubes using a protein lysis buffer and Western blot analysis was carried out using standard procedures as previously described (Miura et al., 2005). For detection of protein levels, samples were run on 5% SDS-PAGE gels and transferred onto PVDF membranes. The monoclonal anti-utrophin

Drp-2 antibody (Vector Laboratories, Burlingame, CA) was used at 1:500 dilution to detect utrophin, followed by incubation with a secondary goat anti-mouse HRP antibody at 1:2500 (Chemicon International, Temecula, CA). To detect GAPDH protein levels, a monoclonal anti-GAPDH antibody (Advanced Immunochemical, Long Beach, CA) was used at 1:10000, followed by a secondary goat anti-mouse antibody at 1:5000 (Jackson Immunoresearch Laboratories, West Grove, PA). Antibody complexes were detected using western lightning chemiluminescent reagent (Perkin Elmer, Waltham, MA) and exposed to Kodak BioMAX MR film (Eastman Kodak, Rochester, NY). The net intensity of the protein bands was quantified using Kodak digital science 1D version 3.0.2 image analysis software.

Plasmids and Transient Transfections- The bicistronic constructs pβGAL/CAT, pβGAL/UtrA/CAT and p(-cmv)βGAL/UtrA/CAT have been previously described (Miura et al., 2005). Progressive deletions of the utrophin A 5'-UTR were amplified by PCR and subcloned into the *Xho1* site of pβGAL/CAT. For creation of the monocistronic constructs pCAT and pUtrA/CAT, the LacZ gene was excised from pβGAL/CAT and pβGAL/UtrA/CAT using *Not1* and the vector was re-ligated. The 1.3 kb utrophin A promoter reporter construct used has been previously described (Gramolini et al., 1997). For promoter reporter transfections, a plasmid encoding the CAT gene driven by the SV40 promoter (Promega, Madison, WI) was co-transfected to control for transfection efficiency. The FGF-II IRES was PCR amplified from an FGF-II bicistronic reporter construct (kindly provided by Dr. A.C. Prats, INSERM, Tolouse, France) (Galy et al., 1999), and subcloned into pβGAL/CAT. A dual luciferase bicistronic construct

harbouring the FGF-IA IRES was kindly provided by Dr. A.C. Prats (Martineau et al., 2004). All transient transfections were carried out using Lipofectamine 2000 (Invitrogen) while the myoblasts were 70-90% confluent as recommended by the manufacturer. For transfections of cells in 35 mm wells, 4 μg of plasmid DNA was combined with 10 μl of Lipofectamine 2000. For experiments involving transfection of monocistronic constructs and analysis of the association of transcripts with polysomes, ten 100mm plates were transfected for each plasmid and drug treatment combination. Each 100 mm plate was transfected using 24 μg of plasmid DNA combined with 60 μl of Lipofectamine 2000.

Reporter Protein Analysis- Cells were harvested in Reporter Lysis Buffer (Promega), and assays for β–galactosidase and CAT protein reporter activity were carried out as previously described (Holcik et al., 2005; Miura et al., 2005). For cells transfected with monocistronic constructs, expression of neomycin was detected using the PathoScreen NPTII ELISA assay detection kit as recommended by the manufacturer (Agdia, Elkhart, IN). For transient transfection experiments using the FGF-IA IRES construct, LucR and LucF activities were measured with the dual luciferase kit (Promega).

Polysome Fractionation Experiments- Polysome fractionation experiments were carried out according to a previously described protocol (Blais et al., 2006). C2C12 myotubes were grown on 100 mm plates coated with matrigel and treated with DMSO or PDN as described above. To isolate polysomes, cells were treated with cycloheximide (CHX) (0.1 mg/mL) for 3 minutes in fresh differentiation media. Cells were washed twice in PBS containing CHX and subsequently lysed in an RNA lysis buffer (0.3M NaCl, 15 mM

$MgCl_2$, 15 mM Tris pH 7.4, 1% Triton X-100, 0.1 mg/mL CHX, 100U/mL RNAsin). Nuclei and cellular debris were removed by centrifugation steps of 3,000 rpm, 5 min, 4°C and then 14,000 rpm, 5 min, 4°C). Three hundred μL of RNA was extracted from part of the lysate for total RNA analysis. Seven hundred μL of the lysate was layered on a continuous sucrose gradient (10-50% sucrose in 15 mM $MgCl_2$, 15 mM Tris pH 7.4, 0.3 M NaCl). Centrifugation was carried out at 39,000 rpm in an SW41-Ti rotor at 4°C for 90 min. Absorbance of the gradients was measured continuously at 254 nm from top to bottom of the gradient via a peristaltic pump operating at a flow rate of 1 ml/min. For some experiments, 1 ml fractions were collected and polysome enriched fractions were pooled prior to RNA extraction.To achieve better separation of polysomes, fractions were collected in 0.5 or 1 mL intervals. For fractions 1-3, ~1 mL was collected per fraction, while for the polysome enriched fractions 4-12, ~0.5 mL was collected per fraction. The polysome fractions were digested with proteinase K. RNA was extracted by phenol chloroform extraction, and then concentrated and treated with DNAse I using the Absolutely RNA miniprep kit (Stratagene, La Jolla, CA). For RT-PCR analysis (see below) on individual fractions obtained from the sucrose gradient, the signal intensity from cDNA products of each band was divided by the sum of the signal intensities of all bands in order to obtain the percentage intensity of signal in each fraction.

RNA Extraction and RT-PCR Analysis- Total RNA was isolated from C2C12 cells using TRIzol reagent (Invitrogen) as recommended by the manufacturer. TRIzol extracted RNA was treated for 1 hour with DNAse I (Invitrogen) to eliminate possible plasmid and genomic DNA contamination. To quantify levels of endogenous utrophin and GAPDH

mRNAs, RT-PCR analysis was employed using previously described protocols and primer sets (Gramolini et al., 1999a; Chakkalakal et al., 2003b). For RT-PCR analysis on cells transfected with the monocistronic pCAT and pUtrA/CAT constructs, CAT was amplified using previously described primers (Miura et al., 2005) and these levels were standardized to neomycin mRNA levels (5'-TGCTCCTGCCGAGAAAGTAT-3'; 5'-AATATCACGGGTAGCCAACG -3'). Cycle numbers varied depending on the primers used and they were all within the linear range of amplification. Controls consisted of RT mixtures in which total RNA was replaced by sterile, diethylpyrocarbonate-treated water. PCR products were separated on 1.5% agarose gels containing ethidium bromide, and the intensity of the signal, which is linearly related to the amount of cDNAs, was quantified using Kodak digital science 1D version 3.0.2 image analysis software.

In separate experiments, we also performed real-time PCR. For these, analysis of endogenous utrophin A and GAPDH levels was carried out using the following primer sets: utrA (5'-ATCTTGTCGGGCTTTCCAC-3'; 5'- ATCCAAAGGCTTTCCCAGAT-3'); GAPDH (5'-GGGTGTGAACCACGAGAA-AT-3'; 5'-CCTTCCACAATGCCAAAGTT-3'). To control for aberrant splicing or the presence of an internal promoter in the utrophin A 5'-UTR, qRT-PCR control experiments were conducted as previously described to detect expression of portions of βGAL and CAT from the bicistronic mRNA transcript (Holcik et al., 2005; Miura et al., 2005; Lewis et al., 2007b). For these experiments, the TRIzol extracted RNA was treated for 1 hour with DNAse I (Invitrogen) to prevent DNA contamination from the transfected plasmid. Reverse transcription for all qRT-PCR experiments was carried out using the First-Strand

cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ) with NotI-d(T)18 primers. Quantitative real time PCR was performed using the QuantiTect SYBR green PCR kit (Qiagen, Mississauga, ON, Canada). Analysis was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) with ABI Prism 7000 SDS Software. To control for DNA contamination, quantitative PCR was performed directly on the RNA samples.

Statistical Analysis- The presence of significant differences between group means was determined using Student's t test. The level of significance was set at $p < 0.05$. Means +/- S.E. are shown throughout.

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CHAPTER 5

The utrophin A 5'-UTR drives cap-independent translation exclusively in skeletal muscles of transgenic mice and interacts with eEF1A2

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Conceived and designed the experiments: PM MH BJJ. Performed the experiments: PM AC GB. Performed microinjections: YD. Analyzed the data: PM AC MH BJJ. Provided Reagents & expertise: RK JL MH. Wrote the paper: PM BJJ

ABSTRACT

The molecular mechanisms regulating expression of utrophin A are of therapeutic interest since upregulating its expression at the sarcolemma can compensate for the lack of dystrophin in animal models of Duchenne Muscular Dystrophy (DMD). The 5'-UTR of utrophin A has been previously shown to drive cap-independent internal ribosome entry site (IRES)-mediated translation in response to muscle regeneration and glucocorticoid treatment. To determine whether the utrophin A IRES displays tissue specific activity, we generated transgenic mice harboring control (CMV/βGAL/CAT) or utrophin A 5'-UTR (CMV/βGAL/UtrA/CAT) bicistronic reporter transgenes. Examination of multiple tissues from two CMV/βGAL/UtrA/CAT lines revealed that the utrophin A 5'-UTR drives cap-independent translation of the reporter gene exclusively in skeletal muscles and no other examined tissues. This expression pattern suggested that skeletal muscle-specific factors are involved in IRES-mediated translation of utrophin A. We performed RNA-affinity chromatography experiments combined with mass spectrometry to identify *trans*-factors that bind the utrophin A 5'-UTR and identified eukaryotic elongation factor 1A2 (eEF1A2). UV-crosslinking experiments confirmed the specificity of this interaction. Regions of the utrophin A 5'-UTR that bound eEF1A2 also mediated cap-independent translation in C2C12 muscle cells. Cultured cells lacking eEF1A2 had reduced IRES activity compared to cells overexpressing eEF1A2. Together, these results suggest an important role for eEF1A2 in driving cap-independent translation of utrophin A in skeletal muscle. The *trans*-factors and signaling pathways driving skeletal-muscle specific IRES-mediated translation of utrophin A could provide unique targets for developing pharmacological-based DMD therapies.

INTRODUCTION

The fatal neuromuscular disease Duchenne Muscular Dystrophy (DMD) is caused by loss of dystrophin expression in the muscle fibers of affected patients (Blake et al., 2002). Utrophin is the autosomal homologue of dystrophin, and through multiple approaches it has been demonstrated that enhancing its expression can compensate for the lack of dystrophin in animal models of DMD and alleviate the muscle pathology (Tinsley et al., 1996; Cerletti et al., 2003; Chakkalakal et al., 2004; Sonnemann et al., 2009). A major difference between dystrophin and utrophin A (the utrophin isoform expressed in skeletal muscle) is that while dystrophin is expressed along the entire sarcolemma, utrophin A expression in mature fibers is primarily restricted to post-synaptic regions of the sarcolemma (Weir et al., 2002; Baby et al., 2009). It is thus of considerable therapeutic interest to identify mechanisms by which utrophin A expression can be enhanced along the entire sarcolemma of DMD patient muscle fibers.

We have found that post-transcriptional mechanisms play an important role in enhancing expression of utrophin A at the sarcolemma. For instance, in addition to transcriptional mechanisms involving calcineurin/NFAT signaling, post-transcriptional events targeting the utrophin A 3'-UTR can modulate the stability of utrophin A transcripts, thus contributing to the enhanced expression of utrophin A in extrasynaptic regions of slow-twitch, oxidative fibers as compared to fast-twitch, glycolytic fibers (Chakkalakal et al., 2003b; Chakkalakal et al., 2008). Increased sarcolemmal expression of utrophin A also occurs in skeletal muscles undergoing regeneration. Interestingly, this enhancement of utrophin protein expression is not accompanied by concomitant increases in utrophin A mRNA levels, as observed both in regenerating versus control mouse

skeletal muscles and in muscle biopsies of DMD patients compared to healthy subjects (Gramolini et al., 1999a). Enhancement of utrophin A protein expression in response to muscle regeneration can be at least partially explained by increased translation initiation mediated by an internal ribosome entry site (IRES) located in the utrophin A 5'-UTR (Miura et al., 2005).

