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Molecular Mechanisms Contributing to the Expression of Utrophin at the Mammalian Neuromuscular Synapse

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THESIS FORMAT

In accordance with the guidelines established by the University of Ottawa School of Graduate Studies, the present Thesis is a compilation of published journal articles preceded by a general review of the relevant literature (Chapter 1) and followed by an overall discussion (Chapter 7). Chapters 1 and 7 are largely based on the following articles: "Regulation and functional significance of utrophin expression at the mammalian neuromuscular synapse" by A.O. Gramolini, J. Wu. and B.J. Jasmin published in *Microscopy Research and Techniques* (In press); "Molecular mechanisms and putative signaling events controlling utrophin expression in mammalian skeletal muscle fibers" by A.O. Gramolini and B.J. Jasmin *Neuromuscular Disorders* **8**, 351-361 (1998) and is reprinted with permission from Elsevier Science; and "Duchenne muscular dystrophy and the neuromuscular junction: The utrophin link." by A.O. Gramolini and B.J. Jasmin *BioEssays* **19**, 747-750 (1997) and is reprinted with permission from John Wiley and Sons, Inc. Chapters 2 through 6 are the original journal articles each containing an abstract, introduction, experimental procedures, results, discussion and references. All references for Chapter 1 and 7 are listed in first author and year format within the text, with the complete reference found in alphabetical order in Chapter 8. For the manuscripts with multiple authors, the contributions of other authors are detailed on the title page of each chapter.

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ABSTRACT

Duchenne muscular dystrophy (DMD) is the most severe and prevalent primary myopathy. This disease is characterized by repeated cycles of muscle fiber degeneration and regeneration with an eventual failure to regenerate leading to the progressive replacement of myofibers by adipose and connective tissues. The genetic defects responsible for DMD are mutations in the short arm of the X chromosome which prevent the production of normal size dystrophin, a large cytoskeletal protein of 427 kDa. In 1989, Love and colleagues showed the existence of a gene on chromosome 6q24 that encodes a cytoskeletal protein, called utrophin, which displays a high degree of sequence similarity with dystrophin (Love, D.R., Hill, D.F., Dickson, G., Spurr, N.K., Byth, B.C., Marsden, R.F., Walsh, F.S., Edwards, Y.H. and Davies, K.E. (1989) An autosomal transcript in skeletal muscle with homology to dystrophin. *Nature* **339**, 55-58). However, in contrast to the homogeneous distribution of dystrophin along muscle fibers, utrophin preferentially accumulates at the neuromuscular junction. Due to this sequence similarity between dystrophin and utrophin, it has been suggested that increased expression of utrophin into extrasynaptic regions of dystrophic muscle fibers may represent a therapeutic strategy for DMD. Recently, it has been confirmed that the upregulation of utrophin can, indeed, functionally compensate for the lack of dystrophin and alleviate the muscle pathology. In this context, it thus becomes essential to determine the cellular and molecular mechanisms presiding over utrophin expression in attempts to overexpress the endogenous gene product throughout skeletal muscle fibers.

In this Thesis, I explore the mechanisms underlying the selective accumulation of utrophin at the postsynaptic membrane of the neuromuscular synapse. We determined by *in situ* hybridization that local transcription contributes to the accumulation of utrophin at the neuromuscular junction. Using direct injections of utrophin promoter-reporter constructs into skeletal muscle, we also defined the promoter elements involved in this local transcription and determined that the N-box element is a key consensus sequence that directs transcriptional control of utrophin expression at the neuromuscular junction. Furthermore, additional experiments revealed that utrophin gene transcription is dependent on the extracellular matrix proteins agrin and ARIA/heregulin, and this regulation is dependent upon the N-box element. Indeed, *in vitro* transfection assays and electromobility shift assays indicated that agrin and ARIA/heregulin may ultimately initiate a cell signaling cascade that activates the ETS-related transcription factor, GA-binding protein (GABP) which binds and activates the N-box element. In a separate series of studies, we also examined the effect of myogenesis in culture on the transcriptional regulation of utrophin gene expression. In these experiments, we determined by RT-PCR, immunoblotting, and nuclear run on assays that, in contrast to the large changes in AChR, utrophin expression was only marginally increased under these conditions.

In addition to these transcriptional events that control the levels and localization of utrophin, it also became apparent that transcription alone could not account for the complete regulation of utrophin expression under certain conditions. Indeed, we observed a discordant relationship between utrophin transcript levels and protein levels in regenerating muscles or muscles obtained from DMD patients, indicating that utrophin expression may be controlled by

post-transcriptional events. Altogether, it appears likely that the regulation of utrophin levels and localization are coordinately regulated both by transcriptional and post-transcriptional events, ultimately leading to the preferential accumulation of utrophin at the neuromuscular junction.

Together, these observations are therefore relevant for our basic understanding of the events involved in the assembly and maintenance of the postsynaptic membrane domain of the neuromuscular junction and for the potential use of utrophin as a therapeutic strategy to counteract the effects of DMD.

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LIST OF ABBREVIATIONS

3' UTR	3' untranslated region
5' UTR	5' untranslated region
AChE	acetylcholinesterase
AChR	acetylcholine receptor
ARIA	acetylcholine-receptor-inducing-activity/heregulin
β-gal	β-galactosidase
bHLH	basic helix-loop-helix
bp	base pairs
C-$Ag_{12,0,0}$	recombinant muscle agrin
C-$Ag_{12,4,8}$	recombinant neural agrin
CAT	chloramphenical acetyltransferase
Catx	cardiotoxin
CGRP	calcitonin gene-related peptide
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified essential medium
ETS	E26 transformation specific
FBS	fetal bovine serum
FITC	fluorescein-isothiocyanate-conjugated
GABP	GA-binding protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HSPG	heparan sulfate proteoglycans
MB	myoblasts
MT	myotubes
MuSK	muscle specific kinase
NCAM	neural cell adhesion molecule

nlsLacZ	LacZ gene with a nuclear localization signal
NMJ	neuromuscular junction
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PM	polymyositis
PVDF	polyvinylidene-difluoride
RIPA	radio-immune precipitation assay
RT-PCR	reverse-transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel
TA	tibialis anterior
UTR	untranslated region

CHAPTER 1

INTRODUCTION

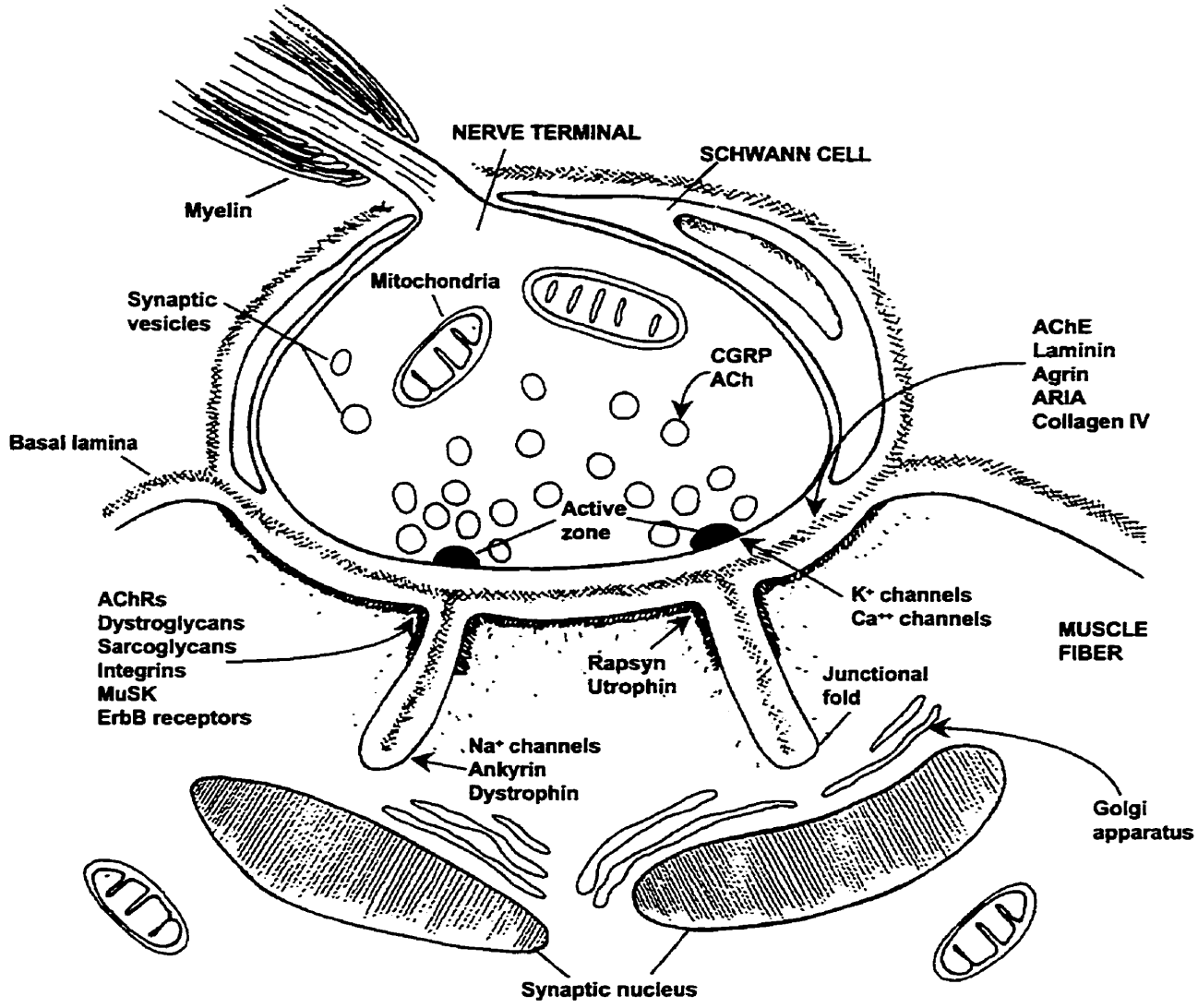
I. The Neuromuscular Junction

Synaptic transmission, the process by which neural signals are communicated from a neuron to its target cell, is a fundamental function of neurons. Proper synaptic transmission is required for: i) the determination of synaptic specificity; ii) the elementary mechanisms of synaptic plasticity; and iii) the establishment of specialized neural networks. The effective transmission at chemical synapses depends upon the coordinated function of a variety of factors, including proper neurotransmitter release, along with a sufficiently high concentration of the appropriate receptors in the postsynaptic membrane. Although a considerable amount of information has become available concerning the general architecture and biochemistry of the synapse, less information is available concerning the mechanisms that lead to the formation and stabilization of this specialized structure. Indeed, the complexity of neurons found within the central nervous system has made it difficult to investigate the mechanisms involved in synaptic transmission. However, a particularly useful model to study these mechanisms has emerged and is derived from a relatively simple synapse, the vertebrate neuromuscular junction. The neuromuscular junction has proven to be a relevant model for examining synapse formation and maintenance for several reasons: i) it is a relatively simple synapse where its overall structure and components have been extensively studied by electron microscopy and immunofluorescence (reviewed in Hall and Sanes, 1993); ii) developing and regenerating synapses can be

experimentally manipulated (see for example, Frank *et al.*, 1975; van Kempen *et al.*, 1994); iii) its biochemistry and physiology have been well characterized (reviewed in Burden, 1998); and iv) gene expression can be altered and studied in detail using transgenic and mutant mice (see for example, Gautam *et al.*, 1995; 1996; Grady *et al.*, 1997a; 1997b; Deconinck *et al.*, 1997a, 1997b; Fromm and Burden, 1998; Feng *et al.*, 1999).

Although the neuromuscular junction represents less than 0.1% of the total area of the muscle fiber, it is a highly differentiated region between skeletal muscle fibers and motor nerves (see for review, Couteux, 1973, Hall and Sanes, 1993; Duclert and Changeux, 1995). Indeed, electron micrographs and thin-section immunofluorescence have revealed that the presynaptic nerve terminal, the muscle fiber, and the surrounding basal lamina are all highly specialized for their role in synaptic transmission (Couteux, 1973, Hall and Sanes, 1993; see also Figure 1.1 and Table 1.1). Large numbers of synaptic vesicles containing, for example, the neurotransmitter acetylcholine (ACh) or the calcitonin gene- related peptide (CGRP), are found within the nerve terminals of motor neurons. These synaptic vesicles are clustered at specialized sites within the motor neuron terminal known as "active zones", a specialized region where the vesicles are ultimately released when an action potential elicits an influx of calcium within the nerve terminal. Contained within this area is the molecular machinery necessary for proper vesicle storage, docking, and release, including for example, a high concentration of sodium channels, calcium channels, and numerous mitochondria (see for review, Hall and Sanes, 1993; Sanes and Lichtman, 1999).

Figure 1.1. The adult neuromuscular junction. Shown is a schematic of the general structure and architecture of the neuromuscular junction. Note that several distinct membrane and cytoskeletal proteins as well as numerous organelles accumulate within this specialized region. (Figure modified from Hall and Sanes, 1993)


NERVE TERMINAL
SCHWANN CELL
Myelin
Mitochondria
Synaptic vesicles
CGRP
ACh
AChE
Laminin
Agrin
ARIA
Collagen IV
Basal lamina
Active zone
AChRs
Dystroglycans
Sarcoglycans
Integrins
MuSK
ErbB receptors
K+ channels
Ca++ channels
MUSCLE FIBER
Rapsyn
Utrophin
Junctional fold
Na+ channels
Ankyrin
Dystrophin
Golgi apparatus
Synaptic nucleus

NEUROMUSCULAR JUNCTION PROTEINS			
Presynaptic Terminal	**Synaptic Basal Lamina**	**Postsynaptic membrane**	**Subsynaptic Cyotskeleton**
synapsins	agrin	acetylcholine receptors (AChR)	dystrophin
syntaxin	s-laminin	ErbB receptors	utrophin
synaptobrevin	collagen IV	integrins	ankyrin
synaptophysin	heparin sulfate proteoglycan	sodium channels	desmin
neuregulins	entactin	MuSK	syntrophins
integrin α1	acetylcholinesterase	N-CAM	β-spectrin
choline acetyltransferase	neuregulins	dystroglycans	talin
calcium channels	Heparin-binding growth-associated molecule (HB-GAM)	sarcoglycans	vincullin
potassium channels	N-acetyl-D-galactosamine (GAL NAc)	rapsyn	actin
acetylcholine			tubulin
calcitonin gene-related protein (CGRP)			dystrobrevins

Table 1.1. Some molecular components of the neuromuscular junction (reviewed in Hall and Sanes, 1993; Duclert and Changeux, 1995; Meier and Wallace, 1998; Sanes and Lichtman, 1999).

Each muscle fiber is enveloped by a basal lamina which not only fully encompasses the fiber but extends into the membrane folds found at the neuromuscular junction. The major components of the muscle basal lamina are consistent with those found within basal lamina of various cell types. For example, collagen IV, laminin, entactin and heparan sulfate proteoglycans (HSPGs) are all found to be present within the muscle basal lamina (Anderson and Fambrough *et al.*, 1983; Sanes *et al.*, 1990). However, the extracellular matrix of muscle fibers becomes highly specialized at the neuromuscular junction. In particular, the synaptic basal lamina is enriched in several additional components including various glyoconjugates (Scott *et al.*, 1988), a collagen-tailed form of acetylcholinesterase (Krecji *et al.*, 1997), and several other molecules, such as agrin and acetylcholine receptor aggregating activity (ARIA: also referred to as heuregulin/neuregulin/neu differentiation factor), two molecules which are expressed both by the motor nerve and the muscle fiber and are known to regulate the expression and localization of various components of the neuromuscular junction (Anderson and Cohen, 1977; Burden *et al.*, 1979; McMahan, 1990) (discussed in detail below).

The postsynaptic membrane of the neuromuscular junction formed by the muscle fiber is also highly specialized for its role in synaptic transmission. For instance, this region of the muscle fiber is characterized by 1 μm invaginations of the muscle membrane, termed postjunctional folds, which serve to dramatically increase membrane surface area at the neuromuscular junction. It is within the crests of these junctional folds that a high concentration of acetylcholine receptors (AChR) (> 10,000 molecules/μm^2) are found (Salpeter *et al.*, 1983). In addition to the junctional folds, other postsynaptic specializations are also evident at the

neuromuscular junction. For example, present beneath the postsynaptic membrane domain of the neuromuscular junction are myonuclei that are morphologically distinct from their extrasynaptic counterparts (Ranvier, 1888). These nuclei transcribe a selective subset of genes encoding various synaptic molecules including for example, AChR (Merlie and Sanes, 1985; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Klarsfeld *et al.*, 1991; Sanes *et al.*, 1991; Simon *et al.*, 1992) and AChE (Jasmin *et al.*, 1993; Michel *et al.*, 1994; Chan *et al.*, 1999). Also present within the postsynaptic membrane domain is a high concentration of mitochondria to meet the energy demands of neurotransmission (Jasmin *et al.*, 1995a; Campbell *et al.*, 1996; see for review, Ogata, 1988; Engel, 1994) along with a specialized golgi apparatus and microtubule network to facilitate sorting and targeting of synaptic proteins (Jasmin *et al.*, 1989; Jasmin *et al.*, 1995b). Finally, various other proteins implicated in maintaining the structure and function of the neuromuscular junction are also present at the postsynaptic membrane domain including for example, actin, tubulin, neural cell adhesion molecules (N-CAM), voltage-gated sodium channels, dystrophin and its homologue, utrophin (see for review, Hall and Sanes, 1993; Sanes and Lichtmann, 1999).

Although considerable information is available concerning the general architecture of the neuromuscular junction, less information is available regarding the mechanisms that lead to the formation and maintenance of this specialized region of the muscle fiber. However, one of the best studied components of the neuromuscular junction is the AChR. Indeed, numerous studies have examined the cellular factors that underlie the localization of the AChR at the postsynaptic membrane domain (see for review, Duclert and Changeux, 1995). It has been demonstrated that

three distinct processes contribute to the localization of AChR at developing and adult neuromuscular junctions including: i) the local clustering of diffusely presynthesized AChR in the postsynaptic membrane; ii) local transcriptional control of AChR gene expression at the neuromuscular junction; and iii) transcriptional repression of AChR genes in extrasynaptic regions (Hall and Sanes, 1993; Duclert and Changeux, 1995; Sanes and Lichtman, 1999). Ultimately, these three mechanisms act to cooperatively ensure the local expression of AChR at the neuromuscular junction. These distinct processes are controlled by the motor nerve in separate but complementary mechanisms, discussed in further detail below (see Figure 1.2).

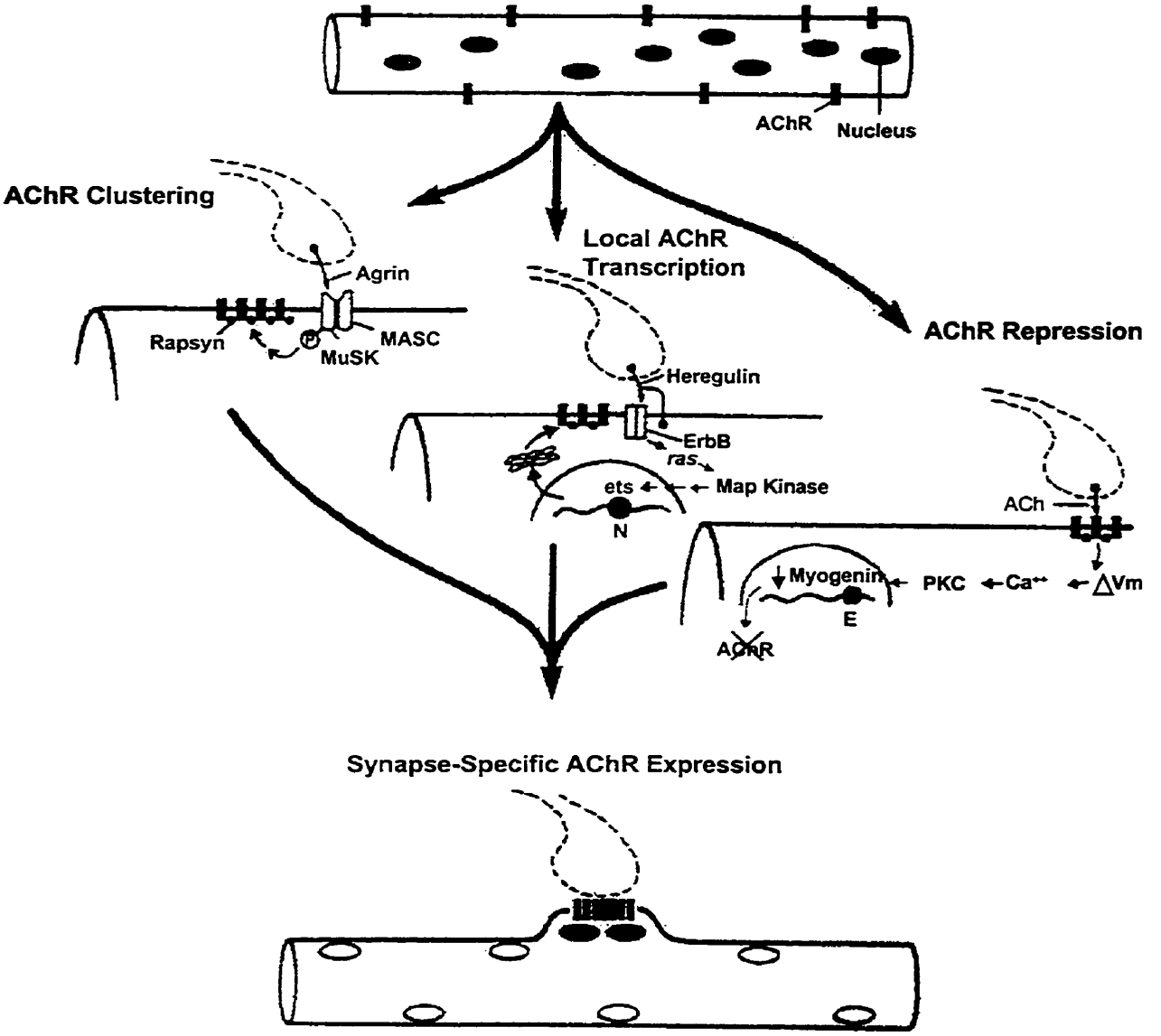
I.A. Local Clustering of Synaptic Proteins

The earliest stages of synaptic differentiation involve the local clustering of proteins at the developing synaptic regions. Initial studies into the mechanisms responsible for this local clustering revealed that the accumulation of synaptic proteins could occur at preexisting synaptic sites, in the absence of the motor nerve (Anglister and McMahan, 1985; Nitkin *et al.*, 1987; see also Burden *et al.*, 1979). Detailed investigation into the factors that induced these synaptic clusters revealed the important contribution of the basal lamina, and specifically the extracellular matrix protein called agrin (see for review, Bowe and Fallon, 1995). Subsequent analysis of agrin revealed it to be ~200 kD proteoglycan that is synthesized by motor nerves, transported to nerve terminals and inserted into the basal lamina (Magill-Solc and McMahan, 1988, 1990). Following its identification, McMahan (1990) originally proposed that agrin was a critical nerve-derived organizer of postsynaptic differentiation. Consistent with such a model, purified or

recombinant agrin added to muscle cells in culture induces specializations at which AChRs and other components of the postsynaptic apparatus accumulate (McMahan, 1990; Reist *et al.*, 1992; Campanelli *et al.*, 1994). Furthermore, postsynaptic AChR aggregates are markedly reduced in number, size, and density in muscles from agrin-deficient mice, resulting in the death of these animals immediately after birth from the inability to breathe (Gautam *et al.*, 1996). Together, these results support a central role for agrin in postsynaptic formation, as proposed by McMahan (1990).

The agrin gene has been localized to human chromosome 1p32 and mouse chromosome 4 (Rupp *et al.*, 1992). This gene gives rise to several alternatively spliced mRNAs which generate multiple agrin isoforms that differ in their carboxyl termini (Ferns *et al.*, 1992, 1993; Ruegg *et al.*, 1992; Hoch *et al.*, 1993). These isoforms are generated by three splicing sites (termed x, y and z) where extra amino acids (up to 12, 4 or 19, respectively) can be inserted (Ferns *et al.*, 1992, 1993; Ruegg *et al.*, 1992; Hoch *et al.*, 1993). The agrin isoforms that are the most active in clustering AChRs contain inserts at the y and z sites, while the agrin isoforms that are least active in AChR clustering lack inserts at either one or both of the y and z sites (Fallon and Gelfman, 1989; Ferns *et al.*, 1992, 1993; Ruegg *et al.*, 1992; Hoch *et al.*, 1993; Gesemann *et al.*, 1995). Importantly, the expression of distinct subsets of these agrin isoforms appear to be restricted to either muscle or nerve. For instance, the highly active forms of agrin are made exclusively by motor neurons and are deposited into the synaptic basal lamina, while the agrin isoforms that lack the inserts are made predominately by the muscle (Ruegg *et al.*, 1992; Ferns

Figure 1.2. Selective accumulation of synaptic proteins in the postsynaptic membrane. The compartmentalization of AChR is dependent on the motor nerve via three distinct mechanisms: initial clustering, local transcription and extrajunctional repression. Agrin interacting with MuSK organizes rapsyn-mediated AChR clustering. Local transcription appears to be regulated by heregulin and the ErbB receptor kinases to induce selective gene expression within synaptic nuclei. This local transcription appears to be dependent upon the ETS- family of transcription factors binding to the N-box DNA element. Finally, extrajunctional repression is mediated by ACh release which activates AChR and generates action potentials that repress AChR subunit gene expression in extrasynaptic nuclei. This effect is mediated in part by the inactivation and reduction of myogenic regulatory proteins, termed the MyoD transcription factors, which bind and activate the E-box sequence. Together, these signals lead to the selective synthesis and precise accumulation of AChRs in the postsynaptic membrane. (Figure modified from Sanes and Lichtman, 1999).



Synapse-Specific AChR Expression

et al., 1992; Hoch *et al.*, 1993). Indeed, the ability of agrin isoforms to cluster AChR in muscle cells can vary by up to ten thousand fold (Ferns *et al.*, 1992, 1993; Ruegg *et al.*, 1992; Hoch *et al.*, 1993; Gesemann *et al.*, 1995). The function of all of the various agrin isoforms, has yet to be fully addressed, however, it appears that at least some of the non-neural isoforms may act to modulate the neural agrin signaling pathway (Lieth *et al.*, 1993; Deyst *et al.*, 1998; Meier *et al.*, 1998).

Further characterization of the effects of agrin on muscle cells revealed two apparently distinct phases of agrin-induced molecular redistributions: an early stage and a late stage (Bowe and Fallon, 1995). The early stage begins immediately upon agrin treatment of cultured myotubes and is characterized by the clustering of several membrane and membrane-associated proteins including AChR and globular cholinesterases (Wallace, 1989; Nastuk *et al.*, 1991). The aggregation of these molecules results from the recruitment of pre-existing molecules to developing synaptic sites, along with a localized targeting of newly synthesized molecules to these sites, and not necessarily due to increased protein synthesis (Wallace, 1989). It was also observed that a second set of molecules becomes concentrated at agrin-induced AChR aggregates following several hours of agrin treatment. These later-phase elements include various components of the dystrophin-associated glycoprotein complex, such as utrophin, along with additional basal lamina components, including the muscle isoform of agrin (Wallace, 1989; Nitkin and Rothschild, 1990; Lieth *et al.*, 1993). In contrast to the first stage of agrin-induced AChR clustering, the appearance of these molecules has been suggested to involve new protein synthesis (Bowe and Fallon, 1995). Coincidentally, the expression of these late-phase molecules

correlates with increased AChR cluster stability, indicating that these particular molecules may be important for synaptic maturation (Bowe and Fallon, 1995). Thus, the multi-step nature of synapse formation and agrin-induced clustering may function by initially clustering AChR and then stabilizing a synapse-specific membrane cytoskeletal scaffold upon which synaptic molecules are anchored and concentrated (Campanelli *et al.*, 1994).

Although the entire signaling pathway involved in the agrin-induced AChR clustering remains to be demonstrated (McMahan, 1990; Bowe and Fallon, 1995) converging lines of evidence implicate the involvement of tyrosine phosphorylation (Wallace, 1995; Meier *et al.*, 1995; Ferns *et al.*, 1996). A muscle-specific tyrosine kinase receptor designated as MuSK has been identified and shown to interact with agrin (Valenzuela *et al.*, 1995; Glass *et al.*, 1996). Several lines of evidence now support a central role for MuSK in agrin-induced synaptic differentiation including: i) MuSK is abundantly expressed in skeletal muscle fibers of newborn rats and becomes concentrated at the neuromuscular junction in adult animals (Valenzuela *et al.*, 1995); ii) recent analysis of MuSK-deficient mice (Valenzuela *et al.*, 1995) which, similar to the agrin-deficient mice (Gautam *et al.*, 1996), lack specialization at the neuromuscular junction; iii) cultured MuSK mutant muscle cells are not able to cluster AChRs in response to agrin (Glass *et al.*, 1996); iv) agrin can be cross-linked to MuSK (Glass *et al.*, 1996); and v) constitutively active MuSK is capable of inducing postsynaptic specializations and can increase AChR gene expression (Jones *et al.*, 1999). Therefore, one putative signaling pathway involved in AChR clustering by agrin may involve binding of agrin to a complex of proteins in the postsynaptic sarcoplasm that includes MuSK and a myotube-specific accessory component (MASC) that

appears to be required for MuSK to bind agrin (Valenzuela *et al.*, 1995; Glass *et al.*, 1996; Glass and Yancopoulos, 1997). Additional molecules that appear to be involved downstream of MuSK include a 43 kD protein, called rapsyn, which has been shown to play an important role in agrin-mediated AChR clustering (Sanes and Lichtman, 1999). Rapsyn is a peripheral membrane protein that is present at the earliest stages of AChR clustering (Noakes *et al.*, 1993) and can induce the formation of AChR clusters in cultured cells (Froehner *et al.*, 1990; Phillips *et al.*, 1993). Recently, the normal localization of AChR along with other synaptic proteins was shown to be significantly disrupted in rapsyn-deficient mice (Gautam *et al.*, 1995), supporting a central role for rapsyn in synaptic formation. Altogether, MuSK is a critical component of a primary synaptic scaffold to which rapsyn is recruited which subsequently leads to the assembly of other synaptic components, such as AChR, onto this scaffold.

In addition to a MuSK-dependent regulation of AChR clustering, other agrin-binding proteins of the muscle membrane have also been identified. These include the heparin-binding growth-associated molecule (HB-GAM) (Peng *et al.*, 1995), heparan sulfate proteoglycans (HPSGs) (Meier *et al.*, 1998) and α-dystroglycan (Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Hemler, 1999). The most abundant of these molecules and first identified agrin-binding protein is α-dystroglycan. α-dystroglycan is a peripheral membrane protein which binds both agrin and laminin in the extracellular matrix and is linked to β-dystroglycan, an integral membrane membrane that associates intracellularly with dystrophin (Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Hemler, 1999) or utrophin (Matsumura *et al.*, 1992). Although it is known that agrin binds α-dystroglycan with high affinity (Bowe *et al.*, 1994;

Campanelli *et al.*, 1994; Sugiyama *et al.*, 1994; Gee *et al.*, 1994), the exact role of α-dystroglycan in AChR cluster formation is still unclear. There is accumulating evidence that supports a role for α-dystroglycan in agrin-induced synaptic formation including experiments where α-dystroglycan antibodies impaired agrin-induced AChR cluster formation (Campanelli *et al.*, 1994; Gee *et al.*, 1994). The generation of α-dystroglycan knockout mice failed to further elucidate the role of α-dystroglycan in neuromuscular junction formation since these animals do not survive long enough to develop peripheral synapses (Williamson *et al.*, 1997). However, chimeric mice with a marked deficiency in α-dystroglycan have recently been generated and these animals survive through postnatal development (Côté *et al.*, 1999). It is also interesting that these animals possess aberrant neuromuscular junctions with a marked disruption of the localization of several critical synaptic proteins, including AChR and AChE (Côté *et al.*, 1999). Based on these results, it is likely that α-dystroglycan is involved in mediating critical steps in the formation and maintenance of the neuromuscular junction and may act in concert with MuSK-dependent signaling mechanisms to ensure proper synaptic formation and differentiation.

I.B. Synapse-Specific Gene Transcription

Following the initial accumulation and stabilization of AChR clusters at the developing neuromuscular junction, continued maintenance of synapse-specific expression appears to depend on the selective transcription of certain genes by postsynaptic myonuclei. For instance, numerous studies have observed the preferential accumulation of transcripts encoding several

neuromuscular proteins specifically enriched within postsynaptic membrane domains (Merlie and Sanes, 1985; Goldman and Staple, 1989; Klarsfeld *et al.*, 1991; Sanes *et al.*, 1991; Simon *et al.*, 1992; Michel *et al.*, 1994; Moscoso *et al.*, 1995; Imaizumi-Scherrer *et al.*, 1996; reviewed in Duclert and Changeux, 1995). It has been proposed that this local accumulation of transcripts results in the preferential expression of synaptic proteins (see for review, Duclert and Changeux, 1995). Although the mechanisms responsible for this local mRNA expression are not completely understood, it appears that factors derived from the motor nerve account for most of this selective mRNA localization (Klarsfeld *et al.*, 1991; Sanes *et al.*, 1991; Duclert *et al.*, 1993; Tang *et al.*, 1994). Specifically, substantial evidence has revealed the important contribution of several nerve-derived factors including calcitonin gene-related peptide and acetylcholine receptor-aggregating-activity (ARIA/heregulin) to the transcriptional regulation of synaptic proteins.

I.B.i. Nerve-Derived Trophic Factors

Calcitonin gene-related peptide (CGRP) is a neuropeptide which is synthesized in spinal motor neurons and found to be enriched at the motor endplate (Popper and Micevych, 1989). Originally, due to its synaptic location it was suggested that CGRP may be a key factor in regulating synaptic differentiation (Popper and Micevych, 1989). Since then, numerous studies have been performed to ascertain the role of CGRP in synaptic formation (see for review, Duclert and Changeux, 1995). For instance, in cultured muscle cells CGRP has been demonstrated to be a potent inducer of AChR gene expression, likely via the activation of a cAMP-dependent

protein kinase (New and Mudge, 1986; Fontaine *et al.*, 1987; Osterlund *et al.*, 1989). Thus, it has been suggested that the local presence of CGRP at the motor endplate may ultimately contribute to the local expression of synaptic proteins by affecting gene expression (Changeux *et al.*, 1992). To directly assess the role of CGRP in neuromuscular junction formation, CGRP-deficient mice have recently been generated and their neuromuscular junctions examined (Lu *et al.*, 1999). Detailed characterization of these animals including an assessment of nicotinic receptor localization, terminal sprouting in response to denervation, developmental regulation of AChR subunit expression, and synapse elimination revealed no major differences in CGRP-deficient animals as compared to control littermates (Lu *et al.*, 1999). These results suggest that CGRP may not be required for the initial development of the neuromuscular junction, however, this does not preclude the possibility that CGRP may still function as a modulator of synaptic differentiation under certain conditions (Lu *et al.*, 1999).

Acetylcholine-receptor inducing activity (ARIA/heregulin) is another growth/trophic factor that is found to be enriched within the synaptic basal lamina. ARIA/heregulin is ~45 kD protein initially purified from chick brain (Usdin and Fischbach, 1986) on the basis of its ability to stimulate AChR synthesis in cultured myotubes (Harris *et al.*, 1988; Martinou *et al.*, 1991; Chu *et al.*, 1995; Ahn Jo *et al.*, 1995; Lemke, 1996). Identification and sequence analysis of the ARIA gene revealed that it is encoded by the same gene that encodes various ligands for the neu-proto-oncogene (heregulin/neu) (Holmes *et al.*, 1992; Marchionni *et al.*, 1993). To date, a minimum of fourteen different cDNAs for neuregulin have been identified (Peles and Yarden, 1993; Ben-Baruch and Yarden, 1994; Fischbach and Rosen, 1997). The neuregulin isoforms are

generated from alternative promoters and splicing events, but one major structural feature that is conserved among all isoforms is a common EGF-like domain (Fischbach and Rosen, 1997). Despite the molecular differences of the various isoforms it appears that they are all similar, if not identical, with respect to signal transduction (Fischbach and Rosen, 1997), likely as a result of the conserved EGF-like domain (Yang *et al.*, 1997). Based on their extensive similarity and origin from the same gene, these related isoforms are now collectively referred to as the neuregulins (Peles and Yarden, 1993; Ben-Baruch and Yarden, 1994; Fischbach and Rosen, 1997).

It has been well established that the members of the neuregulin family are ligands for the EGF-receptor-related (ErbB) tyrosine kinase receptors and can regulate gene expression (Peles and Yarden, 1993; Ben-Baruch and Yarden, 1994; Fischbach and Rosen, 1997). In particular, neuregulin signaling via the ErbB family of receptors is known to involve the recruitment of various cytoplasmic proteins, such as Grb2 or SHC, to the receptor through SH2-binding domains which can subsequently activate the GTP/GDP-binding protein called RAS (McCormick *et al.*, 1994; Burgering and Bos, 1995). A downstream target of activated RAS is the serine/threonine protein kinase, termed RAF, which, in turn, activates the mitogen-activated protein (MAP) kinase kinase (ERK), also referred to as MEK for MAP-kinase/ERK-activating kinase (Blenis, 1993). Finally, ERK/MEK can activate MAP kinase which is known to control gene expression and protein synthesis of various transcription factors (see for review, Marshall, 1994; Robbins *et al.*, 1994). Therefore, one signaling pathway initiated by neuregulin family

members is activated by ErbB receptors and, ultimately, mediates gene transcription via the activation of MAP kinase.

Accumulating evidence suggests that members of the neuregulin family may regulate synaptic gene expression in skeletal muscle fibers. Initial studies attempted to ascertain the role of neuregulin signaling in neuromuscular junction formation, however, mice deficient in the neuregulins or the ErbB receptors die during embryogenesis prior to neuromuscular junction formation, making it difficult to determine the contribution of neuregulins to regulating synaptic differentiation (Meyer and Birhmeier, 1995). Nonetheless, heterozygous mice with low levels of neuregulins have also been generated and these animals display a mild deficiency in synaptic transmission and possess significantly reduced AChR numbers at the neuromuscular junction (Sandrock *et al.*, 1997), indicating that neuregulins may indeed be required for synaptic formation and maintenance. In fact, the neuregulin isoform, ARIA/heregulin, is found to be preferentially expressed at the neuromuscular junction (Ahn Jo *et al.*, 1995; Goodearl *et al.*, 1995; Moscoso *et al.*, 1995b), along with the ErbB receptors (Altiok *et al.*, 1995; Zhu *et al.*, 1995). Furthermore, treatment of muscle cells in culture with ARIA/heregulin is known to markedly influence the expression of the ε-subunit of AChR subunit genes (Gunderson *et al.*, 1993; Tang *et al.*, 1994; Ahn Jo *et al.*, 1995; Chu *et al.*, 1995; Schaeffer *et al.*, 1998; Fromm and Burden, 1998; Si and Mei, 1999), and has recently been implicated in the regulation of several muscle-specific genes, including for example, myosin and tropomyosin (Kim *et al.*, 1999; Fu *et al.*, 1999). The regulation of gene expression for these proteins appears to be a direct result of activation and tyrosine phosphorylation of the ErbB receptors (Ahn Jo *et al.*, 1995; Altiok *et al.*,

1995). Since ARIA/heregulin has previously been documented to activate the RAS-dependent signal cascade (Ben Levy *et al.*, 1994; Marte *et al.*, 1995; for review see Schlessinger, 1994), it is possible that ARIA/heregulin may regulate AChR gene expression via this pathway. Indeed, several studies have determined that ARIA/heregulin does activate the RAS/MAP-kinase signal cascade in skeletal muscle cells, resulting in an increased AChR gene expression (Tansey *et al.*, 1996; Si and Mei., 1999; Tanowitz *et al.*, 1999; Won *et al.*, 1999).

I.B.ii. DNA Regulatory Elements

In addition to the elucidation of the role of nerve-derived trophic factors, recent studies using *in vivo* DNA injection techniques and transgenic animals have defined the DNA promoter elements critical for the local transcription of the AChR within synaptic myonuclei (Koike *et al.*, 1995; Duclert *et al.*, 1996; Schaeffer *et al.*, 1998; Fromm and Burden, 1998). In these studies, the DNA element consisting of the core sequence of TTCCGG, called an N-box element, has been identified on the basis of its ability to direct the local transcriptional activation of both the δ- and ε-subunit of AChR at the neuromuscular junction (Koike *et al.*, 1995; Duclert *et al.*, 1996). Although several recent reports have indicated that the N-box motif binds a protein complex from muscle extracts (Koike *et al.*, 1995; Duclert *et al.*, 1996), the identity of this factor(s) has remained largely unknown. However, ETS (E26 transformation specific) transcription factors are known to bind to the core sequence of C/A GGA A/T in the middle of ~10 bp sequence of DNA and transcriptionally activate genes containing ETS-binding sites (Wasylyk *et al.*, 1993). Coincidentally, the δ- and ε-subunit genes of AChR contain this

sequence which directly overlaps the region of the N-box along with its flanking sequence (Schaeffer *et al*, 1998; Fromm and Burden, 1998). Based on the presence of this consensus sequence within the AChR subunit genes and the fact that this sequence overlaps the N-box motif, which has previously been shown to be important in synapse-specific gene regulation (Koike *et al.*, 1995; Duclert *et al.*, 1996), it appears likely that ETS-transcription factors may regulate the expression of synaptic proteins. In fact, two recent studies have confirmed that the synaptic transcriptional activation via the N-box element is due to the binding of an ETS-related transcription factor, called GA-binding protein or GABP (Brown and McKnight, 1992), to this DNA region (Schaeffer *et al*, 1998; Fromm and Burden, 1998). GABP is a multimeric ETS-related transcription factor that consists of a 58 kD α-subunit containing the DNA-binding ETS motif, and a 43 kD β-subunit required to obtain efficient DNA binding (LaMarco *et al.*, 1991; Brown and McKnight, 1992; Sawa *et al.*, 1996; Batchelor *et al.*, 1998). Indeed, although the β-subunit of GABP is found to be evenly expressed along the entire length of skeletal muscle fibers, the α-subunit is preferentially enriched within postsynaptic myonuclei (Schaeffer *et al.*, 1998), a finding entirely consistent with GABP being a transcription factor that would regulate synaptic gene expression. Taken together, the downstream events of transcriptional regulation of gene expression at the neuromuscular junction appear to involve the N-box DNA element and the transcription factor GABP (Schaeffer *et al.*,1998; Fromm and Burden, 1998).

Interestingly, it is now known that the downstream events of ARIA/heregulin signaling involve the same cis-acting region that regulates the preferential synaptic expression of AChR. In fact, ARIA/heregulin has recently been shown to activate the N-box element (Schaeffer al.,

1998; Fromm and Burden, 1998). Detailed investigation into the mechanism of action of ARIA/heregulin has revealed that it increases the protein level of the GABP α-subunit along with increasing the phosphorylated state of both subunits (Schaeffer *et al.*, 1998; Burden *et al.*,1998). Altogether, it is apparent that synapse-specific gene expression is controlled by ARIA/heregulin acting on its receptors, and ultimately involves the ETS-related transcription factor, GABP, interacting with the N-box consensus sequence (Schaeffer *et al.*, 1998; Fromm and Burden, 1998).

I.C. Extrajunctional Repression of Synaptic Expression

In order to ensure preferential synapse-specific gene expression, a mechanism also exists by which extrajunctional expression of synaptic proteins is repressed. For example, following the initial formation of synaptic specializations, the nerve appears to exert a pronounced repressive effect on the synthesis of extrajunctional AChRs via nerve-derived electrical activity (for review see, Lomo and Westgaard, 1975; Hall and Sanes, 1993). This electrical activity-dependent gene repression is the result of electrical activity being transmitted along the length of the sarcolemma initiating electrical potentials along the muscle fiber. These depolarizations of the muscle membrane subsequently lead to elevated levels of intracellular calcium through voltage activated calcium channels which, in turn, activate the intracellular signaling molecule, protein kinase C (PKC) (Klarsfeld *et al.*, 1989; Laufer *et al.*, 1991; Huang *et al.*, 1992). Protein kinase C subsequently inactivates and downregulates the expression of a family of proteins, termed the MyoD family of transcription activation factors (Huang *et al.*, 1992).

The MyoD family of transcription regulatory proteins consists of four members including MRF4, myogenin, Myf-5 and MyoD (Weintraub *et al.*, 1991; Weintraub *et al.*, 1993; Rudnicki and Jaenisch, 1995; Ludolph and Konieczny, 1995; Arnold and Winter, 1998). These proteins are characterized by a basic helix-loop-helix (bHLH) domain which is involved in dimerization with a ubiquitous class of bHLH proteins known as E proteins (see Molkentin and Olsen, 1996). The resulting heterodimers bind the consensus sequence termed an E-box, which consists of the sequence CANNTG, and subsequently regulate muscle-specific gene expression (Weintraub *et al.*, 1991; Weintraub *et al.*, 1993; Rudnicki and Jaenisch, 1995; Ludolph and Konieczny, 1995; Arnold and Winter, 1998). Therefore, the inactivation and reduction in MyoD levels caused by electrical activity results in the downregulation of genes that are regulated by these factors. Coincidentally, E-box motifs have been identified in numerous skeletal muscle-specific genes, including the genes encoding the various AChR subunits (Piette *et al.*, 1990; Jia *et al.*, 1992; Prody and Merlie, 1992; Simon and Burden, 1993; see Duclert and Changeux, 1995). Coherent with this model of electrically-derived extrasynaptic repression, the inhibition of electrical activity during muscle cell development using chemical agents can prevent the downregulation of AChR within extrajunctional regions (Burden, 1977). Similarly, the denervation of adult muscle induces the reappearance of extrajunctional expression of AChR via a transcriptional activation of AChR gene expression (Miledi, 1960; Tsay and Schmidt, 1989; reviewed in Duclert and Changeux, 1995). Finally, the chronic stimulation of denervated muscles can reverse the reappearance of AChR within extrajunctional regions (Lomo and Rosenthal, 1972; Lomo and Westgaard, 1975). Altogether, electrical activity, the MyoD family

of transcription factors and the E-box element play a central role in the repression of extrajunctional AChR.

However, in addition to the regulation of extrajunctional repression of synaptic proteins, the E-box and the MyoD family of transcription factors are also involved in the regulation of gene expression during muscle cell development, or myogenesis (Weintraub *et al.*, 1991; Weintraub *et al.*, 1993; Rudnicki and Jaenisch, 1995; Ludolph and Konieczny, 1995; Arnold and Winter, 1998). The formation of skeletal muscle during vertebrate embryogenesis requires: i) the commitment of precursor cells to the skeletal muscle lineage; ii) the withdrawal of myoblasts from the cell cycle; iii) the fusion with other myoblasts; and iv) the transcriptional activation of muscle-specific genes (Weintraub *et al.*, 1991; Weintraub *et al.*, 1993; Rudnicki and Jaenisch, 1995; Arnold and Winter, 1998). The elucidation of the molecular mechanisms that control myogenesis has revealed that the MyoD family of transcription factors along with the myocyte enhancer factor 2 (MEF2) play a pivotal role in this process of lineage commitment and differentiation (Molkentin and Olson, 1996; Arnold and Winter, 1998). Indeed, during skeletal muscle development, the expression of the MyoD family of transcription factors is significantly increased and, as a result of the elevated levels of these transcription factors, other genes that contain E-box elements are significantly upregulated during skeletal muscle formation (Weintraub *et al.*, 1991; Weintraub *et al.*, 1993; Rudnicki and Jaenisch, 1995; Ludolph and Konieczny, 1995; Arnold and Winter, 1998). For example, the transcription of genes regulated by the MyoD family of transcription factors, which includes myosin, dystrophin and AChR, can be increased up to 30-fold during myogenic differentiation (Medford *et al.*, 1983; Lev *et al.*,

1987; Nudel *et al.*, 1988; Passaquin *et al.*, 1993; Tennyson *et al.*, 1996a; Tennyson *et al.*, 1996b). Thus, the E-box element and MyoD transcription factors are critical regulators of gene expression during skeletal muscle development.

II. Dystrophin and Duchenne Muscular Dystrophy

Muscular dystrophy is a term encompassing a wide range of congenital disorders which are characterized by progressive skeletal muscle wasting. Amongst the various forms of dystrophies, Duchenne muscular dystrophy (DMD) is the most prevalent affecting approximately 1 out of every 3,500 male births (Emery, 1991). The disease is also extremely severe since DMD patients usually become confined to a wheelchair by adolescence and die of respiratory or cardiac failure in their third decade of life. The gene responsible for DMD was identified several years ago and designated as the dystrophin gene (for review, see Ahn and Kunkel, 1993; Matsumura and Campbell, 1994; Worton, 1995). Located on the short arm of the X chromosome at Xp21, dystrophin constitutes the largest gene identified to date with 79 exons that span more than 2.5 megabases in the human genome (Monaco *et al.*, 1986; Koenig *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988). The dystrophin gene encodes a 14 kb transcript that is translated into a 427 kD protein which is predominately expressed in skeletal, cardiac and smooth muscle, with lower levels in the brain (Monaco *et al.*, 1986; Burghes *et al.*, 1987; Chamberlain *et al.*, 1988; Nudel *et al.*, 1988). Indeed, a detailed investigation into the structure and function of dystrophin has revealed four major domains: i) an N-terminal region which contains a functional actin-binding region (Winder and Kendrick-Jones, 1995; Winder, 1997; Amman *et al.*, 1998); ii) a central rod

domain which consists of a number of amino acid repeats that show similarity to spectrin, likely contributing to the flexible rod-shape structure of dystrophin (Pons *et al.*, 1990); iii) a cysteine rich segment which contains putative calcium binding sites (Koenig *et al.*, 1988); and iv) a carboxy terminal region which binds a complex of membrane-associated proteins, collectively referred to as the dystrophin-associated protein (DAP) complex (Matsumura *et al.*, 1992). Furthermore, in recent years it has become increasingly evident that the structure of the dystrophin gene is also extremely complex. Indeed, use of tissue-specific and internal promoters can give rise to multiple isoforms of dystrophin which are expressed in a variety of tissues (Table 1.2) (Bar *et al.*, 1990; Gorecki *et al.*, 1992; Lambert *et al.*, 1993; Schofield *et al.*, 1994; D'Souza *et al.*, 1995; Lidov *et al.*, 1995).

In skeletal muscle fibers, full-length dystrophin is known to accumulate along the cytoplasmic face of the sarcolemma and also to be enriched at the neuromuscular and myotendinous junctions (Arahata *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988; Bonilla *et al.*, 1988; Watkins *et al.*, 1988; Byers *et al.*, 1991; Sealock *et al.*, 1991). Although the precise function of dystrophin remains elusive, biochemical experiments have led to the notion that dystrophin links the internal cytoskeleton of muscle fibers to the extracellular matrix via interactions with the oligomeric dystrophin-associated protein (DAP) complex (Ervasti *et al.*, 1990; Matsumura *et al.*, 1992; Blake *et al.*, 1994; Sadoulet-Puccio and Kunkel, 1996; Winder, 1997; see also Figure 1.3). This subcellular organization suggests that dystrophin plays an essential role in maintaining the integrity of the sarcolemma during repeated cycles of muscle contraction and relaxation (Petrof *et al.*, 1993; Pasternak *et al.*, 1995; Decrouy *et al.*, 1997). Mutations and/or deletions in the

dystrophin gene, as seen in DMD, result in a complete absence of functional dystrophin molecules as well as in a significant reduction of DAPs from the sarcolemma (Ervasti *et al.*, 1990; Matsumura *et al.*, 1992; Straub *et al.*, 1997; and see for reviews, Matsumura and Campbell, 1994; Worton, 1995). Accordingly, dystrophic muscle fibers demonstrate increased sarcolemmal instability (Straub *et al.*, 1997) and are therefore, highly susceptible to the effects of mechanical stress (Petrof *et al.*, 1993; Pasternak *et al.*, 1995; Decrouy *et al.*, 1997).

