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The Role of the 5'-Untranslated Region in Regulating the Expression of Utrophin in Skeletal Muscle Cells

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The Role of the 5'-Untranslated Region in Regulating the Expression of Utrophin in Skeletal Muscle Cells

by

© Jennifer Margaret Thompson

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Abstract

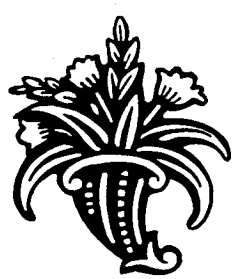
Up-regulating utrophin expression at the sarcolemma is thought to be a key therapeutic approach in the fight against DMD. Utrophin was found to have two isoforms which differ mainly in their 5'-untranslated regions. Effects of the utrophin 5'UTRs on reporter gene expression in cell culture and muscle injection experiments were assayed by measuring both reporter transcript and protein levels. Translation efficiencies were estimated by calculating transcript to protein ratios. Our results indicate that there is considerable translation inhibition in control muscle. In comparison, significantly divergent translation efficiencies in regenerating muscle indicate a direct correlation between transcript and protein levels, as seen in cell culture. Furthermore, low levels of translational inhibition are present in adult *mdx* mice, yet it is significantly de-repressed in young *mdx* muscle. Taken together, our results suggest that, via the utrophin 5'UTRs, translational regulation is involved in regulating utrophin expression.

Dedication

I would like to dedicate this work to my mom and dad
for their love and support through these challenging years.
I would also like to dedicate it to my sister who has become my
closest and dearest friend in the recent past and is now
embarking on her own academic journey.
I will try to be there for you as much as you were for me.

To my very special friends, Tania and Cheryl,
who have helped me through the good and the bad,
I cherish your friendship and appreciate your encouragement.

Without any of you, I would not be where I am today.
To all of you, thank you.



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List of Abbreviations

AChR / AChE	Acetylcholine Receptor / Acetylcholine Esterase
AUG/ATG	Translation Initiation Start Site
ATP	Adenosine Triphosphate
BiP	Immunoglobin Heavy-Chain Binding Protein
BMD	Becker Muscular Dystrophy
bp	Base Pairs
cDNA	Complementary Deoxyribonucleic Acid
DAP	Dystrophin-Associated-Proteins
DMD	Duchenne Muscular Dystrophy
DRP	Dystrophin-Related Protein
EDL	Extensor Digitorum Longus
eIF	Eukaryotic Initiation Factor
HIF-1a	Hypoxia-Inducible Factor-1α
GTP	Guanosine Triphosphate
IRES	Internal Ribosome Entry Site
LGMD	Autosomal-recessive Limb Girdle Muscular Dystrophy
kb	Kilo Bases
kD	Kilo Dalton
mRNA	Messenger Ribonucleic Acid
MTT	Myoblast Transfer Therapy
NMJ	Neuromuscular Junction
pCMV	Cytomegalovirus Promoter

PM	Polymyositis
RACE	Rapid Amplification of cDNA Ends
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SOL	Soleus
TA	Tibialis Anterior
UTR	Untranslated Region of mRNA

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A special acknowledgment to thank Lindsay for our "little" early morning chats. They were very encouraging and helped me get through the tough decisions and obstacles I was faced with, in all aspects of my life. I will definitely miss our discussions and wish you all the best.

A special thanks to the lab gang, that was there through thick and thin and made me smile through it all (or almost). We never did get the sign "RAM lab", but it was there in spirit. John, thank you for your continuous help and knowledge. You put your work on hold to help me with mine. I always did/will appreciate it. Thank you to all the BJ^2 lab members. I wish you all the best in your life endeavors.

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Thank you all, it was appreciated more than you will ever know!

Chapter 1

Introduction

1.1 Duchenne Muscular Dystrophy

1.1.1 Clinical features of the disease

Duchenne muscular dystrophy (DMD) is the most prevalent and severe degenerative disorder of skeletal and cardiac muscle (reviewed in Brown and Hoffman, 1988) affecting 1 in 3500 male births (Emery, 1991). DMD patients characteristically display progressive muscle weakness, which begins in early childhood (Mokri and Engel, 1975). Proximal muscles seem to be affected initially, followed by distal muscles (Mokri and Engel, 1975).

Even though DMD is present at birth, clinical symptoms are not evident until the age of 3-5 years (Hoffman *et al.*, 1987a; Anderson and Kunkel, 1992). Initial symptoms are usually leg weakness, increasing convex curvature of the spine and a waddle-like gait (Anderson and Kunkel, 1992). Continuous muscle wasting results in progressively weaker muscles which usually leaves DMD patients wheelchair bound by the age of 11-12 years (Hoffman *et al.*, 1987a; Anderson and Kunkel, 1992). Those affected usually die from respiratory failure by the second decade of life (Hoffman *et al.*, 1987a; Anderson and Kunkel, 1992; Emery, 1993). A similar, yet milder dystrophy, has been identified and is known as Becker's muscular dystrophy (BMD). This disease is known to be more variable phenotypically and generally follows a less severe course than DMD. BMD patients usually remain ambulatory throughout their lives (Ahn and Kunkel, 1993).

Histologically, it has been well established that patterns of degeneration and regeneration of individual muscle fibers as well as elevated levels of serum creatine

kinase (Emery and Holloway, 1977) are characteristic to both diseases. DMD and BMD muscles progressively lose their ability to regenerate which allows for the accumulation of adipose and connective tissues (Watkins and Cullen, 1985). It has been suggested that the increase in connective tissue and the decline in muscle regeneration result in a gradual decrease in the number of muscle fibers (Carpenter and Karpati, 1979; Watkins and Cullen, 1985; Anderson and Kunkel, 1992).

1.1.2 Duchenne and Becker muscular dystrophies are genetic disorders

As mentioned, DMD is prevalent in males, therefore it was thought that DMD was gender-linked. Mokri and Engel (1975) reported that in DMD muscle biopsies, 5 out of 7 exhibited an X-linked inheritance. In fact, the gene responsible for DMD was later found to be located on chromosome Xp21 in humans (Burghes *et al.*, 1987) and to code for a 14-16 kb RNA transcript (Monaco *et al.*, 1986). The DMD gene was cloned and was shown to display a high degree of sequence homology from mouse to human, as well as being equally expressed in skeletal and cardiac muscle in both species (Hoffman *et al.*, 1987b). The DMD gene is the largest gene identified to date, which could account for the high incidence and increased rate of mutations seen causing the disease (Moser, 1984).

BMD is less frequent and severe than DMD, yet it is considered to be an allelic disorder to DMD (Monaco *et al.*, 1988). Interestingly, it was discovered that BMD was caused by the same gene as DMD. The main difference between the

two diseases is that the DMD gene product is present in BMD fibers in a truncated form due to significant deletions. Both DMD and BMD result from mutations which are mostly deletions of more than 137 kb (Kunkel *et al.*, 1986). It is thought that the effect the mutation or deletion has on the open reading frame determines whether it will cause DMD or BMD (Monaco *et al.*, 1988; England *et al.*, 1990). Mutations in the DMD gene that ensue a shift in the translation reading frame generally result in DMD, whereas deletions resulting in the absence of large portions of the gene without affecting the reading frame give rise to BMD (Monaco *et al.*, 1988; England *et al.*, 1990). In a study of 7 patients affected with DMD or IMD (intermediate muscular dystrophy), it was determined that all cases were due to a mutation (Roberts *et al.*, 1992). Resulting in a precocious translation termination, this mutation either introduced a premature translation stop codon or shifted the reading frame. The mutations identified all resulted in the loss of the C-terminus of the DMD gene product and presumably, were the cause of the diseases (Roberts *et al.*, 1992).

1.2. Dystrophin : The DMD Gene Product

The DMD gene product, found in low abundance in tissues, is known as dystrophin (Hoffman *et al.*, 1987a). Coded by a 14 kb transcript, this 3 685 amino acid protein has a molecular weight of 427 kD (Koenig *et al.*, 1988). Dystrophin is a protein composed of four domains: 1) an N-terminal "actin-binding" domain, 2) a

middle "rod" domain, consisting of 26 spectrin-like repeats, 3) a cysteine-rich domain and 4) a carboxy terminal (Koenig *et al.*, 1988) (Figure 1A).

The dystrophin actin-binding and cysteine-rich domains show significant similarity to those of α-actinin and spectrin (Davison and Critchley, 1988; Koenig *et al.*, 1988; Byers *et al.*, 1989). The actin-binding domain binds actin filaments, suggesting that this protein is also a cytoskeletal protein. The rod domain contains repeats which are distinct yet homologous to α-actinin and spectrin which provide flexibility through 4 putative hinges (Davison and Critchley, 1988). Homologous to a similar domain in α-actinin, the cysteine-rich region is putatively implicated in calcium homeostasis and associates with plasma membranes (reviewed in Ahn and Kunkel, 1993). The carboxy terminus has been shown to associate with proteins involved in development or homeostasis (reviewed in Ahn and Kunkel, 1993). Due to their high homology, dystrophin, α-actinin and spectrin are part of a family of cytoskeletal proteins (Davison and Critchley, 1988). The high sequence similarity suggests that structural and functional properties are conserved within this superfamily (Byers *et al.*, 1989).

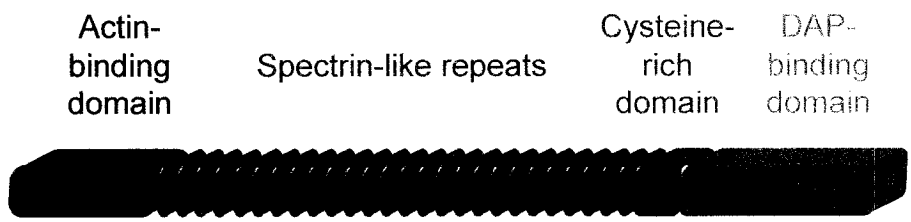
1.2.1 Tissue distribution of dystrophin

In mice, dystrophin is expressed in brain (Nudel *et al.*, 1988; Lidov *et al.*, 1990), muscle (Nudel *et al.*, 1988; Byers *et al.*, 1991) and can be detected in other non-muscle tissues (Nudel *et al.*, 1989). In brain, it is the cerebellum and cerebral cortex that are rich in dystrophin, followed by brain stem and spinal cord (Lidov *et*

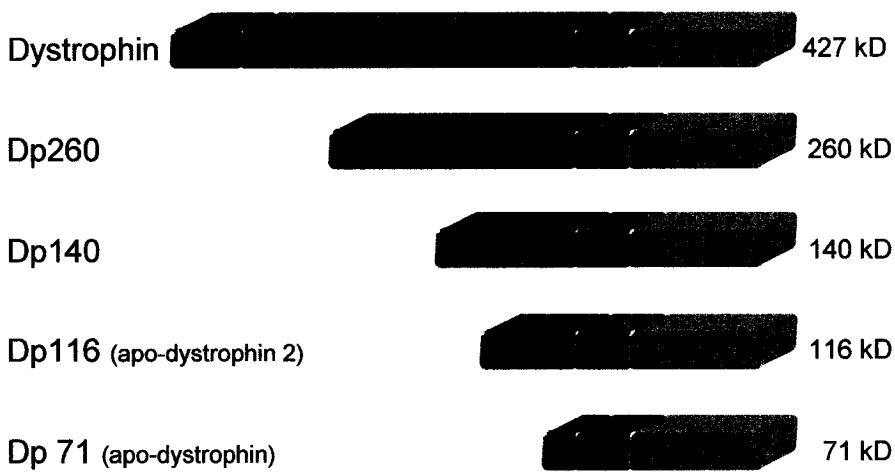
Figure 1. Schematic diagram of A) dystrophin and B) dystrophin isoforms.

Shown are the four main domains of full-length dystrophin: actin-binding domain, rod domain (26 spectrin-like repeats), cysteine-rich domain and a DAP-binding domain. Both the cysteine-rich domain and carboxy terminal are found in all the isoforms. Name and molecular weight are also indicated for each dystrophin isoform.

A)


Actin-binding domain
Spectrin-like repeats
Cysteine-rich domain
DAP-binding domain

B)


Dystrophin
427 kD
Dp260
260 kD
Dp140
140 kD
Dp116 (apo-dystrophin 2)
116 kD
Dp 71 (apo-dystrophin)
71 kD

al., 1990). In the cerebellum, Purkinje cells show punctate staining of dystrophin along the somites and dendrites, with no intra-cytoplasmic dystrophin. In the cerebral cortex, cortical neurons show similar yet more attenuated punctate staining compared to Purkinje cells (Lidov *et al.*, 1990). These results suggest that dystrophin expression in brain is specific to both cell-type and sub-cellular location. It is thought that dystrophin may play a role in anchoring receptors or other elements of the post-synaptic apparatus to specific locations in neuronal membranes (Lidov *et al.*, 1990).

In muscle, dystrophin has a very specific distribution. It is found throughout the sarcolemma but not on internal structures or membranes (Arahata *et al.*, 1988; Bonilla *et al.*, 1988; Watkins *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988). Enriched at myotendinous junctions, dystrophin also has a slightly higher abundance at the troughs of neuromuscular junctions (Nudel *et al.*, 1988; 1989). Dystrophin is expressed in all muscle types; skeletal, cardiac and smooth muscle (Byers *et al.*, 1991). Skeletal and cardiac muscles show similar expression levels. Dystrophin expression in smooth muscle is significantly lower and exhibits a more punctate and discontinuous distribution when compared to skeletal or cardiac muscle. Therefore, dystrophin levels and distribution seem to be muscle-type specific (Byers *et al.*, 1991).

1.2.2 Expression of dystrophin in dystrophic muscles

To study the differences in dystrophin expression or the effects of its absence, several studies have examined dystrophin-deficient muscles. One study examined biopsies from DMD, BMD or other neuromuscular disorder patients (Hoffman *et al.*, 1988). This study reported that 95 % of those diagnosed with disorders unrelated to DMD or BMD exhibited normal dystrophin phenotypes. However, 92 % of patients diagnosed with DMD or BMD showed abnormal dystrophin phenotypes. In DMD patients, dystrophin was completely undetectable and only low levels of normal sized dystrophin were seen in intermediate dystrophies. However, in BMD patients, normal levels of an abnormal sized dystrophin were observed. Therefore, it is suggested that the severity of the diseases directly correlate with quantitative abnormalities rather than qualitative abnormalities of dystrophin, since no correlation was detected between deletion size and disease severity (Hoffman *et al.*, 1988). Even though BMD was found to be due to mutations in the dystrophin gene, normal dystrophin distribution at the sarcolemma of all muscle fibers was observed (Bonilla *et al.*, 1988). These findings suggest that the truncated forms of dystrophin found in BMD patients may be able to function similarly to full length dystrophin.

1.2.3 Dystrophin isoforms

Following the localization of dystrophin in brain and muscle, the next step was to determine if the DMD gene product found in each tissue was identical or if other dystrophin isoforms existed. An initial study demonstrated that transcripts found in brain had a different 5' terminal compared to the muscle transcripts, which suggested that dystrophin had at least two different promoters (Nudel *et al.*, 1989).

The "brain" promoter was identified and localized to a genomic exon more than 90 kb upstream of the "muscle" promoter (Boyce *et al.*, 1991). The large distance between the two promoters may allow disruption of one without affecting the other (Boyce *et al.*, 1991; Tennyson et *al.*, 1996a). In one DMD patient, it was observed that the "brain" isoform may have been compensating for the lack of the "muscle" isoform (Boyce *et al.*, 1991). Therefore, both dystrophin promoters do not show absolute tissue-specificity (Boyce *et al.*, 1991).

A 6.5 kb transcript was identified (now referred to as Dp71) and was found to be expressed in non-myogenic tissues such as brain, kidney, liver, testis and lung (Bar *et al.*, 1990). In brain, this isoform is found at similar levels as the 14 kb transcript, which introduces the possibility that disruption or absence of this "brain" isoform may lead to mental retardation seen in some DMD cases (Bar *et al.*, 1990). Dp71 is found in several tissues, which may also explain abnormalities seen in different cell types in DMD (Bar *et al.*,1990).

Additional studies have determined that dystrophin has seven different isoforms, transcribed from seven distinct promoters (Figure 1B). Three isoforms are full-length, 427 kD proteins expressed in muscle (Koenig *et al.*, 1988) and brain

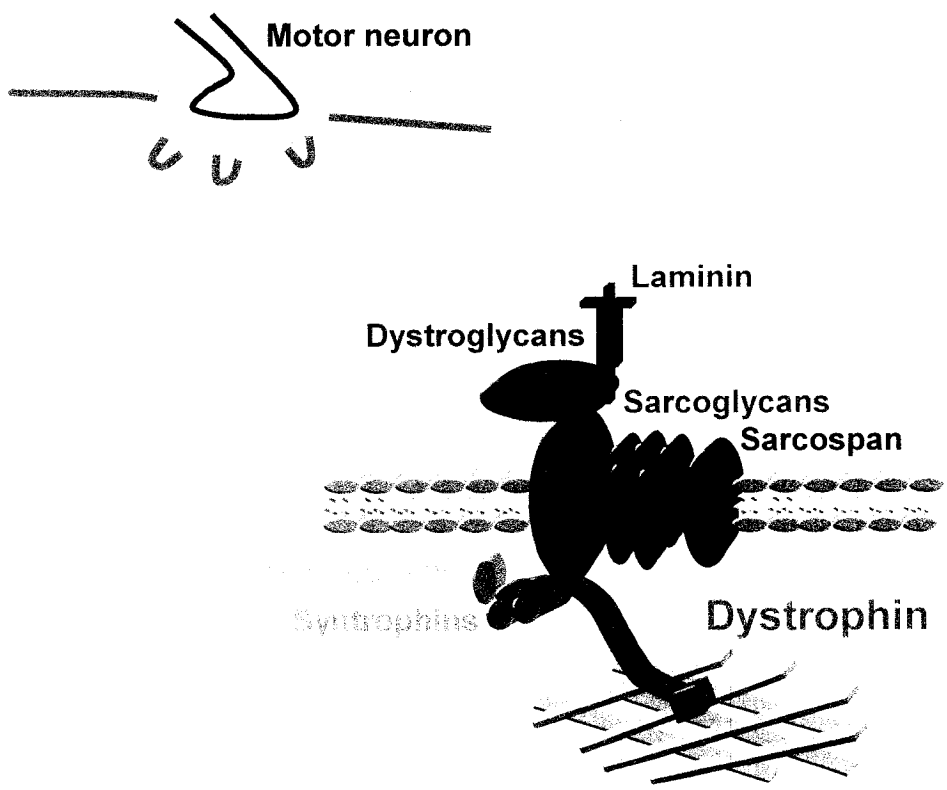
(Boyce *et al.*, 1991; Gorecki *et al.*, 1992). These full-length isoforms are transcribed from three distinct promoters in the 5' end of the gene. The other four isoforms contain unique first exons, are significantly shorter and are also products of distinct promoters. These promoters give rise to a 260 kD (D'Souza *et al.*, 1995), 140 kD (Lidov *et al.*, 1995), 116 kD (Byers *et al.*, 1993) and 71 kD (Lederfein *et al.*, 1992) protein. Dp71 is the only shorter isoform that does not display tissue-specificity and is expressed in several adult non-myogenic tissues (Bar *et al.*, 1990). The shorter isoforms lack the actin-binding domain which suggests that they may have different functions than the full-length isoforms. Further studies may provide evidence that these isoforms could prove to be beneficial in treating DMD.

1.2.4 Dystrophin interacts with membrane glycoproteins

In conjunction with the amino acid sequence of dystrophin and the identification of its four domains, sub-cellular localization of dystrophin at the sarcolemma suggests that dystrophin is a membrane-associated cytoskeletal protein. In fact, dystrophin is part of a glycoprotein complex located at the sarcolemma, known as the dystrophin-associated protein (DAP) complex (Ervasti and Campbell, 1991) (Figure 2). Dystrophin was found to associate with a 156 kD glycoprotein via the trans-membrane glycoprotein complex consisting of three other glycoproteins (50, 43 and 35 kD units) (Ervasti and Campbell, 1991). The DAP complex can be described as three components: 1) cytoskeletal elements, known as dystrophin and a 59 kD protein, 2) sarcolemmal glycoproteins, consisting of 25,

Figure 2. Dystophin localization in muscle

Schematic representation of dystrophin localization in muscle fibers. Dystrophin is found throughout the sarcolemma of adult muscle fibers as well as at troughs of neuromuscular junctions. Dystrophin is thought to link cytoskeletal actin filaments to the extracellular matrix via a complex comprised of DAPs. This complex consists of dystroglycans (α, β), sarcoglycans (α, β, δ, γ, ε), sarcospan, syntrophins (α, β) and dystrobrevin.


Motor neuron
Laminin
Dystroglycans
Sarcoglycans
Sarcospan
Syntrophins
Dystrophin

50, 43 and 35 kD proteins and 3) extracellular glycoprotein, 156 kD (Ervasti and Campbell, 1991). The 156 kD glycoprotein, now referred to as α-dystroglycan, is a co-product of the post-translational modification of a 97 kD protein (Ibraghimov-Beskrovnaya *et al.*, 1992). The other co-product, a 43 kD protein, is a trans-membrane protein known as β-dystroglycan (Ibraghimov-Beskrovnaya *et al.*, 1992). Additional membrane spanning proteins are the sarcoglycans (α, β, γ, δ and ε) and sarcospan. Intracellular DAPs are referred to as syntrophins (α and β) and dystrobrevin. These initial findings further support the idea that, via DAP complexes, dystrophin serves to link actin filaments to extracellular proteins (Ervasti and Campbell, 1991) such as laminin (Ibraghimov-Beskrovnaya *et al.*, 1992).

In *mdx* mice, which are dystrophin-deficient, DAP localization is dramatically affected. In fact, a 80-90 % reduction of DAP levels were reported in *mdx* skeletal muscle (Ohlendieck and Campbell, 1991). DAP staining intensities at the sarcolemma were also significantly lower when compared to control muscles (Ervasti *et al.*, 1990; Ohlendieck and Campbell, 1991). In DMD muscle, a similarly dramatic decrease in DAPs at the sarcolemma was observed (Ohlendieck *et al.*, 1993). Taken together, these data suggest that the absence of dystrophin ensues the loss of DAPs at the sarcolemma, which is thought to render muscle fibers susceptible to degeneration (Ohlendieck *et al.*, 1993). Both instability of DAP complexes and their loss of localization at the sarcolemma are proposed to result in a loss of membrane integrity, which can lead to the muscle damage, necrosis and fibrosis seen in dystrophic tissue (Arahata *et al.*, 1988; Cooper *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988; Matsumura *et al.*, 1992; reviewed in Ahn and Kunkel, 1993; Ohlendieck, 1996; Blake *et al.*, 2002).

Loss or absence of DAPs has been shown to cause other known myopathies. For example, autosomal-recessive limb-girdle muscular dystrophy (LGMD) can be caused by mutations in sarcoglycan subunits. The pattern of secondary deficiencies accompanying the primary sarcoglycan mutations is extremely variable where the clinical course of LGMD can resemble the severe and lethal course of DMD or the milder and progressive course of BMD (for review see Bushby, 1999). Total loss of the DAP complex has been shown to generally occur due to mutations in β- and δ-sarcoglycan (reviewed in Bushby, 1999). The mutations can result in their absence, which putatively leads to the progressive muscle weakness and death (Angelini *et al.*, 1999).

1.2.5 Putative functions of dystrophin

The exact function of dystrophin is not known. However, two main putative functions have been proposed. Dystrophin has been shown to link actin filaments to the extracellular matrix via membrane-associated DAPs (section 1.2.4; for review see Tinsley *et al.*, 1994 and Ohlendieck, 1996). To this effect, dystrophin is thought to both localize and stabilize DAP complexes at the sarcolemma (for review see O'Brien and Kunkel, 2001; Blake *et al.*, 2002). Through its interactions with the DAP complex and its rod domain containing putative "hinges" (Davison and Critchley, 1988), dystrophin was suggested to serve a structural or mechanical role in stabilizing muscle membranes through cycles of contraction and relaxation (Brown and Hoffman, 1988; Koenig and Kunkel, 1990; Ahn and Kunkel, 1993).

Absence of dystrophin is suggested to ensue disruption of the linkage between plasma membrane and extracellular matrix, rendering the fiber susceptible to necrosis.

Dystrophin has also been suggested to play a role in calcium homeostasis, however the precise mechanisms are still unknown (reviewed in Blake *et al.*, 2002). In both *mdx* mice and DMD patients, intracellular calcium levels are elevated compared to controls (Fong *et al.*, 1990; Turner *et al.*, 1991). In cultured DMD and *mdx* myotubes, increased leak channel activity has been reported, possibly resulting in the increased intracellular calcium concentrations (Fong *et al.*, 1990). Dystrophic myotubes are less capable of regulating their intracellular calcium, either due to a decrease in efflux or an increase in influx of calcium (Turner *et al.*, 1991). This suggests that dystrophin may play a role in regulating calcium homeostasis.

1.3 Therapeutic Approaches To Treat DMD

DMD is a monogenic disorder in which the absence of dystrophin leads to the characteristic features of the disease. It is generally accepted that membrane defects seen in DMD and *mdx* mice lead to abnormal calcium flux and tissue damage, thus resulting in the lethal myopathy. Several different dystrophin-deficient animal models such as dog, cat and mouse have been identified or generated and are used to study the effects of dystrophin deficiency (reviewed in Watchko *et al.*, 2002). These models are the result of spontaneous mutations in the conserved

dystrophin gene (for review see Blake *et al.*, 2002). To examine function and localization of dystrophin, as well as the patho-physiology of dystrophic tissue, the most widely used model is the *mdx* mouse. This murine example of dystrophin-deficiency was the first identified DMD model (Bulfield *et al.*, 1984) and lacks full-length dystrophin due to a point mutation in *exon 23* (Hoffman *et al.*, 1987b). The point mutation introduces a premature stop codon that results in a truncated non-functional dystrophin (Sicinski *et al.*, 1989). The *mdx* mouse exhibits a similar pathology to DMD, such as repeated cycles of degeneration and regeneration, high serum creatine kinase levels, atrophy, fibrosis, fiber loss and basic muscle dysfunction (reviewed in Watchko *et al.*, 2002). Features appear to be milder and emerge at a later onset than what is seen in patients suffering from DMD. However, the *mdx* mouse is considered a genetic equivalent to DMD in terms of dystrophin expression and does display a dystrophic phenotype.

To assess the beneficial effects of expressing dystrophin in a dystrophic model, transgenic *mdx* mice expressing a full- or mini-dystrophin transgene were generated. These mice have been reported to be spared of the dystrophic pathology. For example, in transgenic *mdx* mice expressing full-length dystrophin, amelioration of the muscle pathology was observed (Phelps *et al.*, 1995). No dystrophic symptoms were detected in these mice when dystrophin was expressed at 70 % of control levels. A reduction in the degree of pathology was also seen with transgenics expressing even lower levels of dystrophin, suggesting that lower than normal levels of full-length dystrophin can functionally correct dystrophic phenotypes (Phelps *et al.*, 1995; DelloRusso *et al.*, 2002). Micro-dystrophin cDNA

was also shown to ameliorate dystrophic pathology when inserted as a transgene into *mdx* mice (Sakamoto *et al.*, 2002).

To study the effects of over-expressing dystrophin at a later stage in the disease, an inducible system was developed in *mdx* mice (Ahmad *et al.*, 2000). This system consists of generating transgenic *mdx* mice expressing a transgene that is inducible with tetracycline. Inducing transgene expression is physiologically significant as it allows the study of somatic expression of dystrophin. Not all DMD cases are identified *in utero* therefore, it is important to determine if inducing the expression of dystrophin at a later age is as effective. Inducing dystrophin expression in young *mdx* mice (4-5 days after birth) resulted in correct DAP localization and prevented the onset of dystrophic pathology (Ahmad *et al.*, 2000). Preliminary studies in older *mdx* animals (4-30 weeks of age) reported no significant changes in cell morphology with induced dystrophin expression. However no other features were assessed, such as muscle force or membrane stability. Further studies remain to be pursued to determine if dystrophin treatment is beneficial in older dystrophic animals or if there exists a window within which dystrophin treatment is optimal.

Taken together, these results suggest that an effective treatment for DMD would consist of expressing a functional form of dystrophin or related protein. Several strategies are being investigated in the hopes of finding treatments or cures for DMD. Such therapeutic approaches are gene therapy, chimeric oligonucleotide therapy, cell-based therapies such as myoblast transfer and stem cell transplantation, antibiotics and additional pharmacological interventions as well as the up-regulation of related gene products.

1.3.1 *Dystrophin expression via gene therapy*

The reduced or even absent dystrophic pathology observed in transgenic *mdx* mice engineered to express dystrophin, brought forth gene therapy as a plausible treatment for DMD. Many groups have directed their efforts towards developing this therapeutic strategy. The optimal goal of gene therapy is to induce life-long expression of a cDNA-based gene in dystrophin-deficient muscle to diminish the dystrophic pathology. It has been extensively shown that gene therapy, by direct injection of cDNA or viral vectors expressing functional dystrophin, induced recovery in *mdx* muscle (for reviews see Hartigan-O'Connor and Chamberlain, 2000; Scott *et al.*, 2002; Dickson *et al.*, 2002; Wells and Wells, 2002). Studies have also been performed using truncated dystrophin cDNA (Phelps *et al.*, 1995; Decrouy *et al.*, 1998; Roberts *et al.*, 2002; Sakomoto *et al.*, 2002). These truncated forms of dystrophin were based on deletions seen in patients suffering from BMD. In one study, truncated dystrophin did not prove to be as effective as full-length dystrophin in preventing dystrophic symptoms (Phelps *et al.*, 1995). However, another study reported that expression of truncated dystrophin led to the recovery of muscle function in *mdx* mice and coincided with correct sarcolemmal localization of DAP complexes (Decrouy *et al.*, 1998).

A more recent gene therapy approach is the use of hybrid vectors - a combination of adenoviruses and retroviruses. Adenoviruses have been the vectors most commonly used for viral gene therapy since they can incorporate large cDNA fragments. However they do not result in continuous expression due to immunogenic properties of the vectors and their inability to incorporate into host cell

genomes (Roberts *et al.*, 2002). Therefore, these hybrid vectors show promise in gene therapy since they utilize the high infection efficiency of adenoviruses and the capacity of retroviruses to integrate host genome (Roberts *et al.*, 2002). Infecting *mdx* mice with a hybrid adeno-retrovirus vector, containing a micro-dystrophin fragment, restored DAP complexes at the sarcolemma. In addition, less muscle degeneration was observed. Adult *mdx* mice expressed dystrophin in nearly 100% of muscle fibers when infected as neonates. Integration of the micro-dystrophin gene into the host genome also occurred (Roberts *et al.*, 2002).

Gene therapy appears very promising in the treatment of DMD, yet there still remains several aspects to be determined such as optimal levels of expression and transduction efficiencies required to rescue affected muscles. An important drawback of inducing dystrophin expression in DMD patients is that it has been shown to trigger an immunogenic response.

1.3.2 Chimeraplast RNA/DNA oligonucleotides

The most recently described therapeutic approach is the use of chimeraplast oligonucleotides. These chimeric oligonucleotides are used to direct the correction of a mutation by inducing gene conversion (Cole-Strauss *et al.*, 1996; Kren *et al.*, 1998; Santana *et al.*, 1998). Chimeric RNA/DNA oligonucleotides are homologous sequences to a targeted gene, yet include one mismatched base (see Rando, 2002). This approach results in gene repair, such as a point mutation causing DMD or BMD for example, without the use of viral vectors (Kren *et al.*, 1998). However,

the frequency of gene conversion seems to vary among cell types (Santana *et al.*, 1998) in addition to being dose-dependent (Kren *et al.*, 1998).