IRES elements are thought to recruit the ribosome, IRES *trans*-acting factors (ITAFs), and other components of the translational machinery to the 5'-UTRs of some cellular mRNAs (Baird et al., 2006). IRES-mediated translation initiation occurs independently of the 7-methyl guanosine cap at the 5' end of the mRNA, thus providing an alternative to the canonical, cap-dependent mechanism of translation initiation. This alternative mechanism allows for the cell to express a subset of proteins under stress conditions where global cap-dependent translation is suppressed, such as during viral infection, hypoxia, and apoptosis (Spriggs et al., 2008). In the case of the utrophin A IRES, its activity is enhanced during muscle regeneration and glucocorticoid treatment when global cap-dependent translation may be suppressed (Miura et al., 2005; Miura et al., 2008).

In this study, we set out to determine the tissue distribution of utrophin A IRES activity by generating and characterizing utrophin A 5'-UTR reporter transgenic mice. We also initiated experiments to identify ITAFs that interact with the utrophin A 5'-UTR and regulate its IRES activity.

RESULTS

Generation of transgenic mice harboring CMV/βGAL/CAT and CMV/βGAL/UtrA/CAT reporters

Previous work on the FGF-2 IRES has shown that tissue specificity of IRES activity *in vivo* is not necessarily correlated with activity in cell lines (Creancier et al., 2000). We thus generated transgenic mice harboring a bicistronic utrophin A 5'-UTR reporter. For the creation of utrophin A 5'-UTR reporter transgenic mice, we used the CMV/βGAL/CAT bicistronic vector (**Fig. 23a**). This vector was chosen because it does not exhibit splicing or internal promoter activity under a variety of conditions when harboring the utrophin A 5'-UTR (Miura et al., 2005; Miura and Jasmin, 2006), and because previous studies have employed bicistronic reporters driven by the CMV promoter to evaluate IRES activity in transgenic mice (Creancier et al., 2000). From 3 founder mice identified by genotyping, full analysis was performed on two utrophin A 5'-UTR reporter transgenic lines (CMV/βGAL/UtrA/CAT), namely lines 863 and 876. To serve as a control, we generated a transgenic line harboring a control bicistronic reporter (CMV/βGAL/CAT).

The utrophin A 5'-UTR drives muscle-specific translation in transgenic mice

We examined multiple tissues of the transgenic mouse lines for IRES activity. The reporter transgene provides a read-out of cap-dependent translation as β-galactosidase activity (βGAL), whereas cap-independent, IRES-mediated translation, is reported as chloramphenicol acetyltransferase activity (CAT). In three transgenic lines

harboring the utrophin A 5'-UTR reporter, we detected appreciable levels of IRES activity (reported as a ratio of CAT to βGAL) in hindlimb skeletal muscles. Interestingly, we did not detect IRES activity in any other organs examined, including the kidney, heart, lung, liver and brain (**Fig. 23b,c**). In line 863, IRES activity in the tibialis anterior (TA) and gastrocnemius (gastroc) skeletal muscles was 14.5-fold and 10.7-fold above background brain levels (**Fig. 23b**). In line 876, a similar trend was observed, with IRES activity 7.3-fold and 6-fold greater than background brain levels in the TA and gastroc, respectively (**Fig. 23c**). A third line harboring the CMV/βGAL/UtrA/CAT reporter (line 881) also displayed IRES activity exclusively in skeletal muscles (data not shown).

Examination of the above data expressed as individual βGAL (**Fig. 24a-c**) and CAT (**Fig. 24d-f**) values revealed that the increase in CAT to βGAL ratio in TA and gastroc muscles of the 863 and 876 lines is due to enhanced CAT expression, and not merely a result of low βGAL levels stemming from low CMV driven transcription. These findings indicate that, *in vivo*, cap-independent translation driven by the utrophin A IRES occurs exclusively in skeletal muscles and no other tissues.

We performed several control experiments to ensure that the skeletal muscle-specific expression of the CAT reporter could be attributed to genuine utrophin A IRES activity. Examination of the control transgenic line (CMV/βGAL/CAT) revealed that while βGAL activity was detectable in all tissues (**Fig. 24c**), appreciable levels of CAT activity could not be detected (**Fig. 24f**). This result demonstrates that the CAT activity detected in skeletal muscles of transgenic lines 863 and 876 can be attributed solely to the utrophin A 5'-UTR.

Fig. 23. Utrophin A 5'-UTR bicistronic reporter transgenic mice display IRES activity exclusively in skeletal muscles.

A) Schematic representation of utrophin A 5'-UTR (CMV/βGAL/UtrA/CAT) reporter transgene used for generation of transgenic mice. B) Relative IRES activity in various tissues of CMV/βGAL/UtrA/CAT reporter transgenic line 863. Values are standardized to the brain IRES activity ratio. $*P < 0.05$, relative to brain, n=6. C) Relative IRES activity in various tissues of CMV/βGAL/UtrA/CAT reporter transgenic line 876. Values are standardized to the brain IRES activity ratio. $\#P < 0.05$, relative to heart, n=5. See Fig. 24 for individual CAT and βGAL reporter levels. (D,E) qPCR analysis using βGAL and CAT primers was performed on reverse transcriptase-amplified RNA from various tissues of lines 863 and 876. The ratio of amplified βGAL and CAT cDNAs is not significantly different among the different tissues in both lines, providing evidence for the exclusive presence of an intact bicistronic transcript ($P > 0.05$, relative to brain, n=3). TA-tibialis anterior, Gastroc-gastrocnemius.

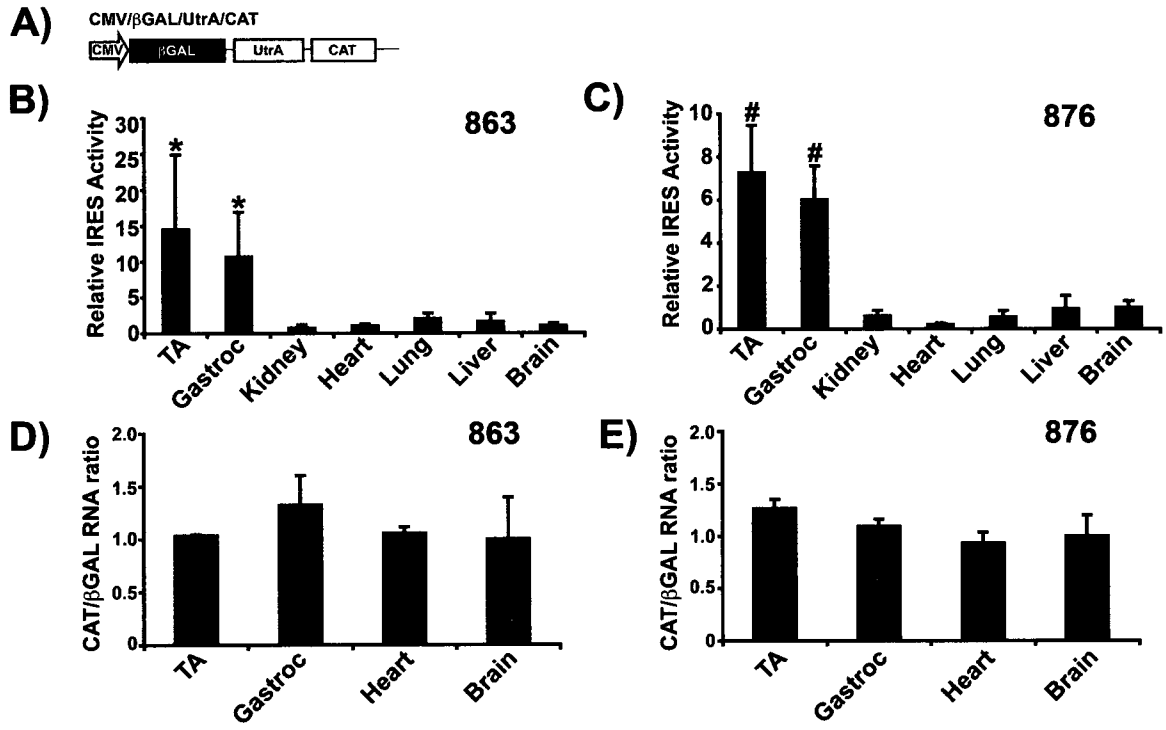
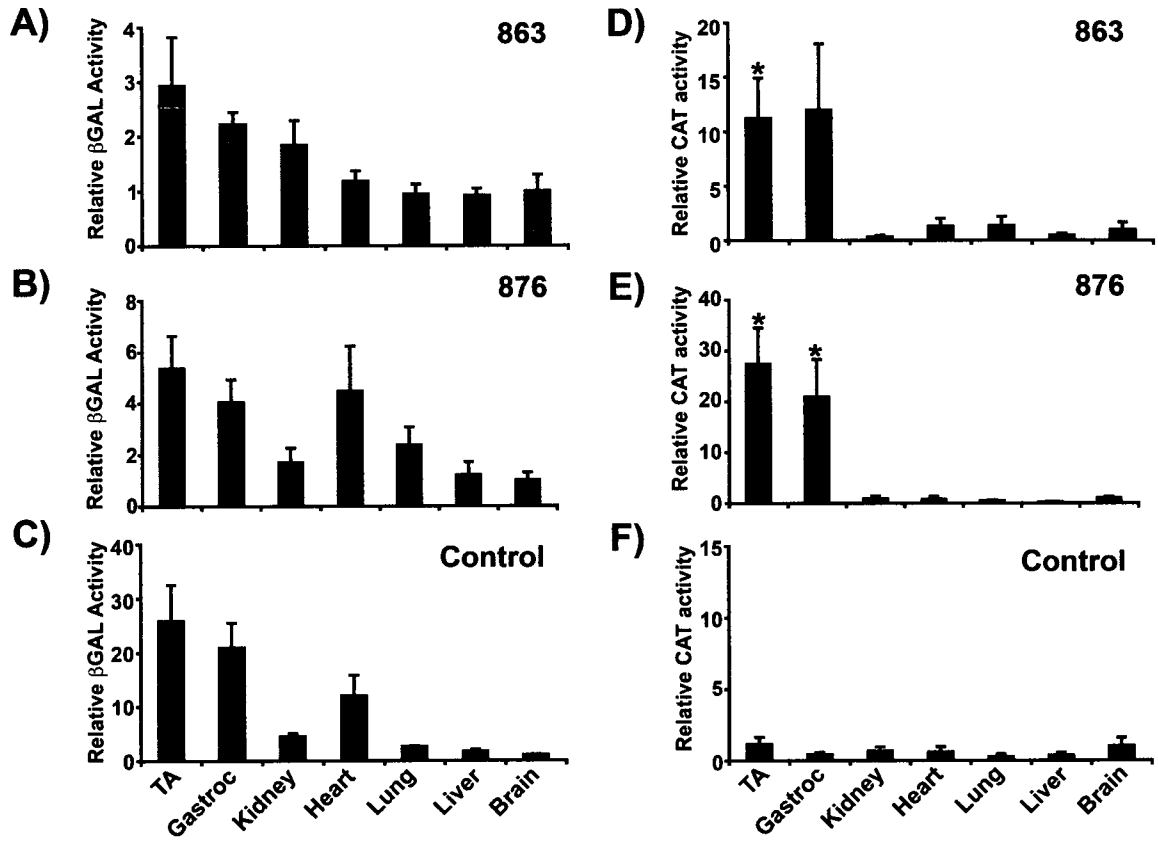

A)
CMV/βGAL/UtrA/CAT
CMV
βGAL
UtrA
CAT
B)
863
Relative IRES Activity
30
25
20
15
10
5
0
*
*
TA
Gastroc
Kidney
Heart
Lung
Liver
Brain
C)
876
Relative IRES Activity
10
8
6
4
2
0
#
#
TA
Gastroc
Kidney
Heart
Lung
Liver
Brain
D)
863
CAT/βGAL RNA ratio
2.0
1.5
1.0
0.5
0
TA
Gastroc
Heart
Brain
E)
876
CAT/βGAL RNA ratio
2.0
1.5
1.0
0.5
0
TA
Gastroc
Heart
Brain

Fig. 24. Individual levels of βGAL and CAT reporter activity in utrophin A 5'-UTR and control bicistronic reporter transgenic mice.