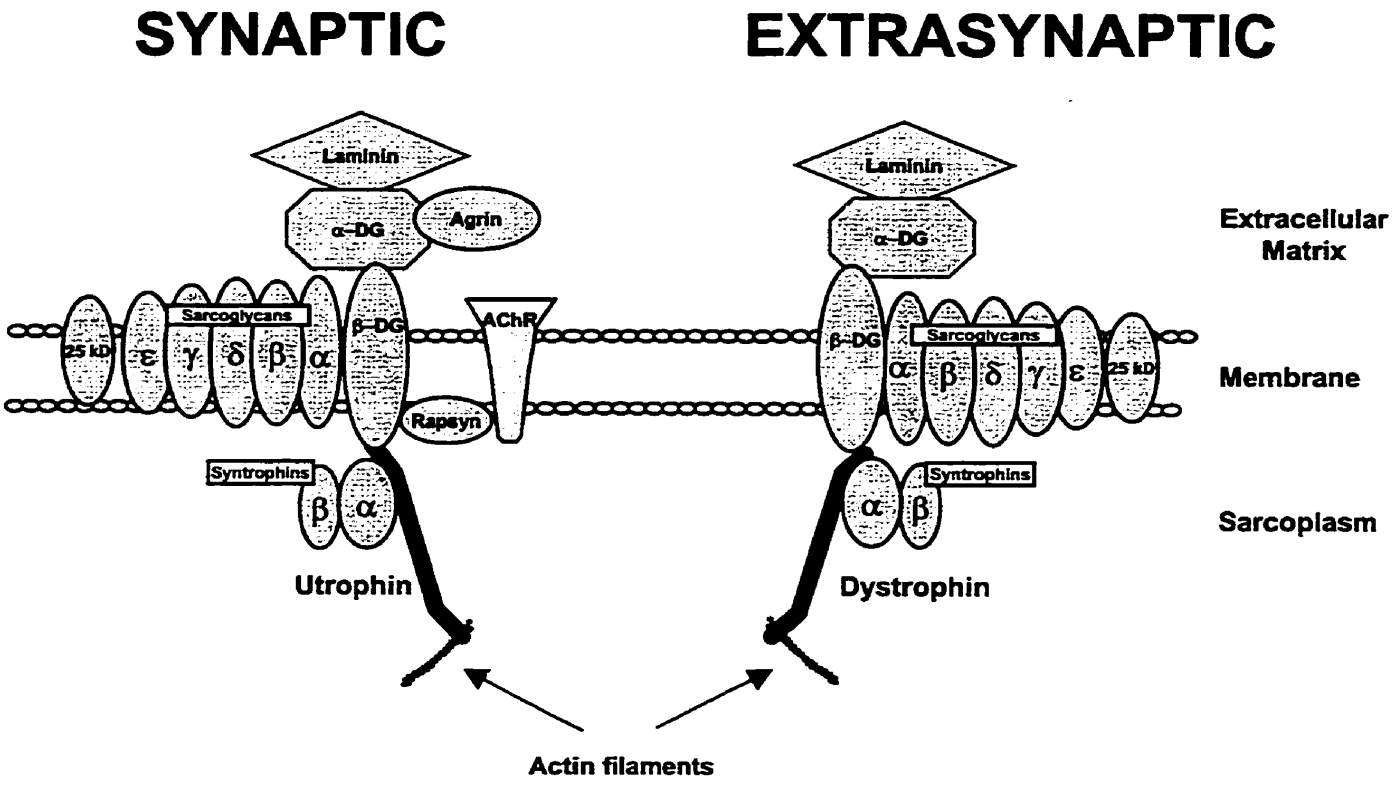
II.A. Utrophin: An Autosomal Homologue to Dystrophin

Utrophin is an autosomal homologue to dystrophin originally discovered by screening a fetal muscle cDNA library under low stringency using oligonucleotide probes from the carboxyl terminal coding region of the dystrophin transcript (Love *et al.*, 1989). A partial clone was first isolated and led, subsequently, to the identification of the utrophin gene (Love *et al.*, 1989; Buckle *et al.*, 1990). In contrast to the X-linked dystrophin gene, the utrophin gene is located on mouse chromosome 10 and human chromosome 6 (Buckle *et al.*, 1990). Similar to dystrophin, the utrophin gene is exceptionally large since it spans more than 1 Mb in the human genome. The gene encodes a large transcript, i.e., 13 kb, which once translated, gives rise to a cytoskeletal protein of ~395 kD. The deduced amino acid sequence of utrophin predicts the existence of several structural motifs similar to those identified in dystrophin. For example, utrophin and dystrophin both possess an NH_2-terminal actin-binding motif, a central rod region containing multiple triple helical repeats, a cysteine-rich domain and a COOH-terminal domain which interacts with DAPs (Matsumura *et al.*, 1992; Tinsley *et al.*, 1992) (Figure 1.4). In fact,

DYSTROPHIN ISOFORMS AND HOMOLOGUES				
Gene (Chromosome)	**Transcript Size (kb)**	**Protein Size (kD)**	**Expression**	**References**
Dystrophin (Xp21)	14	427	Muscle and brain	Koenig *et al.*, 1987; Zubrzycka-Gaarn *et al.*, 1988; (see for review Straub and Campbell, 1997)
Dp260 (Xp21)	not determined	260	Retina	D'Souza *et al.*, 1995; Rodius *et al.*, 1997
Dp140 (Xp21)	7.5	140	Brain and retina	Lidov *et al.*, 1995
Dp116 (Xp21)	5.8	116	Peripheral nerve, embryonic brain	Schofield *et al.*, 1994; Byers *et al.*, 1993
Dp71 (Xp21)	4.8	71	Fetal muscle only, brain, lung, liver, kidney	Lambert *et al.*, 1993
Utrophin (DRP) (6q24)	13	395	Most tissues including muscle	Love *et al.*, 1991; Khurana *et al.*, 1992
Up71 (6q24)	4	71	Most tissues including muscle	Wilson *et al.*, 1999
Up140 (6q24)	6.8	155	Most tissues including muscle	Wilson *et al.*, 1999
G-utrophin (6q24)	5.5	113	Brain	Blake *et al.*, 1995
DRP2 (Xq22)	7.7	110	Brain, spinal cord	Roberts *et al.*, 1996; Dixon *et al.*, 1997

Table 1.2. Dystrophin isoforms and homologues.

Figure 1.3. Schematic diagram of the dystrophin-associated protein complex. Shown is the molecular organization of the dystrophin-glycoprotein complex within synaptic and extrasynaptic regions of normal skeletal muscle fibers. Note the critical role of dystrophin and utrophin in linking the extracellular matrix to the intracellular cytoskeleton via the dystrophin-associated protein complex. Symbols; α-dg, α-dystroglycan; β-dg, β-dystroglycan; AChR, acetylcholine receptor.


SYNAPTIC
EXTRASYNAPTIC
Laminin
α–DG
Agrin
Laminin
α–DG
Extracellular Matrix
Sarcoglycans
25 kD
ε
γ
δ
β
α
β–DG
AChR
Rapsyn
β–DG
Sarcoglycans
α
β
δ
γ
ε
25 kD
Membrane
Syntrophins
β
α
Utrophin
α
β
Syntrophins
Dystrophin
Sarcoplasm
Actin filaments

comparison of the overall nucleotide and deduced amino acid sequence of the cDNAs shows that utrophin presents 65% and 73% homology to dystrophin, respectively, with greater than 80% homology within the COOH region and up to 85% homology within the putative DAP-binding domain (Love *et al.*, 1989; Tinsley *et al.*, 1992). Utrophin was thus initially named "dystrophin-related protein (DRP)" or "Duchenne Muscular Dystrophy-like (DMDL)" protein (Love *et al.*, 1989; Pearce *et al.*, 1993).

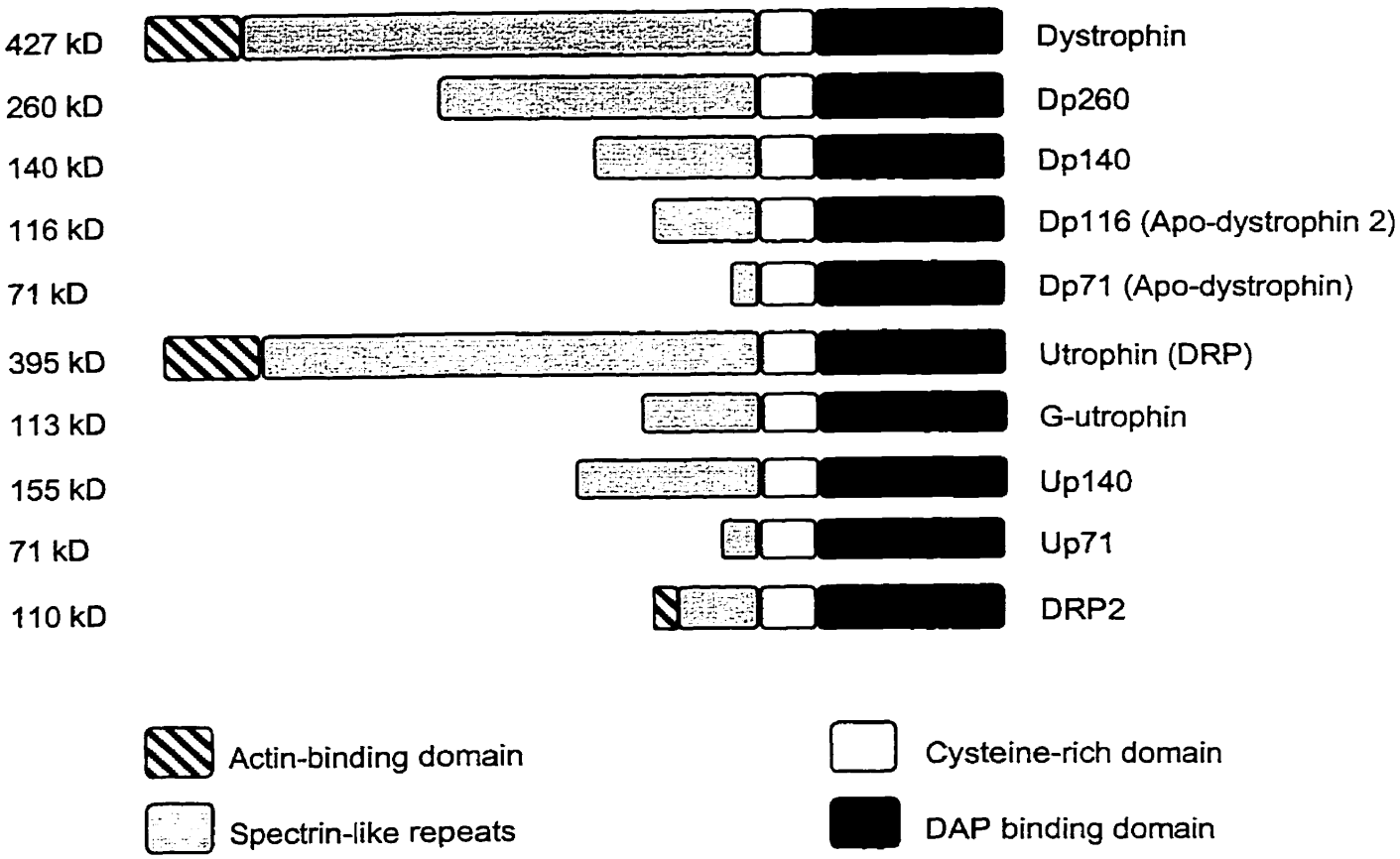
In contrast to the restricted expression of full-length dystrophin in brain and muscle, utrophin is abundantly expressed in nearly all tissues. For example, utrophin expression has been reported in the kidney, liver, spleen, testis, stomach, uterus, smooth, skeletal and cardiac muscles (Love *et al.*, 1989; Khurana *et al.*, 1990, 1991; thiMan *et al.*, 1991). In addition, particularly high levels of utrophin have been observed in the lung, blood vessels and nervous system (Love *et al.*, 1989; Khurana *et al.*, 1992; see also Blake *et al.*, 1996). Such a wide pattern of expression led to the renaming of dystrophin-related protein to "utrophin" to reflect its ubiquitous tissue-distribution profile (Blake *et al.*, 1994).

In addition to utrophin, several utrophin-related proteins have recently been identified. For example, G-utrophin is an isoform that is transcribed from a distal internal promoter within the utrophin locus (Blake *et al.*, 1995). Accordingly, the predicted structure of G-utrophin indicates a truncated version of utrophin that lacks both the actin-binding domain as well as the majority of the central spectrin repeats within the rod domain. In contrast to the wide tissue distribution of utrophin, expression of this isoform appears restricted to specific regions of the

adult brain and developing peripheral neural tissues (Blake *et al.*, 1995). Furthermore, several recent studies have also revealed the existence of other utrophin isoforms (Lumeng *et al.*, 1999; see also Fabbrizio *et al*,. 1995). In fact, western blot analyses using utrophin-specific antibodies revealed the presence of a 78 kD and an 82 kD isoform found specifically within the CNS, a 90 kD isoform found in the testis, spleen and liver, and a 97 kD isoform expressed only in the testis (Fabrizzio *et al.*, 1995; Lumeng *et al.*, 1999). Indeed, the complexity of the utrophin family is further evidenced by the observation that two novel transcripts of utrophin have been identified and termed Up71 and Up140 (to illustrate their relation to the dystrophin homologues, Dp71 and Dp 140) (Wilson *et al.*, 1999). Up71 is a utrophin transcript found in most tissues which predicts a 71 kD protein, and the Up140 transcript also appears to be ubiquitously expressed and predicts a protein product of 150 kD (Wilson *et al.*, 1999). Altogether, these studies indicate that utrophin has several related proteins generated perhaps by alternative promoters or splicing events (Wilson *et al.*, 1999).

In addition to these protein products arising from the utrophin gene, dystrophin-related protein-2 (DRP2) is a protein also highly homologous to dystrophin and utrophin that is encoded, however, by a separate gene located on the X chromosome (Roberts *et al.*, 1996). This gene encodes a 7.7 kb transcript with a predicted protein size of ~110 kD (Roberts *et al.*, 1996). Similar to G-utrophin, this protein initially appeared to present a more restricted pattern of expression since it was found predominately in the brain and spinal cord (Roberts *et al.*, 1996). However, more recent data indicate that DRP2 is also present in several non-neural tissues including the oesophagus, ovary, colon and eye (Dixon *et al.*, 1997).

Figure 1.4. Representative diagram of dystrophin and the dystrophin-related proteins. Shown are actin-binding domains, spectrin-like repeats, cysteine-rich and carboxy-terminal domains. The molecular mass of the proteins are also indicated at left.


427 kD
Dystrophin
260 kD
Dp260
140 kD
Dp140
116 kD
Dp116 (Apo-dystrophin 2)
71 kD
Dp71 (Apo-dystrophin)
395 kD
Utrophin (DRP)
113 kD
G-utrophin
155 kD
Up140
71 kD
Up71
110 kD
DRP2
Actin-binding domain
Spectrin-like repeats
Cysteine-rich domain
DAP binding domain

II.B. Functional Significance of Utrophin Expression in Muscle Fibers

In skeletal muscle, utrophin is found preferentially expressed at the neuromuscular junction in muscle fibers (Fardeau *et al.*, 1990, Khurana *et al.*, 1991, Ohlendieck *et al.*, 1991, thiMan *et al.*, 1991, Helliwell *et al.*, 1992). Indeed, high resolution analysis of the neuromuscular junction has indicated that utrophin is found to be precisely colocalized with acetylcholine receptors at the crests of the junctional folds and excluded from the depths (Bewick *et al.*, 1992). Despite the information regarding the localization of utrophin, however, the precise physiological role of utrophin remains to be determined. It has been suggested that utrophin contributes to the development and/or maintenance of the postsynaptic apparatus by providing a cytoskeletal scaffold necessary for the accumulation of synaptic molecules (Campanelli *et al.*, 1994; Hoch *et al.*, 1994; see also Jasmin *et al.*, 1990). In support of this view, several studies have shown the presence of utrophin at agrin-induced clusters of acetylcholine receptors (AChR) on the surface of myotubes grown in culture (Campanelli *et al.*, 1994; Phillips *et al.*, 1993) as well as at developing postsynaptic membrane domains (Phillips *et al.*, 1993). In addition, it has been demonstrated in tissue culture experiments that antibodies directed against utrophin can significantly impair the formation of AChR clusters following exogenous application of agrin (Namba and Scheller, 1996). Together, these results tend to support a role for utrophin in the formation and/or maintenance of postsynaptic membrane domains.

In order to further examine the contribution of utrophin to the formation of the neuromuscular junction, utrophin-deficient mice were recently generated (Deconinck *et al.*,

1997a; Grady *et al.*, 1997a). Surprisingly, these mice are generally healthy and show no obvious sign of neuromuscular abnormality indicating that utrophin does not appear necessary for the initial stages of synapse formation. However, a detailed analysis of their neuromuscular junctions revealed nonetheless, that the number of acetylcholine receptors (AChR) is decreased by ~ 40% compared to normal mice along with a concomitant reduction in the number of postsynaptic membrane folds (Deconinck *et al.*, 1997a; Grady *et al.*, 1997a). Given that utrophin and dystrophin share extensive homology, it is possible that in these studies, dystrophin compensated for the lack of utrophin and therefore, attenuated the phenotypic manifestation. To examine this possibility, utrophin-deficient mice were cross-bred with *mdx* mice, the mouse model of Duchenne muscular dystrophy, resulting in the generation of a mouse deficient in both utrophin and dystrophin (Deconinck *et al.*, 1997b; Grady *et al.*, 1997b). These mice were found to display a strong dystrophic phenotype, with subtle defects noted in relatively mature muscle fibers. For instance, in the double mutants, the postsynaptic membrane folding was further reduced and an assessment of the sarcolemmal distribution of the dystrophin-associated protein complex revealed that β2-syntrophin, dystrobrevin and β-dystrogylcan were all significantly reduced (Grady *et al.*, 1997b). These findings, therefore, indicate that although utrophin may not be necessary for the initial formation of the neuromuscular junction, it may be required for the full differentiation and maintenance of the postsynaptic membrane domain (Deconinck *et al.*, 1997b; Grady *et al.*, 1997b).

II.C. Upregulation of Utrophin as a Therapeutic Strategy for DMD Treatment

There are several strategies that may be envisaged to counteract the effects of DMD including introduction of functional dystrophin using various gene therapy approaches or stem cell transplantation, as well as prevention of the muscle pathology via pharmacological interventions (Ahn and Kunkel, 1993; Khan, 1993; Matsumura and Campbell, 1994; Gussoni *et al.*, 1999; Barton-Davis *et al.*, 1999). In this context, an alternative therapeutic avenue has recently been receiving increasing attention (Tinsley *et al.*, 1993, 1994; Blake *et al.*, 1996; Karpati, 1996; Roush, 1997). This therapeutic approach consists in utilizing a protein normally expressed in diseased muscle which can assume the functional role of dystrophin. Based on the high degree of sequence similarity between utrophin and dystrophin (Tinsley *et al.*, 1992; Pearce *et al.*, 1993) as well as the capacity of utrophin to interact with DAPs (Matsumura *et al.*, 1992), the possibility of therapy via increased expression of utrophin appears particularly promising. Thus, if utrophin expression could somehow be systematically extended from the synaptic regions of dystrophic muscle fibers into extrasynaptic compartments, it may functionally compensate for the lack of dystrophin and restore muscle function (Tinsley *et al.*, 1993; Blake *et al.*, 1996).

Recently, several studies have directly examined this therapeutic approach by two separate strategies. In one case, lines of transgenic mice that overexpress utrophin full-length or mini-genes were generated and cross-bred with mice from the *mdx* genetic background, a mouse model for DMD (Tinsley *et al.*, 1996; Deconinck *et al.*, 1997; Tinsley *et al.*, 1998; Rafael

et al., 1998). In these dystrophic mice, increased expression of utrophin along muscle fibers was accompanied by a number of specific changes in the dystrophic muscle fibers. In particular, a number of key indices of normal muscle function were recovered following utrophin upregulation, including a restoration of the DAP complex at the sarcolemma, a return toward normal levels of serum creatine kinase, as well as a reduction in the number of muscle fibers that underwent cycles of degeneration-regeneration, as evidenced by the number of centrally located myonuclei (Tinsley *et al.*, 1996; Rafael *et al.*, 1998). In addition to these morphological and biochemical differences, several physiological parameters relating to muscle function were also affected in the utrophin-*mdx* transgenic mice (Deconinck *et al.*, 1997; Tinsley *et al.*, 1998). For example, overexpression of utrophin in extrasynaptic compartments of dystrophic muscle fibers increased the ability of the muscle to generate contractile force, increased the resistance of the sarcolemma to damage induced by lengthening contractions, and improved the regulation of the basal levels of cytosolic calcium (Deconinck *et al.*, 1997; Tinsley *et al.*, 1998). In separate studies, elevated levels of utrophin in adult *mdx* muscle have been achieved by injecting adult muscle fibers with an adenoviral vector containing the utrophin minigene, resulting in restoration of muscle biochemical properties and function (Gilbert *et al.*, 1998, 1999). Taken together, these studies provide convincing evidence that upregulation of utrophin represents a viable approach for the treatment of DMD. Therefore, one of the next steps along this line of investigation is to determine the cellular and molecular mechanisms regulating expression of utrophin in normal muscle fibers in order to ultimately increase expression of the endogenous gene product throughout extrasynaptic regions of dystrophic muscle fibers.

II.D. Regulation of Utrophin Expression in Muscle Fibers

In skeletal muscle fibers, utrophin is found preferentially expressed at the neuromuscular junction, with very low levels detectable in extrajunctional regions (Fardeau *et al.*, 1990; Khurana *et al.*, 1991; Ohlendieck *et al.*, 1991; thiMan *et al.*, 1991; Helliwell *et al.*, 1992). Interestingly, the abundance and localization of utrophin along muscle fibers appears to be developmentally regulated since levels of utrophin are known to be higher in embryonic and neonatal tissues as compared to the levels in adult muscle (Khurana *et al.*, 1992; Schofield *et al.*, 1993; Pons *et al.*, 1994). Specifically, utrophin expression is observed along the length of the sarcolemma in human neonatal muscle fibers (Clerk *et al.*, 1993). At approximately nine weeks of gestation, utrophin gradually becomes enriched within the postsynaptic sarcoplasm although extrajunctional expression can still be observed through early neonatal development (Clerk *et al.*, 1993). Similar findings have been reported using mouse muscle since maximal levels of utrophin expression occur at embryonic day 13, after which time utrophin levels progressively decline (Khurana *et al.*, 1992; Koga *et al.*, 1993).

In addition to this developmental regulation, the levels and localization of utrophin appear to be affected under certain disease conditions. In muscles from DMD patients for example, levels of utrophin are significantly increased and expression extends well into extrasynaptic compartments of muscle fibers (Takemitsu *et al.*, 1991; Helliwell *et al.*, 1992; Karpati *et al.*, 1993). In addition to DMD, utrophin levels have been shown to be significantly elevated in several inflammatory myopathies including polymyositis (PM) and dermatomyositis

(DM) (Helliwell *et al.*, 1992; Karpati *et al.*, 1993). Although the precise mechanism responsible for the increased expression of utrophin in diseased muscle is not well understood, it has been suggested that it involves the contribution of regenerating muscle fibers which are known to express higher levels of utrophin (Helliwell *et al.*, 1992; Karpati *et al.*, 1993; Wilson *et al.*, 1994; Sewry *et al.*, 1994). However, it is becoming apparent that regeneration alone cannot account entirely for the observed increase in utrophin expression in diseased muscles thereby indicating that additional, yet unknown factors must also contribute to this natural upregulation (see Sewry *et al.*, 1994; Taylor *et al.*, 1997).

In addition, since previous studies examining postsynaptic proteins, including for example AChR, have indicated that factors derived from the motor nerve are involved in regulating local expression of these proteins, it is possible that the motor nerve is also involved in controlling the restricted expression of utrophin at the neuromuscular junction. Indeed, based on our knowledge of the cellular and molecular mechanisms presiding over expression of AChR at the postsynaptic membrane of the neuromuscular junction (Hall and Sanes, 1993; Duclert and Changeux, 1995), it may be envisaged that the nerve exerts its effects on utrophin expression via two pathways involving either nerve-evoked electrical activity and/or nerve-derived trophic factors (see Figure 1.2; and section I). These cooperative mechanisms effectively ensure the compartmentalized expression of AChR transcripts at the neuromuscular synapse as well as the subsequent local synthesis and insertion of functional receptor molecules at the level of the postsynaptic membrane. Using this hypothetical model, recent studies have thus begun to explore whether the nerve influences utrophin expression in a manner similar to that regulating

AChR expression. For example, the role of electrical activity in the regulation of utrophin was examined by denervation experiments (Jasmin *et al.*, 1995c; Biral *et al.*, 1996). In these studies, it was determined that the elimination of electrical activity by denervation, which is known to lead to large increases in AChR expression, failed to significantly alter the levels of utrophin and its mRNA in mouse muscle (Jasmin *et al.*, 1995c; Biral *et al.*, 1996). However, despite these initial studies, a detailed study of the contribution of the motor nerve to the regulation of the local expression of utrophin at the neuromuscular junction has yet to be undertaken.

Finally, the utrophin promoter has recently been isolated and cloned, providing additional insight into the mechanisms regulating utrophin expression. Dennis *et al.* (1996) isolated and cloned the promoter for full length utrophin which was shown to consist of ~900 bp with a CpG rich region of ~155 bp essential for maintaining basal levels of expression (Dennis *et al.*, 1996). Further sequence analysis revealed the absence of TATA and CAAT motifs, sequences common to most eukaryotic promoters, along with the presence of various other transcriptional regulatory elements, including Sp1, Sp2, AP1, and AP2 motifs. Of particular interest, however, was the presence of two additional transcription factor consensus sequences which may be important for controlling utrophin expression in skeletal muscle. For instance, the utrophin promoter contains a conserved E-box element (CANNTG) and a recently identified DNA motif termed an N-box element. As previously mentioned, E-box motifs are DNA elements that interact with the MyoD family of transcription factors, shown to be important for the regulation of muscle-specific genes (Weintraub *et al.*, 1991; Weintraub, 1993; Rudnicki and Jaenisch, 1995; Arnold and Winter, 1998. On the other hand, the N-box element has recently been implicated in the regulation of

synapse-specific expression of the δ– and the ε–subunits of the acetylcholine receptor (Duclert *et al.*, 1993; Koike *et al.*, 1995; Duclert *et al.*, 1996). Thus, it is possible that these two elements are critical in the overall regulation of the transcriptional control of the utrophin gene. However, the contribution of these transcriptional regulatory elements to the control of utrophin gene expression has not been addressed.

III. Statement of Problem and Objectives

Although considerable information is available regarding the localization of utrophin in various tissues, including skeletal muscle, our current understanding of the mechanisms that regulate the expression of utrophin is rudimentary. Since utrophin is found preferentially enriched at the neuromuscular junction of skeletal muscle it is possible that nerve-derived factors contribute to this pattern of expression. Furthermore, there is evidence that utrophin expression may also be regulated under other conditions such as myogenic development and regeneration. Therefore, in the present studies we were interested in: i) determining the involvement of nerve-derived signals that may regulate the levels and localization of utrophin expression in skeletal muscle; and ii) examining the contribution of other regulatory mechanisms controlling utrophin expression in skeletal muscle. Thus, the primary objectives of this study are as follows:

1. **To determine the mechanisms responsible for the local expression of utrophin at the neuromuscular junction (Chapter 2).**

From the earliest stages of synaptic formation, utrophin is found to be preferentially localized to the neuromuscular junction (Phillips *et al.*, 1993). As a result of this restricted pattern of expression, it is possible that the motor nerve regulates utrophin expression by inducing local transcription of the utrophin gene within synaptic myonuclei, in a manner similar to that observed for the acetylcholine receptor. Therefore, we will begin to investigate the

mechanisms involved in this local expression of utrophin. In particular, we will determine the contribution of transcriptional regulation of the utrophin gene by examining: i) the localization of utrophin transcripts along skeletal muscle fibers; ii) the expression of utrophin promoter-reporter constructs in skeletal muscle fibers; and iii) the contribution of the motor nerve to the local expression of utrophin. *Hypothesis: Local transcriptional control of the utrophin gene results in the preferential expression of utrophin at the neuromuscular synapse.*

2. To determine the effect of nerve-derived trophic factors on utrophin expression and begin to characterize the transcriptional regulatory pathways involved (Chapters 3 and 4).

As the motor nerve appears critical in the regulation of utrophin, but nerve-derived electrical activity does not seem to be the mechanism by which the motor nerve acts (Jasmin *et al.*, 1995c; Biral *et al.*, 1996), it is likely that utrophin expression is regulated by nerve-derived trophic factors. Several nerve-derived trophic factors, including CGRP, agrin and ARIA/heregulin, are all known to significantly regulate the expression of synapse-specific proteins, including AChR (see for review, Duclert and Changeux, 1995). Thus, we will determine the contribution of these nerve-derived trophic factors to the regulation of utrophin gene expression. In addition, we will explore the mechanisms that ultimately regulate the transcriptional pathways involved in utrophin expression at the neuromuscular junction. In particular, we will explore the DNA regulatory elements and putative transcription factors that

are important in controlling utrophin expression in skeletal muscle. *Hypothesis: Nerve-derived trophic factors regulate the local transcription of the utrophin gene via the N-box element.*

3. **To examine the mechanisms responsible for the elevated levels of utrophin in human skeletal muscle obtained from Duchenne muscular dystrophy patients (Chapter 5).**

Previous studies have revealed that utrophin levels in muscle biopsies obtained from patients with Duchenne's muscular dystrophy or various inflammatory myopathies are elevated and even extend into extrajunctional regions of the muscle fiber (Takemitsu *et al.*, 1991; Matsumura *et al.*, 1992; Helliwill *et al.*, 1992; Karpati *et al.*, 1993; Mizuno *et al.*, 1993; Pons *et al.*, 1993). Thus, we will determine the molecular mechanisms underlying the elevated levels of utrophin in these conditions. Accordingly, we will analyse utrophin mRNA and protein levels in normal individuals and from patients with Duchenne muscular dystrophy and polymyositis; two conditions previously characterized to possess elevated utrophin levels (Helliwell *et al.*, 1992; Karpati *et al.*, 1993). In addition, since it has been suggested that muscle fiber regeneration is associated with the increased utrophin levels under these conditions (Helliwell *et al.*, 1992; Karpati *et al.*, 1993; Wilson *et al.*, 1994; Sewry *et al.*, 1994), we will also examine the levels of utrophin when skeletal muscle fibers are chemically induced to regenerate. *Hypothesis: Utrophin transcript levels are elevated and extend along the length of the muscle fiber in DMD patients.*

4. To determine the effects of myogenic differentiation on utrophin expression (Chapter 6).

The process of myogenic differentiation is accompanied by large increases in the expression of genes encoding various cytoskeletal and membrane proteins, including dystrophin and AChR. In fact, the developmental regulation of these genes is now known to be the result of their activation by transcription factors belonging to the MyoD family of proteins. This family of transcription factors bind to DNA consensus sequences termed E-box elements, which consist of the sequence CANNTG (see for review, Weintraub *et al.*, 1991; Rudnicki and Jaenisch, 1995). Interestingly, the identification and sequencing of the utrophin promoter revealed the presence of such a DNA element, suggesting that the levels and localization of utrophin may be markedly regulated during myogenesis. In order to address the involvement of the myogenic regulatory process to the control of utrophin expression, we will examine the effects of myogenic differentiation on utrophin levels in cultured muscle cells. *Hypothesis: Myogenic differentiation will result in a significant increase in the expression of utrophin.*

CHAPTER 2

LOCAL TRANSCRIPTIONAL CONTROL OF UTROPHIN EXPRESSION AT THE NEUROMUSCULAR SYNAPSE

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For this paper, A. Gramolini performed all of the experimental procedures and the manuscript was subsequently written by A. Gramolini and Dr. Jasmin. Drs. C. Dennis, J. Tinsley, and K. Davies supplied the utrophin promoter constructs (Dennis *et al.*, 1996). Dr. G. Robertson was helpful in establishing the *in situ* hybridization technique in our laboratory. Dr. J. Cartaud collaborated with Dr. Jasmin during the course of this work.

ABSTRACT

Recently, the use of a transgenic mouse model system for Duchenne muscular dystrophy (DMD) has demonstrated the ability of utrophin to functionally replace dystrophin and alleviate the muscle pathology (see Tinsley, J.M., Potter, A.C., Phelps, A.C., Fisher, S.R., Trickett, J.I., and Davies, K.E. (1996) *Nature* 384: 349-353). However, there is currently a clear lack of information concerning the regulatory mechanisms presiding over utrophin expression during normal myogenesis and synaptogenesis. Using *in situ* hybridization, we show that utrophin mRNAs selectively accumulate within the postsynaptic sarcoplasm of adult muscle fibers. In addition, we demonstrate that a 1.3 kb fragment of the human utrophin promoter is sufficient to confer synapse-specific expression to a reporter gene. Deletion of 800 bp from this promoter fragment reduces the overall expression of the reporter gene and abolishes its synapse-specific expression. Finally, we also show that utrophin is present at the postsynaptic membrane of ectopic synapses induced to form at sites distant from the original neuromuscular junctions. Taken together, these results indicate that nerve-derived factors regulate locally the transcriptional activation of the utrophin gene in skeletal muscle fibers and that myonuclei located in extrasynaptic regions are capable of expressing utrophin upon receiving appropriate neuronal cues.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most severe and prevalent primary myopathy. The genetic defect responsible for DMD is located on the short arm of the X chromosome and prevents the production of normal size dystrophin, a large cytoskeletal protein of 427 kDa (1,2). In 1989, Love and colleagues showed the existence of a gene on chromosome 6q24 that encodes a cytoskeletal protein displaying a high degree of sequence similarity with dystrophin (3,4).

In skeletal muscle, the level and localization of utrophin has been shown to vary markedly according to the state of differentiation and innervation of muscle fibers. In embryonic tissue for instance, utrophin localizes to the sarcolemma along the entire length of developing fibers (5,6). As the muscle matures, the amount of utrophin decreases progressively and utrophin becomes preferentially localized to the neuromuscular synapse (7,8). An exception to this occurs in muscle fibers from both DMD patients and *mdx* mice where utrophin persists at the sarcolemma in extrasynaptic regions (9-11). Together, these studies therefore suggest that in addition to therapies based on dystrophin gene transfer, upregulation of utrophin may be envisaged as an alternative strategy to prevent the relentless progression of DMD. In this context, we have recently shown that high expression of a truncated utrophin transgene markedly reduced the dystrophic muscle phenotype in *mdx* hindlimb and diaphragm muscles indicating that systemic upregulation of utrophin may indeed be an effective treatment for DMD (12). In the present study, we have thus initiated a series of experiments focusing on the molecular

mechanisms involved in the restricted expression of utrophin at the neuromuscular synapse by using three distinct, yet complementary approaches.

EXPERIMENTAL PROCEDURES

Surgery. Ectopic synapses were induced to form on soleus muscles from adult control and *mdx* mice. An incision was first made at the mid-calf region and the common peroneal nerve was exposed by blunt dissection. Both branches of this nerve were isolated, cut and transplanted on to the distal surface of the soleus using procedures described elsewhere (13). Fourteen days later, ~ 5 mm of the tibial nerve was cut and removed to denervate the muscle and to allow the foreign nerve to form synaptic contacts with soleus muscle fibers. Two weeks after sectioning the tibial nerve, the sciatic nerve was stimulated at supramaximal voltage with bipolar platinum electrodes. Soleus muscles which demonstrated contractile activity in response to electrical stimulation were excised, mounted with Tissue Tek freezing medium (Miles Inc., Elkhart, IN).

Immunofluorescence. Immunofluorescence experiments were performed on longitudinal serial sections (12 μm) of soleus muscles. The presence of synapsin was monitored using a rabbit anti-synapsin antibody (Alexis Corporation; San Diego, CA). Utrophin immunoreactivity was detected using either a rabbit anti-utrophin antibody (from Dr. Tejvir Khurana, Harvard University) or a monoclonal anti-utrophin antibody (from Dr. Glen Morris, N.E. Wales Institute, UK). Synapsin and utrophin antibodies were applied onto separate serial muscle sections for one hour. Fluorescein isothiocyanate-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR) was used to label the acetylcholine receptor (AChR).

In situ Hybridization. Longitudinal serial cryostat sections (12 μm) of hindlimb muscles from control C57BL/6 and *mdx* mice were placed on alternate Superfrost Plus slides (Fisher Scientific Co; Pittsburgh, PA). Alternate slides were either processed for acetylcholinesterase (AChE) histochemistry (14) to visualize neuromuscular junctions or subjected to *in situ* hybridization using synthetic oligonucleotides for detection of utrophin transcripts. *In situ* hybridization was performed using two anti-sense oligonucleotides complementary to the mouse utrophin mRNA (5',3': #1; ACGACGGACCACCTTGACACCCGGACCCAGTCACAGTTCAC and #2: TGCTGC CTGGTGGAACTGTGGGCCTGGGTCAGTGTCAAGTG) according to Schalling *et al.* (15).

Analysis of *in situ* hybridization labeling was performed using an image analysis system equipped with Image 1.47 software (Wayne Rasband, NIMH) (16). The density of *in situ* hybridization labeling in synaptic versus extrasynaptic regions was determined by measuring the number of labeled pixels within a circular field of constant 100 μm in diameter. To determine the difference in utrophin mRNA levels between control and *mdx* mouse muscles, 1 mm^2 areas of extrasynaptic regions were sampled. For these analyses, both control and *mdx* mouse muscle sections were placed on the same slide and processed for *in situ* hybridization simultaneously. Previous analyses determined that the number of silver grains is linearly related to the optical density using this image analysis system. Thus, optical density values were used as a measure of labelling with higher values indicating greater labelling (17). Twelve muscle sections were processed for each condition and a minimum of four measurements were performed on each section. Three animals were used for each condition.

Expression of Utrophin Promoter-Reporter Gene Constructs. Four human utrophin promoter-reporter gene constructs were used in these experiments: 1.3 kb and 0.5 kb promoter fragments positioned in either the forward or reverse orientations (see Figure 2.1; and Ref 18). These promoter fragments were inserted upstream of the reporter gene LacZ and a nuclear localization signal (nlsLacZ). Plasmid DNA was prepared using the Qiagen mega-prep procedure (Chatsworth, CA) and final pellets were resuspended in sterile PBS to a final concentration of 2 μg/μl.

For direct gene transfer, 25 μl of DNA solution was injected directly into the tibialis anterior (TA) muscle of 4-week old mice (19-21). At different time-intervals thereafter (7 to 42 days), TA muscles were excised and quickly frozen for serial cryostat sectioning. Tissue sections were processed histochemically for the demonstration of β-galactosidase and AChE activity. The position of blue myonuclei indicative of utrophin promoter activity was determined and compared to the presence of neuromuscular synapses using the quantitative procedure established by Duclert *et al.* (21). A region of a muscle fiber containing blue myonuclei was designated as an event. These blue regions were further characterized as synaptic or extrasynaptic according to whether the event coincided with the presence of a neuromuscular synapse identified by AChE histochemical staining.

RESULTS

In a first series of experiments, we examined by *in situ* hybridization the distribution of utrophin mRNAs along muscle fibers from both C57BL/6 and *mdx* mice. Our results disclosed a selective accumulation of utrophin transcripts within the postsynaptic sarcoplasm (Figure 2.2A and B). In these experiments, utrophin mRNAs were also detected in extrasynaptic regions of muscle fibers albeit at significantly lower levels in comparison to synaptic sites. As expected, utrophin transcripts were observed in blood vessels and capillaries (Figure 2.2C). Control experiments performed with synthetic oligonucleotides corresponding to the sense strand of the mouse utrophin mRNA failed to label subcellular structures within these muscle sections (not shown).

Quantitative analyses revealed that of 375 neuromuscular junctions, 313 (~83%) displayed an accumulation of silver grains corresponding to utrophin transcripts. Densitometric analysis further showed that the levels of utrophin mRNAs confined within the postsynaptic sarcoplasm were approximately 12-fold higher than those observed in extrasynaptic regions (Figure 2.3A). In agreement with previous reports showing upregulation of utrophin in *mdx* mouse muscle (for example see Ref. 22), we also noted that in comparison to control mice, levels of utrophin mRNA were significantly elevated (~ 400%) in hindlimb muscle fibers from *mdx* mice (Figure 2.3B). However, the ratio of utrophin transcripts in synaptic versus extrasynaptic regions from *mdx* mouse muscle fibers was similar to that obtained with C57BL/6 mice.

To determine whether selective transcription of the utrophin gene accounts for the preferential accumulation of utrophin transcripts within the postsynaptic sarcoplasm, we performed an additional set of experiments in which human utrophin promoter-reporter gene constructs were directly injected into skeletal muscle. For these studies, 1.3 and 0.5 kb fragments from the utrophin promoter (see Figure 2.1; and Ref. 18) were inserted upstream of the reporter gene nlsLacZ. Seven to 42 days following injections of these constructs into TA muscles, the position of blue myonuclei, indicative of β-galactosidase expression, was determined and compared to the localization of neuromuscular synapses.

Muscles injected with the 1.3 kb utrophin promoter-nlsLacZ construct demonstrated a strong level of expression (Figure 2.4). In fact, quantitative analysis revealed that ~ 72% of muscles injected with this construct contained myonuclei expressing significant levels of β-galactosidase (Figure 2.5A). By contrast, expression of the nlsLacZ construct driven by the 0.5 kb utrophin promoter fragment was markedly reduced since less than 30% of the injected muscles displayed blue myonuclei. These results therefore, highlight the importance of regulatory elements contained within the deleted 800 bp fragment for the overall expression of the reporter gene in skeletal muscle fibers *in vivo* (see Figure 2.1).

Injections of TA muscles with the construct containing the 1.3 kb human utrophin promoter fragment led to the preferential expression of β-galactosidase in myonuclei located in the vicinity of neuromuscular synapses (Figure 2.4). Detailed quantitative analysis showed that in approximately 55% of the cases, the presence of blue myonuclei coincided with synaptic sites

identified by AChE histochemistry (Figures 2.4 and 2.5B). Similar patterns of expression were observed at different time-intervals, i.e. 7, 14 and 42 days, following DNA injection. Deletion of 800 bp 5' of this utrophin promoter fragment led to a marked reduction in the percentage of synaptic events (Figure 2.5B). These results are nearly identical to those recently reported for the synapse-specific expression of AChR subunit gene promoters (50-55%) and for the nonsynapse-specific expression obtained with the muscle creatine kinase promoter (10-12%; Refs.19-21). In our experiments, injections of constructs containing the utrophin promoter fragments cloned in the reverse orientation failed to induce nlsLacZ expression in TA muscles.

Finally, we induced the formation of ectopic synapses at sites distant from the original synaptic regions to: i) examine the contribution of the nerve in the local accumulation of utrophin at the neuromuscular junction; and ii) determine whether utrophin could be expressed in extrasynaptic regions of adult muscle fibers. In these experiments, we observed numerous newly formed ectopic synapses in all soleus muscles that displayed a functional motor response. In fact, co-distribution between the presence of synapsin immunoreactivity and AChR was routinely observed (Figure 2.6A and B). Immunofluorescence experiments performed on both control and *mdx* mouse soleus muscles using either one of the two utrophin antibodies revealed that utrophin was already present at the postsynaptic membrane of these ectopic synapses (Figure 2.6C and D). The expression of utrophin at these newly formed synaptic sites suggests therefore, that myonuclei normally located in extrasynaptic regions are capable of expressing utrophin upon receiving appropriate neuronal cues.

DISCUSSION

We examined the molecular mechanisms underlying the selective accumulation of utrophin at the postsynaptic membrane of the neuromuscular synapse and determined the contribution of the nerve in the local regulation of utrophin. Our data show that utrophin mRNAs are enriched within the postsynaptic sarcoplasm of adult muscle fibers as a result of the preferential activation of the utrophin gene in synaptic myonuclei. In addition, we demonstrate that the nerve exerts a local and profound influence on expression of utrophin and that myonuclei located in extrasynaptic regions of adult muscle fibers are capable of transcribing the utrophin gene upon receiving appropriate neuronal cues.

Synapse-Specific Expression of the Utrophin Gene and its mRNA

The postsynaptic sarcoplasm of the neuromuscular junction represents a highly differentiated domain within muscle fibers in which numerous organelles accumulate. These include morphologically distinct myonuclei referred to as fundamental by Ranvier (23), a synapse-specific Golgi apparatus (24,25) and a stable array of microtubules (26). In recent years, this proposal has received increasing experimental support. Previous studies have shown for example, the selective accumulation of transcripts encoding the various AChR subunits (27,28) as well as AChE (29,30) in the postsynaptic sarcoplasm of adult muscle fibers. In the present study, we show that accumulations of utrophin mRNAs are detectable at 83% of the

neuromuscular junctions. This value is in fact similar to those reported recently for transcripts encoding other synapse-associated proteins (31).

In attempts to elucidate the mechanisms involved in the preferential accumulation of utrophin mRNAs in synaptic regions of muscle fibers (29,31) we injected various utrophin promoter-reporter gene constructs directly into muscle. Similar to the transcriptional activation of the various AChR subunit genes within the fundamental myonuclei (27,28), we observed that injection of constructs containing the 1.3 kb utrophin promoter resulted in synapse-specific expression of the reporter gene. Deletion of 800 bp 5' of this promoter fragment abolished synapse-specific expression indicating therefore that regulatory elements contained within this DNA fragment are necessary for conferring synapse-specific expression.

Sequence analysis of the deleted 800 bp fragment revealed the presence of several sites that may play significant regulatory roles in utrophin expression along muscle fibers. This DNA fragment contains, for example, an E box which is known to bind myogenic transcription factors. Interestingly, this site is the only consensus sequence that has been found common to all AChR promoters to date (28). Although myogenic factors contribute to the activity-dependent regulation of AChR subunit genes in muscle fibers, this binding site is not required for synapse-specific expression of the AChR ε-subunit gene (19). An N box motif constitutes another DNA element which may be involved in the local expression of the utrophin gene within nuclei located in the postsynaptic sarcoplasm (20; and Figure 2.1). The N box motif consists of the core sequence TTCCGG. Deletion and mutagenesis experiments have revealed that this DNA element is sufficient to confer synapse-specific expression to the mouse AChR δ- and ε-subunit

genes and that it binds a protein complex from muscle nuclear extracts in gel retardation assays (20,21). This DNA element may thus be responsible for the synapse-specific expression conferred by the 1.3 kb utrophin promoter fragment. If this is indeed the case, it would indicate that expression of genes encoding synapse-associated membrane and cytoskeletal proteins is therefore co-regulated through the concerted action of common transcription factors and signaling pathways.

Localization of Utrophin at Ectopic Synapses

Ectopic nerve implants have been successfully used to study the development of the neuromuscular junction *in vivo* . Using this approach, we observed numerous ectopic synapses in "old" extrasynaptic regions of soleus muscle fibers. Immunofluorescence experiments further showed that utrophin appeared at these newly formed synaptic sites within two weeks following induction of ectopic synapses. These results are thus in agreement with previous studies which showed the presence of utrophin at agrin-induced AChR clusters in cultured myotubes (32). More importantly, our results indicate that the utrophin gene may be expressed in extrasynaptic regions of muscle fibers upon receiving appropriate neuronal cues. It appears therefore that nerve-derived factors play a crucial role in dictating the local expression of utrophin gene products.

Several nerve-derived factors are known to influence the localization and regulation of AChR. For example, ARIA/heregulin has been shown to markedly influence expression of AChR and in particular, the expression of the ε-subunit gene (33). Since the pattern of expression of the utrophin gene along muscle fibers is similar to that of the ε-subunit gene (21;

and this study) and since both genes appear largely insensitive to abolition of neuromuscular activity (34,35), ARIA/heregulin may thus be considered as a plausible candidate involved in the local regulation of utrophin at the synapse. Agrin represents another factor that may also contribute to the regulation of the utrophin gene within the postsynaptic sarcoplasm. A recent study has in fact shown that substrate-bound agrin induces a 2- to 3-fold increase in the expression of the AChR ε-subunit gene in cultured myotubes (36) thereby providing support to the notion that agrin is also a transcriptional activator. Since utrophin may be involved in the early steps of synaptogenesis, it is thus possible that agrin stimulates expression of utrophin to ensure the presence of a cytoskeletal scaffold necessary for the assembly and stabilization of postsynaptic membrane domains. Preliminary results obtained in our laboratory indicate that indeed, both Torpedo and recombinant agrin increase the levels of utrophin mRNA in cultured myotubes (Jasmin *et al.*, unpublished observations). The identification of nerve-derived factors involved in modulating expression of the utrophin gene will provide key information essential for the upregulation of utrophin as a therapeutic strategy for DMD.

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THESIS REVISIONS

Student's t-tests were performed on the data for Figures 2.3 and 2.5B. With these analyses, both sets of experiments showed significant differences ($P<0.05$).

REFERENCES

1. Ahn, A. H. & Kunkel, L. M. (1993) *Nature (Genetics)* **3**, 283-291.

2. Matsumura, K. & Campbell, K. P. (1994) *Muscle Nerve* **17**, 2-15.

3. Love, D. R., Hill, D. F., Dickson, G., Spurr, N. K., Byth, B. C., Marsden, R. F., Walsh, F. S., Edwards, Y. H. & Davies, K. E. (1989) *Nature* **339**, 55-58.

4. Tinsley, J. M., Blake, D. J., Roche, A., Fairbrother, U., Riss, J., Byth, B. C., Knight, A. E., Kendrick-Jones, J., Suthers, G. K., Love, D. R., Edwards, Y. H. & Davies, K. E. (1992) *Nature* **360**, 591-593.

5. Khurana, T. S., Hoffman, E. P. & Kunkel, L. M. (1990) *J. Biol. Chem.* **265**, 16717-16720.

6. Pons, F., Augier, N., Leger, J., Robert, A., Tome, F. M., Fardeau, M., Voit, T., Nicholson, L. V. B., Mornet, D. & Leger, J. L. (1991) *FEBS Lett.* **282**, 161-165.

7. Fardeau, M., Tome, F. M., Collin, H., Augier, N., Pons, F., Leger, J. & Leger, J. L. (1990) *Compt. Rend. Acad. Sci.* **311**, 197-204.

8. Ohlendieck, K., Ervasti, J. M., Matsumura, K., Kahl, S. D., Leveille, C. J. & Campbell, K. P. (1991) *Neuron* **7**, 499-508.

9. Helliwell, T. R., thi Man, N., Morris, G. E. & Davies, K. E. (1992) *Neuromusc. Dis.* **2**, 177-184.

10. Matsumura, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. D. & Campbell, K. P. (1992) *Nature* **360**, 588-591.

11. Mizuno, Y., Yoshida, M., Yamamoto, H., Hirai, S. & Ozawa, E. (1993) *J. Biochem.* **114**, 936-941.

12. Tinsley, J. M., Potter, A. C., Phelps, S. R., Fisher, R., Trickett, J. I. & Davies, K. E. (1996) *Nature* **384**, 349-353.

13. van Kempen, G. T. H., Molenaar, P. C. & Slater, C. R. (1994) *J. Physiol. (Lond.)* **478**, 229-238.

14. Karnovsky, M. J. & Roots, L. (1996) *J. Histochem. Cytochem.* **12**, 219-221.

15. Schalling, M., Dagerlind, A., Brene, S., Hallman, H., Djurfeld, M., Perrson, H., Terenius, L., Goldstein, M., Schesinger, D. & Hokfelt, T. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8306-8310.

16. Robertson, G. S., Tetzlaff, W., Bedard, A., St-Jean, M. & Wigle, N. (1995) *Neurosci.* **67**, 325-344.

17. Gerfen, C. R. & Young, W. S. (1988) *Brain Res.* **460**, 161-167.

18. Dennis, C. L., Tinsley, J. M., Deconinck, A. E. & Davies, K. E. (1996) *Nucleic Acids Res.* **24**, 1646-1652.

19. Duclert, A., Savatier, N. & Changeux, J. P. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3043-3047.

20. Koike, S., Schaeffer, L. & Changeux, J. P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 10624-10628.

21. Duclert, A., Savatier, N., Schaeffer, L. & Changeux, J. P. (1996) *J. Biol. Chem.* **271**, 17433-17438.

22. Matsumura, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. D. & Campbell, K. P. (1992) *Nature* **360**, 588-591.

23. Ranvier, L. (1888) *Traité technique d'histologie*. Savy, Paris.

24. Jasmin, B. J., Cartaud, J., Bornens, M. & Changeux, J. P. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7218-7222.

25. Jasmin, B. J., Antony, C., Changeux, J. P. & Cartaud, J. (1995) *Eur. J. Neurosci.* **7**, 470-479.

26. Jasmin, B. J., Changeux, J. P. & Cartaud, J. (1990) *Nature* **344**, 673-675.

27. Hall, Z. W. & Sanes, J. R. (1993) *Cell/Neuron* **72**, 99-121.

28. Duclert, A. & Changeux, J. P. (1995) *Physiol. Rev.* **75**, 339-368.

29. Jasmin, B. J., Lee, R. K. & Rotundo, R. L. (1993) *Neuron* **11**, 467-477.

30. Michel, R. N., Vu, C. Q., Tetzlaff, W. & Jasmin, B. J. (1994) *J. Cell Biol.* **127**, 1061-1069.

31. Moscoso, L. M., Merlie, J. P. & Sanes, J. R. (1995) *Mol. Cell. Neurosci.* **6**, 80-89.

32. Phillips, W. D., Noakes, P. G., Roberds, S. L., Campbell, K. P. & Merlie, J. P. (1993) *J. Cell. Biol.* **123**, 729-740.

33. Chu, G. C., Moscoso, L. M., Sliwkowski, M. X. & Merlie, J. P. (1995) *Neuron* **14**, 329-339.

34. Witzemann, V., Brenner, H. R. & Sakmann, B. (1991) *J. Cell Biol.* **114**, 125-141.

35. Jasmin, B. J., Alameddine, H., Lunde, J. A., Stetzkowksi-Marden, F., Collin, H., Tinsley, J. M., Davies, K. E., Tome, F. M. S., Parry, D. J. & Cartaud, J. (1995) *FEBS Lett.* **374**, 393-398.

36. Jones, G., Herczeg, A., Ruegg, M. A., Lichtsteiner, M., Kröger, S. & Brenner, H. R. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 5985-5990.

Figure 2.1. Schematic representation of the human utrophin promoter. Shown are fragments of 1.3 and 0.5 kb. These fragments were inserted upstream of the reporter gene LacZ and a nuclear localization signal (nlsLacZ). Consensus binding sites for several transcription factors are indicated. Arrows indicate transcription start site (see Ref. 18).