In *mdx* mice, injected with a chimeric oligonucleotide directed to correct the point mutation, dystrophin was found to be expressed in muscle fibers surrounding the site of injection (Rando *et al.*, 2000). These results indicate that chimeric oligonucleotide injections can rectify the point mutation in the dystrophin gene in *mdx* mice. Upon further characterization, the dystrophin protein expressed in these mice was reported to be full-length (427 kD) and included all the exons surrounding the former mutation (Rando *et al.*, 2000). A more recent study confirmed these results both *in vitro* and *in vivo* (Bertoni and Rando, 2002). This latter study demonstrated that gene conversion had occurred at the DNA, RNA and protein levels in cell culture. In addition, gene conversion occurred in injected *mdx* muscles suggesting that the injection of chimeric oligonucleotides may be an effective therapy against dystrophies caused by point mutations.

Several aspects still need to be resolved before this approach is considered a therapy. These include for example, establishing the beneficial dose of chimeric oligonucleotides and determining if this approach requires a multiple injection protocol to obtain therapeutic levels of gene conversion. As of yet, this therapeutic venue would only be viable for a subset of patients; those suffering from point mutation-induced dystrophies.

1.3.3 Myoblast transfer therapy (MTT)

Muscle transplantation and myoblast transfer therapy (MTT) were thought to have significant potential as therapies (see Smythe *et al.*, 2000 and Partridge, 2000 for review). Partridge and colleagues (1978) demonstrated that host and donor myoblasts could fuse following muscle grafts in mice. Near-normal contractile properties were produced in adult dystrophic hosts following muscle transplantation, suggesting that muscle transplant may be a possible treatment venue (Law and Yap, 1979). The procedure consisted of grafting a newborn normal mouse muscle into a dystrophic mouse recipient muscle when initial clinical signs of dystrophy appeared. This study was the first to show increased twitch tension in a dystrophic mouse. Precise mechanisms by which the improvement was attained however are still unknown. These results suggest that normal cells in the graft survived and contributed to the improvement (Law and Yap, 1979). A distinct drawback of this procedure is that it requires the use of newborn muscle to overcome problems seen with adult tissue, such as hypoxia due to difficulties in re-innervation and re-vascularization (Law and Yap, 1979). Access to newborn tissue in quantities that would be adequate to make this procedure a suitable therapy for all DMD patients would be hard to attain, not to mention the ethical aspect. Therefore, the use of myoblasts that can be grown in cell culture in significant quantities would be a more conceivable approach.

MTT consists of injecting or transplanting donor muscle precursor cells (myoblasts) into a dystrophic host. The goal of MTT, following the distribution and fusion of donor myoblasts with host muscle, is to induce expression of dystrophin

throughout dystrophic muscles (Partridge *et al.*, 1989). It was shown that fusion of injected cells into host *mdx* myofibers can occur (Partridge *et al.*, 1989). In this latter study, dystrophin was shown to be expressed at 30-40 % of normal levels and was found to localize at the sarcolemma in 10-40 % of muscle fibers. A single injection into regenerating muscles gave way to the synthesis of normal sized dystrophin in substantial amounts (Partridge *et al.*, 1989). It was also reported to be correctly localized at the sarcolemma. However, MTT has many obstacles to overcome before it becomes a suitable therapeutic approach. Such obstacles are 1) attaining sufficient distribution and fusion of donor cells to host muscle, 2) extending the donor myoblast survival period since these cells die soon after transplantation and 3) eliminating the immunological responses to donor myoblasts or dystrophin itself (Partridge *et al.*, 1989; see Smythe *et al.*, 2000 for review).

Human clinical studies were performed on DMD patients, despite a lack of evidence fully supporting this treatment. No significant contribution from donor cells and significant immunological responses were reported, even if cells were said histo-compatible (Gussoni *et al.*, 1992; Karpati *et al.*, 1993; Mendell *et al.*, 1995). Even with the use of multiple injection procedures, MTT efficiency was very low and did not improve muscle strength in these studies. Skuk and colleagues (1999; 2000; 2002) have been performing studies on non-human primates in the hopes of ameliorating this therapy. Several different parameters are being established by this group to increase efficiency of MTT, such as the use of different immuno-suppressive agents, myotoxins and multiple injection protocols. A considerable amount of research still remains to be performed to overcome the important limitations of this approach.

1.3.4 Stem cell therapy

Injection of healthy stem cells into a DMD patient, in anticipation that the host will incorporate donor-derived nuclei into its muscle, is the goal of this approach. This phenomenon was shown to induce partial restoration of dystrophin expression in *mdx* muscles (Gussoni *et al.*, 1999). Direct injection of stem cells into the vascular system allows for more widely disseminated distribution of transplanted stem cells. This approach results in a systemic muscle repair, rather than the local repair seen with intra-muscular injections (Gussoni *et al.*, 1999). Although dystrophin levels obtained via this method were lower than what would presumably be clinically beneficial, this method does show promise as a therapeutic approach (Gussoni *et al.*, 1999). A recent study reported that, following intra-arterial injections of muscle-derived stem cells from newborn mice into *mdx* hindlimbs, donor cells migrated from circulation into host muscle (Torrente *et al.*, 2001). Dystrophin transcripts were expressed in the injected hindlimb muscles. It is speculated that muscle-derived stem cells attach to muscle capillaries and then participate in the regeneration of damaged muscles (Torrente *et al.*, 2001).

In one study, muscle biopsies were collected from a DMD patient that had received a bone marrow transplant (Gussoni *et al.*, 2002). Donor-derived nuclei were still present 13 years after the procedure. Even though the dystrophin expressed was a truncated version of the wild-type, these results are promising in regards to this treatment venue, as fusion of donor-stem cells can persist even after several years (Gussoni *et al.*, 2002).

1.3.5 Treatment with antibiotics

Mutations causing DMD are not only deletions but also consist of introducing premature stop codons (Barton-Davis *et al.*, 1999). As mentioned, the absence of dystrophin in *mdx* mice is due to a mutation that introduces a premature stop codon (Sicinski *et al.*, 1989). It has been previously shown, that aminoglycosides in cultured cells cause suppression of stop codons by causing extensive misreading of RNA codes (Palmer *et al.*, 1979; see Barton-Davis *et al.*, 1999 for review). The misreading can result in the insertion of alternative amino acids, which could omit the premature stop codon.

Treatment with gentamicin, an aminoglycoside antibiotic, led to the synthesis and localization of dystrophin in *mdx* mice, as well restored its functional protection against contraction-induced damage (Barton-Davis *et al.*, 1999). This treatment also re-localized DAPs to the sarcolemma (Barton-Davis *et al.*, 1999). One main advantage of gentamicin is that intra-vascular injections of this antibiotic allow for a systemic delivery, which in turn helps in suppression of the DMD phenotype (Barton-Davis *et al.*, 1999). Only 10-20 % of control dystrophin levels were seen with gentamicin treatment in *mdx* mice, which suggests that misreading RNA codes can not completely restore dystrophin levels (Barton-Davis *et al.*, 1999). However, the increases did appear to be enough to protect the muscles from contraction-induced damage. This treatment may only prove to be effective in muscular dystrophies caused by mutations introducing premature stop codons (Barton-Davis *et al.*, 1999).

1.3.6 Additional pharmacological interventions

Several additional pharmacological interventions have been proposed throughout the years. Various compounds such as allupurinol (Hunter *et al.*, 1983), vitamin E and selenium (Backman *et al.*, 1988) as well as mazindol, a growth hormone inhibitor (Zatz *et al.*, 1986;1988) have been deemed non-effective or non-beneficial against the progression of DMD. Examples of compounds that have been continuously examined are prednisone and a derivative, Deflazacort. These anti-inflammatory compounds have proven to be equally beneficial in slowing the progression of DMD (Mesa *et al.*, 1991; Reitter, 1995; Bonifati *et al.*, 2000). However, Deflazacort seems to circumvent the side effects seen with prednisone. The main effects reported with these steroids consist of improved motor function and muscle strength when compared to untreated DMD patients (Mesa *et al.*, 1991; Reitter, 1995; Bonifati *et al.*, 2000).

More recent studies using Deflazacort demonstrated that pulmonary function was significantly greater in treated boys with limited side effects (Biggar *et al.*, 2001). Protective effects of Deflazacort are suggested to be due to its ability to promote myogenic differentiation, myoblast fusion and laminin expression in dystrophic tissues (Anderson *et al.*, 2000). It has also been suggested that Deflazacort treatment, in conjunction with MTT, may prove to be more therapeutic than either treatment alone, as it may provide a more long-term solution (Anderson *et al.*, 2000).

One of the most recent pharmacological interventions being studied is a myostatin blocker. Myostatin is a negative regulator of skeletal muscle growth

therefore, it was thought that blocking myostatin in DMD patients would lead to an increase in muscle mass. In fact, blocking endogenous myostatin in *mdx* mice resulted in an increase in body weight, muscle mass and size as well as overall muscle strength (Bogdanovich *et al.*, 2002). It was also reported that a significant decrease in both muscle degeneration and serum creatine kinase was seen in the treated animals. This treatment venue is very recent and little is known regarding the putative side effects of treating animals with this compound however, preliminary studies seem promising.

1.3.7 Up-regulation of dystrophin-related proteins

Several above-mentioned therapeutic approaches share a similar constraint: an immunological response triggered by the induction of dystrophin expression. An additional approach which could possibly evade the immunological reaction involves up-regulating a protein that is expressed endogenously in dystrophic muscle. The premise of this approach is that the up-regulated protein would compensate for the lack of dystrophin.

One such protein was thought to be the non-muscle dystrophin isoform Dp71. Transgenic *mdx* mice expressing Dp71 were generated and displayed normal expression of DAPs at the sarcolemma (Cox *et al.*, 1994). However, the dystrophic phenotype was not alleviated in these mice, indicating that Dp71 can not compensate for the lack of dystrophin. These results suggest that C-terminal isoforms may not have the ability to replace dystrophin and mitigate the dystrophic

pathology. This implicates that both amino and carboxy terminals are required in a related protein to function as a therapeutic approach. The only protein containing both homologous terminals to dystrophin, is dystrophin-related protein 1, also known as utrophin (Figure 3A).

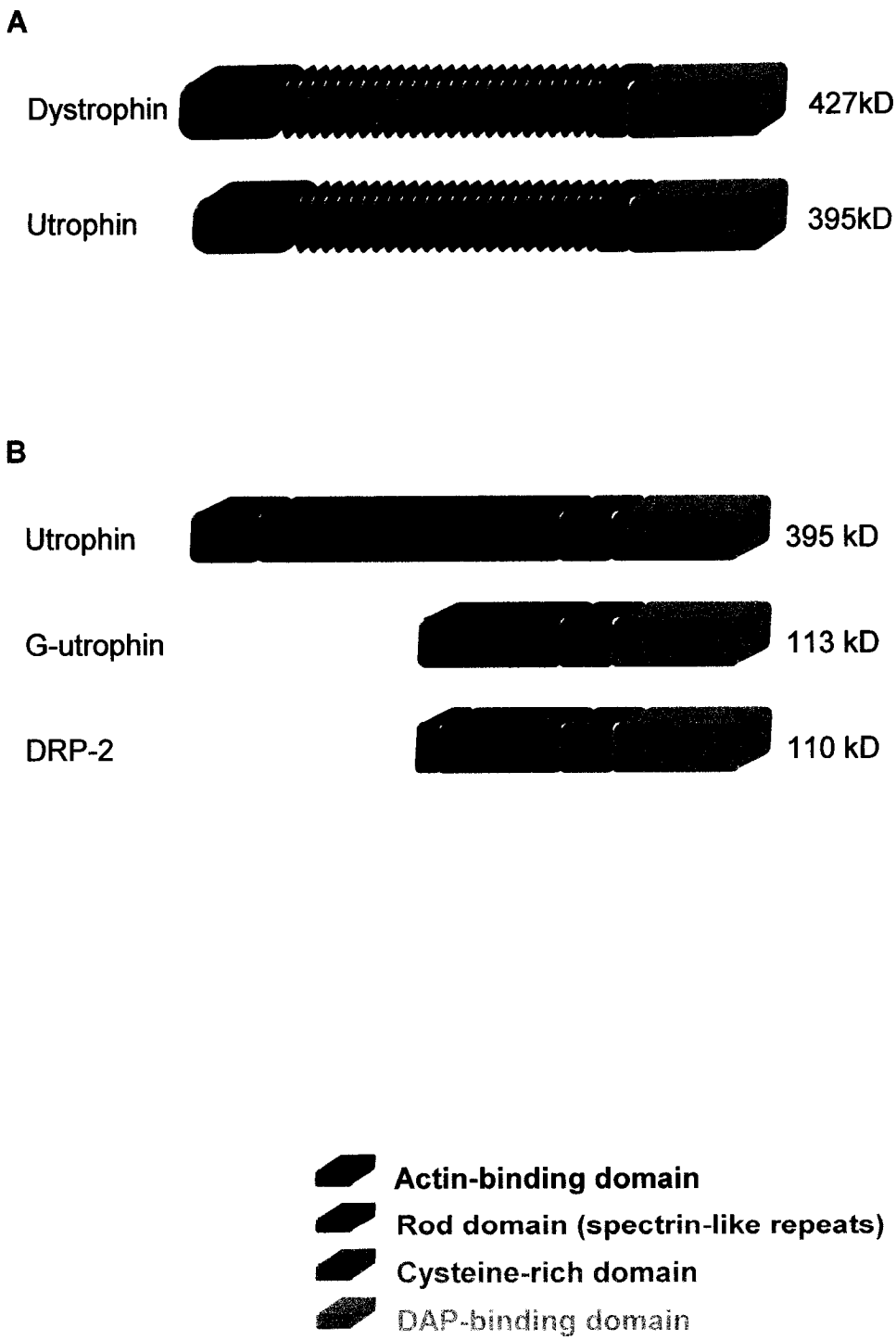
1.4 Utrophin : A Dystrophin-Related Protein

Dystrophin has been found to have an autosomal paralogue, dystrophin-related protein, commonly known as utrophin. In the process of isolating the full-coding sequence of dystrophin, an additional cDNA was identified which was homologous to the dystrophin C-terminal (Love *et al.*, 1989). This gene, coding for a 13 kb transcript, was located to chromosome 6 in humans (Love *et al.*, 1989) and to chromosome 10 in mice (Buckle *et al.*, 1990). Exhibiting a comparable molecular weight to dystrophin, utrophin also displays a similar cellular abundance (< 0.01%) (Khurana *et al.*, 1990). Therefore it was named dystrophin-related protein (DRP). Both genes have been shown to have analogous genomic organization in their 5' end and it has even been suggested that they may derive from a common ancestral gene as a result of genomic duplication (Pearce *et al.*, 1993). This hypothesis was based on the extensive coding sequence similarity and cross-species homology shared by these two proteins.

Characterization of full-length utrophin determined that this protein was homologous to dystrophin throughout its entire length (Tinsley *et al.*, 1992) (Figure

Figure 3. Dystrophin and related proteins

Schematic representation of A) dystrophin and utrophin. Shown are the actin-binding domain, rod domain, cysteine-rich domain and DAP-binding domain. B) Schematic representation of utrophin and its isoforms. Note the similar carboxy terminals of the isoforms. Both name and molecular weight are also indicated for each isoform.


A
Dystrophin
427kD
Utrophin
395kD
B
Utrophin
395 kD
G-utrophin
113 kD
DRP-2
110 kD
Actin-binding domain
Rod domain (spectrin-like repeats)
Cysteine-rich domain
DAP-binding domain

3A). Their overall structures are similar since utrophin also has a putative actin-binding domain in the first 250 amino acids, a long rod domain consisting of 24 spectrin-like repeat units and a cysteine-rich domain (Tinsley *et al.*, 1992). Homology between dystrophin and utrophin was estimated at approximately 80 % in both amino and caboxy terminals (Tinsley *et al.*, 1992; Pearce *et al.*, 1993). However, they do differ significantly in the rod domain (Tinsley *et al.*, 1992; Pearce *et al.*, 1993). The utrophin rod domain is shorter and shows less than 40 % homology to the dystrophin rod domain (Pearce *et al.*, 1993).

1.4.1 Utrophin localization and isoforms

Contrary to the restricted expression of full-length dystrophin in muscle and brain tissues, utrophin has a more ubiquitous distribution. Utrophin is expressed in several human tissues, such as brain, liver, testis, stomach, kidney, placenta, lungs and blood vessels (Ohlendieck *et al.*, 1991; Karpati *et al.*, 1993a; Taylor *et al.*, 1997). More specifically, in adult muscle, utrophin is preferentially localized to neuromuscular junctions (Khurana *et al.*, 1991; Nguyen thiMan *et al.*, 1991; Ohlendieck *et al.*, 1991; Takemitsu *et al.*, 1991; Matsumura *et al.*, 1992; Karpati *et al.*, 1993; Taylor *et al.*, 1997) and myotendinous junctions (Khurana *et al.*, 1991).

Due to the high genomic homology between utrophin and dystrophin, it is plausible that utrophin also has truncated isoforms (Figure 3B). G-utrophin, is such an isoform, where its name depicts the tissue from which it was first detected, the sensory ganglia (Blake *et al.*, 1995). This isoform has a 5.5 kb transcript which

codes for a 113 kD protein (Blake *et al.*, 1995). G-utrophin differs from utrophin at the same point Dp116 diverges from dystrophin. When further examined, the overlap sequence between G-utrophin and utrophin show 98.9 % similarity (97.1 % identity) (Blake *et al.*, 1995). Unlike the ubiquitous expression of full-length utrophin, G-utrophin was found only in brain, where its transcript is found in the cerebral cortex, caudate putamen, amygdala, hypothalamic region and olfactory bulb. G-utrophin is first detected at 11.5 days in cranial nerve and dorsal root ganglia (Blake *et al.*, 1995). At 15.5 days, G-utrophin is present in several brain regions, which would suggest that G-utrophin is developmentally regulated (Blake *et al.*, 1995). In addition, it has been proposed that G-utrophin is the predominant form of utrophin in brain (Blake *et al.*, 1995).

Utrophin was found to have a second isoform, encoded by a separate gene, termed dystrophin-related protein-2 (DRP-2) (figure 3). This isoform was discovered during a phylogenetic study of dystrophin-related protein C-terminals by Roberts and colleagues (1996). DRP-2 is a relatively small protein consisting of only 2 spectrin-like repeats, as well as a cysteine-rich and C-terminal domain homologous to both dystrophin and utrophin (Roberts *et al.*, 1996). The 45 kb gene is localized to chromosome Xq22 in humans and has a similar structure to the dystrophin isoform Dp116 (Roberts *et al.*, 1996; Dixon *et al.*, 1997). DRP-2 was found predominantly expressed in spinal cord and brain (Roberts *et al.*, 1996), where it was found to be concentrated in regions involved in cholinergic synaptic transmission (Roberts and Sheng, 2000). This localization suggests that DRP-2 may be involved in organizing cholinergic synapses (Roberts and Sheng, 2000). In brain, DRP-2 was expressed in regions that were 1) known to express dystroglycan

and 2) expressing various dystrophin isoforms (Dixon *et al.*, 1997). DRP-2 was also found to be associated with membrane fractions as well as enriched at post-synaptic densities (Roberts and Sheng, 2000).

DRP-2 was not only expressed in the central nervous system, but also in peripheral tissues such as eye, kidney, teeth, oesophagus, colon, epididymis and ovaries (Dixon *et al.*, 1997). Interestingly, a dystroglycan-DRP-2 complex was identified at the surface of myelin-forming Schwann cells (Sherman *et al.*, 2001). This complex was found to cluster in response to an interaction between DRP-2 and L-periaxin (Sherman *et al.*, 2001). Absence of L-periaxin was shown to result in mis-localization and depletion of DRP-2 as well as its complex from the membrane (Sherman *et al.*, 2001). This disruption was shown to lead to hyper-myelination and de-stabilization of Schwann cell-axon units (Sherman *et al.*, 2001). Taken together, results obtained in these studies suggest that DRP-2 may be an important element in localization and stabilization of neuronal DAP complexes (Roberts *et al.*, 1996), in addition to playing a role in myelinogenesis in the peripheral nervous system (Sherman *et al.*, 2001).

1.4.2 Utrophin also binds the DAP complex

Since utrophin and dystrophin display significant sequence homology, utrophin was also thought to bind the DAP complex. Correct localization of DAPs at neuromuscular junctions and their loss at the sarcolemma in dystrophic tissues, led to the hypothesis that utrophin interacts with these proteins (Matsumura *et al.*,

1992). In fact, utrophin was found to precipitate with all the DAPs (Matsumura *et al.*, 1992). These results suggest that utrophin and DAPs interact, much like dystrophin and DAPs. Accordingly, this strong similarity and conservation suggests that utrophin could play a similar role to dystrophin in linking extracellular matrix proteins to cytoskeletal actin filaments via DAP complexes (Matsumura *et al.*, 1992; Tinsley *et al.*, 1992).

1.5 Regulation of Utrophin Expression In Skeletal Muscle

1.5.1 Utrophin and muscle development

In culture, significant increases in protein levels (10-100 fold) are observed in response to myogenic differentiation for muscle proteins such as dystrophin (Lev *et al.*, 1987; Nudel *et al.*, 1988) and synaptic proteins such as AChR, AChE and N-CAM (Buonanno and Merlie, 1986; Fuentes and Taylor, 1993; Angus *et al.*, 2001; Moore *et al.*, 1987). However, increases in utrophin levels through myogenic differentiation are moderate compared to other muscle proteins. It was shown that both utrophin transcript and protein levels increase only 2 fold with the differentiation of mononucleated myoblasts into multinucleated myotubes (Schofield *et al.*, 1993; Gramolini and Jasmin, 1999; Perkins *et al.*, 2001). This increase is due to a similarly moderate increase in the rate of transcription (Gramolini and Jasmin, 1999).

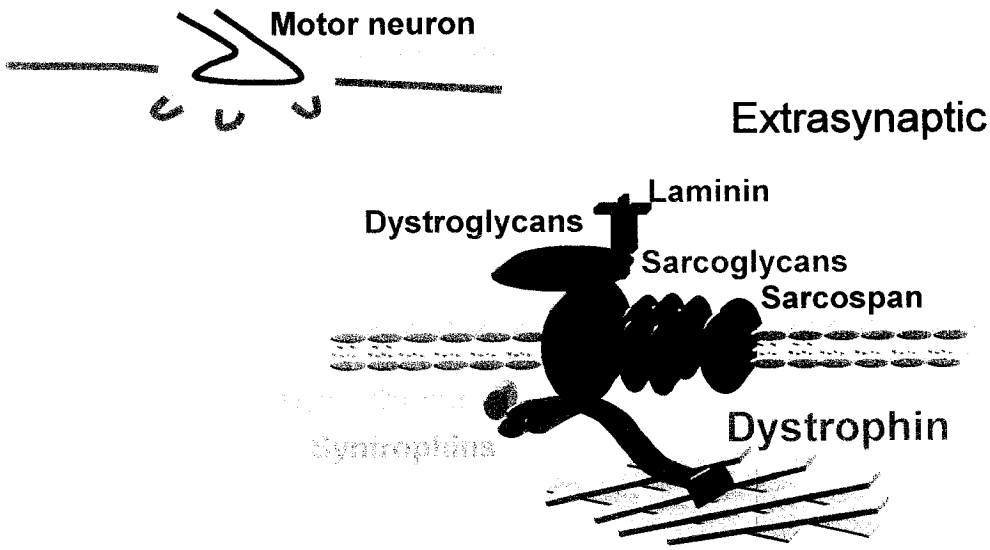
In embryonic muscle, utrophin reaches peak levels and is expressed throughout the sarcolemma (Khurana *et al.*, 1991; Taylor *et al.*, 1997). However, utrophin expression then gradually decreases in extra-synaptic regions. Restricted utrophin expression begins to occur during neonatal development, when it is replaced by dystrophin at the sarcolemma and then gradually accumulates at the crests of neuromuscular junctions (Deconinck *et al.*, 1997a; Taylor *et al.*, 1997) (Figure 4). In addition, necrosis is only detected in *mdx* mice when perinatal utrophin levels decrease to adult levels which would indicate that utrophin may play a protective role in young dystrophic muscle (Khurana *et al.*, 1991). These results indicate that utrophin is developmentally regulated seeing as its sarcolemmal expression is high in developing fibers and decreases as the fibers mature (Helliwell *et al.*, 1992; Clerk *et al.*, 1993; Taylor *et al.*, 1997). Due to its expression pattern in normal muscle development and its protective effects in dystrophin-deficient muscle, utrophin has been suggested to be a fetal form of dystrophin.

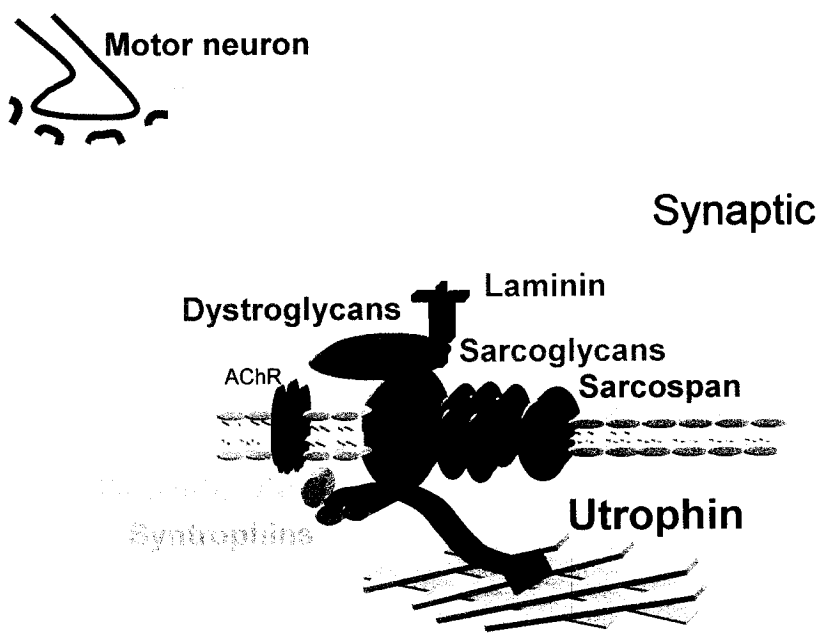
1.5.2 Up-regulated utrophin in mdx and DMD fibers

In muscular dystrophies and other inflammatory diseases, such as polymyositis (PM- a myopathy characterized by inflammation accompanied by severe regeneration), utrophin levels have been shown to be up-regulated (Karpati *et al.*, 1993; Mizuno *et al.*, 1993; Pons *et al.*, 1993). Utrophin is localized at neuromuscular junctions in these diseases however, it can also be detected at the sarcolemma (Khurana *et al.*, 1991; Nguyen thiMan *et al.*, 1991; Takemitsu *et al.*,

Figure 4. Dystrophin and utrophin localization in muscle fibers

Schematic representation of dystrophin and utrophin, in conjunction with the DAP complex at the sarcolemma. Both dystrophin and utrophin link the basal lamina to the actin cytoskeleton via a similar complex. The DAP complex consists of dystroglycans (α, β), sarcoglycans (α, β, γ, δ, ε), sarcospan, syntrophins (α, β) and dystrobrevin. Note the differential localization of dystrophin and utrophin in the muscle fiber. Dystrophin is found throughout the sarcolemma and at troughs of neuromuscular junctions whereas, utrophin is found only at crests of neuromuscular junctions, comparable to AChR.


Motor neuron
Extrasynaptic
Laminin
Dystroglycans
Sarcoglycans
Sarcospan
Dystrophin
Syntrophins


Motor neuron
Synaptic
Laminin
Dystroglycans
Sarcoglycans
AChR
Sarcospan
Utrophin
Syntrophins

1991; Helliwell *et al.*, 1992; Karpati *et al.*, 1993; Pons *et al.*, 1993). This extra-synaptic expression was especially evident in small regenerating fibers. In muscle biopsies from DMD, BMD and PM patients, utrophin levels were significantly higher than control levels (17-, 15- and 4-fold, respectively) (Karpati *et al.*, 1993). Another study confirmed more moderate utrophin increases (3.5 fold) in DMD and PM muscles compared to controls (Gramolini *et al.*, 1999b). Muscle samples chosen for this study were enriched in synapses, therefore higher synaptic utrophin levels, in both control and diseased muscles, resulted in a more moderate increase (Gramolini *et al.*, 1999b).

1.5.3 Utrophin expression during muscle regeneration

Utrophin levels are also substantially increased in regenerating fibers (Helliwell *et al.*, 1992; Taylor *et al.*, 1997; Lin *et al.*, 1998; Gramolini *et al.*, 1999b). These studies reported that extra-synaptic utrophin expression is high in these fibers. Increased utrophin levels in regenerating fibers suggests that utrophin is a regeneration-associated protein (Lin *et al.*, 1998). This attribute could explain the increases seen in diseased muscles such as DMD or PM considering that regeneration is characteristic of these diseases (Lin *et al.*, 1998). It was also observed that regenerating fibers exhibited the extra-synaptic utrophin staining and thus portrayed its up-regulation in the *mdx* and DMD muscles (Helliwell *et al.*, 1992; Taylor *et al.*, 1997). It has been proposed that, with its strong homology to dystrophin, up-regulated utrophin expression throughout the sarcolemma of

diseased and regenerating fibers could be a form of compensation for dystrophin deficiency (Karpati *et al.*, 1993; Deconinck *et al.*, 1997a). These results led to the idea that utrophin may be a plausible candidate to treat DMD.

1.6 Utrophin : A Plausible Therapeutic Approach?

As mentioned, dystrophic tissues display extrajunctional utrophin expression, which could suggest a compensatory mechanism in response to dystrophin-deficiency (Khurana *et al.*, 1991; Mizuno *et al.*, 1991; Nguyen thiMan *et al.*, 1991; Karpati *et al.*, 1993). In fact, up-regulating utrophin expression had beneficial effects in diseased animal models. Using a full-length utrophin construct in *mdx* mice, recovery of muscle mechanical functions and prevention of the development of muscular dystrophy were observed when utrophin was localized at the sarcolemma (Tinsley *et al.*, 1998). Expression of a truncated utrophin transgene in *mdx* mice also improved mechanical muscle performance such as force, resistance and spontaneous activity, as well as restored the maintenance of calcium homeostasis (Deconinck *et al.*, 1997b). Decreases in muscle fibre regeneration (Tinsley *et al.*, 1996), re-localization of DAP complexes at the sarcolemma and normalized expression patterns of muscle proteins were also associated with utrophin over-expression (Rafael *et al.*, 1998). The truncated utrophin transgene contained both the actin-binding and carboxy domains, with the majority of the

spectrin-repeats removed (Deconinck *et al.*, 1997b). These results support the idea that both the amino and carboxy terminals are required to functionally replace dystrophin. Taking this into account, with the fact that both G-utrophin and DRP-2 lack the utrophin amino and carboxy terminals, it seems unlikely that these two isoforms will prove to be as effective therapeutic targets as utrophin, since they seem to have their own distinct functions.

To study the ability of utrophin in preventing the progression of the dystrophic pathology at later stages of the disease, an inducible transgenic system was generated (Squire *et al.*, 2002). These *mdx* mice over-express a utrophin transgene that is induced by tetracycline. Most DMD cases are only diagnosed after birth therefore, expressing utrophin at a later stage in the disease further supports the physiological relevance of this approach. Following the induction of the transgene, utrophin over-expression resulted in the correction of DMD features such as calcium channel activity, membrane localization of DAPs, susceptibility to contractions, muscle force, serum creatine kinase levels and muscle hypertrophy (Squire *et al.*, 2002). Utrophin levels seem to determine the extent of rescue, which indicates that the therapeutic effects are dose-dependent (Squire *et al.*, 2002).

Additional gene therapy studies have been extensively performed to over-express utrophin in dystrophic tissue (for review, see Perkins and Davies, 2002; Dickson *et al.*, 2002; Wells and Wells, 2002). For example, gene therapy was studied using utrophin inserted into adenoviruses. Over-expression of utrophin, following the injection of the utrophin adenoviruses into *mdx* mice, led to a homogeneous distribution of utrophin throughout muscle fibers as well as restored DAP localization to the sarcolemma (Gilbert *et al.*, 1998). Taken together, these

studies support the hypothesis that up-regulating extra-junctional utrophin expression in dystrophic tissues is a plausible therapeutic venue. Accordingly, it becomes imperative to elucidate the mechanisms presiding over utrophin expression in muscle.