A,B,C) Relative βGAL activity in transgenic mice from transgenic lines 863, 876 and control normalized to reporter levels in brain. D,E,F) Relative CAT activity in transgenic mice from transgenic lines 863, 876 and control normalized to reporter levels in brain. Note that CAT activity is found at high levels only in skeletal muscles (TA and Gastroc) in both 863 and 876 lines and that no tissue of the control line displays CAT activity, although βGAL activity can be detected. $*P < 0.05$, relative to brain. Control line, n=4; 876 line, n=5; 863 line, n=6.


A)
863
Relative βGAL Activity
0
1
2
3
4
D)
863
Relative CAT activity
0
5
10
15
20
*
B)
876
Relative βGAL Activity
0
2
4
6
8
E)
876
Relative CAT activity
0
10
20
30
40
*
*
C)
Control
Relative βGAL Activity
0
10
20
30
40
F)
Control
Relative CAT activity
0
5
10
15
TA
Gastroc
Kidney
Heart
Lung
Liver
Brain

Additional control experiments were performed to ensure that IRES activity observed in skeletal muscle was not due to an aberrant splicing event or cryptic promoter activity. The presence of internal promoter activity or aberrant splicing could result in the expression of transcripts other than the intact, full-length bicistronic mRNA, potentially leading to a false-positive indication of IRES activity. We performed qRT-PCR analysis on RNA extracted from the 876 and 863 lines (CMV/βGAL/UtrA/CAT) to ensure that βGAL and CAT cDNA could be amplified in an equal ratio in both tissues that displayed IRES activity (gastroc and TA) and tissues that had no IRES activity (heart and brain). Indeed, we found that the ratio of amplified CAT to βGAL cDNA in all tissues examined was not statistically different ($P > 0.05$) (**Fig. 23d,e**). The enhanced CAT activity in skeletal muscles of the CMV/βGAL/UtrA/CAT reporter mice can thus be attributed to increased cap-independent translation driven by events that target the utrophin A 5'-UTR.

Identification of RNA-binding proteins that interact with the utrophin A 5'-UTR

Since IRES-mediated translation is regulated by the binding of *trans*-factors, we set out to identify RNA-binding proteins that interact with the utrophin A 5'-UTR. We first examined whether proteins isolated from regenerating muscle versus control muscle could bind selectively to the utrophin A 5'-UTR since utrophin A IRES activity is enhanced in regenerating muscle (Miura et al., 2005). To induce skeletal muscle degeneration and regeneration, we injected TA muscles of mice with the snake venom cardiotoxin and isolated the muscles after 7 days. At this time-point, utrophin A IRES

activity is induced and its protein levels are ~14-fold above control levels (Miura et al., 2005). Using northwestern analysis, we found that a RNA probe corresponding to a portion of the utrophin A 5'-UTR (nucleotides 147-363) associated with several proteins preferentially in regenerating muscle extracts (CTX) (**Fig. 24a**). Thus, conditions under which the utrophin A IRES is highly activated are associated with the binding of multiple proteins to the utrophin A 5'-UTR.

eEF1A2 interacts with the utrophin A 5'-UTR

Various RNA-binding proteins are involved in cellular IRES-mediated translation; however, each particular IRES appears to be regulated by a distinct set of *trans*-factors, and no single universal factor has been shown to be necessary for cap-independent translation of all IRES. An RNA affinity chromatography approach (Kim et al., 2004; Lewis et al., 2007a) was employed to isolate and identify proteins that interact with the utrophin A 5'-UTR. For these experiments, we used a biotinylated RNA probe containing the same region of the utrophin A 5'-UTR found to associate with several proteins in regenerating skeletal muscles (nucleotides 147-363, **Fig. 25a**). The probe was linked to avidin-agarose beads and incubated with regenerating skeletal muscle protein lysates. After extensive washing, SDS PAGE was performed. Analysis of the SYPRO ruby-stained gel revealed the presence of a band, migrating at ~50 kDa, that was only present in the sample containing the 5'-UTR probe and not in the sample lacking the biotinylated RNA (**Fig. 25b**). The band was excised and analyzed it by MALDI-TOF, which identified the protein to be eukaryotic elongation factor 1A2 (eEF1A2).

To confirm that the 50 kDA protein was indeed eEF1A2, we performed additional RNA affinity chromatography experiments, transferred the denatured proteins to a PVDF membrane and performed western analysis using an anti-eEF1A antibody that recognizes both eEF1A1 and eEF1A2. Western analysis revealed the presence of a band at the expected molecular mass in samples that were incubated with the utrophin A 5'-UTR probe (**Fig. 25c**). We verified that this interaction between the utrophin A 5'-UTR and eEF1A protein(s) was not caused by non-specific binding to RNA by performing additional experiments in which a non-related RNA probe was used (**Fig. 25c**). We also performed the chromatography experiment using lysates from HEK293T cells. Association of the probe to eEF1A was not detected using these lysates (**Fig. 25d**). Since HEK293T cells express eEF1A1 and not, or very limited amounts of, eEF1A2 (~12-fold difference as determined by qRT-PCR), these experiments provide evidence that eEF1A2 is a factor that interacts with the utrophin A 5'-UTR.

Regions of the utrophin A 5'-UTR that contain IRES activity also bind eEF1A2

In order to delineate the regions of the utrophin A 5'-UTR that interact with eEF1A2, purified eEF1A2 was incubated with radiolabelled RNA probes containing various truncations of the utrophin A 5'-UTR and then cross-linked by UV radiation. A specific interaction was identified for probes 1-228 and 147-363, but not 1-70, as indicated by the presence of a shifted complex (**Fig. 26**). This interaction was specific since the shifted complexes detected using the 1-228 and 147-363 probes could be competed away by the addition of cold probe.

Fig. 25. eEF1A-2 interacts with the utrophin A 5'-UTR.

A) Northwestern analysis of control and cardiotoxin (CTX)- injected tibialis anterior (TA) muscles using a [α-^{32}P]UTP-labeled RNA probe corresponding to nucleotides 147-363 of the utrophin A 5'-UTR. Note the presence of several bands in regenerating muscles. Blot is representative of experiments performed with muscles of three mice. B) RNA-affinity chromatography isolation of utrophin A 5'-UTR-binding proteins. Precleared extracts from cardiotoxin-treated TA muscles were incubated with agarose beads coated with biotinylated utrophin A 5'-UTR RNA (147-363) or agarose beads alone. Beads were washed extensively, eluted by boiling and resolved by SDS-PAGE. Sypro Ruby stained gel shows a 50 kDA protein species that was identified as eukaryotic elongation factor 1A2 (eEF1A2) by mass spectrometry analysis. C) Samples prepared as in B) were separated by SDS-PAGE, transferred to PVDF membrane, and western blot was performed using an anti-EF1A antibody. This antibody detects both eEF1A1 and eEF1A2 isoforms. eEF1A was detected in CTX muscle lysate incubated with the utrophin A 5'-UTR biotinylated probe, but not to a no RNA control, or an unrelated biotinylated RNA probe (corresponding to the utrophin A 3'-UTR). D) Biotinylated 5'-UTR probe (147-363) does not bind to eEF1A from HEK293T protein lysate (HEK).

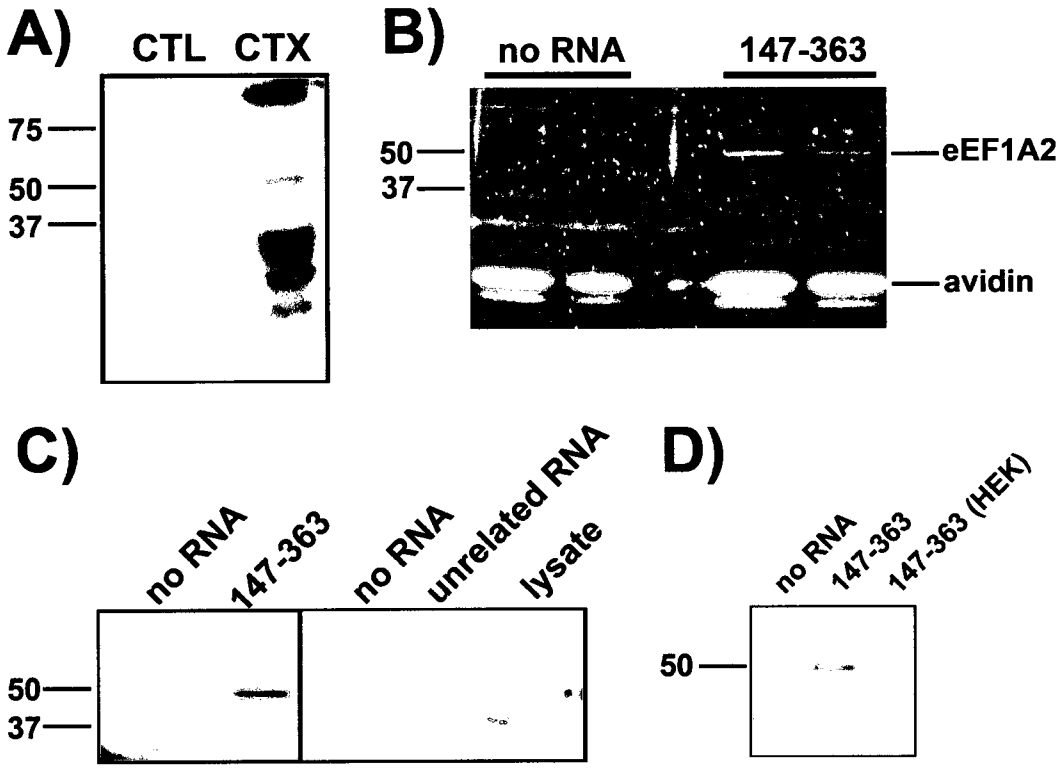
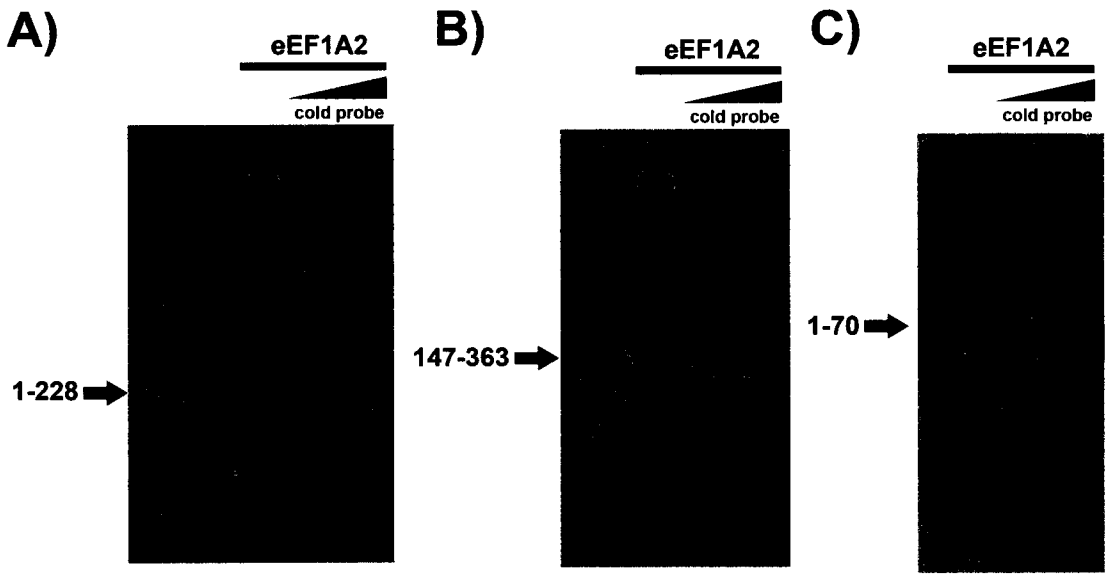