Human Utrophin Promoter-Reporter Constructs

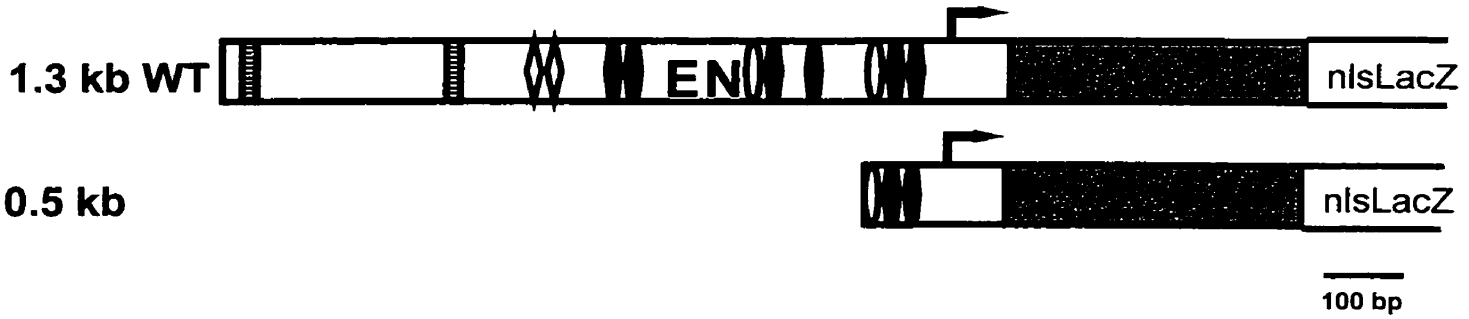


Figure 2.2. Localization of utrophin mRNA in mouse skeletal muscle by *in situ* hybridization. (A) shows a representative bright field photomicrograph of a longitudinal cryostat section stained for AChE to visualize neuromuscular junctions. (B) represents the corresponding serial section processed for *in situ* hybridization. Comparison of these two panels reveals the selective accumulation of utrophin mRNAs within the postsynaptic sarcoplasm. (C) shows a blood vessel labeled with the oligonucleotide specific for utrophin mRNAs. Closed and open arrows indicate the accumulations of utrophin transcripts at neuromuscular junctions and in a capillary, respectively. Bar = 70 μm.

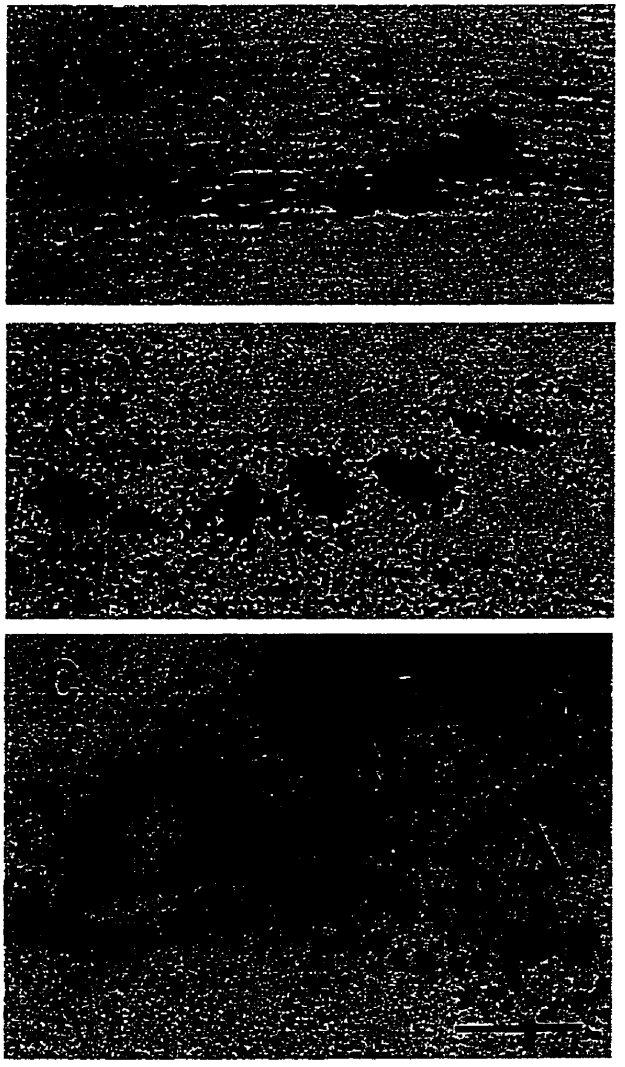
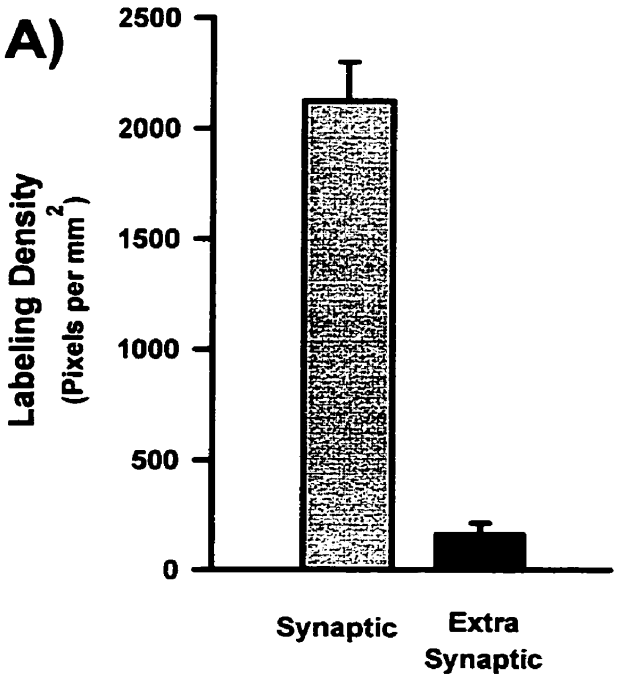


Figure 2.3. Quantitation of the levels of utrophin mRNA in skeletal muscle fibers. Note the higher level of utrophin mRNA in synaptic vs extrasynaptic regions of muscle fibers from control mice (A) as well as in extrasynaptic regions of fibers from *mdx* vs control mice (B).


A)
2500
2000
1500
1000
500
0
Labeling Density
(Pixels per mm 2)
Synaptic
Extra
Synaptic

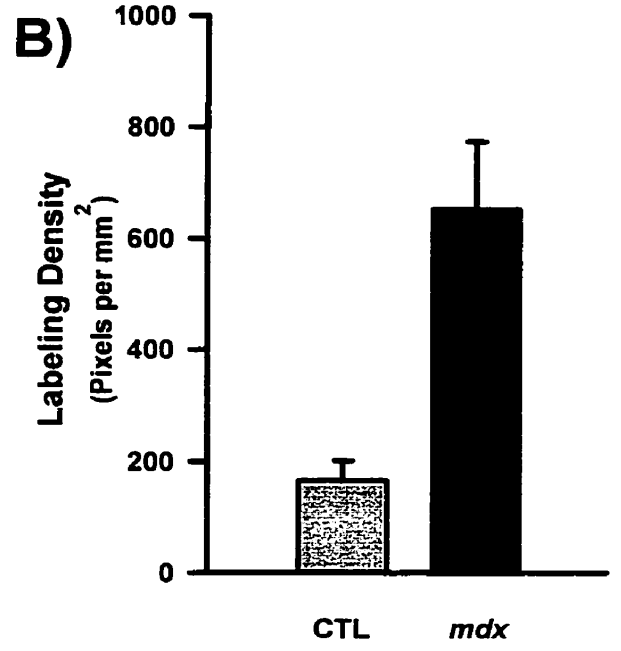

B)
1000
800
600
400
200
0
Labeling Density
(Pixels per mm 2)
CTL
mdx

Figure 2.4. Localization of utrophin promoter-reporter expression in muscle fibers. (A) and (B) show examples of TA muscles injected with plasmids containing the 1.3 kb utrophin promoter fragment and nlsLacZ. Brown precipitates correspond to AChE histochemistry indicating the presence of neuromuscular junctions. Note the co-localization between the presence of β-galactosidase-positive myonuclei and neuromuscular synapses following injections with this utrophin promoter fragment. (C) and (D) represent TA muscles injected with plasmids containing the 0.5 kb utrophin promoter fragment. Note that blue myonuclei are observed in extrasynaptic regions of muscle fibers. Bar = 60 μm.

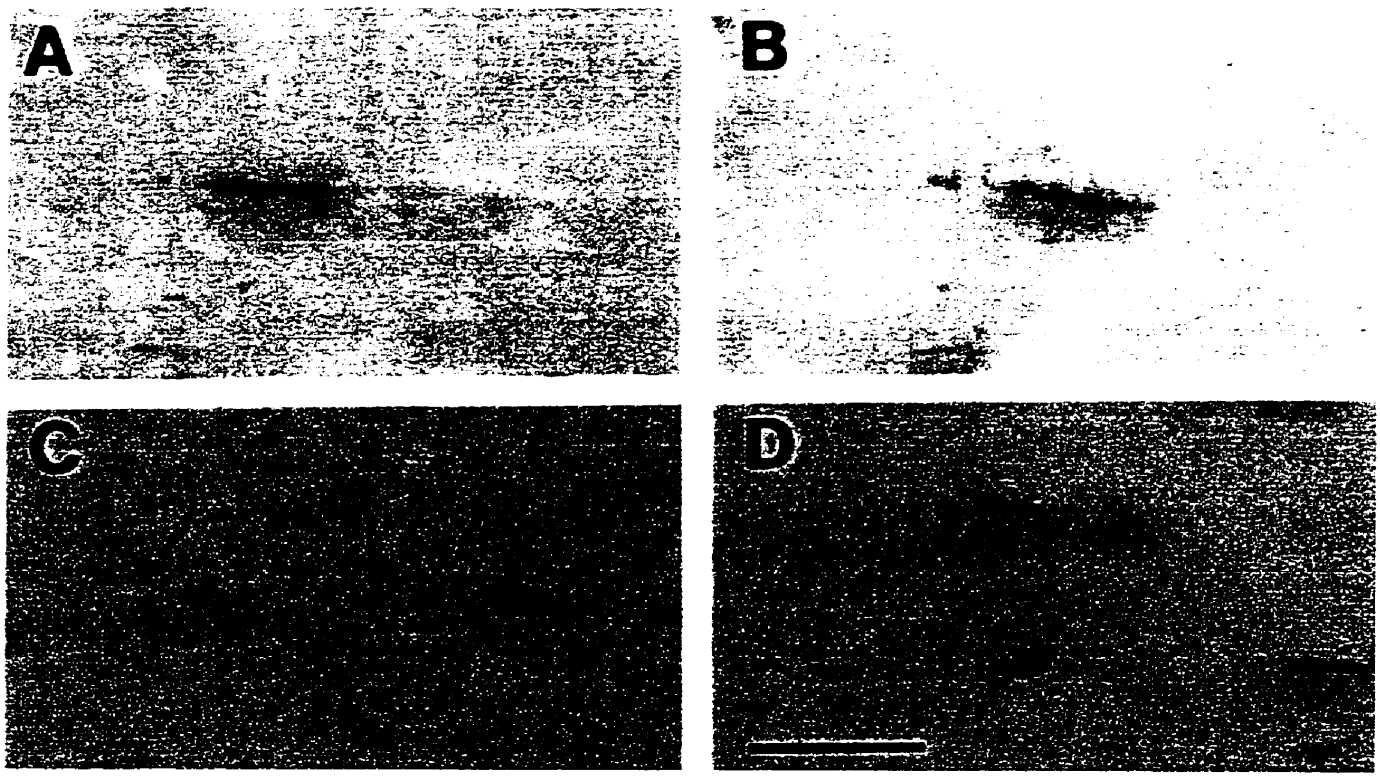

A
B
C
D

Figure 2.5. Expression of utrophin promoter-reporter gene constructs in muscle fibers. (A) shows the percentage of TA muscles expressing the construct following injections with plasmids containing either the 1.3 or 0.5 kb utrophin promoter fragment. Note that deletion of 800 bp from the 5' region of the 1.3 kb fragment reduced the percentage of muscles expressing the reporter gene. (B) shows the percentage of synaptic events (see Materials and Methods) following injections with the two different constructs. Note that the 1.3 kb utrophin promoter fragment confers preferential synaptic expression to the reporter gene nlsLacZ.

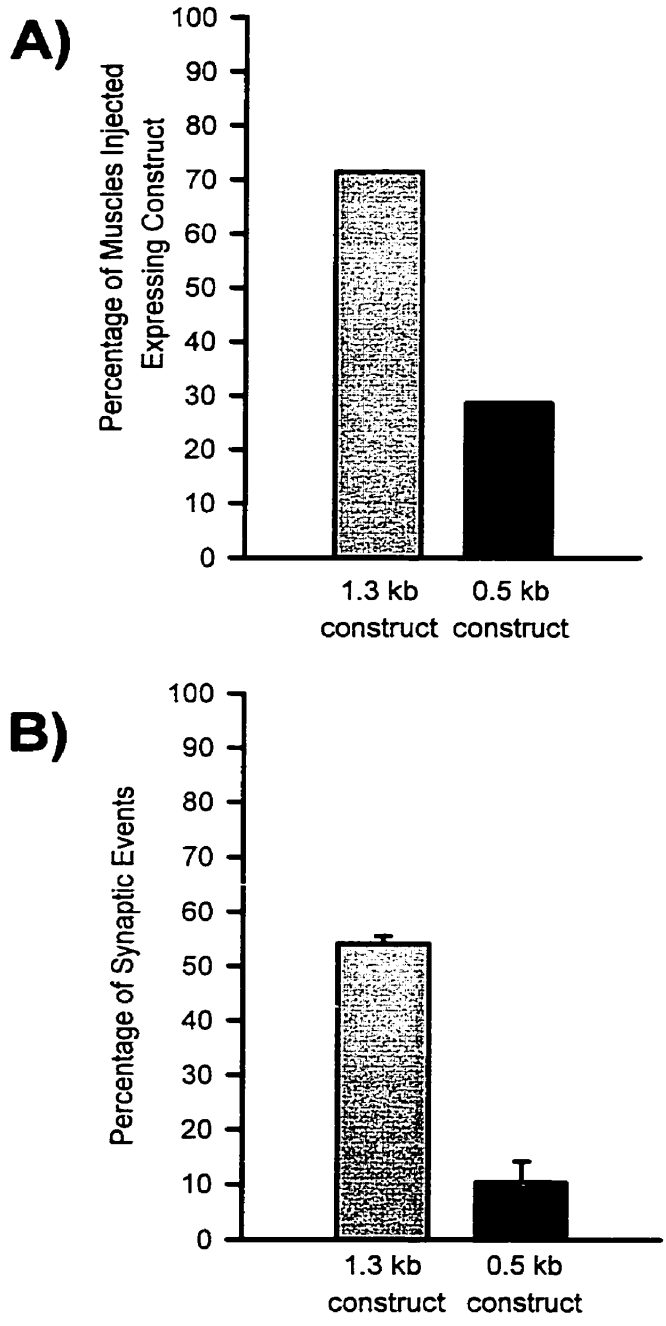
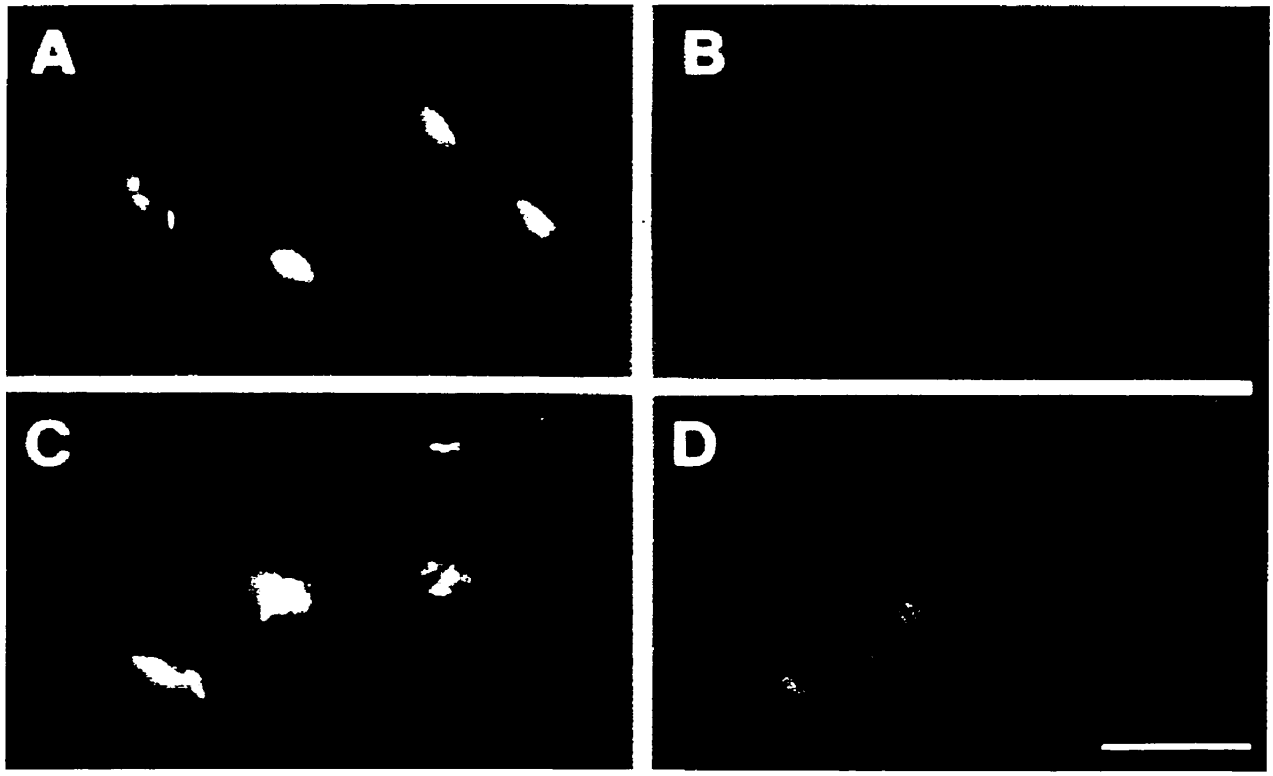

A)
Percentage of Muscles Injected Expressing Construct
100
90
80
70
60
50
40
30
20
10
0
1.3 kb construct
0.5 kb construct
B)
Percentage of Synaptic Events
100
90
80
70
60
50
40
30
20
10
0
1.3 kb construct
0.5 kb construct

Figure 2.6. Localization of utrophin at newly formed ectopic synapses. Ectopic synapses were induced to form at sites distant from the original neuromuscular junctions. Serial cryostat sections were analyzed for the presence of AChR using fluorescein-conjugated α-bungarotoxin (A,C), and synapsin (B) or utrophin (D) by immunofluorescence. Note the presence of post (A) and presynaptic (B) structures at these ectopic synapses as well as the co-localization between AChR (C) and utrophin (D). Bar = 70 μm.


A
B
C
D

CHAPTER 3

MUSCLE AND NEURAL ISOFORMS OF AGRIN INCREASE UTROPHIN EXPRESSION IN CULTURED MYOTUBES VIA A TRANSCRIPTIONAL REGULATORY MECHANISM

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A. Gramolini performed all of the experimental procedures, with technical assistance from J. Lunde for the plasmid DNA injections. The manuscript was written by A. Gramolini and Dr. Jasmin. As part of an ongoing collaboration, J.M. Tinsley, E.A. Burton, and K.E. Davies supplied the utrophin promoter-reporter constructs. Drs. M. Ferns, A. Cartaud, and J. Cartaud supplied the recombinant and *Torpedo* agrin and provided comments on the written manuscript.

ABSTRACT

Duchenne muscular dystrophy (DMD) is a prevalent X-linked neuromuscular disease for which there is currently no cure. Recently, it was demonstrated in a transgenic mouse model that utrophin could functionally compensate for the lack of dystrophin and alleviate the muscle pathology (Tinsley, J.M., Potter, A.C., Phelps, A.C., Fisher, S.R., Trickett, J.I., and Davies, K.E. (1996) *Nature* 384, 349-353). In this context, it thus becomes essential to determine the cellular and molecular mechanisms presiding over utrophin expression in attempts to overexpress the endogenous gene product throughout skeletal muscle fibers. In a recent study, we showed that the nerve exerts a profound influence on utrophin gene expression and postulated that nerve-derived trophic factors mediate the local transcriptional activation of the utrophin gene within nuclei located in the postsynaptic sarcoplasm (Gramolini, A.O., Dennis, C.L., Tinsley, J.M., Robertson,G.S., Cartaud, J., Davies, K.E. and Jasmin, B.J. (1997) *J. Biol. Chem.* 272, 8117-8120). In the present study, we have therefore focused on the effect of agrin on utrophin expression in cultured C2 myotubes. In response to Torpedo, muscle- or nerve-derived agrin, we observed a significant 2-fold increase in utrophin mRNAs. By contrast, CGRP treatment failed to affect expression of utrophin transcripts. Western blotting experiments also revealed that the increase in utrophin mRNAs was accompanied by an increase in the levels of utrophin. To determine whether these changes were caused by parallel increases in the transcriptional activity of the utrophin gene, we transfected muscle cells with a 1.3 kb utrophin promoter-reporter (nlsLacZ) gene construct and treated them with agrin for 24 to 48 hr. Under these

conditions, both muscle- and nerve-derived agrin increased the activity of β-galactosidase indicating that agrin treatment led, directly or indirectly, to the transcriptional activation of the utrophin gene. Furthermore, this increase in transcriptional activity in response to agrin resulted from a greater number of myonuclei expressing the 1.3 kb utrophin promoter-nlsLacZ construct. Deletion of 800 bp 5' from this fragment decreased the basal levels of nlsLacZ expression and abolished the sensitivity of the utrophin promoter to exogenously applied agrin. In addition, site-directed mutagenesis of an N-box motif contained within this 800 bp fragment demonstrated its essential contribution in this regulatory mechanism. Finally, direct gene transfer studies performed *in vivo* further revealed the importance of this DNA element for the synapse-specific expression of the utrophin gene along multinucleated muscle fibers. These data show that both muscle and neural isoforms of agrin can regulate expression of the utrophin gene and further indicate that agrin is not only involved in the mechanisms leading to the formation of clusters containing pre-synthesized synaptic molecules but that it can also participate in the local regulation of genes encoding synaptic proteins. Together, these observations are therefore relevant for our basic understanding of the events involved in the assembly and maintenance of the postsynaptic membrane domain of the neuromuscular junction and for the potential use of utrophin as a therapeutic strategy to counteract the effects of DMD.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most severe and prevalent neuromuscular disease affecting one in 3,500 male births (1). This disease is characterized by repeated cycles of muscle fiber degeneration and regeneration with an eventual failure to regenerate thereby leading to a loss of muscle mass and function. The genetic defect underlying DMD has been located on the short arm of the X chromosome and prevents the production of dystrophin, a large cytoskeletal protein of the spectrin superfamily (2,3). Previous studies have shown that in muscle, dystrophin is located at the cytoplasmic face of the sarcolemma where it links the intracellular cytoskeleton network to the extracellular matrix via a complex of dystrophin-associated proteins (for reviews, see 4–7).

Several years ago, an autosomal homologue to dystrophin was identified on chromosome 6q24 (8). This gene, now referred to as utrophin, presents a genomic organization similar to that of the dystrophin gene indicating that both genes evolved from an ancestral duplication event (9). Cloning of a full-length cDNA and subsequent analysis of its deduced amino acid sequence revealed in fact, that utrophin shares considerable similarity with dystrophin particularly in the actin binding domain and carboxy terminus (10). However, in comparison to high molecular mass isoforms of dystrophin which are predominantly expressed in brain and muscle, utrophin displays a ubiquitous pattern of expression since it can be detected in most tissues (11-13).

In normal skeletal muscle, expression of utrophin is known to be influenced by the state of differentiation and innervation of muscle fibers. In developing myotubes for example, utrophin is first localized to the entire length of the sarcolemma (14-17). Following the establishment of synaptic contacts, utrophin becomes highly enriched within the postsynaptic membrane domain of the neuromuscular junction (18.19). However, several studies have shown that in dystrophic muscles, utrophin expression is not restricted to postsynaptic compartments since it extends well into extrasynaptic regions of adult muscle fibers (14, 20-23). Such modulations in the pattern of expression indicate that distinct cellular and molecular mechanisms must exist in order to maintain the uneven distribution of utrophin along normal adult muscle fibers and to alter its levels and localization in developing and diseased muscles.

Despite these recent advances however, our knowledge of the regulatory mechanisms presiding over utrophin expression in muscle is clearly lacking. A better understanding of these mechanisms appears important particularly since upregulation of utrophin is currently envisaged as a therapeutic strategy to prevent the relentless progression of DMD (24,25). In this context, we have recently shown that the nerve exerts a profound influence on utrophin gene expression (26). Since our previous experiments also demonstrated that nerve-derived electrical activity is not a key factor regulating utrophin expression (27), we postulated in these initial studies, that nerve-derived trophic factors likely mediate the local transcriptional activation of the utrophin gene within nuclei of the postsynaptic membrane domain (26). In the present study, we have therefore determined the effects of nerve-derived trophic factors on utrophin expression in

cultured myotubes. A preliminary account of this work has previously appeared in abstract form (28).

EXPERIMENTAL PROCEDURES

Tissue Culture. C2 cells were cultured on Matrigel-coated (Collaborative Biomedical Products, Bedford, MA) 35 mm culture plates and kept at 37°C in a water-saturated atmosphere containing 5% CO_2. Myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% horse serum, 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin and 292 ng/ml L-glutamine until they reached confluence. At this stage, the concentration of horse serum was reduced to 5% and FBS was eliminated to promote myotube formation. Myoblasts were allowed to fuse into multinucleated myotubes for 3 to 4 days and were then used for experiments. To examine the effects of nerve-derived trophic factors, 0.1 μM of rat CGRP (Sigma, St.Louis, MO) or 10 ng/ml of purified *Torpedo* agrin (29) was added directly to the culture media for 24 to 48 hr. Additionally, the effects of 1 nM recombinant neural ($C\text{-}Ag_{12,4,8}$) or muscle ($C\text{-}Ag_{12,0,0}$) isoforms of agrin were also examined (30).

Immunofluorescence and Quantitation of AChR Clusters. Differentiated C2 myotubes were treated with 10 ng/ml of Torpedo or recombinant agrin for 24 to 48 hr. Cultures were subsequently fixed for 10 min in 4% paraformaldehyde. Clusters of acetylcholine receptors (AChR) were visualized with fluorescein isothiocyanate (FITC)-conjugated α-bungarotoxin used at a final concentration of 4 ng/ml in phosphate-buffered saline (PBS). Following thorough washing with PBS, the myotubes were covered with a glycerol:PBS solution and a cover slip, and they were then examined by epifluorescence using a Zeiss photomicroscope. For the

determination of agrin-induced AChR clusters, the numbers of myotubes and AChR aggregates were determined in 10 fields of view per culture at a 400 X magnification as described in detail in Gee *et al.* (31). A minimum of four cultures were quantitated for each experimental condition. Photographs were taken with Kodak T-MAX 400 black and white films.

Immunoblotting. C2 myotubes were treated with agrin for 48 hr, washed in PBS and then solubilized in RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 0.5% Triton X-100, 1 mM PMSF, 5 mM iodoacetamide, 2 μg/ml aprotinin, 0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, and 0.025% NaN_3) (32). Samples were centrifuged, and the supernatant collected and stored at -20°C until analysis. The resulting pellet was further solubilized in RIPA buffer containing 5% SDS. Following centrifugation, the supernatant was collected and stored at -20°C. The concentration of SDS-solubilized protein was determined using the bicinchoninic acid (BCA) Protein Assay Reagent protocol (Pierce Laboratories; Rockford, IL). Equivalent amounts of cell extracts (70 μg) were separated on a 6% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Sigma; Toronto, ON). To ensure that equivalent amounts of proteins were loaded for each sample, membranes were also stained with Ponceau S (Sigma). Membranes were subsequently incubated with monoclonal antibodies directed against either utrophin (MANCHO-7; kindly supplied by Dr. Glen Morris, N.E. Wales Institute, UK), α-actinin (Sigma), or sarcomeric myosin (MF-20, Developmental Hybridoma Bank; see Ref. 33). Bound antibodies were detected by secondary antibodies linked to horseradish peroxidase and revealed via chemiluminescence using a commercially available kit (New England Nuclear;

Boston, MA). Membranes were then exposed onto BioMax autoradiographic films (Kodak; Rochester, NY), developed and scanned at 200 dpi using a Hewlett-Packard Scanjet 4C.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted using Trizol as recommended by the manufacturer (Gibco; Burlington, ON). Briefly, cells were scraped into 1 ml of Trizol. Following addition of 200 µl of chloroform, the samples were mixed vigorously and centrifuged at 12,000 X g for 15 min at 4°C. The aqueous layer was then transferred to a fresh tube and 500 µl of ice-cold isopropanol was added. For RNA precipitation, the isopropanol mixture was spun and the resultant pellets washed twice with ice-cold 75% ethanol.

For all samples, total RNA was redissolved into 20 µl of RNase-free water. From each of these stocks, the RNA was further diluted to a final concentration of 50 ng/µl and only 2 µl of this dilution was used for RT-PCR as described in detail in Jasmin *et al.* (27,34). Briefly, a RT master mix was prepared containing 5 mM $MgCl_2$, 1 X PCR buffer II (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM dNTPs, 20 U RNase inhibitor, 50 U reverse transcriptase and 2.5 mM of random hexamers (GeneAmp RNA PCR kit; Perkin Elmer Cetus; Foster City, CA). The master mix was aliquoted and the appropriate RNA sample subsequently added. Negative controls consisted of RT mixtures in which the RNA sample was replaced with RNase-free water. RT was performed for 45 minutes at 42°C and heated to 99°C for 5 minutes to terminate the reaction.

Complementary DNAs encoding utrophin and dystrophin were specifically amplified using primers designed on the basis of available mouse cDNA sequences (see Refs. 27,34). Amplification of the selected cDNAs was performed in a DNA thermal cycler (Perkin Elmer) by adding 4 μl of the RT mixture to 16 μl of a PCR master mix. Each cycle of amplification for utrophin cDNAs consisted of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min. For dystrophin amplification, each cycle consisted of denaturation at 94 °C for 1 min, followed by primer annealing and extension at 72°C for 3 minutes. The number of cycles for utrophin and dystrophin was 26 and 44, respectively. PCR products were visualized on a 1.5% agarose gel containing ethidium bromide. The 100 bp molecular mass marker (Gibco BRL) was used to estimate the molecular mass of the PCR products. Quantitative PCR experiments were performed in order to strictly determine the relative abundance of transcripts following different experimental treatments. These experiments were carried out using either one of two methods. In one case, 1.5 X 10^6 cpm per sample of ^{32}P end-labeled primers were added to the PCR master mix. PCR products were visualized and carefully excised from the agarose gel using a scalpel. The level of radioactivity present in these gel bands was determined by Cerenkov counting. Alternatively, PCR products were separated in 1.5% agarose gels containing the fluorescent dye Vistra Green (Amersham; Arlington Heights, IL) and the labeling intensity of the PCR product, which is linearly related to the amount of DNA, was quantitated using a Storm PhosphorImager (Molecular Dynamics; Sunnyvale, CA).

Expression of Utrophin Promoter-Reporter Gene Constructs. Several human utrophin promoter-reporter gene constructs were used in these experiments (35). These 1.3 and 0.5 kb promoter fragments were inserted upstream of the reporter gene LacZ and a nuclear localization signal (26). Additionally, two other 1.3 kb constructs were generated with mutations of the N-box. The 1.3 kb HindIII human utrophin promoter clone (35) was digested with XhoI and PstI liberating a 300 bp fragment containing the N-box which was then further cloned into pBSSKII(-) (Stratagene; Cambridge, UK) generating the clone pBSXP. Mutagenesis was performed using Quick Change (Stratagene) essentially following the manufacturers instructions except for using cloned Pfu polymerase (Stratagene). Two pairs of complementary primers were generated with a single or double point mutation in the N-box (N5F:5U-GTG GGG CTG ATC TTC CAG AAC AAA GTT GC, N5R: 5U-GCA ACT TTG TGG AAG ATC AGC CCC AC, N34F: 5U-GGG GCT GAT CTT TTG GAA CAA AGT TGC TGG G, N34R: 5U-CCC AGC AAC TTT CTT CCA AAA GAT CAG CCC C). pBSXP was used as the template for synthesis of the mutations using these oligonucleotide primer pairs. Following 15 cycles of 95°C for 30 sec, 56°C for 1 min, 68°C for 7 min, the wild type plasmid template was destroyed using the methylation sensitive restriction endonuclease DpnI. The mutant plasmids were cloned and sequenced to verify the addition of the mutations in the N-box and to confirm that no new mutations had been introduced into other sequences. The 300 bp XhoI/PstI was released and used to replace the equivalent non-mutated fragment at the same sites in the plasmid 1.3 kb nlsLacZ (26). The new promoter mutant/reporter constructs were then sequenced to check for no further mutations. For transfection and direct gene transfer experiments, plasmid DNA was prepared using the Qiagen mega-prep procedure (Chatsworth, CA).

C2 myoblasts were transfected with 3 μg of the appropriate utrophin promoter-reporter gene construct using the Mammalian Transfection System-Calcium Phosphate kit (Promega; Madison, WI). Once the cultures became confluent, the media was switched to the differentiation media (see above) to stimulate myotube formation. Three to 4 days later, agrin was added to the media for 48 hours. Levels of β-galactosidase activity were then determined using either a histochemical staining procedure (26) or a biochemical assay (Promega β-Galactosidase Enzyme system). For the biochemical assays, the levels of β-galactosidase activity were normalized according to a cotransfected chloramphenicol acetyltransferase (CAT) plasmid (Promega) and protein content. In these experiments, the cotransfected CAT plasmid allowed for the correction of any variation due to differences in transfection efficiency between culture wells. CAT activity was determined using a CAT Enzyme Assay system (Promega) while protein content was determined by the BCA method (see above).

For direct gene transfer into mouse tibialis anterior (TA) muscles, experiments were performed as described previously (26). Briefly, 25 μl of DNA solution (2 μg/μl) was injected directly into the muscles of 4 week-old mice. Muscles were excised 2 weeks following injection and they were quickly frozen in melting isopentane precooled with liquid nitrogen. Cryostat tissue sections were then processed for β-galactosidase and acetylcholinesterase (AChE) histochemistry (26). The position of blue myonuclei clusters indicative of utrophin promoter activity and designated as an event, was determined and compared to the presence of

neuromuscular junctions using the quantitative procedure recently established by Duclert *et al.* (36).

Statistical Analysis. Paired Student's *t*-tests were performed to evaluate the effects of agrin on utrophin expression. These tests were used to strictly compare the effects of agrin-treated vs non-treated myotubes. The level of significance was set at $P < 0.05$. Data are expressed as mean ± SEM throughout.

RESULTS

Agrin Increases Expression of Utrophin in Cultured Myotubes. In an initial series of experiments, 3 to 4 day-old myotubes were treated with agrin purified from *Torpedo* electric tissue or with recombinant agrin isoforms in attempts to identify putative extracellular cues capable of regulating utrophin gene expression. As expected, agrin treatment increased the number of AChR clusters present on the surface of these C2 myotubes (Figure 3.1). Quantitative analyses revealed that the number of AChR clusters per myotube increased by approximately 15-fold ($P < 0.05$) following *Torpedo* agrin treatment (Figure 3.1). Immunofluorescence experiments using the monoclonal antibody MANCHO 7 showed that utrophin was present at these AChR clusters but only at the largest ones (data not shown). As expected, treatment of myotubes with the predominant isoform of agrin expressed in muscle ($C\text{-}Ag_{12,0,0}$) failed to induce the formation of AChR clusters above the levels normally detected in non-treated cultures.

Next, we examined whether agrin treatment which not only led to AChR clustering but also to the reorganization of the subsarcolemmal cytoskeleton, also influenced expression of utrophin in C2 myotubes. To this end, myotubes were treated with agrin and 48 hr later, they were solubilized sequentially in RIPA buffer containing either 0.1% or 5% SDS (see Experimental Procedures). Western blotting experiments showed that agrin treatment increased the levels of utrophin in 0.1% SDS-extracted proteins (Figure 3.2A). Ponceau staining of the membranes prior to immunoblotting confirmed that an equal amount of proteins had been loaded

in each lane of the gel (Figure 3.2C). To further ensure that similar amounts of proteins were present in each lane, the same membranes were also processed to determine the levels of sarcomeric myosin and α-actinin. In these experiments, we observed that the amount of sarcomeric myosin (Figure 3.2A) and α-actinin (data not shown) were similar between agrin-treated vs non-treated myotubes. By contrast, the levels of utrophin extracted from the initial pellets with RIPA buffer containing a higher concentration of SDS was not affected by agrin treatment (Figure 3.2B). These results suggest that with the initial extraction buffer containing low levels of SDS, we primarily extracted utrophin not yet incorporated into the cytoskeleton which may thus reflect newly synthesized molecules. The observation that agrin increases the levels of utrophin in a readily extractable fraction indicates that agrin not only leads to a redistribution of preexisting synaptic molecules onto the surface of myotubes but that it can also increase expression of these synaptic components.

Agrin Stimulates Transcription of the Utrophin Gene. In order to determine if the increase in utrophin following agrin treatment resulted from enhanced transcriptional activation of the utrophin gene, we first examined the levels of utrophin transcripts in agrin-treated vs non-treated myotube cultures by RT-PCR. Quantitative analysis revealed that utrophin mRNA levels increased significantly ($P < 0.05$) following Torpedo agrin treatment (Figures 3.3 and 3.4). Recombinant neural agrin (C-$Ag_{12,4,8}$) had a similar effect (Figure 3.4) thus ruling out the possibility that the increased expression of utrophin transcripts seen after treatment with Torpedo agrin was caused by contaminants present in this purified extract. Interestingly, treatment of

myotubes with the muscle isoform of agrin ($C\text{-}Ag_{12,0,0}$) also increased the expression of utrophin mRNAs by approximately 2-fold (Figure 3.4). Myotubes treated for 48 hr with Torpedo or recombinant isoforms of agrin showed slightly higher increases in the levels of utrophin transcripts in comparison to those observed following 24 hr-treatments (data not shown). In these experiments, agrin did not affect the levels of dystrophin transcripts (Figure 3.3).

In separate experiments, we also determined the effects of calcitonin gene-related peptide (CGRP), a neuropeptide enriched at the motor endplate and known to affect expression of AChR in cultured myotubes (for review, see Refs. 37,38). In contrast to the effects seen with agrin, CGRP treatment of C2 myotubes failed to induce expression of utrophin mRNA (Figures 3.3 and 3.4). Consistent with previous reports however (39), we nonetheless consistently observed in these experiments, a small but significant 1.4-fold increase in the levels of transcripts encoding the AChR α-subunit following CGRP treatment (data not shown).

We next performed a series of experiments in which human utrophin promoter-reporter gene constructs were transfected into C2 myoblasts. Three to 4 day-old myotubes were then treated with agrin and 48 hr later, the activity of β-galactosidase was determined and normalized to CAT activity and protein content. As illustrated in Figure 3.5, we observed a marked increase in the expression of the reporter gene in cultures transfected with the construct containing the 1.3 kb utrophin promoter fragment and treated with agrin. In fact, quantitative analyses showed that both muscle- ($C\text{-}Ag_{12,0,0}$) and nerve-derived ($C\text{-}Ag_{12,4,8}$) isoforms of agrin increased the expression of β-galactosidase by more than 2-fold ($P < 0.05$). In contrast, agrin treatment of

myotubes transfected with the 0.5 kb utrophin promoter-reporter gene construct failed to induce expression of β-galactosidase above basal levels. Taken together, these results indicate therefore, that regulatory sequences contained within the deleted 800 bp fragment of the utrophin promoter are essential for transcriptional activation of the utrophin gene following agrin treatment.

On the basis of these findings, it became important to determine whether the increase in the activity of β-galactosidase was due to an increase in the number of myonuclei expressing detectable levels of the reporter gene or, alternatively, to an enhanced level of expression in myonuclei already expressing β-galactosidase. To address this issue, we histochemically stained transfected cultures for β-galactosidase and counted the number of positive myonuclei in control versus agrin-treated myotube cultures. This analysis was justified and statistically valid for two reasons. First, our biochemical experiments (see above) showed that transfection efficiency did not vary markedly from one culture dish to another as evidenced by the relatively constant levels of CAT used to normalize β-galactosidase activity. In fact, we noted in these experiments that CAT levels varied by less than 15% between transfected culture dishes. Second, quantitative analysis showed that the number of β-galactosidase-positive myonuclei increased *significantly* ($P < 0.05$) following agrin treatment (Figure 3.6) thereby eliminating the contribution of a random experimental event such as transfection efficiency, to the overall results. Similar to our data obtained by determining biochemically the activity of β-galactosidase and normalizing it to CAT activity and protein content (Figure 3.5), this effect was observed with both muscle (C-$Ag_{12,0,0}$) and neural (C-$Ag_{12,4,8}$) isoforms of agrin (Figure 3.6). Taken together, these data show

therefore that the increase in β-galactosidase activity observed in our biochemical assays resulted primarily from a greater number of nuclei expressing the 1.3 kb utrophin promoter-reporter gene construct. In agreement with our biochemical data, we also observed that agrin treatment of myotubes transfected with the construct containing the 0.5 kb utrophin promoter fragment failed to increase the number of β-galactosidase-positive nuclei thereby further highlighting the importance of regulatory elements contained within the deleted 800 bp promoter fragment for the transcriptional activation of the utrophin gene in response to agrin.

Role of the N-box Motif in Regulating Utrophin Gene Expression. Based on recent studies which have shown that the N-box motif plays a crucial role in regulating the expression of genes encoding the δ- and ε-subunits of the AChR (36,40), we examined the contribution of this DNA element in the transcriptional regulation of the utrophin gene by agrin. For these studies, site-directed mutagenesis was used to introduce single or double-base pair mutations into the N-box motif contained within the utrophin promoter (26,35). Two different mutants were generated and differed from the wild-type N-box (TTCCGG) by one (N5 = TTCCAG) or two bases (N34 = TTTTGG). The mutant utrophin promoter fragments were inserted upstream of the nlsLacZ reporter gene.

In contrast to the 2- to 3- fold induction in the activity of β-galactosidase driven by the wild-type 1.3 kb utrophin promoter fragment seen following agrin treatment (Figures 3.5 and 3.7), both N-box mutant constructs failed to display a similar responsiveness to agrin (Figure

3.7). Quantitative analyses revealed that expression of β-galactosidase driven by either one of the two N-box mutant promoter fragments was not significantly ($P>0.05$) different between agrin-treated vs non-treated myotube cultures. These results strongly indicate therefore that the N-box motif is involved in the regulatory mechanism governing expression of the utrophin gene in response to agrin.

The N-Box Motif Regulates the Synaptic Expression of the Utrophin Gene In Vivo. To determine whether the N-box motif participates also in the regulation of the utrophin gene *in vivo* (see Refs. 26,36,40), we injected directly into mouse TA muscles constructs containing either the 1.3 kb wild-type utrophin promoter fragment or the N-box mutants. In agreement with our previous findings (26), we observed that ~55% of all blue myonuclei clusters seen in muscles injected with constructs containing the wild-type 1.3 kb promoter fragment coincided with the presence of neuromuscular junctions (Figure 3.8). Mutations of the N-box however, led to a marked reduction in the percentage of synaptic events. In fact, quantitative analysis revealed that in muscles injected with either one of the mutant constructs, less than 20% of all blue myonuclei clusters were located in the vicinity of neuromuscular junctions (Figure 3.8). These results indicate therefore, that the N-box motif regulates also *in vivo* expression of the utrophin gene since it modulates its pattern of synaptic expression.

Finally, to gain insights into the mechanisms contributing to the local transcriptional regulation of the utrophin gene along muscle fibers *in vivo* , we determined the total number of synaptic vs extrasynaptic events per muscle following injection of constructs containing the

wild-type 1.3 kb utrophin promoter fragment or the N5 mutant. In these experiments, we focused our analysis on the N5 mutant since the total number of β-galactosidase-positive fibers seen after injection with this construct was similar to that observed following injection with the construct containing the wild-type promoter fragment (Figure 3.9A). Interestingly, we observed a significantly lower number of synaptic events per muscle following injection of the N5 mutant construct as compared to the wild-type 1.3 kb utrophin promoter fragment (Figure 3.9B). By contrast, the number of events in extrasynaptic regions of muscle fibers was similar between these two constructs (Figure 3.9C). Therefore, these results suggest that the N-box motif contributes to the local transcriptional activation of the utrophin gene within myonuclei of the postsynaptic sarcoplasm by increasing its expression in this specialized region of muscle fibers as opposed to repressing its activity in extrasynaptic compartments (see Duclert *et al.* (36) for further discussion).

DISCUSSION

In a recent study, we demonstrated that utrophin transcripts accumulate preferentially within the postsynaptic sarcoplasm of muscle fibers and that this accumulation resulted from the local transcriptional activation of the utrophin gene in myonuclei concentrated beneath the neuromuscular junction (26). Induction of ectopic synapses at sites distant from the original neuromuscular junctions further revealed that nuclei located in extrasynaptic regions were capable of expressing utrophin upon receiving appropriate neuronal cues. Together with the demonstration that levels of utrophin in muscle are largely insensitive to elimination of nerve-evoked electrical activity (19,27), these experiments led us to postulate that nerve-derived trophic factors regulate locally the expression of the utrophin gene (25,26). Among the molecules known to regulate the expression or localization of AChR (for review, see Refs. 37,38), agrin appeared as a plausible candidate for several reasons. For example, detailed analysis of agrin- (41) and MuSK- (42) deficient mice has led to the suggestion that *in vivo* , agrin may ultimately affect transcription of genes encoding synaptic proteins such as AChR. Moreover, in response to exogenously applied agrin, cultured myotubes show increase numbers of AChR clusters with only large ones containing utrophin (43,44). Although agrin treatment leads to a redistribution of normally diffusing AChR molecules, it is unlikely that it causes a similar clustering of pre-synthesized, membrane-attached utrophin. The presence of utrophin in large AChR clusters may thus result from compartmentalized de novo expression of utrophin

by nuclei located in the vicinity of the growing clusters. In the present study, we have therefore focused on the effect of agrin on utrophin expression.

In attempts to determine whether agrin treatment induced utrophin expression, we initially measured levels of utrophin and its mRNA in cultures of treated versus non-treated myotubes. In addition to causing the clustering of AChR, agrin treatment also increased the levels of utrophin. In these experiments, we observed that utrophin levels increased within an easily dissociated cellular fraction thereby suggesting that this increase resulted from a newly synthesized pool of utrophin not yet intertwined within the existing cytoskeleton. Similarly, we also noted that agrin treatment induced a significant 2-fold increase in the levels of utrophin transcripts. Interestingly, both nerve- and muscle-derived isoforms of agrin had a comparable stimulatory effect on utrophin expression. These increases are in fact of similar magnitude to those reported recently by Jones *et al.* (45) who examined the impact of both muscle and neural isoforms of agrin on expression of transcripts encoding the AChR ε-subunit. However, a major difference between the two studies is that we were able to observe an effect on utrophin gene expression without the necessity of agrin being substrate-bound (45). Although the reason for this difference remains currently obscure, it appears reasonable to assume that it likely arises from differences in culture conditions. In particular, recent experiments have revealed that Matrigel™ is capable of binding agrin (46,47). Since in our experiments, myotube cultures are plated on Matrigel-coated plates, it appears likely that Torpedo agrin as well as recombinant agrin fragments may become bound to this substrate via an unknown mechanism (see Denzer *et al.* (46,47) for further discussion) and therefore do not remain in a "soluble" form (see Ref.

45). Nonetheless, since the pattern of expression of the utrophin gene along muscle fibers resembles that of the ε-subunit gene (26,36,48), these results are coherent with the notion that expression of genes encoding membrane and cytoskeletal proteins of the postsynaptic membrane are co-regulated and therefore involve a common signal transduction pathway.

Transfection experiments with utrophin promoter-reporter gene constructs indicated that the increase in utrophin mRNA levels following agrin treatment resulted from the transcriptional activation of the utrophin gene. In agreement with our previous *in vivo* studies (26), deletion of 800 bp from the 3' region of the 1.3 kb promoter fragment significantly reduced the activity of the reporter gene in transfected cells. More importantly, it also abolished the response to agrin treatment. Together, these results indicate that DNA elements contained within the deleted 800 bp are not only regulating the basal level of utrophin gene expression in muscle cells *in vivo* (26) and *in vitro* (this study), but they also confer to the utrophin promoter its sensitivity to neuronal cues including agrin. Among the putative elements that may play a crucial role in this regulatory mechanism is the N-box motif (26,35,40) which was shown recently to be essential for the synapse-specific expression of AChR δ- and ε-subunit genes (36,40). In the present study, site-directed mutagenesis confirmed that the N-box motif is indeed essential in this regulatory mechanism. These results further suggest that the N-box motif may in fact represent the ultimate target within the utrophin promoter which mediates the agrin effect in cultured myotubes. In addition, it appears that this DNA element also plays an essential role *in vivo* in the regulation of the utrophin gene since direct injection of constructs containing mutant utrophin promoter

fragments into TA muscles failed to induce synapse-specific expression of the reporter gene as observed with the wild-type 1.3 kb utrophin promoter fragment (26).

The molecular mechanism by which nerve- and muscle-derived isoforms of agrin lead to the transcriptional activation of the utrophin gene remains to be established. In this context however, there are several pathways that may be currently envisaged. One signaling pathway involves binding of agrin to a complex that includes the tyrosine kinase receptor MuSK and a myotube-specific accessory component (49). This binding is known to trigger a series of biochemical events that culminate in the clustering of AChR on the surface of myotubes and in a reorganization of the underlying cytoskeleton. However, this pathway is probably not directly involved since only neural agrin activates MuSK and induces AChR clustering (49).

A more likely mechanism responsible for the agrin-induced effects on utrophin gene expression involves not only clustering of AChR but also of other postsynaptic membrane proteins which in turn, may directly participate in the regulation of utrophin. For example, it has been recently demonstrated that intramuscular injections of plasmid DNA encoding agrin into extrasynaptic regions of denervated soleus muscle fibers induced, in addition to AChR clustering, the aggregation of muscle-derived ARIA along with its receptors, erbB2 and erbB3 (50). Since these molecules are known to regulate expression of AChR subunit genes (51-53), agrin treatment may thus ultimately stimulate ARIA-dependent gene expression via an autocrine mechanism involving muscle ARIA and its receptors (45,50). Accordingly, agrin may be sufficient for: i) the initial events underlying AChR clustering; and ii) the positioning of other

molecules involved in regulating expression of synaptic proteins. Such a role for agrin would thereby ensure the proper growth of developing postsynaptic membrane domains as well as their long-term maintenance. Furthermore, it could also explain the presence of utrophin only in large AChR clusters since recruitment of all necessary components would parallel the growth of the clusters. In fact, this mechanism is consistent with our statistical analysis demonstrating that the agrin effect on the activity of the reporter gene was caused by a significantly greater number of nuclei expressing the 1.3 kb construct as opposed to a similar number of nuclei increasing their level of expression. These results indicate therefore, that the effect of agrin is to stimulate transcription of the utrophin gene in normally quiescent nuclei; an expected effect given that agrin increases the number of clusters containing AChR and other synaptic proteins on the surface of these myotubes. In the case of muscle-derived agrin however, the effect on utrophin gene expression likely occurs via a mechanism altogether distinct from that involving the MuSK-dependent pathway (see also Ref. 45). Finally, it is also conceivable that the effects of both muscle and neural isoforms of agrin occurs via a distinct and unique pathway involving therefore a MuSK-independent mechanism. For example, as a protein of the extracellular matrix, agrin may activate transcription of synaptic genes by first binding to other receptors such as the integrins or α-dystroglycan which are known to accumulate at developing postsynaptic membrane domains (54,55). We are currently examining these possibilities using several experimental approaches.

In a recent study, Tinsley *et al.* (24) showed that expression of utrophin in extrasynaptic regions of muscle fibers from *mdx* mice functionally compensated for the lack of dystrophin and

alleviated the dystrophic pathology. These findings demonstrate that upregulation of utrophin may indeed represent an effective treatment for DMD. In this context, the next logical step is naturally to identify molecules capable of increasing utrophin gene expression in skeletal muscle fibers. Our observation that agrin increases levels of utrophin protein and mRNA via a transcriptional regulatory mechanism is therefore not only relevant for our basic understanding of the events involved in the assembly and maintenance of the postsynaptic membrane domain of the neuromuscular junction but also, for the potential use of utrophin as a therapeutic strategy for DMD.

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THESIS REVISIONS

Additional student's t-tests and analyses of variance (ANOVA) statistical tests were performed on the data for Figures 3.1, 3.4, 3.5B, 3.6, 3.7, and 3.8. With these analyses, all sets of experiments showed significant differences ($P<0.05$) consistent with our original statistical observations.

REFERENCES

1. Emery, A.E. (1991) *Neuromuscular Disorders* **1**, 19-29.

2. Hoffman, E.P, Knudson C.M, Campbell K.P., & Kunkel L.M. (1987) *Nature* **330**, 754-758.

3. Koenig, M., Monaco, A.P., & Kunkel, L.M. (1988) *Cell* **53**, 219-226

4. Matsumura, K. & Campbell, K. P. (1994) *Muscle Nerve* **17**, 2-15.

5. Tinsley, J.M., Blake, D.J., Zuellig, R.A., & Davies, K.E. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8307-8713.

6. Blake, D.J., Tinsley, J.M., & Davies, K.E. (1996) *Brain Pathol.* **6**, 37-47.

7. Sadoulet-Puccio, H.M., & Kunkel, L.M. (1996) *Brain Pathol.* **6**, 25-35.

8. Love, D. R., Hill, D. F., Dickson, G., Spurr, N. K., Byth, B. C., Marsden, R. F., Walsh, F. S., Edwards, Y. H. & Davies, K. E. (1989) *Nature* **339**, 55-58.