1.7 Molecular Mechanisms Regulating Utrophin Expression

1.7.1 Transcriptional mechanisms regulate utrophin expression

Distinct accumulations of utrophin mRNA are seen at neuromuscular junctions yet transcripts can also be detected in extra-synaptic regions (Gramolini *et al.*, 1997; Vater *et al.*, 1998). Eighty-three percent of neuromuscular junctions exhibited accumulation of utrophin transcripts, which is consistent with other synaptic proteins (Gramolini and Jasmin, 1999). Selective accumulation of mRNA has been reported for other synaptic proteins, such as AChE (Jasmin *et al.*, 1993), rapsyn and laminin (Moscoso *et al.*, 1995). This suggests that synaptic proteins may be preferentially transcribed in synaptic nuclei. Accumulation of utrophin message at synapses was shown to result from an increase in transcription activity in synaptic myonuclei compared to non-synaptic nuclei (Gramolini *et al.*, 1997; 1998).

Increased transcription in sub-synaptic nuclei was due to regulatory elements found in an 800 bp fragment of the utrophin promoter (Gramolini *et al.*, 1997).

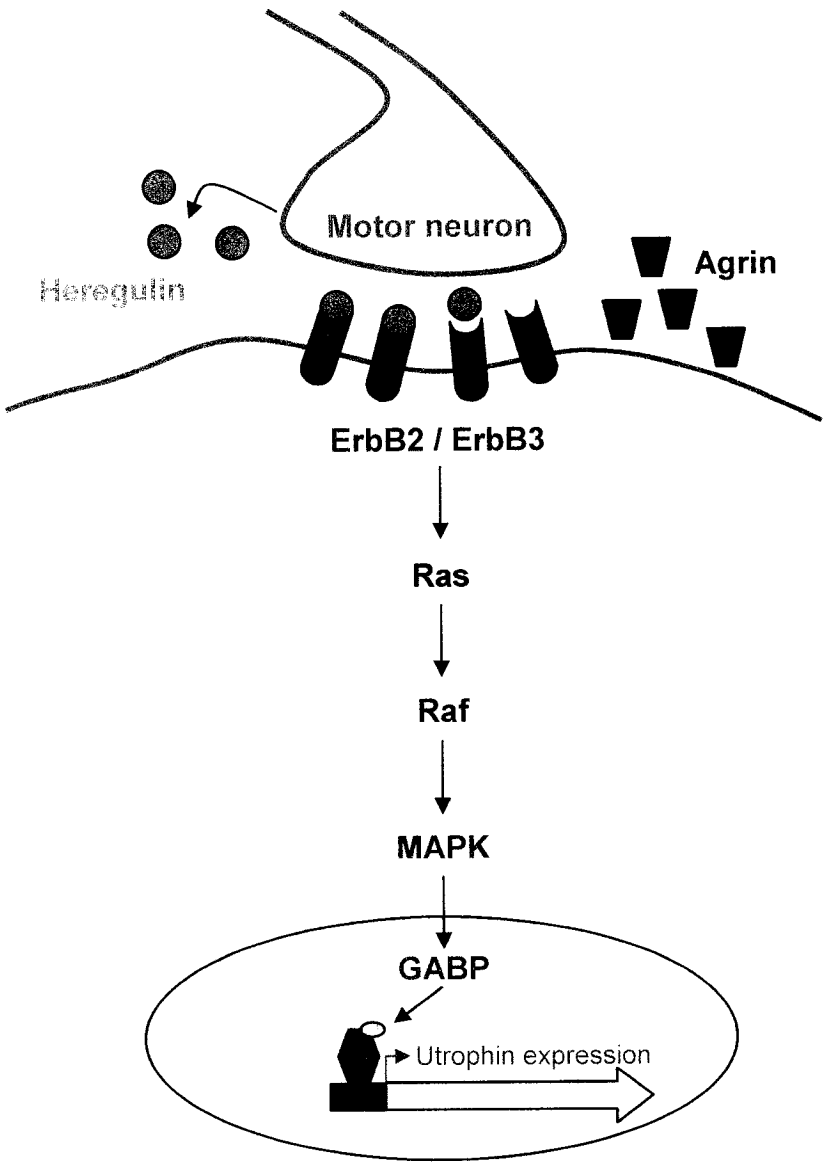
When this fragment was removed, synaptic expression was abolished. It had been previously observed that a DNA regulatory element, known as the N-box motif, was found in the utrophin promoter (Dennis *et al.*,1996) and seems to be required for synapse-specific expression (Gramolini *et al.*, 1997; 1998).

It has been suggested that the nerve can exert effects on gene expression via two putative pathways: 1) nerve-derived trophic factors and/or 2) nerve-evoked electrical activity. Agrin, a motor neuron derived proteoglycan, has been shown to stimulate protein reorganization at muscle cell membranes such as inducing AChR clusters, as well as play a critical role in both synapse formation and maintenance (see Hall and Sanes, 1993; Sanes and Lichtman, 1999). Agrin was also shown to contribute to the synaptic transcription of several proteins (Gautam *et al.*, 1996) such as the AChR ε-subunit gene (Jones *et al.*, 1996). In fact, agrin increased both utrophin transcript and protein levels in cell culture (Gramolini *et al.*, 1998). The exact pathway by which agrin increases utrophin transcription is unknown. However, there is evidence in culture, that agrin exerts its effect on utrophin expression via the N-box (Gramolini *et al.*, 1998). A reporter construct containing a mutated N-box confirmed that this element was required for both the response to agrin in cell culture (Gramolini *et al.*, 1998).

Another nerve-derived trophic factor which has been shown to activate transcription via N-box motifs is heregulin. Treatment of muscle cells with heregulin was shown to increase utrophin expression (Gramolini *et al.*, 1999a). The exact mechanism by which heregulin increases utrophin expression is still unclear. One putative pathway requires the binding of heregulin to its receptor, HER/erbB, which then activates several putative signalling cascades (Figure 5). Activation of these

Figure 5. Schematic diagram depicting regulatory mechanisms mediating utrophin expression at NMJs

Several studies suggest that local utrophin transcription is influenced by nerve-derived trophic factors such as heregulin and agrin. Heregulin has been shown to bind ErbB receptors and thus activate the MAPK signaling cascade. This pathway leads to phosphorylation of *Ets*-related transcription factor GABP in synaptic nuclei. Once phosphorylated, GABP binds the N-box region of the utrophin promoter, an interaction that activates utrophin transcription. Agrin has also been shown to increase utrophin expression via the N-box motif.


Motor neuron
Heregulin
Agrin
ErbB2 / ErbB3
Ras
Raf
MAPK
GABP
Utrophin expression

pathways leads to phosphorylation of the GA-binding protein (GABP), an *Ets*-related transcription factor. Phosphorylated GABP then recognizes the N-box motif and its interaction with the N-box stimulates transcription in subsynaptic nuclei (Fromm and Burden, 1998; Schaeffer *et al.*, 1998; Gramolini *et al.*, 1999a; Khurana *et al.*, 1999; reviewed in Gramolini *et al.,* 2000; Buonanno and Fischbach, 2001).

The second pathway by which the nerve is suggested to affect gene expression is via its activity. Nerve-evoked electrical activity was initially thought to influence synaptic and non-synaptic utrophin expression since AChR δ-subunit, MyoD and myogenin expression have all been shown to be dependent on electrical activity (Dutton *et al.*, 1993). In addition, the decrease in extra-junctional utrophin expression seems to coincide with the time of innervation by the motor nerve, which would suggest that the motor nerve is involved in regulating utrophin expression (see Gramolini and Jasmin, 1998). However, electrical activity was shown to have little or no effect on both utrophin transcript (Jasmin *et al.*, 1995a) and protein (Ohlendieck *et al.*, 1991; Takemitsu *et al.*, 1991; Jasmin *et al.*, 1995a) levels, suggesting that utrophin and AChR are distinctly regulated. Since nerve-evoked electrical activity does not seem to affect utrophin expression, other mechanisms must be involved in regulating utrophin expression in non-synaptic regions.

1.7.2 *Post-transcriptional events regulating utrophin expression*

Gene expression is an event consisting of several different steps such as transcription; mRNA splicing, targeting and stability; translation; and post-translational events (i.e. protein stability and modifications) (for review see Day and Tuite, 1998). Few studies have been performed to examine utrophin expression at the level of transcription as well as message stability and targeting. In addition, no studies have been performed to elucidate translational and post-translational regulation of utrophin expression. Determining their influence on utrophin expression may be key towards developing methods to up-regulate utrophin.

A form of post-transcriptional regulation, known as mRNA splicing, can generate additional isoforms. Several splice variants have been identified for dystrophin, where alternative splicing occurs in the 3' end of its transcript (Feener *et al.*, 1989). Polypeptides, generated by splicing of the 14 kb transcript, differ in their carboxy end and therefore, could potentially interact with different proteins, as well as have different functions than full-length dystrophin. As of yet, no alternatively spliced isoforms have been reported for utrophin. Alternative splicing in the utrophin transcript could affect utrophin expression by providing alternatively spliced proteins that may not perform similar functions.

Stability and targeting of transcripts have been shown to influence subsequent steps in protein synthesis. In fact, the utrophin 3'-untranslated region (UTR) has been found to play a role in both targeting and stability of its transcript (Gramolini *et al.*, 2001a). The utrophin 3'UTR corresponds to a 2 kb fragment which is clearly distinct from the dystrophin 3'UTR (Gramolini *et al.*, 2001a). Following the

generation of deletion constructs, a distinct region was pinpointed as being required to target utrophin transcripts to the cytoskeleton (Gramolini *et al.*, 2001a). Using these same constructs, a completely different region was found to be implicated in transcript stability. This 171 nucleotide fragment conferred the 20 hour half-life previously measured for the whole utrophin transcript (Gramolini and Jasmin, 1999), suggesting that elements required for transcript stability reside in this region (Gramolini *et al.*, 2001a).

Utrophin transcript and protein levels were shown to be higher in slow muscles compared to fast muscles, with no apparent differences in transcriptional activity (Gramolini *et al.*, 2001b). The discrepancy in transcript levels seen between these two muscle types was shown to be primarily due to message stability (Gramolini *et al.*, 2001b). It was observed that slow twitch muscles display low levels of both utrophin transcript and protein in extra-synaptic regions, suggesting that the higher utrophin levels are due to an increase in extra-synaptic utrophin expression (Gramolini *et al.*, 2001b). This study also used reporter constructs including the utrophin 3'UTR, since the latter had been implicated in message stability. Reporter transcript levels were higher in slow muscles compared to fast muscles. These data suggest that utrophin can be regulated post-transcriptionally via its 3'UTR.

1.7.3 *Translational regulation of utrophin expression*

As mentioned, utrophin levels are elevated in *mdx*, DMD and regenerating fibers (section 1.5.3 and 1.5.4). Utrophin levels in *mdx* mice were shown to be increased by 2 and 4 fold in heart and skeletal muscle, respectively (Weir *et al.*, 2002). However, transcript levels did not mirror the significant increases in protein levels. Compared to controls, utrophin transcript levels did not change in *mdx* hearts and transcript levels were only 50 % higher in skeletal muscle (Weir *et al.*, 2002). Therefore, the slight elevation in transcript levels does not explain the increase in protein.

To determine if the increase in protein levels in diseased muscles were due to an increase in transcription as seen with myogenic differentiation, Gramolini and colleagues (1999b) assayed utrophin mRNA levels in DMD and PM affected muscles. Interestingly, mRNA levels were similar in control and diseased muscles, whereas protein levels were shown to increase by 3.5 fold. A recent study performed expression profiling on DMD muscle biopsies to determine the changes in gene expression encountered with the disease (Chen *et al.*, 2000). The results confirmed the lack of change in utrophin mRNA levels in DMD muscles compared to controls. In regenerating muscle, utrophin transcript levels were also compared to controls (Gramolini *et al.*, 1999b; Galvagni *et al.*, 2002). These studies also reported a discrepancy between message and protein levels. Messenger RNA levels in regenerating fibers were not significantly different from controls. Increased protein levels and steady mRNA levels in regenerating fibers and diseased muscles, suggest that translational regulation may be involved. It is also plausible that post-

translational events such as protein stability may play a role in regulating utrophin expression however, no studies have been performed to elucidate these mechanisms.

1.7.3.1 Additional promoters give rise to different 5'UTRs

As mentioned, dystrophin and utrophin show similar genomic structure at their 5' end (section 1.4), therefore utrophin may also have several isoforms derived from different promoters analogous to dystrophin. In both genes, a long intron is found separating the second and third exon suggesting that additional promoters, comparable to dystrophin and associated with novel 5'-untranslated regions (UTRs), may reside within this sequence for utrophin (Pearce *et al.*, 1993).

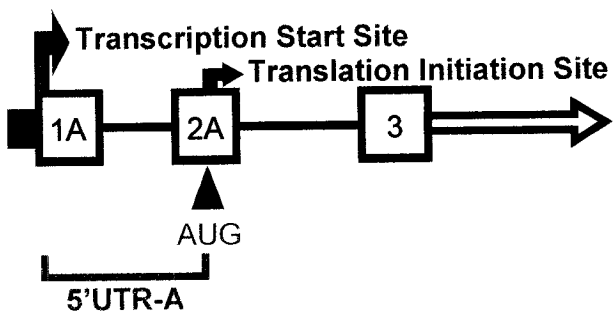
The first utrophin promoter identified resides at the 5' end of the gene and is known as promoter A (Figure 6). The corresponding 5'UTR expands through the first exon and the translation start site is located in the second exon (Pearce *et al.*, 1993; Dennis *et al.*, 1996; Burton *et al.*, 1999). Containing a sequence that targets synaptic expression, an N-box motif (Dennis *et al.*, 1996; Gramolini *et al.*, 1998), this promoter drives the expression for a 13 kb transcript (Dennis *et al.*, 1996). The full length utrophin A 5'UTR is believed to be between 500 and 1000 nucleotides in length (Dennis *et al.*, 1996).

A second promoter, promoter B, was identified and located in intron 2 (Burton *et al.*, 1999). This additional promoter drives the expression of a 13 kb, full length utrophin transcript which has a 5'UTR that is 74 bases in length (Burton *et al.*,

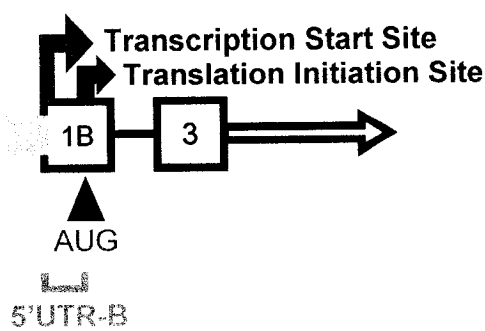
Figure 6. Schematic representation of the nomenclature used to depict the utrophin promoters, exons and transcripts

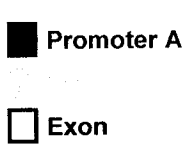
The 5'UTRs are the sequences between the transcription start site and the translation initiation site, AUG). The utrophin A 5'UTR, provided by promoter A, corresponds to a 509 nucleotide fragment. The utrophin B 5'UTR, provided by promoter B, corresponds to a 74 nucleotide fragment. The utrophin transcripts and proteins differ only in their 5' ends. The utrophin A protein contains the remaining predicted 26 amino acids of exon 2A whereas the 5' end of the utrophin B protein consists of the 31 predicted amino acids from exon 1B. Both transcripts splice into a common 13 kb mRNA at exon 3.

Utrophin A



Utrophin B





1999). The utrophin A and B messages only differ in their 5' end, since they splice into a common transcript at exon 3 (Perkins *et al.*, 2001). Both utrophin A and B transcripts generate 395 kD proteins. Twenty-six amino acids provided by exon 2A in the utrophin A transcript differ from the 31 amino acids provided by exon 1B in the utrophin B transcript (Burton *et al.*, 1999). Expression of the utrophin isoforms has been reported to be tissue-specific. Utrophin A expression is more prominent in kidney, utrophin B expression predominates in heart and equal amounts of each are expressed in brain and skeletal muscle, as determined by RNA protection assays (Burton *et al.*, 1999). Moreover, the sub-cellular localization of utrophin A is predominantly at neuromuscular junctions, peripheral nerves and vascular smooth muscle whereas utrophin B is in vascular endothelium (Weir *et al.*, 2002). The two utrophin 5'UTRs, derived from independent promoters, differ extensively in length and sequence which could suggest different regulatory effects at the level of translation.

1.8 Translation Initiation Can Affect Gene Expression

Translation can be divided into three main steps: initiation (assembly of ribosomal subunits at the translation initiation start site, AUG), elongation (decoding mRNA to form a polypeptide chain) and termination (release of ribosomal complex at a stop codon) (Day and Tuite, 1998). The first step in translation is initiation,

which is thought to be the critical step (Guan and Weiner, 1989; Day and Tuite, 1998).

Translation initiation consists of recruiting ribosomal subunits and eukaryotic translation initiation factors (eIFs) followed by the recognition of the translation start site. However, it is a complex process in which several steps are involved and have been elucidated previously (see Gray and Wickens, 1998 and Pestova *et al.*, 2001 for reviews). The first stage is characterized by the grouping of a ternary complex (eIF-2, GTP and Met-tRNA$_i$) and the subsequent formation of the 43S pre-initiation complex (the ternary complex with the 40S ribosomal subunit and eIF-3). eIF-4G, eIF-4E and eIF-4A compose a trimer, eIF-4F, that associates with eIF-4B to form the eIF-4 complex. This complex then recognizes the methylated cap on the 5' end of 5'UTRs (see section 1.8.1). The 43S complex binds to the eIF-4/cap which then forms the 48S pre-initiation complex. This complex, in the presence of ATP, migrates linearly along the 5'UTR until it recognizes an AUG and pauses. The initiation factors are then released, the 60S ribosomal subunit joins the 40S and the elongation process is induced (see Kozak, 1989a; Gray and Wickens, 1998; Mignone *et al.*, 2002 for review). Since recruitment of the translational machinery and recognition of translation initiation sites involve the 5'UTR, the latter thus plays an important role in translation initiation.

1.8.1 *Characteristic structural features of the 5' untranslated-region*

The 5'UTR can modulate translation initiation via at least five characteristic structural features (for review see Kozak, 1991; Day and Tuite, 1998). The first feature is a methylated-guanylated cap on the 5'-end of transcripts and is found on almost all eukaryotic mRNAs. It serves to protect mRNA from 5'-3' exonucleolytic degradation as well as stimulate translation efficiency (reviewed in Kozak, 1991). This cap is also the region that binds eIF-4E, one of three subunits constituting eIF-4F. It is generally accepted that the role of eIF-4F is to unwind secondary structures found in 5'UTRs by helicase activity. It also aids in the recruitment and binding of ribosomal subunits to 5'UTRs (for review see Gray and Wickens, 1998).

Translation initiation was originally thought to be strictly cap-dependent. However, cap-independent translation has also been shown to occur in eukaryotes. This type of initiation requires a regulatory element known as an internal ribosome entry site (IRES) (Vagner *et al.*, 2001 for review). Several cellular mRNAs have been shown to contain an IRES in their 5'UTRs, such as immunoglobin heavy-chain binding protein (BiP), basic fibroblast growth factor and *c-myc* (see van der Velden and Thomas, 1999 for review). IRES elements are known as long sequences that form secondary structures which can recruit the required ribosomal subunits and thus promote translation initiation (see Vagner *et al.*, 2001 for review).

Secondly, the primary sequence surrounding the initiation start site has also proved to be important. An optimal sequence for the context within which resides the initiation start site has been proposed, (**A/G**)CCAUG**G** (Kozak, 1986a). With the A of the AUG being +1, it is a purine, preferably an adenine, found in position -3 and

a guanine in position +4 that have been found to be the most important nucleotides (Kozak, 1986a; see Mignone *et al.*, 2002 for review). It has been suggested that this sequence would increase the efficiency with which the 43S pre-initiation complex recognizes the translation initiation site (Day and Tuite, 1998). Even though an optimal context has been proposed and shown to influence initiation, it is not the primary regulator of translation initiation. Many other features or characteristics have also been shown to come into play since translation start sites in poor contexts can still be translated (for review see Gray and Wickens, 1999).

Thirdly, the presence of upstream translation start sites can hinder translation initiation of the wanted transcript, seeing as the ribosomal scanning complex generally initiates translation at the first AUG codon it encounters (Kozak, 1987). The presence of upstream out of frame AUGs was shown to inhibit translation, unless a premature termination codon in the same reading frame was present. In this case, the ribosomal complex can possibly reinitiate at the main AUG downstream, a process known as *leaky scanning* (Kozak, 1989a). However, without the premature stop codon, translation can result in non-functional or aberrant proteins.

The fourth feature, the presence of secondary structures has been found to influence initiation. In addition, the stability of these structures influences translation efficiency. Stable structures found between the cap and AUG can inhibit translation initiation as a result of their position in relation to the cap or the AUG, as well as the free energy of the hairpin structures themselves (Kozak, 1986b; 1989b). A structure found close to the cap hinders the formation of initiation complexes, whereas a secondary structure found surrounding the AUG, hinders the recognition of the

translation start site (Day and Tuite, 1998). The inhibitory effect has been found to be stronger however when stable hairpin structures are found closer to the cap than the AUG (Kozak, 1989b).

The fifth feature is the length of the 5'UTR, known as leader length, which can decrease or increase translation efficiency. A long 5'UTR can introduce stable secondary structures which can be inhibitory, as mentioned above. It was also shown that very short 5'UTRs can inhibit initiation as well. This was proposed to be due to the ribosomal complex skipping the favourable AUG when the leader length was only 12 nucleotides (reviewed in Kozak, 1991). However, when the short 5'UTR was increased to 20 nucleotides, translation efficiency was increased. Therefore, translation efficiency can be decreased with a very short or very long 5'UTR (reviewed in Kozak, 1991).

These five characteristics play roles in regulating translation initiation efficiency. Although these characteristics seem fairly simple, initiation can be modulated through a number of combinations of these features. The regulation of translation initiation itself, when one includes the five structural features of 5'UTRs as well as involved proteins and ribosomal subunits, is consequently a very complex, multi-step process.

1.9 Statement of the Problem, Hypothesis and Objectives

DMD is the most prevalent and severe degenerative disorder of skeletal muscle. Characterized by a progressive muscle weakness, this disease is due to the absence of dystrophin, which leads to plasma membrane defects, repeated cycles of degeneration and regeneration as well as an actual loss of muscle fibers. Utrophin, an autosomal homologue, has been reported to be up-regulated intrinsically in *mdx* and DMD muscles. Utrophin up-regulation has been shown to alleviate the dystrophic pathology in *mdx* mice, suggesting it is a plausible therapeutic venue.

Several studies have reported discrepancies between transcript and protein levels in different muscle conditions. In *mdx*, DMD and regenerating fibers, utrophin protein levels increase however, transcript levels do not change compared to controls. We hypothesized that, based on the previous findings, the utrophin up-regulation seen in *mdx*, DMD and regenerating fibers is due to translational regulation.

Typically, 5'UTRs have been shown to affect translation, therefore, characterization and study of both utrophin 5'UTRs could lead to determining their effect(s) as well as mechanism(s) regulating utrophin translation. Some studies have examined the different stages of utrophin expression, yet little is known regarding the translational regulation of utrophin. To this end, our main objectives are to:

1) Clone the utrophin A and B 5'UTRs isolated from C2C12 myotube RNA into the pCMV•SPORT-βgal reporter vector

2) Determine the effects of the utrophin 5'UTRs on message stability in C2C12 myotubes by measuring transcript half-lives following Actinomycin D treatment

3) Study the effects of each utrophin 5'UTR on reporter gene expression in C2C12 myoblasts and myotubes

4) Examine the effects of the utrophin 5'UTRs on reporter gene expression in young (5-6 week-old) control and regenerating muscles

5) Compare the effects of the utrophin A 5'UTR on reporter gene expression in young (5-6 week-old) and adult (13-18 week-old) *mdx* mice.

Chapter 2

Methods

2.1 Amplification of Utrophin A and B 5'UTRs and Generation of Reporter Gene Constructs

Utrophin A and B 5'UTRs were inserted into a cytomegalovirus (CMV) promoter driven plasmid, pCMV•SPORT-βgal (Gibco/BRL, Burlington, ON) and used for reporter gene experiments, both in cell culture and animal studies. As a control plasmid, the intact pCMV•SPORT-βgal was used in each experiment and referred to as the parental plasmid. RT-PCR was performed on RNA extracted from 4 day-old myotubes to obtain utrophin A and B 5'UTRs. All primers and PCR conditions are expressed in Table I. The full utrophin A 5'UTR sequence has not been published therefore the 3' end of this region is unknown. We designed primers which amplified a fragment that contained the first 16 nucleotides of the coding region (Table I - primer set 1). To obtain the full utrophin A 5'UTR, without incorporating a second translation initiation site since the Lac Z reporter contains its own, utrophin A 5'UTR specific primers, that exclude the utrophin ATG, were designed based on the subcloned 5'UTR sequence obtained (Table I - primer set 2). For subcloning purposes, these latter primers include restriction sites, *Nco*I on the 5' primer and *EcoR*I on the 3' primer.

To obtain the utrophin B 5'UTR, specific primers were designed against the published sequence (Burton *et al.*, 1999), where the upstream primer resides at the transcription start site and the downstream primer just upstream from the translation start site (Table I - primer set 3). For cloning purposes, restriction sites were added to each primer.

Table I

PCR conditions

Primers, fragment size, cycling parameters and required number of cycles.

Amplified fragment	Upstream primer (5', 3')	Downstream primer (5', 3')	Fragment size (bp)	Cycling parameters			# of cycles
				Denaturation	Annealing	Extension	
β-gal	GTGACGGCAGTTATC-TGG	TTGGCAGTGCTCGTA-GTA	506	94 °C 45 s	55 °C 1 min	72 °C 1 min	20-27
CAT	TGGCAATGAAAGACG-GTGAG	GAAAACGGGGGCGA-AGAAGT	290	94 °C 45 s	55 °C 1 min	72 °C 1 min	23-28
Total endogenous utrophin (A & B)	GGGGAAGATGTGAG-AGATTT	GTGTGGTGAGGAGAT-ACGAT	548	94 °C 1 min	60 °C 1 min	72 °C 1 min	22-28
Utrophin A 5'UTR (including ATG)	GTTGTGGAGTCGCCCT	CCCCATACTTGGCCAT	525	94 °C 1 min	60 °C 1 min	72 °C 1 min	30-40
Utrophin A 5'UTR (cloning)	CATGCCATGGCATGT-GTTGTGGAGTCGCCCT	CGGAATTCCGCTTGA-ATGAGTTTCAGT	533	94 °C 1 min	63 °C 1 min	72 °C 1 min	30-40
Utrophin B 5'UTR (cloning)	CATGCCCATGGCATG-CCCAGTGTGCAGTTCG	CGGAATTCCGCACCT-ACAGTGGCTGAG	128	94 °C 1 min	60 °C 1 min	72 °C 1 min	30-40
Endogenous utrophin A	CCTTTTCTTTCGGGT-CATTCCT	AATCCAAAGGCTTTC-CCAGATC	360	94 °C 1 min	63 °C 1 min	72 °C 1 min	22-28
Endogenous utrophin B	CGGAATTCCGCCCAG-TGTGCAGTTCG	GGATCTCCTGCAAGC-CTGG	174	94 °C 1 min	63 °C 1 min	72 °C 1 min	24-30
Endogenous rRNA	GGAAGGCATAGCTGC-TGG	CCTCGATGACATCCT-TGG	368	94 °C 1 min	54 °C 1 min	72 °C 2 min	20-27

* All PCR reactions were preceded with a 5 min denaturation cycle at 94 °C. Following the last cycle of each reaction, a 10 min extension period at 72 °C was performed.

** Primers used for cloning purposes included restrictions sites (NcoI and EcoRI).

PCR products were visualized on ethidium-bromide stained 1.5 % agarose gels and excised using a Qiagen Gel extraction kit (Chatsworth, CA). The cDNA was then ligated to a cloning vector, pUC57 (InsT/Aclone™ PCR Product Cloning Kit; MBI Fermentas, Burlington, ON), where ligation solutions were incubated overnight at room temperature. Ligated products were used to transform *E. coli* DH5-α™ competent cells (Gibco/BRL, Burlington, ON). Transformed cells were then plated on Luria-Bertani (LB)-agar plates (with 50 μg/ml ampicillin) and incubated overnight at 37 °C. Positive clones, indicated by blue staining, were selected and grown in LB-broth (with 50 μg/ml ampicillin) overnight at 37 °C. cDNA was prepared using Qiagen (Chatsworth, CA) mini-prep kit and pellets were re-suspended in sterile water. Positive clones were identified by restriction enzyme digests with *EcoR*I (MBI Fermentas, Burlington, ON) and *Nco*I (Gibco/BRL, Burlington, ON). Subsequent sequencing was performed (Institut de recherche en Biotechnologie, University of Ottawa, Ottawa, ON) on clones positive for the correct sized fragments visualized on an ethidium-bromide stained gel.

Inserts were excised from positive sequenced clones by restriction digest with *EcoR*I and *Nco*I and isolated from ethidium-bromide gels. The native 5'UTR was excised from the pCMV•SPORT-βgal plasmid by restriction digest with *EcoR*I and *Nco*I. Utrophin A and B 5'UTR inserts were ligated to the pCMV•SPORT-βgal, lacking its 5'UTR, overnight at room temperature. Ligated products were used to transform *E. coli* DH5-α™ competent cells and positive clones were selected and grown. Positive clones were further identified by restriction enzyme digest and confirmed by sequencing.

Plasmid DNA was prepared using Qiagen (Chatsworth, CA) mega- or midi-prep protocols. Pellets were re-suspended in sterile water for transient transfections or in sterile PBS for muscle injections. Plasmids used for transfections and muscle injections were: *Parental* (pCMV•SPORT-βgal), *UTR A* (pCMV•SPORT-UTR A-βgal) and *UTR B* (pCMV•SPORT-UTR B-βgal) (Figure 7 and Table II).

2.2 Cell Culture

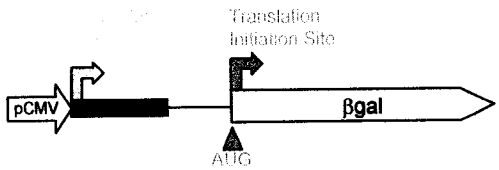
Mouse C2C12 cells (ATCC, Manassas, VA) were cultured on Matrigel (Collaborative Biomedical Products, Bedford, MA)-coated culture dishes in Dulbecco's modified Eagle medium (D-MEM high-glucose, with L-glutamine; Gibco/BRL, Burlington, ON) supplemented with 10 % fetal bovine serum (Gibco/BRL, Burlington, ON), 20 % horse serum (Gibco/BRL, Burlington, ON) and 100 U/ml penicillin-streptomycin (Gibco/BRL, Burlington, ON). Cells were maintained in a humidified chamber at 37 °C containing 5 % CO_2.

Myoblasts were passaged upon reaching 80-90 % confluence. Following removal of media, 1 ml of trypsin-EDTA (Gibco/BRL, Burlington, On) was added to a 35 mm or 60 mm plate and incubated for approximately 2-5 min at 37 °C. Cells were triturated in trypsin-EDTA to ensure that all cells were removed from the wells. Four ml of growth media were added to inhibit trypsin. Cells were centrifuged at 1 200 x g for 15 min. Cells were then re-suspended in fresh growth media, plated

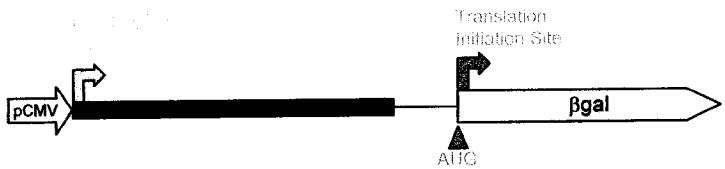
Figure 7. 5'UTR reporter constructs used for transfections and muscle injections

Representative diagram of reporter gene constructs. The parental construct is the intact pCMV•SPORT-βGal vector. Following the removal of the parental 5'UTR, mouse utrophin 5'UTRs, A or B, were inserted into the reporter vector. The yellow block arrows represent the constitutively active CMV promoter. Colored rectangles represent the 5'UTRs; parental, utrophin A and utrophin B. Both angled arrows are the transcription or translation (AUG) start sites. Horizontal pickets portray the reporter gene, LacZ.