A)
CTL
CTX
75
50
37
B)
no RNA
147-363
50
37
eEF1A2
avidin
C)
no RNA
147-363
no RNA
unrelated RNA
lysate
50
37
D)
no RNA
147-363
147-363 (HEK)
50

Fig. 26. eEF1A-2 directly interacts with particular regions of the utrophin A 5'-UTR.

Recombinant eEF1A2 was incubated with [α-^{32}P]UTP probes corresponding to various regions of the utrophin A 5'-UTR, including A) 1-228, B) 147-363 and C) 1-70. Samples were UV cross-linked and separated on Tris-glycine-polyacrylamide gels. The presence of a shifted complex is indicative of a specific interaction between the probe and the purified eEF1A2. Note that the addition of an increasing concentration of cold probe (150 ng to 600 ng) successfully competed away shifted bands (A, B).


A)
eEF1A2
cold probe
1-228
B)
eEF1A2
cold probe
147-363
C)
eEF1A2
cold probe
1-70

To determine whether the regions of the utrophin A 5'-UTR that bind to eEF1A2 also harbor IRES activity, we performed transient transfections of utrophin A 5'-UTR bicistronic reporters or an empty vector control in C2C12 myoblasts. The full-length utrophin A 5'-UTR displayed ~14-fold IRES activity (normalized to empty vector control) (**Fig. 27a**). The 1-228 region showed 59% of the activity of the full-length utrophin A 5'-UTR and the 147-363 region showed 25% of full-length activity (8.3-fold for 1-228, 3.4-fold for 147-363 above empty vector control levels) ($P < 0.05$). In contrast, the 1-70 region showed no significant IRES activity over control levels ($P > 0.05$). These experiments demonstrate that regions of the utrophin A 5'-UTR that bind to eEF1A2 are the same regions able to drive cap-independent translation in C2C12 myoblasts.

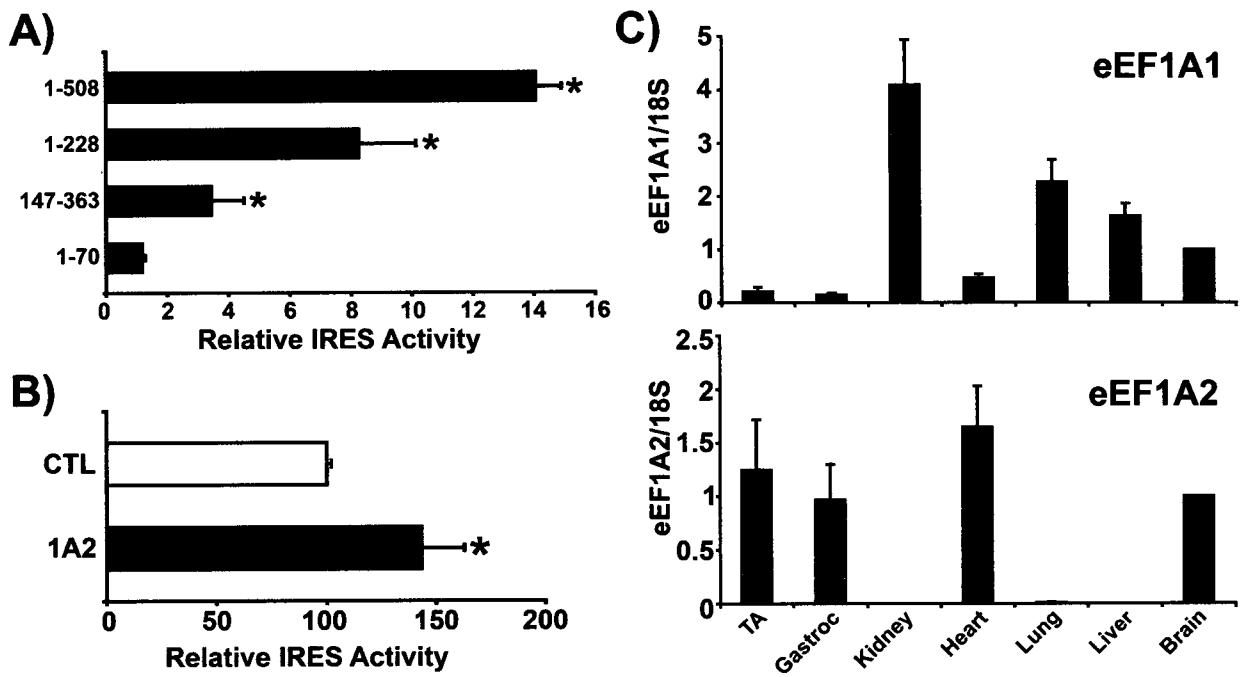
eEF1A2 enhances utrophin A IRES activity and its expression pattern correlates with IRES activity in transgenic mice

To ascertain whether eEF1A2 can directly regulate utrophin A IRES activity, we attempted to knockdown eEF1A2 in C2C12 cells by using siRNA and shRNA based protocols. Due to the abundant expression of eEF1A2 in C2C12 cells, we were unable to achieve significant knockdown of this protein (data not shown). As an alternative approach, we obtained a rat fibroblast cell line that lacks eEF1A2, and one that is stably transfected to overexpress eEF1A2 (Amiri et al., 2007). Transient transfection experiments revealed that the utrophin A IRES had ~1.4 fold greater activity in the cells expressing eEF1A2 (1A2) compared to controls lacking eEF1A2 (CTL) ($P < 0.05$) (**Fig. 27b**).

To assess whether eEF1A2 expression correlates with utrophin A IRES activity in tissues of the utrophin A 5'-UTR reporter transgenic mice, we examined mRNA levels of eEF1A2 and eEF1A1 in various tissues by real-time quantitative RT-PCR. This technique was used because antibodies that distinguish between the two isoforms are not available. In accordance with previous studies (Lee et al., 1992; Chambers et al., 1998), we found that eEF1A2 was expressed in skeletal muscle, heart and brain, but not kidney, lung and liver (**Fig. 27c**). Examination of the relative abundance of eEF1A2 to eEF1A1 revealed that although eEF1A2 is expressed in heart and brain (tissues that do not contain utrophin A IRES activity), there are higher levels of eEF1A1 in these organs compared to skeletal muscle, which contains nearly undetectable levels of eEF1A1 (**Fig. 27c**). The relative abundance of the two eEF1A isoforms shown as a ratio in **table 3** reveals that skeletal muscles contain ~ 2- and 6- fold the ratio of eEF1A2 to eEF1A1 as compared to heart and brain tissues, respectively.

Fig. 27. Role of eEF1A-2 in regulating utrophin A IRES-mediated translation.

A) Transient transfection of bicistronic vectors harboring various truncations of the utrophin A 5'-UTR into C2C12 myoblasts reveals that regions of the utrophin A 5'-UTR that bind to eEF1A2 (see Fig. 26) also display significant levels of IRES activity. IRES activity is reported as a ratio of CAT to βGAL, values are standardized to control reporter levels. *, $P < 0.05$. B) Rat2 fibroblast cells (CTL) or Rat2 fibroblast cells stably transfected with eEF1A2 (1A2) were transfected with pβGAL/UtrA/CAT and relative IRES activity was measured. *$P < 0.05$. For A and B, values were determined from two or more independent transfections. C) qRT-PCR was performed on various tissues of mice from the 863 line to determine expression of eEF1A-1 or eEF1A-2 relative to 18S ribosomal RNA. Note the lowest levels of eEF1A1 are found in the skeletal muscle samples (n=3). Ratios of eEF1A2 to eEF1A1 mRNA are shown in table 3.


A)
1-508
1-228
147-363
1-70
0 2 4 6 8 10 12 14 16
Relative IRES Activity
B)
CTL
1A2
0 50 100 150 200
Relative IRES Activity
C)
eEF1A1
eEF1A1/18S
0 1 2 3 4 5
eEF1A2
eEF1A2/18S
0 0.5 1 1.5 2 2.5
TA
Gastroc
Kidney
Heart
Lung
Liver
Brain

Tissue	eEF1A2/eEF1A1 mRNA ratio ± sem
TA	6.6 ± 0.5
Gastroc	7.8 ± 3.9
Kidney	0
Heart	3.4 ± 1.1
Lung	0
Liver	0
Brain	1

Table 3: Ratio of eEF1A2 to eEF1A1 mRNA levels in various tissues of transgenic mice.

Data was obtained by qRT-PCR. Ratio of eEF1A2 to eEF1A1 was calculated for each individual mouse prior to calculating the mean (n=3). For raw data of eEF1A1 and eEF1A2 standardized to 18S ribosomal RNA, see Fig. 27c.

DISCUSSION

In recent work, we have shown that the utrophin A 5'-UTR contains an IRES that is activated in response to a variety of conditions (Miura et al., 2005; Miura et al., 2008). Here, we analyzed transgenic mice harboring utrophin A 5'-UTR reporter constructs to determine whether the activity of the utrophin A IRES is tissue specific. Interestingly, we found that the utrophin A 5'-UTR can direct cap-independent translation exclusively in skeletal muscles, and no other tissues. This expression pattern suggested that factors specifically expressed or activated in skeletal muscle could be important in mediating utrophin A IRES activity. In accordance with this prediction, RNA-affinity chromatography experiments identified eEF1A2, the isoform of eEF1A expressed in mature skeletal muscle, as a factor that interacts with the utrophin A 5'-UTR. Additional binding experiments and reporter activity assays demonstrated the importance of this interaction in mediating utrophin A IRES activity.

Utrophin A is expressed in a variety of different organs and tissues, including lungs, kidney, brain, heart and skeletal muscle (Matsumura et al., 1992; Weir et al., 2002; Baby et al., 2009). At the transcriptional level, *cis*-elements in the promoter region direct expression of utrophin A to multiple tissues, and an intronic enhancer appears to contribute to utrophin A mRNA expression in heart and skeletal muscle (Stocksley et al., 2005; Takahashi et al., 2005; Tanihata et al., 2008). In contrast to the wide expression pattern of utrophin A mRNA and protein, here we found utrophin A IRES activity only in skeletal muscles. While this observation does not rule out the involvement of translational control mechanisms in regulating utrophin A in other tissues, it does strongly suggest that translational regulation of utrophin A by its 5'-UTR is particularly

important in skeletal muscle. Indeed, we have already uncovered that, like several other IRES regulated transcripts, utrophin A cap-independent translation can be driven under conditions of stress where the overall levels of cap-dependent translation are reduced (Miura et al., 2005; Miura et al., 2008; Spriggs et al., 2008). Given these results, we predict that in skeletal muscle, additional physiologically important "stress" stimuli might target the utrophin A 5'-UTR to allow for the rapid synthesis of protein from a pre-existing pool of utrophin A transcripts.