9. Pearce, M., Blake, D.J., Tinsley, J.M., Byth, B.C., Campbell, L., Monaco, A.P., & Davies, K.E. (1993) *Hum. Mol. Genet.* **2**, 1765-1772.

10. Tinsley, J. M., Blake, D. J., Roche, A., Fairbrother, U., Riss, J., Byth, B. C., Knight, A. E., Kendrick-Jones, J., Suthers, G. K., Love, D. R., Edwards, Y. H. & Davies, K. E. (1992) *Nature* **360**, 591-593.

11. Khurana, T. S., Hoffman, E. P. & Kunkel, L. M. (1990) *J. Biol. Chem.* **265**, 16717-16720.

12. Love, D. R., Morris, G. E., Ellis, J. M., Fairbrother, U., Marsden, R. F., Bloomfield, J. F., Edward, Y. H., Slater, C. P., Parry, D. J. & Davies, K. E. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3243-3247.

13. thi Man, N., Ellis, J. M., Love, D. R., Davies, K. E., Gatter, K. C., Dickson, G. & Morris, G. E. (1991) *J. Cell Biol.* **115**, 1695-1700.

14. Takemitsu, M., Ishiura, S., Koga, R., Kamakura, K., Arahata, K., Nonaka, I. & Sugita, H. (1991) *Biochem. Biophys. Res. Commun.* **180**, 1179-1186.

15. Khurana, T. S., Watkins, S. C., Chafey, P., Chelly, J., Tome, F. M., Fardeau, M., Kaplan, J. & Kunkel, L. M. (1991) *Neuromusc. Dis.* **1**, 185-194.

16. Zhao, J., Yoshioka, K., Miike, T. & Miyatake, M. (1993) *J. Neurol. Sci.* **114**, 104-108

17. Pons, F., Robert, A., Marini, J. F. & Leger, J. (1994) *J. Neurol. Sci.* **122**, 162-170.

18. Fardeau, M., Tome, F. M., Collin, H., Augier, N., Pons, F., Leger, J. & Leger, J. L. (1990) *Compt. Rend. Acad. Sci.* **311**, 197-204.

19. Ohlendieck, K., Ervasti, J. M., Matsumura, K., Kahl, S. D., Leveille, C. J. & Campbell, K. P. (1991) *Neuron* **7**, 499-508.

20. Helliwell, T. R., thi Man, N., Morris, G. E. & Davies, K. E. (1992) *Neuromusc. Dis.* **2**, 177-184.

21. Matsumura, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. D. & Campbell, K. P. (1992) *Nature* **360**, 588-591.

22. Karpati, G., Carpenter, S., Morris, G. E., Davies, K. E., Guerin, C. & Holland, P. (1993) *J. Neuropathol. Exp. Neurol.* **52**, 119-128.

23. Mizuno, Y., Yoshida, M., Yamamoto, H., Hirai, S. & Ozawa, E. (1993) *J. Biochem.* **114**, 936-941.

24. Tinsley, J. M., Potter, A. C., Phelps, S. R., Fisher, R., Trickett, J. I. & Davies, K. E. (1996) *Nature* **384**, 349-353.

25. Gramolini, A.O., & Jasmin, B.J. (1997) *BioEssays* **19**, 747-750.

26 Gramolini, A.O., Dennis, C.L., Tinsley, J.M., Robertson, G.S., Davies, K.E, Cartaud, J., & Jasmin, B.J. (1997) *J. Biol. Chem.* **272**, 8117-8120.

27. Jasmin, B. J., Alameddine, H., Lunde, J. A., Stetzkowksi-Marden, F., Collin, H., Tinsley, J. M., Davies, K. E., Tome, F. M. S., Parry, D. J. & Cartaud, J. (1995) *FEBS Lett.* **374**, 393-398.

28. Gramolini, A.O., Tinsley, J.M., Robertson, G.S., Davies, K.E, Cartaud, J., & Jasmin, B.J. (1996) *Soc. for Neurosci.* **22**, 535.

29. Cartaud, A., Ludosky, M.A., Haasemann, M., Jung, D., Campbell, K., & Cartaud, J. (1996) *J. Cell Sci.* **109**: 1837-1846.

30. Ferns, M.J., Campanelli, J.T., Hoch, W., Scheller, R.H., & Hall, Z. (1993) *Neuron* **11**, 491-502.

31. Gee, S.H., Montanaro, F., Lindenbaum, M.H., & Carbonetto, S. (1994) *Cell* **77**, 675-686.

32. Springer, T.A. In: *Current Protocols in Molecular Biology*. Edited by Ausubel, F. M., Brent, R., Kingston, R.E., Moore, M.M., Seidman, J.G., Smith, J. A., & Struhl, K. John Wiley and Sons, Inc. Cambridge, Massachusetts, 1995.

33. Bader, D., Masaki, T., & Fischman, D.A. (1982) *J Cell Biol.* **95**, 763-770.

34. Jasmin, B.J., Lee, R.K., & Rotundo, R.L. (1993) *Neuron* **11**, 467-477.

35. Dennis, C. L., Tinsley, J. M., Deconinck, A. E. & Davies, K. E. (1996) *Nucleic Acids Res.* **24**, 1646-1652.

36. Duclert, A., Savatier, N., Schaeffer, L. & Changeux, J. P. (1996) *J. Biol. Chem.* **271**, 17433-17438.

37. Hall, Z. W. & Sanes, J. R. (1993) *Cell/Neuron* **72**, 99-121.

38. Duclert, A. & Changeux, J. P. (1995) *Physiol. Rev.* **75**, 339-368.

39. Duclert, A., Piette, J., & Changeux, J.P. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1391-1395.

40. Koike, S., Schaeffer, L. & Changeux, J. P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 10624-10628.

41. Gautam, M., Noakes, P.G., Moscoso, L., Rupp, F., Scheller, R.H., Merlie, J.P., & Sanes, J.R. (1996) *Cell* **85**, 525-535.

42. DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T., Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., Smith, C., Distefano, P.S., Glass, D.J., Burden, S.J., & Yancopoulos, G.D. (1996) *Cell* **85**, 513-523.

43. Phillips, W. D., Noakes, P. G., Roberds, S. L., Campbell, K. P. & Merlie, J. P. (1993) *J. Cell. Biol.* **123**, 729-740.

44. Campanelli, J.T., Roberds, S.L., Campbell, K.P., & Scheller, R.H. (1994) *Cell* 77, 663-674.

45. Jones, G., Herczeg, A., Ruegg, M. A., Lichtsteiner, M., Kröger, S. & Brenner, H. R. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 5985-5990.

46. Denzer, A.J., Gesemann, M., Schumacher, B., & Ruegg, M.A. *J. Cell Biol.* **131**, 1547-1560.

47. Denzer, A.J., Brandenberger, R., Gesemann, M., Chiquet, M., & Ruegg, M.A. (1997) *J. Cell Biol.* **137**, 671-683.

48. Gundersen, K., Sanes, J.R., & Merlie, J.P. (1993) *J. Cell Biol.* **123**, 1535-1344.

49. Glass, D.J., Bowen, D.C., Stitt, T.N., Radziejewski, C., Bruno, J., Ryan, T.E., Gies, D.R., Shah, S., Mattson, K., Burden, S.J., Distefano, P.S., Valenzuela, D.M., Dechiara, T.M., & Yancopoulos, G.D. (1996) *Cell* **85**, 513-523.

50. Rimer, M., Cohen, I., Burden, S.J., & McMahan, U.J. (1996) *Soc. for Neurosci.* **22**, 1689.

51. Altiok, N., Bessereau, J.L., Changeux, J.P. (1995) *EMBO J.* **14**, 4258-4566.

52. Chu, G. C., Moscoso, L. M., Sliwkowski, M. X. & Merlie, J. P. (1995) *Neuron* **14**, 329-339.

53. Jo, S.A., Zhu, X., & Burden, S.J. (1995) *Nature* **373**, 158-161.

54. Cohen, M.W., Jacobson, C., Godfrey, E.W., Campbell, K.P. & Carbonetto, S. (1995) *J.Cell Biol.* **129**, 1093-1101.

55. Martin, P.T., Kaufman, S.J., Kramer, R.H. and Sanes, J.R. (1996) *Dev. Biol.* **174**, 125-139.

Figure 3.1. Agrin induces AChR clustering. (A) and (B) are representative examples of control and *Torpedo* agrin-treated myotubes labeled with FITC-conjugated α-bungarotoxin, respectively. Note the presence of numerous AChR clusters following agrin treatment. For quantitation (C), the number of AChR clusters was determined and expressed per myotubes. Bar = 45 μm.

C)

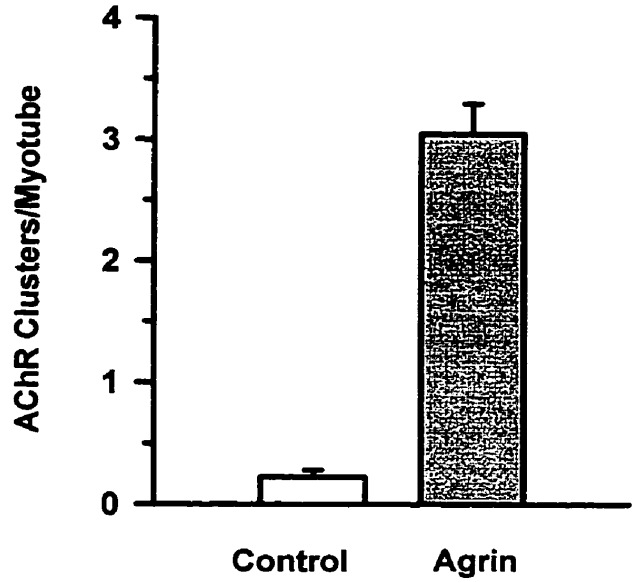


Figure 3.2. Utrophin protein levels are increased in agrin-treated myotubes. Myotubes were incubated with agrin purified from the electric organ of *Torpedo marmorata* for 48 hr. Cells were solubilized in RIPA buffer containing 0.1% SDS and protein extracts were then subjected to immunoblotting. The upper panel in (A) is a representative example of a blot showing utrophin levels in non-treated (CTL) vs agrin-treated myotubes. In the lower panel, the same membrane was subsequently striped and reprocessed for immunoblotting using the MF-20 antibody against sarcomeric myosin. Note the relative increase in utrophin following agrin treatment. (B) shows the result of an immunoblot performed using protein extracted from the initial pellet with RIPA buffer containing 5% SDS. Note that within this cellular fraction, utrophin levels were not affected by the agrin treatment.

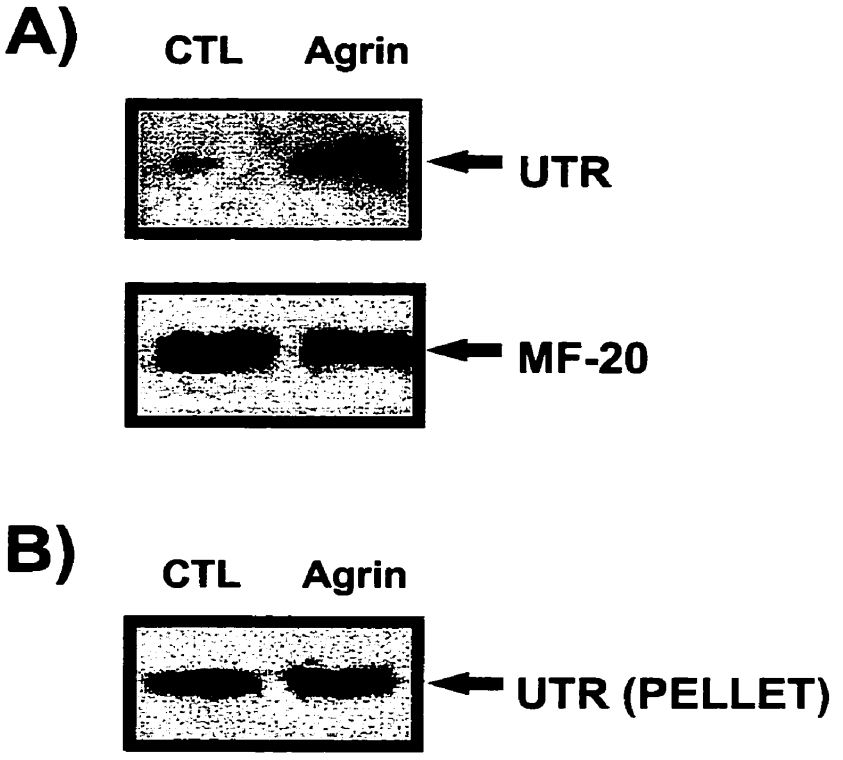

A)
CTL
Agrin
UTR
MF-20
B)
CTL
Agrin
UTR (PELLET)

Figure 3.3. Representative example of utrophin mRNA levels in control and agrin-treated cultured myotubes. Shown are examples of ethidium bromide-stained gels of RT-PCR products obtained from non-treated (CTL) vs agrin-treated myotubes. (A) and (B) show the effect of agrin on utrophin and dystrophin mRNA levels, respectively. Note the relative increase in utrophin mRNA levels following agrin treatment. (C) shows the level of utrophin mRNAs in control (CTL) and CGRP-treated (CGRP) myotubes. As shown, CGRP did not affect utrophin mRNA levels in these cultured myotubes. In all panels, the negative control lane is marked with a minus sign. The molecular mass of the PCR products is shown in bp.

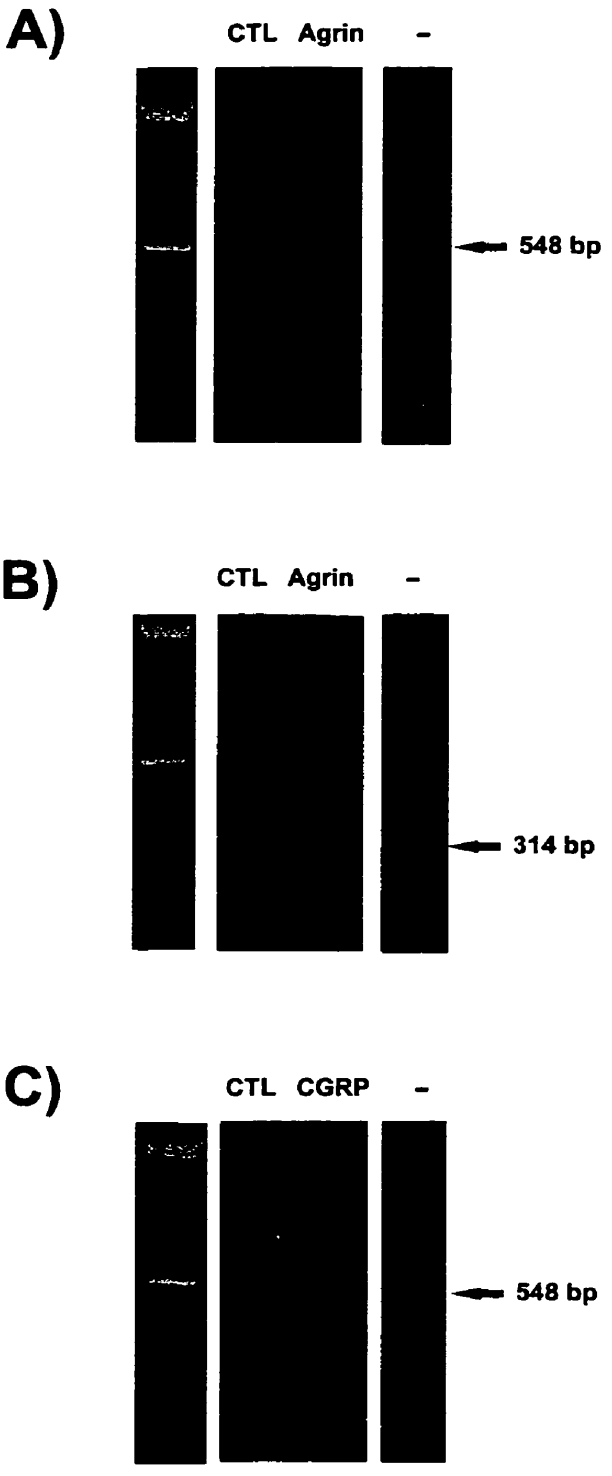

A)
CTL
Agrin
-
548 bp
B)
CTL
Agrin
-
314 bp
C)
CTL
CGRP
-
548 bp

Figure 3.4. Agrin increases utrophin transcript levels in cultured myotubes. Myotubes were incubated with either CGRP (C), purified *Torpedo* agrin (T), muscle- (0,0), or nerve-derived agrin (4,8) for 24 and 48 hr. Total RNA was extracted and subjected to RT-PCR. Utrophin transcript levels were determined and are expressed as percent of control, denoted by the hatched line. Shown are the results obtained with a minimum of 5 independent experiments. Asterisks denote significant differences from control levels ($P < 0.05$).

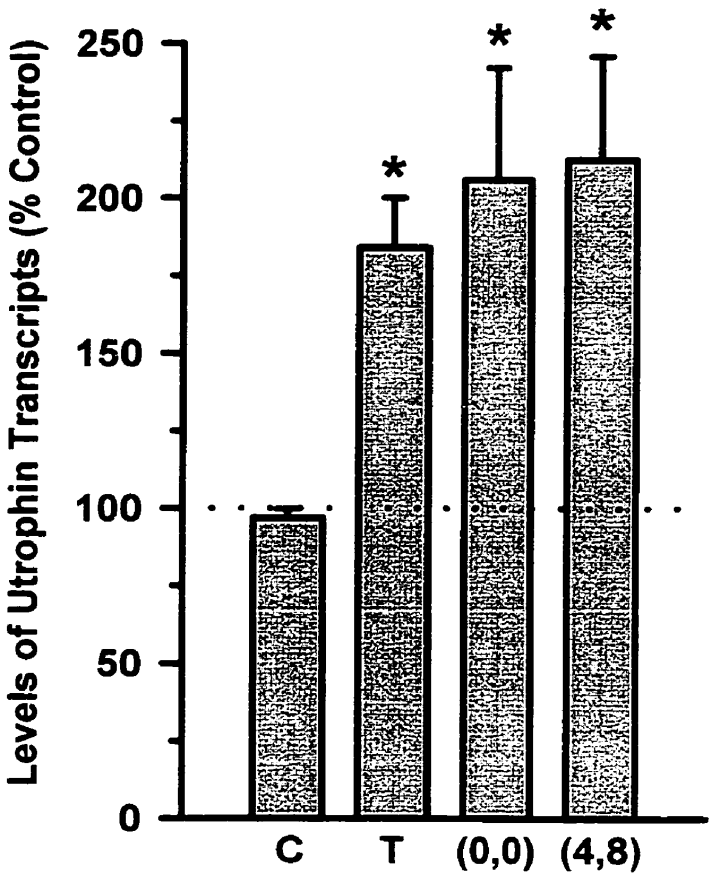

Levels of Utrophin Transcripts (% Control)
250
200
150
100
50
0
*
*
*
C
T
(0,0)
(4,8)

Figure 3.5. Utrophin promoter-reporter expression is increased in agrin-treated cultured myotubes. Human utrophin promoter fragments (1.3 or 0.5 kb) were inserted upstream of the reporter gene nlsLacZ and transfected in myoblasts. Myotubes were then incubated with agrin and 48 hr later, the levels of β-galactosidase activity were determined and normalized to CAT activity and protein content. Shown are the results of a minimum of 5 independent experiments. Symbols are 1.3, 1.3 kb promoter fragment; 0.5, 0.5 kb promoter fragment; (0,0), muscle-derived agrin; and (4,8), nerve-derived agrin. Data are presented as a percentage of the activity seen with the 1.3 kb promoter fragment which served as the control level. Note the increase in activity of the reporter gene following treatment with both isoforms of agrin. Asterisks denote significant differences from control levels ($P < 0.05$).

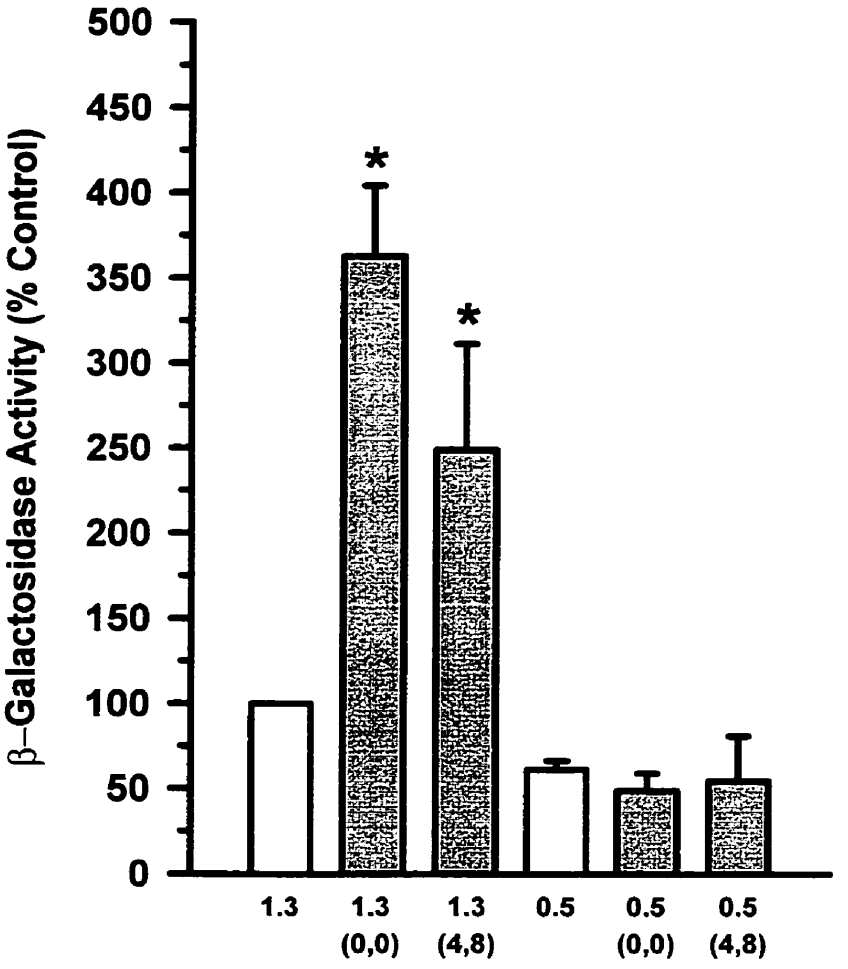

β–Galactosidase Activity (% Control)
500
450
400
350
300
250
200
150
100
50
0
*
*
1.3
1.3 (0,0)
1.3 (4,8)
0.5
0.5 (0,0)
0.5 (4,8)

Figure 3.6. Agrin increases the number of β-galactosidase-positive nuclei per culture dish. Since transfection efficiency did not significantly differ between cultures (see results), the number of β-galactosidase-positive nuclei were counted. Symbols are 1.3, 1.3 kb promoter fragment; 0.5, 0.5 kb promoter fragment; (0,0), muscle-derived agrin; and (4,8), nerve-derived agrin. Note the 2- to 3-fold increases seen in the number of positive nuclei following transfection with the construct containing the 1.3 kb utrophin promoter fragment and treated with agrin. Shown are the results of a minimum of 6 independent experiments. Asterisks denote significant differences from levels seen with the 1.3 kb fragment ($P < 0.05$).

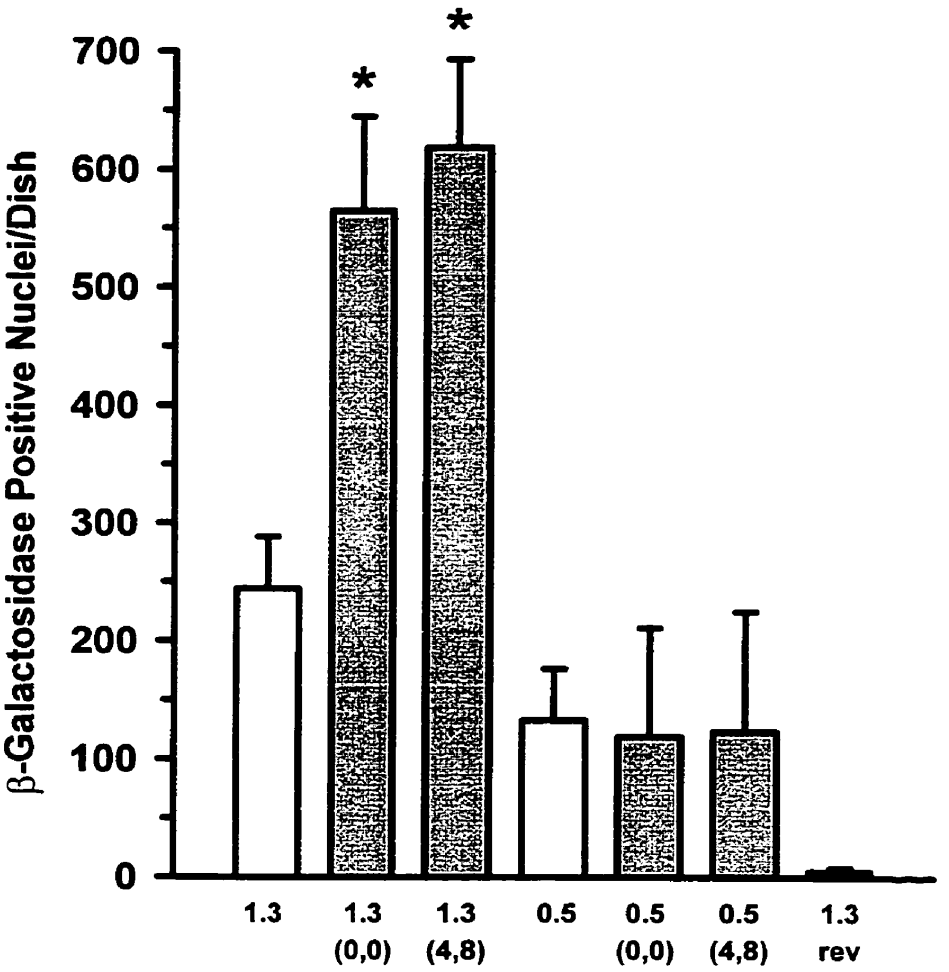

β-Galactosidase Positive Nuclei/Dish
700
600
500
400
300
200
100
0
*
*
1.3
1.3 (0,0)
1.3 (4,8)
0.5
0.5 (0,0)
0.5 (4,8)
1.3 rev

Figure 3.7. The N-box motif is critical for mediating the response to agrin. Human utrophin promoter constructs (wild-type 1.3 kb or N-box mutants N5 and N34) were inserted upstream of the reporter gene nlsLacZ and transfected in myoblasts. Myotubes were then incubated with agrin and 48 hr later, the levels of β-galactosidase activity were determined and normalized to CAT activity and protein content. Note that the increase in the activity of the reporter gene driven by the 1.3 kb utrophin promoter fragment following treatment with both muscle ($C\text{-}Ag_{12,0,0}$) and neural ($C\text{-}Ag_{12,4,8}$) isoforms of agrin is abolished in myotubes transfected with constructs containing the N-box mutants (N5 and N34). Shown are the results of a minimum of 5 independent experiments. Asterisks denote significant differences from control levels ($P < 0.05$).

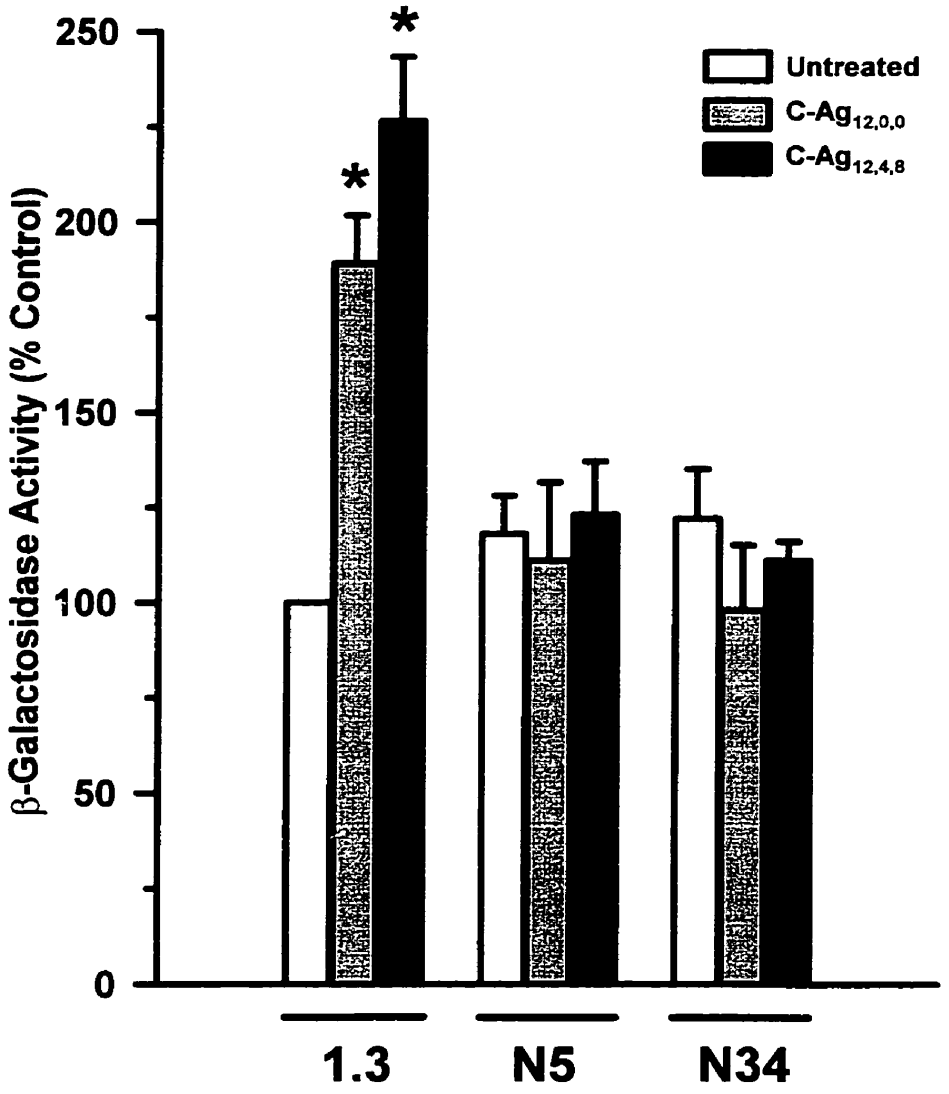

β-Galactosidase Activity (% Control)
250
200
150
100
50
0
*
*
Untreated
C-Ag12,0,0
C-Ag12,4,8
1.3
N5
N34

Figure 3.8. The N-box motif is responsible for synaptic expression of the utrophin gene *in vivo* . Note that mutating the N-box significantly reduced the percentage of synaptic events as determined by comparing the location of clusters of blue myonuclei with the presence of neuromuscular junctions identified by AChE histochemistry. Shown are the results obtained with a minimum of 17 injected muscles per construct. Asterisks denote significant differences from the levels seen with the wild-type 1.3 kb utrophin promoter fragment ($P < 0.05$).

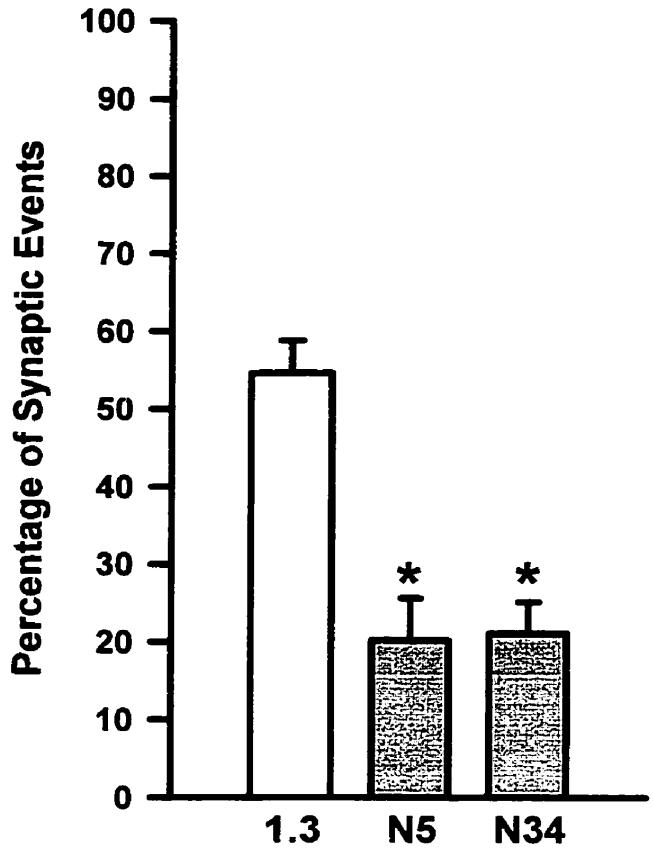
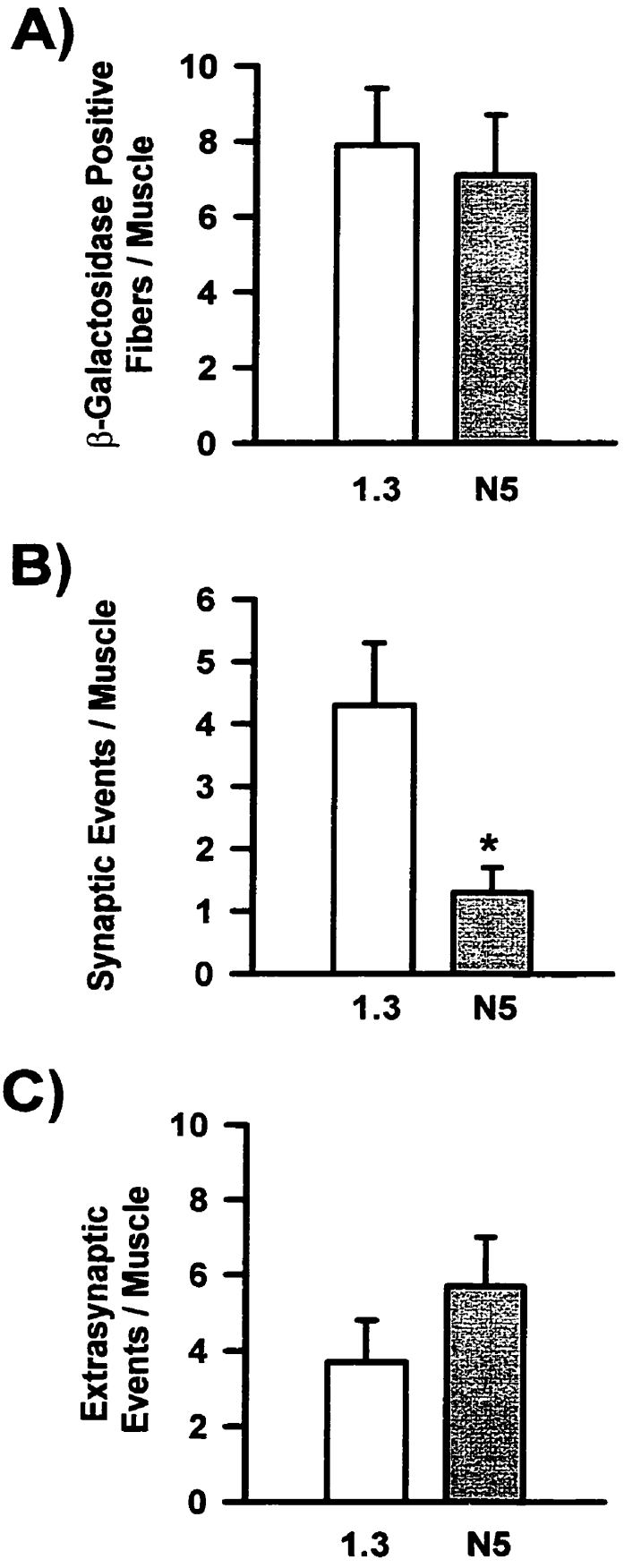

Percentage of Synaptic Events
100
90
80
70
60
50
40
30
20
10
0
*
*
1.3
N5
N34

Figure 3.9. The N-box motif increases expression of the utrophin gene in synaptic regions of muscle fibers. (A) shows the absolute number of β-galactosidase-positive fibers per muscle injected with constructs containing either the wild-type 1.3 kb utrophin promoter fragment or the N-box mutant N5. (B) represents the number of synaptic events per muscle for each construct. Note that the amount of synaptic events was significantly decreased in the N5 mutant-injected muscles. Conversely, there was no difference in the number of extrasynaptic events per muscle between muscles injected with the wild-type 1.3 kb promoter fragment and the N5 mutant (C). Shown are the results obtained with a minimum of 17 injected muscles per construct. Asterisk denotes a significant difference between the two constructs ($P < 0.05$).


A)
β-Galactosidase Positive Fibers / Muscle
10
8
6
4
2
0
1.3
N5
B)
Synaptic Events / Muscle
6
5
4
3
2
1
0
*
1.3
N5
C)
Extrasynaptic Events / Muscle
10
8
6
4
2
0
1.3
N5

CHAPTER 4

INDUCTION OF UTROPHIN GENE EXPRESSION BY HEREGULIN IN SKELETAL MUSCLE CELLS: ROLE OF THE N-BOX MOTIF AND GABP

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For this paper, A. Gramolini performed all experimental procedures, with the exception of the gel shift assay. The manuscript was written by A. Gramolini and Dr. Jasmin. L. Angus performed the electrophoresis mobility shift assay. Drs. L. Schaeffer and J.P. Changeux supplied antibodies to GABP, cDNAs encoding GABP and heregulin, and provided comments on the manuscript. Drs. J. Tinsley, E. Burton and K. Davies supplied the utrophin promoter-reporter constructs (see Chapters 3 and 4).

ABSTRACT

The modulation of utrophin gene expression in muscle by the nerve-derived factor agrin plausibly involves the trophic factor ARIA/heregulin. Here we show that heregulin treatment of mouse and human cultured myotubes caused a ~2.5-fold increase ($P < 0.05$) in utrophin mRNA levels. Transient transfection experiments with utrophin promoter-reporter gene constructs showed that this increase resulted from an enhanced transcription of the utrophin gene. In the case of the nicotinic acetylcholine receptor δ- and ε-subunit genes, heregulin was previously reported to stimulate transcription via a conserved promoter element, the N-box, which binds the multimeric ETS-related transcription factor GA-binding protein (GABP). Accordingly, site-directed mutagenesis of a single N-box motif in the utrophin promoter abolished the transcriptional response to heregulin. In addition, overexpression of heregulin or of the two GABP subunits in cultured myotubes caused an N-box dependent increase in utrophin promoter activity. *In vivo*, direct gene transfer into muscle fibers confirmed that heregulin regulates utrophin gene expression. Finally, electrophoretic mobility shift assays and supershift experiments performed with muscle extracts revealed that the N-box of the utrophin promoter binds GABP. These findings suggest that the subsynaptic activation of transcription by heregulin via the N-box motif and GABP are conserved among genes expressed at the neuromuscular junction. Because utrophin can functionally compensate for the lack of dystrophin, the elucidation of the molecular mechanisms regulating utrophin gene transcription may ultimately lead to therapies based on utrophin expression throughout the muscle fibers of Duchenne muscular dystrophy patients.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most prevalent primary myopathy since it affects approximately 1 out of every 3,500 male births (1). The disease is characterized by repeated cycles of muscle degeneration/regeneration with an eventual failure to regenerate leading to the replacement of muscle fibers by fat and connective tissues. DMD progresses rapidly since patients are functionally impaired before their teen years and death usually occurs in the second or third decade of life most often as a result of respiratory or cardiac failure. The genetic defect underlying DMD was simultaneously mapped to chromosome Xp21 in different laboratories (for review, see Refs. 2,3). The gene responsible for this disease was termed dystrophin, and it codes for a large cytoskeletal protein known to accumulate at the sarcolemma of muscle fibers. Mutations and/or deletions of this gene as seen in DMD, lead to an absence of full-length dystrophin thereby making muscle fibers extremely fragile to the effects of repetitive cycles of muscle contraction and relaxation. Although several therapeutic strategies have been envisaged including dystrophin gene replacement and pharmacological interventions (4-6), there is currently no cure available for DMD.

Several years ago, an autosomal homologue to dystrophin was identified (7). This gene, now referred to as utrophin, also codes for a large cytoskeletal protein (8). In contrast to the homogeneous distribution of dystrophin along muscle fibers, utrophin preferentially accumulates within the postsynaptic domain of the neuromuscular junction in both normal and DMD muscle fibers (9-12). Because of the high degree of sequence similarity between dystrophin and utrophin as well as their ability to both bind a group of proteins referred to as the dystrophin-

associated proteins (13), it has been suggested that increased expression of utrophin into extrasynaptic regions of dystrophic muscle fibers may represent an alternate therapeutic strategy for DMD (14,15). Recent studies using transgenic mouse model systems have clearly demonstrated that expression of utrophin throughout muscle fibers can indeed functionally compensate for the lack of dystrophin and hence, prevent the muscle pathology (16-18). It thus becomes important to elucidate the molecular and cellular mechanisms presiding over utrophin expression at the neuromuscular junction in order to ultimately modulate specific regulatory events which could therefore lead to expression of the endogenous gene product along the length of dystrophic muscle fibers.

In a recent series of studies, we began to examine the role of the nerve in regulating utrophin expression at the neuromuscular junction. Initially, we showed that local transcriptional activation of the utrophin gene in myonuclei located within the postsynaptic sarcoplasm accounts for the synaptic localization of utrophin (19). We have next examined the contribution of specific nerve-derived trophic factors in the regulation of utrophin in muscle cells and showed that agrin induced the expression of utrophin in cultured muscle cells via a transcriptional regulatory mechanism (20). ARIA/heregulin, another nerve-derived trophic factor, is known to regulate acetylcholine receptor (AChR) subunit genes (see for review Refs. 21,22). In addition, agrin is though to stimulate transcription of the AChR gene via heregulin. In the present study, we demonstrate that heregulin modulates utrophin gene expression and analyze the molecular mechanisms of this regulation in cultured myotubes and muscle fibers *in vivo*.

EXPERIMENTAL PROCEDURES

Tissue Culture. Mouse C2 muscle cells were cultured as described previously (20). Normal human skeletal muscle cells were obtained from Clonetics-BioWhittaker Inc. (San Diego, California), and they were grown and maintained according to the supplier's recommendations. Three- to five-day old myotubes were treated with 3 or 30 nM heregulin (kindly supplied by M. Sliwkowski, Genentech Inc., San Francisco, CA) for 48 hours.

RNA extraction, Reverse Transcription and Polymerase Chain Reaction. Total RNA was extracted from samples using Tripure as recommended by the manufacturer (Boehringer Mannheim Corp., Indianapolis, USA). The RNA concentration was determined using a GeneQuant II RNA/DNA spectrophotometer (Pharmacia, Quebec, Canada) and the samples were rediluted to a final concentration of 50 ng/µl. Only 2 µl (100 ng of total RNA) of this dilution was used for reverse-transcription and amplification with the polymerase chain reaction (RT-PCR).

RT-PCR analysis was performed in order to strictly determine the relative abundance of transcripts under different experimental conditions. Utrophin cDNAs were specifically amplified using primers synthesized on the basis of available sequences for human (10) and mouse (20) cDNAs. PCR experiments were performed as described elsewhere (20). Typically, 30 to 36 cycles of amplification were performed since control experiments showed that these cycle numbers were within the linear range of amplification. Following amplification, PCR products were separated on ethidium bromide-stained agarose gels and the size of the products was

estimated using the 100 bp molecular mass marker (Gibco, BRL). For quantitative assays, the PCR products were separated on Vistragreen (Amersham Corp.)-containing gels and the fluorescent intensity of the products which is linearly related to the amount of DNA, was quantitated using a Storm PhosphorImager (Molecular Dynamics; Sunnyvale, CA) and analyzed using the accompanying ImageQuant software. In these assays, we verified that equal amounts of total RNA were indeed used for each sample by monitoring from the same RT mixtures, the abundance of either S12 rRNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (see Gramolini *et al.*, 1999).

Expression of Utrophin Promoter-Reporter Gene Constructs. In these experiments, we used the same human utrophin promoter-reporter gene constructs that we recently described (19,20). Specifically, we used the 1.3 kb utrophin promoter fragment and the N5 N-box mutant. These promoter fragments were inserted upstream of the reporter gene LacZ and a nuclear localization signal (nls). In addition, we used plasmids containing the heregulin β cDNA driven by the CMV promoter (kindly supplied by M. Sliwkowski, Genentech Inc., San Francisco, CA), and the GABP α and β cDNAs placed downstream of the MSV promoter (24). Plasmid DNA was prepared using the Qiagen Mega-Prep procedure (Chatsworth, CA).

C2 myoblasts were transfected with 3 μg of the appropriate utrophin promoter-reporter gene construct using the Mammalian Transfection System-Calcium Phosphate kit (Promega; Madison, WI). Once the cultures became confluent, the media was switched to the differentiation media and treated with heregulin as described above. Forty-eight hours later, cells were harvested into 300 μl of 1X Reporter Lysis buffer (Promega, Madison, WI) and freeze-

thawed twice. After centrifugation, the supernatants were collected and the level of β-galactosidase activity was determined using a luminescent assay (Luminescent β-gal Enzyme Kit; Clonetech) and normalized to a cotransfected chloramphenicol acetyltransferase (CAT) plasmid (Promega) and protein content. CAT activity was determined using a CAT Enzyme Assay system (Promega) while protein content was determined by the bicinchoninic acid method (Pierce Laboratories; Rockford, IL).

For direct gene transfer into mouse tibialis anterior (TA) muscles, experiments were performed as described previously (19,20,25-27). Briefly, 25 μl of DNA solution was injected directly into TA muscles of 4 week-old mice. Muscles were excised 2 weeks later, frozen in liquid nitrogen and homogenized in 500 μl of 1X Reporter Lysis buffer (Promega) using a Polytron. After centrifugation, the supernatants were collected and the activities of β-galactosidase and CAT were determined as described above.

Muscle Extracts and Electrophoretic Mobility Shift Assays. Muscle extracts were prepared as described previously (26). Electrophoretic mobility shift assays (EMSA) were performed using ^{32}P-labelled probes encompassing the utrophin N-box region (sense: 5'-GGCTGATCTTCCGGAACAAAGT-3 and antisense: 5'-ACTTTGTTCCGGAAGATCAGCC-3'). The binding reaction mixture included 0.2 ng labelled probes, 1.0 μg of poly (dI-dC) and 20 μg of muscle extract and was incubated for 30 minutes on ice prior to electrophoresis in a 5% polyacrylamide gel. The specificity of the binding reaction was assessed by adding a 50- and 500-fold molar excess of unlabeled probe in the reaction mixture. For the supershift assays, antibodies to GABPα and GABPβ were kindly provided by Dr. Steve McNight (Tularik Inc., San

Francisco CA). These antibodies were added to the reaction mixture for 20 min on ice after the initial 30 min incubation and prior to electrophoresis.

RESULTS

In a first set of experiments, we examined whether heregulin increased utrophin gene expression in cultured myotubes. In comparison to untreated cultures, we found that the levels of utrophin transcripts were increased by heregulin treatment (Figure 4.1A). In fact, the abundance of utrophin mRNA was approximately 2.5-fold higher ($P < 0.05$) following treatment with either 3 or 30 nM heregulin (Figure 4.1B). By contrast, treatment of myotubes with EGF, PDGF, IGF-I or IGF-II which are known to influence expression of muscle proteins in tissue culture (28), failed to alter expression of utrophin transcripts (data not shown). Consistent with our results obtained with mouse muscle cells, we also noted that treatment of human myotubes with either 3 or 30 nM heregulin also led to a ~2-fold increase in utrophin mRNA levels (Figure 4.2).

To determine whether the increase in utrophin transcripts following heregulin treatment resulted from enhanced transcriptional activation of the utrophin gene, we next transfected C2 myoblasts with plasmids containing the reporter gene LacZ driven by the 1.3 kb wildtype utrophin promoter or its N-box mutated counterpart (see N5 mutant construct in Gramolini *et al.* 1998), and treated myotubes for 48 hours with heregulin. As illustrated in Figure 4.3A, we observed a significant increase ($P < 0.05$) in the expression of β-galactosidase in cells transfected with the construct containing the wildtype promoter fragment and treated with heregulin. However, expression of the reporter gene was not affected following heregulin treatment in cultures transfected with constructs containing the N5-mutated utrophin promoter fragment. Similarly, cotransfection of C2 cultures with plasmids containing the heregulin or both subunits

of GABP cDNAs driven by constitutive promoters and the wildtype utrophin promoter fragment induced an increase in the expression of the reporter gene (Figure 4.3B). In parallel cultures transfected with plasmids containing the N-box mutant promoter construct, overexpression of heregulin or GABP α and β failed to affect expression of β-galactosidase.

To verify that similar regulatory mechanisms could contribute to the regulation of the utrophin gene *in vivo* , we performed a series of experiments in which plasmid DNA was directly injected into mouse TA muscles. In comparison to injection of the 1.3 kb utrophin promoter–reporter gene constructs, co-injection with a plasmid containing the heregulin cDNA constitutively expressed led to a ~2-fold increase ($P < 0.05$) in the expression of β-galactosidase (Figure 4.4A). In these experiments, we also examined the contribution of GABP α and β in the control of utrophin gene expression *in vivo* . As shown in Figure 4.4A, co-injection of the wildtype utrophin promoter construct with plasmids constitutively overexpressing GABP α and β also induced a significant increase ($P < 0.05$) in the activity of the reporter gene.

Finally, to confirm the binding of GABP to the N-box present in the utrophin promoter, we performed a series of EMSA using muscle nuclear extracts. In these experiments, we observed specific protein binding activity which could be competed by an excess of unlabeled oligonucleotides (Figure 4.4B). Furthermore, this binding activity was supershifted by incubating the reaction mixtures with antibodies against GABP α or β thereby confirming the involvement of these subunits from an Ets-related protein in the transcriptional regulation of the utrophin gene.

DISCUSSION

Recently, we showed that maintenance of high levels of utrophin at the neuromuscular junction involved the local transcriptional regulation of the utrophin gene in myonuclei of the postsynaptic sarcoplasm (19,20). In addition, we demonstrated in these initial studies, the important contribution of the nerve in maintaining utrophin expression since induction of ectopic synapses at sites distant from the original neuromuscular junctions resulted in the appearance of utrophin at these newly formed synaptic contacts (19). Since expression of utrophin is largely insensitive to nerve-evoked electrical activity (29,30), we postulated that expression of utrophin in muscle is strictly positively regulated by nerve-derived trophic factors (15). In the present study, we now show that heregulin, which is concentrated at the neuromuscular junction (see 21), can increase utrophin gene expression in cultured muscle cells as well as in muscle fibers *in vivo*

.

It is well established that ARIA/neuregulin/heregulin exerts a profound influence on expression of the AChR subunit genes in myogenic cells maintained in culture (for review see Refs. 21,22). Such observation has in fact led to the notion that the release of this molecule from nerve terminals and its subsequent interaction with ErbB receptors located on the postsynaptic membrane of the neuromuscular junction, trigger a signaling cascade that culminates in the local activation of specific AChR subunit genes within myonuclei of the postsynaptic sarcoplasm (31-33). Until recently, there was a clear lack of information on the nature of the signaling pathway involved in this trans-synaptic control of gene expression. However, promoter analysis has led to the identification of a DNA element termed N-box, that is critical for directing the synapse-

specific expression of AChR δ and ε subunit genes (26,27). Additional studies have shown that the N-box plays a central role in the transcriptional activation of AChR genes by heregulin. These studies demonstrated that the response to heregulin involved binding of Ets transcription factors to the N-box (24,34). The candidate factor implicated in this regulation was shown to be the multimeric Ets-related factor GABP (24), a finding recently confirmed by Fromm and Burden (35). Interestingly, it was also shown that the Ras/MAP kinase pathway, through which heregulin stimulates AChR gene transcription (36,37), controls the N-box dependent response to heregulin and modulates phosphorylation of GABP (24). Taken together, these data are consistent with a model whereby selective expression of AChR subunit genes at the neuromuscular junction is achieved via interaction of ARIA/neuregulin/heregulin with ErbB receptors which in turn, results in the transactivation of AChR subunit promoters through Ets-related transcription factors binding to the N-box motif. Our current results showing that heregulin and GABP α and β increase utrophin gene expression in muscle cells via the N-box, are therefore entirely consistent with this model. A conserved mechanism involving the N-box and GABP may thus regulate the expression of multiple synapse-specific genes at the level of the fundamental nuclei.

In a recent study, we determined that treatment of myogenic cells in culture with agrin increased the expression of utrophin via a transcriptional regulatory mechanism involving the N-box (20). However, the exact nature of the regulatory events underlying this increase in utrophin expression remained unclear. In this context, it is noteworthy that agrin treatment has also been shown to induce the transcriptional activation of the AChR ε subunit gene in cultured muscle cells (38,39). Interestingly, Brenner and colleagues have recently deciphered some of

the key steps involved in this regulatory mechanism since they showed that agrin treatment acted by first inducing the local accumulation of muscle-derived ARIA/neuregulin/heregulin and its ErbB tyrosine kinase receptors on the surface of myotubes which led subsequently to a transcriptional activation of the AChR ε subunit gene via an autocrine/paracrine pathway (39). Based on our current findings showing, in particular, that overexpression of heregulin in muscle cells increases utrophin gene expression, it seems likely therefore that a similar mechanism accounts for the increase in utrophin expression following agrin treatment.