Parental 5'UTR (71 nt)



Utrophin A 5'UTR (509 nt)



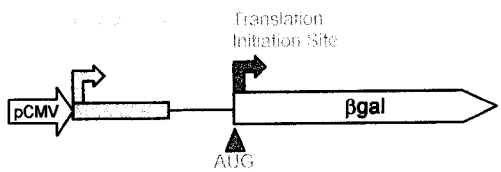


Table II

Reporter Constructs Generated and Length of 5'UTR inserts

Construct	Common name	Length of 5'UTR insert
pCMV•SPORT-βgal	Parental	71 bp (native)
pCMV•SPORT-UTR A-βgal	UTR A	533 bp (including restriction sites)
pCMV•SPORT-UTR B-βgal	UTR B	128 bp (including restriction sites)

on Matrigel-coated plates and allowed to recover for 24 hr in the humidified chamber. Culture media were changed every 48 hr. Myotubes were obtained by substituting growth media with a low serum media (D-MEM supplemented with 5 % horse serum and 100 U/ml penicillin-streptomycin) once cells reached confluence. The first day in differentiation media was considered to be day 0. For myogenic studies, cells were harvested as myoblasts at 50 and 100 % confluence as well as 2 and 4 day-old myotubes.

2.3 Transient Transfections

Cells were transfected using Lipofectamine Reagent (Life Technologies, Inc., Burlington, ON). They were plated in 6 well, Matrigel-coated plates and transfected 18-24 hr later at 50-60 % confluence. Transfection solutions consisted of 1 ml of Optimem Reduced Serum (Gibco/BRL, Burlington, ON), 2 μg of reporter gene construct (Parental, UTR A or UTR B), 2 μg of chloramphenicol acetyltransferase (CAT) plasmid (to account for transfection efficiency) and 15 μg of Lipofectamine, for each well. Transfection solutions were incubated at room temperature for 30 min. Cells were rinsed twice with Optimem and transfection media were added to the cells. Transfection media were removed following a 5 hr incubation in the humidified chamber and replaced with growth media to allow cells to recover for 18-24 hr. Cells were then harvested as myoblasts or induced to differentiate by replacing growth media with differentiation media for 4-5 days.

2.4 Animal Care, Direct Gene Transfer and Surgery

C57BL mice (3-4 week-old) were obtained from Charles River Laboratories (St. Constant, Québec, Canada) and their care and treatment was in accordance with guidelines established by the Canadian Council on Animal Care. Young (5-6 week-old) and adult (13-18 week-old) *mdx* mice were obtained from our own colony. For injection experiments, mice were anesthetized with gaseous halothane. Twenty-five μl of a 2 μg/μl plasmid solution containing reporter gene construct (parental, UTR A or UTR B) and CAT plasmid were co-injected into each tibialis anterior (TA) muscle. Seven days later, muscles were dissected, excised and rapidly frozen in liquid nitrogen. Samples were kept at -80 °C until further analysis.

To induce muscle degeneration, C57BL mouse muscles were treated with cardiotoxin. Cardiotoxin treatment consisted of injecting 25 μl of 10^{-5} M cardiotoxin (LATOXAN: Laboratoire des Toxines Animales et Animaux Venimeux, Rosans, France) into TA muscles of 4-5 week-old mice as described (Gramolini *et al.*, 1999b). Plasmid DNA was injected into regenerating muscles 3 days after cardiotoxin treatment. Muscles were excised four days following plasmid injections.

2.5 Determination of β-Galactosidase and Chloramphenicol Acetyltransferase (CAT) Activity

Transfected cells were lysed in 400 µl of 1 X Reporter Lysis buffer (Promega) and triturated through a pipette tip. Whole TA muscles were homogenized in 1 ml of 1 X Reporter Lysis Buffer (RLB) using a Polytron set at maximum speed for 2 x 15 s. Following two freeze-thaw cycles in a dry ice/ethanol bath, extracts were centrifuged at 15 000 x g for 20 min at 4 °C. Supernatant was collected and kept at -80 °C until further analysis. β-Galactosidase and CAT activity were measured in each lysate.

β-Galactosidase activity was measured using the β-Galactosidase Enzyme Assay system (Promega, Madison, WI). Each sample reaction consisted of 150 µl of 2 X Assay Buffer and 150 µl of diluted sample (in 1 X RLB). Due to the high expression of the parental pCMV•SPORT-βgal plasmid, only 3 µl of extract was used for Parental lysates, whereas 15 µl was used for UTR A and UTR B samples. Lysates were diluted to a final volume of 150 µl in 1 X RLB. Reactions were then incubated at 37 °C for 20-30 min and terminated by adding 500 µl of 1 M sodium carbonate (BDH Inc., Toronto, ON). Absorbance was read at 420 nm in a standard spectrophotometer (Beckman DU® 640, Spectrophotometer, Palto Alto, CA). Absorbance values obtained with the parental pCMV•SPORT-βgal plasmid were multiplied by 5, since only one-fifth volume of extract was used per reaction. β-galactosidase activities were then normalized to corresponding CAT activities.

Chloramphenicol acetyltransferase (CAT) activity was measured using the CAT Enzyme Assay (Promega, Madison, WI). Fiftyt μl of lysates were heated for 10 min at 60 °C. To each sample, 0.05 μCi/ml of ^{14}C-chloramphenicol and 5 μg of n-Butyryl Coenzyme A (Sigma Aldrich) were added and brought to a final volume of 125 μl with distilled water. Reactions were incubated at 37 °C for 2 hr and then spun in a microcentrifuge at 15 000 x g for 3 min. Reactions were terminated by adding 300 μl of mixed xylenes (BDH Inc., Toronto, ON), vortexed and centrifuged at 15 000 x g for 3 min. Two-hundred μl of supernatant were transferred to a fresh tube and 200 μl of fresh 0.25 M Tris-HCl, pH 8.0 (EM Science, Germany) were added. Samples were vortexed and centrifuged at 15 000 x g for 3 min. One hundred μl of supernatant were carefully transferred to a scintillation vial, to which 2 ml of scintillation fluid (CytoScint, ICN) were added. Samples were counted in a scintillation counter (Wallac 1414 Liquid Scintillation Counter with 1414 Winspectral Windows software), where counts per minute (CPMs) correspond to butyrylated chloramphenicol products. Both negative (1 X RLB) and positive (0.25 μl of chloramphenicol acetyltransferase with 1 X RLB) controls were used in each assay. Counts measured were used to normalize β-galactosidase activity.

2.6 RNA Isolation and DNase I Treatment

RNA was isolated from myoblasts, myotubes and TA muscles using Total RNA Isolation Reagent (TRIzol) (Life Technologies, Burlington, ON). RNA was extracted from cells by adding 1 ml of TRIzol Reagent to each well. Cells were homogenized by passing them through a pipette several times. For whole muscle samples, 1 ml of TRIzol was added to a tube and muscles were homogenized for 2 x 15 s with a Polytron set at maximum speed. Homogenized samples were transferred to fresh eppendorf tubes. To each sample, 200 µl of chloroform were added. Samples were vortexed and centrifuged at 14 000 x g for 15 min at 4 °C. For each sample, the aqueous supernatant was carefully transferred to a fresh tube and 500 µl of isopropanol were added. Samples were mixed and kept at -80 °C overnight. Samples were then incubated at room temperature for 10 min and centrifuged at 14 000 x g for 15 min at 4 °C. From each sample, the supernatant was carefully removed and the pellet was washed with 75 % ethanol in RNase-free water. All RNA pellets were then vortexed and centrifuged at 14 000 x g for 5 min at 4 °C. Supernatants were removed carefully and pellets were air dried for 5 min. Dried pellets were re-suspended in RNase-free water.

RNA extracted from both transfected cells and injected muscles were DNase I treated (Message Clean kit; GenHunter Corporation, Nashville, TN) to ensure that no plasmid DNA contamination was present in RNA samples. For each sample, 10-50 µg of total RNA, 1 X Reaction Buffer and 10 U/µl of DNase I were added. Reaction volumes were brought to a total volume of 56.7 µl with RNase-free water.

Samples were mixed and incubated for 30 min at 37 °C. Forty µl of phenol/chloroform (3:1) were added to each sample and vortexed. This step ensures removal of protein contamination and DNase I from the RNA. Samples were incubated on ice for 10 min and centrifuged at 14 000 x g for 5 min at 4 °C. The upper phase was collected in a fresh tube to which 200 µl of 100 % ethanol and 5 µl of 3 M NaOAc was added. Samples were mixed and kept at -80 °C overnight. Once thawed, samples were centrifuged at 14 000 x g for 10 min at 4 °C. Pellets were washed, re-suspended in RNase-free water and kept at -80 °C until further analysis.

2.7 Quantitative Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

For each sample, relative RNA content was measured using a GeneQuant II RNA/DNA spectrophotometer (Pharmacia, Québec, Canada). RNA was standardized to 50 ng/µl and 100 ng were subjected to RT-PCR as described (Jasmin *et al.*, 1993; Michel *et al.*, 1994; Gramolini *et al.*, 1998) . For reverse transcription (RT), RNA was added to an RT master mix consisting of 5 mM $MgCl_2$, 1 X PCR Buffer II (50 mM KCl, 10 mM Tris-HCl (pH 8.3)), 1 mM dNTPs, 1 U/µl RNase inhibitor, 2.5 U/µl reverse transcriptase and 2.5 mM random hexamers (GeneAMP RNA PCR kit; Perkin Elmer Cetus). Two negative controls, 1) RT mix

lacking reverse transcriptase and 2) RT mix with RNase-free water and no RNA, were prepared for each RT reaction. The RT conditions consisted of 45 min at 42 °C and the reaction was terminated by heating samples at 99 °C for 5 min in a PCR Eppendorf Mastercycler *gradient* (Hamburg, Germany).

PCR master mixes were prepared with final concentrations of 2 mM $MgCl_2$, 1 X PCR Buffer II, 2.5 U/100 µl of AmpliTaq DNA polymerase and 15 µM of each primer. Desired transcripts were amplified by using primers specific for the desired transcript. β-Galactosidase cDNAs (Table I - primer set 4) (Gramolini *et al.*, 2001a, 2001b) and CAT cDNAs (Table I - primer set 5) (Gramolini *et al.*, 2001a, 2001b) were amplified in all cell culture and muscle injection experiments.

Using primers that do not discriminate between A and B utrophin transcripts, total utrophin cDNAs were amplified (Jasmin *et al.*, 1995a). These primers amplify a fragment common to both A and B utrophin transcripts (Table I - primer set 6). Endogenous utrophin A transcripts were measured throughout myogenic differentiation using primers designed within the utrophin A 5'UTR (Table I - primer set 7). Utrophin B transcripts were amplified using primers which reside at the transcription start site (5' primer) and in the coding region (3' primer) (Table I - primer set 8).

To confirm the constant loading of RNA in RT reactions, s12 ribosomal RNA levels were also measured. As an internal control, rRNA cDNAs were amplified using primers previously described (Table I - primer set 9) (Forster *et al.*, 1993). rRNA transcript levels were used to normalize endogenous utrophin transcript levels measured in myogenic differentiation experiments.

All RT-PCR products were visualized on 1.5 % agarose ethidium bromide-stained gels. A 100 bp ladder (Gibco/BRL, Burlington, ON) was used to estimate the fragment size of PCR products. Intensity of the RT-PCR product bands, which reflects relative transcript abundance, was measured and calculated using a Kodak Digital Science Image Station 440 CF (Eastman Kodak Company, New Haven, CT) with the appropriate software (Kodak Digital Science 1D Scientific Imaging Systems - Image Analysis Software).

2.8 Histochemical Stain For β-Galactosidase

Transfected myoblasts or myotubes were stained for β-galactosidase as described by Dannenberg and Suga (1981). Cells were fixed with 1% gluteraldehyde in phosphate-buffered saline (PBS; 0.1M, pH 7.4) for 15 min at 4 °C. Following 3 x 5 min washes in PBS at 4 °C, cells were incubated in an X-gal solution (1 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, X-gal (400 μg/ml), 0.01 % sodium desoxycholate and 0.02 % Nonidet NP40 in PBS) for 2 ½ hr at 37 °C . Cells were rinsed with PBS at room temperature 2 x 5-10 min. Fresh PBS was added and culture dishes were scanned (Hewlett Packard ScanJet 6300C; HP Precision Scan Pro Software) or analyzed under a microscope (Zeiss Axiophot fluorescence photomicroscope; Oberkochen, West Germany). Photographs were taken using a Sony Power HAD, 3CCD Color Video Cam in conjunction with Northern Eclipse software (Empix Imaging Inc., Missisauga, ON)

to visualize the pictures. Presence of β-galactosidase was indicated by blue staining.

2.9 Determination of Transcript Half-life

Cells were plated in 6 well, Matrigel-coated dishes and transfected as described previously (section 2.3). Four day-old myotubes were treated with Actinomycin D (Sigma/Aldrich, St. Louis, MO), an antibiotic compound that inhibits cell proliferation by forming complexes with DNA and interfering with RNA synthesis. Four 4 μg/ml of Actinomycin D were added to differentiation media (Taormino and Fambrough, 1990; Tennyson *et al.*, 1996a, 1996b; Gramolini and Jasmin, 1999c). Treatment medium was added to each well and cells were incubated in the humidified chamber. Treated cells were harvested at different time-points (0, 1, 2, 4, 10 and 24 hr) and RNA was extracted (Tennyson *et al.*, 1996a, 1996b; Gramolini and Jasmin, 1999c).

2.10 Immunofluorescence

Extensor digitorum longus (EDL) and soleus muscles were excised from control C57BL mice, mounted in Tissue-Tek® OCT compound embedding medium (Miles Inc., Elkhart, IN) and frozen in liquid nitrogen-cooled isopentane. Muscles were kept at -80 °C until sectioned. Cross-sections (10 μm) were washed with Buffer A (PBS containing 0.5 % bovine serum albumin (BSA) (EM Science, Germany) and 0.15 % glycine (EM Science, Germany)). Sections were then blocked for 15 min at room temperature with Buffer A containing 5 % horse serum (Gibco/BRL, Burlington, ON). Polyclonal goat purified antibodies raised to the C-terminus of eukaryotic initiation factor-4E (eIF-4E; Santa Cruz Biotechnology Inc.) or to eukaryotic initiation factor-2α (eIF-2α; Santa Cruz Biotechnology Inc.) were diluted to 1:50 in PBS. Sections were exposed to primary antibodies overnight at 4 °C. Sections were then washed 3 x 10 min with Buffer A. A Cy™3-conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, (West Grove, PA) was diluted (1:500) in Buffer A and applied to sections for 1 hr at room temperature in a dark room. Fluorescein-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR) was added (1:500) to secondary antibody solution to identify NMJs by labeling AChRs. Sections were washed 3 x 20 min with PBS and mounted in aqueous anti-fading fluorescence medium, Fluoromount G (Electron Microscopy Sciences, Washington, PA). Photographs were taken using the Zeiss Axiophot fluorescence photomicroscope and a Sony Power HAD, 3CCD Color Video Cam in conjunction with Northern Eclipse software (Empix Imaging Inc., Mississauga, ON).

2.11 Statistical Analysis

One-way analysis of variance (ANOVA), followed by Tukey post-hoc analysis, were performed to assess if differences seen between group means were statistically significant. The level of significance was set at $p < 0.05$.

Chapter 3

Results

3.1 Eukaryotic Translation Initiation Factors Co-Localize With Neuromuscular Junctions

Utrophin is preferentially expressed at NMJs in adult muscle (Nguyen *et al.*, 1991; Ohlendieck *et al.*, 1991). To assay if synaptic expression could result from enhanced translational capacity at NMJs, we wanted to examine the distribution of translation initiation factors in mouse muscle fibers.

Eukaryotic initiation factor-4E (eIF-4E) is thought to be the limiting factor in translation. Therefore, we examined its distribution in mouse muscle. EDL and SOL muscles from C57BL mice were sectioned and acetylcholine receptors (AChR) were labelled using α-bungarotoxin to indicate NMJs. eIF-4E was labelled and displayed limited extra-synaptic expression (figure 8). However, it appeared to have important accumulations at neuromuscular junctions as indicated by arrows. Co-localization was also observed in sections labelled with eukaryotic initiation factor-2α (figure 9), which had very little expression in extra-junctional regions. eIF-2α is part of a heterotrimeric complex whose phosphorylation inhibits translation initiation (Gray and Wickens, 1998), indicating that it is also an important regulator of translation initiation. Synaptic enrichment of these translation initiation factors at mouse NMJs could possibly indicate that translational regulation may play an important role in selective synaptic expression.

Figure 8. Co-localization of translation initiation factor eIF-4E with neuromuscular junctions in mouse SOL and EDL muscles

Alpha-bungarotoxin was used to indicate the presence of neuromuscular junctions by labelling acetylcholine receptors in SOL (A-D) and EDL (E-J) sections of C57/BL mice. Sections were also immunolabelled with a eIF-4E specific antibody. No primary antibody was added as negative controls to indicate antibody specificity (I, J). Note co-localization of the translation initiation factor with neuromuscular junctions. Bar = 100 μm.

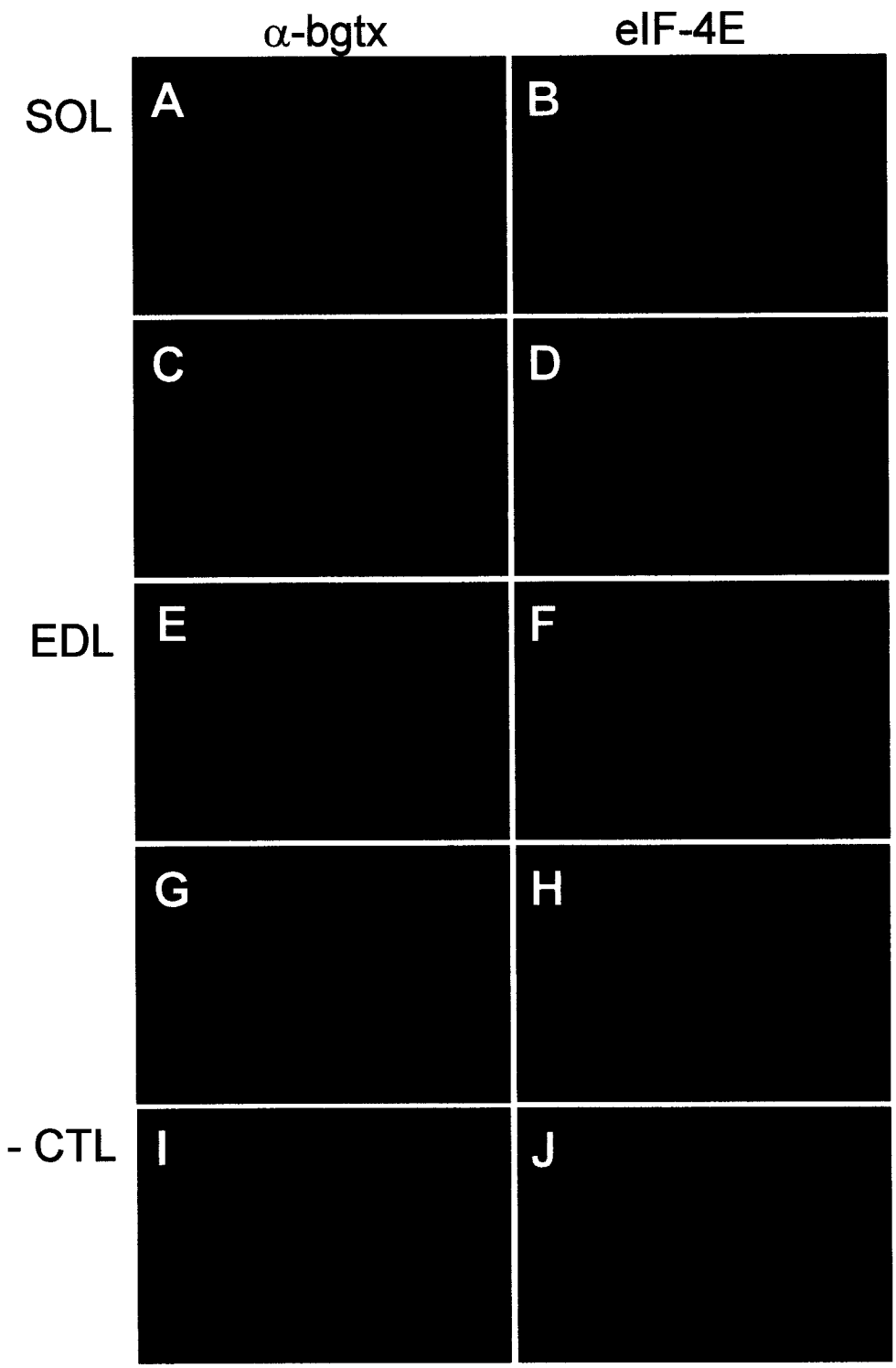
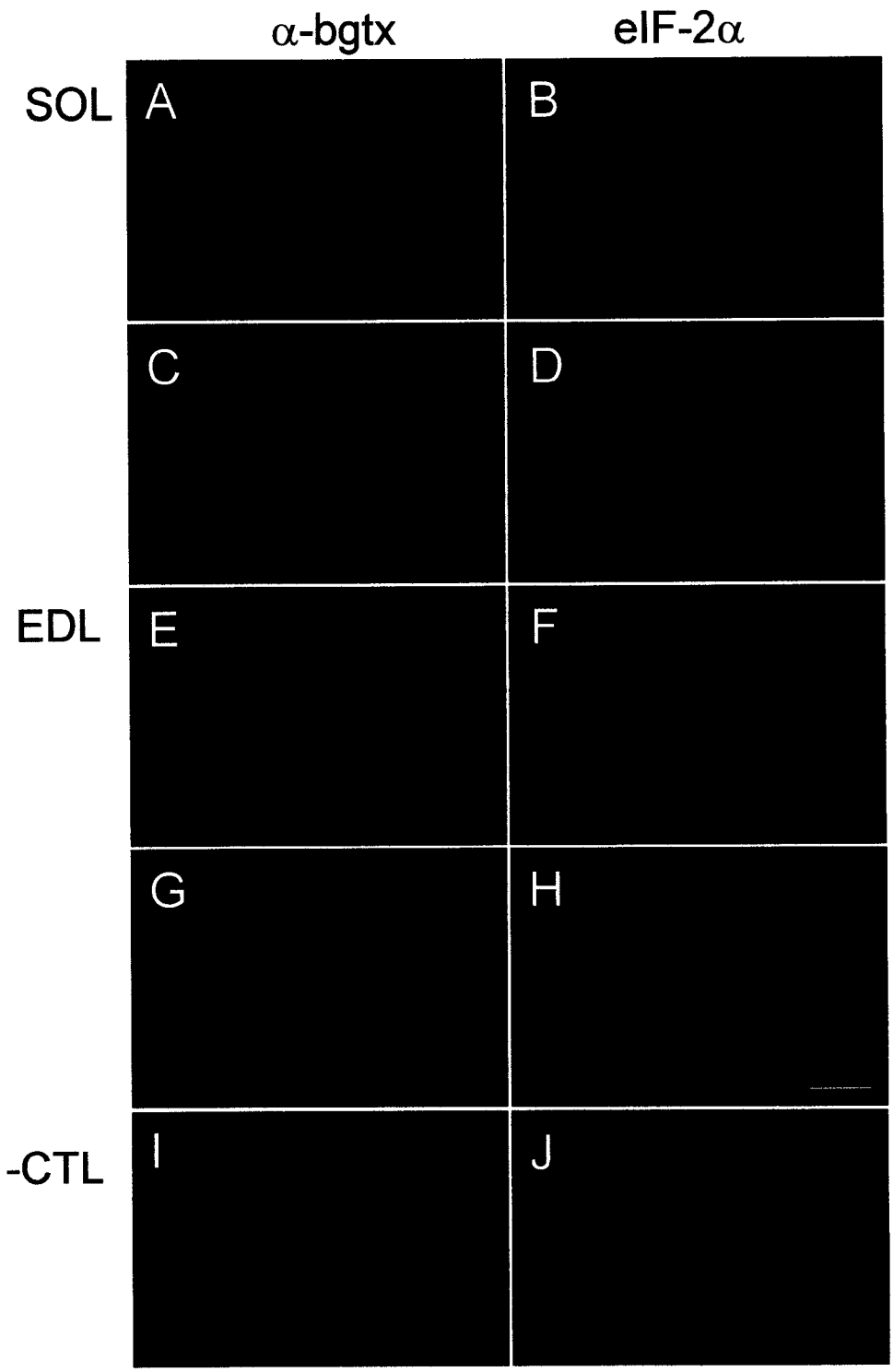

α-bgtx
eIF-4E
SOL
A
B
C
D
EDL
E
F
G
H
- CTL
I
J

Figure 9. Co-localization of translation initiation factor eIF-2α with neuromuscular junctions in mouse SOL and EDL muscles

Alpha-bungarotoxin was used to indicate the presence of neuromuscular junctions by labelling acetylcholine receptors in SOL (A-D) and EDL (E-J) sections of C57/BL mice. Sections were also immunolabelled with a eIF-2α specific antibody. No primary antibody was added as negative controls to indicate antibody specificity (I, J). Note distinct co-localization of the translation initiation factor with neuromuscular junctions. Bar = 150 μm.


α-bgtx
eIF-2α
SOL
A
B
C
D
EDL
E
F
G
H
-CTL
I
J

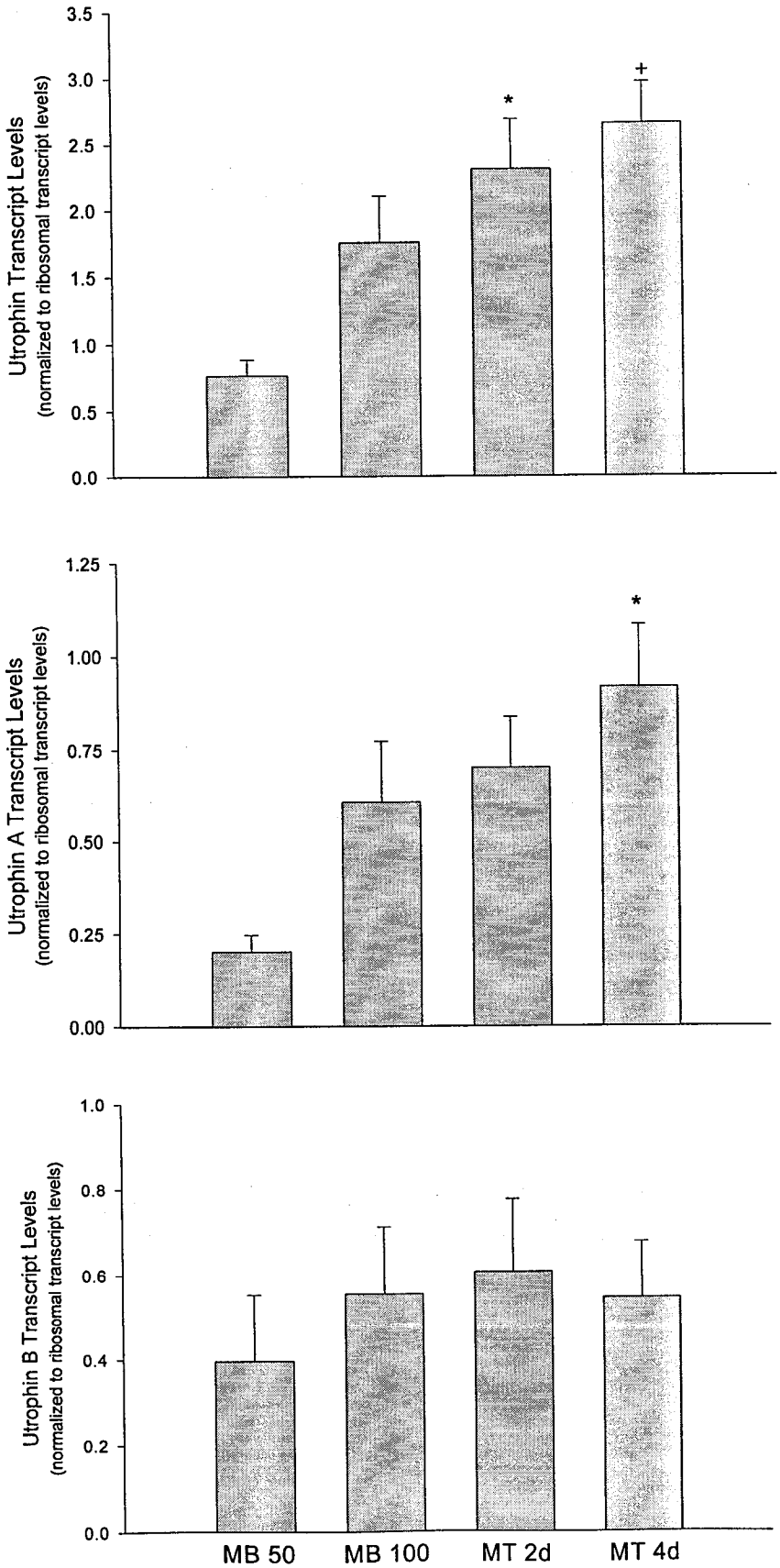
3.2 Effects of Myogenic Differentiation On Utrophin Transcript Levels

In previous studies (Gramolini *et al.*, 1999c; Perkins *et al.*, 2001), total utrophin transcript levels were shown to increase with myogenic differentiation. To determine which transcript was responsible for this increase, RNA was extracted from myoblasts at 50 and 100% confluence, as well as from two and four-day-old myotubes. RT-PCR was performed to amplify total utrophin, utrophin A or utrophin B transcripts specifically (Figure 10).

Total utrophin transcript levels were shown to increase significantly by approximately 2-3 folds at two days of differentiation compared to myoblasts at 50% confluence, as reported previously (Gramolini and Jasmin, 1999; Perkins *et al.*, 2001). Total utrophin transcripts were even higher in four-day-old myotubes compared to myoblasts at 50% confluence. Utrophin A transcript levels increased in parallel with total utrophin transcript levels. Utrophin A transcript levels were significantly higher in four-day-old myotubes than in myoblasts at 50% confluence. Utrophin B transcript levels did not change significantly with myogenic differentiation. These results indicate that the observed increase in total utrophin transcript levels is most likely due to the similar increase in utrophin A transcript levels and the expression of utrophin A seems to be influenced by factors involved in myogenic differentiation. The transition from myoblasts to myotubes appears to have no significant impact on utrophin B transcript levels, further suggesting that utrophin transcripts are differentially regulated.

Figure 10. Transcript levels throughout myogenic differentiation

C2C12 myoblasts were maintained and harvested once they reached 50 (MB 50) and 100 (MB 100) % confluency or upon reaching confluence, myoblasts were induced to differentiate by adding low serum media (differentiation media) and harvested 2 (MT 2d) or 4 (MT 4d) days following addition of differentiation media. RNA was extracted and RT-PCR was performed using primers specific for total utrophin, utrophin A or utrophin B transcripts. Transcript levels were normalized to ribosomal message levels. Mean $\pm$ Std. error are shown. Results represent 3 independent experiments performed in duplicate for all time-points. *Asterisks* denotes significant difference ($p < 0.05$ *versus* Myoblasts 50 % confluence). *Cross* denotes significant difference ($p < 0.01$ *versus* Myoblasts 50 % confluence).