It is well established that utrophin A is more highly expressed in slow-oxidative muscles, such as the soleus, compared to fast-glycolytic muscles, such as the extensor digitorum longus (EDL) (Chakkalakal et al., 2003b; Chakkalakal et al., 2008). Interestingly, we observed that IRES-activity in line 876 was 6.8-fold greater in the more fast-glycolytic EDL muscles as compared to soleus muscles ($P < 0.05$). A similar trend was observed in line 863, however the difference was not statistically significant. These observations suggest that utrophin A IRES activity is greater in muscle fibers expressing fast, type II myosin heavy chain isoforms. Future experiments using bicistronic vectors harboring fluorescent protein reporters will be necessary to confirm whether there are clear fiber type differences in utrophin A IRES activity. This question could not be addressed using our transgenic mice since antibodies do not reliably detect the CAT reporter by indirect immunofluorescence. Fluorescent bicistronic reporters will also be useful to investigate the subcellular localization of IRES activity within skeletal muscles; in particular to determine whether IRES-activity is enhanced at the neuromuscular junction.

A limited number of studies have examined the relevance of cellular IRES elements *in vivo*. Studies performed on *Drosophila* harboring bicistronic reporters for ultrabithorax and antennapedia 5'-UTRs display spatial and temporal regulation of IRES activity (Ye et al., 1997). Mice harboring a c-myc IRES reporter display high activity in embryonic tissues, but low or undetectable activity in adult tissues (Creancier et al., 2001). Several studies have examined activity of the FGF-2 IRES in transgenic mice. IRES-activity in these mice is regulated in response to hyperglycemia and aging (Teshima-Kondo et al., 2004; Gonzalez-Herrera et al., 2006), and activity is particularly high in the brain, with certain structures displaying more activity than others (Creancier et al., 2000; Audigier et al., 2008). Interestingly, a factor highly expressed in brain, hnRNP A1, is important for FGF-2 IRES activity (Bonnal et al., 2005). From these limited number of studies, it appears that cellular IRES elements are highly regulated in a tissue-specific manner, suggesting an important role for *trans*-factors that have tightly controlled expression patterns.

Since the utrophin A 5'-UTR drives IRES-mediated translation only in skeletal muscles, it is likely that the complex of proteins that permits this type of translation is present or active exclusively in skeletal muscles. We provide evidence suggesting that one of the factors is eEF1A2. The two eEF1A isoforms are eEF1A1 and eEF1A2. These share 92% sequence identity and appear to be functionally equivalent with regard to their effects on protein synthesis activity *in vitro* (Kahns et al., 1998). While the canonical role for eEF1A isoforms is in shuttling aminoacyl-tRNA during translation elongation, distinct "moonlighting" roles for these proteins have also been identified (Ejiri, 2002). eEF1A1 is important in regulating cytoskeletal organization (Gross and Kinzy, 2005),

protein nuclear export (Khacho et al., 2008) and mRNA stability (Yan et al., 2008; Zhang et al., 2009). In contrast, eEF1A2 protects against apoptosis (Ruest et al., 2002; Chang and Wang, 2007), and plays a role in actin remodeling and oncogenesis (Amiri et al., 2007; Jeganathan et al., 2008). Distinct roles for these two proteins are also suggested by their tissue specific expression pattern. While eEF1A1 is expressed almost ubiquitously, eEF1A2 is expressed in skeletal muscle, heart and brain (Lee et al., 1992; Knudsen et al., 1993). Interestingly, mice lacking functional eEF1A2 (wasted mice, *wst/wst*) exhibit muscle wasting and motor neuron degeneration resulting in premature death (Chambers et al., 1998; Newbery et al., 2007).

It could be expected that since eEF1A2 is expressed in skeletal muscle, heart and brain, utrophin A 5'-UTR reporter transgenic mice would show IRES activity in all these tissues. One explanation for the lack of IRES activity in brain and heart tissue can be inferred from the greater ratio of eEF1A2 to eEF1A1 found in skeletal muscle compared to heart and brain (see **Fig. 27c** and **table 3**). In tissues that express both isoforms, competition for binding to the utrophin A 5'-UTR may occur, with eEF1A1 opposing the positive effect of eEF1A2 on IRES-mediated translation. On the other hand, skeletal muscle-specific accessory factors that bind eEF1A2 (for example, see (Mansilla et al., 2008)), might be needed for IRES-meditated translation to occur. Alternatively, eEF1A2 might be only one of several proteins needed for skeletal muscle-specific utrophin A IRES activity. Based on expression pattern, it seems that eEF1A2 is not responsible for the enhancement of utrophin A IRES activity during muscle regeneration, since it is not upregulated after myotoxin injection (Miura, Coriati & Jasmin, unpublished observations;

(Khalyfa et al., 1999)). Thus, additional factors or protein modifications are likely required for enhancement of utrophin A IRES activity under various stress conditions.

It is unknown how eEF1A2 is involved in the mechanism of utrophin A IRES-mediated translation and what additional factors are required. Given the ability of eEF1A2 to bind aminoacyl-tRNA, and the presence of tRNA like structures in several viral IRES elements (Wilson et al., 2000; Lyons and Robertson, 2003), we hypothesized that the protein might interact with structural elements in the utrophin A 5'-UTR that resemble tRNA; however, using the bioinformatic analysis of Baird et al., (Baird et al., 2006) we could detect no such structural elements. Cricket paralysis virus (CrPV) and hepatitis C virus (HCV) both contain tRNA-like structures and can interestingly be translated via their IRES elements in an *in vitro* system that contains ribosomes, tRNA and elongation factors, but lacks translation initiation factors (Pestova and Hellen, 2003; Lancaster et al., 2006). In light of our findings presented here, one could speculate that eEF1A2 plays a role in translation initiation for these viruses. Further investigation into regulation of IRES-mediated translation by eEF1A2 may provide insights into the mechanistic commonalities of translation initiation of viral and cellular IRESes.

Several groups, including our own, have been performing pre-clinical screening of small molecules for their ability to enhance activity of the utrophin A promoter in hopes of finding an effective drug treatment for DMD. Our results presented here, combined with previous work demonstrating that glucocorticoid treatment can stimulate utrophin A translation via its 5'-UTR (Miura et al., 2008), provide a rationale to screen for compounds that can activate expression of utrophin A at the translational level. In this regard, utrophin A 5'-UTR reporter transgenic mice could serve as a useful tool to

validate promising compounds *in vivo*. Given the skeletal-muscle specific pattern of utrophin A IRES activity, therapeutics that target the utrophin A 5'-UTR would have the advantage of stimulating protein expression specifically in skeletal muscles and no other tissues.

MATERIALS AND METHODS

Cell culture and Transfections

C2C12 cells were cultured under standard conditions (Miura et al., 2005). Rat2 cells and Rat2 cells overexpressing eEF1A2 were cultured as previously described (Amiri et al., 2007). The bicistronic reporter constructs containing various truncations of the utrophin A 5'-UTR were transiently transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously described (Miura et al., 2008).

Bicistronic Transgenic mice

Six to eight week old C6B3F1 mice were purchased from Charles River Laboratories (Boston, MA). They were cared for in approval with the University of Ottawa Animal Care and Use Committee who is compliant with the Guidelines of the Canadian Council on Animal Care and the Animals for Research Act. The bicistronic vectors, pβGAL/UtrA/CAT and pβGAL/CAT were digested with SalI and ApaI restriction enzymes to generate 6528 and 6020 nucleotide products respectively. These fragments included the CMV promoter and both reporter genes. Products were electrophoresed on 1% agarose gels containing ethidium bromide. Bands were excised and purified using Qiagen gel extraction kit (Qiagen, Chatsworth, CA). To generate transgenic mice, hybrid C6B3F1 mice were used as donors for fertilized one-cell embryos. CMV/βGAL/UtrA/CAT and CMV/βGAL/CAT DNA fragments were separately microinjected into the pronucleus of donor embryos. Pseudopregnant females were used as recipients for the modified zygotes. Potential founders were weaned at 3 weeks after birth. Tail biopsies were collected, minced and incubated overnight in

proteinase K (20 mg/ml) at 55°C, genomic DNA was extracted by a standard phenol/chloroform/isoamyl alcohol extraction and subjected to PCR genotyping. PCR was performed using a 5' primer spanning 142 nucleotides of the LacZ gene and a 3' primer spanning 459 nucleotides of the CAT gene (5'-TTTTTCCCGATTTGGCTACA-3'; 5'-TGAAACTCACCCAGGGATTG-3'). PCR products were visualized on 2% agarose gel containing ethidium bromide. Founders were bred with C6B3F1 wild-type mice and their progeny were examined for transgene expression using β-galactosidase assay. The three CMV/βGAL/UtrA/CAT (876, 881, and 863) and one CMV/βGAL/CAT transgenic lines were backcrossed with C6B3F1 wild-type mice for several generations.

Reporter Activity Assays

Various tissues were excised from transgenic mice and immediately frozen in liquid nitrogen after euthanizing the mice with CO_2. For reporter assays, protein from tissues was extracted with reporter lysis buffer (Promega) as previously described (Miura et al., 2005) and protein concentration was quantified by Bradford Standard Assay. Protein samples were diluted to a final concentration of 4 μg/μl prior to reporter assays. β-galactosidase (βGAL) enzymatic assays were performed using the β-galactosidase enzyme assay system as recommended by the manufacturer (Promega). To measure chloramphenicol acetyltransferase (CAT) activity, we analyzed the conversion of chloramphenicol to butyryl chloramphenicol by incorporation of [^{14}C] butyryl coenzyme A (Miura et al., 2005). Background levels for both reporter assays were determined by analyzing reporter activity in tissues from mice not harbouring a transgene.

RNA Extraction and qRT-PCR Analysis

Total RNA was isolated from tissues of CMV/βGAL/UtrA/CAT and CMV/βGAL/CAT transgenic mice using TRIzol reagent (Invitrogen) as recommended by the manufacturer. TRIzol extraction was followed by a one hour DNAse I (Invitrogen) treatment to eliminate possible plasmid and genomic DNA contamination. To control for the presence of an intact bicistronic reporter transcript in CMV/βGAL/UtrA/CAT transgenic mice, qRT-PCR was performed with previously described βGAL and CAT primers (Miura et al., 2005). Negative controls consisted of an RT mixture that had the reverse transcriptase replaced by sterile water. Quantitative real-time RT-PCR was performed on reverse transcribed RNA using QuantiTect SYBR green PCR kit (Qiagen) on a Stratagene MX3005p, and the delta delta Ct method was used to quantify expression of endogenous mRNAs relative to 18S ribosomal RNA. The following primer sets were used to detect eEF1A2- 5'-CCAGGCCGGAGGATACCG-3'; 5'-GAAATGCAAGCGGCGTGT-3'; and eEF1A1- 5'-GGCAGTGCCAGTGGCACCA-3'; 5'-CCAGGCTTGAGAACACCAGT-3'. 18S ribosomal RNA was detected using previously described primers (Ma et al., 2007). For detection of human eEF1A1 and eEF1A2 in HEK293T cells, previously published primer sequences were used (Kim et al., 2009).