Recent studies performed with transgenic mouse model systems have revealed that an increase in the expression of utrophin in extrasynaptic compartments of dystrophic muscle fibers could prevent the occurrence of the muscle pathology (16-18) thereby indicating that upregulation of utrophin is indeed a viable approach for treating DMD. Therefore, the results demonstrating that both agrin and heregulin can modulate expression of the utrophin gene in myogenic cells in culture (this study; and Ref. 20) as well as in muscle fibers *in vivo* (this study; and Refs. 41,42) have definite implications for the treatment of DMD since they offer the possibility of pharmacologically stimulating the signaling cascade that links membrane events to alterations in utrophin gene expression. In this context, our current results showing that heregulin treatment increased expression of utrophin transcripts not only in mouse muscle cells but also in human myotubes, is particularly relevant since they now provide the necessary basis to begin designing specific pharmacological interventions in a clinically relevant system.

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THESIS REVISIONS

Additional analyses of variance (ANOVA) statistical tests were performed on the data for Figures 4.1B, 4.3, and 4.4A. With these analyses, all sets of experiments showed significant differences ($P<0.05$).

REFERENCES

1. Emery, A. (1991) *Neuromusc. Disord.* **1**, 19-29.

2. Sadoulet-Puccio, H.M. & Kunkel, L.M. (1996) *Brain Pathol.* **6**, 25-35.

3. Worton, R. (1995) *Science* **270**, 755-760.

4. Ahn, A.H. & Kunkel, L.M. (1993) *Nature Genet.* **3**, 283-291.

5. Khan, M.A. (1993) *J. Neurol. Sci.* **120**, 8-14.

6. Matsumura, K. & Campbell, K.P. (1994) *Muscle Nerve* **17**, 2-15.

7. Love, D.R., Hill, D.F., Dickson, G., Spurr, N.K., Byth, B.C., Marsden, R.F., Walsh, F.S., Edwards, Y.H. & Davies, K.E. (1989) *Nature* **339**, 55-58.

8. Tinsley, J.M., Blake, D.J., Roche, A., Fairbrother, U., Riss, J., Byth, B.C., Knight, A.E., Kendrick-Jones, J., Suthers, G.K., Love, D.R., Edwards, Y.H., & Davies, K.E. (1992) *Nature* **360**, 591-593.

9. Fardeau, M. Tome, F.M., Collin, H., Augier, N., Pons, F., Leger, J., & Leger, J. (1990) *Compt. Rend. Acad.Sci.* **311**, 197-294

10. Khurana, T.S., Watkins, S.C., Chafey, P., Chelly, J., Tome, F.M., Fardeau, M., Kaplan, J. & Kunkel, L.M. (1991) *Neuromusc. Disord.* **1**, 185-194.

11. thi Man, N., Ellis, J.M., Love, D.R., Davies, K.E., Gatter, K.C., Dickson, G., & Morris, G.E. (1991) *J. Cell Biol.* **115**, 1695-1700.

12. Ohlendieck, K., Ervasti, J.M., Matsumura, K., Kahl, S.D., Leveille, C.J., & Campbell, K.P. (1991) *Neuron* **7**, 499-508.

13. Matsumura, K., Ervasti, J.M., Ohlendieck, K., Kahl, S.D., & Campbell, K.P. (1992) *Nature* **360**, 588-591.

14. Blake, D.J., Tinsley, J.M., & Davies, K.E. (1996) *Brain Pathol.* **6**, 37-47.

15. Gramolini, A.O. & Jasmin, B.J. (1998) *Neuromusc. Disord.* **8**, 351-361.

16. Tinsley, J.M., Potter, A.C., Phelps, S.R., Fisher, R., Trickett, J.I., & Davies, K.E. (1996) *Nature* **384**, 349-353.

17. Deconinck, N., Tinsley, J.M., DeBacker, F., Fisher, R., Kahn, D., Phelps, S., Davies, K., & Gillis, J.M. (1997) *Nature Med.* **3**, 1216-1221.

18. Tinsley, J., Deconinck, N., Fisher, R., Kahn, D., Phelps, S., Gillis, J.M., & Davies K.E. (1998) *Nature Med.* **4**, 1441-1444.

19. Gramolini, A.O., Dennis, C.L., Tinsley, J.M., Roberston, G.S., Cartaud, J., Davies, K.E., & Jasmin, B.J. (1997) *J. Biol. Chem.* **272**, 8117-8120.

20. Gramolini, A.O., Burton, E.A., Tinsley, J.M., Ferns, M.J., Cartaud, A., Cartaud, J., Davies, K.E., Lunde, J.A., & Jasmin, B.J. (1998) *J. Biol. Chem.* **273**, 736-743.

21. Duclert, A. & Changeux, J.P. (1995) *Physiol. Rev.* **75**, 339-368.

22. Burden, S.J. (1998) *Genes Dev.* **12**, 133-148.

23. Gramolini, A.O., Karpati, G., & Jasmin, B.J. (1999) *J.Neuropath.Exp. Neurol.* (In Press)

24. Schaeffer, L., Duclert, N., Huchet, M., & Changeux, J.P. (1998) *EMBO J.* **17**, 3078-3090.

25. Duclert, A., Savatier, N. & Changeux, J.P. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3043-3047.

26. Koike S., Schaeffer, L., & Changeux, J.P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 10624-10628.

27. Duclert, A., Savatier, N., Schaeffer, L., & Changeux, J.P. (1996) *J. Biol. Chem.* **271**, 17433-17438.

28. Husmann I., Soulet L., Gautron J., Martelly I., & Barritault D. (1996) *Cytokine Growth Factor Rev.* **7**, 249-258.

29. Jasmin, B.J., Alameddine, H., Lunde, J.A., Stetzkowksi-Marden, F., Collin, H., Tinsley, J.M., Davies, K.E., Tome, F.M.S., Parry, D.J. & Cartaud, J. (1995) *FEBS* **374**, 393-398.

30. Biral, D., Senter, L., & Salviati, G. (1996) *J. Muscle Res. Cell Motil.* **17**, 523-532.

31. Altiok, N., Bessereau, J., & Changeux, J.P. (1995) *EMBO J.* **14**, 4258-4266.

32. Ahn Jo, S., Zhu, X., Marchionni, M.A., & Burden, S.J. (1995) *Nature* **373**, 158-161.

33. Moscoso L.M., Chu G.C., Gautam M., Noakes P.G., Merlie J.P., & Sanes, J.R. (1995) *Dev. Biol.* **172**, 158-169.

34. Fromm, L. & Burden, S.J. (1998) *Genes Dev* **12**, 3074-3083.

35. Sapru, M., Florence, S.K., Kirk, C., & Goldman, D. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 1289-1294.

36. Tansey, M.G., Chu, G.C., & Merlie, J.P. (1996) *J. Cell Biol.* **134**, 465-476.

37. Jones, G., Herczeg, A., Ruegg, M.A., Lichtsteiner, M., Kröger, S., & Brenner, H.R. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 5985-5990.

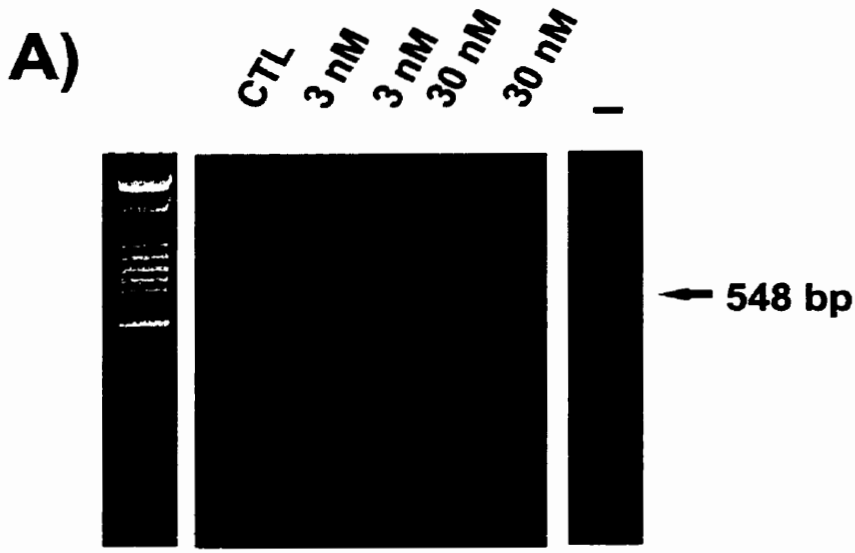
38. Jones, G., Lichtsteiner, M., Witzemann, V., Sakmann, B., & Brenner, H.R. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 2654-2659.

39. Meier, T., Masciulli, F., Moore, C., Schoumacher, F., Eppenberger, U., Denzer, A.J., Jones, G., & Brenner, H.R. (1998) *J. Cell Biol.* **141**, 715-726.

40. Meier, T., Hauser, D.M., Chiquet, M., Landman, L., Ruegg, M.A. & Brenner, H.R. (1997) *J. Neurosci.* **17**, 6534-6544.

41. Cohen I., Rimer M., Lomo T. & McMahan U.J. (1997) *Mol. Cell. Neurosci.* **9**, 237-253.

Figure 4.1. Heregulin increases utrophin mRNA levels in cultured myotubes. (A) is a representative example of an ethidium bromide-stained gel of utrophin PCR products (548 bp) obtained from non-treated (control; CTL) versus heregulin-treated (3 or 30 nM) mouse myotubes. Note the increase in utrophin mRNA levels following heregulin treatment. The negative control lane is marked with a minus sign. The left panel is the 100 bp marker (Gibco BRL). (B) shows quantitative analysis of utrophin mRNA levels in control and heregulin-treated myotubes. Utrophin transcript levels are expressed as a percent of control. Asterisks denote significant differences from control (CTL) levels (student's t-test, $P < 0.05$).


A)
CTL
3 nM
3 nM
30 nM
30 nM
–
548 bp

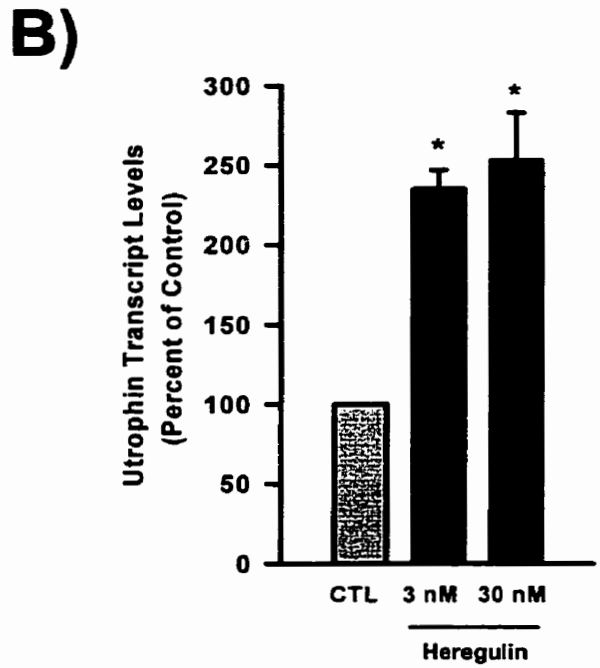

B)
Utrophin Transcript Levels
(Percent of Control)
300
250
200
150
100
50
0
*
*
CTL
3 nM
30 nM
Heregulin

Figure 4.2. Heregulin increases utrophin mRNA levels in primary cultures derived from human skeletal muscle. Representative example of an ethidium bromide-stained gel of utrophin PCR products (410 bp) obtained from non-treated (control; CTL) versus heregulin-treated (3 and 30 nM) human myotubes. Note the increase in utrophin mRNA levels following heregulin treatment. The negative control lane is marked with a minus sign. The left panel is the 100 bp marker (Gibco BRL).

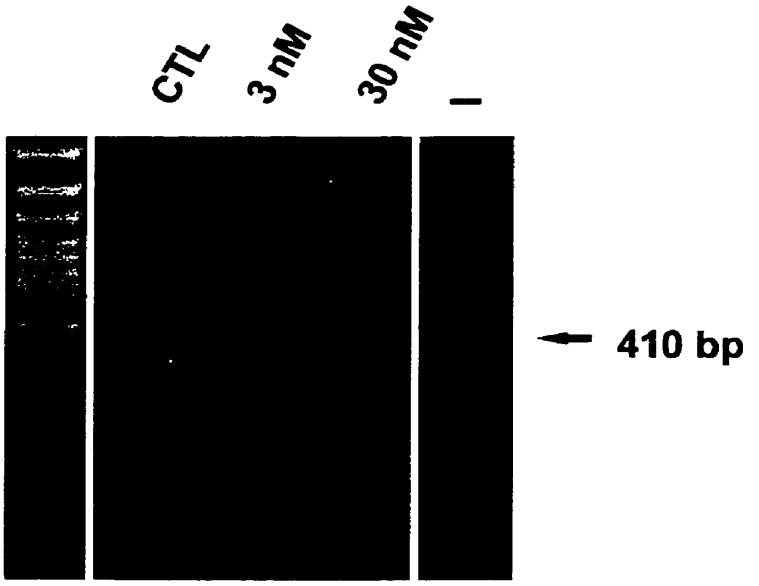

CTL
3 nM
30 nM
–
410 bp

Figure 4.3. Heregulin and the transcription factor, GABP, increase utrophin promoter activity in culture. (A) Mouse myotubes transfected with plasmids containing human utrophin promoter fragments (either the 1.3 kb wildtype or the N5 mutant; see Gramolini *et al.*, 1998) and the reporter gene LacZ were treated with heregulin. Note the increase in activity in cultures transfected with the wildtype utrophin promoter fragment. (B) Cotransfection of the utrophin wildtype or N5 mutant promoter fragments with cDNAs encoding heregulin or GABP α and β. Note the increase in activity of the reporter gene driven by the 1.3 kb wildtype promoter following overexpression of heregulin or GABP α and β. For all these experiments, the levels of β-galactosidase activity were determined and normalized to CAT and protein content. Asterisks denote significant differences from control levels (student's t-test, $P < 0.05$).

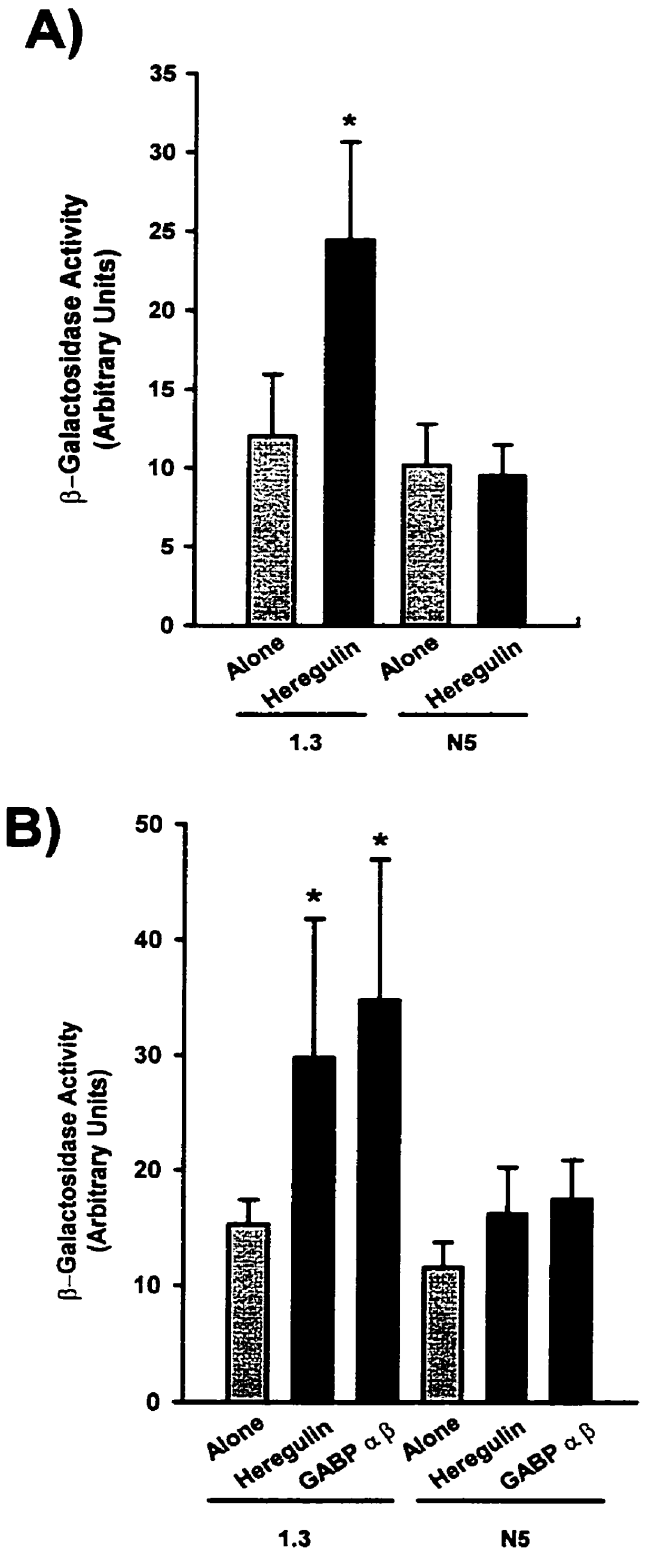
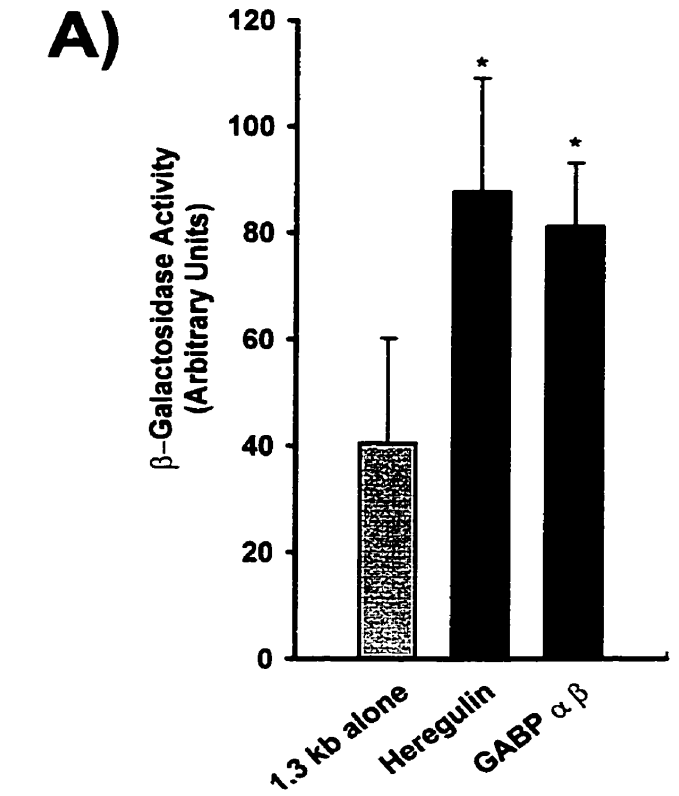
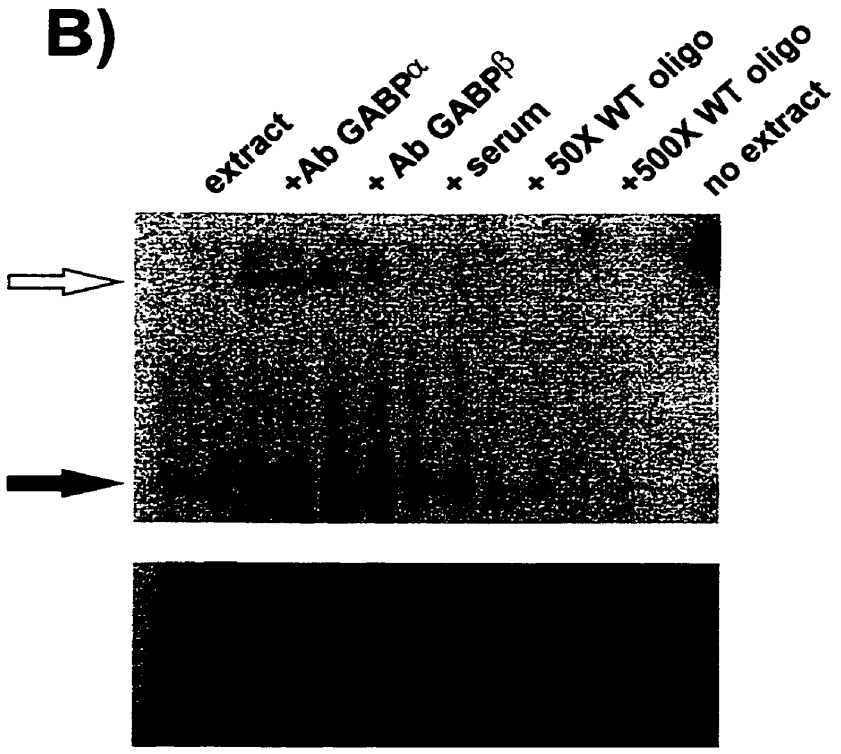

A)
β-Galactosidase Activity (Arbitrary Units)
35
30
25
20
15
10
5
0
*
Alone
Heregulin
Alone
Heregulin
1.3
N5
B)
β-Galactosidase Activity (Arbitrary Units)
50
40
30
20
10
0
*
*
Alone
Heregulin
GABP αβ
Alone
Heregulin
GABP αβ
1.3
N5

Figure 4.4. Ectopic overexpression of heregulin or GABP α and β increases expression of the wildtype utrophin promoter-reporter gene construct. (A) Mouse TA muscles were coinjected with plasmids containing the wildtype human utrophin promoter fragment along with plasmids encoding heregulin or GABP α and β, and the level of β-galactosidase activity was determined two weeks later and normalized to CAT and protein content. Asterisks denote significant differences from control levels (student's t-test, $P < 0.05$). (B) The Ets-related transcription factor GABP binds to the N-box motif contained within the utrophin promoter. DNA-binding activity to the N-box motif (black arrow) was detected using EMSA and extracts from TA muscles. This band was competed by incubation with either 50 or 500 M excess of wildtype (WT) unlabeled probe. In addition, this band was supershifted (white arrow) by an additional incubation with antibodies against either GABP α and β, but not by incubation with the pre-immune serum. Lower panel shows the unbound radioactivity.


A)
β-Galactosidase Activity
(Arbitrary Units)
120
100
80
60
40
20
0
*
*
1.3 kb alone
Heregulin
GABP α β


B)
extract
+Ab GABPα
+ Ab GABPβ
+ serum
+ 50X WT oligo
+500X WT oligo
no extract

CHAPTER 5

DISCORDANT EXPRESSION OF UTROPHIN AND ITS TRANSCRIPT IN HUMAN AND MOUSE SKELETAL MUSCLES

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A. Gramolini performed all of the experimental procedures. The manuscript was written by A. Gramolini and Dr. Jasmin. Dr. G. Karpati supplied the human muscle biopsies and provided comments on the manuscript.

ABSTRACT

In order to determine the mechanisms regulating utrophin expression in human skeletal muscle, we examined the expression and distribution of utrophin and its transcript in biopsies from normal subjects as well as from Duchenne muscular dystrophy (DMD) and polymyositis (PM) patients. We first determined by immunoblotting that in comparison to biopsies from normal subjects, utrophin levels were indeed higher in muscle samples from both DMD and PM patients as previously shown. By contrast, levels of utrophin mRNAs as determined by both RT-PCR assays and *in situ* hybridization, were identical in muscle samples obtained from normal subjects versus DMD and PM patients. In these experiments, we also noted that while utrophin transcripts had a clear tendency to accumulate within the postsynaptic sarcoplasm of normal human muscle fibers, the extent of synaptic accumulation was considerably less than that which we recently observed in mouse muscle fibers. The distribution of utrophin transcripts in synaptic and extrasynaptic compartments of muscle fibers obtained from DMD and PM patients was similar to that seen along muscle fibers from normal subjects. Finally, we also monitored expression of utrophin and its transcripts during regeneration of mouse muscle induced to degenerate by cardiotoxin injections. In these regenerating muscles, we observed by both immunoblotting and immunofluorescence, a large increase (4- to 7-fold) in the levels of utrophin. In agreement with our results obtained with human muscle, the increase in utrophin levels in regenerating mouse muscle was not accompanied by parallel changes in the abundance of utrophin transcripts. Taken together, these results indicate that the levels of utrophin and its transcript in muscle are discordantly regulated under certain conditions thereby highlighting the

important contribution of post-transcriptional regulatory mechanisms in the control of utrophin levels in skeletal muscle fibers.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe and fatal X-linked myopathy with an incidence of approximately 1 in 3,500 male births (1). The gene responsible for DMD codes for dystrophin, a large cytoskeletal protein of the spectrin superfamily predominantly expressed in brain and muscle (2-4). Although the precise function of dystrophin still remains elusive, biochemical and immunocytochemical experiments have led to the notion that dystrophin links the internal cytoskeleton of muscle fibers to the extracellular matrix via a complex of dystrophin-associated proteins (5,6). In turn, this subcellular organization suggests that dystrophin plays an essential role in maintaining the mechanical integrity of the sarcolemma during repeated cycles of muscle contraction and relaxation (7,8). The absence of dystrophin as seen in DMD induces cycles of muscle fiber necrosis and regeneration but as the disease progresses, the regenerative capacity weakens and muscle wasting begins to occur. DMD patients will usually die in their second or third decade of life most often as a result of respiratory or cardiac failure.

Several years ago, Love and colleagues showed the existence of a large multi-exonic gene on chromosome 6q24, that encodes a large cytoskeletal protein displaying extensive sequence similarity with dystrophin (9-11). This protein, called utrophin, is present in most tissues including skeletal muscle, with particularly high levels of expression in lung, blood vessels and nervous system (12-19). In adult normal muscle fibers, utrophin accumulates selectively at the neuromuscular junction (20-24) where it may participate in the full differentiation and/or maintenance of a mature postsynaptic membrane domain (25-28). Interestingly however, the pattern of utrophin expression in muscle is also known to be markedly affected under certain

conditions (for review, see Ref. 29). In particular, muscle fibers obtained from DMD patients and from patients afflicted with inflammatory myopathies such as polymyositis (PM), contain larger amounts of utrophin in comparison to muscle from normal subjects (6,24,30-34). In addition, it is known that in these diseased muscles, utrophin expression is not restricted to synaptic regions of muscle fibers since it also extends well into extrasynaptic regions.

In comparison to the recent progress made in the elucidation of some of the mechanisms underlying the synaptic accumulation of utrophin along muscle fibers (29,35,36), little is known about the mechanisms that lead to an increase in utrophin content in DMD and PM muscles. In the present study, we have therefore begun to examine this issue by determining whether these changes in the abundance of utrophin in diseased muscles were in fact accompanied by alterations in the levels of utrophin transcripts. In addition, we also examined the subcellular distribution of utrophin mRNAs in muscle fibers from normal subjects as well as from DMD and PM patients. For comparison, we also monitored expression of both utrophin and its transcript in regenerating mouse muscles.

EXPERIMENTAL PROCEDURES

Patients. Muscle biopsies were collected from normal subjects (n = 10) as well as from DMD (n = 6) and PM (n = 6) patients ranging in age from 3 to 72 years. For the experiments in which age-matched comparisons were made, additional muscle biopsies from normal subjects and DMD patients from 3 to 6 years of age were used (n = 4). The diagnosis in each case was made rigorously using the usual clinical and laboratory criteria. In all cases, muscle biopsies were obtained following appropriate informed consent.

RNA Extraction. Total RNA was extracted from the muscle samples using TriPure as recommended by the manufacturer (Boehringer Mannheim Corp.; Indianapolis, IN). Briefly, samples were first homogenized using a Polytron set at maximum speed, in 0.5 to 2.0 ml of TriPure. Following addition of chloroform, the samples were mixed and centrifuged at 4°C for 10 min at 12,000 X g. The aqueous phase was then transferred into a fresh microfuge tube and an appropriate volume of ice-cold isopropanol was added. RNA pellets were obtained by centrifuging the samples at 12,000 X g for 15 min. The pellets were subsequently washed with 75% ethanol, air-dried and resuspended in RNase-free water.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR). Total RNA levels were first determined by a GeneQuant II RNA/DNA spectrophotometer (Pharmacia, Quebec, Canada) and standardized to 50 ng/μl. RT-PCR experiments were performed by subjecting 100 ng of total RNA to RT-PCR as described in detail elsewhere (36-38). Briefly, a RT master mixture was prepared containing 5 mM $MgCl_2$, 1 X PCR buffer II (50 mM KCl, 10 mM Tris-HCl; pH 8.3),

1 mM dNTPs, 20 U RNase inhibitor, 50 U reverse transcriptase and 2.5 mM of random hexamers (GeneAmp RNA PCR kit; Perkin Elmer Cetus Co.; Norwalk, CT). The master mix was aliquoted into separate microcentrifuge tubes and the appropriate RNA sample was added into each tube. Negative controls consisted of RT mixtures in which the total RNA sample was replaced with RNase-free water. RT was performed for 45 minutes at 42°C, and the reaction was terminated by heating the samples at 99°C for 5 min.

A PCR master mix was then prepared with final concentrations of 2.5 U AmpliTaq Gold DNA polymerase, 2 mM $MgCl_2$ and 1X PCR buffer II. Utrophin cDNAs were specifically amplified using primers designed on the basis of the available human utrophin sequence: 5' primer (5',3': TGTCGGTTCACCGCCAGAGT) and 3' primer (5',3': GTGGCCTGCTGGGAAC ATTT) (13). These primers amplify a 410 bp target sequence. cDNAs encoding mouse utrophin were amplified using primers and procedures described recently (36). PCR was performed in a DNA thermal cycler (Perkin Elmer Cetus Co.) by adding 4 µl of the RT mixture to 16 µl of the PCR master mix. For all cDNAs, each cycle of amplification consisted of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min. Typically, 30 to 36 cycles of amplification were performed since control experiments showed that these number of cycles were within the linear range of amplification (data not shown). In separate experiments, we verified that equivalent amounts of total RNA were used in our RT-PCR experiments by examining the levels of two well-established loading controls, S12 ribosomal RNA (39) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (40,41). In these assays, we determined that their abundance was consistent from sample to sample since we observed

less than a 10% variation between them (results not shown) thereby indicating that equivalent amounts of total RNA were indeed analyzed.

PCR products were visualized on a 1.5% agarose gel containing ethidium bromide. The 100 bp markers (Gibco BRL; Burlington, Ontario) were used to estimate the molecular mass of the PCR products. Quantitative PCR experiments were performed in order to strictly determine the relative abundance of utrophin transcripts under different experimental conditions. These experiments were carried out as described above except that PCR products were separated in 1.5% agarose gels containing the fluorescent dye VistraGreen (Amersham; Arlington Heights, IL). The labeling intensity of the PCR products, which is linearly related to the amount of DNA, was quantitated using a Storm PhosphorImager and analyzed with the accompanying ImageQuant software program (Molecular Dynamics; Sunnyvale, CA).

In situ Hybridization. Longitudinal serial cryostat sections (12 μm) of muscles from normal subjects and from DMD and PM patients were placed on alternate slides and immediately fixed in 4% paraformaldehyde for 10 minutes. Slides were processed for acetylcholinesterase (AChE) histochemistry (42) and the regions containing neuromuscular junctions were photographed and then subjected to *in situ* hybridization using synthetic oligonucleotides for detection of utrophin transcripts as described previously (35). *In situ* hybridization was performed using two antisense oligonucleotides complementary to the human utrophin cDNA (5' - 3': #1; AGAGATCAGGTTTATGTCGGTGGAGGACAGCAT TAAAGGCGA and #2; TTCTGTCCCATTTTGCATTCAGCTGA GTAAGTGTA TCTCTG) as well as a sense strand as described previously (35). To verify that our oligonucleotides specifically detected utrophin

transcripts, we performed Northern blot analyses with PolyA+ RNA collected from the mouse myogenic C_2C_{12} cell line and from primary cultures of human endothelial cells. Using the ^{32}P-labeled utrophin oligonucleotides, we observed in these experiments, the presence of a single, high molecular mass (>12 kb) band thereby indicating that our synthetic oligonucleotides were indeed specific for utrophin mRNAs (data not shown).

Analysis of *in situ* hybridization labeling was performed using an image analysis system equipped with Image 1.47 software (Wayne Rasband, NIMH) as described previously 35). The labeling density in synaptic versus extrasynaptic regions was determined by measuring the optical density within a circular field of constant 100 μm in diameter. Circular fields were chosen to represent areas approximately covering the neuromuscular junction as the imaging software did not allow for the freeform tracing of the area underlying the AChE histochemistry. To determine whether differences existed between utrophin mRNA levels in muscle fibers from normal subjects and DMD and PM patients, 1 mm^2 square areas of extrasynaptic regions were sampled. These regions were carefully selected to ensure the absence of large blood vessels and neuromuscular junctions contained within these areas. For these analyses, both normal and patient muscle sections were placed on the same slide and processed for *in situ* hybridization simultaneously. Previous analyses determined that the number of silver grains is linearly related to the optical density using this image analysis system (43). Thus, optical density values were used as a measure of labeling with higher values indicating greater labeling. A minimum of twelve muscle sections were processed for each condition and a minimum of four measurements were performed on each section. For our *in situ* hybridization experiments, background values

were determined to be the optical density of regions external to the muscle fibers, ie the values obtained from the blank slide, and these values were subtracted from all subsequent measurements. Control experiments performed with a synthetic oligonucleotide corresponding to the sense strand of the human utrophin cDNA failed to label subcellular structures above background levels.

Immunoblotting. For these experiments, protein extracts from muscle biopsies were obtained using two separate methods. In one case, total RNA and proteins were extracted from the same biopsy using TriPure (Boehringer Mannheim) according to the manufacturer's specifications. Additionally, we also isolated proteins from cryostat sections of biopsies as recently described (44). For these experiments, the concentration of proteins was determined using the bicinchoninic acid (BCA) Protein Assay Reagent protocol (Pierce Laboratories; Rockford, IL). Up to 50 μg of extracted proteins were separated on 6% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Sigma; Toronto, ON). To ensure that equivalent amounts of proteins were loaded for each sample, the membranes were stained with Ponceau S (Sigma). Membranes were subsequently incubated with the NCL-DRP2 monoclonal antibody directed against the N-terminus of utrophin (Novocastra Laboratories; Newcastle upon Tyne, UK). Bound antibodies were detected using secondary antibodies linked to horseradish peroxidase and revealed via chemiluminescence using a commercially available kit (New England Nuclear; Boston, MA). Membranes were then exposed onto BioMax autoradiographic films (Kodak; Rochester, NY), developed and scanned by densitometry.

Cardiotoxin Injections Into Mouse Muscle. To induce muscle degeneration followed by a period of muscle regeneration in mice, 25 μl of 10^{-5} M cardiotoxin (LATOXAN: Laboratoire des

Toxines Animales et Animaux Venimeux; Rosans, France) were directly injected into upper and lower regions of tibialis anterior (TA) muscles of C57/BL mice anesthetized with halothane (45). Regenerating muscles were then excised 2 and 5 days later. They were either frozen in liquid nitrogen for immunoblotting and RT-PCR assays or in melting isopentane cooled with liquid nitrogen for histological and immunofluorescence analyses.

Histology and Immunofluorescence. Haematoxylin and eosin (H and E) staining was performed to determine the morphological changes in mouse TA muscles following cardiotoxin injections. Briefly, longitudinal cryostat sections (10 to 12 μm) were incubated in haematoxylin for 5 minutes followed by thorough washing in water. Sections were then counterstained and dipped for 2 min in eosin. After thorough washing in water, the slides were dehydrated in a series of ethanol solutions and mounted in xylene/Permount (Sigma).

Detection of utrophin in these regenerating muscle fibers was performed by immunofluorescence experiments using the monoclonal utrophin antibody (see above) followed by a Cy3-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories). In some experiments, the presence of neuromuscular junctions was also examined by incubating the tissue sections with fluorescein-conjugated α-bungarotoxin (Molecular Probes; Eugene, OR).

Statistical Analysis. Paired Student's *t*-tests were performed to evaluate whether the differences in utrophin levels between normal subjects versus DMD and PM patients were statistically significant. A one-way analysis of variance was performed to compare utrophin levels in mouse

regenerating muscles over time. The level of significance was set at $P < 0.05$. All data are expressed as mean ± SEM throughout the text.

RESULTS

Utrophin Levels in Human Muscle Biopsies. We initially performed immunoblotting experiments on biopsies obtained from normal subjects as well as from DMD and PM patients. In agreement with previous results (24,31,34,46), we observed that utrophin protein levels were indeed higher in both DMD and PM patients. Densitometric analyses revealed that in muscle samples from these patients, utrophin levels were approximately 3.5-fold higher ($P < 0.05$) than those seen in biopsies from normal subjects (Figure 6.1).

Utrophin mRNA Levels in Human Muscle Biopsies. In order to begin exploring the mechanisms that govern utrophin expression in human skeletal muscle, we examined utrophin mRNA levels in muscle biopsies from normal subjects as well as from DMD and PM patients. For these experiments, equivalent amounts of total RNA were subjected to RT-PCR analysis and the relative abundance of utrophin transcripts was determined. Our analysis revealed that levels of utrophin transcripts in biopsies from DMD and PM patients were not significantly different from the levels seen in normal subjects (Figure 5.2A). Quantitative analysis showed, in fact, that transcript levels in muscle samples from DMD and PM patients were approximately 94% and 97% ($P > 0.05$) of the levels observed in normal individuals, respectively (Figure 5.2). Additional experiments indicated that the levels of utrophin mRNAs were also similar ($P > 0.05$) in muscle biopsies obtained from age-matched normal subjects and DMD patients (results not shown).

Localization of Utrophin Transcripts in Human Skeletal Muscle Fibers. In a separate series of experiments, we examined by *in situ* hybridization, the distribution of utrophin mRNAs along muscle fibers from normal subjects as well as from DMD and PM patients. These experiments were undertaken to determine whether single muscle fibers from these patients expressed greater amounts of utrophin mRNAs. For these analyses, we determined utrophin transcript levels in each of the three conditions (normal, DMD and PM) by quantitating 1 mm^2 areas of extrajunctional regions of muscle fibers. Concordant with our RT-PCR results, we did not observe any significant changes ($P > 0.05$) in the intensity of labeling in normal subjects versus DMD and PM patients (Figure 5.3). In addition, the pattern of labeling within and between individual muscle fibers present in these cryostat sections from normal subjects and these patients, was very similar. As expected, we also detected utrophin mRNAs in large blood vessels and capillaries present in these muscle biopsies (data not show).

We also examined whether utrophin mRNAs were more abundant in synaptic versus extrasynaptic regions of human muscles fibers as we recently observed in mouse muscle (35). Our quantitative analysis revealed that out of 168 neuromuscular junctions identified by AChE histochemistry, 111 or 66%, displayed an enrichment of silver grains corresponding to utrophin transcripts within the postsynaptic sarcoplasm (Figure 5.4). Densitometric analysis revealed that in comparison to extrasynaptic regions, utrophin mRNA levels were indeed more abundant in synaptic compartments of muscle fibers (Figure 5.3). In addition, the extent of synaptic accumulation of utrophin transcripts in muscle fibers from DMD and PM patients was similar to that seen in muscle samples from normal subjects ($P > 0.05$) (Figure 5.3).

Expression of Utrophin in Regenerating Mouse Skeletal Muscle. Since our results obtained with human muscles indicated that levels of utrophin and of its transcript were not modified in parallel in biopsies from normal subjects versus DMD and PM patients, we examined in a last set of experiments, expression of utrophin in mouse regenerating muscles. To this end, we injected cardiotoxin into mouse TA muscles as a way to induce severe muscle necrosis and, subsequently, to significantly increase the number of regenerating fibers in a given muscle. By H and E staining of cryostat sections, we observed that 2 days following cardiotoxin injections, a substantial number of necrotic fibers were present as evidenced by a lack of intact muscle fibers and a complete disarray of the myonuclei (data not shown). Five days following injections, numerous regenerating fibers were present in these TA muscles as determined by the presence of centrally-located nuclei.

During this period of muscle regeneration, we observed a substantial increase in utrophin along the sarcolemma of regenerating fibers (Figure 5.5). To quantitate the expression of utrophin and its transcript in these regenerating muscles, total RNA and protein were first isolated from the same muscle as described above, and immunoblotting and RT-PCR experiments were then performed. In agreement with our immunofluorescence analysis, we determined that in comparison to intact muscles, utrophin levels were significantly ($P < 0.05$) elevated during muscle regeneration (Figure 5.6A). Densitometric analysis revealed that utrophin levels were approximately 4- and 7-fold higher 2 and 5 days following cardiotoxin injections, respectively (Table 5.1). By contrast, utrophin mRNA levels were nearly identical in control and regenerating muscles as shown in Figure 5.6B and Table 5.1. Together, these

results indicate therefore that the increase in utrophin observed by immunofluorescence and immunoblotting occurs independently of any pronounced changes in transcript levels.

DISCUSSION

In the present study, we began to examine the molecular mechanisms controlling utrophin expression in human skeletal muscle. Since previous studies have shown that utrophin levels in muscle biopsies obtained from DMD and PM patients are significantly elevated (24,31,34,44), we initially focused on this observation and determined whether utrophin transcripts are also increased in these diseased muscles. To complement our quantitative RT-PCR analysis, we have, in addition, examined the distribution of utrophin mRNAs along muscle fibers by *in situ* hybridization, and compared the pattern of expression between normal, DMD and PM muscles. Finally, we also quantitated the levels of utrophin and its transcript in regenerating mouse muscles in attempts to determine whether they varied in parallel during a phase of massive muscle regeneration. Taken together, our results indicate that the levels of utrophin and its transcript in muscle are discordantly regulated under certain conditions thereby highlighting the important contribution of post-transcriptional regulatory mechanisms in the control of utrophin levels in skeletal muscle fibers.

Utrophin mRNA levels Are Not Affected in DMD and PM Muscles. The observation in this study that utrophin levels are elevated in muscle biopsies obtained from DMD and PM patients confirms previous findings which first demonstrated the existence of such differences (24,31,34,44). However, the more modest upregulation seen in our study (~4-fold) as compared to the larger increases (~ 10-fold) previously reported (31), can be explained by the fact that we purposely analyzed muscle biopsies with a high neuromuscular junction content since as part of

our experiments, we also examined the distribution of utrophin transcripts in synaptic versus extrasynaptic compartments of muscle fibers. Such selection of samples may have therefore increased the levels of utrophin in muscle samples from both normal subjects and patients thereby reducing the magnitude of the utrophin up-regulation in diseased muscles. Nonetheless, the greater amount of utrophin seen in DMD and PM muscles which results, in part, from the regenerative process (47), appears to occur independently of any significant alterations in the levels of utrophin mRNAs as revealed by our RT-PCR analysis and *in situ* hybridization experiments (see also Figure 4D in Ref. 13). Altogether, these results suggest therefore that the accumulation of utrophin in extrajunctional regions of DMD and PM muscles is mediated by mechanisms controlling protein expression and/or stability. This view is in fact strongly supported by the experiments performed with mouse regenerating muscles in which we observed a substantial increase in utrophin expression with little modifications in mRNA levels. Thus, it appears that under these conditions, utrophin expression is largely regulated by mechanisms altogether distinct from those involved in the transcriptional regulation of the utrophin gene. Although surprising at first, these findings are entirely coherent with the demonstration that the utrophin promoter displays features characteristic of housekeeping genes (48) which are constitutively and ubiquitously expressed.

In a previous study, we demonstrated that dexamethasone treatment of dystrophic myotubes maintained in culture, increased utrophin expression without affecting mRNA levels (49). Based on these findings, we speculated that in normal muscle, utrophin and dystrophin compete for available β-dystroglycan bindings sites along the sarcolemma since it is known that both can bind this membrane glycoprotein (6). The absence of dystrophin in DMD muscle may

therefore allow a pool of newly synthesized utrophin molecules which is normally cytoplasmic and undergoing rapid degradation, to bind available β-dystroglycan bindings sites. Accordingly, this binding stabilizes utrophin molecules by incorporating them into the complex of dystrophin-associated molecules at the sarcolemma.

Although this appears as an attractive hypothesis to explain the enhanced levels of utrophin in DMD muscles, it cannot account for the similar changes seen in PM muscles where dystrophin is normally expressed. A corollary to our hypothesis could have been that in PM muscles, expression of β-dystroglycan is increased thereby making more binding sites available. However, we tested this in separate experiments and failed to detect any changes in the levels of β-dystroglycan in muscle biopsies obtained from normal subjects versus PM patients (data not shown). Therefore, in the case of inflammatory myopathies, a different mechanism must operate.

Synaptic Accumulation of Utrophin mRNAs. Previous studies performed with various mouse and rat models have shown that several transcripts encoding synaptic proteins such as the AChR subunits, accumulate selectively within the postsynaptic sarcoplasm of muscle fibers as a result of the compartmentalized transcriptional activation of their respective genes (for review, see Refs. 50, 51). In this context, we have recently demonstrated in mouse skeletal muscle fibers that a similar transcriptional regulatory mechanism underlies the preferential accumulation of utrophin at the postsynaptic membrane of the neuromuscular junction (35,36). Until recently

however, it was unknown whether a similar mechanism governed the local expression of proteins of the postsynaptic membrane in human muscle fibers.

Our *in situ* hybridization experiments allowed us to directly address this issue. The quantitative analysis indicated that utrophin mRNAs accumulate within the postsynaptic sarcoplasm of human muscle fibers as previously seen in mouse muscle (35). By comparing the labeling intensity in synaptic versus extrasynaptic compartments of muscle fibers, we observed however, that the magnitude of the synaptic accumulation of utrophin mRNAs in human skeletal muscle fibers is considerably less than that seen along mouse muscle fibers (2- to 3-fold versus 12-fold). There are several explanations that may be envisaged to account for this difference. For instance, it is possible that the myonuclei located within the postsynaptic sarcoplasm of human muscle fibers display a lower degree of transcriptional specialization. This, however, seems unlikely given the recent data showing the pronounced synaptic accumulation of transcripts encoding the AChR ε-subunit in human muscle fibers (52). Alternatively, it is also possible that this difference in the extent of synaptic accumulation between human and mouse muscle fibers depends upon the type of muscle fibers. Specifically, in our previous *in situ* hybridization experiments using mouse hindlimb muscles, we focused on fast-contracting muscles for our analyses (35) whereas typically, human muscles contains a much larger proportion of slow fibers (see for example, Ref. 53). In this context, it is noteworthy that previous studies have revealed that specific rodent muscles also display different extent of synaptic mRNA accumulations. In particular, both AChE and AChR α-subunit transcripts exhibit a less striking synaptic accumulation in the slow soleus muscle as compared to that seen in fast muscles (38,54). Therefore, it appears that fast-twitch fibers display a pronounced

synaptic enrichment of transcripts while slow-twitch fibers exhibit a more homogeneous distribution along their entire length. The high percentage of slow fibers in human skeletal muscle that we sampled may thus explain the modest synaptic accumulation of utrophin transcripts as compared to that seen in mouse muscle fibers.

In conclusion, our results indicate that in addition to transcriptional events (35,36), there are other mechanisms contributing to the regulation and localization of utrophin along skeletal muscle fibers. Specifically, since utrophin protein levels can be modulated without alterations in the abundance of its transcript, our data show that additional regulatory steps such as translational control, post-translational processing, intracellular sorting and targeting may also be involved in controlling the expression of utrophin. Currently, there is considerable interest in upregulating utrophin protein levels in attempts to alleviate the muscle pathology seen in DMD (55,56). In this context, a thorough understanding of post-transcriptional regulatory mechanisms appears warranted since this may provide an additional opportunity, aside from transcriptional regulation, by which utrophin expression can be systematically augmented in dystrophic muscle fibers.

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REFERENCES

(1) Emery A. Population frequencies of inherited neuromuscular disorders--A world survey. Neuromusc Disord 1991;1:19-29

(2) Ahn AH, Kunkel LM. The structural and functional diversity of dystrophin. Nature Genet 1993;3:283-291

(3) Matsumura K, Campbell KP. Dystrophin-associated complex: Its role in the molecular pathogenesis of muscular dystrophies. Muscle Nerve 1994; 7:2-15

(4) Worton R. Muscular dystrophies: Diseases of the dystrophin-glycoprotein complex. Science 1995;270:755-760

(5) Ervasti JM, Ohlendieck K, Kahl SD, Gaver MG, Campbell KP. Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature 1990;345:315-319

(6) Matsumura K, Ervasti JM, Ohlendieck K, Kahl SD, Campbell KP. Association of dystrophin-related protein with dystrophin-associated proteins in *mdx* mouse muscle. Nature 1992;360:588-591

(7) Sadoulet-Puccio HM, Kunkel LM. Dystrophin and its isoforms. Brain Pathol 1996;6:25-35

(8) Winder SJ. The membrane-cytoskeleton interface: The role of dystrophin and utrophin. J. Muscle Res Cell Motil 1997;18: 617-629

(9) Love DR, Hill DF, Dickson G, *et al.* An autosomal transcript in skeletal muscle with homology to dystrophin. Nature 1989;339:55-58

(10) Buckle VJ, Guenet JL, Simon-Chazottes D, Love DR, Davies KE. Localisation of a dystrophin-related autosomal gene to 6q24 in man and to mouse chromosome 10 in the region of the dystrophia muscularis (dy) locus. Hum Genet 1990;85:324-326

(11) Tinsley JM, Blake DJ, Roche A, *et al.* Primary structure of dystrophin-related protein. Nature 1992;360:591-593

(12) Love DR, Morris GE, Ellis JM, *et al.* Tissue distribution of the dystrophin-related gene product and expression in the *mdx* and dy mouse. Proc Natl Acad Sci USA 1991;88:3243-3247

(13) Khurana TS, Watkins SC, Chafey P, *et al.* Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle. Neuromusc Disord 1991;1:185-194

(14) Khurana TS, Watkins SC, Kunkel LM. The subcellular distribution of chromosome-6-encoded dystrophin-related protein in the brain. J Cell Biol 1992;119:357-366

(15) Zhao J, Yoshioka K, Miyatake M, Miike T. Dystrophin and a dystrophin-related protein in intrafusal muscle fibers, and neuromuscular and myotendinous junctions. Acta Neuropathol 1992;84:141-146

(16) Matsumura K, Shasby DM, Campbell KP. Purification of dystrophin-related protein (utrophin) from lung and its identification in pulmonary artery endothelial cells. FEBS 1993;326:289-293

(17) Matsumura K, Yamada H, Shimizu T, Campbell KP. Differential expression of dystrophin, utrophin and dystrophin-associated proteins in peripheral nerve. FEBS 1993;334:281-285

(18) Uchino M, Yoshioka K, Miike T, *et al.* Dystrophin and dystrophin-related protein in the brains of normal and *mdx* mice. Muscle Nerve 1994;17:533-538

(19) Pons F, Robert A, Fabbrizio E, *et al.* Utrophin localization in normal and dystrophin-deficient heart. Circ 1994;90:369-374

(20) Fardeau M, Tomé FM, Collin H, Augier N, Pons F, Léger J, Léger J. Présence d'une protéine de type dystrophine au niveau de la jonction neuromusculaire dans la dystrophie musculaire de Duchenne et la souris mutante "*mdx*". Comptes Rendus de l'Academie des Sciences 1990;311:197-204

(21) Khurana TS, Hoffman EP, Kunkel LM. Identification of a chromosome-6-encoded dystrophin-related protein. J Biol Chem 1990;265:16717-16720

(22) thi Man N, Ellis JM, Love DR, Davies KE, Gatter KC, Dickson G, Morris GE. Localization of the DMDL gene-encoded dystrophin-related protein using a panel of nineteen monoclonal antibodies: Presence at neuromuscular junctions, in the sarcolemma of dystrophic skeletal muscle, in vascular and other smooth muscles, and in proliferating brain cell lines. J Cell Biol 1991;115:1695-1700

(23) Ohlendieck K, Ervasti JM, Matsumura K, Kahl SD, Leveille CJ, Campbell KP. Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. Neuron 1991;7:499-508

(24) Helliwell TR, thi Man N, Morris GE, Davies KE. The dystrophin-related protein, utrophin, is expressed on the sarcolemma of regenerating human skeletal muscle fibers in dystrophies and inflammatory myopathies. Neuromusc Disord 1992;2:177-184

(25) Jasmin BJ, Cartaud A, Ludosky MA, Changeux JP, Cartaud J. Asymmetric distribution of dystrophin in developing and adult Torpedo marmorata electrocyte: Evidence for its association with the acetylcholine receptor-rich membrane. Proc Natl Acad Sci USA 1990;87:3938-3941

(26) Hoch W, Campanelli JT, Scheller RH. Agrin-induced clustering of acetylcholine receptors: A cytoskeletal link. J Cell Biol 1994;126:1-4

(27) Grady RM, Merlie JP, Sanes JR. Subtle neuromuscular defects in utrophin-deficient mice. J Cell Biol 1997;136:871-882

(28) Deconinck AE, Potter AC, Tinsley JM, *et al.* Postsynaptic abnormalities at the neuromuscular junctions of utrophin-deficient mice. J Cell Biol 1997;136:883-894

(29) Gramolini AO, Jasmin BJ. Molecular mechanisms and putative signaling events controlling utrophin expression in mammalian skeletal muscle fibers. Neuromusc Disord 1998; 8:351-361

(30) Takemitsu M, Ishiura S, Koga R, Kamakura K, Arahata K, Nonaka I, Sugita H. Dystrophin-related protein in the fetal and denervated skeletal muscles of normal and *mdx* mice. Biochem Biophys Res Commun 1991;180:1179-1186

(31) Karpati G, Carpenter S, Morris GE, Davies KE, Guerin C, Holland P. Localization and quantitation of the chromosome 6-encoded dystrophin-related protein in normal and pathological human muscle. J Neuropath Exp Neurol 1993;52:119-128

(32) Mizuno Y, Yoshida M, Yamamoto H, Hirai S, Ozawa E. Distribution of dystrophin isoforms and dystrophin-associated proteins 43DAG (A3a) and 50DAG (A2) in various monkey tissues. J Biochem 1993;114:936-941

(33) Schofield J, Houzelstein D, Davies KE, Buckingham M, Edwards YH. Expression of the dystrophin-related protein (utrophin) gene expression during mouse embryogenesis. Develop Dynamics 1993;198:254-264

(34) Pons F, Nicholson LVB, Robert A, Voit T, Leger JJ Dystrophin and dystrophin-related protein (utrophin) distribution in normal and dystrophin-deficient skeletal muscles. Neuromusc Disord 1993;3:507-514

(35) Gramolini AO, Dennis CL, Tinsley JM, Roberston GS, Cartaud J, Davies KE, Jasmin BJ Local transcriptional control of utrophin expression at the neuromuscular synapse. J Biol Chem 1997;272:8117-8120

(36) Gramolini AO, Burton EA, Tinsley JM, *et al.* Muscle and neural isoforms of agrin increase utrophin expression in cultured myotubes via a transcriptional regulatory mechanism. J Biol Chem 1998;273:736-743

(37) Jasmin BJ, Lee RK, Rotundo RL. Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction. Neuron 1993;11:467-477

(38) Michel RN, Vu CQ, Tetzlaff W, Jasmin BJ. Neural regulation of acetylcholinesterase mRNAs at mammalian neuromuscular synapses. J Cell Biol 1994;127:1061-1069

(39) Amoureux MC, van Gool D, Herrero MT, Dom R, Colpaert FC, Pauwels PJ. Regulation of metallothionein-III (GIG) mRNA in the brain of patients with Alzheimer disease is not impaired. Mol Chem Neuropath 1997;32:101-121

(40) Sharma V, Xu M, Vail J, Campbell R. Comparative analysis of multiple techniques for semi-quantitation of RT-PCR amplicons. Biotech Tech 1998;12:521-524

(41) Nirde P, Georget V, Terouanne B, Galifer RB, Belon C, Sultan C. Quantitation of androgen receptor messenger RNA from genital skin fibroblasts by reverse transcription - competitive polymerase chain reaction. J Steroid Biochem Mol Biol 1998;66:35-43

(42) Karnovsky MJ, Roots L. A "direct coloring" thiocholine method for cholinesterase. J Histochem Cytochem 1996;12:219-221

(43) Gerfen CR, Young WS. Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an *in situ* hybridization histochemistry and fluorescent retrograde tracing study. Brain Res 1988;460:161-167

(44) Gilbert R, Nalbanoglu J, Tinsley JM, Massie B, Davies KE, Karpati G. Efficient utrophin expression following adenovirus gene transfer in dystrophic muscle. Biochem Biophys Res Commun 1998;242:244-247

(45) d'Albis A, Couteaux R, Janmot C, Mira, JC. Myosin isoform transitions in regeneration of fast and slow muscles during postnatal development of the rat. Dev Biol 1989;135:320-5

(46) Mizuno Y, Nonaka I, Hirai S, Ozawa E. Reciprocal expression of dystrophin and utrophin in muscles of Duchenne muscular dystrophy patients, female DMD-carriers and control subjects. J Neurol Sci 1993;119:43-52

(47) Taylor J, Muntoni F, Dubowitz V, Sewry CA. The abnormal expression of utrophin in Duchenne and Becker muscular dystrophy is age related. Neuropath Appl Neurol 1997;23:399-405

(48) Dennis CL, Tinsley JM, Deconinck AE, Davies KE. Molecular and functional analysis of the utrophin promoter. Nucl Acids Res 1996;24:1646-1652

(49) Pasquini F, Guerin C, Blake D, Davies KE, Karpati G, Holland P. The effect of glucocorticoids on the accumulation of utrophin by cultured normal and dystrophic human skeletal muscle satellite cells. Neuromusc Disord 1995;5:105-114

(50) Hall ZW, Sanes JR. Synaptic structure and development: the neuromuscular junction. Cell/Neuron 1993;72:99-121

(51) Duclert A, Changeux JP. Acetylcholine receptor gene expression at the developing neuromuscular junction. Physiol Rev 1995;75:339-368

(52) Young C, Lindsay S, Vater R, Slater CR. An improved method for the simultaneous demonstration of mRNA and esterase activity at the human neuromuscular junction. Histochem J 1998;30:7-11

(53) Simoneau JA, Bouchard C. Human variation in skeletal muscle fiber-type proportion and enzyme activities. Am J Physiol 1989;257:E567-72

(54) Kues WA, Sakmann B, Witzemann V. Differential expression patterns of five acetylcholine receptor subunit genes in rat muscle during development. Eur J Neurosci 1995;7:1376-1385

(55) Tinsley JM, Potter AC, Phelps SR, Fisher R, Trickett JI, Davies KE. Amelioration of the dystrophic phenotype of *mdx* mice using a truncated utrophin transgene. Nature 1996;384:349-353.