Utrophin Transcript Levels
(normalized to ribosomal transcript levels)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
+
Utrophin A Transcript Levels
(normalized to ribosomal transcript levels)
1.25
1.00
0.75
0.50
0.25
0.00
*
Utrophin B Transcript Levels
(normalized to ribosomal transcript levels)
1.0
0.8
0.6
0.4
0.2
0.0
MB 50
MB 100
MT 2d
MT 4d

3.3 Utrophin A and B 5'UTRs Are Very Different From Each Other

Reporter gene constructs were generated to examine the effects of the different utrophin 5'UTRs on gene expression. The parental vector, used in all experiments, was a LacZ expressing reporter gene driven by a constitutively active CMV promoter. The parental 5'UTR was removed and the utrophin A or B 5'UTR was inserted using primer-generated restriction sites. The full utrophin A 5'UTR sequence has not been published and required additional steps in the cloning process. Utrophin A 5'UTR was amplified using a 5' primer that recognized the transcription initiation site and a 3' primer that included the first few codons in the coding region. The fragment was sequenced and re-amplified with new primers that excluded the ATG. Full utrophin A 5'UTR consists of a 509-nucleotide fragment from the transcription start site to the last nucleotide before the translation initiation site (figure 11). The utrophin B 5'UTR sequence has been previously published (Burton *et al.*, 1999) and was amplified using the reported primers, to which restriction sites were added for cloning purposes. Utrophin B 5'UTR was cloned and was identical to the previously reported 74 nucleotide fragment (figure 11). Both fragments were each inserted into a pCMV•SPORT-βgal vector lacking its own 5'UTR (see figure 7 and Table II). These constructs were used in all cell culture and animal experiments.

UTRdb (Pesole *et al.*, 2000, 2002) is a specialized database that contains sequences of functional elements that have been found in untranslated regions of eukaryotic mRNAs. Both utrophin 5'UTR sequences were submitted to this database to identify if they contained any known functional elements. No matches

Figure 11. Utrophin A and B 5'UTR sequences.

RT-PCR was performed on muscle RNA using specific primers for each 5'UTR. Utrophin A primers (red) amplified a 509 nucleotide fragment and utrophin B primers (blue), a 74 nucleotide fragment. The 5' primers contain an *Nco*I restriction site and the 3' primers an *EcoR*I restriction site that were used for cloning purposes. The putative IRES (purple) consists of the 93 nucleotides upstream of the translation initiation site.

Utrophin A 5'UTR (509 nucleotides)

UGUUGGGAGUCGCCCUUCCCCCCGAGGAGGGGAGCGGGCAGCGCCCGGGC
GGCGGGAUUUCUCACCCCAGGGCCGGAGGAAGCCCUGGGCUCCUCAACC
GAUCGGGCCAACCUCCCGGGAGCGGCGCCCCUUUUUCUUUCGGGUCAUU
CCUGCAGAGGGAAAACUCCUGCGUGCGCCUGCCCCCAGGCUAGCGUUUG
GACUGUUUUGUUUUUGGCGGAACUACCGGGCAGGAAGAUUGCACAAGUC
AGGGGGCGUUUCCAAUCGGGUGUCAAUUUUGGAUCUCCCCCCCCCCACC
CCCCUUUGGAUCUUGUCGGGCUUUCCACGUUUCACUUAAAAAAAAUUCU
UGUAACGUCCAAGGCAAGCUCCCCACGACCCGAACACGCCCAGAGGAAC
CUUGGGGCCUCUGGGACUUGGCUA**GCAGGUAUUCAUGCUAGCCUGGACC**
AUUUUUCAGAUUUAGCCUUCAGAAGAGGAUCUGGGAAAGCCUUUGGAUU
AUACUGAAACUCAUUCAAG

Utrophin B 5'UTR (74 nucleotides)

CCCAGUGUGCAGUUCGCGGGCGGCUUUUGUGUUGAUUUCCUUCACAGUU
UCCCUCAUCUCAGCCACUGUAGGUG

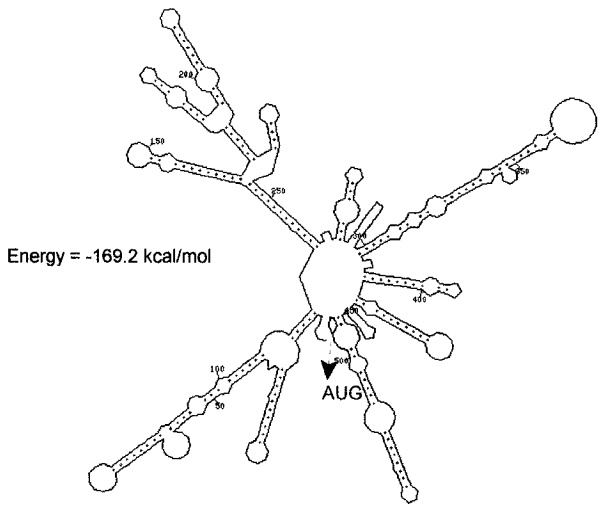
were recognized in the utrophin B 5'UTR sequence. The database did identify a possible IRES in the 93 nucleotides 5' of the AUG of utrophin A 5'UTR. Activity of this element on utrophin expression, if any, remains to be elucidated.

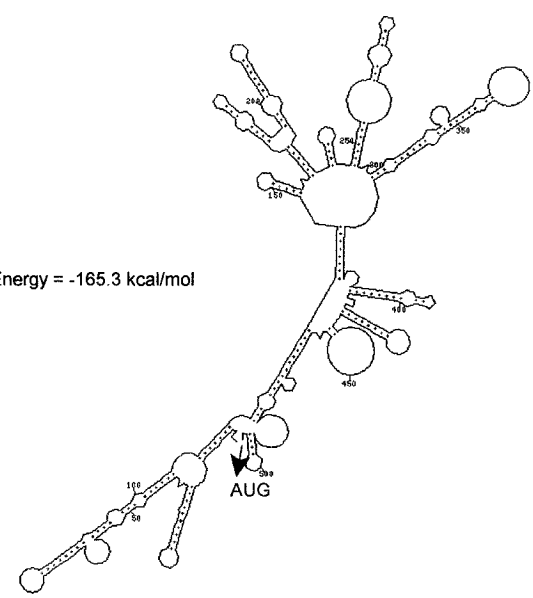
To examine if utrophin 5'UTRs could form stable secondary structures which hinder translation, both sequences were analyzed with *mfold* using the Zuker algorithm (Zuker *et al.*, 1981, 1991; Mathews *et al.*, 1999) . Twenty-two plausible structures were suggested for utrophin A 5'UTR whereas utrophin B 5'UTR had four predicted structures. It has been well established that secondary structures with the lowest free energy are favoured. These structures are only suggested and several others could exist (for examples, see figure 12). The coding sequence following the AUG could also affect secondary structure. Therefore, these diagrams can only be used to portray the general idea that the utrophin A 5'UTR has the potential to form more intricate secondary structures than the utrophin B 5'UTR.

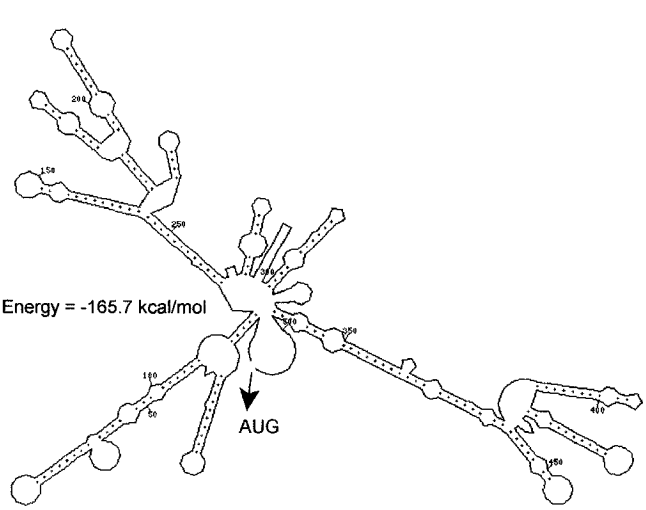
Figure 12. Predicted secondary structures of the 5'-untranslated regions of mouse A and B utrophin mRNA

Schematic representation of plausible secondary structures that can be formed based on their sequence. Only 3 examples are shown for each utrophin 5'UTR. Note the intricate secondary structures utrophin A 5'UTR can form compared to utrophin B 5'UTR. Translation initiation start sites are indicated by ATG with an arrow. Corresponding free energies are indicated to the left of each structure. Most probable structures have the lowest free energies.

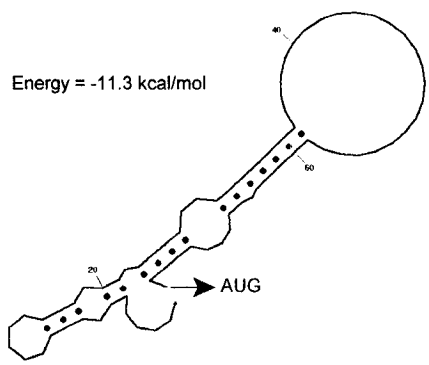
Utrophin A 5'UTR

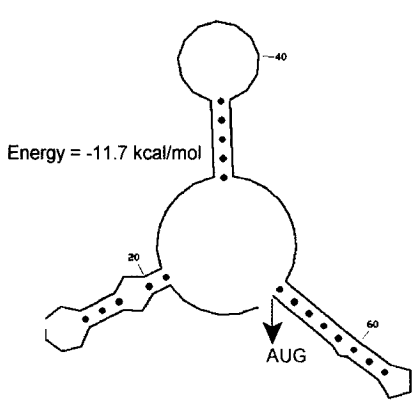


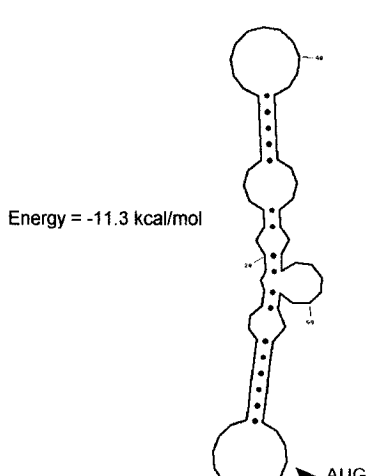




Utrophin B 5'UTR







3.4 Utrophin A and B 5'UTRs Do Not Have Differential Effects On Message Stability

In several studies, 5'UTRs have been found to affect message stability (for review see Linz *et al.*, 1997) by containing stabilizing/destabilizing elements (Arnold *et al.*, 1998; Vilela *et al.*, 1999) that interact with RNA-binding proteins (Ruiz-Echevarria and Peltz, 2000). We wanted to determine if the utrophin 5'UTRs had differential effects on β-galactosidase message stability.

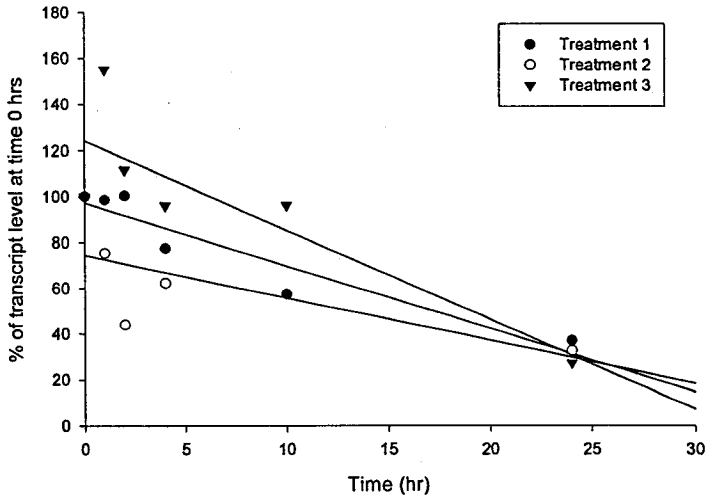
C2C12 myoblasts were transfected with utrophin 5'UTR constructs and induced to differentiate for four days in differentiation media. Reporter transcript half-lives were then determined by treating cells with Actinomycin D, an antibiotic compound that inhibits cell proliferation by forming complexes with DNA and interfering with RNA synthesis. Following the addition of Actinomycin D, RNA was extracted from treated cells at different time-points (0, 1, 2, 4, 10 and 24 hours of treatment) and RT-PCR was performed (figure 13 and 14).

Transcript half-lives for both utrophin 5'UTR reporter constructs were very similar, suggesting that utrophin 5'UTRs do not affect message stability differentially in cell culture. The half-life measured for the utrophin A 5'UTR construct was 14 hr whereas the utrophin B 5'UTR gave way to a 16-hr half-life. No significant differences were seen between the two utrophin 5'UTR half-lives.

Figure 13. Transcript half-life in myotubes

Myoblasts were transfected with appropriate constructs (UTR A or UTR B) and allowed to differentiate for 4 days. Myotubes were treated with Actinomycin D (4 μg/ml) and RNA was extracted at different time-points. RT-PCR was performed with β-galactosidase specific primers. Three independent experiments were performed in duplicate for each construct and linear regression was performed for each treatment. Calculated transcript half-lives for the utrophin A 5'UTR and utrophin B 5'UTR were approximately 14 and 16 hours, respectively. No significant differences were seen between both utrophin 5'UTRs.

A) pCMV-UTR A



B) pCMV-UTR B

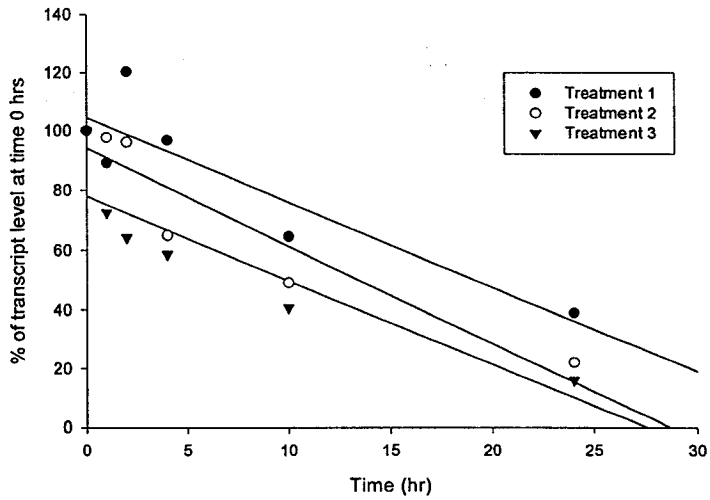
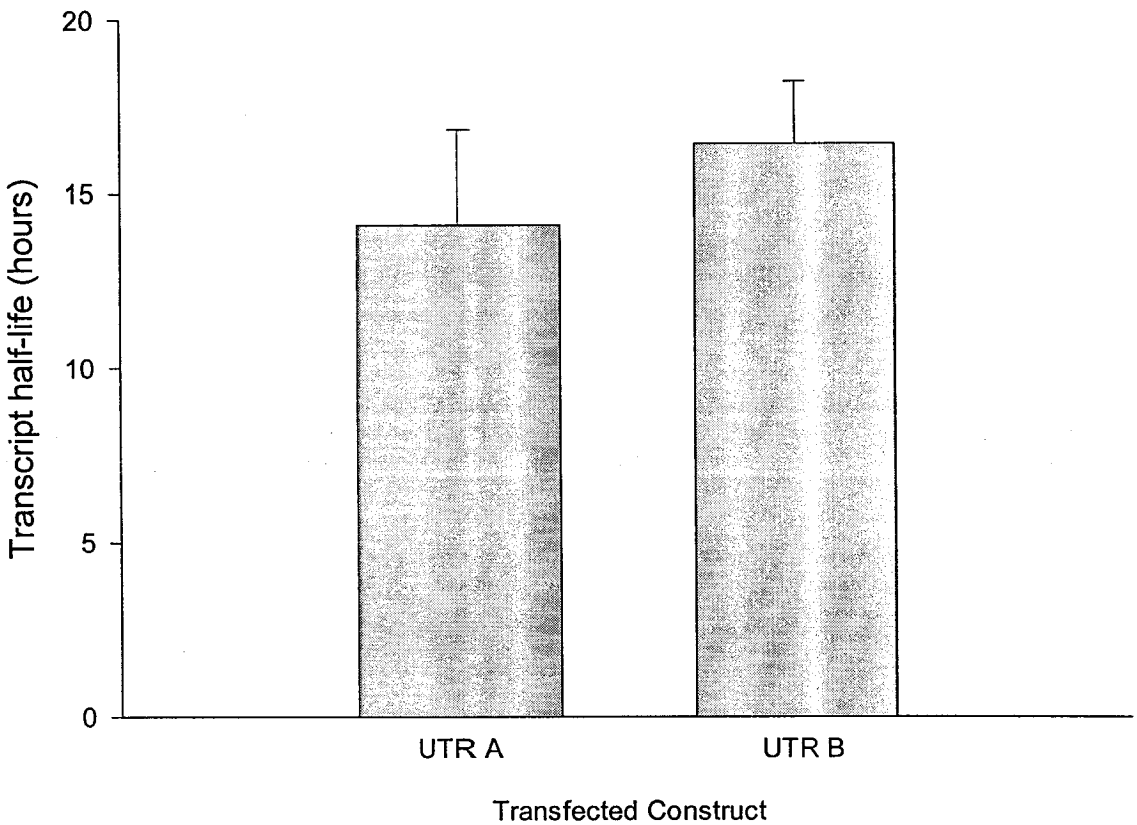


Figure 14. Utrophin A and B 5'UTR transcript half-life

Four day-old transfected myotubes were treated with Actinomycin D (4 μg/ml) to determine the transcript half-life. For both utrophin 5'UTR constructs, half-lives represent the average of 3 independent experiments performed in duplicate. Linear regression was performed for each treatment. No significant differences on transcript stability were seen between the two utrophin 5'UTR constructs ($p > 0.05$).


20
15
10
5
0
Transcript half-life (hours)
UTR A
UTR B
Transfected Construct

3.5 No Indication of Translational Regulation In Cell Culture

3.5.1 Effects of utrophin 5'UTRs in myoblasts

Following the study of utrophin 5'UTR constructs and their half-lives, we wanted to examine their effects on both transcript and protein levels in myoblasts. C2C12 myoblasts were transfected and harvested either for RNA or protein assays. RT-PCR was performed with primers specific for either β-galactosidase or CAT message.

CAT levels were uniform with either of the co-transfected reporter plasmids, which demonstrated constant transfection efficiency (figure 15). However, transcript levels were different for each construct (figure 16). The parental construct had very high transcript levels compared to the two utrophin 5'UTR constructs. Utrophin A 5'UTR transcript levels were significantly lower than parental transcript levels. Utrophin B 5'UTR transcript levels were moderately higher, approximately two folds, than utrophin A 5'UTR levels. Yet these were not significantly lower than parental transcript levels. These results were consistent within five independent experiments. Both mouse utrophin 5'UTRs resulted in lower transcript levels in myoblasts when compared to parental transfected cells.

To verify if protein levels were a direct correlation of transcript levels, β-galactosidase activity assays were performed and normalized to CAT activity levels. C2C12 cells were transfected, harvested and activities were measured. Protein levels seem to reflect transcript levels, where measured activity was significantly higher in parental transfected cells than utrophin A 5'UTR transfected cells. To a

Figure 15. Effects of mouse utrophin 5'UTRs on β-galactosidase transcript levels in myoblasts

Representative example of ethidium-bromide stained agarose gels of (A) β-galactosidase and (B) CAT PCR products. RNA samples were pooled from 5 independent duplicate transfections. A negative control for transfection (1- no plasmid in the transfection treatment) and for contamination in the RT-PCR (2- no RNA and 3- no Reverse Transcriptase) were also performed. Note the difference between parental transcript level and the two utrophin 5'UTR constructs.

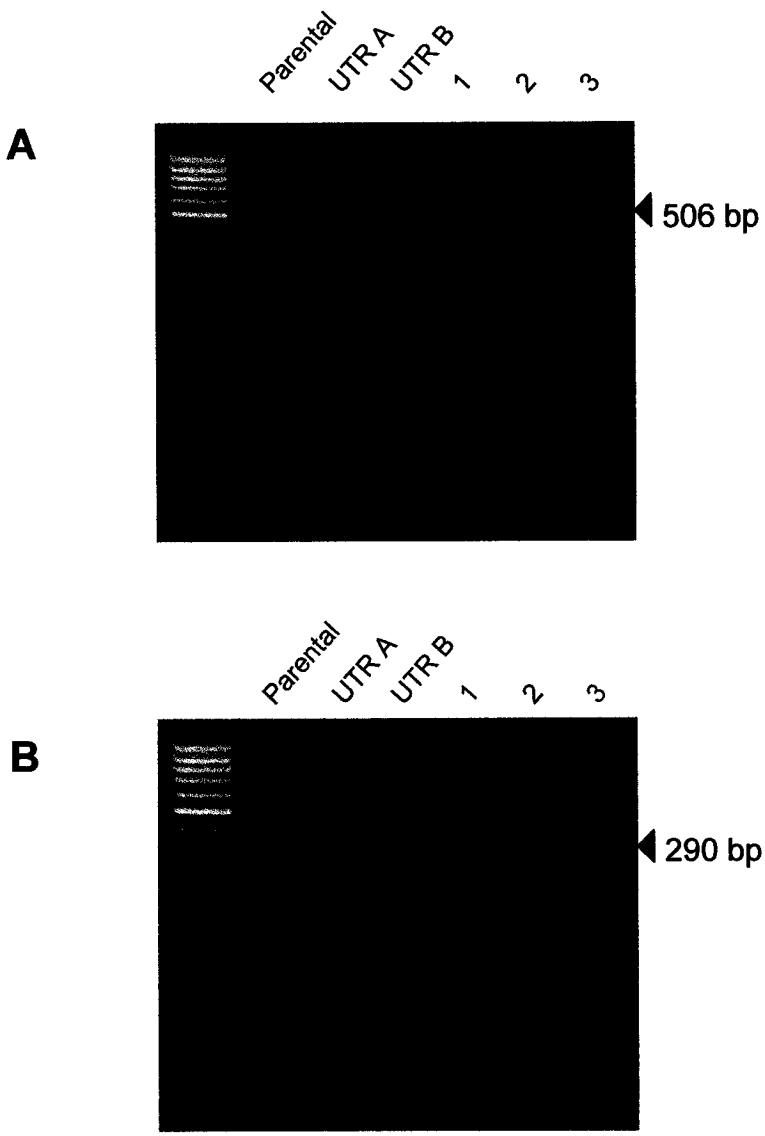
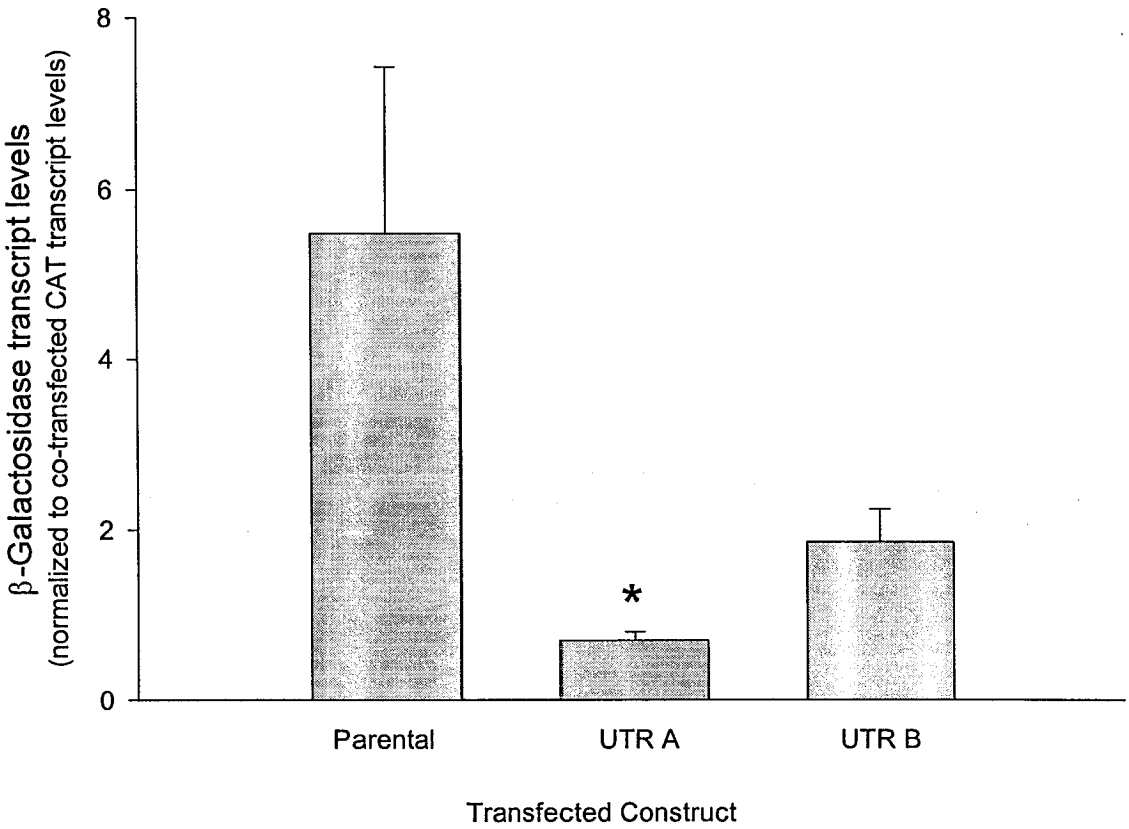

A
Parental
UTR A
UTR B
1
2
3
506 bp
B
Parental
UTR A
UTR B
1
2
3
290 bp

Figure 16. Mouse utrophin A 5'UTR affects transcript levels of reporter gene in myoblasts

Myoblasts were co-transfected with reporter gene constructs and a CAT plasmid. RNA was extracted from myoblasts with TRIzol and DNase I treated. Transcript levels were measured by RT-PCR and values were normalized to measured CAT transcript levels. *Asterisks* denotes significant difference ($p < 0.05$ *versus* Parental). Mean $\pm$ Std. error are shown; results represent 5 independent experiments performed in duplicate for all constructs.


β-Galactosidase transcript levels
(normalized to co-transfected CAT transcript levels)
8
6
4
2
0
*
Parental
UTR A
UTR B
Transfected Construct

lesser degree, measured activities in utrophin B 5'UTR transfected cells were also lower than parental activity levels (figure 17). Activity levels in utrophin A 5'UTR transfected cells were approximately two-fold lower than in cells transfected with utrophin B 5'UTR, but no significant differences were established.

To confirm protein activity assays, transfected cells were also stained for β-galactosidase. These cells were scanned (figure 18) and observed more closely with a microscope (figure 19). Staining intensity appeared to mirror measured activity. These results suggest that in myoblasts, utrophin 5'UTRs may have little or no translational regulation, since protein levels reflect transcript levels for each construct.

Figure 17. Mouse utrophin A 5'UTR affects β-galactosidase activity in myoblasts

Myoblasts were co-transfected with reporter gene constructs and a CAT plasmid. Cells were harvested as myoblasts. β-galactosidase activity was measured and values were normalized to measured CAT activity. *Asterisks* denotes significant difference ($p < 0.01$ *versus* Parental). Mean ± Std. error are shown; results represent 5 independent experiments performed in duplicate for all constructs.

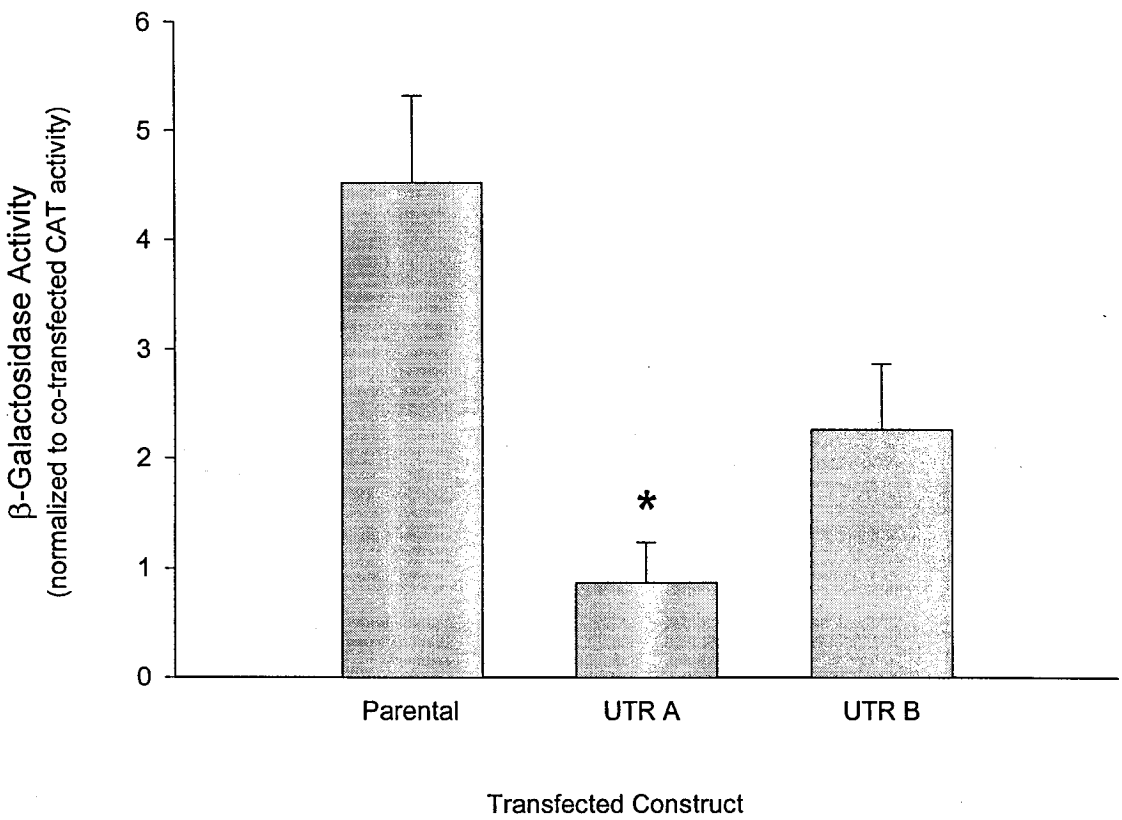

β-Galactosidase Activity
(normalized to co-transfected CAT activity)
6
5
4
3
2
1
0
*
Parental
UTR A
UTR B
Transfected Construct

Figure 18. Histochemical β-galactosidase staining of transfected myoblasts

Myoblasts were transfected in duplicate with appropriate constructs. Cells were then fixed and stained for β-galactosidase using an X-gal solution. P1 and P2 correspond to cells transfected with Parental; AF1 and AF2, UTR A; and BF1 and BF2, UTR B. Note decrease in β-galactosidase staining in the utrophin A 5'UTR transfected cells.