RNA binding experiments

Cardiotoxin-treatment of C57BL/10 mice was performed as previously described (Miura et al., 2005) and tibialis anterior (TA) muscles were excised 7 days after toxin injection. Isolation of proteins binding the utrophin A 5'-UTR was performed using an RNA-affinity chromatography protocol (Lewis et al., 2007a). For these experiments, 60 μg of biotinylated RNA was conjugated to avidin-agarose beads and incubated with 4 mg

of protein extract from regenerating TA muscle extracts. In-gel trypsin digestion and mass peptide fingerprinting was performed at the Protein Function Discovery Centre (Queen's University, Kingston, ON, Canada). To confirm eEF1A2 as an interacting protein, we performed RNA-affinity chromatography using 15 μg of biotinylated RNA and 1.6 mg of TA muscle or HEK293T cell extracts, transferred proteins from the gel to a PVDF membrane, and performed western blot using an eEF1A antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

RNA probes were transcribed from PCR templates containing T7 RNA polymerase binding site using the MAXIScript in vitro transcription kit (Ambion, Austin, TX, USA), and [α-^{32}P]UTP (800 Ci/mmol; Perkin Elmer, Boston, MA, USA). For northwestern analysis, 60 μg of cardiotoxin-treated TA muscle extract was run on a 10% SDS PAGE gel and transferred to PVDF membrane. Equal loading was confirmed by staining with Ponceau S. Membranes were presoaked, incubated with the $1x10^6$ cpm [α-32P] labeled utrophin A 5'-UTR RNA probes and washed as previously described (Sela-Brown et al., 2000). Binding of the RNA probe to proteins was visualized by autoradiography. For UV-crosslinking experiments, recombinant eEF1A2 was produced as previously described (Kulkarni et al., 2007), desalted with a Sepharose G25 column (Sigma, USA) and quantified by BCA kit (Pierce, Rockford, IL, USA). MEGAShort Script kit (Ambion, Austin, TX, USA) was used to produce non-radiolabelled RNA probes. Unincorporated nucleotides were removed by Sepharose G25 column and purified by electrophoresis on 6% Tris-glycine-polyacrylamide gels. Desalted eEF1A2 protein (100 ng) and 50000 cpm of RNA probe were pre-incubated for 30 minutes at room temperature, in UV-binding buffer (10 mM Tris-HCl pH 7.5, 50 nM KCl, 10%

glycerol, 5 mM DTT and 10 ng/ul of yeast tRNA). For competition experiments, non-radiolabelled RNA was added 15 min prior to the end of the pre-incubation step. Samples were then exposed to 254 nm UV light for 30 minutes at 4°C and resuspended in RNA loading buffer. Samples were separated on Tris-glycine-polyacrylamide gels using 4% w/v acrylamide/bis-acrylamide (19:1). Following electrophoresis, the gels were dried and subjected to autoradiography.

Statistical Analysis

For transgenic mice reporter activity analysis, one-tailed *t*-tests were used to determine statistical significance. For all other experiments, two tailed *t*-tests were used. Level of significance was set at $P < 0.05$.

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CHAPTER 6

GENERAL DISCUSSION

I. Summary

In this thesis, we investigated regulatory mechanisms controlling utrophin A expression in skeletal muscle. We first presented a manuscript that focused on regulation of utrophin A at the level of transcription (Chapter 2). The utrophin A promoter region is the most studied regulatory element of the utrophin gene, and it has been envisioned that the design, or screening of small molecules that activate transcription of utrophin A through this element could lead to a therapy for DMD. We uncovered a novel regulator of the utrophin A promoter, PPARβ/δ, and found that a clinically relevant agonist of PPARβ/δ, GW501516, stimulated utrophin A expression at the sarcolemma of the *mdx* mouse. As expected based on previous findings by ourselves and others, this modest increase of utrophin A expression was sufficient to confer functional benefits to dystrophic muscles. The remaining chapters of this thesis describe the discovery and characterization of a new element that can control the synthesis of utrophin A at the translational level. We found that this element, an Internal Ribosome Entry Site (IRES), translationally regulates utrophin A in response to skeletal muscle regeneration (Chapter 3) and glucocorticoid treatment (Chapter 4). We also found, using utrophin A 5'UTR reporter transgenic mice, that utrophin A IRES activity is present in skeletal muscles and no other tissues (Chapter 5). Finally, we showed that eEF1A2 is a *trans*-acting factor that interacts with the IRES, and presented evidence suggesting that it regulates the skeletal muscle-specific IRES activity pattern (Chapter 5).

II. PPARβ/δ regulation of utrophin A and its clinical implications

As part of the objectives outlined in this thesis, we investigated whether PPARβ/δ activation by a synthetic agonist can stimulate utrophin A expression (Chapter 2). Previous studies that examined the effects of calcineurin activation and PGC-1α on utrophin A expression and the dystrophic phenotype provided the rationale for pursuing this avenue (Chakkalakal et al., 2004; Angus et al., 2005; Stupka et al., 2006; Handschin et al., 2007a). The study we present here is unique in that we had the opportunity to modulate an effector of the slow myofiber program using a clinically relevant drug. In this regard, the work represents the culmination of ~10 years of investigation into how regulation of the slow myogenic program can modulate utrophin expression and lead to functional improvements in dystrophic skeletal muscles. The benefits conferred to *mdx* skeletal muscles as a result of GW501516 treatment included upregulated utrophin A expression, improved sarcolemmal stability and restoration of DAPC proteins at the sarcolemma. Notably, an improvement in muscle function was also demonstrated by the reduced susceptibility of the skeletal muscles of treated mice to lengthening contractions.

We determined that GW501516 exerts its effects on utrophin A transcription through a PPRE located in the utrophin A promoter. It remains to be seen what other factors accompany PPARβ/δ when it binds this element and what modifications of these factors occur in response to GW501516 treatment. Previous work has established that the transcriptional co-factor PGC-1α forms a complex with GABP and Host Cell Factor (HCF) to regulate utrophin A and other genes that are preferentially expressed at the NMJ (Handschin et al., 2007a). There is some evidence that PGC-1α might cooperate with

PPARβ/δ to regulate utrophin; for instance, a study showed that PPARβ/δ coimmunoprecipitates with PGC-1α in skeletal muscle nuclear extracts (Wang et al., 2003). Thus, in addition to regulating utrophin A expression as part of the PGC-1α-GABP-HCF complex at the N-box motif, PGC-1α- might also exert its effects through the upstream PPRE site in conjunction with PPARβ/δ (see **Fig 4** for utrophin A promoter schematic). An investigation into this possibility should include assessing the contribution of post-translational modifications of PGC-1α, including phosphorylation and acetylation (Lerin et al., 2006), on its association with PPARβ/δ and activation of the utrophin A promoter. Indeed, the effects of neuregulin on utrophin expression at the NMJ appear to require phosphorylation of PGC-1α (Handschin et al., 2007a). In addition to the potential cooperation between PPARβ/δ and PGC-1α, it is possible that calcineurin/NFAT signaling is involved in the regulation of utrophin A by PPARβ/δ activation. In this regard, it has recently been shown that activation of PPARβ/δ in mice stimulates calcineurin transcriptional activity (Gaudel et al., 2008; Wagner et al., 2009).

The influence of PPARβ/δ on the endogenous expression pattern of utrophin A in the absence of synthetic agonist activation remains to be determined. PPARβ/δ is expressed in many tissues, and is in fact the most ubiquitously expressed of the PPAR nuclear receptors (Braissant et al., 1996). GW501516 activation of PPARβ/δ in *mdx* mice resulted in enhanced expression of utrophin A along the sarcolemma, suggesting that this nuclear receptor is expressed in nuclei throughout skeletal muscle fibers of these mice. It remains to be seen whether PPARβ/δ is specifically involved in the restricted junctional expression pattern of utrophin A in healthy mature skeletal muscle fibers. To this end, immunofluoresence analysis of muscle fibers with appropriate antibodies should

determine whether PPARβ/δ is found at enhanced levels in post-synaptic nuclei. If PPARβ/δ expression is indeed enhanced at the NMJ in skeletal muscle, perhaps it also regulates expression of other genes with specific roles at the NMJ. Further investigation into the role of PPARβ/δ in regulating expression of utrophin A and other synaptic genes could employ the use of PPARβ/δ muscle specific knockout mice (Schuler et al., 2006). In addition to expression levels of PPARβ/δ, the activity of the receptor, regulated by endogenous ligands such as triglycerides, fatty acids, and retinoic acid (Michalik et al., 2006; Wagner and Wagner, 2009), likely influences utrophin A expression. It will be interesting to assess the contribution of these endogenous ligands to the activity of PPARβ/δ in dystrophic versus healthy muscles and in slow- versus fast- twitch muscle fibers.

PPARβ/δ plays important regulatory roles in several tissues, including heart, skin, adipose tissue, and skeletal muscle. Several synthetic agonists to this receptor are under investigation for the treatment of dyslipidemia, metabolic syndrome, diabetes and cardiovascular disease (Wagner and Wagner, 2009). The wide range of potential applications makes it likely that PPARβ/δ agonists will soon be accepted for clinical use. GW501516 in particular has already undergone safety evaluation in the clinic and has been found to ameliorate metabolic abnormalities in obese men (Sprecher et al., 2007; Riserus et al., 2008). This makes it an ideal candidate for a DMD therapeutic, as trials for the treatment of DMD could be streamlined. However, additional experiments must be performed before one can consider treating DMD patients with GW501516. It will be necessary to assess the efficacy of the drug on *mdx* mice at different ages, using varying dosages and treatment durations. Treating *mdx* mice at the pre-necrotic phase (<3 weeks

old) might in fact reveal more pronounced benefits than we observed in our study of mature (5-7 week old) mice, including potentially counteracting muscle necrosis and promoting muscle regeneration. This speculation is supported by a recent finding that short term PPARβ/δ activation increases myonuclear accretion, which was suggested to result from increased fusion of muscle progenitor cells to myofibers (Giordano et al., 2009). As with all potential DMD treatments, further investigation into the effects of GW501516 treatment should also include assessment of additional skeletal muscles including the diaphragm, and also cardiac muscles (Townsend et al., 2008). This is especially important given that PPARβ/δ activation has recently been shown to increase vascularization and cardiac growth (Wagner et al., 2009). Further pre-clinical investigations of GW501516 would also include treatment of the GRMD dog; a DMD model that exhibits a disease progression more closely reminiscent of the human pathology (Willmann et al., 2009).

Given the numerous studies describing the benefits of modest utrophin A upregulation in *mdx* mice, it is likely that the therapeutic effects of GW501516 described here are in fact mediated through utrophin A. On the other hand, it is possible that some of the beneficial effects observed could be attributed to other processes. For instance, PPARβ/δ has an direct inhibitory effect on NF-κB signaling in cardiac (Planavila et al., 2005) and adipose tissue (Rodriguez-Calvo et al., 2008); perhaps this inhibition might be involved in the amelioration of dystrophic symptoms given that NF-κB contributes to the dystrophic pathology by increasing inflammatory gene expression (Evans et al., 2009). Many oxidative genes are regulated by PPARβ/δ agonist treatment in skeletal muscle (Tanaka et al., 2003; Narkar et al., 2008). To conclusively determine whether the

beneficial effects depend on utrophin A upregulation, it will be important to treat mice deficient for both utrophin and dystrophin (*dko* mice) with GW501516; if the benefits are mediated by utrophin A, GW501516 treatment should cause no functional improvement in muscle function of these mice.