(56) Deconinck N, Tinsley JM, DeBacker F, *et al.* Expression of truncated utrophin leads to major functional improvements in dystrophin-deficient muscles of mice. Nature Med 1997; 3:1216-1221.

Figure 5.1. Utrophin protein levels in muscle biopsies obtained from normal patients as well as patients with inflammatory myopathies. Immunoblots of human muscle biopsy homogenates (50 μg of total protein per well) incubated with a utrophin antibody. A shows a representative example of samples obtained from normal subjects (CTL), and from DMD and PM patients. B represents the quantitation of these results indicating that utrophin levels are increased in both PM and DMD muscles. Shown are the results obtained with a minimum of 5 biopsies. Asterisks denote significant differences from normal subjects ($P < 0.05$).

A)


CTL
PM
DMD

B)

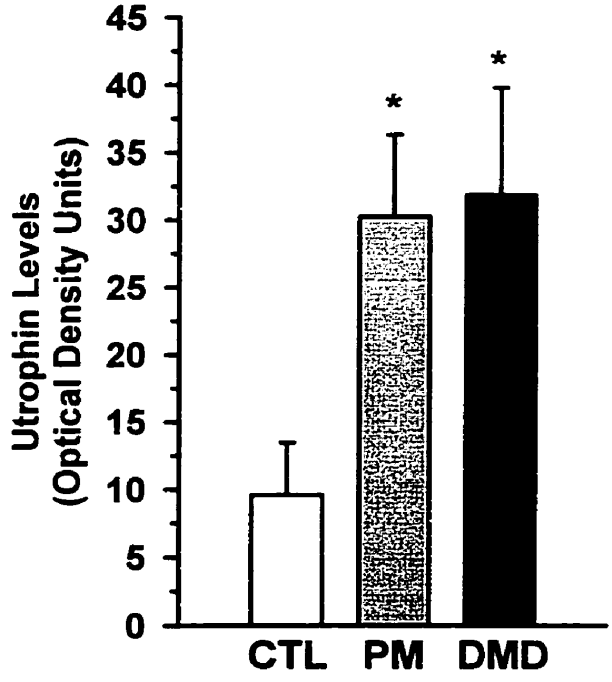
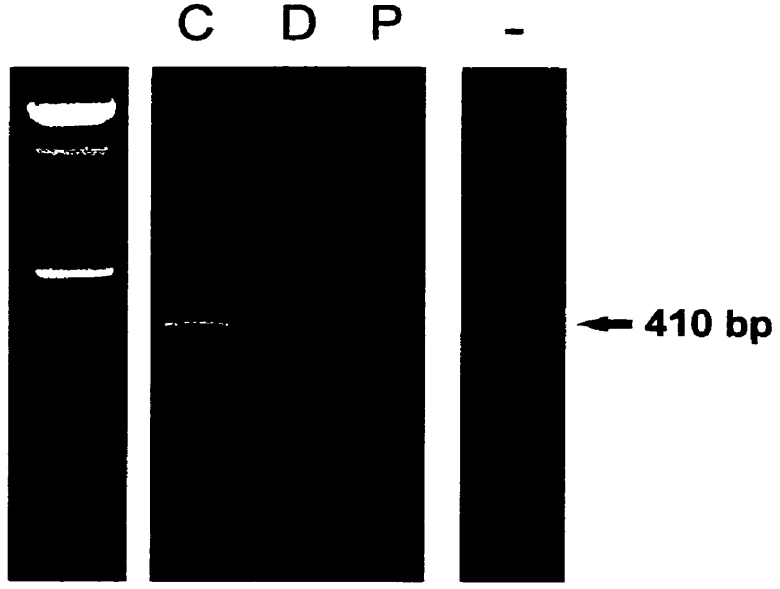

Utrophin Levels
(Optical Density Units)
45
40
35
30
25
20
15
10
5
0
*
*
CTL
PM
DMD

Figure 5.2. Utrophin transcript levels in biopsies from DMD and PM patients are not different than those from normal subjects. A is a representative example of ethidium-bromide stained agarose gel showing utrophin PCR products obtained from normal subjects (C) as well as DMD (D) and PM (P) patients. The negative control lane is marked with a minus sign. B shows the quantitation of these results highlighting the lack of difference between normal subjects versus DMD and PM patients. Shown are the results obtained with a minimum of 6 biopsies.

A)


C
D
P
-
410 bp

B)

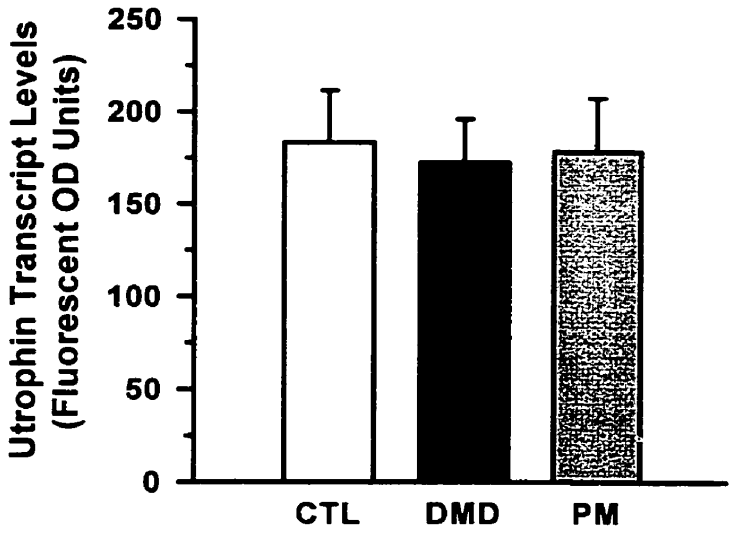

Utrophin Transcript Levels
(Fluorescent OD Units)
250
200
150
100
50
0
CTL
DMD
PM

Figure 5.3. Quantitation of the levels of utrophin mRNAs in skeletal muscle fibers by *in situ* hybridization. Shown is the quantitation of utrophin transcripts in synaptic (S) and extrasynaptic (E) regions of muscle fibers from normal subjects (CTL) as well as from DMD and PM patients. Note the higher levels of utrophin mRNAs within the synaptic region and the presence in the extrajunctional regions of a significant amount of utrophin transcripts. * denote significant differences from extrasynaptic levels ($P < 0.05$).

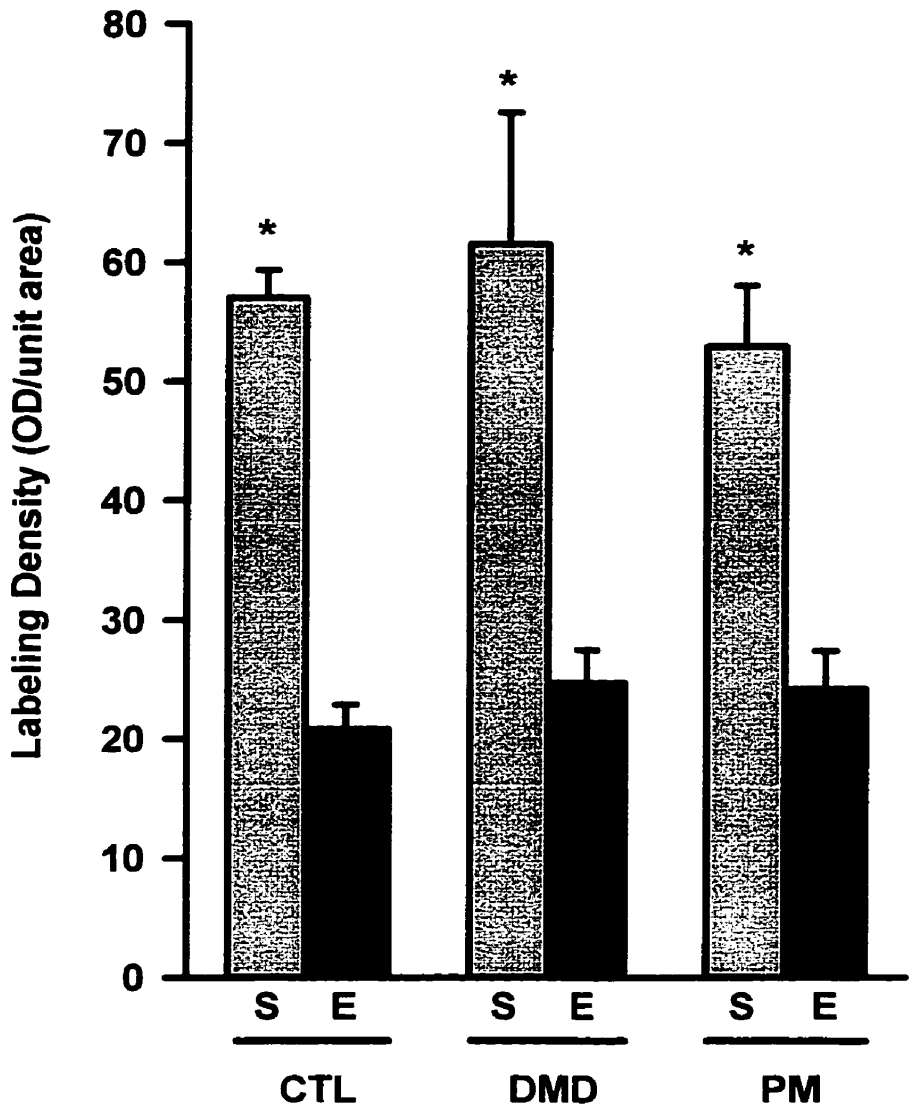

Labeling Density (OD/unit area)
80
70
60
50
40
30
20
10
0
*
*
*
S
E
S
E
S
E
CTL
DMD
PM

Figure 5.4. Localization of utrophin mRNAs along human skeletal muscle fibers by *in situ* hybridization. A is a representative bright-field photomicrographs of longitudinal cryostat sections stained for AChE to visualize neuromuscular junctions. B shows the same muscle section processed for *in situ* hybridization with oligonucleotides specific for human utrophin mRNAs. A detailed comparison of these two panels reveals the selective accumulation of utrophin mRNAs within the postsynpatic compartment, although the accumulation appears less striking than that reported for other synaptically enriched transcripts (see Text for further discussion). Closed arrows point to examples of co-localization whereas the open arrow shows a neuromuscular junction without an accumulation of utrophin transcripts. Bar = 75 μm.

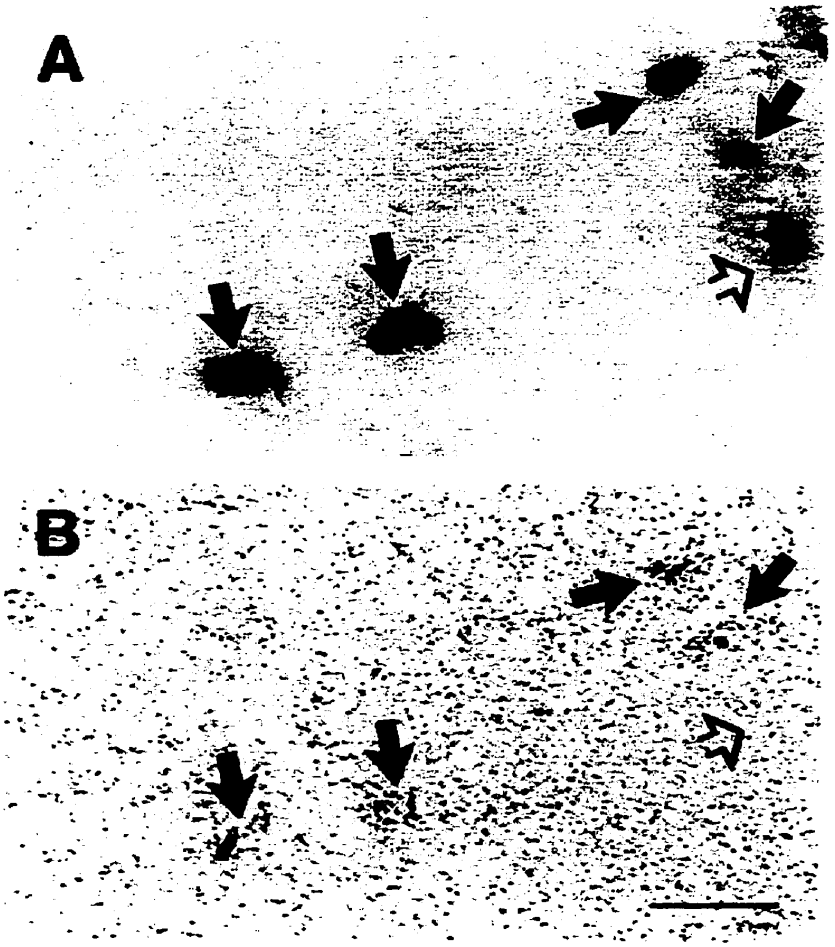

A
B

Figure 5.5. Injection of cardiotoxin into mouse muscle leads to a cycle of severe degeneration and regeneration which is accompanied by an increase in utrophin expression. Shown are representative photomicrographs of muscles processed for immunofluorescence with an antibody against utrophin. Note the restricted expression of utrophin at the neuromuscular junction in control muscle (A) and the pronounced increase in utrophin levels at the sarcolemma of 5 day-regenerating muscles (B and C). Bar = 220 μm.

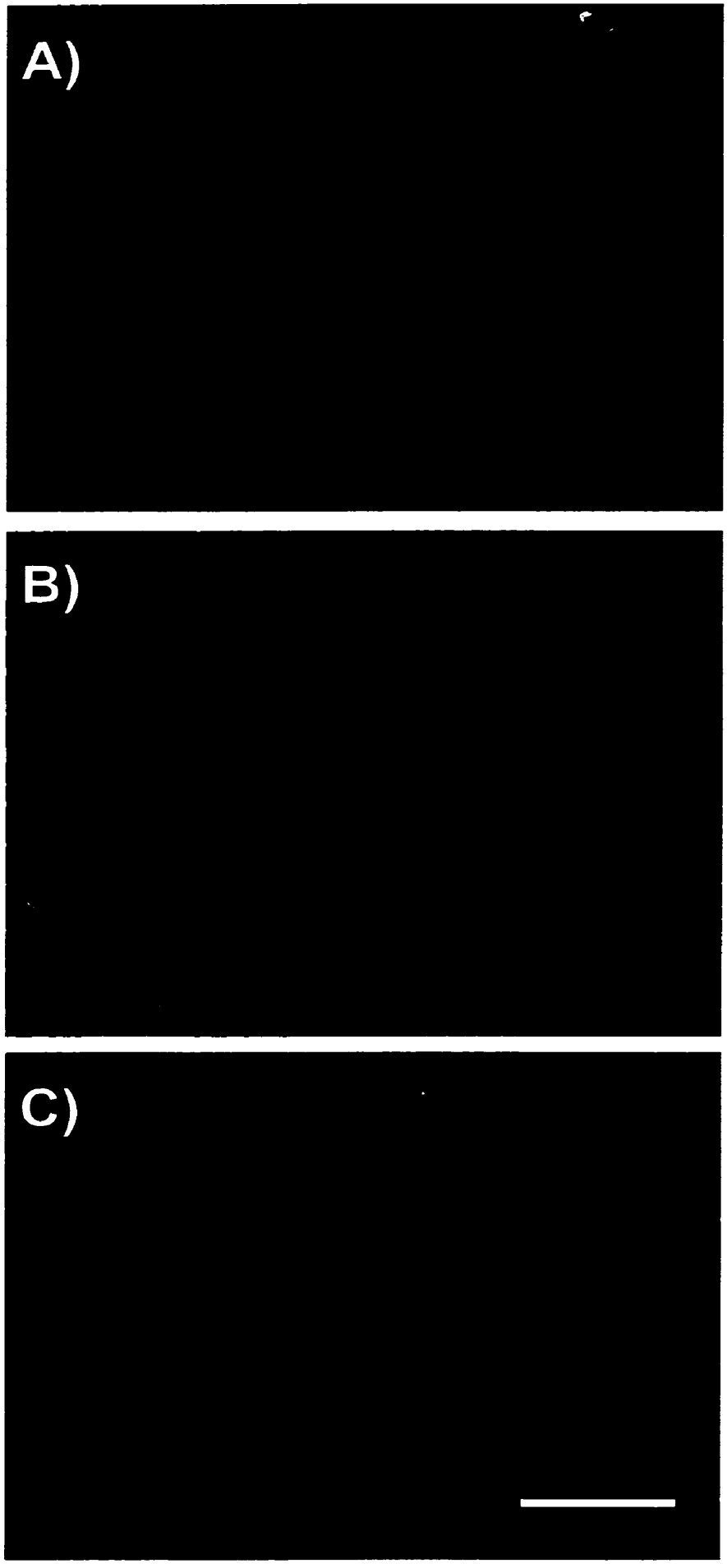
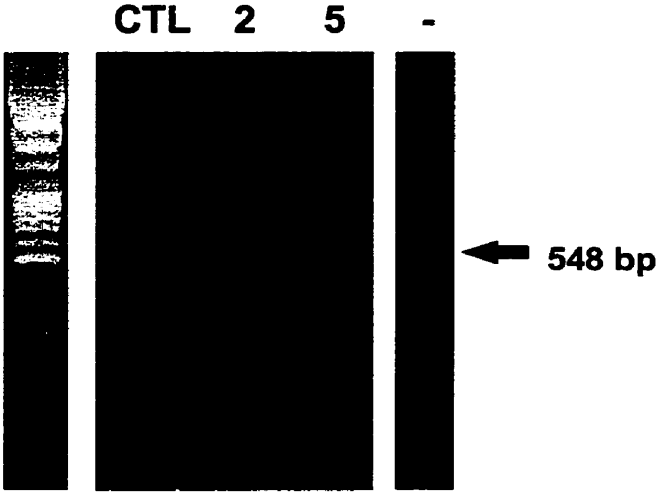

A)
B)
C)

Figure 5.6. Regeneration of mouse skeletal muscle leads to a large increase in utrophin levels without concomitant changes in the abundance of utrophin mRNAs. Upper panel in A is a representative immunoblot showing that utrophin expression increases significantly at 2 and 5 days following cardiotoxin injections. Lower panel corresponds to the Ponceau S staining indicating that similar amounts of proteins were loaded into each well. B is a representative ethidium-stained agarose gel of utrophin PCR products. Total RNA was harvested using a procedure which allows for the isolation of proteins and RNA from the same samples (see Methods). Note the lack of any significant changes in utrophin transcript levels despite the substantial increase in protein levels. The negative control lane is marked with a minus sign. CTL refers to control and 2 and 5 correspond to 2 and 5 days following injection of cardiotoxin.

A)

B)



Utrophin Expression in Mouse Skeletal Muscle During Cardiotoxin-Induced Regeneration

	Utrophin Protein Levels	Utrophin mRNA Levels
Control muscles	6.2 ● 1.0	191.1 ± 15.8
2 days post-cardiotoxin	24.4 ± 5.2*	197.8 ± 7.8
5 days post-cardiotoxin	47.2 ± 11.0*	201.9 ± 28.5

Table 5.1. Muscle fiber regeneration leads to an increase in utrophin protein levels without significant changes in utrophin mRNA levels. Mouse hindlimb muscles were injected with 25 μl of 10^{-5} M cardiotoxin to induce muscle fiber degeneration followed by regeneration, and muscles were then collected 2 and 5 days following the cardiotoxin injection. Protein and total RNA were extracted from the same muscle as described in the Methods section. Utrophin protein levels were determined by immunoblotting and densitometry and are expressed in optical density units. Utrophin mRNA levels were determined using a phosporimager and are expressed in fluorescent optical density units. * denote significant differences ($P < 0.05$) from control values, $n = 4$.

CHAPTER 6

EXPRESSION OF THE UTROPHIN GENE DURING MYOGENIC DIFFERENTIATION

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A. Gramolini performed all of the experimental procedures. The manuscript was written by A. Gramolini and Dr. Jasmin.

ABSTRACT

The process of myogenic differentiation is known to be accompanied by large increases (~10-fold) in the expression of genes encoding cytoskeletal and membrane proteins including dystrophin and the acetylcholine receptor (AChR) subunits, via the effects of transcription factors belonging to the MyoD family. Since in skeletal muscle; i) utrophin is a synaptic homologue to dystrophin; and ii) the utrophin promoter contains an E-box, we examined in the present study, expression of the utrophin gene during myogenic differentiation using the mouse C2 muscle cell line. We observed that in comparison to myoblasts, the levels of utrophin and its transcript were ~2-fold higher in differentiated myotubes. In order to address whether a greater rate of transcription contributed to the elevated levels of utrophin transcripts, we performed nuclear run-on assays. In these studies, we determined that the rate of transcription of the utrophin gene was ~2-fold greater in myotubes as compared to myoblasts. Finally, we examined the stability of utrophin mRNAs in muscle cultures by two separate methods; following transcription blockade with actinomycin D and by pulse-chase experiments. Under these conditions, we determined that the half-life of utrophin mRNAs in myoblasts was ~20 hours and that it remained largely unaffected during myogenic differentiation. Altogether, these results show that in comparison to other synaptic proteins and to dystrophin, expression of the utrophin gene is only moderately increased during myogenic differentiation.

INTRODUCTION

The process of myogenesis is characterized by a series of morphological and biochemical changes that result in the fusion and differentiation of mononucleated myoblasts into postmitotic myotubes (1,2). These changes are known to be accompanied by coordinated increases in the expression of several muscle proteins. For example, expression of cytoskeletal and contractile proteins such as dystrophin and myosin, is increased by ~10-fold during myogenic differentiation (3-8). In addition, many of the synapse-associated proteins including the acetycholine receptor (AChR), the neural cell-adhesion molecule (NCAM) and the enzyme acetycholinesterase (AChE), become highly expressed in multinucleated myotubes (9-18). In recent years, there has been considerable interest in unravelling the cellular and molecular events that underlie myogenic differentiation and in fact, some of the crucial steps have already been characterized (for review, see 19). In particular, the contribution of basic helix-loop-helix (bHLH) transcription factors from the MyoD family interacting with the E-box element, is now well recognized (see 19-22).

In 1989, Love and colleagues (23) identified an autosomal homologue to dystrophin, the gene involved in Duchenne muscular dystrophy (DMD) (24-26). This gene, now referred to as utrophin, encodes a large cytoskeletal protein of the spectrin superfamily that is ubiquitously expressed in most tissues (23, 26-31). In mature skeletal muscle, utrophin accumulates preferentially at the postsynaptic membrane of the neuromuscular junctions in both normal and dystrophic muscles (29, 32-35). Because of this compartmentalized expression, we began in a recent series of studies to examine the mechanisms involved in the expression of utrophin at the

neuromuscular junction. Using a combination of approaches, we showed that local transcriptional activation of the utrophin gene via nerve-derived factors such as agrin and ARIA/heregulin, contributes to the preferential localization of utrophin at the neuromuscular junction (36-38). In contrast to these recent developments however, there is currently less information available on the events contributing to the expression of utrophin during muscle differentiation. In the present study, we have therefore examined the expression of utrophin during myogenesis. Our main objective in these experiments was to determine whether expression of the utrophin gene was subject to regulatory mechanisms similar to those previously described for dystrophin (3-7, see also 24,25) and other synaptic proteins such as the AChR (9-14, see also 39-41) during myogenic differentiation. This appeared particularly important since; i) utrophin is a synaptic homologue to dystrophin; and ii) the utrophin promoter contains an E-box (42).

EXPERIMENTAL PROCEDURES

Tissue Culture. C2C12 muscle cells were cultured and maintained as described previously (see 37). Experiments were performed on either undifferentiated myoblasts (~50% confluency), confluent myoblasts or differentiated myotubes. For experiments involving the inhibition of RNA synthesis, 4 μg/ml of actinomycin D was added to the culture media (6,7,43) and samples were collected at different time-intervals thereafter. Normal human skeletal muscle cells were obtained from Clonetics-BioWhittaker Inc. (San Diego, California) and maintained according to the supplier's recommendations.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from cultured cells using Tripure as recommended by the manufacturer (Boehringer Mannheim, Indianapolis). The RNA concentration for each sample was determined using a Genequant II RNA/DNA spectrophotometer (Pharmacia, Quebec, Canada) and all samples were adjusted with RNase-free water to a final concentration of 50 ng/μl. Only 2 μl (100 ng of total RNA) of this dilution was used for RT-PCR as described (37, 44, 45). RT was performed for 45 minutes at 42 °C and the mixture was heated to 99 °C for 5 minutes to terminate the reaction. Negative controls were prepared by substituting the 2 μl of total RNA for RNase-free water. Utrophin cDNAs of 548 bp and 410 bp were specifically amplified using primers synthesized on the basis of available sequences for mouse (37) and human (29) cDNAs, respectively, as described in detail elsewhere (37,44,45). Amplification of the selected cDNAs was performed in a DNA thermal cycler (Perkin Elmer Cetus Co.; Norwalk, CT). Each cycle of amplification consisted of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min,

and extension at 72°C for 1 min. Typically, 30 to 34 cycles of amplication were performed since control experiments showed that these number of cycles were within the linear range of amplification (data not shown). cDNAs encoding the AChR α-subunit were amplified using primers based on the mouse sequence (46) (5', 5' GACTATGGAGGA GTGAAAAA 3'; and 3', 5' TGGAGGTGGAAGGGATTAGC 3') and they generate a 576 bp cDNA PCR product. Dystrophin cDNAs were amplified as described previously (37). In separate experiments, we verified that equivalent amounts of total RNA were used in our RT-PCR experiments by examining the levels of two well-established loading controls, S12 ribosomal RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (45). In these assays, we determined that their abundance was relatively consistent from sample to sample, since we observed less than 10% variation between them (data not shown) indicating that equivalent amounts of total RNA were indeed analysed.

PCR products were visualized on 1% agarose gel containing ethidium bromide. The 100-bp molecular mass marker (Life Technologies, Inc.; Burlington, ON) was used to estimate the molecular mass of the PCR products. For quantitative PCR experiments, PCR products were separated and visualized on 1.5% agarose gels containing the fluorescent dye Vistra Green (Amersham; Arlington Heights, IL) (37). The labeling intensity of the PCR product, which is linearly related to the amount of DNA, was subsequently quantitated using a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and accompanying ImageQuant software.

Immunoblotting. Cells were washed with phosphate-buffered saline (PBS), solubilized in Tris-HCl (1% sodium deoxycholate, 5% SDS, 0.5% Triton X-100, 1 mM PMSF, 5 mM iodoacetamide, 2 mg/ml aprotinin, 100 mM Tris-HCl, pH 8.0, 140 mM NaCl, and 0.025% NaN_3) and subjected to immunoblotting as described (37). Briefly, equivalent amounts of cell extracts (70 μg) were separated on a 6% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Sigma; St. Louis, MO). For immunoblotting, membranes were incubated with monoclonal antibodies directed against utrophin (dilution 1 into 100; Novocastra Laboratories, Newcastle upon Tyne, UK) and revealed using a commercially available chemifluorescence kit from New England Nuclear (NEN) Life Sciences (Boston, MA). To ensure that equivalent amounts of proteins were loaded for each sample, membranes were also stained with Ponceau S (Sigma).

Isolation of Nuclei and Run-on Assays. Nuclei were isolated and run-on transcription assays were performed as described (47-49). Briefly, ~10^7 cells (five 60 mm culture plates) cultures were washed with PBS, homogenized with a Dounce homogenizer in a solution containing 10% sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES, 0.5 mM EGTA, 2 mM EDTA, 0.1 mM spermine, 0.5 mM spermidine, 0.5 mM DTT, and 1 μM PMSF and nuclei were then isolated by centrifugation. Nuclei were resuspended in a solution containing 50% glycerol, 20 mM Tris, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 μM PMSF, and 10 U/μl RNase inhibitor and subjected to *in vitro* transcription by adding 200 μCi of [α-^{32}P]-UTP (Amersham) to label nascent transcripts for 30 min at 27 °C. Following DNase I digestion and protein denaturation, radiolabeled RNA was extracted using TriPure (see above) and hybridized to Protran nitrocellulose membranes (Schleicher and Schuell; Keene, NH) containing 10 μg of immobilized

genomic DNA, and cDNAs encoding utrophin, the AChR α–subunit, and GAPDH (49). Following hybridization, membranes were washed thoroughly (1X SSC, 0.1 % SDS) at 42 °C, and subjected to autoradiography. Signal intensities were quantitated using a Storm PhosphorImager and subsequently standardized to the genomic signal. For these experiments, utrophin cDNAs corresponded to the 548 bp mouse PCR product which was subcloned into the pCR 2.1 vector using the TA Cloning Kit (Invitrogen; San Diego, CA). The AChR α-subunit cDNA was kindly supplied by Dr. J.R. Sanes (Washington University, St. Louis, MO).

Pulse-chase analyses. Pulse-chase analyses were performed to measure the half-life of utrophin transcripts. To label cellular RNA, the cultures were exposed to [5,6-^{3}H] uridine (New England Nuclear; Boston, MA) for 4 hours (50,51). To terminate radioactive labeling, the cells were washed twice with DMEM, followed by two additional washes with DMEM containing 5 mM uridine and 2.5 mM cytidine. Cultures were then incubated with their appropriate media containing uridine and cytidine. At various time points thereafter (up to 36 hours), total RNA was isolated as described above. Radiolabeled RNA was subsequently hybridized to filters containing 5 μg of immobilized cDNAs encoding utrophin. Filters were then sprayed with Enhance spray (NEN) and subjected to autoradiography (BioMax; Kodak, Rochester, NY). The labeling intensity of the hybridization signal was then quantitated using a Storm PhosphorImager (Molecular Dynamics) and accompanying ImageQuant software.

RESULTS

Since expression of the AChR is known to increase markedly during myogenic differentiation (9-14), we initially verified that under our culture conditions AChR α-subunit expression was significantly increased in myotubes. Consistent with previous reports (9,11,14), we observed that during myogenic differentiation AChR α-subunit mRNA levels increased by ~9-fold ($P < 0.05$) (Figure 6.1A), and that the rate of transcription for this gene increased similarly under these conditions (up to 8-fold; $P<0.05$, $n=9$) (Figure 6.1B).

We next examined the levels of utrophin in confluent myoblasts and differentiated myotubes. In these experiments, we observed by immunoblotting that the levels of utrophin in myotubes were higher than those observed in confluent myoblasts (Figure 6.2A). As shown in Figure 2C, quantitative analysis revealed however, that utrophin levels increased by only ~2-fold during differentiation of myoblasts into myotubes. Ponceau staining of the membranes confirmed that an equal amount of total protein had been loaded onto each lane of the gel (Figure 6.2B).

To determine whether the increase in utrophin levels involved an accumulation of utrophin transcripts, we measured the abundance of utrophin mRNAs in undifferentiated myoblasts (~50% confluency), confluent myoblasts and myotubes. Utrophin mRNAs were already present in undifferentiated myoblasts and their level increased by only 12% once the cells had reached confluence (Figure 6.3B). Differentiation of the myoblasts into myotubes resulted in a further increase in the levels of utrophin transcripts (Figure 6.3A). In agreement

with the immunoblot data, the abundance of utrophin transcripts increased by ~2-fold in myotubes as compared to myoblasts (Figure 6.3B). Consistent with these results obtained with mouse myotubes, we noted that myogenic differentiation of human skeletal muscle cells also led to a 1.5- to 2-fold increase in utrophin (Figure 6.4A) and its mRNA (Figure 6.4B) (see also 52,53).

In order to elucidate the mechanisms responsible for the increased expression of utrophin during muscle cell development, we next performed nuclear run-on assays to measure the transcriptional activity of specific genes during myogenesis. In agreement with the mRNA data (Figures 6.3A and 6.3B), we determined that expression of the utrophin gene increased during myogenic differentiation (Figure 6.5A). Indeed, quantitation of these results revealed that the transcriptional activity of the utrophin gene in myotubes was ~2-fold higher ($P<0.05$) than the activity observed in myoblasts (Figure 6.5B). By contrast, the rate of transcription of the GAPDH gene remained largely unchanged during myogenesis (see also Ref. 9).

In separate studies, we also determined the half-life of utrophin transcripts in skeletal muscle cells in culture using two separate methods. In one case, cultures were exposed to actinomycin D for up to 40 hours and RNA samples were collected and analysed by RT-PCR. Consistent with two recent studies examining the stability of dystrophin mRNA using actinomycin D (6,7), we determined that the half-life of dystrophin transcripts was ~16 hours. In addition, we observed that the half-life of utrophin transcripts was ~20 hours in myoblasts and that it remained largely unaffected in myotubes (Figure 6.6A and B). In a second experimental

approach, we performed pulse-chase experiments. In these assays, we determined that the half -life of utrophin mRNAs was also ~20 hours in both myoblasts and myotubes (Figure 6.6C). The findings that both actinomycin D and the pulse-chase experiments yielded similar results are consistent with a previous study comparing these distinct methods to determine mRNA half-lives (54).

DISCUSSION

Previous studies have indicated that utrophin expression during embryological development is increased along the length of the muscle fiber and remains elevated until early postnatal development, at which point utrophin becomes preferentially localized to the neuromuscular and myotendinous junctions (29, 55-57). However, the exact mechanisms that regulate the levels and localization of utrophin during development are currently not well understood. In the present study, we have begun to examine this issue by determining initially the mechanisms controlling utrophin expression in C2 cells undergoing myogenic differentiation in culture.

In agreement with our findings obtained with the AChR α-subunit gene, myogenic differentiation of muscle cells is known to lead to large increases (~10-fold) in the levels of various transcripts encoding for example, dystrophin (3-7) and several of the AChR subunits (9-14). In this context, it appears well established that during myogenic differentiation, expression of several genes is regulated at least partially, by MyoD family members that interact with E-box motifs located within the 5' flanking region of these genes (see for reviews 19-22). Since the utrophin promoter contains one E-box consensus sequence (42), we expected to observe a substantial increase in the expression of this gene. However, in contrast to the large changes seen in dystrophin and AChR expression during myogenesis, we determined that utrophin mRNA and protein levels were only increased by ~2-fold during myogenic differentiation.

It is well established that denervation of skeletal muscle leads to a large increase in the expression of AChR subunit genes via a transcriptional induction involving the E-box motif (see for review 40). By contrast, it has been shown that denervation, which also leads to a significant increase in the expression of myogenic factors (58-60), does not have a significant impact on utrophin expression (44,61). Therefore, our results showing that myogenic differentiation is accompanied by a rather modest increase in utrophin expression, are in fact entirely consistent with these previous findings observed with the denervation model and hence, further support the view that the MyoD family of transcription factors are not major regulators of utrophin expression. However, since it is known that multiple E-box elements located in close proximity to each other are necessary for myogenic factors to transcriptionally activate muscle genes (62-66), it remains plausible that under specific conditions, a second DNA regulatory element within the utrophin promoter may act in cooperation with the single E-box to regulate expression of the utrophin gene.

Recently, we demonstrated that expression of utrophin in skeletal muscle fibers was dependent upon the presence of an intact N-box element and on the Ets-related transcription factor GA-binding protein (GABP), which binds to this consensus sequence (37,38, see also 67). Interestingly, analysis of the utrophin promoter reveals that the E-box element is in fact located in the immediate vicinity of the N-box motif (36,42). Since Ets-related proteins including GABP, may possess a conserved domain with homology to the bHLH transcription factors such as myogenic factors (68) and since Ets proteins usually act in cooperation with other transcription factors (69), it appears possible therefore, that the E- and N-box elements along with their respective transcription factors, act in a synergistic manner to regulate expression of

the utrophin gene (see further discussion in 70). This view is particularly attractive especially if we consider that these two DNA regulatory elements are also found in close proximity to each other in the AChR δ- and ε-subunit promoters (70-74) as well as in an intronic region of the AChE gene shown recently to be critical for regulating expression of this gene (75).

Together with the data obtained using the denervation model (see above) and the observation that utrophin is found in a wide range of tissues (23,26-31), our results are entirely coherent with the fact that the utrophin gene displays features characteristic of housekeeping genes (42) which are constitutively and ubiquitously expressed (see also 45). Therefore, it may be assumed that, with the exception of the synaptic regions of muscle fibers where utrophin expression appears enhanced via the effects of basal lamina-associated components (37,38), expression of this gene does not vary markedly according to the state of differentiation and innervation of muscle fibers. If indeed transcription of the utrophin gene remains rather constant throughout the lifespan of a muscle fiber then, one has to wonder about the mechanisms involved in the accumulation of the utrophin protein at the sarcolemma of embryonic muscle fibers (57). Given that both dystrophin and utrophin interact with a complex of dystrophin-associated proteins (DAP) (76), one possibility is that the simple competition between dystrophin and utrophin for available binding sites may dictate the levels of utrophin present at the sarcolemma. This view is particularly attractive since the number of DAP-binding sites appears relatively constant during myogenesis (77) whereas expression of dystrophin is greatly enhanced (3-7). Therefore, when dystrophin levels are low such as during the early stages of myogenic differentiation, utrophin may be sufficiently expressed to bind to a large number of available

DAP-binding sites at the sarcolemma. At later stages of muscle fiber development, the significant increase in dystrophin expression with no parallel changes in the availability of DAP-binding sites, would therefore result in dystrophin out-competing utrophin. In this context, it is important to note that a similar competition-based model has previously been proposed to explain the presence of distinct spectrin isoforms within the membrane cytoskeleton of developing erythrocytes (78,79). Furthermore, this model is also consistent with the previously reported increase in utrophin expression at the sarcolemma of DMD muscle fibers (29, 34, 53, 80) in the absence of a concomitant increase in the levels of its mRNA (45) and with the presence of utrophin mRNAs in extrasynaptic regions of muscle fibers (36, 81). Together, these data clearly highlight the important contribution of post-translational mechanisms in the overall regulation of the levels and localization of utrophin expression along developing and mature skeletal muscle fibers.

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REFERENCES

1. Olson, E.N., Brennan, T.J., Chakroborty, T., Cheng, T.C., Cserjesi, P., Edmondson, D., James, G. and Li, J. (1991) *Mol. Cell Biochem.* **104**, 7-13.

2. Ludolph, D.C. and Konieczny, S.F. (1995) *FASEB J.* **9**, 1595-1604.

3. Lev, A.A., Feener, C.C., Kunkel, L.M. and Brown, R.H. Jr. (1987) *J. Biol. Chem.* **262**, 15817-15820.

4. Nudel, U., Robzyk, K. and Yaffe, D. (1988) *Nature* **331**, 635-639.

5. Passaquin, A.C., Metzinger, L., Leger, J.J., Warter, J.M. and Poindron, P. (1993) *J. Neurosci. Res.* **35**, 363-372.

6. Tennyson, C.N., Shi, Q.W. and Worton, R.G. (1996) *Nucl. Acids Res.* **24**, 3059-3064.

7. Tennyson, C.N., Dally, G.Y., Ray, P.N. and Worton, R.G. (1996) *Hum. Mol. Genet.* **5**, 1559-1566.

8. Medford, R.M., Nguyen, H.T. and Nadal-Ginard, B. (1983) *J. Biol. Chem.* **258**, 11063-11073.

9. Buonanno, A. and Merlie, J.P. (1986) *J. Biol. Chem.* **261**, 11452-11455.

10. Evans, S. Goldman, D., Heinemann, S. and Patrick, S.J. (1987) *J. Biol. Chem.* **262**, 4911-4916.

11. Crowder, C.M. and Merlie, J.P. (1988) *Mol. Cell Biol.* **8**, 5257-5267.

12. Baldwin, T.J. and Burden, S.J. (1988) *J. Cell Biol.* **107**, 2271-2279.

13. Fontaine, B. and Changeux, J.P. (1989) *J. Cell Biol.* **108**, 1025-1037.

14. Prody, C.A. and Merlie, J.P. (1991) *J. Biol. Chem.* **266**, 22588-22596.

15. Fuentes, M. E. and Taylor, P. (1993) *Neuron* **10**, 679-687.

16. Grubic, Z., Komel, R., Walker, W.F. and Miranda, A.F. (1995) *Neuron* **14**, 317-327.

17. Moore, S.E., Thompson, J. Kirkness, V. Dickson, J.G. and Walsh, F.S. (1987) *J. Cell Biol.* **105**, 1377-1386.

18. Roubin, R. and Goridis, C. (1995) *Exp. Cell Res.* **200**, 500-505.

19. Arnold, A.H. and Winter, B. (1998) *Curr. Opin. Genet. Dev.* **8**, 539-544.

20. Weintraub,H. Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T.K., Turner, D., Rupp, R. and Hollenberg, S. (1991) *Science* **251**, 761-766

21. Weintraub, H. (1993) *Cell* **75**, 1241-1244.

22. Rudnicki, M.A. and Jaenisch, R. (1995) *BioEssays* **17**, 203-209.

23. Love, D.R., Hill, D.F., Dickson, G., Spurr, N.K., Byth, B.C., Marsden, R.F., Walsh, F.S., Edwards, Y.H. and Davies, K.E. (1989) *Nature* **339**, 55-58.

24. Ahn, A.H. and Kunkel, L.M. (1993) *Nature Genet.* **3**, 283-291.

25. Worton, R. (1995) *Science* **270**, 755-760.

26. Gramolini, A.O. and Jasmin, B.J. (1998) *Neuromusc. Disord.* **8**, 351-361.

27. Love, D.R., Morris, G.E., Ellis, J.M., Fairbrother, U., Marsden, R.F., Bloomfield, J.F., Edward, Y.H., Slater, C.P., Parry, D.J. and Davies, K.E. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3243-3247.

28. Khurana, T.S., Hoffman, E.P. and Kunkel, L.M. (1990) *J. Biol. Chem.* **265**, 16717-16720.

29. Khurana, T.S., Watkins, S.C., Chafey, P., Chelly, J., Tome, F.M., Fardeau, M., Kaplan, J. and Kunkel, L.M. (1991) *Neuromusc. Disord.* **1**, 185-194.

30. Khurana, T.S., Watkins, S.C. and Kunkel, L.M. (1992) *J. Cell Biol.* **119**, 357-366.

31. Matsumura, K., Shasby, D.M. and Campbell, K.P. (1993) *FEBS Lett.* **326**, 289-293.

32. Fardeau, M., Tome, F. M., Collin, H., Augier, N., Pons, F., Leger, J. and Leger, J. L. (1990) *Compt. Rend. Acad. Sci.* **311**, 197-204.

33. Ohlendieck, K., Ervasti, J.M., Matsumura, K., Kahl, S.D., Leveille, C.J. and Campbell, K.P. (1991) *Neuron* **7**, 499-508.

34. Helliwell, T.R., thi Man, N., Morris, G.E. and Davies, K.E. (1992) *Neuromusc. Disord.* **2**, 177-184.

35. thi Man, N., Ellis, J.M., Love, D.R., Davies, K.E., Gatter, K.C., Dickson, G. and Morris, G.E. (1991) *J. Cell Biol.* **115**, 1695-1700.

36. Gramolini, A.O., Dennis, C.L.,Tinsley, J.M., Robertson, G.S., Cartaud, J., Davies, K.E. and Jasmin, B.J. (1997) *J. Biol. Chem.* **272**, 8117-8120.

37. Gramolini, A.O., Burton, E.A., Tinsley, J.T., Ferns, M.F., Cartaud, A., Cartaud, J., Davies, K.E., Lunde, J.A. and Jasmin, B.J. (1998) *J. Biol. Chem.* **273,** 736-743.

38. Gramolini, A.O., Angus, L.A., Schaeffer, L., Burton, E.A., Tinsley, J.T., Davies, K.E., Changeux, J.P. and Jasmin, B.J. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 3223-3227.

39. Hall, Z. W. and Sanes, J. R. (1993) *Cell* **72**, 99-121.

40. Duclert, A. and Changeux, J.P. (1995) *Physiol. Rev.* **75**, 339-368.

41. Burden, S.J. (1998) *Genes Dev.* **12**, 133-148.

42. Dennis, C. L., Tinsley, J. M., Deconinck, A. E. and Davies, K. E. (1996) *Nucl. Acids Res.* **24**, 1646-1652.

43. Taormino, J.P. and Fambrough, D.M. (1990) *J. Biol. Chem.* **265**, 4116-4123.

44. Jasmin, B.J., Alameddine, H., Lunde, J.A., Stetzkowksi-Marden, F., Collin, H., Tinsley, J.M., Davies, K.E., Tome, F.M.S., Parry, D.J. and Cartaud, J. (1995) *FEBS Lett.* **374**, 393-398.

45. Gramolini, A.O., Karpati, G. and Jasmin, B.J. (1999) *J. Neuropath. Exp. Neurol.* **58**, 235–244.

46. Boulter, J., Luyten, W., Evans, K., Mason, P., Ballivet, M., Goldman, D., Stengelin, S., Martin, G., Heineman, S. and Patrick, J. (1985) *J. Neurosci.* **5**, 2545-2552.

47. Ray,A., Kyselovic, J., Leddy, J.J., Wigle, J.T., Jasmin, B.J. and Tuana, B.S. (1995) *J. Biol. Chem.* **270**, 25837-25844.

48. Boudreau-Lariviere, C. and Jasmin, B.J. (1999) *FEBS Lett.* **444**, 22-26.

49. Chan, R.Y.Y., Adatia, F.A., Krupa, A.M. and Jasmin, B.J. (1998) *J. Biol. Chem.* **273**, 9727-9733.

50. Chedrese, P.J., Kay, T.W.H. and Jameson, J.L. (1994) *Endocrinol.* **134**, 2475-2481.

51. Hod, Y. and Hanson, R.W. (1988) *J. Biol. Chem.* **263**, 7747-7752.

52. Karpati, G., Carpenter, S., Morris, G.E., Davies, K.E., Guerin, C. and Holland, P. (1993) *J. Neuropath. Exp. Neurol.* **52**, 119-128.

53. Pasquini, F., Guerin, C., Blake, D., Davies, K., Karpati, G. and Holland, P. (1995) *Neuromusc. Disord.* **5**, 105-114.

54. Harrold, S., Genovese, C., Kobrin, B., Morrison, S.L. and Milcarek, C. (1991) *Anal. Biochem.* **198**, 19-29.

55. Schofield, J., Houzelstein, D., Davies, K.E., Buckingham, M. and Edwards, Y.H. (1993) *Dev. Dynamics* **198**, 254-264.

56. Mora, M., Di Blasi, C., Barresi, R., Morandi, L., Brambati, B., Jarre, L. and Cornelio, F. (1996) *Dev. Brain Res.* **91**, 70-82.

57. Clerk, A., Morris, G.E., Dubowitz, V., Davies, K.E. and Sewry, C. (1993) *Histochem. J.* **25**, 554-561.

58. Duclert, A., Piette, J. and Changeux, J.P. (1991) *Neuroreport* **2**, 25-28.

59. Eftimie, R., Brenner, H.R. and Buonanno, A. (1991) *Proc. Natl. Acad. Sci. USA* **88**,1349-1353.

60. Witzemann, V., Brenner, H.R. and Sakmann, B. (1991) *J. Cell Biol.* **114**, 125-141.

61. Biral, D., Senter, L. and Salviati, G. (1996) *J. Muscle Res. Cell Motil.* **17**, 523-532.

62. Berberich, C., Durr, I., Koenen, M. and Witzemann, V. (1993) *Eur. J. Biochem.* **216**, 395-404.

63. Durr, I., Numberger, M., Berberich, C. and Witzemann, V. (1994) *Eur. J. Biochem.* **224**, 353-64.

64. Bessereau, J.L., Stratford-Perricaudet, L.D., Piette, J., Le Poupon, C. and Changeux, J.P. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 1304-8.

65. Kraner, S.D., Rich, M.M., Kallen, R.G. and Barchi, R.L. (1998) *J. Biol. Chem.* **273**, 11327-11334.

66. Kraner, S.D., Rich, M.M., Sholl, M.A., Zhou, H., Zorc, C.S., Kallen, G. and Barchi, R.L. (1999) *J. Biol. Chem.* **274**, 8129-8136.

67. Khurana, T.S., Rosmarin, A.G., Shang, T., Krog, T.O.B., Das, S. and Gammeltoft, S. (1999) *Mol. Biol. Cell* **10**, 2075-2086.

68. Seth, A. and Papas, T. (1990) *Oncogene* **5**, 1761-1767.

69. Crepieux, P., Coll, J. and Stehlin, D. (1994) *Crit. Rev. Oncogene* **5**, 615-638.

70. Schaeffer, L., Duclert, N., Huchet-Dymanus, M. and Changeux, J.P. (1998) *EMBO J.* **17**, 3078-3090.

71. Duclert, A., Savatier, N. and Changeux, J. P. (1993) *Proc. Natl. Acad. Sci.USA* **90**, 3043-3047.

72. Koike, S., Schaeffer, L. and Changeux, J.P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 10624-10628.

73. Duclert, A., Savatier, N., Schaeffer, L. and Changeux, J.P. (1996) *J. Biol. Chem.* **271**, 17433-17438.

74. Fromm, L. and Burden, S.J. (1998) *Genes Dev.* **12**, 3074-3083.

75. Chan, R.Y.Y., Boudreau-Lariviere, C., Angus, L.M., Mankal, F.A. and Jasmin, B.J. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 4627-4632.