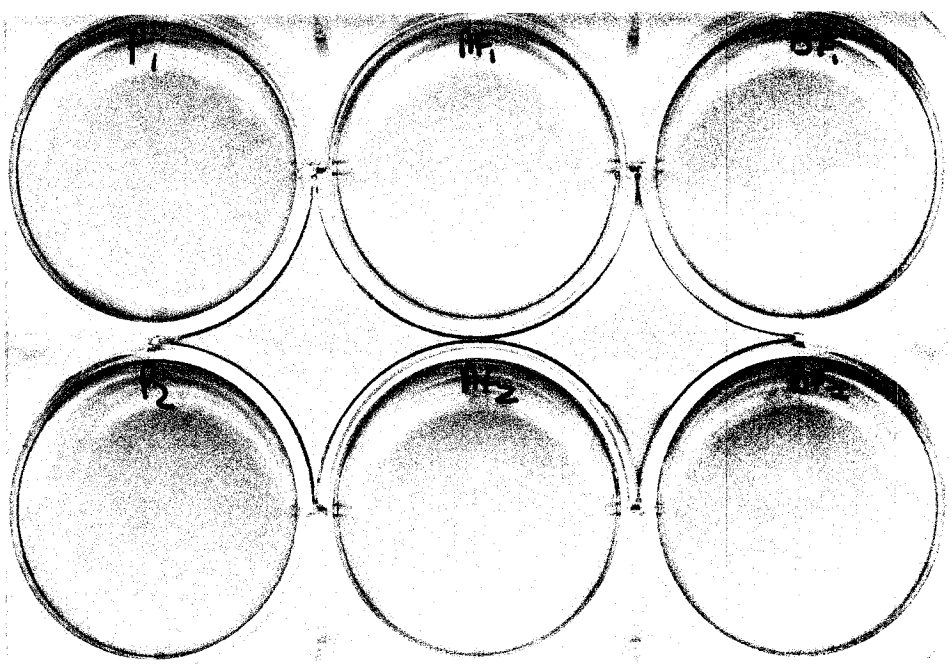
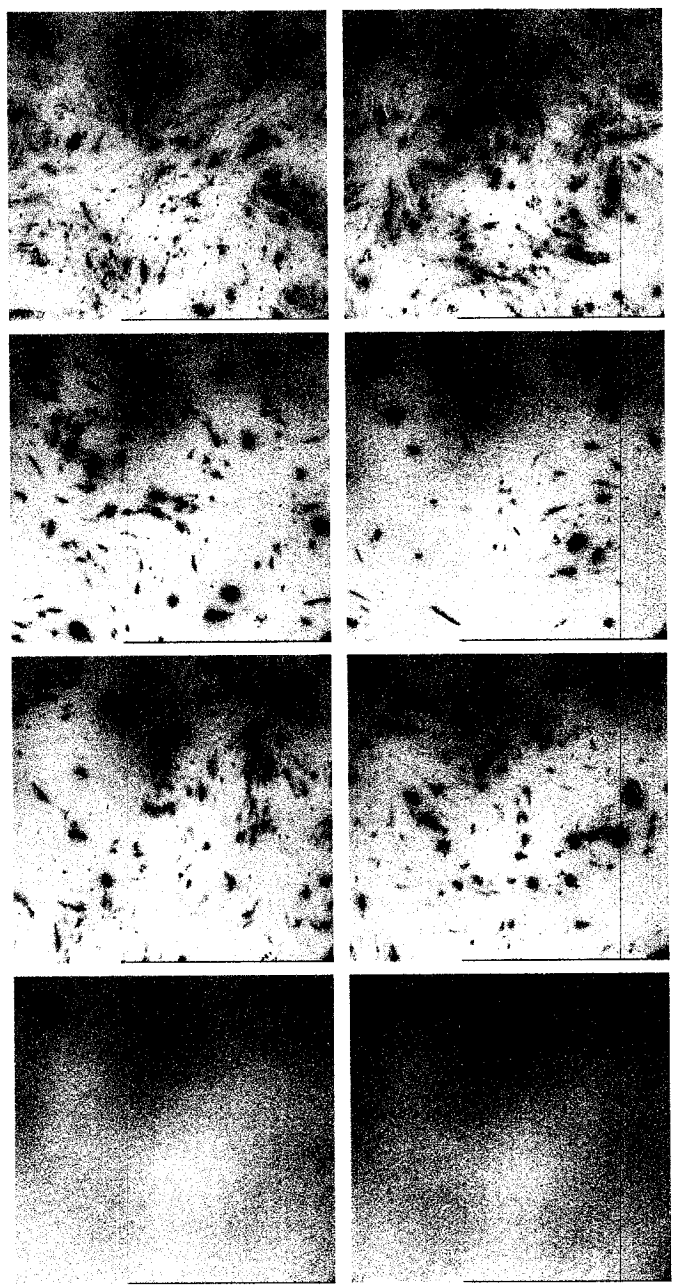


Figure 19. Expression of reporter gene constructs in transfected myoblasts

Cells were transfected with the appropriate construct; parental vector (A, B), utrophin A 5'UTR construct (C, D) and utrophin B 5'UTR construct (E, F). Controls (G, H) were exposed to same transfection treatment, but in the absence of reporter plasmid. Histochemical staining for β-galactosidase was performed on myoblasts one day after transfection. (Mag. = 20x).



3.5.2 Effects of utrophin 5'UTRs in myotubes

Since utrophin A transcript levels were shown to increase significantly through myogenic differentiation, we wanted to compare the effects of utrophin 5'UTRs in myotubes and determine if they differed from those seen in myoblasts. C2C12 cells were transfected as myoblasts, allowed to differentiate for four days and harvested for RNA or protein assays.

Transcript levels (figure 20) in myotubes were not the same as was seen in myoblasts. Parental transcript levels were higher than in utrophin A 5'UTR transfected cells, as seen previously in myoblasts. However, transcript levels for utrophin B 5'UTR transfected cells were similar to parental levels. No significant differences were seen between either utrophin 5'UTR and the parental plasmid.

Activity levels were assayed to determine if translational regulation would be seen in myotubes. Once again, protein levels mirrored transcript levels (figure 21), suggesting a direct correlation between transcript levels and protein activity levels. Utrophin A 5'UTR transfected cells showed very low activity levels, approximately 4-fold less than the parental transfected cells. Cells transfected with the utrophin B 5'UTR exhibited similar protein activity levels to parental transfected cells. β-galactosidase staining was also performed on transfected myotubes (figure 22 and 23) which confirmed results obtained in protein activity assays.

Figure 20. Transcript levels in transfected myotubes

Myoblasts were co-transfected with the appropriate reporter construct and CAT plasmid. Cells were then induced to differentiate in low serum media for 4 days. Myotubes were harvested, RNA extracted and RT-PCR performed. β-galactosidase transcript levels were normalized to CAT transcript levels. Results represent 5 independent duplicate transfections for each construct. Mean ± Std. error are shown. No significant differences were seen for either utrophin 5'UTR.

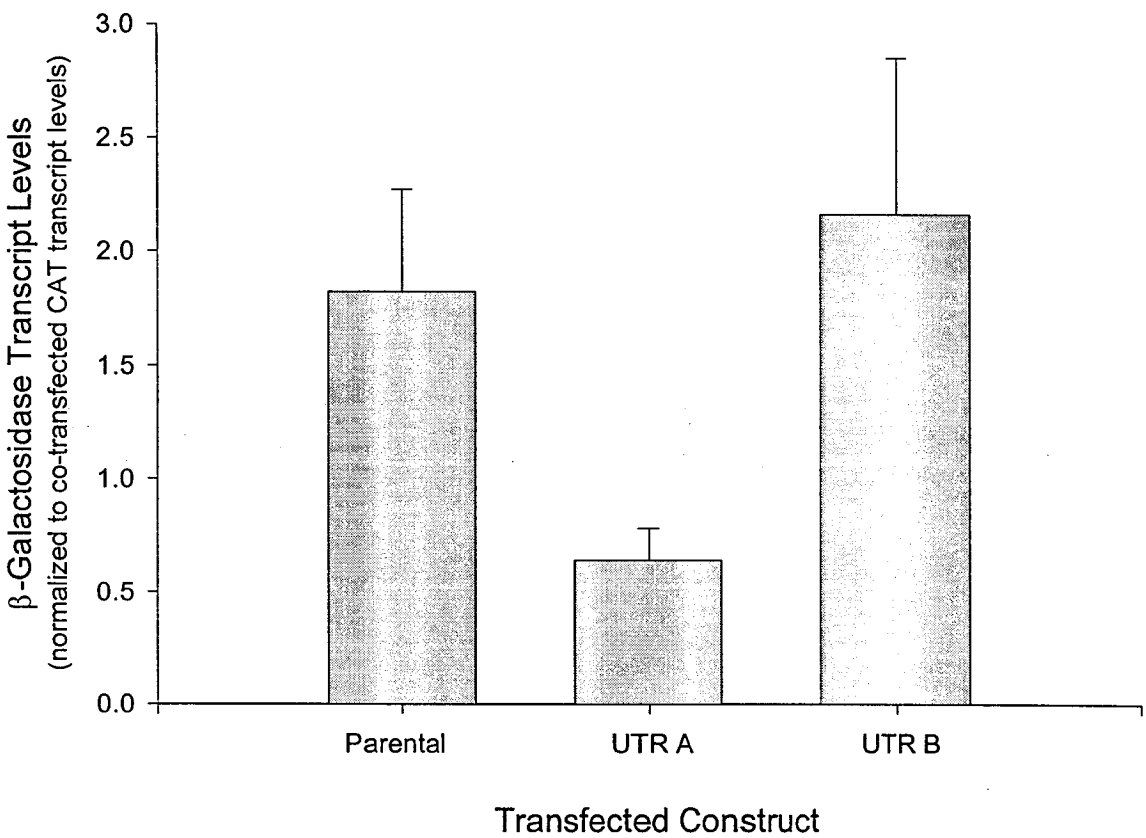

β-Galactosidase Transcript Levels
(normalized to co-transfected CAT transcript levels)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Parental
UTR A
UTR B
Transfected Construct

Figure 21. Mouse utrophin A 5'UTR affects expression of reporter gene construct

Myoblasts were co-transfected with appropriate reporter gene and CAT plasmid. Myotubes were harvested after cells were in differentiation media for 4 days. β-galactosidase activity was measured and values were normalized to measured CAT activity levels. *Asterisks* denotes significant difference ($p < 0.01$ *versus* Parental). Mean ± Std. error are shown; results represent 5 independent duplicate experiments.

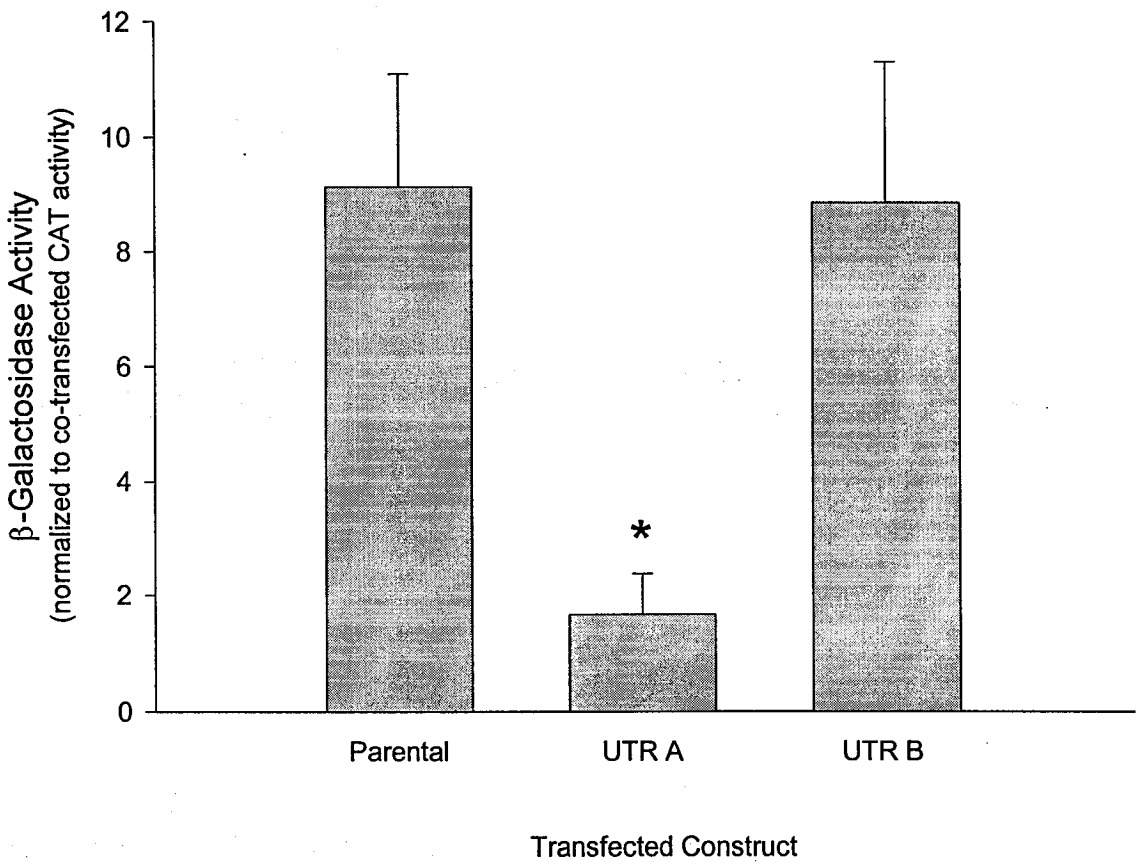

12
10
8
6
4
2
0
β-Galactosidase Activity
(normalized to co-transfected CAT activity)
*
Parental
UTR A
UTR B
Transfected Construct

Figure 22. Histochemical β-galactosidase staining of transfected myotubes

Myoblasts were transfected in duplicate, with the appropriate constructs and induced to differentiate into myotubes for 4 days. Cells were then fixed and stained for β-galactosidase using an X-gal solution. P1 and P2 correspond to cells transfected Parental; AF1 and AF2, UTR A; and BF1 and BF2, UTR B. Note the considerable decrease in staining in utrophin A 5'UTR transfected cells.

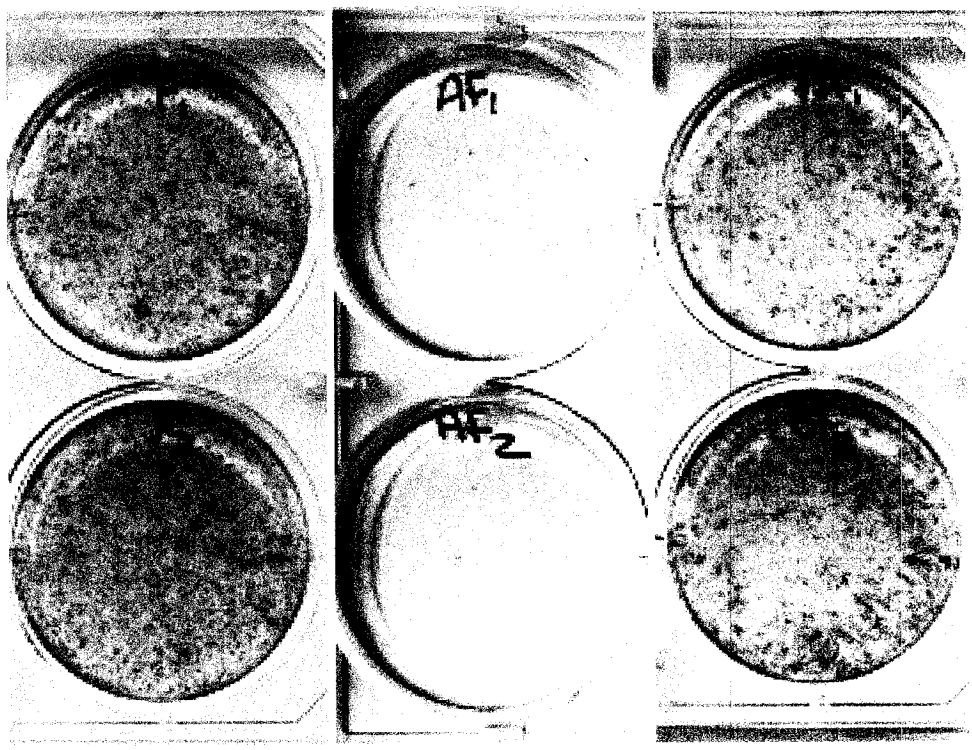
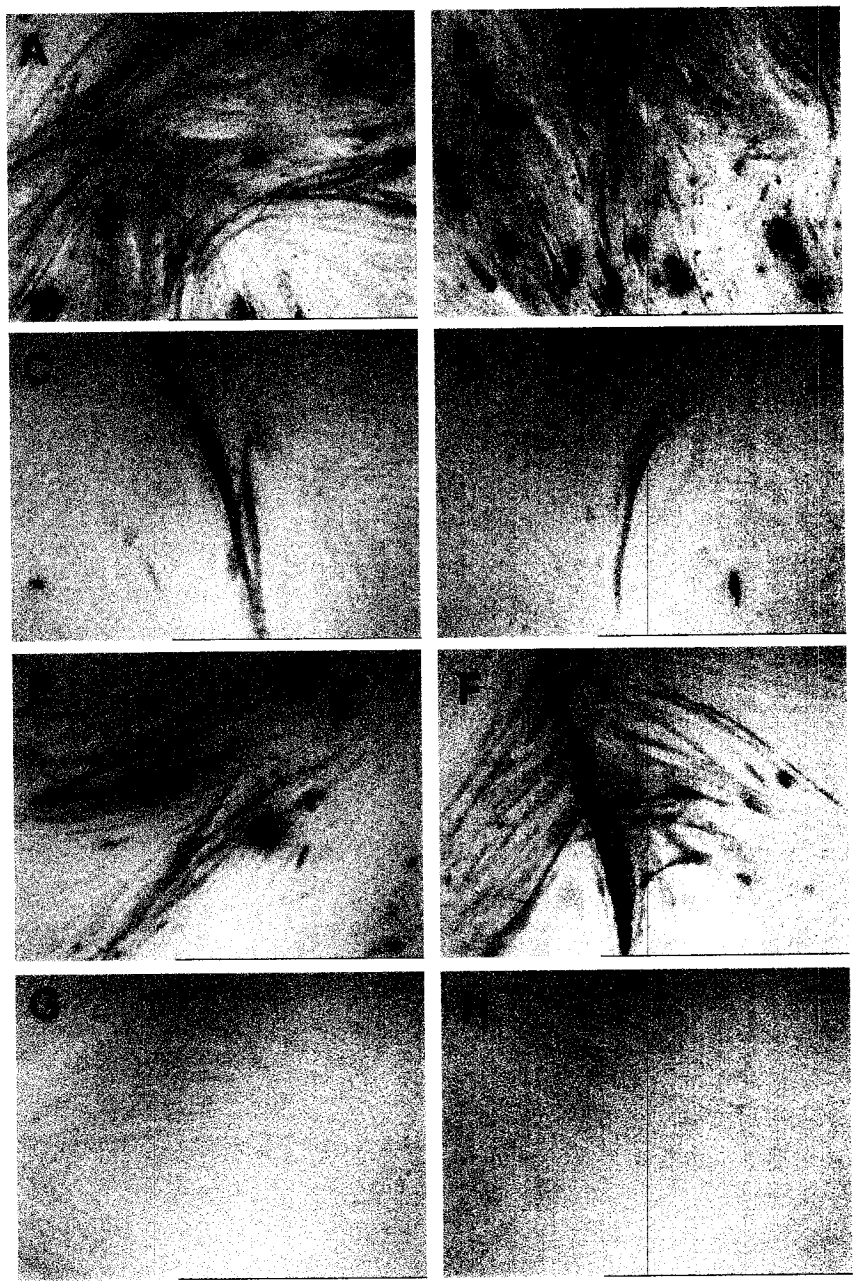

AF1
AF2

Figure 23. Expression of reporter gene constructs in transfected myotubes

Myoblasts were transfected with the appropriate construct; Parental (A, B), UTR A (C, D) and UTR B (E, F). Cells were induced to differentiate in low serum media for 4 days. Controls (G, H) were exposed to the same transfection treatment but in the absence of a plasmid. Histochemical staining for β-galactosidase was performed on the fourth day of differentiation. Note the considerably lower staining in utrophin A 5'UTR transfected cells than with the other two vectors (Mag. = 20x).


A
F

3.5.3 Direct correlations between transcript and protein levels in cell culture

An idea of the translatability of a transcript can be assessed by taking the ratio between RNA levels and protein levels, which gives rise to an estimate of the translation efficiency of the particular message (see Cok and Morrison, 2001; Sakurai and Ishihama, 2001; Shao and Ismail-Beigi, 2001). When a ratio has an approximate value of one, protein levels are a direct correlation of transcript levels. The ratio of one suggests that there is no translational inhibition, indicating that protein levels are controlled by transcript levels and are not regulated at the level of translation (Sakurai and Ishihama, 2001; Shao and Ismail-Beigi, 2001). A higher ratio however, suggests that there may be translational inhibition since protein levels are lower than the transcript levels.

Transcript (figure 16 and 20) and protein (figure 17 and 21) levels of the reporter constructs, in both myoblasts and myotubes respectively, are expressed as ratios in Table III. Utrophin A and B 5'UTR transcript and activity levels were expressed as a percentage of parental levels. Ratios were approximately equal to one for both utrophin 5'UTRs in myoblasts, which indicate that β-galactosidase activity seen in these transfected cells were most likely due to transcript levels and not to translational regulation. Similarly, ratios seen in myotubes do not suggest translational regulation as they were 1.9 and 1.2 for utrophin A and B 5'UTRs, respectively. These results suggest that there is no apparent translational inhibition in myoblasts or myotubes for either utrophin 5'UTR.

Table III

Comparison of β-galactosidase transcript levels and β-galactosidase activity levels of reporter gene constructs in cell culture

To assess the contribution of utrophin 5'UTRs on translation regulation in cell culture, β-galactosidase transcript levels (Fig. 15 and 19) and β-galactosidase activity (Fig. 16 and 20), from myoblast and myotube experiments respectively, are expressed as ratios. Global expression of the reporter gene is affected mainly by translation inhibition with a higher ratio. However, a ratio of 1 indicates a direct correlation between transcript levels and protein activity levels.

Myoblasts

Construct	β-gal transcript level	β-gal activity level	Ratio
Parental	100.0	100.0	1.0
UTR A	87.1	80.7	1.1
UTR B	66.2	50.2	1.3

Myotubes

Construct	β-gal transcript level	β-gal activity level	Ratio
Parental	100.0	100.0	1.0
UTR A	34.9	18.3	1.9
UTR B	118.6	96.9	1.2

3.6 Translational Regulation In Control and Regenerating Muscle

3.6.1 Effects of different utrophin 5'UTRs in control muscle

One might expect that the different utrophin 5'UTRs would have some impact on translational regulation due to their differences in sequence, length and secondary structures. Even though cell culture experiments indicated no apparent translational regulation in the presence of either utrophin 5'UTR, experiments were performed in mice.

Mouse TA muscles were injected with plasmid DNA and excised one week later. For each muscle, RNA was isolated and RT-PCR was performed. Both utrophin 5'UTRs significantly affected β-galactosidase transcript levels compared to the parental 5'UTR (figure 24). As seen in cell culture, transcript levels measured in parental 5'UTR injected control muscles were significantly higher than in utrophin A or B 5'UTR injected muscles.

Additionally, protein activity levels were measured. Another set of mouse TA muscles were injected and excised one week later. Protein lysates were extracted, from which β-galactosidase and CAT activities were assayed (figure 25). Normalized activity levels in muscles injected with the parental construct were significantly higher than both utrophin A and B 5'UTR injected muscles. No significant differences were seen between the two utrophin 5'UTR constructs.

Figure 24. Both mouse utrophin 5'UTRs affect β-galactosidase transcript levels in injected muscles

Mouse TA muscles were injected with the appropriate constructs. The CAT plasmid was co-injected to control for injection efficiency. Fifty μg of plasmid DNA were injected per mouse. One week later, muscles were excised, frozen and kept at -80 °C until use. RNA was extracted and DNase I treated. Transcript levels were measured by RT-PCR and values were normalized to measured CAT transcript levels. *Asterisks* denotes significant difference ($p < 0.001$ *versus* Parental). Mean ± Std. error are shown; results represent transcript levels measured in 10 injected muscles for Parental and in 12 injected muscles for each utrophin 5'UTR construct.

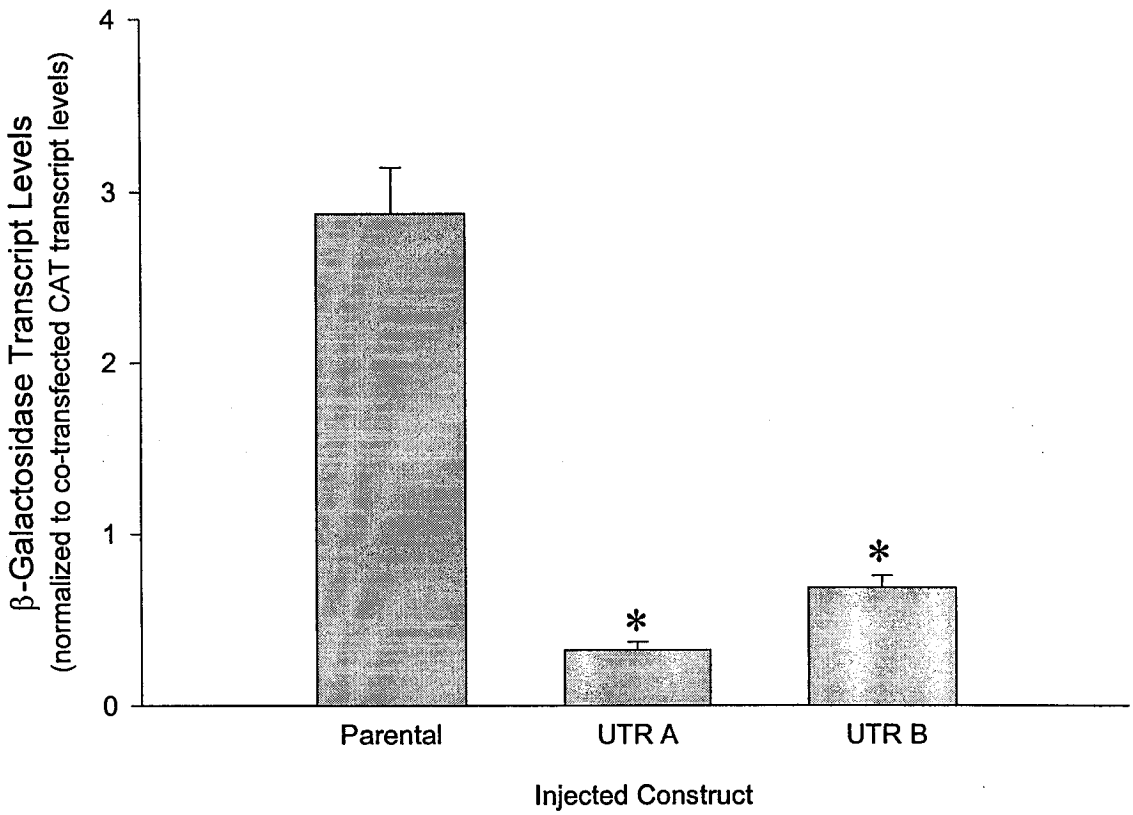
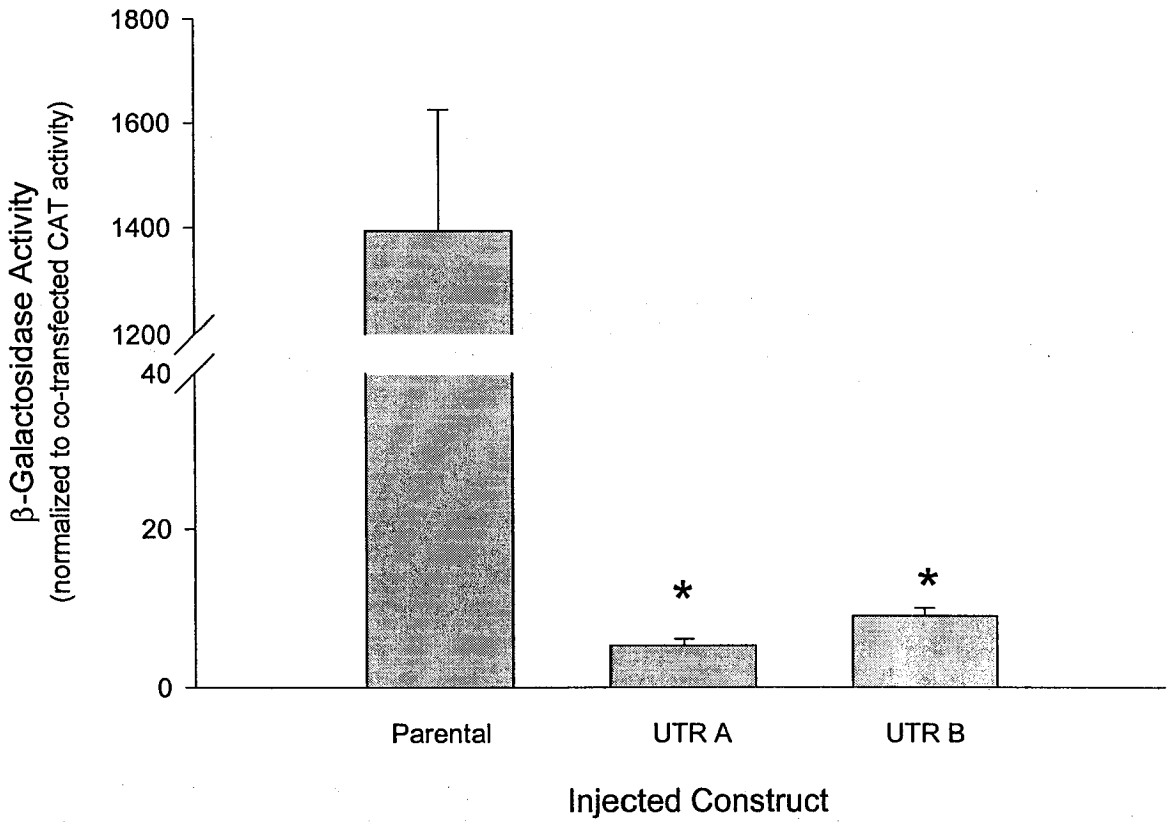

β-Galactosidase Transcript Levels
(normalized to co-transfected CAT transcript levels)
4
3
2
1
0
*
*
Parental
UTR A
UTR B
Injected Construct

Figure 25. Both mouse utrophin A and B 5'UTRs significantly affect β-galactosidase activity in mouse muscle

Each construct was co-injected into both TA muscles of 6 mice with a CAT plasmid. Fifty μg of total DNA were injected per animal. Muscles were excised 7 days following injections. Protein was extracted, assayed for β-galactosidase activity and normalized to CAT activity. *Asterisks* denotes significant difference ($p < 0.001$ *versus* Parental). Mean ± Std. error are shown; results represent activity measured in 12 muscles for each construct.


1800
1600
1400
1200
40
20
0
β-Galactosidase Activity
(normalized to co-transfected CAT activity)
*
*
Parental
UTR A
UTR B
Injected Construct

3.6.2 Effects of utrophin 5'UTRs in regenerating muscle

Since utrophin has been shown to be up-regulated in regenerating muscle (Gramolini *et al.*, 1999b; Galvagni *et al.*, 2002), we wanted to examine if the different utrophin 5'UTRs generate distinct expression profiles in regenerating muscle than seen in control muscles. Cardiotoxin was injected into mouse TA muscles to induce muscle degeneration. Three days later, muscles were injected with plasmid DNA and excised four days after plasmid injection. For each mouse, RNA was extracted from one muscle and protein from the other.

Parental transcript levels were once again significantly higher than in utrophin A or B 5'UTR injected regenerating muscles (figure 26). Transcript levels for both utrophin 5'UTRs were found to be very similar. Protein activity levels were also assayed (figure 27) where significant differences were once again observed between utrophin A or B 5'UTR and parental injected muscles. No significant differences were seen between the utrophin 5'UTR injected muscles.

Figure 26. Mouse utrophin A and B 5'UTRs express similar β-galactosidase transcript levels in regenerating muscle

Each construct was co-injected into both regenerating TA muscles of mice with a CAT plasmid. RNA was extracted and DNase I treated. Transcript levels were measured by RT-PCR and values were normalized to co-injected CAT transcript levels. *Asterisks* denotes significant difference ($p < 0.001$ *versus* Parental). Mean $\pm$ Std. error are shown; results represent levels measured in one TA muscle from 6 different mice for each construct.