One of the reasons others have studied the effect of PPARβ/δ agonists is because of their endurance enhancing effects that have been demonstrated in mice (Narkar et al., 2008). Such molecules target signaling pathways that are important in the adaptive response of skeletal muscle to exercise, and hence can be referred to as exercise mimetics. Given our findings presented here, and the increased susceptibility of fast muscles to damage in muscular dystrophy (Webster et al., 1988; Moens et al., 1993), it will be worthwhile to investigate whether other exercise mimetics, such as the AMPK activator AICAR (Jager et al., 2007; Narkar et al., 2008), can confer benefits to *mdx* mice through stimulating the slow myogenic program.

III. IRES-mediated translation of utrophin A

In our investigations of translational regulation of utrophin A, we were driven by earlier observations that demonstrated a discrepancy between utrophin protein and mRNA levels in response to various conditions, including muscle regeneration and glucocorticoid treatment. We found that both of these "stresses" cause an enhancement of utrophin A IRES activity, demonstrating that enhanced cap-independent translation initiation can at least partially account for the enhanced utrophin A protein levels (Chapters 3 & 4). Both of these conditions were found to be associated with a general decrease in cap-dependent translation. These observations are in agreement with data for several other IRES elements that are regulated in response to particular stresses

associated with a decrease in global, cap-dependent translation rates, such as hypoxia and apoptosis (Blais et al., 2006; Bushell et al., 2006b). Investigation into the expression patterns of additional transcripts in response to muscle regeneration and glucocorticoid treatment will be needed to identify whether IRES-mediated translation serves as a general mechanism of translational regulation under these conditions.

Although the standard method to assess IRES activity uses bicistronic reporter assays, it should be noted that the use of these reporters to assess IRES activity is viewed with controversy by some investigators (Kozak, 2005; Bert et al., 2006). Part of this controversy stems from the finding that the luciferase bicistronic reporter system is subject to splicing events that can lead to false positive indications of IRES activity (James et al., 2000; Holcik et al., 2005). In our investigations, we did not use the luciferase system, and performed extensive control experiments to ensure that utrophin A IRES activity reported from our bicistronic constructs was genuine. We also complemented our investigations by measuring protein reporter and RNA reporter levels from transfected monocistronic reporters, and in the case of glucocorticoid treatment, supported our conclusions with polysome profiling experiments. As new cellular IRES elements continue to be identified, it will be important for the scientific community to establish a generally agreed upon criteria for classifying a cellular transcript as being translated via an IRES (Van Eden et al., 2004).

As mentioned in the general introduction, viral IRES elements can be classified based in part by defined structural motifs, but classification of cellular IRESes is much less straightforward. The sequence and structure requirements as well as the *trans*-acting factors required for IRES-mediated translation appear to be for the most part, transcript-

specific. The details of the translation initiation mechanism for cellular IRESes are poor in comparison to those described for viral IRES elements. However, similarities between some viral and cellular IRES elements have been described. For instance, human BiP IRES and picornaviruses have similarities in structural motifs (Le and Maizel, 1997). In addition, the mechanism of translation initiation for c-Src IRES and the hepatitis C virus appear to be similar, as they both can assemble the 80S initiation complex independently of eIF2 (Allam and Ali, 2009). Currently, the most promise for identifying general mechanisms of cellular IRES-mediated translation comes from the identification of *trans*-acting factors, or ITAFs, that play a common role for multiple IRES elements, such as is the case for PTB-regulation of cellular IRES-mediated translation during apoptosis (Bushell et al., 2006b). As the techniques for studying alternative mechanisms for translation initiation are refined, initiation mechanisms generally classified as IRES-mediated today might eventually be classified as multiple distinct mechanisms of initiation that have particular requirements of cellular context, translational machinery components, and alternative *trans*-acting factors.

We employed an unbiased search for proteins that interact with the utrophin A 5'-UTR using RNA-affinity chromatography combined with mass spectrometry analysis, and we presented evidence suggesting that the multifunctional protein eEF1A2 is one *trans*-acting factor that contributes to IRES-mediated translation of utrophin A (Chapter 5). We uncovered that specific regions of the 5'-UTR able to drive IRES activity in cultured cells were also those that bound to eEF1A2. In chapter 4, we also identified particular regions that are important for glucocorticoid-enhanced IRES activity. Given the importance of 5'-UTR secondary and tertiary structure to IRES-mediated translation

(Baird et al., 2006), it was not surprising that we did not find regions in the primary sequences that correspond with known consensus regulatory elements. With the identification, presented in this thesis, of the 5'UTR sequences and an ITAF that contribute to IRES-mediated translation of utrophin A in different contexts, it would be worthwhile at this point to investigate the secondary and tertiary structure characteristics of the utrophin A 5'-UTR. Identification of structural elements for other cellular IRESes has been achieved by using a variety of approaches, including enzymatic structural probing combined with mutational analysis and bioinformatic folding predictions (Baird et al., 2007; Graber et al., 2009). Such experiments could be useful to identify what structure(s) in the utrophin A 5'-UTR interact with eEF1A2, the ribosome, components of the canonical translation initiation machinery, and other, as of yet unidentified ITAFs. It is likely that additional ITAFs are involved given the modest regulation of utrophin A IRES activity by eEF1A2. For the identification of additional utrophin A 5'-UTR associated ITAFs, the use of newly developed methods of determining RNA-protein interactions that employ quantitative proteomics could be beneficial (Butter et al., 2009). It is likely that these additional ITAFs will either be enhanced or preferentially activated in skeletal muscle because utrophin A IRES-activity in transgenic mice was skeletal muscle-specific. Although other proteins that have roles in skeletal muscle are regulated by IRES elements, including FGF-II (Creancier et al., 2000), IGF-I receptor (Giraud et al., 2001), and IGF-II (Pedersen et al., 2002), the utrophin A IRES is the only IRES known to display muscle-specific activity.

The utrophin A 5'UTR transgenic reporter mice will prove useful to confirm the involvement of the IRES during skeletal muscle regeneration. The mice will also be

useful to assess whether other situations where a discrepancy between utrophin protein and mRNA levels are observed involve enhanced utrophin A IRES activity, such as during development and aging (Roma et al., 2004). Heightened utrophin A protein, but not mRNA expression has also been reported in skeletal muscle, brain and heart of *mdx* mice compared to wild-type mice (Weir et al., 2002; Baby et al., 2009). The contribution of the IRES to this expression pattern could be assessed by crossing the transgenic bicistronic reporter mice with *mdx* mice. Although skeletal muscles of *mdx* mice undergo progressive cycles of muscle cell necrosis and regeneration, it has been suggested that the upregulation of utrophin A in striated muscles of *mdx* mice occurs independently of muscle regeneration (Weir et al., 2004). By examining non-regenerating tissues of the *mdx*/IRES-reporter mice, and/or by performing gamma irradiation to inhibit regeneration in limb muscles, we could assess the relative contribution of regeneration-induced IRES activity to the utrophin A expression pattern in *mdx* mice.

Utrophin A protein expression is not particularly high in skeletal muscles compared to other tissues. It was thus surprising to find that utrophin A IRES-activity was skeletal muscle-specific. At the same time, the skeletal-muscle specific activity pattern might prove useful for the development of utrophin-enhancing therapeutics. In theory, systemically delivered molecules that enhance utrophin A IRES activity would exert their functions exclusively in skeletal muscles. The transgenic bicistronic reporter mouse could be used to search for such utrophin A IRES enhancing drugs. In order to demonstrate the suitability of the mouse for this purpose, we could examine whether glucocorticoid treatment stimulates utrophin A IRES reporter activity in skeletal muscles. With regard to the suitability of the IRES as a target for upregulating utrophin expression

at the sarcolemma, it remains to be seen whether this element is strong enough to drive translation of this massive protein in mature muscle fibers. It is also likely that an IRES-mediated therapeutic approach would have to be complemented with other strategies (such as GW501516-treatment) to enhance the extrasynaptic expression of utrophin A mRNA, given that bulk of utrophin transcripts are localized to the NMJ in mature muscle fibers.

IV. Concluding Remarks

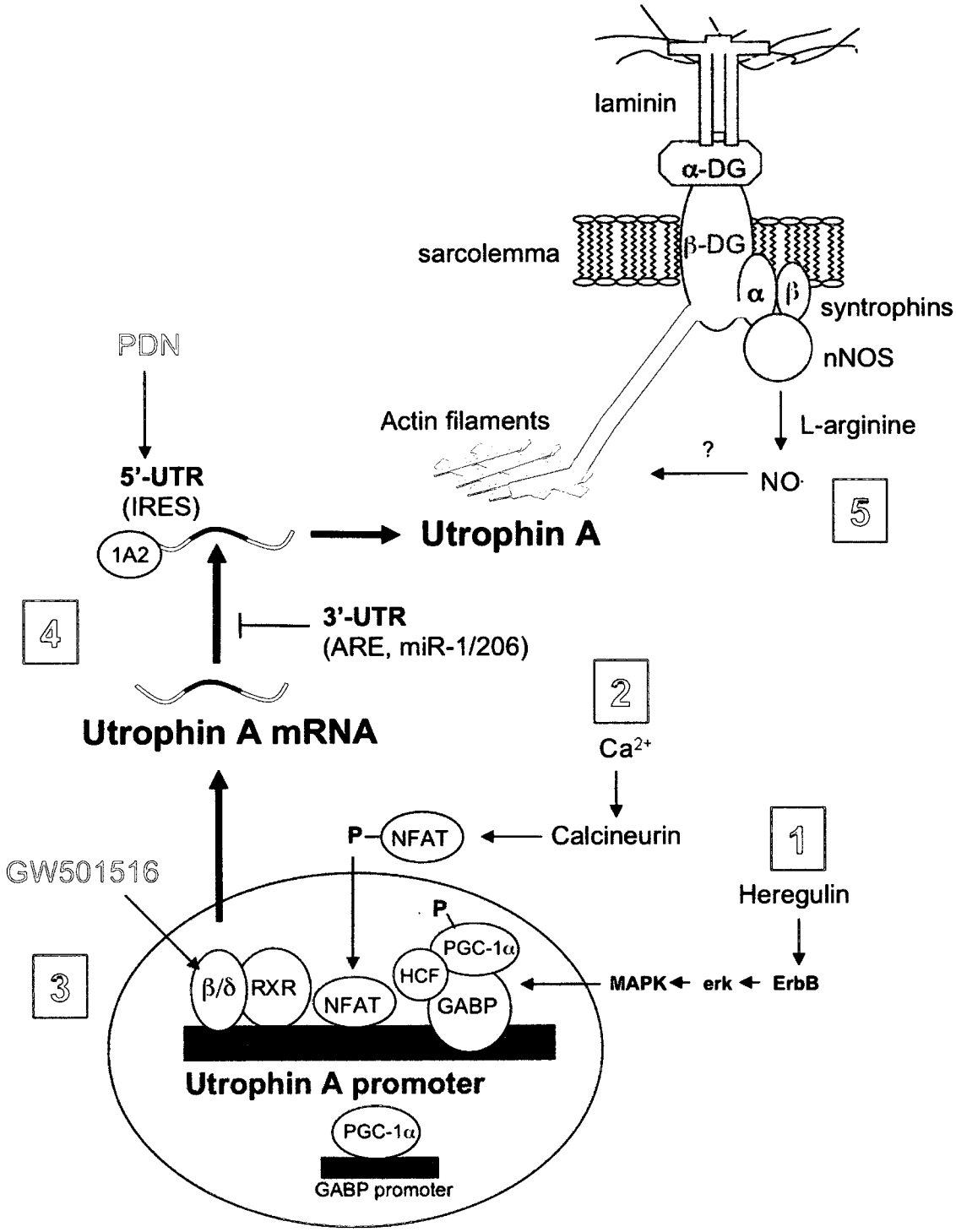
The manuscripts presented here add to the work of many groups that have been investigating the molecular mechanisms governing the expression of utrophin (Miura and Jasmin, 2006). These regulatory events range, for example, from transcriptional regulatory pathways under the control of Ca^{2+} signaling, to ARE and microRNA mediated regulation of mRNA stability, to translational regulation via an IRES (**Fig. 28**). In our investigations into the transcriptional and post-transcriptional mechanisms regulating utrophin A, we operated from a theoretical framework that emphasizes physiological and therapeutic relevance. Starting from the initial observation that there is some extrasynaptic sarcolemmal utrophin expression in slow-muscle fibers (Gramolini et al., 2001b), we and others proceeded to identify factors and signaling pathways important to regulating this expression pattern. This has culminated in the identification here of a medically relevant drug that can confer therapeutic benefits to the *mdx* mouse. In a similar manner, our investigations into translational regulation of utrophin A were prompted by early measurements of utrophin mRNA and protein in muscle biopsies from DMD patients (Gramolini et al., 1999a). Hopefully continued insights into the regulatory

mechanism of utrophin A IRES-mediated translation might also one day lead to the development of novel utrophin-based DMD therapeutics.