76. Matsumura, K., Ervasti, J.M., Ohlendieck, K., Kahl, S.D. and Campbell, K.P. (1992) *Nature* **360**, 588-591.

77. Gee, S.H., Montanaro, F., Lindenbaum, M.H. and Carbonetto, S. (1994) *Cell* **77**, 675-686.

78. Woods, C.M. and Lazarides, E. (1988) *Ann. Rev. Med.* **39**, 107-22.

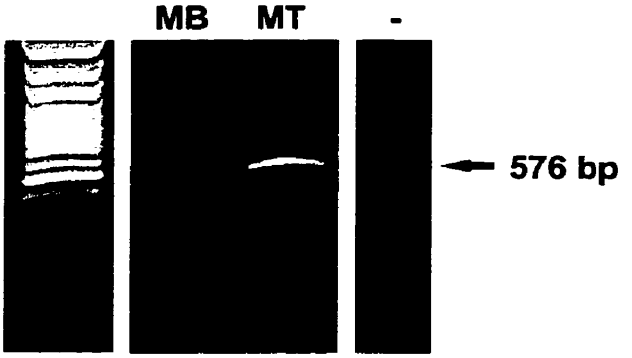
79. Lazarides, E. and Woods, C. (1989) *Ann. Rev. Cell Biol.* **5**, 427-52.

80. Taylor, J., Muntoni, F. Dubowitz, V. and Sewry, C.A. (1997) *Neuropath. Appl. Neurobiol.* **23**, 399-405.

(81) Vater, R., Young.C., Anderson, L.V.B., Lindsay, S., Blake, D.J., Davies, K.E., Zuellig, R. and Slater, C.R. (1998) *Mol. Cell. Neurosci.* **10**, 229-242.

Figure 6.1. Differentiation of C2C12 muscle cells leads to a significant increase in AChR α-subunit expression. (A) Shown is a representative example of an ethidium bromide-stained agarose gel of RT-PCR products corresponding to AChR α-subunit cDNAs obtained from myoblasts (MB) and myotubes (MT). Left lane is the 100 bp molecular mass marker (Life Technologies). The negative control lane is marked with a minus sign. Similar results were obtained in five independent experiments. (B) Nuclear run-on assays reveal that AChR α-subunit gene transcription is significantly increased during myogenic differentiation. Shown are representative examples of nine independent experiments.

A)



AChRα

B)

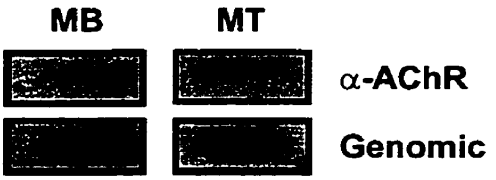


Figure 6.2. Utrophin protein levels increase during myogenesis. Muscle cells were solubilized and protein extracts were subjected to immunoblotting. (A) is a representative example of a western blot showing utrophin levels in myoblasts (MB) vs myotubes (MT). (B) The same membrane was reprocessed for ponceau staining to stain total protein. Relative molecular masses are indicated at right. (C) Utrophin levels were quantitated and expressed as percent of the levels seen in confluent myoblasts. Shown are the results obtained with four independent experiments. All data are expressed as mean ± SEM. Asterisk denotes a significant difference (Student's *t*-test, $P < 0.05$).

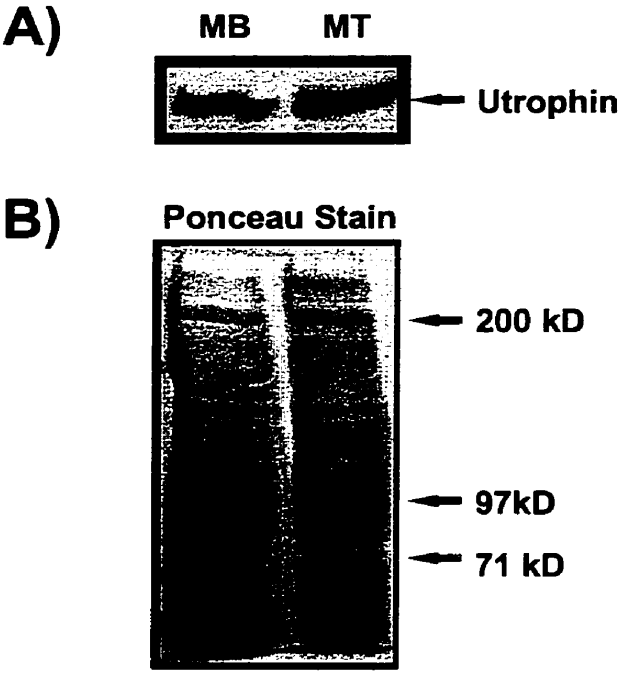

A)
MB
MT
Utrophin
B)
Ponceau Stain
200 kD
97kD
71 kD
C)
Utrophin Levels (% Myoblast)
250
200
150
100
50
0
*
MB
MT

Figure 6.3. Myogenic differentiation increases utrophin transcript levels. (A) A representative ethidium bromide-stained agarose gel of RT-PCR products corresponding to utrophin cDNAs obtained from myoblasts (MB) and myotubes (MT). The negative control lane is marked with a minus sign. Left lane is the 100 bp molecular mass marker (Life Technologies). (B) Utrophin transcript levels were quantitated and expressed as a percent of the levels seen in non-confluent myoblasts (undifferentiated; U). Shown are the results obtained with a minimum of five independent experiments. Asterisk denotes a significant difference from undifferentiated myoblasts (Student's *t*-test, $P < 0.05$).

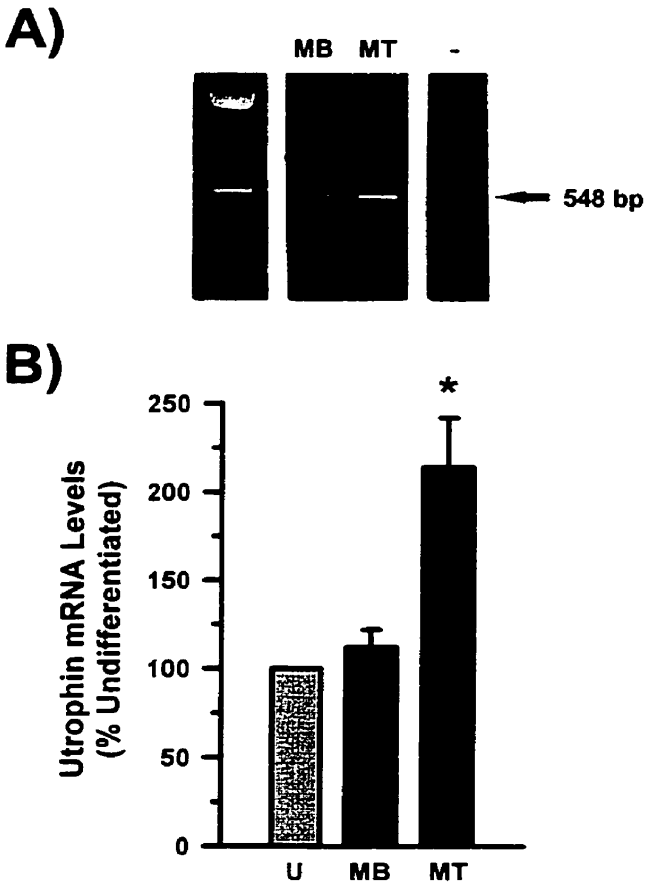

A)
MB
MT
-
548 bp
B)
Utrophin mRNA Levels
(% Undifferentiated)
250
200
150
100
50
0
*
U
MB
MT

Figure 6.4. Utrophin protein and mRNA levels in human skeletal muscle cells are increased during myogenic differentiation. (A) is a representative immunoblot revealing that utrophin levels increase during muscle cell development from undifferentiated myoblasts (U) to myotubes (MT). (B) is a representative ethidium bromide-stained agarose gel of utrophin PCR products showing the increase in utrophin transcript levels with differentiation from undifferentiated myoblasts (U) into myotubes (MT). The negative control lane is marked with a minus sign. Left lane is the 100 bp molecular mass marker (Life Technologies). Shown are representative results obtained from four independent experiments.

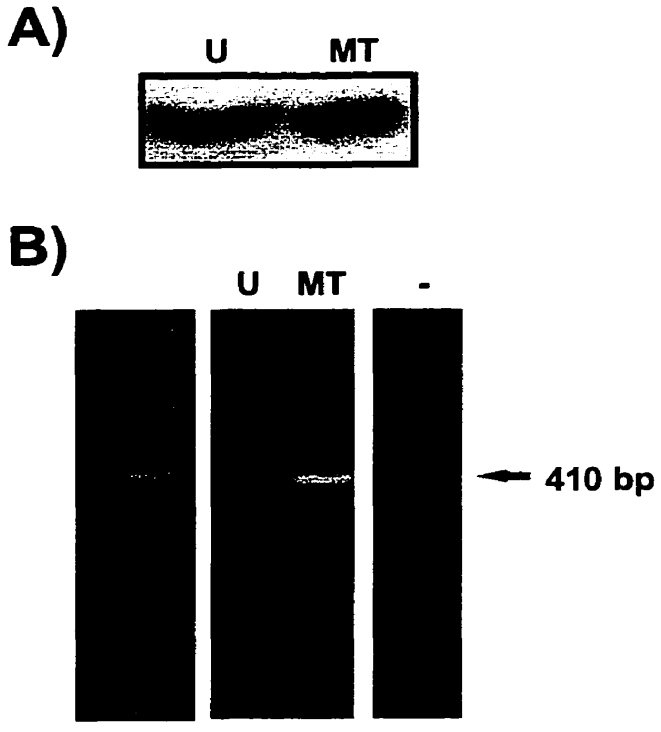
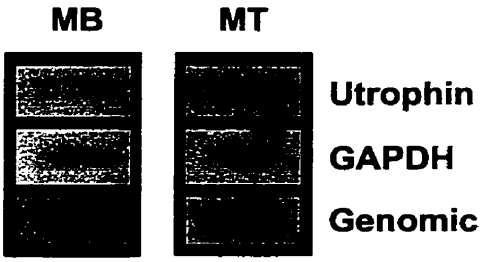

A)
U
MT
B)
U
MT
-
410 bp

Figure 6.5. Myogenic differentiation results in an increase in transcription of the utrophin gene. (A) Shown are representative autoradiograms of run-on assays using nuclei obtained from myoblasts (MB) and myotubes (MT). (B) Quantitation of the nuclear run-on assays. Hybridization signals were determined using a Storm Phosphorimager and are normalized to the genomic hybridization signal. Shown are the results obtained from six independent experiments. Asterisks denote significant differences from myoblast levels (Student's *t*-test, $P < 0.05$).

A)


MB
MT
Utrophin
GAPDH
Genomic

B)

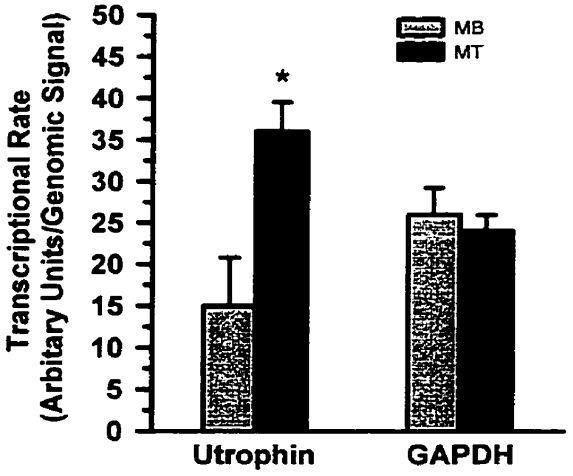
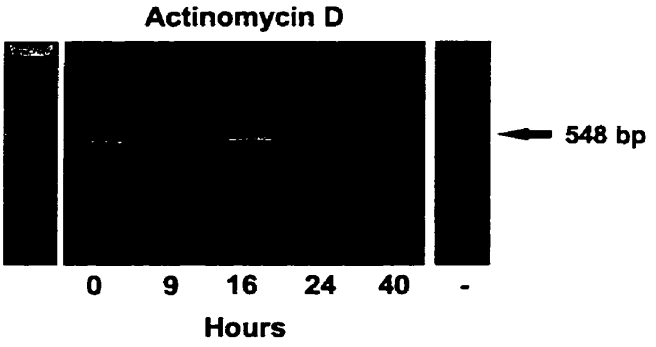
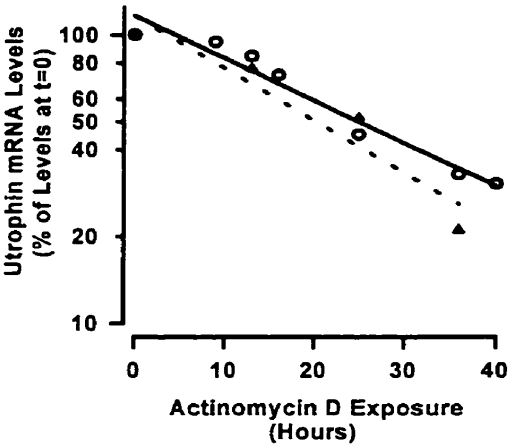

Transcriptional Rate
(Arbitary Units/Genomic Signal)
50
45
40
35
30
25
20
15
10
5
0
MB
MT
*
Utrophin
GAPDH

Figure 6.6. Half-life determination of utrophin transcripts in myogenic cultures. (A) Inhibition of RNA synthesis was achieved by exposing cultures to actinomycin D at time zero. Shown is a representative ethidium bromide-stained agarose gel of utrophin PCR products following actinomycin D exposure for different time periods. (B) Quantitation of the half-life of utrophin transcripts by regression analysis. Note that the half-life for utrophin mRNAs is ~20 hours in undifferentiated myoblasts and is largely unchanged in myotubes (~24 hours). Symbols; ●, utrophin transcript levels at time zero for both myoblasts and myotubes; ▲, myoblasts; dashed line, linear regression for myoblast data; ○, myotubes; solid line, linear regression for myotube data. Shown are the data obtained using pooled samples from five independent experiments. (C) Pulse-chase analysis of utrophin mRNAs in muscle cells. ^{3}H-labeled RNA was incubated with immobilized cDNAs encoding utrophin and subjected to autoradiography. Shown are representative examples obtained using RNA harvested from cultures up to 36 hours following exposure to ^{3}H-uridine. See text for quantitation.

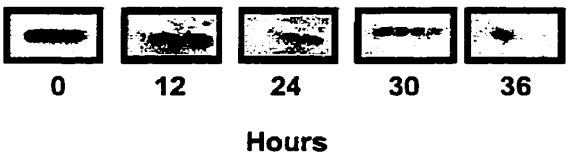
A)


Actinomycin D
548 bp
0
9
16
24
40
-
Hours

B)


Utrophin mRNA Levels
(% of Levels at t=0)
100
80
60
50
40
20
10
0
10
20
30
40
Actinomycin D Exposure
(Hours)

C)


0
12
24
30
36
Hours

CHAPTER 7

DISCUSSION

Although utrophin is known to accumulate selectively at the neuromuscular junction, the cellular and molecular mechanisms contributing to this compartmentalized expression have remained largely unknown. Therefore, the purpose of the present studies was to investigate the mechanisms underlying the selective accumulation of utrophin at the postsynaptic membrane of the neuromuscular synapse. We determined that local transcription contributes to the accumulation of utrophin at the neuromuscular junction. We also defined the promoter elements involved in this local transcription and determined that the N-box element is a key consensus sequence that directs transcriptional control of utrophin synaptic expression. Furthermore, utrophin gene transcription was shown to be dependent on the extracellular matrix proteins agrin and ARIA/heregulin, and this regulation was dependent upon the N-box element. Indeed, agrin and ARIA/heregulin may ultimately initiate a cell signaling cascade that activates the ETS-related transcription factor, GA-binding protein (GABP) which binds and activates the N-box element. In separate studies, we also examined the effect of myogenesis on the transcriptional regulation of utrophin gene expression. In these experiments, we determined that, in contrast to the large changes in AChR, utrophin gene expression was only marginally increased.

In addition to these transcriptional events that control utrophin levels and localization, it also became apparent that transcription alone could not account for the complete regulation of utrophin expression under certain conditions. Indeed, we observed a discordant relationship between utrophin transcript levels and protein levels in regenerating muscles or muscles obtained

from DMD patients, indicating that utrophin expression may be controlled by post-transcriptional events. Altogether, it appears likely that the regulation of utrophin levels and localization are coordinately regulated both by transcriptional and post-transcriptional events, ultimately leading to the preferential accumulation of utrophin at the neuromuscular junction.

I. Additional Putative Signaling Mechanisms Regulating Utrophin Expression

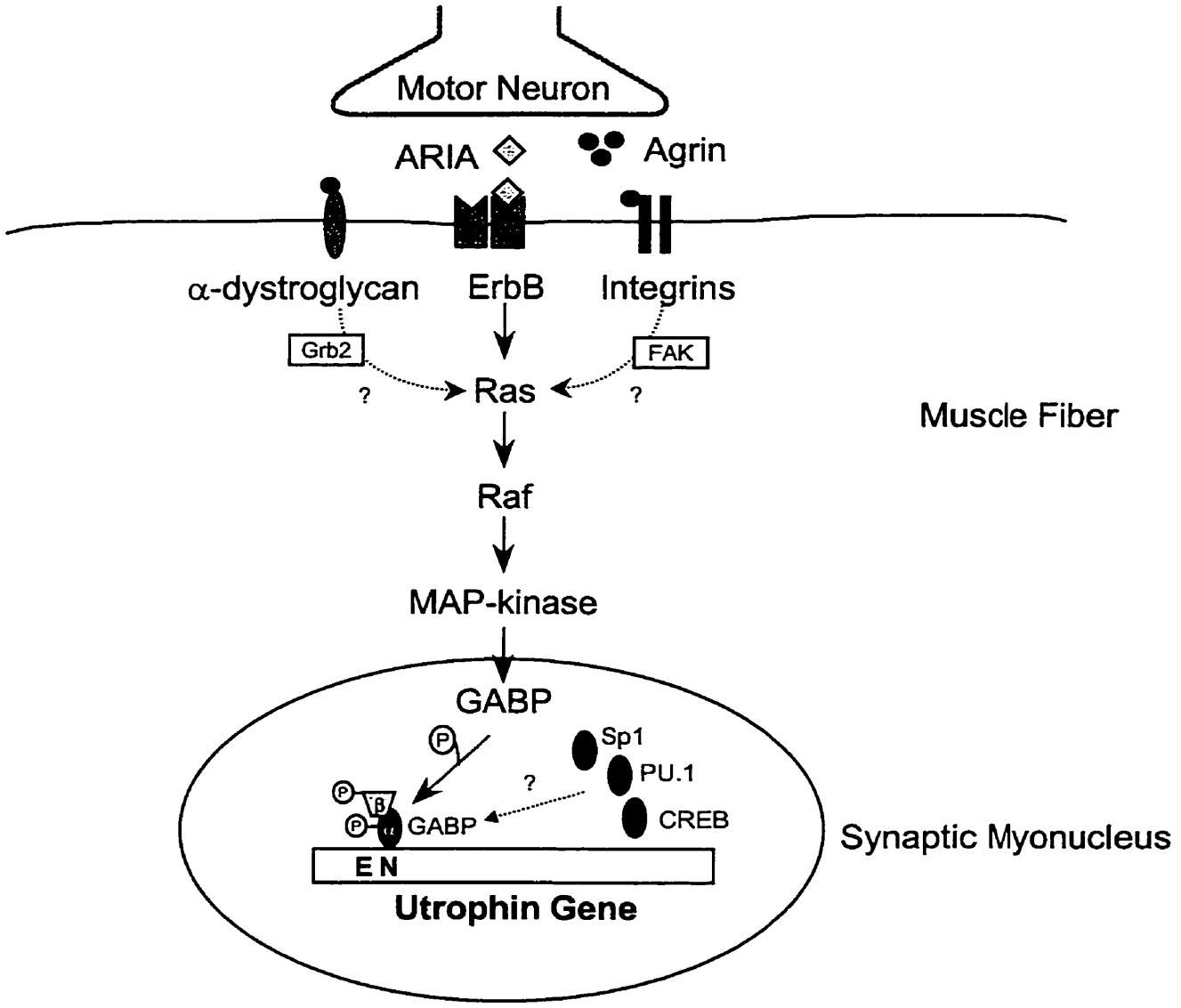
It appears that maintenance of utrophin expression at the neuromuscular junction involves several basal lamina-associated factors which converge to ultimately phosphorylate and activate, via the Ras/MAP-kinase pathway (Tansey *et al.*, 1996; Sapru *et al.*, 1998; Schaeffer *et al.*, 1998; Fromm and Burden, 1998), the Ets-related transcription factor GABP (Figure 7.1; see also Khurana *et al.*, 1999). In fact, these results are also coherent with the observations that ETS proteins are known targets of the MAP kinase pathway (Marais *et al.*, 1993; Brunner *et al.*, 1994; O'Hagan and Hassell, 1999), and that GABP can be phosphorylated by MAP kinase (Flory *et al.*, 1996). In addition, GABP α-subunit mRNA preferentially accumulates in synaptic regions *in vivo* (Schaeffer *et al.*, 1998), further supporting the conclusion that GABP is the factor that binds to the N-box and stimulates synaptic transcription. Therefore, these results support the participation of GABP in regulating local activation of gene expression at the neuromuscular junction.

The involvement of ETS-related transcription factors in utrophin gene regulation raises the possibility that other transcription factors may also contribute to the preferential transcription of the utrophin gene within the postsynaptic membrane domain. For example, ETS-related

transcription factors have been observed to function in cooperation with other transcription factors (Crepieux *et al.*, 1994). Indeed, there is evidence that GABP can directly bind additional transcription factors, such as cAMP response-binding element (CREB/p300) (Bannert *et al.*, 1999), Sp1 (Rosmarin *et al.*, 1998) or PU.1 (Rosmarin *et al.*, 1995) to regulate gene expression. It also may be of particular importance that the ETS-related family of transcription factors have been suggested to interact with the myogenic regulatory factors via a conserved domain with homology to basic helix-loop-helix (bHLH) domains (Seth and Papas, 1990). Therefore, it is possible that GABP may interact with members of the MyoD family of transcription factors and thus regulate utrophin expression. Such a mechanism appears justifiable since the E-box element which binds the MyoD family of transcription factors is located in the immediate vicinity of the N-box in the utrophin gene (Dennis *et al.*, 1996), the δ- and ε-subunit genes of AChR (Koike *et al.*, 1995; Duclert *et al.*, 1996) and the AChE gene (Chan *et al.*, 1999), perhaps indicative of such regulatory mechanisms.

Although the role of agrin interacting with MuSK and clustering other proteins in the regulation of gene expression at developing synapse is appealing, it is nonetheless complicated by the observations that the muscle isoform of agrin increases expression of utrophin (Chapter 2) and the AChR ε-subunit genes (Jones *et al.*, 1996) but only the neural isoform of agrin appears to efficiently cluster synaptic proteins and to induce the tyrosine phosphorylation of MuSK (Glass *et al.*, 1996). Therefore, MuSK alone is unlikely to be the mechanism by which utrophin and the ε-subunit of AChR genes can be regulated by agrin.

Figure 7.1. Schematic diagram of putative regulatory mechanisms controlling utrophin expression at the neuromuscular junction. The presence of agrin at the neuromuscular junction leads to the clustering of numerous synaptic proteins including the acetylcholine receptor (not shown), heregulin and the ErbB receptors, on the surface of the developing myotubes via binding and activation of MuSK. Subsequent activation of ErbB receptors by heregulin triggers, in turn, a signaling cascade involving RAS-MAP kinase which ultimately phosphorylates and activates the ETS-related transcription factor GABP. Since GABP is known to bind to the N-box element, this agrin-heregulin dependent regulatory mechanism may thus induce the enhanced expression of utrophin at the neuromuscular junction via transactivation of this gene. In addition, it is possible that utrophin gene expression is also controlled by agrin interacting with: i) the synaptic integrins activating FAK; or ii) α-dystroglycan activating Grb2. Finally, the involvement of additional transcriptional factors, such as Sp1, CREB, or PU.1 in GABP-mediated regulation of utrophin expression has yet to be determined. Symbols: E, E-box element; CREB, cAMP-response element binding; GABP, GA-binding protein; N, N-box element.


Motor Neuron
ARIA
Agrin
α-dystroglycan
ErbB
Integrins
Grb2
FAK
?
Ras
?
Muscle Fiber
Raf
MAP-kinase
GABP
Sp1
PU.1
?
GABP
CREB
E N
Utrophin Gene
Synaptic Myonucleus

It is possible that agrin binds and activates other receptors which may not discriminate between the various isoforms of agrin. For instance, it is known that specific integrins, i.e., α3, α7, αv, and β1 are found to accumulate at the neuromuscular junction (Bozyczko *et al.*, 1989; Martin *et al.*, 1996); loss of specific integrins such as α7β1 (Mayer *et al.*, 1997; Hodges *et al.*, 1997) or αv (Taverna *et al.*, 1998) can result in forms of muscular dystrophy; and that agrin isoforms can bind with high affinity to integrins (Martin and Sanes, 1997). As the integrins are well known signal transducing receptors for extracellular matrix proteins, agrin binding may thus initiate a signal mechanism controlling synaptic expression. Indeed, the integrins are known to stimulate a cell signaling pathway which involves focal adhesion kinase (FAK) and other members of the src family of tyrosine kinases (Hynes *et al.*, 1992; Clark and Brugge, 1995; Lafrenie and Yamada, 1996). In fact, it may be of particular relevance that integrin signaling via FAK can activate MAP kinase (Renshaw *et al.*, 1999). Therefore, it is possible that integrins may transduce the agrin signals from the extracellular matrix to the nucleus and activate transcription of the utrophin gene within synaptic myonuclei as a result of MAP kinase and GABP activation.

In addition to the integrins, agrin may act to regulate synaptic expression via binding to different cell surface receptors, such as α-dystroglycan. Indeed, α-dystroglycan may be a likely candidate to regulate synaptic gene expression for several reasons. Firstly, chimeric mice deficient in α-dystroglycan possess aberrant neuromuscular junctions highlighting a central role for this molecule in synaptic organization and differentiation (Côté *et al.*, 1999). Secondly, α-

dystroglycan is known to bind both neural and muscle isoforms of agrin with high affinity (Bowe *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994). Thirdly, recent studies have indicated that α-dystroglycan, via the DAP complex, is associated with the signaling molecules Grb2 and nNOS (Yang *et al.*, 1995; Brenman *et al.*, 1996), suggesting that α-dystroglycan may serve to transduce agrin signaling via these molecules. In fact, Grb2 signaling is known to activate the RAS/MAP kinase signal transduction pathway (see for review, Blenis, 1993). Altogether, both the integrin and α-dystroglycan signaling mechanisms could account for the observation that both isoforms of agrin activate utrophin transcription with equal efficiency and these mechanisms also account for the observation that MAP-kinase phosphorylation of GABP is the final step in regulating utrophin gene expression (see Figure 7.1).

A recent report has also suggested that agrin-induced AChR gene regulation may be due to agrin interacting with heparan sulfate proteoglycans (HSPGs) present in the extracellular matrix of skeletal muscle (Meier *et al.*, 1998). The local enrichment of agrin at the neuromuscular junction would subsequently bind to the HSPGs and result in their sequestration at the neuromuscular junction. Interestingly, this binding depends on the glycosaminoglycan side chains of agrin and HSPGs and occurs independently of the AChR clustering activity of agrin (Meier *et al.*, 1998). As a result, HSPGs may become locally enriched at the synapse regardless of the isoform of agrin. Since HSPGs can bind with high affinity to neuregulins, the local accumulation of HSPGs therefore, may then lead to the localization of neuregulins at the developing synapse (Meier *et al.*,1998).

In addition to the regulation of utrophin expression by the N-box element and nerve derived factors, separate studies have recently described the presence of additional regulatory elements that control utrophin gene expression which may be independent of these mechanisms. In one report, an internal promoter for the utrophin gene has recently been identified (Burton *et al.*, 1999). This alternative promoter, which lies in the second intron of the utrophin gene ~50 kb from exon 2,drives the expression of a unique utrophin transcript that contains a novel exon 1 which subsequently splices into exon 3 (Burton *et al.*, 1999). The levels of this alternative transcript in skeletal muscle are similar to the levels of transcripts derived from the utrophin promoter first described by Dennis *et al.* (1996) (Burton *et al.*, 1999). Interestingly, a detailed analysis of this region has revealed that it lacks an N-box element, indicating that this promoter is likely regulated by signaling pathways that are distinct from those that regulate the original promoter (Burton *et al.*, 1999). In a second report, the presence of a 128 bp intronic enhancer has been identified to regulate utrophin transcription (Galvagni and Oliviero, 2000). Based on the presence of this element within the second intron, it is likely that this element contributes to the expression of the second utrophin promoter. However, in both cases, the involvement of these additional mechanisms in the overall regulation of utrophin expression in skeletal muscle remains largely unknown.

II. Extrajunctional Repression of Utrophin Expression

Although the mechanisms discussed above may account for the preferential activation of utrophin transcription within postsynaptic myonuclei, they do not provide an explanation for

the mechanisms by which utrophin expression is repressed within extrajunctional myonuclei. At least for AChR, extrajunctional repression of AChR expression has been clearly established to involve the MyoD family of transcription factors and the E-box element coordinately downregulating AChR expression outside of the neuromuscular junction (Duclert and Changeux, 1995). However, accumulating evidence suggests that utrophin expression is not regulated by similar mechanisms. In particular, utrophin expression is not affected by muscle denervation (Jasmin *et al.*, 1995c; Biral *et al.*, 1996), nor is it markedly upregulated during muscle cell development (Chapter 6), two conditions which are known to be regulated by the MyoD family of transcription factors. These results indicate that the E-box and the MyoD family of transcription factors are not likely to mediate the repression of utrophin expression in muscle and, therefore, the mechanism by which utrophin expression is repressed within extrajunctional regions, if any exist, has yet to be identified.

III. Additional Regulatory Mechanisms Controlling Utrophin Expression

The focus of these studies (Chapters 2-4) has largely been on the transcriptional regulation of utrophin within skeletal muscle fibers. In fact, considerable effort has been focused on elucidating the transcriptional regulatory mechanisms for most of the proteins found at the neuromuscular junction. However, it is becoming increasingly apparent that transcription alone can not account for the complete control of proteins that are preferentially expressed within the synaptic region. Particularly, post-transcriptional regulatory mechanisms have been shown to play pivotal roles in the development, function, and plasticity of numerous cell types, including neurons and skeletal muscle (Willis, 1999; Schuman *et al.*, 1999). Indeed, during the course of

this work, it became apparent that transcriptional regulatory mechanisms were not the only mechanisms controlling the levels and localization of utrophin expression in skeletal muscle. In particular, we determined that utrophin protein levels in DMD or regenerating muscle were found to be significantly increased and extend along the length of the muscle membrane while the corresponding transcript levels and distribution were unchanged (Chapter 5).

One mechanism that may be involved in the post-transcriptional regulation of utrophin expression under these conditions is an increased level of translation. For instance, preliminary evidence from our lab has revealed that the 5' untranslated region (UTR) of the utrophin transcript significantly contributes to the overall efficiency of utrophin translation (Thompson, Wu and Jasmin, unpublished observations). Given that this region is instrumental in the control of translation (Gray and Wickens, 1998; van der Velden and Thomas, 1999; Willis, 1999), it is possible that under certain conditions, i.e., in regenerating muscle fibers, that utrophin is translated at a greater rate resulting in elevated protein levels. Since translation can be regulated by specific RNA-binding proteins (Gray and Wickens, 1998; van der Velden and Thomas, 1999; Willis, 1999), it is possible that these proteins are affected within extrajunctional regions, resulting in the translation of the very low levels of utrophin transcripts which are present within extrajunctional regions (Chapters 2 and 5; see also Vater *et al.*, 1998).

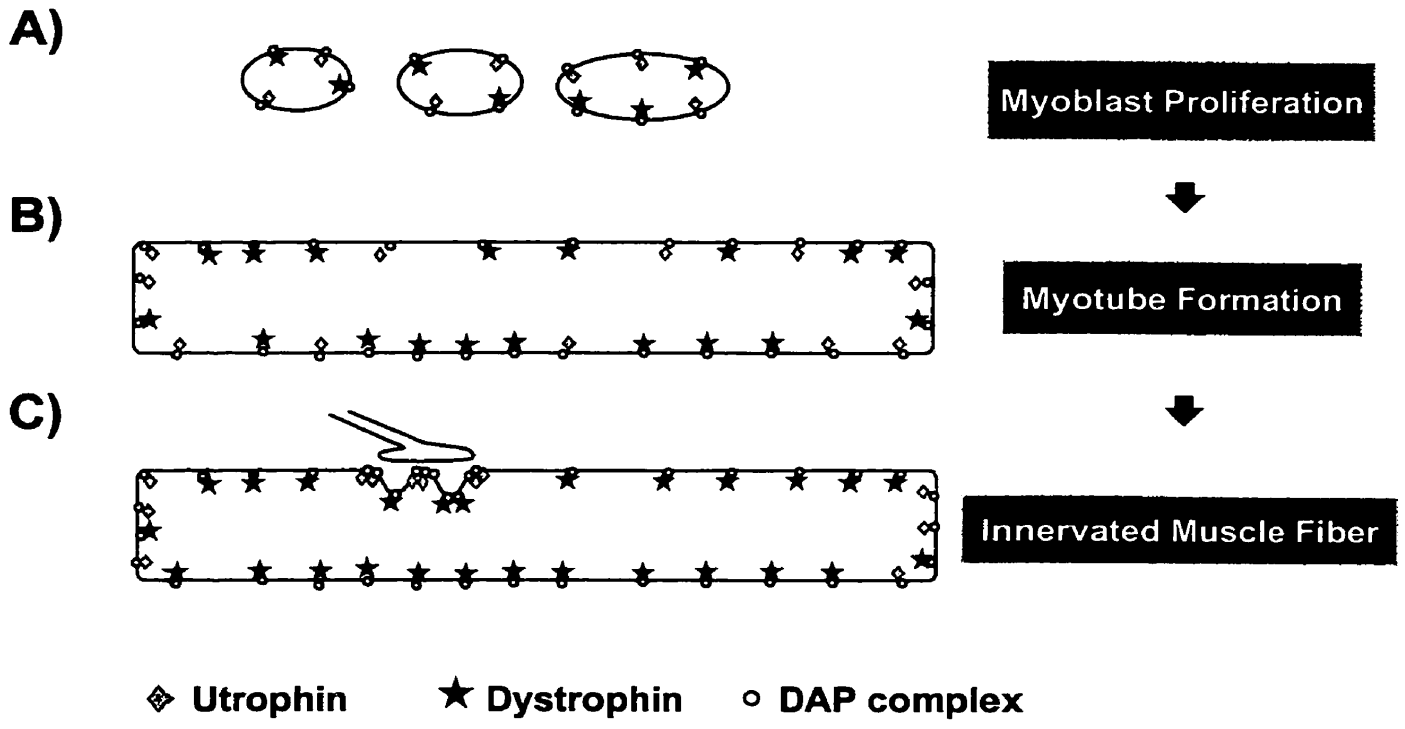
Alternatively, the 3' UTR is known to affect the subcellular localization of various transcripts in oocytes, neurons and developing muscle and can participate in controlling local protein synthesis (see Schuman *et al.*, 1999). In fact, specific regions within the 3' UTR, recently

termed "zip codes" (Fulton, 1993), appear to direct mRNAs to the proper "address" within the cell via an interaction with the intracellular cytoskeleton (Davis *et al.*, 1991; Mowry and Melton, 1992; Kim-Ha *et al.*, 1993; Hesketh *et al.*, 1994; Kislauskis *et al.*, 1994; Veyrune *et al.*, 1997; Gray and Wickens, 1998; van der Velden and Thomas, 1999; Willis; 1999). Interestingly, we have determined that utrophin mRNAs preferentially associate with the actin cytoskeleton, suggesting that utrophin mRNAs may be subject to similar targeting mechanisms (Gramolini and Jasmin; unpublished observations). Furthermore, it has also been suggested that the 3' UTR region may confer stability of the transcripts through binding to the actin cytoskeleton (Bassell and Singer, 1997). As a result, the stability of the transcript at the proper subcellular site would ensure production of the protein in the correct location within the cell. If similar mechanisms control utrophin expression then the interaction of utrophin mRNAs with the actin cytoskeleton may contribute not only to the stability of utrophin mRNAs, but may also contribute to the localization of utrophin within the muscle fiber. Together, the detailed characterization of the 3' UTR and the 5' UTR along with their corresponding binding proteins will likely yield valuable information concerning the mechanisms regulating the levels and localization of utrophin.

It is also likely that additional post-transcriptional mechanisms which involve protein binding interactions may influence utrophin expression. For instance, it has been suggested that competition between dystrophin and utrophin may result in binding to the dystrophin-associated protein complex and which may affect the stability of these proteins (Karpati *et al.*, 1993). In addition, a similar model can be proposed to explain the preferential localization of utrophin during skeletal muscle development (Khurana *et al.*, 1992; Schofield *et al.*, 1993; Clerk *et al.*, 1993; Koga *et al.*, 1993; Pons *et al.*, 1994; see Chapter 6 and Figure 7.2). However, although

a competition model may explain the presence of utrophin along the length of developing muscle fibers and within extrajunctional regions, it can not account for the preferential localization of utrophin at the crests of the junctional folds (Bewick *et al.*, 1992). Therefore, additional factors must also be considered to contribute to the preferential localization of utrophin within the junctional folds. For instance, a recent study has indicated that utrophin may have a greater binding affinity for junctional DAP complexes than dystrophin (Lumeng *et al.*, 1999). Accordingly, this greater affinity may allow dystrophin to become expressed along the length of muscle fiber, while utrophin is selectively enriched at the neuromuscular junction. It is also possible that this differential affinity may, in fact, even be extended between DAP complexes found at the crests versus the troughs of the junctional folds. Coherent with such a model, recent findings have indicated that utrophin localization may be dictated to, in part, by protein interactions (Amann *et al.*, 1999; Winder and James, 1999). For example, tyrosine phosphorylation of β-dystroglycan has recently been shown to inhibit binding to utrophin (Winder and James, 1999). Accordingly, it is possible that such protein stability mechanisms may regulate the affinity of utrophin binding between junctional and extrajunctional complexes, perhaps according to the state of phosphorylation of β-dystroglycan.

Figure 7.2. Schematic representation of the effects of myogenesis and innervation on utrophin and dystrophin expression. (A) During myoblast proliferation, similarly low levels of dystrophin and utrophin expression are observed. (B) However, during myoblast fusion and differentiation, the levels of utrophin and of the dystrophin-associated proteins (DAPs) do not substantially change, whereas dystrophin levels can increase by up to 10-fold. The greater amount of dystrophin subsequently binds to more DAP complexes based on competition for these sites. (C) When the exploratory motor axons reach the surface of the muscle fiber, specific molecules including agrin and heregulin become enriched at the neuromuscular junction and they positively regulate locally the transcriptional activity of the utrophin gene. In addition, greater membrane surface area at the neuromuscular junction due to junctional folds may also contribute to the local accumulation of utrophin at the neuromuscular junction because it results in more available DAP binding sites.


A)
B)
C)
Myoblast Proliferation
Myotube Formation
Innervated Muscle Fiber
Utrophin
Dystrophin
DAP complex

IV. Regulation of Utrophin Expression in Other Cell Types

Since a major goal in utrophin research has been to identify mechanisms by which utrophin can be upregulated throughout the muscle fiber of dystrophic patients, considerable attention has been focused on utrophin expression in skeletal muscle fibers. As a result, the regulation of utrophin expression in other non-muscle cells has largely been neglected. Given that utrophin is present in numerous other tissues (Love *et al.*, 1989; Khurana *et al.*, 1990, 1991; thiMan *et al.*, 1991) with particularly high levels of utrophin observed in the lung, blood vessels and nervous system (Love *et al.*, 1989; Khurana *et al.*, 1992, 1995; Kamakura *et al.*, 1994), the regulatory mechanisms controlling utrophin expression in these other tissues remains to be determined.

There is evidence that suggests there may be conserved mechanisms of utrophin regulation between synapses in the central nervous system and neuromuscular synapses. Since agrin and ARIA/heregulin along with their respective receptors are abundantly expressed in the nervous system (see for review, Sanes and Lichtman, 1999), these proteins may regulate the expression of utrophin in neurons as they do in skeletal muscle. Indeed, although agrin does not affect hippocampal neuron structure (Serpinskaya *et al.*, 1999), it can influence the intracellular signaling events in hippocampal neurons (Ji *et al.*, 1998; Ferreira, 1999) and in cortical neurons (Hilgenberg *et al.*, 1999). In addition, ARIA/heregulin was recently demonstrated to regulate the gene expression of synaptic proteins in neurons (Ozaki *et al.*, 1997; Yang *et al.*, 1998; Rieff *et al.*, 1999), so it is possible that ARIA/heregulin may also affect utrophin expression in neurons

as it does in skeletal muscle. Moreover, the ETS-related transcription factor, GABP has been shown to be expressed in neuronal cells (Brown and McKnight, 1992; Schaeffer *et al.*, 1998), further supporting the argument that a conserved mechanism of utrophin regulation may exist between neurons and skeletal muscle.

The elucidation of utrophin regulatory mechanisms in the CNS is also complicated by the presence of additional isoforms, including G-utrophin (Blake *et al.*, 1995), a 78 kD and an 82 kD utrophin isoform (Wilson *et al.*, 1999). Specifically, G-utrophin is found in the cortex, olfactory bulb, and the basal ganglia, while the 78 and 82 kD isoforms are detected in whole brain extracts (Blake *et al.*, 1995; Wilson *et al.*, 1999). Since these utrophin isoforms are restricted to the central nervous system, there appears to be distinct regulatory mechanisms that control alternative promoter or splicing events in these cells that are not evident in skeletal muscle fibers (Blake *et al.*, 1995; Wilson *et al.*, 1999). However, the mechanisms resulting in the generation of these nervous system-specific isoforms have not been characterized.

In addition, despite the fact that utrophin expression can be detected in the brain and spinal cord (Love *et al.*, 1991; thiMan *et al.*, 1991; Khurana *et al.*, 1992; Khurana *et al.*, 1993; Matsumura *et al.*, 1993; Khurana *et al.*, 1995), utrophin levels are highest in the microvasculature and astrocytes within the brain (thiMan *et al.*, 1991; Khurana *et al.*, 1992; Khurana *et al.*, 1995; Lumeng *et al.*, 1999). It is interesting to note that utrophin expression in astrocytes appears to be modulated by specific components of the extracellular matrix (Khurana *et al.*, 1995). In particular, cells that were cultured on the extracellular component, laminin, displayed significantly higher levels of utrophin expression, suggesting that laminin may

regulate utrophin gene expression in these cells (Khurana *et al.*, 1995). Furthermore, it was suggested that laminin binds to specific membrane receptors, such as the integrins or other laminin-binding proteins, to ultimately regulate utrophin expression in these cells (Khurana *et al.*, 1995). These results are particularly intriguing given the observation that extracellular components of the synaptic basal lamina, i.e., agrin and ARIA/heregulin are critical regulators of utrophin expression at the postsynaptic membrane domain of muscle fibers (Chapters 3 and 4). Taken together, these results suggest that there may be some similarities in the overall mechanisms controlling utrophin expression via the extracellular matrix in different cell types, particularly neurons and astrocytes.

V. Utrophin Upregulation in DMD Skeletal Muscle Fibers

There are several therapeutic strategies that are currently envisioned and are actively being pursued to counteract the effects of DMD. These therapies include the introduction of functional dystrophin using various gene therapy approaches, stem cell transplantation, as well as the prevention of the muscle pathology via pharmacological interventions (Ahn and Kunkel, 1993; Khan, 1993; Matsumura and Campbell, 1994; Gussoni *et al.*, 1999; Barton-Davis *et al.*, 1999). The introduction of dystrophin into dystrophic muscle via plasmid DNA injections (Ascadi *et al.*, 1991; Danko *et al.*, 1993; Fritz *et al.*, 1995; Decrouy *et al.*, 1997) or by a viral delivery system (Ragot *et al.*, 1993; Alameddine *et al.*,1994; Chen *et al.*, 1997; Zhao *et al.*, 1997) may lead to the expression of high levels of dystrophin, but it is complicated by the natural immune system of the body. Since dystrophic muscle has never expressed functional dystrophin,

the immune system has a tendency to recognize dystrophin as a foreign material and mounts a systemic immune response against dystrophin (Lochmuller *et al.*, 1996; Tripathy *et al.*, 1996; Michou *et al.*, 1997; Morral *et al.*, 1997; Ohtsuka *et al.*, 1998). Stem cell therapy is a new and exciting approach to treat dystrophic patients since early precursor stem cells could be systemically injected into the blood stream of dystrophic patients and would eventually fuse with the hosts skeletal muscle cells (Gussoni *et al.*, 1999). Once fused, the chimeric muscle fiber could then begin to produce functional dystrophin. Although this therapy is still in its infancy, it may also face the same challenges in overcoming the immune rejection of both the foreign stem cells as well as the foreign dystrophin protein. Pharmacological interventions are generally aimed at: i) inducing the misreading of dystrophin mRNAs to produce a functional protein (Barton-Davis *et al.*, 1999); or ii) increasing the capacity of the muscle fibers to regenerate despite the massive cycles of degeneration (Khan, 1993). Although this type of therapy holds promise, the identification of clinically relevant compounds may prove to be extremely laborious due to the large number of potential candidates to screen.

The upregulation of utrophin is another therapeutic strategy that is receiving increasing attention (see Tinsley *et al.*, 1993; Blake *et al.*, 1994; Blake *et al.*, 1996; Karpati, 1997; Roush, 1997) since it has been clearly established that the systemic overexpression of utrophin along the length of skeletal muscle fibers can functionally compensate for the loss of dystrophin (Tinsley *et al.*, 1996; Deconinck *et al.*, 1997; Rafael *et al.*, 1998; Tinsley *et al.*, 1998; Gilbert *et al.*, 1998; Gilbert *et al.*, 1999). Furthermore, the difficulties with an immune rejection of utrophin should not exist since utrophin is normally expressed in diseased muscle (see for example, Matsumura *et al.*, 1992; Karpati *et al.*, 1993) and therefore would not be recognized as a foreign protein.

Therefore, what remains to be determined is the best method to upregulate endogenous levels of utrophin and how can this be achieved in dystrophic muscle fibers. Utrophin upregulation may be achieved using current gene therapy techniques (see Gilbert *et al.*, 1998, 1999). In addition, the elucidation of the cellular mechanisms regulating utrophin expression at the neuromuscular synapse may yield valuable information to design additional clinically relevant therapies. Since pharmacological compounds can now be specifically designed to regulate gene expression (Gottesfeld *et al.*, 1997), the transcriptional and the posttranscriptional mechanisms that regulate utrophin expression could be targeted by specially designed pharmacological compounds to upregulate the expression of utrophin (Roush, 1997).

In addition to the utrophin regulatory mechanisms that we have elucidated, separate studies have recently reported additional mechanisms by which utrophin levels can be increased in skeletal muscle (Kammesheidt and Martin, 1999; Chaubourt *et al.*, 1999). For example, muscles cells in culture treated with L-arginine, the substrate of nitric oxide synthase (NOS) or an exogenous nitric oxide (NO) donor showed a significant increase in utrophin protein expression (Chaubourt *et al.*, 1999). Since NOS has recently been reported to be part of the dystrophin-associated protein complex (Brenman *et al.*, 1995; Brenman *et al.*, 1996), these results indicate that NOS localization within this complex may act as a critical regulator of utrophin expression. However, the therapeutic application in upregulating NO or NOS expression in skeletal muscle is seriously complicated by the widespread function of NO in skeletal muscle. Indeed, functional studies have implicated nitric oxide as a modulator of skeletal muscle contractility, mitochondrial respiration, carbohydrate metabolism, and

neuromuscular transmission (Grozdanovic and Baumgarten, 1999). Nonetheless, further studies are needed to further clarify the role of NO in utrophin regulation. In addition, a preliminary abstract report has indicated that the overexpression of N-acetylgalactosamine (GAL-NAc), a synaptic carbohydrate, in control and dystrophic muscle cells can result in a significant increase in utrophin expression (Kammesheidt and Martin, 1999). Interestingly, this carbohydrate appears to be involved in agrin-induced AChR clustering by a mechanism that remains largely unclear (Martin and Sanes, 1995). Nonetheless, these results tend to suggest that GAL-NAc regulation of utrophin expression may involve a mechanism which likely converges with the agrin and ARIA/heregulin signal transduction pathways. Altogether, a more detailed understanding of the molecular mechanisms by which GAL-Nac and NO regulate utrophin expression may yield valuable information regarding alternative methods to increase utrophin expression into extrajunctional regions of muscles.

VI. Additional Therapeutic Considerations

One question that still remains regarding upregulating utrophin into extrajunctional regions of dystrophic muscle fibers is how much utrophin will be needed to ameliorate the muscle pathology. Although this remains to be established, there may be an indication of the amount of utrophin required from studies using fast and slow twitch muscle fibers. For instance, fast twitch muscles express approximately 70% less utrophin than slow twitch fibers (Gramolini and Jasmin; unpublished observations). Since slow twitch muscle fibers appear to be more resistant to the deleterious effects of DMD (Webster *et al.*, 1988), it is possible that the elevated levels of utrophin present in these fibers slows the progression of the disease. Therefore, one

could hypothesize that increasing utrophin levels in fast twitch fibers to the levels seen in slow twitch fibers should significantly slow the progression of the disease. Furthermore, if increasing the levels of utrophin by ~3-fold would lead to a slower progression of DMD, then to begin to significantly ameliorate the muscle pathology, the levels of utrophin may likely only need to be increased within an order of magnitude. Nonetheless, the clarification of the amount of utrophin needed to ameliorate the pathology of dystrophin muscle will have to be determined as this value may dictate whether a potential therapy to upregulate utrophin will be successful or not. Similarly, it remains to be determined whether the overexpression of utrophin will be required in all muscles of the body, or if a restricted group of muscles can be selectively targeted. Given that the phenotype of DMD is largely a result of the failure of postural muscles, along with respiratory failure due to degeneration of the diaphragm and intercostal muscles, it may be necessary to upregulate utrophin only in these muscles groups.

A final problem that has yet to be addressed is whether the upregulation of utrophin will be able to reverse some of the pathology of dystrophic muscles or will it only be sufficient to halt the progression of the disease. Given that dystrophic patients are usually diagnosed in their first decade of life, the severe progression of the disease has already commenced resulting in dystrophic infants having difficulty with normal tasks (Emery, 1991). So, in the case of these patients, utrophin upregulation may stop the progression of the disease and also may allow the infant to develop 'normal' skeletal muscle. However, there are currently a number of teenagers and young adults who are physically disabled due to the severe progression of DMD. Therefore, it is unclear whether utrophin upregulation in these cases will be able to restore normal muscle

function in these individuals. Altogether, it appears likely that the best strategy for utrophin therapy in DMD patients lies in the early diagnosis and prevention of the muscle deterioration, rather than attempting to reverse the damage.

VII. Concluding Remarks

The extensive similarity between utrophin and dystrophin has led to the idea that utrophin could functionally compensate for the absence of dystrophin in DMD muscle fibers. Recent mouse model systems have revealed that indeed, upregulation of utrophin into extrajunctional compartments of dystrophic muscle fibers can compensate for the lack of dystrophin and prevent the development of the muscle pathology (Tinsley *et al.*, 1996; Deconinck *et al.*, 1997; Gilbert *et al.*, 1998; Tinsley *et al.*, 1998; Rafael *et al.*, 1998; Gilbert *et al.*, 1999). Therefore considerable attention has been directed toward the identification and understanding of the various cellular and molecular mechanisms ultimately regulating the preferential expression of utrophin at the neuromuscular junction. In this study, our data clearly indicate that both transcriptional and posttranscriptional regulatory mechanisms ultimately contribute to the local expression of utrophin in skeletal muscle fibers. Altogether, the study of these mechanisms may lead to a potential therapeutic strategy for DMD while providing, in addition, information useful for our understanding of the events involved in the formation, maintenance and plasticity of the neuromuscular synapse.

CHAPTER 8

REFERENCES

Acsadi, G., Dickson, G., Love, D.R., Jani, A., Walsh, F.S., Gurusinghe, A., Wolff, J.A. and Davies, K.E. (1991) Human dystrophin expression in *mdx* mice after intramuscular injection of DNA constructs. *Nature* **352**, 815-818.

Adams, M.E., Dwyer, T.M., Dowler, L.L., White, R.A. and Froehner, S.C. (1995) Mouse alpha 1- and beta 2-syntrophin gene structure, chromosome localization, and homology with a discs large domain. *J. Biol. Chem.* **270**, 25859-25865.

Ahn Jo, S., Zhu, X., Marchionni, M.A. and Burden, S.J. (1995) Neuregulins are concentrated at nerve-muscle synapses and activate ACh-receptor gene expression. *Nature* **373**, 158-161.

Ahn, A.H. and Kunkel, L.M. (1993) The structural and functional diversity of dystrophin. *Nature Genet.* **3**, 283-291.

Alameddine, H.S., Quantin, B., Cartaud, A., Dehaupas, M., Mandel, J.L. and Fardeau, M. (1994) Expression of a recombinant dystrophin in *mdx* mice using adenovirus vector. *Neuromusc. Disord.* 4,193-203.