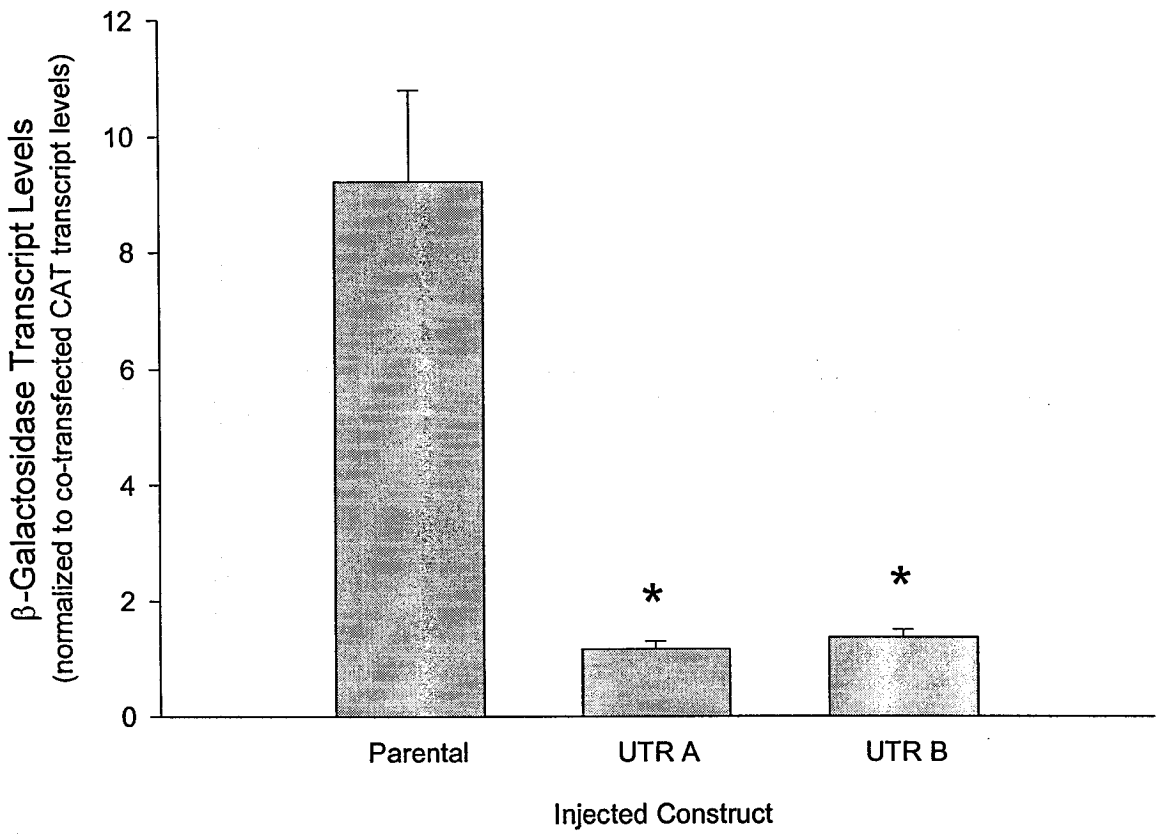
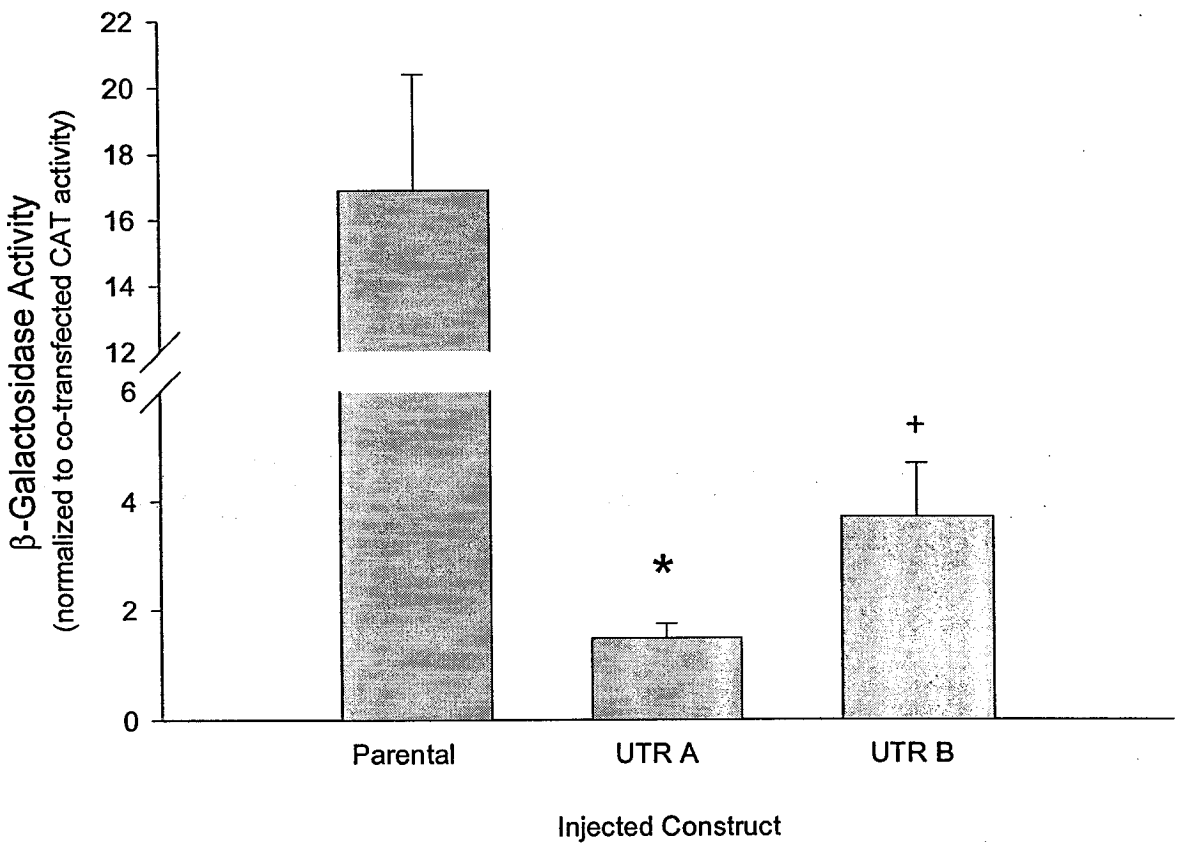

β-Galactosidase Transcript Levels
(normalized to co-transfected CAT transcript levels)
12
10
8
6
4
2
0
*
*
Parental
UTR A
UTR B
Injected Construct

Figure 27. Mouse utrophin 5'UTRs significantly affect β-galactosidase activity in regenerating muscle

Each construct was co-injected with a CAT plasmid into cardiotoxin treated TA muscles of 6 mice. Protein was extracted from excised muscles, assayed for β-galactosidase activity and normalized to CAT activity. *Asterisks* denotes very significant difference ($p < 0.001$ *versus* Parental) and *cross* denotes significant difference ($p < 0.01$ *versus* Parental). Mean $\pm$ Std. error are shown; results represent activity levels assayed in 6 muscles for each construct.


β-Galactosidase Activity
(normalized to co-transfected CAT activity)
22
20
18
16
14
12
6
4
2
0
*
+
Parental
UTR A
UTR B
Injected Construct

3.6.3 *Translational efficiencies in animal studies indicate translational regulation*

For control and regenerating muscles, transcript profiles and/or protein profiles were different from those obtained in cell culture. Translation efficiencies were estimated by calculating ratios between transcript and protein levels (figure 24 and 25 for control; figure 26 and 27 for regenerating muscle). The higher the ratio, the more translation inhibition is present, where lack of protein is not due to transcript levels but possibly to translation of transcripts themselves. Ratios for control muscles are expressed in Table IV. The high ratios suggest that utrophin A and B 5'UTRs exhibit significant translation inhibition in control injected muscles. In normal muscle, low protein activity levels are most likely indicative of translation inhibition due to the utrophin 5'UTRs.

Interestingly, these ratios were not seen in regenerating muscle (Table IV). Similar to ratios seen in cell culture, both utrophin 5'UTRs had ratios close to one, suggesting that protein levels directly correlate to transcript levels. These results propose that translational inhibition by utrophin 5'UTRs is possibly de-repressed in regenerating muscle resulting in increased protein expression. These results could suggest that increased utrophin levels seen in regenerating muscles may reflect a de-repression of translation inhibition.

Table IV

Comparison of β-galactosidase transcript levels and β-galactosidase activity levels of reporter gene constructs in muscle

To assess the contribution of utrophin 5'UTRs on translation regulation, β-galactosidase transcript levels and β-galactosidase activity levels in control TA muscles (Fig. 24 and 25) and regenerating TA muscle (Fig. 26 and 27) are expressed as ratios. Global expression of the reporter gene is affected mainly by translation inhibition with a higher ratio. However, a ratio of 1 indicates a direct correlation between transcript levels and protein activity levels.

Control TA

Construct	β-gal transcript level	β-gal activity level	Ratio
Parental	100.0	100.0	1.0
UTR A	11.3	0.4	28.2
UTR B	14.4	0.6	24.0

Regenerating TA

Construct	β-gal transcript level	β-gal activity level	Ratio
Parental	100.0	100.0	1.0
UTR A	11.6	8.8	1.3
UTR B	13.8	21.9	0.6

3.7 Differential Translation Regulation In *Mdx* Muscle

3.7.1 Utrophin A 5'UTR has an effect on translation in both young and adult mdx muscle

In a recent study (Weir *et al.*, 2002), utrophin A protein levels were 2-4 fold higher in *mdx* muscle compared to healthy muscle. We wanted to examine if increased utrophin A protein levels were due to its 5'UTR. Utrophin B protein levels were similar in both *mdx* and control muscles (Weir *et al.*, 2002), therefore we did not study the effects of utrophin B 5'UTR on gene expression in *mdx* muscle.

In young *mdx* mice (6-7 week-old), the transcript profile was very similar to what was seen in control and regenerating muscles (figure 28). Parental transcript levels differed significantly from utrophin A 5'UTR transcript levels. The protein profile (figure 29) also appeared to reflect results seen in control and regenerating muscles, where the utrophin A 5'UTR resulted in significantly lower activity levels than the parental 5'UTR.

We then examined if utrophin A 5'UTR displayed age-dependent effects. Since it has been suggested that adult *mdx* mice have surpassed critical stages of degeneration/regeneration and utrophin is known as a regeneration-associated protein, plasmid injections were performed on adult (13-18 week-old) *mdx* mice. TA muscles were injected with appropriate constructs and excised as described with control muscles. For each mouse, RNA was extracted from one TA muscle and protein from the other. Transcript levels measured in utrophin A 5'UTR injected muscles were not significantly different from parental injected muscles (figure 30).

Figure 28. Mouse utrophin A 5'UTR significantly affects β-galactosidase transcript levels in young *mdx* mice

Each construct was co-injected with a CAT plasmid into TA muscles of 6 young (5-6 week-old) *mdx* mice. Muscles were excised one week later and RNA was extracted. RT-PCR was performed using primers amplifying a β-gal fragment or a CAT fragment. β-gal transcript levels were normalized to CAT transcript levels. *Asterisks* denotes very significant difference ($p < 0.001$ *versus* Parental). Mean ± Std. error are shown; each bar represents transcript levels measured in 6 muscles.

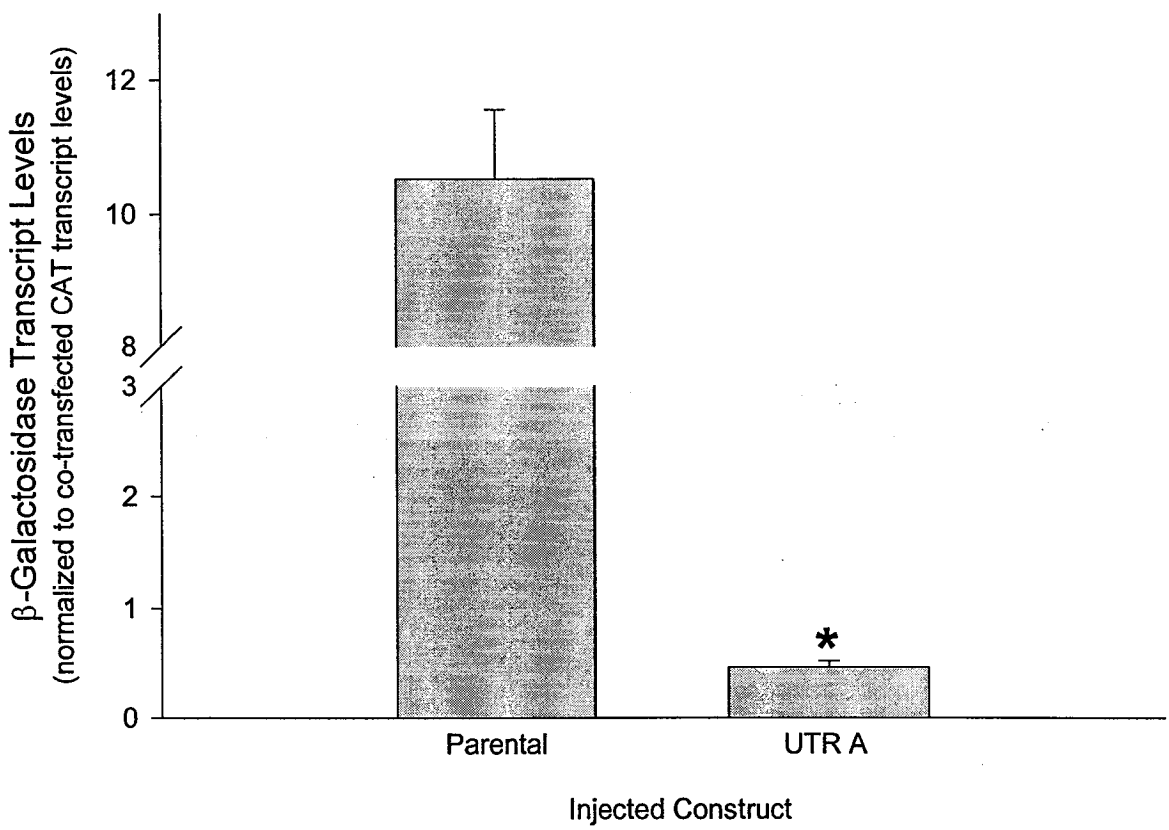

β-Galactosidase Transcript Levels
(normalized to co-transfected CAT transcript levels)
12
10
8
3
2
1
0
*
Parental
UTR A
Injected Construct

Figure 29. Mouse utrophin A 5'UTR affects β-galactosidase activity in young *mdx* mice

Young (5-6 week-old) *mdx* TA muscles were co-injected with either parental or UTR A construct and a CAT plasmid. Muscles were excised one week later and protein was extracted. Measured β-galactosidase activity was normalized to measured CAT activity. *Asterisks* denotes significant difference ($p < 0.05$ *versus* Parental). Mean ± Std. error are shown. Each bar represents activity levels measured in 6 TA muscles.

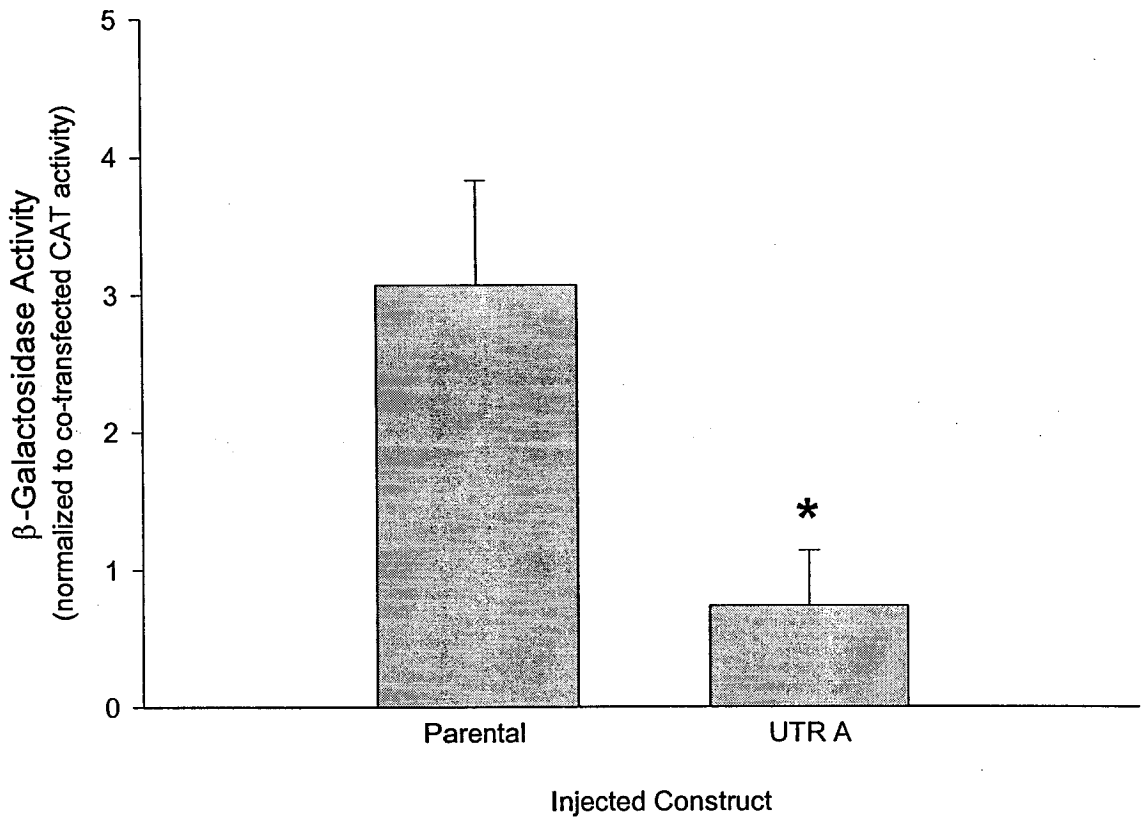
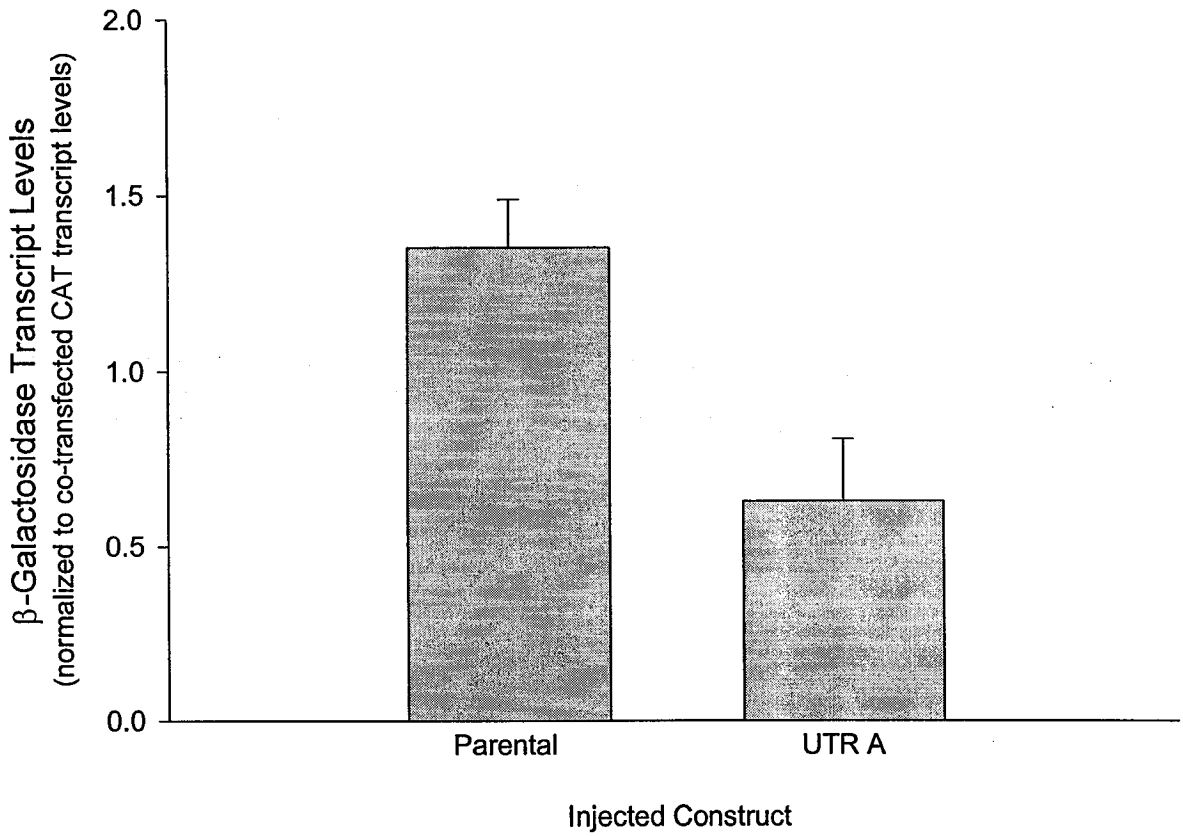

β-Galactosidase Activity
(normalized to co-transfected CAT activity)
5
4
3
2
1
0
*
Parental
UTR A
Injected Construct

Figure 30. Mouse utrophin A 5'UTR does not affect transcript levels in adult *mdx* TA muscle

Adult (13-18 week-old) *mdx* mouse TA muscles were injected with appropriate constructs and a CAT plasmid. Fifty μg of plasmid DNA were injected per animal. One week later, muscles were excised, frozen and kept at -80 °C until use. RNA was extracted and RT-PCR was performed. Mean ± Std. error are shown; results represent transcript levels measured in 3 injected TA muscles for Parental and 4 injected TA muscles for UTR A.


β-Galactosidase Transcript Levels
(normalized to co-transfected CAT transcript levels)
2.0
1.5
1.0
0.5
0.0
Parental
UTR A
Injected Construct

However, a significant difference was seen between protein activity levels in parental and in utrophin A 5'UTR samples (Figure 31). Utrophin A 5'UTR lead to significantly lower protein activity levels than the parental 5'UTR.

3.7.2 Increased translation efficiencies in mdx muscle

Even though trends in transcript levels and protein activities were very similar to those obtained in control and regenerating muscle experiments, estimated RNA (figure 28) to protein (figure 29) ratios were considerably different in young *mdx* muscles (Table V). As mentioned previously, *mdx* muscles have increased utrophin levels. Ratios obtained in these experiments clearly support this increased expression. Since a ratio of one indicates a direct correlation between protein activity and transcript levels, a ratio of 0.2 obtained in utrophin A 5'UTR injected *mdx* muscles suggests that there is considerably more protein activity measured in respect to transcript levels. These high protein activity levels are presumably due to increased translation of the existing message.

Notable differences were seen when transcript and protein levels were compared for adult *mdx* mice. Transcript (figure 30) to protein (figure 31) ratios are expressed in Table V. Translation efficiencies are quite divergent from those seen in young *mdx* mice. Utrophin A 5'UTR resulted in translation inhibition by displaying a ratio of six. Interestingly, translation efficiency in adult *mdx* mice suggests that

Figure 31. Mouse utrophin A 5'UTR significantly affects β-galactosidase activity in adult *mdx* muscle

Adult (13-18 week-old) *mdx* TA muscles were injected with appropriate constructs in conjunction with a CAT plasmid. Muscles were excised 7 days following injections and proteins were extracted. Lysates were assayed for β-galactosidase activity and normalized to CAT activity. *Asterisks* denotes significant difference ($p < 0.01$ *versus* Parental). Mean ± Std. error are shown; results represent protein levels measured in 3 injected TA muscles for Parental and 4 injected TA muscles for UTR A.

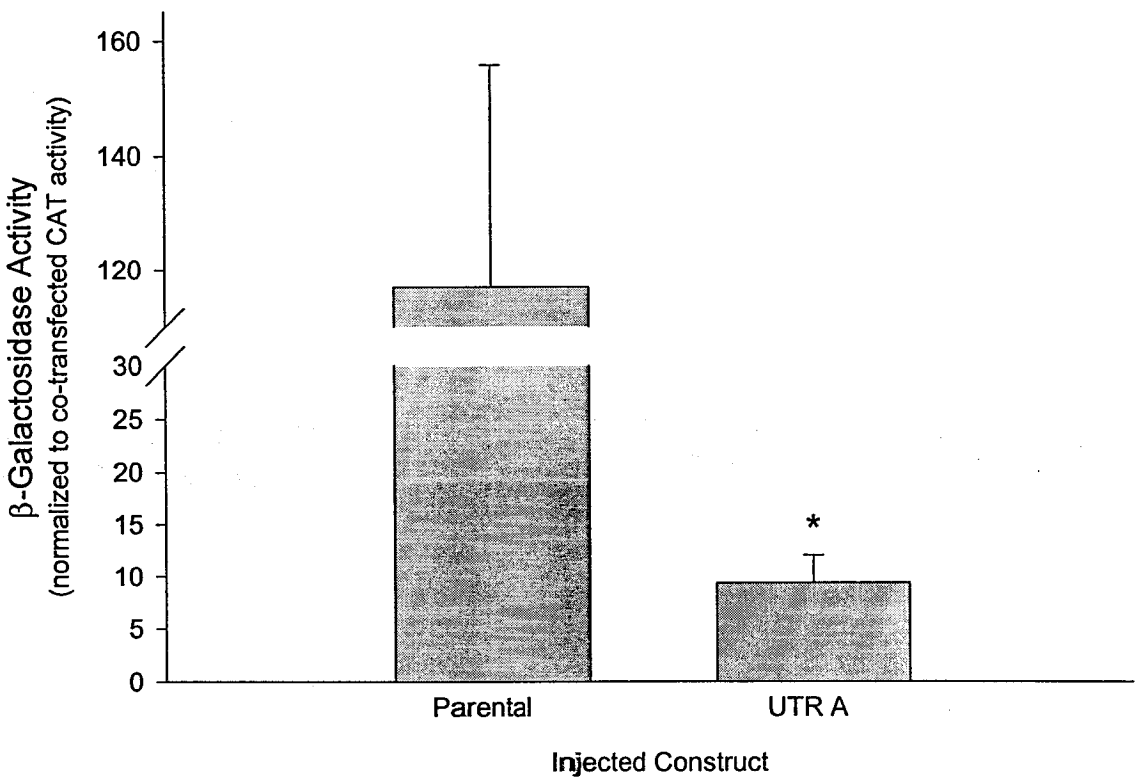

β-Galactosidase Activity
(normalized to co-transfected CAT activity)
160
140
120
30
25
20
15
10
5
0
*
Parental
UTR A
Injected Construct

Table V

Comparison of β-galactosidase transcript levels and β-galactosidase activity levels of reporter gene constructs in mdx muscle

To assess the contribution of the utrophin A 5'UTR on translation regulation, β-galactosidase transcript levels and β-galactosidase activity levels in young *mdx* TA muscles (Fig. 7and Fig. 28) and adult *mdx* TA muscles (Fig. 29 and 30) are expressed as ratios. Global expression of the reporter gene is affected mainly by translation inhibition with a higher ratio. Whereas global expression of the reporter gene is affected mainly by an increase in protein expression, without an increase in transcript levels with a lower ratio.

Young *mdx* TA

Construct	β-gal transcript level	β-gal activity level	Ratio
Parental	100.0	100.0	1.0
UTR A	4.4	24.0	0.2

Adult *mdx* TA

Construct	β-gal transcript level	β-gal activity level	Ratio
Parental	100.0	100.0	1.0
UTR A	46.4	8.0	5.8

translation inhibition is present in adult which was not evident in young *mdx* mice. These results hint differential translation regulation between adult and young *mdx* mice in respect to the utrophin A 5'UTR.

Chapter 4

Discussion

Up-regulating utrophin expression at the sarcolemma is thought to be a key therapeutic approach in the fight against DMD. However, mechanisms involved in the highly regulated utrophin expression at crests of neuromuscular junctions, as well as the more fundamental stages of utrophin synthesis, have yet to be fully elucidated. This study has examined the effects of the utrophin 5'UTRs on reporter gene expression in cell culture and in different mouse muscle conditions, in an attempt to determine if the utrophin 5'UTRs regulate translation differentially in distinct muscle conditions.

Contrary to the lack of translational regulation in cell culture experiments, the utrophin 5'UTRs significantly affected reporter gene expression in mouse muscle. In control muscles, both utrophin 5'UTRs manifested significant inhibitory roles on reporter gene translation and as seen in cell culture, no signs of translational silencing were seen in regenerating muscles. In addition, a significant de-repression was seen with the utrophin A 5'UTR in young *mdx* muscle which could suggest an increase in the translation of existing transcripts. However, in adult *mdx* mice the utrophin A 5'UTR had a moderate inhibitory effect. Taken together, these results imply that utrophin expression may be regulated at the level of translation and possibly via its 5'UTRs. Furthermore, translational regulation is evident and seems to be dependent on the state of the muscle. We also observed that translation initiation factors accumulate at neuromuscular junctions, which further supports the idea of increased translational capacity at synapses. Therefore, it is plausible that translational regulation may be a factor involved in the synaptic expression of utrophin.

4.1 Translational Regulation and Myogenic Differentiation

Myogenesis is known to be a very complex multi-step process and the expression of several genes is altered in response to myogenic differentiation. The effects of myogenic differentiation on utrophin expression consist of two fold increases of transcript and protein levels in differentiated myotubes compared to proliferating myoblasts (Gramolini and Jasmin, 1999). This moderate increase was surprising because both dystrophin (Lev *et al.*, 1987) and AChR (Buonanno and Merlie, 1986) have been shown to increase more than 10-fold with differentiation. In addition, the elevation of utrophin transcript and protein levels in differentiated myotubes was determined to result from a heightened rate of transcription (Gramolini and Jasmin, 1999).

The combined effects of transcription and translation regulation have been previously reported for several proteins. For example, dystrophin and its several isoforms have been shown to be regulated at both the transcription and translation levels. It has been well established that dystrophin is transcriptionally regulated via its multiple promoters in a tissue-specific manner (reviewed in Blake *et al.*, 2002). Furthermore, one of the dystrophin isoforms, Dp71, is post-transcriptionally regulated. This was demonstrated as transcript levels did not change in response to myogenic differentiation though protein levels were significantly higher in differentiated myogenic cells (Tennyson *et al.*, 1996a). A lack of change in transcript levels in conjunction with an increase in protein synthesis suggests that translational regulation may be involved (Tennyson *et al.*, 1996a).

It is well known that utrophin is a dystrophin homologue. Therefore it was initially thought that utrophin may also be regulated at both the level of transcription and translation. However, there is strong evidence suggesting that the increase in utrophin expression in myotubes is primarily a result of transcriptional regulation. Since the transcription rate as well as transcript and protein levels all increase by approximately 2-fold, we initially considered that translational regulation does not play an important role in myogenic differentiation.

Foremost, because utrophin was found to have two full-length isoforms, we wanted to determine if the greater total utrophin levels in myotubes were specific to one isoform or a combination of both. Therefore, utrophin A and B transcript levels were measured through myogenic differentiation. As reported previously (Gramolini and Jasmin, 1999; Perkins *et al.*, 2001), a moderate increase in total utrophin transcript levels was observed in myotubes when compared to myoblasts. Our results also indicate that the elevation of utrophin transcripts through myogenic differentiation is primarily a reflection of an increase in utrophin A transcript levels, since utrophin B levels did not change. Utrophin B is thought to be the vascular endothelium isoform (Weir *et al.*, 2002), yet transcripts were detectable in the C2C12 cell line. Additional experiments would be required to determine if these transcripts are even translated to full-length proteins in these cell lines.

Moreover, the utrophin 5'UTRs did not exhibit any translational regulation through myogenic differentiation resulting in protein activity levels that mirror transcript levels. Even though endogenous transcript levels increase moderately with myogenic differentiation, transcript levels of the reporter genes containing the utrophin 5'UTRS are slightly higher in myotubes when compared to myoblasts.

However, the activity levels are also slightly higher in myotubes than in myoblasts suggesting that it is not a translational effect. These results would suggest that translation does not play an important role in regulating utrophin expression in cultured muscle cells.

4.2 Translational Regulation In Control Muscle

Following the cell culture studies, it was important to assay the effects of each utrophin 5'UTR on reporter gene expression *in vivo*, particularly since utrophin accumulates at the neuromuscular junction in mature muscle. Post-synaptic membranes of neuromuscular junctions have been described as highly differentiated regions of skeletal muscle fibers (see Hall and Sanes, 1993). Indeed, Golgi apparatus (Jasmin *et al.*, 1995b; Ralston *et al.*, 1999) and specialized microtubule networks (Jasmin *et al.*, 1991; Ralston *et al.*, 1999) appear restricted to neuromuscular junctions. During myogenesis, remodeling of the Golgi apparatus results in functional compartmentalization at neuromuscular junctions with no well-organized Golgi complexes in extrasynaptic regions (Jasmin *et al.*, 1990, 1995b). In addition, the structure of the Golgi apparatus can be influenced by the state of innervation where denervation leads to expansion and reorganization of the Golgi (Jasmin *et al.*, 1989, 1995b). Microtubule networks have also been shown to preferentially converge toward troughs of neuromuscular junctions, though some do extend to the membranes (Jasmin *et al.*, 1995b). Additionally, vesicles have been found to accumulate at the end of these microtubules (Jasmin *et al.*, 1995b).