The exciting and rapid development of new experimental strategies to treat DMD and other muscle-related illnesses gives hope that multiple approaches will eventually reach the clinic. Given the heterogeneity of the gene defects and symptoms of BMD/DMD patients as well as the progressive nature of the disease, an effective treatment strategy in the future might turn out to be personalized to individual patients. These personalized treatments will likely involve a combinatorial strategy. In this regard, an effective DMD therapy might include a drug treatment to stimulate endogenous utrophin A expression combined with treatments that enhance muscle growth or decrease inflammation. Hopefully, such a therapy will soon be realized as treatments to improve the quality of life and lifespan of DMD patients are desperately needed.

Fig. 28. Regulatory mechanisms governing utrophin A expression.

In mature muscle, utrophin is primarily expressed at the postsynaptic sarcoplasm. At the sarcolemma, utrophin associates with members of the dystrophin-associated protein complex (DAPC) and the actin cytoskeleton. **1)** The nerve-derived-trophic factor heregulin binds to ErbB receptors and activates a cascade that culminates in transcriptional activation of the utrophin promoter A via GABP α/β and the N-box motif. Phosphorylated PGC-1α can form a complex with phosphorylated GABP and host cell factor (HCF) to mediate the response to heregulin. **2)** Within extrasynaptic regions of slow muscle fibers, calcineurin dephosphorylates NFAT, allowing it to enter the myonucleus and activate the utrophin A promoter. **3)** PPARβ/δ (β/δ) heterodimerizes with RXR to activate utrophin A transcription via a PPRE half site. GW501516 is a synthetic agonist of PPARβ/δ. **4)** The 3'-UTR confers stability to the utrophin transcript and controls its sub-cellular localization. An AU-rich element (ARE) and microRNA-1/206 complementary sites provide negative post-transcriptional control. An Internal Ribosome Entry Site (IRES) in the 5'-UTR drives translation during muscle regeneration, and in response to prednisone treatment (PDN). eEF1A2 (1A2) is factor involved in IRES-mediated translation of utrophin A. **5)** L-arginine, a substrate for neuronal nitric oxide synthase (nNOS), causes increased production of nitric oxide (NO) which stimulates utrophin expression. Drugs studied in this thesis that regulate utrophin A are shown in red. For simplicity, only some of the members of the DAPC are shown. NFAT, Nuclear Factor of activated T cells; β-DG, β-dystroglycan; α-DG α-dystroglycan; ? denotes that the mechanism of action is undetermined.


laminin
α-DG
β-DG
sarcolemma
α
β
syntrophins
nNOS
L-arginine
NO·
?
Actin filaments
PDN
5'-UTR
(IRES)
1A2
Utrophin A
5
3'-UTR
(ARE, miR-1/206)
4
Utrophin A mRNA
2
Ca2+
Calcineurin
P
NFAT
1
Heregulin
GW501516
P
PGC-1α
HCF
MAPK
erk
ErbB
3
β/δ
RXR
NFAT
GABP
Utrophin A promoter
PGC-1α
GABP promoter

CHAPTER 7

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APPENDIX

CV for Pedro Miura

1. EDUCATION

- Ph.D. student, Neuroscience, University of Ottawa 2004-2010
- M.Sc. student, Neuroscience, University of Ottawa 2003-2004
- Certificate in Philosophy, Dominican College, Ottawa 2002-2003
- Bachelor of Science, Queen's University, Kingston 1997-2002

2. RESEARCH EXPERIENCE

- **Department of Cellular and Molecular Medicine, University of Ottawa** 09/03-02/10
  Ph.D. Thesis Project (Transferred from M.Sc.)
- **Queen's University Biochemistry Department** 01/01-05/01
  Honours Thesis Project
- **University of Notre Dame Biology Department** 05/00-12/00
  Co-op Student, South Bend, Indiana, USA
- **University of Ottawa Heart Institute** 05/99-08/99 &
  Summer Student, Cardiac Cell and Molecular Biology Laboratory 07/97-08/97

3. AWARDS

- University of Ottawa Faculty of Medicine Award of Excellence 2007
- Canadian Institutes of Health Research National Poster Competition. Award of Excellence (Silver Category) 2005
- Outstanding Student Seminar. Ph.D. level. Cellular and Molecular Medicine, 2005
- The Gerry Taichman Award. The best research achievement by a graduate student. Doctoral level poster presentation. Cellular and Molecular Medicine, University of Ottawa 2005
- Outstanding Student Seminar. Master's level. Cellular and Molecular Medicine, University of Ottawa 2004
- The Gerry Taichman Award. The best research achievement by a 2004

graduate student. Master's level poster presentation. Cellular and Molecular Medicine, University of Ottawa

4. PUBLICATIONS

4.1 Manuscripts in Preparation

Miura, P., Clow, C., Jasmin B.J. *MicroRNA-206 Regulates BDNF Expression During Myogenic Differentiation.* In preparation, 2010.

4.2 Peer Reviewed Manuscripts

Nasrallah R, Clark J, Corinaldi J, Paris G, **Miura P**, Jasmin BJ, Hebert RL. *Thiazolidinediones alter growth and epithelial cell integrity, independent of PPAR{gamma} and MAPK activation, in mouse M1 Cortical Collecting Duct Cells.* **Am J Physiol Renal Physiol.** Feb 17. [Epub ahead of print] 2010.

Miura, P., Coriati, A., Bélanger, G., Lee, J., Holcik, M., Kothary, R., Jasmin, B.J. *The utrophin A 5'UTR drives cap-independent translation exclusively in skeletal muscles of transgenic mice and interacts with eEF1A2.* **Hum Mol Genet**, 19(7):1211-1220, 2010.

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Miura, P., Andrews, M., Holcik, M., Jasmin, B.J. *IRES-Mediated Translation of Utrophin A is Enhanced by Glucocorticoid Treatment in Skeletal Muscle Cells.* **PLoS ONE**, Jun 11;3(6):e2309, 2008.

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Miura P., and Jasmin B.J. *Utrophin upregulation for treating Duchenne or Becker muscular dystrophy: How close are we?* **Trends Mol Med.** 12:122-9, 2006.

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Stocksley, M.A., Chakkalakal, J.V., Bradford, A., **Miura, P.**, De Repentigny, Y., Kothary, R., Jasmin, B.J. *A 1.3 kb promoter fragment confers spatial and temporal expression of utrophin A mRNA in mouse skeletal muscle fibers.* **Neuromuscul.Disord.** 15, 437-449, 2005.

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4.3 Abstracts

Miura, P., Clow, C., Jasmin, B.J. *MicroRNA-206 Regulates BDNF Expression During Myogenic Differentiation.* FASEB- Making Muscle in the Embryo and Adult, New York, NY, May 2009.

Bélanger, G., **Miura, P.,** Chakkalakal, J.V., Jasmin, B.J. *Role of Cis- and Trans-acting Factors in the Regulation of Utrophin mRNA Stability in Skeletal Muscle in vivo.* RNA Society Annual Meeting, Berlin, Germany, July 2008.

Miura, P., Coriati, A., Sarkar, M., Andrews, M., Holcik, M., Jasmin, B.J. *Regulation of Utrophin A IRES-Mediated Translation by Glucocorticoid Treatment in Skeletal Muscle Cells.* Association Française contre les Myopathies (AFM) International Congress of Myology, Marseille, France, May, 2008.

Miura, P., Andrews, M., Holcik, M., Jasmin, B.J. *IRES-Mediated Translation of Utrophin A is Enhanced by Glucocorticoid Treatment in Skeletal Muscle Cells.* Keystone Symposia on Translational Regulatory Mechanisms, Coeur D'Alene, Idaho. January, 2008.

Miura, P., Andrews, M., Ungureanu, N., Lunde, J., Shaver, A., Holcik, M.H., Jasmin, B.J. *IRES Mediated Translation of Utrophin A is Enhanced by Glucocorticoid Treatment in Muscle Cells.* The 20th Anniversary of the DMD Gene Discovery: Impact on Muscle Biology, Disease and Therapy. Ottawa, Canada. May, 2007.

Miura P., Chakkalakal J.V., Lunde, J., Shaver, A., Belanger, G., Jasmin, B.J. *Activation of PPARδ stimulates utrophin A expression in skeletal muscle cells.* FASEB Experimental Biology meeting. Washington D.C. April, 2007.

Miura P., Lee, J., Holcik, M., Jasmin, B.J. *Functional Characterization of the Utrophin A IRES and Identification of the Eukaryotic Elongation Factor 1A-2 (eEF1A-2) as a potential IRES Trans-Activating Factor.* CSHL meeting on translational control. Cold Spring Harbor, New York, September, 2006.

Miura P., Thompson J., Chakkalakal J.V., Holcik M. and Jasmin B.J. *Utrophin A is Translationally Regulated in Regenerating Skeletal Muscle via an IRES Dependent Mechanism.* Canadian Student Health Research Forum. Winnepeg, Manitoba, May 2005.

Miura P., Thompson J., Chakkalakal J.V., Holcik M. and Jasmin B.J. *Utrophin A is Translationally Regulated in Regenerating Skeletal Muscle via an IRES Dependent Mechanism.* International Congress on Myology. Nantes, France, May 2005.

Vaughan P.S., **Miura P.**, Henderson M., Byrne B. and Vaughan K.T. *A Role for Regulated Binding of p150^{Glued} to Microtubule Plus-Ends in Organelle Transport.* Mol. Biol. Cell 13:42a, 2002.

Vaughan P.S., **Miura P.E.**, Henderson M. and Vaughan K.T.*Regulation of Dynactin Targeting to Microtubule Plus-Ends by PKA.* Mol. Biol. Cell 11: 365a, 2000.

5. TEACHING EXPERIENCE

- Part-time professor, University of Ottawa Faculty of Health Sciences 05/08-12/09
  PHS4300- Pathophysiology. 58.5 hours. Lectures included:
  Blood, Cardiovascular System, Respiratory System, Nervous System

- Seminar Presenter, "How to Write your First Research Proposal". 11/08
  CMM/NSC Graduate Council, University of Ottawa

- Seminar Presenter, "How To Write Your First Grant". 10/07
  CMM/NSC Graduate Council, University of Ottawa

- Let's Talk Science Volunteer 2003-2005