Together, these structures are thought to contribute to the specific localization of synaptic proteins. For instance, AChRs are considered to be sorted intracellularly by the Golgi apparatus (Camus *et al.*, 1998) and transported directly to post-synaptic membranes (Jasmin *et al.*, 1990; 1995b) via microtubule networks (Camus *et al.*, 1998).

These highly structured organelles partake in the local accumulation of synaptic proteins. However, additional mechanisms of regulation have been elucidated. For instance, local transcriptional regulation of synaptic genes has emerged as an important factor by which transcripts of synaptic proteins seem to selectively accumulate at neuromuscular junctions. Such proteins are utrophin (Gramolini and Jasmin, 1999), AChR α and ε subunits (see Brenner *et al.*, 1990) as well as AChE (see Jasmin *et al.*, 1993 and Grubic *et al.*, 1995). Transcriptional regulation of these genes as well as their sorting and shuttling via microtubule networks are evident. However, they do not seem to be the only elements contributing to synaptic protein expression.

Indeed, Sigrist and colleagues (2000) imply that the development and maintenance of *drosophila melanogaster* neuromuscular junctions are also regulated via post-transcriptional mechanisms. They believe that neuromuscular junctions are highly localized, active post-synaptic translation sites that can be identified by translation factor aggregates (elf-4E/poly(A)-binding protein, PABP) found to accumulate close to neuromuscular junctions. They reported that messenger RNAs, as well as protein aggregates, were found to associate with neuromuscular junctions and modifications affecting aggregate formation altered synaptic protein levels. For example, they observed that the accumulation of post-

synaptic glutamate receptor DGluR-IIA mRNA was the result of an increase in subsynaptic synthesis in areas with higher incidences of eIF-4E/PABP aggregates (Sigrist *et al.*, 2000). It is also suggested that heightened neuronal activity gives rise to increased synaptic transmission, which in turn, prompts the formation of active subsynaptic regions (Sigrist *et al.*, 2000). In fact, the stimulated sub-synaptic translation sites were recently shown to lead to the previously reported accumulation of the glutamate receptor subunit DGluR-IIA (Sigrist *et al.*, 2002).

In accordance with the aggregation of translation initiation factors at drosophila neuromuscular junctions, we observed similar accumulations of translation machinery in mouse tissue. In addition to the co-localization of eIF-4E with neuromuscular junctions, we also observed accumulations of eIF-2α in synaptic areas. These results further support the hypothesis that neuromuscular junctions may have an increased translational capacity, thus possibly playing an important part in the restricted expression of synaptic proteins, such as utrophin.

Although the utrophin isoforms are driven by different promoters, they only vary in their N-termini, giving rise to transcripts with very distinct 5'UTRs. The utrophin A 5'UTR consists of a 509-nucleotide fragment whereas the utrophin B 5'UTR is a 74-nucleotide fragment. Based on their length, sequence and secondary structures, the utrophin 5'UTRs should exhibit distinct effects on translation. Differences between the effects seen with either utrophin 5'UTR were not a result of differential message stability in cell culture, therefore one could hypothesize that they were induced by the sequences themselves.

Both utrophin 5'UTRs exhibited significant translational inhibition on reporter gene expression in control muscle when compared to the parental 5'UTR. The

utrophin A 5'UTR should be more inhibitory than the utrophin B 5'UTR when taking into account that it is incompatible with efficient ribosomal scanning since long 5'UTRs are able to form secondary structures and/or can contain upstream open-reading frames which hinder translation initiation (for review, see Day and Tuite, 1998; Gray and Wickens, 1998; Kozak, 2002). However, even though the utrophin A 5'UTR is a long and G-C rich sequence which can form stable secondary structures and the utrophin B 5'UTR is a short fragment containing no putative regulatory elements, the degree of translation silencing was similar for both utrophin 5'UTRs. Therefore, other mechanisms or molecules that interact with the different 5'UTRs, issuing similar translational inhibition ratios, must be involved.

4.3 Translational Regulation Is Evident In Regenerating Muscle

Gene expression has been reported to be altered in regenerating fibers compared to control tissues. For example, both cofilin and vimentin transcript levels are significantly affected during regeneration (Akkila *et al.*, 1997). Cofilin transcript levels are very low in control muscles yet increase by approximately 19-fold in regenerating muscles. Vimentin also undergoes a similar increase compared to control levels, yet protein levels peak at a later onset. In addition, transcripts coding for myogenic regulatory factors such as Myf-5, MyoD and MRF4 were also shown to increase in the early stages of regeneration (Launay *et al.*, 2001). However, several other myogenic elements only increase in protein levels, with no change in

transcript levels when compared to control muscles. For example, in regenerating muscles, skeletal-muscle actin, human Fus-like protein, proα_1-collagen type III, human neuroleukin (Launay *et al.*, 2001) and utrophin (Gramolini *et al.*, 1999b) all display similar transcript levels to control tissues. However, elevated protein levels indicate that post-transcriptional mechanisms are involved in the up-regulation of utrophin in these fibers.

Utrophin has been suggested to be a regeneration-associated protein since its expression is increased in regenerating fibers (Lin *et al.*, 1998). Indeed, utrophin levels are significantly higher in regenerating fibers (Helliwell *et al.*, 1992; Taylor *et al.*, 1997; Lin *et al.*, 1998; Gramolini *et al.*, 1999b) yet transcript levels are similar to those in control muscle fibers (Gramolini *et al.*, 1999b; Galvagni *et al.*, 2002). Discordant transcript and protein levels could implicate post-transcriptional regulation in utrophin expression.

Discrepancy between transcript and protein levels could be an indication of increased protein stability. However our approach utilizing reporter constructs containing the utrophin 5'UTRs indicate that it is translational regulation via the inserted 5'UTRs. Indeed, transcript levels of the reporter gene constructs are similar in both control and regenerating muscle yet protein levels are quite different, where more activity is observed in the regenerating muscles. The considerable translational inhibition seen with both utrophin 5'UTRs in control muscle was absent in these muscles. This decreased translational silencing could explain the elevated utrophin levels seen in regenerating fibers where increased translation efficiency would result in the synthesis of more protein. However, the precise mechanisms

by which the utrophin 5'UTR translation efficiencies are amplified in regenerating fibers still remain to be determined.

Both utrophin 5'UTRs exhibited increased translation efficiencies in regenerating muscles, yet they are very different in sequence. One of the few similarities between these two transcripts would be their interactions with eukaryotic translation initiation factors. Increased protein expression could be the result of an accumulation of eIFs, thus resulting in higher levels of translation. Moreover, the over-expression of eIF-4E was shown to result in the more efficient translation of structured 5'UTRs (see Gray and Wickens, 1998 for review). This could suggest that the utrophin A 5'UTR would be translated more effectively with increased levels of eIF-4E. It would be interesting to determine if there is such an increase of translation machinery at muscle sarcolemma in regenerating fibers, considering that elevated aggregation of translation machinery could result in the increased translation seen in these fibers.

4.4 Translational Regulation May Be Age-Dependent In *Mdx* Muscle

A recent study by Weir and colleagues (2002) reported that post-transcriptional regulation is responsible for increased utrophin A expression in *mdx* muscle. Utrophin A protein levels were 2- and 4-fold higher in *mdx* heart and skeletal muscles respectively, yet messenger RNA levels did not increase more than 50% in either tissue when compared to controls. The lack of significant

increases in transcript levels to mirror protein expression, further emphasize the involvement of post-transcriptional regulation in utrophin expression. They also reported that in these tissues, the distribution pattern of utrophin A was also affected. Utrophin A was expressed along skeletal and cardiac muscle sarcolemma in dystrophic tissues rather than its synaptic localization in control muscles (Weir *et al.*, 2002). Interestingly, utrophin B expression and distribution patterns were not altered in the dystrophic tissues studied, when compared to controls. As mentioned, endogenous utrophin transcript levels in *mdx* mice are similar to those in control muscles yet in the presence of the utrophin A 5'UTR, reporter gene transcript levels were lower in young *mdx* and higher in adult *mdx* mice when compared to control muscle. Therefore, other mechanisms are involved in maintaining utrophin levels which may not be present in the utrophin A 5'UTR.

The translational inhibition seen in control muscles was higher than observed in *mdx* mice with the utrophin A 5'UTR construct. A plausible mechanism by which the utrophin A 5'UTR could circumvent the inhibitory effect on translation seen in control muscles may be the presence of an internal ribosomal entry site (IRES). When the utrophin 5'UTRs were submitted to a database that identifies regulatory elements found in other eukaryotic UTRs, a putative IRES was identified in the utrophin A 5'UTR sequence. IRES is known as a secondary structure in the 5'UTR that can recruit ribosomal machinery to the transcript in a cap-independent manner. They have also been shown to be key players in the translation of highly regulated proteins (for review see Vagner *et al.*, 2001).

IRES was initially discovered in picornaviruses, where viral gene translation is cap-independent. Most picornaviral 5'UTRs are uncapped and have specific

characteristics that impede classical ribosome recruitment and scanning, such as long 5'UTRs, stable secondary structures and potential upstream initiation codons (see Vagner *et al.*, 2001 for review). However, several cellular mRNAs have been shown to contain an IRES in their 5'UTRs, such as immunoglobin heavy-chain binding protein (BiP), basic fibroblast growth factor and *c-myc* (see van der Velden and Thomas, 1999 for review). The purpose of IRES in eukaryotic 5'UTRs was not initially understood, considering eukaryotic translation systems were thought to only be initiated in a cap-dependent manner (see Vagner *et al.*, 2001). The presence of IRES in these mRNAs is starting to be accepted, since genes containing this element have tightly regulated expression and/or are required when cap-dependent translation is inhibited (Vagner *et al.*, 2001).

Cap-dependent translation has been shown to be suppressed in several instances when cells experience various forms of stress (Vagner *et al.*, 2001). For example, IRES activity was shown to be present in hypoxic conditions with hypoxia-inducible factor-1α (HIF-1α), a condition known to have impaired cap-dependent translation. HIF-1α, a key protein involved in adaptation to hypoxia, was shown to be translated equally in normoxic and hypoxic conditions suggesting that the presence of an IRES would ensure adequate protein expression when cap-dependent translation is impeded (Lang *et al.*, 2002). It was also observed that not only hypoxic-induced genes were equally expressed in hypoxic conditions. The *c-myc* IRES was also studied in those same conditions and was equally expressed when cap-dependent translation was suppressed (Lang *et al.*, 2002). Mitosis, amino acid starvation, glucose starvation and apoptosis are other known instances where cap-dependent translation is impaired, resulting in a down-regulation of

protein synthesis. Proteins involved in these conditions are still expressed and upon further examination, several contain an IRES in their 5'UTR (Holcik *et al.*, 1999; Stoneley *et al.*, 2000). It is also suggested that G-C rich 5'UTRs longer than ~250 bases are deemed to contain an IRES (Lang *et al.*, 2002). The precise role of IRES remains unclear, yet it is thought that the preservation of translation during cellular stress may be a common purpose of IRES in cellular mRNAs (Lang *et al.*, 2002).

Accordingly, if myogenic cells consider dystrophin-deficiency as a "cellular stress," transcripts containing IRES elements could be translated in a cap-independent manner. This model offers a possible explanation for the de-repression of translation inhibition seen with the utrophin A 5'UTR in young *mdx* muscles. Increased translation of the utrophin A transcript would result in higher utrophin A protein levels, thus supporting our results. The presence of a putative IRES in the utrophin A 5'UTR makes this region very enticing to study, especially if it proves to be active in dystrophic muscle conditions.

Therefore, a possible explanation for the effects seen with utrophin A 5'UTR in *mdx* mice would be that the absence of dystrophin is considered a stress condition by the cells. It has been well established that dystrophin-deficiency affects sarcolemma integrity, which may be considered a stress environment and result in suppression of cap-dependent translation. The utrophin A 5'UTR contains a potential IRES just upstream of the AUG which would permit cap-independent translation of its transcript, even when cap-dependent translation is suppressed. This putative IRES may explain why there is less translational inhibition in *mdx* muscle, resulting in increased protein expression compared to controls. The

presence of an IRES in the utrophin A and not the utrophin B 5'UTR can also explain the lack of change in utrophin B expression. Lacking a potential IRES, translation of the utrophin B message would be inhibited when cap-dependent translation is suppressed. The *mdx* mouse may be a condition that provides a "stressful" environment for cells, resulting in suppression of cap-dependent translation. Therefore any transcript lacking an IRES would not be translated or, translated less efficiently, such as utrophin B for instance.

Our results also suggest that the utrophin A 5'UTR may exert age-dependent translational affects on gene expression. Contrary to the translation silencing seen in adult *mdx* mice, the utrophin A 5'UTR displayed a significant increase in translation efficiency in young *mdx* muscle. The significant de-repression could result from increased muscle regeneration in young *mdx* muscle, which is less evident in the adult dystrophic mouse. Taken together, the utrophin A 5'UTR seems to play an important part in the translation of its transcript, especially in young *mdx* muscle. These results could suggest that increased utrophin seen in *mdx* mice may be caused by the utrophin A 5'UTR.

In addition to the putative IRES found in the utrophin A 5'UTR, other mechanisms may also play a part in the increased translation efficiencies seen in *mdx* mice. Among other proposed alternative mechanisms regulating translation is upstream open reading frames (uORFs) and mRNA splicing. The long utrophin A 5'UTR conceivably contains one upstream AUG with three stop codons which could act to regulate translation. Under different conditions, these elements may display altered effects on translation depending on the start site utilized. Such translational regulation is seen with *GCN4*, a transcriptional activator in

Saccharomyces cerevisiae, whose transcript contains uORFs. By modulating initiation at the main translation initiation site, the four uORFs regulate *GCN4* expression (Gaba *et al.*, 2001). It was shown that ribosomes initiating at uORF1 were able to reinitiate translation at the main start codon, whereas those initiating at uORF4 showed little capacity of re-initiation at the main start codon (Gaba *et al.*, 2001). Interestingly, the levels of eukaryotic translation initiation factors may also contribute to the site of re-initiation in 5'UTRs containing uORFs (Gaba *et al.*, 2001). Moreover, mRNA splicing within the 5'UTR may also contribute to utrophin expression. Uncommon in mammalian mRNA, alternative splicing within the 5'UTR has been shown to regulate the translation of human neuronal nitric-oxide synthase (Newton *et al.*, 2003). Therefore, several mechanisms may regulate translation via the utrophin 5'UTRs, which render these sequences very enticing to study. Identifying which events are involved is important in determining all the processes involved in regulating synaptic utrophin expression.

4.5 Model of Utrophin Expression At the Neuromuscular Junction

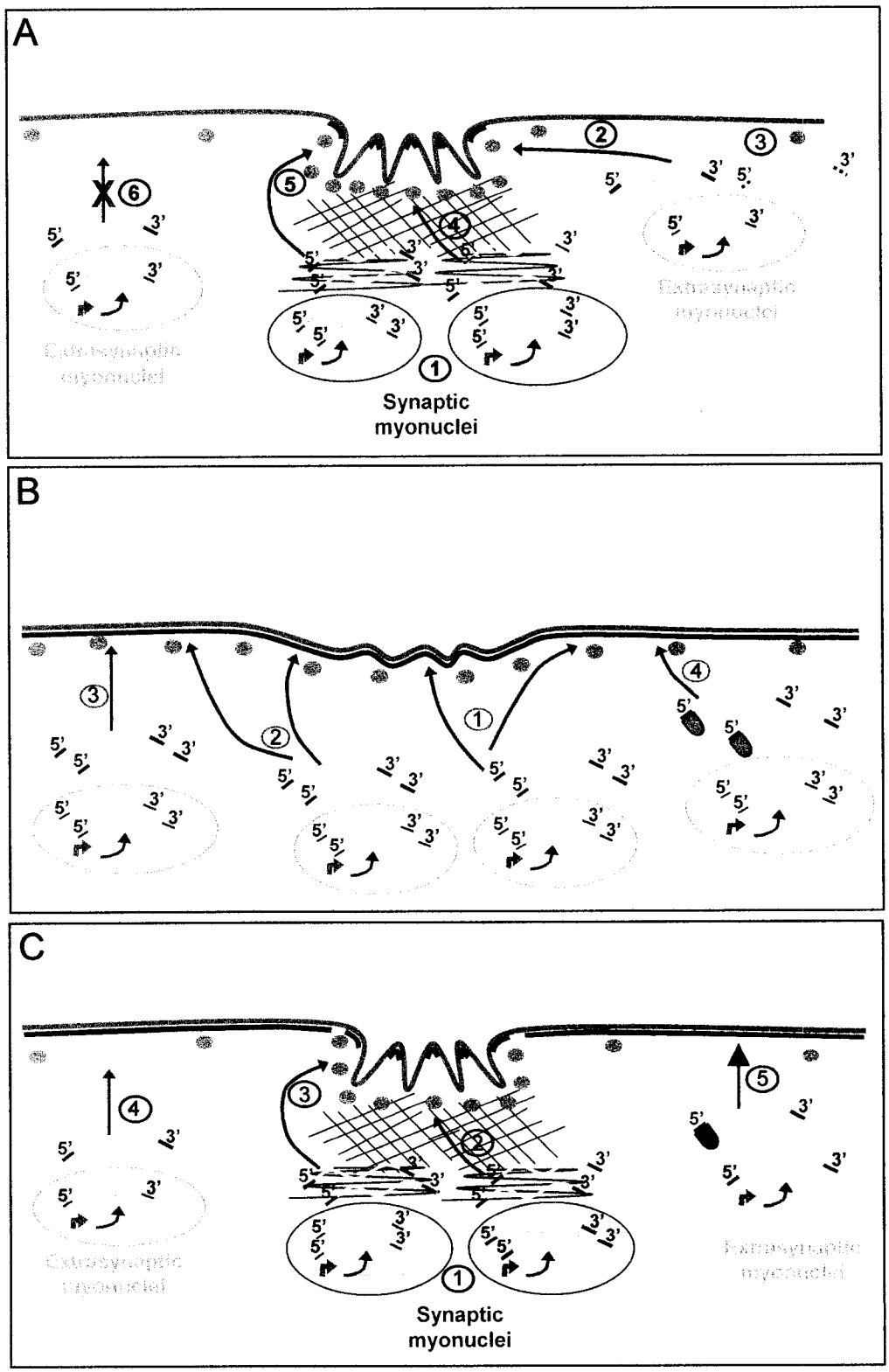
4.5.1 Utrophin Expression in Normal Muscle

Utrophin expression seems to be highly regulated both at the transcriptional and translational level in skeletal muscle. In normal muscle, utrophin is expressed in synaptic regions where it is found specifically at crests of neuromuscular

junctions. Previous studies as well as this study suggest that several different mechanisms are involved in the synaptic expression of utrophin (Figure 32 A). 1) It has been shown that transcriptional regulation (section 1.7.1) does contribute to the synaptic expression of utrophin, where synaptic myonuclei have increased transcription when compared to extra-synaptic nuclei (Gramolini *et al.*, 1997, 1998). Utrophin A also has an N-box which contributes to its synaptic expression (Dennis *et al.*, 1996; Gramolini *et al.*, 1997, 1998). 2) The utrophin 3'UTR (section 1.7.2) has been shown to contain an element that is involved in the targeting of its message to the cytoskeleton (Gramolini *et al.*, 2001a) as well as 3) an element involved in the stability of its transcript (Gramolini *et al.*, 2001a). 4) Neuromuscular junctions have very well organized Golgi apparatus and microtubule networks which would be implicated in the sorting and shuttling of utrophin transcripts to the neuromuscular junction (Jasmin *et al.*, 1991, 1995b; Ralston *et al.*, 1999) (section 4.2). 5) Our study also showed that there seems to be an accumulation of translation initiation factors at neuromuscular junctions suggesting increased translational capacity in these regions and thus providing increased synaptic utrophin (section 3.1 and 4.2). 6) In addition, the utrophin 5'UTRs displayed inhibitory effects on translation in control muscle (section 3.6.1, 3.6.3 and 4.2). Utrophin may not be translated in extra-synaptic regions due to this inhibitory 5'UTR. Taken together, these data suggest that utrophin expression is highly regulated and that several mechanisms are involved in the preferential expression of utrophin at the neuromuscular junction.

Figure 32. Model depicting the transcriptional and post-transcriptional mechanisms at the neuromuscular junction in skeletal muscle.

In control muscle (**A**), utrophin is found predominantly at crests of neuromuscular junctions (*fuschia*). Increased transcription in synaptic myonuclei (1), targeting of utrophin message via its 3'UTR (2), degradation of the utrophin transcript in extra-synaptic regions (3), sorting and shuttling of synaptic transcripts via organized Golgi apparatus and microtubule networks (4), accumulation of translation initiation factors (*orange circles*) at neuromuscular junctions (5) as well as the inhibitory effect of the utrophin 5'UTRs (6) are all mechanisms that could contribute to the synaptic expression of utrophin (see text section 4.6.1). In regenerating fibers (**B**), the lack of organized Golgi apparatus and microtubule networks (1), a more diffuse distribution of translation initiation factors (2), no specific synaptic nuclei (3) and possibly the presence of an RNA-binding protein enabling the translation of the transcript are all possible mechanisms leading to utrophin expression throughout the sarcolemma (section 4.6.2). In *mdx* muscle (**C**), utrophin is found at the neuromuscular junction possibly via similar events than in control muscle, such as increased transcription in synaptic myonuclei (1), organized Golgi apparatus and microtubule networks (2) as well as accumulations of translation initiation factors (3). However, utrophin is also expressed in extra-synaptic regions in dystrophic muscle. In young *mdx* muscle, fiber regeneration is present and therefore events explained above (B) may contribute to extrasynaptic expression. In addition, a putative IRES in the utrophin A 5'UTR may also contribute to the increase in extra-synaptic utrophin due to a decrease in cap-dependent translation (4) (section 4.6.3).


A
Extrasynaptic myonuclei
Synaptic myonuclei
Extrasynaptic myonuclei
B
C
Extrasynaptic myonuclei
Synaptic myonuclei
Extrasynaptic myonuclei

4.5.2 Increased Utrophin Levels in Regenerating Muscle May be Via Translational Mechanisms

In regenerating muscle (Figure 32 B), there is an increase in utrophin expression (Helliwell *et al.*, 1992; Taylor *et al.*, 1997; Gramolini *et al.*, 1999b) where it can be found throughout the sarcolemma. However, utrophin transcripts do not increase in regenerating muscle when compared to controls (Gramolini *et al.*, 1999b; Galvagni *et al.*, 2002). 1) It has been shown that during myogenesis, which is induced in regeneration, the Golgi apparatus and microtubule networks are being remodeled as well as compartmentalized (Jasmin *et al.*, 1990, 1995b). This lack of organized organelles, could thus lead to a more diffuse distribution of utrophin transcripts. 2) There could also be an RNA-binding protein that may be present or absent in regenerating muscle resulting in increased transcript stability throughout the sarcolemma. 3) Since the neuromuscular junctions are not yet organized in regenerating fibers, translation initiation factors may be scattered at the sarcolemma and thus result in a more diffuse distribution of utrophin. 4) Our data suggests that there is a de-repression of the utrophin 5'UTR inhibition seen in control muscle (section 3.6.2 and 4.3). There may be an RNA-binding protein that binds the utrophin 5'UTRs and leads to more efficient translation. This de-repression could also contribute to the dispersed distribution of utrophin in these regenerating fibers as well as the elevated protein levels when compared to age-matched controls.

4.5.3 *Regeneration and A Putative Internal Ribosome Entry Site May Lead To Increased Utrophin in Mdx Muscle*

Similar to normal muscle, *mdx* muscle displays the increased utrophin at neuromuscular junctions. Therefore, mechanisms involved in the synaptic expression of utrophin in control muscle may also be involved in these dystrophic tissues (Figure 32 C). For example, 1) transcriptional control in the subsynaptic myonuclei as well as 2) the sorting and shuttling of the utrophin transcript to the neuromuscular junction via Golgi apparatus and microtubule networks, may contribute to the synaptic utrophin expression. 3) Since there are functional neuromuscular junctions in *mdx* mice, accumulations of translation initiation factors may also occur in this dystrophic model, thus leading to the translation of utrophin in synaptic areas.

Not only is utrophin found at the neuromuscular junction, it is also expressed in extra-synaptic regions in *mdx* mice. It is possible that a putative IRES in the utrophin A 5'UTR may be active in these mice. The lack of dystrophin may provide a "stress environment" and thus decrease cap-dependent translation. The presence of an IRES in the utrophin A 5'UTR would allow the preservation of its translation during the cellular stress. The increased translation seen in the presence of the utrophin A 5'UTR in young *mdx* mice may be the result of an additive effect. The presence of muscle regeneration in young *mdx* would suggest that the mechanisms involved in the up-regulation of utrophin in regenerating fibers would contribute similarly in young dystrophic tissue. Additionally, the presence of a putative IRES in the utrophin A 5'UTR would contribute to increased utrophin

expression in dystrophic tissue. This additive effect in young *mdx* mice could explain the discrepancy observed between the young and adult *mdx* mice since regeneration is absent in the adult. Taken together, these models implicate several transcriptional as well as post-transcriptional events regulating utrophin expression in different muscle conditions.

4.6 Up-Regulation of Utrophin As A Therapeutic Strategy

Up-regulating utrophin expression in extrajunctional regions has been shown to be a valid putative therapy for DMD (Tinsley *et al.*, 1996; Deconinck *et al.*, 1997b; Gilbert *et al.*, 1998; Rafael *et al.*, 1998; Tinsley *et al.*, 1998). Using nerve-derived factors, such as agrin and heregulin, utrophin expression can be up-regulated via transcriptional pathways (Gramolini *et al.*, 1998a, 1999a). Utrophin expression in both diseased and regenerating fibers is regulated via post-transcriptional pathways that have yet to be fully elucidated. Foremost, the utrophin 3'UTR has been shown to be involved in utrophin transcript stability (Gramolini *et al.*, 2001a) and to date, no studies have been performed on the translation regulation of utrophin.

Several studies have shown that over-expression of utrophin in *mdx* mice is therapeutically beneficial. Muscle pathology is protected and DAP localization is restored in the presence of utrophin (Tinsley *et al.*, 1996; Deconinck *et al.*, 1997b; Gilbert *et al.*, 1998; Rafael *et al.*, 1998; Tinsley *et al.*, 1998; Squire *et al.*, 2002). Although therapeutic expression levels of utrophin still remain to be elucidated, this venue shows great promise. Therefore, studying factors regulating utrophin

expression is still of utmost importance. Pharmacologically, it would be interesting to determine if there exists a protein or drug that would bind to either the utrophin A or B 5'UTR and increase their translation efficiency. This substance could act, for example, by unwinding the secondary structures in the utrophin A 5'UTR to increase scanning fluency, or bind the utrophin B 5'UTR and simply increase its translation efficiency. Additionally, pharmacological interventions could affect the translation machinery to enhance gene expression. For instance, leucine (Anthony *et al.*, 2000) and insulin-growth factor I (Vary *et al.*, 2000) have been shown to affect translation initiation in skeletal muscle by stimulating the formation of the eIF-4E/eIF-4G complex, as well as affect the phosphorylation states of other translation factors, resulting in increased protein synthesis. Therefore, further characterization of the utrophin 5'UTRs, as well as other venues that can increase its expression at the level of translation, could add therapeutic targets to treat DMD.

4.7 Future Experiments

Utrophin can be found in several tissues where utrophin A transcripts are found mainly in kidney, brain and muscle and utrophin B transcripts are preferentially expressed in heart, brain and muscle (Burton *et al.*, 1999). The expression pattern of each utrophin isoform within a tissue seems to also be cell-specific. For example, utrophin A is found at neuromuscular junctions, peripheral nerves and smooth muscles whereas utrophin B is considered the vascular

endothelial isoform (Weir *et al.*, 2002). Therefore it would be essential to examine the effects of the different utrophin 5'UTRs on translation within different tissues by repeating the described experiments but in different cell-types and tissues. To further characterize the utrophin 5'UTRs, *in vitro* transcription/translation, UV-cross linking as well as electrophoretic mobility shift assays would be important to execute. These experiments would allow us to examine if the utrophin 5'UTRs interact with RNA-binding proteins and if these are specific to one transcript or cell-type. These experiments would also be of interest to determine if RNA-binding proteins are present differentially in specific conditions (such as myogenic differentiation or in control, regenerating or *mdx* muscles).

This study has shed some light on the differential translation regulation by the utrophin 5'UTRs in different animal studies (control, regenerating and *mdx* TA muscles). However, further studies of the utrophin 5'UTRs and their effects still remain to be performed. It would be necessary to generate deletion studies on the different 5'UTRs to examine whether there are specific fragments that are required to regulate translation initiation and determine which fragments are responsible for the inhibitory role these 5'UTRs have on utrophin expression.

To characterize the putative IRES found in the utrophin A 5'UTR, a bicistronic plasmid should be designed. This vector would be useful to verify if the potential IRES in the utrophin A 5'UTR is indeed active. Bicistronic plasmids are considered the "gold standard" to test IRES activity (Sachs, 2000). The whole utrophin A 5'UTR or the actual sequence that is the plausible IRES can be cloned into this plasmid between two genes. The first gene is expressed normally due to its promoter and resulting capped transcript, yet the second cistron is not translated because it lacks

the methylated cap thereby the ribosomal complex dissociates from the transcript once it reaches the stop codon from the first cistron (van der Velden and Thomas, 1999; Sachs *et al.*, 2000). Unless there is an IRES upstream of this second cistron, the latter is not translated. If the second cistron is expressed, the inserted sequence contains an active IRES which allows the recruitment of the translational machinery in a cap-independent manner. This would be the first experiment suggesting the presence and characterization of a potential IRES regulating its translation in the utrophin A 5'UTR.

There are several experiments that are still required to be performed to define the mechanisms involved in the regulation of utrophin expression. One such experiment to elucidate additional factors at the post-transcriptional level would be the generation of a reporter construct that contains both the 5' and 3' utrophin UTRs. It would be interesting to study the presence of both UTRs to determine if they act synergistically, if one region inhibits the other or if there is stimulation when both are present. It would also be important to repeat the cell culture and muscle injection experiments described in this study utilizing the construct containing both utrophin 5' and 3' UTRs to ascertain the combined regulation of utrophin expression by its UTRs.

4.8 Conclusion

In the present study, we examined the utrophin A and B 5'UTRs by transient transfections in cell culture and muscle injections. The latter were performed on different muscle conditions such as control, regenerating and dystrophic mouse tissues. Our goal was to determine if these 5'UTRs had differential inhibitory effects on the translation of its message in order to better understand the factors that regulate utrophin expression. The results obtained in this study have led to five main conclusions: 1) The utrophin 5'UTRs do not appear to modulate transcript levels by affecting message stability differentially in cell culture; 2) Both utrophin 5'UTRs show no significant indication of translational control in cell culture, nor do they respond to myogenic differentiation; 3) Both utrophin 5'UTRs show considerable inhibitory translational regulation in control muscle; 4) Translation inhibition is absent in regenerating muscle and 5) Translational regulation seems age-dependent in *mdx* tissue where it is undeniably de-repressed in young *mdx* mice. In addition to previous studies implicating transcriptional regulation of utrophin, our results indicate that utrophin expression also undergoes translational control. By which the degree of translational regulation may depend on the muscle condition, where it is significantly inhibitory in control muscle, absent in regenerating muscle and considerably de-repressed in young dystrophic muscle. Together, the study of mechanisms regulating utrophin expression could result in potential therapeutic venues, while subsequently providing information elucidating the factors contributing to the synaptic expression of utrophin.

Chapter 5

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