Altiok, N., Bessereau, J. and Changeux, J.P. (1995) ErbB3 and ErbB2/neu mediate the effect of heregulin on acetylcholine receptor gene expression in muscle: Differential expression at the endplate. *EMBO J.* **14,** 4258-4266.

Altiok, N., Altiol, S. and Changeux, J.P. (1997) Heregulin-stimulated acetylcholine receptor gene expression in muscle–requirements for MAP kinase and evidence for a parallel inhibitory pathway independent of electrical activity. *EMBO J.* **16,** 717-725.

Amann, K.J., Guo, A.W. and Ervasti, J.M. (1999) Utrophin lacks the rod domain actin binding activity of dystrophin. *J. Biol. Chem.* **274,** 35375-35380.

Anderson, M.J. and Fambrough, D.M. (1983) Aggregates of acetylcholine receptors are associated with plaques of a basal lamina heparan sulfate proteoglycan on the surface of skeletal muscle fibers. *J. Cell Biol.* **97,** 1396-1411.

Anderson, M.J. and Cohen, M.W. (1977) Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268,** 757-783.

Anglister, L. and McMahan, U.J. (1985) Basal lamina directs acetylcholinesterase accumulation at synaptic sites in regenerating muscle. *J. Cell Biol.* **101,** 735-743.

Arahata, K., Ishiura, S. and Ishiguro, T. (1988) Immunostaining of skeletal and cardiac muscle surface membrane with antibody against Duchenne muscular dystrophy peptide. *Nature* **333**, 861-863.

Arnold, H.H. and Winter, B. (1998) Muscle differentiation–more complexity to the network of myogenic regulator. *Curr. Op. Genet. Develop.* **8**, 539-544.

Baldwin, T.J. and Burden, S.J. (1988) Isolation and characterization of the mouse acetylcholine receptor delta subunit gene: Identification of a 148-bp cis-acting region that confers myotube-specific expression. *J. Cell Biol.* **107**, 2271-2279.

Bannert, N., Avots, A., Baier, M., Serfling, E. and Kurth, R. (1999) GA-binding protein factors, in concert with the coactivator CREB binding protein/p300, control the induction of the interleukin 16 promoter in T lymphocytes. *Proc. Natl. Acad. Sci. USA* **96**,1541-1546.

Bar, S., Barnea, E., Levy, Z., Neuman, S., Yaffe, D. and Nudel, U. (1990) A novel product of the Duchenne muscular dystrophy gene which greatly differs from the known isoforms in its structure and tissue distribution. *Biochem. J.* **272**, 557-560.

Barton-Davis, E.R., Cordier, L., Shoturma, D.I., Leland, S.E. and Sweeney, H.L. (1999) Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of *mdx* mice. *J. Clin. Invest.* **104**, 375-381.

Bassell, G.J. and Singer, R.H. (1997) mRNA and cytoskeletal filaments. *Curr. Op. Cell Biol.* **9**, 109-115.

Batchelor, A.H., Piper, D.E., de la Brousse, F.C., McKnight, S.L. and Wolberger, C. (1998) The structure of GABPalpha/beta: an ETS domain- ankyrin repeat heterodimer bound to DNA. *Science* **279**, 1037-1041.

Ben-Baruch, N. and Yarden, Y. (1994) Neu differentiation factors: a family of alternatively spliced neuronal and mesenchymal factors. *Proc. Soc. Exp. Biol. Med.* **206**, 221-227.

Ben-Levy, R., Paterson, H.F., Marshal, C.J. and Yarden, Y. (1994) A single autophosphorylation site confers oncogenicity to the neu/ErbB2 receptor and enables coupling to the MAP kinase pathway. *EMBO J.* **13**, 3302-3311.

Bewick, G.S., Nicholson, L.V.B., Young, C., O'Donnell, E. and Slater, C.R. (1992) Different distributions of dystrophin and related proteins at nerve-muscle junctions. *NeuroReport* **3**, 857-860.

Biral, D., Senter, L. and Salviati, G. (1996) Increased expression of dystrophin, β-dystroglycan and adhalin in denervated rat muscles. *J. Muscle Res. Cell Motil.* **17**, 523-532.

Blake, D.J., Tinsley, J.M. and Davies, K.E. (1996) Utrophin: A structural and functional comparison to dystrophin. *Brain Pathol.* **6**, 37-47.

Blake, D.J., Tinsley, J.M. and Davies, K.E. (1994) The emerging family of dystrophin-related proteins. *Trends Cell Biol.* **4**, 19-23.

Blake, D.J., Schofield, J., Zuellig, R.A., Gorecki, D.C., Phelps, S.R., Barnard, E.A., Edwards, Y.H. and Davies, K.E. (1995) G-utrophin, the autosomal homologue of dystrophin Dp116, is expressed in sensory ganglia and brain. *Proc. Natl. Acad. Sci. USA* **92**, 3697-3701.

Blenis, J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. USA* **90**, 5889-5892.

Bonilla, E., Samitt, C.E., Miranda, A.F., Hays, A.P., Salviati, G., DiMauro, S., Kunkel, L.M., Hoffman, E.P. and Rowland, L.P. (1988) Duchenne muscular dystrophy: Deficiency of dystrophin at the muscle cell surface. *Cell* **54**, 447-452.

Bowe, M.A., Deyst, K.A., Leszyk, J.D. and Fallon, J.R. (1994) Identification and purification of an agrin receptor from Torpedo postsynaptic membranes: A heteromeric complex related to the dystroglycans. *Neuron* **12**, 1173-1180.

Bowe, M.A. and Fallon, J.R. (1995) The role of agrin in synaptogenesis. *Annu. Rev. Neurosci.* **18**, 443-462.

Bozyczko, D., Decker, C., Muschler, J. and Horwitz, A.F. (1989) Integrin on developing and adult skeletal muscle. *Exp. Cell Res.* **183**, 72-91.

Brenman, J.E., Chao, D.S., Xia, H., Aldape, K. and Bredt, D.S. (1995) Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* **82**, 743-752.

Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., Froehner, S.C. and Bredt, D.S. (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α1-syntrophin mediated by PDZ domains. *Cell* **84**, 757-767.

Brown, T.A. and Mcknight, S.L. (1992) Specifications of protein-protein and protein-DNA interaction of GABP alpha and two newly defined ETS-related proteins. *Genes Dev.* 6, 2502-2512.

Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H., and Klambt, C. (1994) The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. *Nature* **370**, 386-389

Buckle, V.J., Guenet, J.L., Simon-Chazottes, D., Love, D.R. and Davies, K.E. (1990) Localisation of a dystrophin-related autosomal gene to 6q24 in man and to mouse chromosome 10 in the region of the dystrophia muscularis (dy) locus. *Hum. Genet.* **85**, 324-326.

Burden, S.J., Sargent, P.B. and McMahan, U.J. (1979) Acetylcholine receptors in regenerating muscle accumulate at original synaptic sites in the absence of the nerve. *J. Cell Biol.* **82**, 412-425.

Burden, S.J. (1998). The formation of neuromuscular synapses. *Genes Dev.* **12**, 133-148.

Burgering, B.M. and Bos, J.L. (1995) Regulation of Ras-mediated signaling: more than one way to skin a cat. *Trends Biochem. Sci.* **20**, 18-22.

Burghes, A.H.M., Logan, C., Hu, X., Belfall, B., Worton, R.G. and Ray, P.N. (1987) A cDNA clone from the Duchenne /Becker muscular dystrophy gene. *Nature* **328**, 434-437.

Burton, E.A., Tinsley, J.M., Holzfeind, P.J., Rodrigues, N.R. and Davies, K.E. (1999) A second promoter provides an alternative target for therapeutic up-regulation of utrophin in Duchenne muscular dystrophy. *Proc. Natl. Acad. Sci. USA* **96**, 14025-14030.

Byers, T.J., Lidov, H.G. and Kunkel, L.M. (1993) An alternative dystrophin transcript specific to peripheral nerve. *Nature Genet.* **4**, 77-81.

Campanelli, J.T., Roberds, S.L., Campbell, K.P. and Scheller, R.H. (1994) A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. *Cell* **77**, 663-674.

Campbell, R.J., Jasmin, B.J. and Michel RN. (1996) Succinate dehydrogenase activity within synaptic and extrasynaptic compartments of functionally-overloaded rat skeletal muscle fibers. *Pflugers Archiv. Eur. J. Physiol.* **431**, 797-799.

Chahine, K.G., Walke, W. and Goldman, D. (1992) A 102 base pair sequence of the nicotinic acetylcholine receptor delta-subunit gene confers regulation by muscle electrical activity. *Development* **115**, 213-219.

Chamberlain, J.S., Pearlman, J.A., Muzny, D.M. Gibbs, R.A., Ranier, J.E., Reeves, A.A. and Caskey, C.T. (1988) Expression of the murine Duchenne muscular dystrophy gene in muscle and brain. *Science* **239**, 1416-1418.

Chan, R.Y., Boudreau-Lariviere, C., Angus, L.M.., Mankal, F.A. and Jasmin, B.J. (1999)A n intronic enhancer containing an N-box motif is required for synapse- and tissue-specific expression of the acetylcholinesterase gene in skeletal muscle fibers. *Proc. Natl. Acad. Sci.USA* **96**, 4627-46232.

Changeux, J.P., Duclert, A. and Sekine, S. (1992). Calcitonin gene-related peptides at the neuromuscular junction. *Ann. NY. Acad. Sci.* **657**, 361-378.

Chao, D.S., Silvagno, F., Xia, H., Cornwell, T.L., Lincoln, T.M. and Bredt, D.S. (1997) Nitric oxide synthase and cyclic GMP-dependent protein kinase concentrated at the neuromuscular endplate. *Neurosci.* **76**, 665-672.

Chaubourt, E., Fossier, P., Baux, G., Leprince, C., Israel, M. and De la Forte, S. (1999) Nitric oxide and L-arginine cause an accumulation of utrophin at the sarcolemma: A possible compensation for dystrophin loss in Duchenne muscular dystrophy. *Neurobiol. Disease.* **6**, 499-507.

Chen, H.H., Mack, L.M., Kelly, R., Ontell, M., Kochanek, S. and Clemens P.R. (1997) Persistence in muscle of an adenoviral vector that lacks all viral genes. *Proc. Natl. Acad. Sci. USA* **94**,1645-1650.

Chu, G.C., Moscoso, L.M., Sliwkowski, M.X. and Merlie, J.P. (1995) Regulation of the acetylcholine receptor ε subunit gene by recombinant ARIA: An *in vitro* model for transynaptic gene regulation. *Neuron* **14**, 329-339.

Clark, E.A. and Brugge, J.S. (1995) Integrins and signal transduction pathways: The road taken. *Science* **268**, 233-239.

Clerk, A., Morris, G.E., Dubowitz, V., Davies, K.E. and Sewry, C. (1993) Dystrophin-related protein, utrophin, in normal and dystrophic human fetal skeletal muscle. *Histochem. J.* **25**, 554-561.

Cohen, I., Rimer, M., Lomo, T. and McMahan, U.J. (1997) Agrin-induced postsynaptic-like apparatus in skeletal muscle fibers in Vivo. *Mol. Cell. Neurosci.* **9**, 237-253.

Cohen, M.W., Jacobson, C., Godfrey, E.W., Campbell, K.P. and Carbonetto, S. (1995) Distribution of α-dystroglycan during embryonic nerve-muscle synaptogenesis. *J. Cell Biol.* **129**, 1093-1101.

Côté, P.D., Moukhles, H., Lindenbaum, M., and Carbonetto, S. (1999) Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. *Nature Genet.* **23**, 338-342.

Couteux, R. (1973) Motor endplate structure. In *Structure and Function of Muscle*, Vol., 2 ed. GH Bourne , pp 483-530. New York: Academic Press.

Crowder, C.M. and Merlie, J.P. (1988) Stepwise activation of the mouse acetylcholine receptor delta- and gamma-subunit genes in clonal cell lines. *Mol. Cell. Biol.* **8**, 5257-5267.

D'Souza, V.N., Man, N.T., Morris, G.E., Karges, W., Pillers, D.M. and Ray, P.N. (1995) A novel dystrophin isoform is required for normal retinal electrophysiology. *Hum. Mol. Genet.* **4**, 837-842.

Danko, I., Fritz, J.D., Latendresse, J.S., Herweijer, H., Schultz, E. and Wolff, J.A. (1993) Dystrophin expression improves myofiber survival in *mdx* muscle following intramuscular plasmid DNA injection. *Hum. Mol. Genet.* **2**, 2055-2061.

Davis, I. and Ish-Horowitz, D. (1991) Apical localization of pair-rule transcripts requires 3' sequence and limits protein diffusion in the *Drosophila* blastoderm embryo. *Cell* **67**, 927-940.

DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T., Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., Smith, C., Distefano, P.S., Glass, D.J., Burden, S.J. and Yancopoulos, G.D. (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* **85**, 501-512.

Deconinck, N., Tinsley, J.M., DeBacker, F., Fisher, R., Kahn, D., Phelps, S., Davies, K. and Gillis, J.M. (1997) Expression of truncated utrophin leads to major functional improvements in dystrophin-deficient muscles of mice. *Nature Med.* **3**, 1216-1221.

Deconinck, A.E., Potter, A.C., Tinsley, J.M., Wood, S.J., Vater, R., Young, C., Metzinger, L., Vincent, A., Slater, C.R. and Davies, K.E. (1997a) Postsynaptic abnormalities at the neuromuscular junctions of utrophin-deficient mice. *J. Cell Biol.* **136**, 883-894.

Deconinck, A.E., Rafael, J.A., Skinner, J.A., Brown, S.C., Potter, A.C., Metzinger, L., Watt, D.J., Dickson, G., Tinsley, J.M. and Davies, K.E. (1997b) Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. *Cell* **90**, 717-727.

Decrouy, A., Renaud, J.M., Davis, H.L. Lunde, J.A., Dickson, G. and Jasmin, B.J. (1997) Mini-dystrophin gene transfer in *mdx*4cv diaphragm muscle fibers increases sarcolemmal stability. *Gene Ther.* **4**, 401-408.

Decrouy, A., Renaud,. J.-M, Davis, H.L., Lunde, J.A. Dickson, G. and Jasmin, B.J. (1997) Mini-dystrophin gene transfer in *mdx*4cv diaphragm muscle fibers increases sarcolemmal stability. *Gene Ther.* **4**, 401-408.

Dennis, C.L., Tinsley, J.M., Deconinck, A.E. and Davies, K.E. (1996) Molecular and functional analysis of the utrophin promoter. *Nucl. Acids Res.* **24**, 1646-1652.

Denzer, A.J., Hauser, D.M., Gesemann, M. and Ruegg, M.A. (1997) Synaptic differentiation: The role of agrin in the formation and maintenance of the neuromuscular junction. *Cell Tissue Res.* **290**, 357-365.

Deyst, K.A.., McKechnie, B.A. and Fallon, J.R. (1998) The role of alternative splicing in regulating agrin binding to muscle cells. *Brain Res. (Dev. Brain Res.)* **110**, 185-191.

Dixon, A.K., Tait, T.-M., Campbell, E.A., Bobrow, M., Roberts, R.G. and Freeman, T.C. (1997) Expression of the dystrophin-related protein 2 (DRP2) transcript in the mouse. *J. Mol. Biol.* **270**, 551-558.

Duclert, A., Savatier, N. and Changeux, J.P. (1993) An 83-nucleotide promoter of the acetylcholine receptor ε-subunit gene confers preferential synaptic expression in mouse muscle. *Proc. Natl. Acad. Sci. USA* **90**, 3043-3047.

Duclert, A. and Changeux, J.P. (1995) Acetylcholine receptor gene expression at the developing neuromuscular junction. *Physiol. Rev.* **75**, 339-368.

Duclert, A., Savatier, N., Schaeffer, L. and Changeux, J.P. (1996) Identification of an element crucial for the subsynaptic expression of the acetylcholine receptor ε-subunit gene. *J. Biol. Chem.* **271**, 17433-17438.

Emery, A. (1991) Population frequencies of inherited neuromuscular disorders--A world survey. *Neuromusc. Disord.* **1**, 19-29.

Engel, A.G. (1994) The neuromuscular junction. In *Myology: Basics and Clinical*, Vol. 1, ed. AG Engel, C Franzini-Armstrong, pp. 261-302. New York: McGraw-Hill.

Ervasti, J.M., Ohlendieck, K. Kahl, S.D., Gaver, M.G. and Campbell, K.P. (1990) Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* **345**, 315-9.

Ervasti, J.M. and Campbell, K.P. (1991) Membrane organization of the dystrophin-glycoprotein complex. *Cell.* **66**, 1121-1131.

Fabbrizio, D., Latouche, J., Rivier, F., Hugon, G. and Mornet, D. (1995) Re-evaluation of the distributions of dystrophin and utrophin in sciatic nerve. *Biochem. J.* **312**, 309-314.

Fallon, J.R. and Gelfman, C.E. (1989) Agrin-related molecules are concentrated at acetylcholine receptor clusters in normal and aneural developing muscle. *J.Cell Biol.* **108**, 1527-1535.

Fardeau, M., Tomé, F.M., Collin, H., Augier, N., Pons, F., Léger, J. and Léger, J. (1990) Présence d'une protéine de type dystrophine au niveau de la jonction neuromusculaire dans la dystrophie musculaire de Duchenne et la souris mutante "*mdx*". *Comptes Rendus de l'Academie des Sciences* **311**, 197-204.

Feng, G., Steinbach, J.H. and Sanes, J.R. (1998) Rapsyn clusters neuronal acetylcholine receptors but is inessential for formation of an interneuronal cholinergic synapse. *J. Neurosci.* **18**, 4166-4176.

Feng, G., Krejci, E., Molgo, J., Cunningham, J.C., Massoulie, J., and Sanes, J.R. (1999) Genetic analysis of collagen Q: Roles of acetylcholinesterase and butyrylcholinesterase assembly and in synaptic structure and function. *J. Cell Biol.* **144**,1349-1360.

Ferns, M., Deiner, M. and Hall, Z.W. (1996) Agrin-induced acetylcholine receptor clustering in mammalian muscle requires tyrosine phosphorylation. *J. Cell Biol.* **132**, 937-944.

Ferns, M., Hoch, W., Campanelli, J.T., Rupp, F., Hall, Z. and Scheller, R.H. (1992) RNA splicing regulates agrin-mediated acetylcholine receptor clustering ability in cultured myotubes. *Neuron* **8**, 1079-1086.

Ferns, M.J., Campanelli, J.T., Hoch, W., Scheller, R.H. and Hall, Z. (1993) The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. *Neuron* **11**, 491-502.

Ferreira, A. (1999) Abnormal synapse formation in agrin-depleted hippocampal neurons. *J. Cell Sci.* **112**, 4729-4738.

Fischbach, G.D. and Rosen, K.M. (1997) ARIA: A neuromuscular junction neuregulin. *Ann. Rev. Neurosci.* **20**, 429-458.

Flory, E., Hoffmeyer, A., Smala, V., Rapp, U.R. and Bruder, J.T. (1996) Raf-1 kinase targets GA-binding protein in transcriptional regulation of the human immunodeficiency virus type 1 promoter. *J. Virol.* **70**, 2260-2268.

Fontaine, B., Klarsfeld, A., and Changeux, J.P. (1987) Calcitonin gene related peptide and muscle activity regulate acetylcholine receptor α-subunit mRNA levels by distinct intracellular pathways. *J.Cell Biol.* **105**, 1337-1342.

Fontaine, B. and Changeux, J.P. (1989) Localization of nicotinic acetylcholine receptor α-subunit transcripts during myogenesis and motor endplate development in chick. *J. Cell Biol.* **101**, 1025-1037.

Frank, E., Jansen, J. K., Lomo, T. and Westgaard, R. H. (1975) The interaction between foreign and original motor nerves innervating the soleus muscle of rats. *J. Physiol. (Lond.)* **247**, 725-743.

Fritz, J.D., Danko, I., Roberds, S.L., Campbell, K.P., Latendresse, J.S. and Wolff, J.A. (1995) Expression of deletion-containing dystrophins in *mdx* muscle: implications for gene therapy and dystrophin function. *Ped. Res.* **37**, 693-700.

Froehner, S.C. , Luetje, C.W., Scotland, P.B., and Patrick J. (1990) The postsynaptic 43K protein clusters muscle nicotinic acetylcholine receptors in Xenopus oocytes. *Neuron* **5**, 403-410.

Fromm, L. and Burden, S.J. (1998) Synapse-specific and neuregulin-induced transcription required an Ets site that binds GABPa/GABPb. *Genes Dev.* **12**, 3074-3083.

Fu, A.K.Y., Cheung, W.M.W., Ip, F.C.F. and Ip, N.Y. (1999) Identification of genes induced by neuregulin in cultured myotubes. *Mol. Cell. Neurosci.* **14**, 241-253.

Fulton, A.B. (1993) Spatial organization of the synthesis of cytoskeletal proteins. *J.Cell Biochem.* **52**, 148-152.

Galvani, F. and S. Oliviero (2000) Utrophin transcription is activated by an intronic enhancer. *J. Biol. Chem.* **275**, 3168-3172.

Gautam, M., Noakes, P.G., Moscoso, L.M., Rupp, F., Scheller, R.H., Merlie, J.P. and Sanes, J.R. (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* **85**, 525-535.

Gautam, M., Noakes, P.G., Mudd, J., Nichol, M., Chu, G.C., Sanes, J.R. and Merlie, J.P. (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* **377**, 232-236.

Gee, S.H., Montanaro, F., Lindenbaum, M.H. and Carbonetto, S. (1994) Dystroglycan-α, a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell* **77**, 675-686.

Gerfen, C.R. and Young, W.S. (1988) Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an *in situ* hybridization histochemistry and fluorescent retrograde tracing study. *Brain Res.* **460**, 161-167.

Gesemann, M., Cavalli, V., Denzer, A.J., Brancaccio, A., Schumacher, B. and Ruegg, M.A. (1996) Alternative splicing of agrin alters its binding to heparin, dystroglycan, and the putative agrin receptor. *Neuron* **16**, 755-767.

Gesemann, M., Denzer, A.J. and Ruegg, M.A. (1995) Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. *J. Cell Biol.* **128**, 625-636.

Gilbert, R., Nalbanoglu, J., Petrof, B., Ebinhara, S., Guibinga, G.H., Tinsley, J.M., Kamen, A., Massie, B., Davies, K.E. and Karpati, G. (1999) Adenovirus-mediated utrophin gene transfer mitigates the dystrophic phenotype of *mdx* mouse muscle. *Hum. Gene Ther.* **10**, 1299-1310.

Gilbert, R., Nalbanoglu, J., Tinsley, J.M., Massie, B., Davies, K.E. and Karpati, G. (1998) Efficient utrophin expression following adenovirus gene transfer in dystrophic muscle. *Biochem. Biophys. Res. Commun.* **242**, 244-247.

Glass, D.J., Bowen, D.C., Stitt, T.N., Radziejewski, C., Bruno, J., Ryan, T.E., Gies, D.R., Shah, S., Mattson, K., Burden, S.J., Distefano, P.S., Valenzuela, D.M., DeChiara, T.M. and Yancopoulos, G.D. (1996) Agrin acts via a MuSK receptor complex. *Cell* **85**, 513-523.

Glass, D.J. and Yancopoulos, G.D. (1997) Sequential roles of agrin, MuSK and rapsyn during neuromuscular junction formation. *Curr. Op. Neurobiol.* **7**, 379- 384.

Goldman, D.B. and Staple, J. (1989) Spatial and temporal expression of adult-type nicotinic acetylcholine receptor NRAs in innervated and denervated rat soleus muscle. *Neuron* **3**, 219-228.

Goodearl, A.P., Yee, A.G., Sandrock, A.W., Corfas, G., and Fischbach, G.D. (1995) ARIA is concentrated in the synaptic basal lamina of the developing chick neuromuscular junction. *J. Cell Biol.* **130**, 1423-1434.

Gorecki, D.C., Monaco, A.P., Derry, J.M.J., Walker, A.P., Barnard, E.A. and Barnard, P.J. (1992) Expression of four alternative dystrophin transcripts in brain regions regulated by different promoters. *Hum. Mol. Genet.* **1**, 505-510.

Gottesfeld, J.M., Neely, L., Trauger, J.W., Baird, E.E. and Dervan, P.B. (1997) Regulation of gene expression by small molecules. *Nature* **387**, 202-205.

Grady, R.M., Teng, H., Nichol, M.C., Cunningham, J.C., Wilkinson, R.S. and Sanes, J.R. (1997b) Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: A model for Duchenne muscular dystrophy. *Cell* **90**, 729-738.

Grady, R.M., Merlie, J.P. and Sanes, J.R. (1997a) Subtle neuromuscular defects in utrophin-deficient mice. *J. Cell Biol.* **136**, 871-882.

Gray, N.K. and Wickens, M. (1998) Control of translation initiation in animals. *Ann. Rev.Cell Dev. Biol.* **14**, 399-458.

Grozdanovic, Z. and Baumgarten, H.G. (1999) Nitric oxide synthase in skeletal muscle fibers: a signaling component of the dystrophin-glycoprotein complex. *Histol. Histopathol.* **14**, 243-256.

Gundersen, K., Sanes, J.R. and Merlie, J.P. (1993) Regulation of muscle acetylcholine receptor ε- and α- subunit gene promoters in transgenic mice. *J. Cell Biol.* **123**, 1535-1544.

Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L.M. and Mulligan, R.C. (1999) Dystrophin expression in the *mdx* mouse restored by stem cell transplantation. *Nature* **401,** 390-394.

Hall, Z.W. and Sanes, J.R. (1993) Synaptic structure and development: the neuromuscular junction. *Cell/Neuron* **72/10**, 99-121.

Harris, D.A., Falls, D.L., Dill-Devor, R.M. and Fischbach, G.D. (1988) Acetylcholine receptor-inducing factor from chicken brain increases the level of mRNA encoding the receptor a-subunit. *Proc.Natl.Acad.Sci.USA* **85,**1983-1987.

Helliwell, T.R., thi Man, N., Morris, G.E. and Davies, K.E. (1992) The dystrophin-related protein, utrophin, is expressed on the sarcolemma of regenerating human skeletal muscle fibers in dystrophies and inflammatory myopathies. *Neuromusc. Disord.* **2**, 177-184.

Hemler, M.E. (1999) Dystroglycan versatility. *Cell* **97**, 543-546.

Hesketh, J. (1996) Sorting of messenger RNAs in the cytoplasm: mRNA localization and the cytoskeleton. *Exp. Cell Res.* **225**, 219-236.

Hesketh, J., Campbell, G., Piechacyzk, M. and Blanchard, J.M. (1994) Targeting of c-myc and β-globin coding sequences to cytoskeletal-bound polysomes by c-myc 3' untranslated region. *Biochem.J.* **298** , 143-148.

Hilgenberg, L.G., Hoover, C.L. and Smith, M.A. (1999) Evidence of an agrin receptor in cortical neurons. *J. Neurosci.* **19**, 7384-7393.

Hoch, W., Campanelli, J.T. and Scheller, R.H. (1994) Agrin-induced clustering of acetylcholine receptors: A cytoskeletal link. *J. Cell Biol.* **126**, 1-4.

Hoch, W., Ferns, M., Campanelli, J.T., Hall, Z.W. and Scheller, R.H. (1993) Developmental regulation of highly active alternatively spliced forms of agrin. *Neuron* **11**, 479-490.

Hodges, B.L., Hayashi, Y.K., Nonaka, I., Wang, W., Arahata, K. and Kaufman, S.J. (1997) Altered expression of the alpha7beta1 integrin in human and murine muscular dystrophies. *J. Cell Sci.* **110**, 2873-2881.

Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis G.D. and Sheppard, H.M., Kuang, W.J., Wood, W.I., Goeddel, D.V. and Vandelen, R.L. (1992) Identification of heregulin, a specific activator of p185erbB2. *Science* **256**, 1205-1210.

Hopf, C. and Hoch, W. (1996) Agrin binding to α-dystroglycan. *J. Biol. Chem.* **271**, 5231-5236.

Huang, R.I., Tong, J. and Schmidt, M.M. (1992) Protein kinase C couples membrane excitation to acetylcholine receptor gene inactivation in chick skeletal muscle. *Neuron* **9**, 671-678.

Hynes, R.O. (1992) Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Imaizumi-Scherrer, T., Faust, D.M., Benichou, J.C., Hellio, R. and Weiss, M.C. (1996) Accumulation in fetal muscle and localization to the neuromuscular junction of cAMP-dependent protein kinase A regulatory and catalytic subunits RIa and Ca. *J. Cell Biol.* **134**, 1241-1254.

Janknecht, R. and Nordheim, A. (1993) Gene regulation by Ets proteins. *Biochim. Biophys. Acta* **1155**, 346-356.

Jasmin, B.J., Changeux, J.P. and Cartaud, J. (1989) Compartmentalization of cold-stable and acetylated microtubules in the subsynaptic domain of chick skeletal muscle fiber. *Nature* **344**, 673-675.

Jasmin, B.J., Cartaud, A., Ludosky, M.A., Changeux, J.P. and Cartaud, J. (1990) Asymmetric distribution of dystrophin in developing and adult Torpedo marmorata electrocyte: Evidence for its association with the acetylcholine receptor-rich membrane. *Proc. Natl. Acad. Sci. USA* **87**, 3938-3941.

Jasmin, B.J., Lee, R.K. and Rotundo, R.L. (1993) Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction. *Neuron* **11**, 467-477.

Jasmin, B.J., Campbell, R.J. and Michel R.N. (1995a) Nerve-dependent regulation of succinate dehydrogenase in junctional and extrajunctional compartments of rat muscle fibers. *J. Physiol.* **484**,155-164.

Jasmin, B.J., Antony, C., Changeux, J.P. and Cartaud, J. (1995b) Nerve-dependent plasticity of the Golgi complex in skeletal muscle fibers: Compartmentalization within the subneural sarcoplasm. *Eur. J. Neurosci.* **7**, 470-479.

Jasmin, B.J., Alameddine, H., Lunde, J.A., Stetzkowksi-Marden, F., Collin, H., Tinsley, J.M., Davies, K.E., Tome, F.M.S., Parry, D.J. and Cartaud, J. (1995c) Expression of utrophin and its mRNA in denervated *mdx* mouse muscle. *FEBS Lett.* **374**, 393-398.

Ji, R.R., Bose, C.M., Lesuisse, C., Qiu, D., Huang, J.C., Zhang, Q. and Rupp, F. (1998) Specific agrin isoforms induce cAMP response element binding protein phosphorylation in hippocampal neurons. *J. Neurosci.* **18**, 9695-96702.

Jia, H.-T., Tsay, H.-J. and Schmidt, J. (1992) Analysis of binding and activating functions of the chick muscle acetylcholine receptor γ-subunit upstream sequence. *Cell. Mol. Neurobiol.* **12**, 241-258.

Jones, G., Moore, C., Hashemolhosseini, S. and Brenner, H.R. (1999) Constitutively active MuSK is clustered in the absence of agrin and induces ectopic postsynaptic-like membranes in skeletal muscle fibers. *J. Neurosci.* **19**, 3376-3383.

Kammesheidt, A. and Martin, P.T. (1999) Manipulation of N-acetyl-D-galactosamine, a synaptic carbohydrate, affects utrophin expression, distribution, and function in dystrophic muscles. *Soc. Neurosci.* **25**, 1840.

Jones, G., Herczeg, A., Ruegg, M.A., Lichtsteiner, M., Kröger, S. and Brenner, H.R. (1996) Substrate-bound agrin induces expression of acetylcholine receptor ε-subunit gene in cultured mammalian muscle cells. *Proc. Natl. Acad. Sci. USA* **93**, 5985-5990.

Jones, G., Lichtsteiner, M., Witzemann, V., Sakmann, B. and Brenner, H.R. (1997) Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle. *Proc. Natl. Acad. Sci. USA* **94**, 2654-2659.

Kandel, E.R., Schwartz, J.H., Jessell, T.M. (1991) *Principles of Neural Science 3rd Edition*. New York: Elsevier Press.

Karnovsky, M.J. and Roots, L. (1964) A "direct coloring" thiocholine method for cholinesterase. *J. Histochem. Cytochem.* **12**, 219-221.

Karpati, G., Carpenter, S., Morris, G.E., Davies, K.E., Guerin, C. and Holland, P. (1993) Localization and quantitation of the chromosome 6-encoded dystrophin-related protein in normal and pathological human muscle. *J. Neuropath. Exp. Neurol.* **52**, 119-128.

Khan, M.A. (1993) Corticosteroid therapy in Duchenne muscular dystrophy. *J. Neurol. Sci.* **120**, 8-14.

Khurana, T.S., Watkins, S.C. and Kunkel, L.M. (1992) The subcellular distribution of chromosome-6-encoded dystrophin-related protein in the brain. *J. Cell Biol.* **119**, 357-366.

Khurana, T.S., Kunkel, L.M., Frederickson, A.D., Carbonetto, S. and Watkins, S.C. (1995) Interaction of chromosome-6-encoded dystrophin related protein with the extracellular matrix. *J. Cell. Sci.* **108**, 173-185.

Khurana, T.S., Hoffman, E.P. and Kunkel, L.M. (1990) Identification of a chromosome-6-encoded dystrophin-related protein. *J. Biol. Chem.* **265**, 16717-16720.

Khurana, T.S., Rosmarin, A.G., Shang, J., Krag, T.O.B., Das, S. and Gammeltoft, S. (1999) Activation of utrophin promoter by heregulin via the ets-related transcription factor complex GA-binding protein α/β. *Mol. Biol. Cell* **10**, 2075-2086.

Khurana, T.S., Watkins, S.C., Chafey, P., Chelly, J., Tome, F.M., Fardeau, M., Kaplan, J. and Kunkel, L.M. (1991) Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle. *Neuromusc. Disord.* **1**, 185-194.

Kim, D., Chi, S., Lee, K.H., Rhee, S., Kwon, Y.K., Chung, C.H., Kwon, H. and Kang, M.-S. (1999) Neuregulin stimulates myogenic differentiation in an autocrine manner. *J. Biol. Chem.* **274**, 15395-15400.

Kim-Ha, J., Webster, P., Smith, J. and MacDonald, P. (1993) Multiple RNA regulatory elements mediate distinct steps in localization of osker mRNA. *Development* **119**, 169-178.

Kislauskis, E.H., Zhu, X. and Singer, R.H. (1994) Sequences responsible for intracellular localization of β-actin messenger RNA also affect cell phenotype. *J. Cell Biol.* **127**, 441-451.

Klarsfeld, A., Laufer, R., Fontaine, B., Devillers-Theiry,A., Dubreuil, C. and Changeux, J.P. (1989) Regulation of the muscle AChR α-subunit gene expression by electrical activity: Involvement of protein kinase C and Ca++. *Neuron* **2**, 1229-12236.

Klarsfeld, A., Bessereau, J.L., Salmon, A.M. Triller, A., Babinet, C. and Changeux, J.P. (1991) An acetylcholine receptor a-subunit promoter conferrin preferential synaptic expression in muscles of transgenic mice. *EMBO J.* **10**, 625-632.

Koenig, M., Monaco, A.P. and Kunkel, L.M. (1988) The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* **53**, 219-228.

Koga, R., Ishiura, S., Takemitsu, M., Kamakura, K., Matsuzaki, T., Arahata, K., Nonaka, I. and Sugita, H. (1993) Immunoblot analysis of dystrophin-related protein (DRP). *Biochim. Biophys. Acta* **1180**, 257-261.

Koike, S., Schaeffer, L. and Changeux, J.P. (1995) Identification of a DNA element determining synaptic expression of the mouse acetylcholine receptor δ-subunit gene. *Proc. Natl. Acad. Sci. USA* **92**, 10624-10628.

Krejci, E., Thomine, S., Boschetti, N., Legay, C., Sketelj, J., Massoulie, J. (1997) The mammalian gene of acetylcholinesterase-associated collagen. *J. Biol. Chem.* **272**, 22840-22847.

Kues, W.A., Sakmann, B. and Witzemann, V. (1995) Differential expression patterns of five acetylcholine receptor subunit genes in rat muscle during development. *Eur. J. Neurosci.* **7**, 1376-1385.

Kuhse, J., Betz, H., and Kirsch, J. (1995) The inhibitory glycine receptor: architecture, synaptic localization and molecular pathology of a postsynaptic ion-channel complex. *Curr. Op. Neurobiol.* **5**, 318-323.

Lafrenie, R.M. and Yamada, K.M. (1996) Integrin-dependent signal transduction. *J. Cell Biochem.* **61**, 543-553.

Lamarco, K., Thompson, C.C., Byers, B.P.,Walton, E.M. and McKnight, S.L. (1991) Identification of Ets- and Notch-related subunits in GA binding protein. *Science* **253**, 789-792.

Lambert, M., Chafey, P., Hugnot, J.P., Koulakoff, A., Berwald-Netter, Y., Billard, C., Morris, G.E., Kahn, A., Kaplan, J. and Gilgenkrantz, H. (1993) Expression of the transcripts in the 62nd intron of the dystrophin gene. *Neuromusc. Disord.* **3**, 519-524.

Laufer, R., Klarsfeld, A. and Changeux, J.P. (1991) Phorbol esters inhibit the activity of the chicken acetylcholine receptor alpha-subunit gene promoter. *Eur. J. Biochem.* **202**, 813-818.

Lemmon, M.A. and Schlesinger, J. (1994) Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem. Sci.* **19**, 459-463.

Lev, A.A., Feener, C.C., Kunkel, L.M. and Brown, R.H. (1987) Expression of the Duchennes's muscular dystrophy gene in cultured muscle cells. *J. Biol. Chem.* **262**,15817-15820.

Lidov, H.G., Seling, S. and Kunkel, L.M. (1995) Dp140: A novel 140 kDa CNS transcript from the dystrophin locus. *Hum. Mol. Genet.* **4**, 329-335.

Lieth, E. and Fallon, J.R. (1993) Muscle agrin: Neural regulation and localization at nerve-induced acetylcholine receptor clusters. *J. Neurosci.* 13, 2509-2515.

Lomo, T. and Rosenthal, J. (1972) Control of ACh sensitivity by muscle activity. *J. Physiol.* **221**, 496-513.

Lomo, T. and Westgaard, R.H. (1975) Control of Ach sensitivity in rat muscle fibers. *Cold Spring Harbor Symp. Quant. Biol.* **40**, 263-274.

Love, D.R., Morris, G.E., Ellis, J.M., Fairbrother, U., Marsden, R.F., Bloomfield, J.F., Edward, Y.H., Slater, C.P., Parry, D.J. and Davies, K.E. (1991) Tissue distribution of the dystrophin-related gene product and expression in the *mdx* and dy mouse. *Proc. Natl. Acad. Sci. USA* **88**, 3243-3247.

Love, D.R., Hill, D.F., Dickson, G., Spurr, N.K., Byth, B.C., Marsden, R.F., Walsh, F.S., Edwards, Y.H. and Davies, K.E. (1989) An autosomal transcript in skeletal muscle with homology to dystrophin. *Nature* **339**, 55-58.

Lu, J.T., Son, Y.J., Lee, J., Jetton, T.L., Shiota, M., Moscoso, L., Niswender, K.D., Lewy. A,D., Magnuson, M.A., Sanes, J.R. and Emeson, R.B. (1999) Mice lacking alpha-calcitonin gene-related peptide exhibit normal cardiovascular regulation and neuromuscular development. *Mol. Cell. Neurosci.* **14**, 99-120.

Ludolph, D.C. and Konieczny, S.E. (1995) Transcription factor families: Muscling in on the myogenic program. *FASEB Journal* **9**, 1595-1604.

Lumeng, C., Phelps, S., Rafael, J.A., Cox, G.A., Hutchinson, T.L., Begy, C.R., Adkins, E., Wiltshire, R. and Chamberlain, J.S. (1999) Characterization of dystrophin and utrophin diversity in the mouse. *Hum. Mol. Genet.* **8**, 593-599.

Magill-Solc, C. and McMahan, U.J. (1988) Motor neurons contain agrin-like molecules. *J. Cell Biol.* **107**, 1825-1833.

Magill-Solc, C. and McMahan, U.J. (1990) Synthesis and transport of agrin-like molecules in motor neurons. *J. Exp. Biol.* **153**, 1-10

Marais, R., Wynne, J., and Treisman, R. (1993) The SRF accesory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**, 381-393.

Marchionni, M.A., Goodearl, A.D., Chen, O., Berminghal-McDonough, M.S., Kirk,C., Hendridcks, M., Danehy,F, Misumi, D, Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassre, M., Hiles, I., Davis, J.B., Hsuam, J.J., Totty, N.F., Otsu, M., McBurney, R.N., Waterfield, M.D., Strootbent, P., Gwynne, D. (1993) Glial growth factors are alternatively spliced ErbB2 ligands expressed in the nervous system. *Nature* **362**, 312-318.

Marshall, C.J. (1994) MAP kinase kinase kinase: MAP kinase kinase and MAP kinase *Curr. Op. Genet. Dev.* **4**, 82-89.

Marte, B.M. Graus-Porta, D., Jeschke, M., Doriano, F., Nynes, N.E., Taverna, D. (1995) NDF/heregulin activates MAP kinase and p70/p85 S6 kinase during proliferation or differentiation of mammary epithelial cells. *Oncogene* **10**, 167-175.

Martin, P.T., Kaufman, S.J., Kramer, R.H. and Sanes, J.R. (1996) Synaptic integrins in developing, adult, and mutant mice: Selective association of α1, α7A and α7B integrins with the neuromuscular junction. *Dev. Biol.* **174**, 125-139.

Martin, P.T. and Sanes, J.R. (1995) Role for a synapse-specific carbohydrate in agrin-induced clustering of acetylcholine receptors. *Neuron* **14**, 743-754.

Martin, P.T. and Sanes, J.R. (1997) Integrins mediate adhesion to agrin and modulate agrin signaling. *Development* **124**, 3909-3917.

Martinou, J.C., Falls, D.L., Fischbach, G. and Merlie, J.P. (1991) Acetylcholine-receptor-inducing activity stimulates expression of the e-subunit gene of the muscle nicotinic receptor. *Proc. Natl. Acad. Sci. USA* **88**, 7699-7773.

Matsumura, K. and Campbell, K.P. (1994) Dystrophin-associated complex: Its role in the molecular pathogenesis of muscular dystrophies. *Muscle Nerve* **17**, 2-15.

Matsumura, K., Ervasti, J.M., Ohlendieck, K., Kahl, S.D. and Campbell, K.P. (1992) Association of dystrophin-related protein with dystrophin-associated proteins in *mdx* mouse muscle. *Nature* **360**, 588-591.

Matsumura, K., Yamada, H., Shimizu, T. and Campbell, K.P. (1993b) Differential expression of dystrophin, utrophin and dystrophin-associated proteins in peripheral nerve. *FEBS Lett.* **334**, 281-285.

Matsumura, K., Shasby, D.M. and Campbell, K.P. (1993a) Purification of dystrophin-related protein (utrophin) from lung and its identification in pulmonary artery endothelial cells. *FEBS Lett.* **326**, 289-293.

Mayer, U., Saher, G., Fassler, R., Bornemann, A., Echtermeyer, F., von der Mark, H., Miosge, N., Poschl, E. and von der Mark, K. (1997) Absence of integrin alpha 7 causes a novel form of muscular dystrophy. *Nature Genet.* **17**, 318-323.

Mckormick, F. (1994) Activators and effectors of ras p21 proteins. *Curr. Op. Genet. Dev.* **4**, 71-76.

McMahan, U.J. (1990) The agrin hypothesis. *Cold Spring Harbor Symp. Quant. Biol.* **55**, 407-418.

Medford, R.M., Nguyen, H.T. and Nadal-Ginard, B. (1983) Transcriptional and cell cycle-mediated regulation of myosin heavy chain gene expression during muscle cell differentiation. *J. Biol. Chem.* **258**, 11063-11073.

Meier, T., Hauser, D.M., Chiquet, M., Landman, L., Ruegg, M.A. and Brenner, H.R. (1997) Neural agrin induces ectopic postsynaptic specializations in innervated muscle fibers. *J. Neurosci.* **17**, 6534-6544.

Meier, T., Masciulli, F., Moore, C., Schoumacher, F., Eppenberger, U., Denzer, A.J., Jones, G., Brenner, H. (1998) Agrin can mediate AChR gene expression in muscle by aggregation of muscle-derived neuregulins. *J. Cell Biol.* **141**, 715-726.

Meier, T., Perez, G.M. and Wallace, B.G. (1995) Immobilization of nicotinic acetylcholine receptors in mouse C2 myotubes by agrin-induced protein tyrosine phosphorylation. *J. Cell Biol.* **131**, 441-451.

Merlie, J.,P. and Sanes, J.R. (1985) Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibers. *Nature* **317**, 66-68.

Meyer, D. and Birchmeier, C. (1995) Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390.

Michel, R.N., Vu, C.Q., Tetzlaff, W. and Jasmin, B.J. (1994) Neural regulation of acetylcholinesterase mRNAs at mammalian neuromuscular synapses. *J. Cell Biol.* **127**, 1061-1069.

Miledi, R. (1960) The acetylcholine sensitivity of frog muscle fibers after complete and partial denervation. *J. Physiol.* **151**, 1-23.

Mizuno, Y., Nonaka, I., Hirai, S. and Ozawa, E. (1993) Reciprocal expression of dystrophin and utrophin in muscles of Duchenne muscular dystrophy patients, female DMD-carriers and control subjects. *J. Neurol. Sci.* **119**, 43-52.

Mizuno, Y., Yoshida, M., Yamamoto, H., Hirai, S. and Ozawa, E. (1993) Distribution of dystrophin isoforms and dystrophin-associated proteins 43DAG (A3a) and 50DAG (A2) in various monkey tissues. *J. Biochem.* **114**, 936-941.

Molentin, J.D. and Olson, E.N. (1996) Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. *Proc. Natl. Acad. Sci. USA* **93**, 9366-9373.

Monaco, A.P., Neve, L.P. Coletti-Feener, C., Bertelson, C.J., Kurnit, D.M. and Kunkel, L.M. (1986) Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. *Nature* **323**, 646-650.

Moscoso, L.M., Merlie, J.P. and Sanes, J.R. (1995a) N-CAM, 43K-rapsyn, and S-laminin mRNAs are concentrated at synaptic sites in muscle fibers. *Mol. Cell. Neurosci.* **6**, 80-89.

Moscoso, L.M., Chu, G.C., Gautam, M., Noakes, P.G., Merlie, J.P. and Sanes, J.R. (1995b) Synapse-associated expression of an acetylcholine receptor inducing protein, ARIA/heregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. *Dev. Biol.* **172**, 158-169.

Mowry, K.L. and Melton, D.A. (1992) Vegetal messenger RNA localization directed by 340 nt RNA sequence in Xenopus oocyte. *Science* **255**, 991-994.

Namba, T. and Scheller, R.H. (1996) Inhibition of agrin-mediated acetylcholine receptor clustering by utrophin C-terminal peptides. *Genes to Cells* **1**, 755-764.

Nastuk, M.A. Lieth, E., Ma, J., Cardasis,C.A., Moynihan, E.B. and Fallon, J.R. (1991) The putative agrin receptor binds ligand in a calcium-dependent manner and aggregates during agrin-induced acetylcholine receptor clustering. *Neuron* 7, 807-818.

New, H.V. and Mudge, A.W. (1986) Calcitonin gene related peptide regulates muscle acetylcholine receptor biosynthesis. *Nature* **323**, 809-811.

Nicholson, L.V.B., Johnson, M.A., Davison, K., O'Donnell, E., Falkous, G., Barron, M. and Harris, J.B. (1992) Dystrophin or a "related protein' in Duchenne muscular dystrophy? *Acta Neurol. Scand.* **86**, 8-14.

Nirde, P., Georget, V., Terouanne, B., Galifer, R.B., Belon, C. and Sultan, C. (1998) Quantitation of androgen receptor messenger RNA from genital skin fibroblasts by reverse transcription- competitive polymerase chain reaction. *J. Steroid Biochem. Mol. Biol.* **66**, 35-43.

Nitkin, R.M., Smith, M.A., Magill, C. and Fallon, J.R. (1987) Identification of agrin, a synaptic organizing protein from Torpedo electric organ. *J. Cell Biol.* **105**, 2471-2478.

Nitkin, M.A and Rothschild,T.C. (1990) Agrin-induced reorganization of extracellular matrix componenets on cultured myotubes: Relationship to AChR-aggregation. *J. Cell Biol.* **105**, 2471-2478.

Noakes, P.G., Phillips, W.D., Hanley, T.A., Sanes, J.R. and Merlie, J.P. (1993) 43K protein and acetylcholine receptors colocalize during the initial stages of neuromuscular synapse formation in vivo. *Dev. Biol.* **155**, 275-280.

Noguchi, S., Wakabayashi, E., Imamura, M., Yoshida, M. and Ozawa, E. (1999) Developmental expression of sarcoglycan gene products in cultured myocytes. *Biochem. Biophys. Res. Comm.* **262,** 88-93.

Nudel, U., Robzyk, K. and Yaffe, D. (1988) Expression of putative Duchenne muscular dystrophy gene in differentiated myogenic cell cultures and in the brain. *Nature* **331**, 635-638.

O'Hagan, R.C. and Hassell, J.A. (1999) The PEA3 Ets transcription factor is a downstream target of the HER2/neu receptor tyrosine kinase. *Oncogene* **16**, 301-310.

Ogata, T. (1988) Structure of motor endplates in the different muscle fiber types of vertebrate skeletal muscles. *Arch. Histol. Cytol.* **51**, 385-424.

Ohlendieck, K., Ervasti, J.M., Matsumura, K., Kahl, S.D., Leveille, C.J. and Campbell, K.P. (1991) Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. *Neuron* **7**, 499-508.

Ohtsuka, Y., Udaka, K., Yamashiro, Y., Yagita, H. and Okumura, K. (1998) Dystrophin acts as a transplantation rejection antigen in dystrophin-deficient mice: implication for gene therapy. *J. Immunol.* **160**, 4635-40.

Osterland, M., Fontaine, B., Devillers-Thiery, A., Geoffroy, B. and Changeux, J.P. (1989). Acetylcholine receptor expression in primary cultures of embryonic myotubes-I.

Discoordinate regulation of α, g, and δ-subunit gene expression by calcitonin gene-related peptide and by muscle electrical activity. *Neurosci.* **32**, 279-287.

Ozaki, M., Sasner, M., Yano, R., Lu, H.S. and Buonanno, A. (1997) Neuregulin-beta induces expression of an NMDA-receptor subunit. *Nature* **390**, 691-694.

Pasquini, F., Guerin, C., Blake, D., Davies, K.E., Karpati, G. and Holland, P. (1995) The effect of glucocorticoids on the accumulation of utrophin by cultured normal and dystrophic human skeletal muscle satellite cells. *Neuromusc. Disord.* **5**, 105-114.

Passaquin, A.C., Metzinger, L., Leger, J.J., Warter, J. and Poindron, P. (1993) Prednisolone enhances myogenesis and dystrophin-related protein in skeletal muscle cell cultures from *mdx* mouse. *J. Neurosci. Res* **35**, 363-372.

Pasternak, C., Wong, S. and Elson, E.L. (1995) Mechanical function of dystrophin in muscle cells. *J. Cell Biol.* **128**, 355-361.

Pearce, M., Blake, D.J., Tinsley, J.M., Byth, B.C., Campbell, L., Monaco, A.P. and Davies, K.E. (1993) The utrophin and dystrophin genes share similarities in genomic structure. *Hum. Mol. Genet.* **2**, 1765-1772.

Peles, E. and Yarden, Y. (1993) Neu and its ligands: From an oncogene to neural factors. *BioEssays* **15**, 815-824.

Peng, H.B., Ali, A.A., Dai, Z., Daggett, D.F., Raulo, E. and Rauvala, H. (1995) The role of heparin-binding growth-associated molecule (HB-GAM) in the postsynaptic induction in cultured muscle cells. *J. Neurosci.* **15**, 3027-3038.

Peters, M.F., Kramarcy, N.R., Sealock, R. and Froehner, S.C. (1994) beta 2-syntrophin: Localization at the neuromuscular junction in skeletal muscle. *NeuroReport* **5**, 1577-1580.

Petrof, B.J., Shrager, J.B., Stedman, H.H., Kelly, A.M. and Sweeney, H.L. (1993) Dystrophin protects the sarcolemma from stress developed during muscle contraction. *Proc. Natl. Acad. Sci. USA* **90**, 3710-3714.

Phillips, W.D., Noakes, P.G., Roberds, S.L., Campbell, K.P. and Merlie, J.P. (1993) Clustering and immobilization of acetylcholine receptors by the 43-kD protein: a possible role for dystrophin-related protein. *J. Cell Biol.* **123**, 729-740.

Piette, J. Bessereau, J.L., Huchet, M. and Changeux, J.P. (1990) Two adjacent MyoD1-binding sites regulate expression of the acetylcholine receptor α-subunit gene. *Nature* **345**, 353-355.

Pons, F., Robert, A., Fabbrizio, E., Hugon, G., Califnao, J., Fehrentz, J.A., Marinez, J and Mornet, D. (1994) Utrophin localization in normal and dystrophin-deficient heart. *Circ.* **90**, 369-374.

Pons, F., Robert, A., Marinin, J.F. and Leger, J.J. (1994) Does utrophin expression in muscles of *mdx* mice during postnatal development functionally compensate for dystrophin deficiency. *J. Neurol. Sci.* **122**, 162-170.

Pons, F., Nicholson, L.V.B., Robert, A., Voit, T. and Leger, J.J. (1993) Dystrophin and dystrophin-related protein (utrophin) distribution in normal and dystrophin-deficient skeletal muscles. *Neuromusc. Disord.* **3**, 507-514.

Popper, P. and Micevych, P.E. (1989) Localization of calcitonin gene-related peptide and its receptors in a striated muscle. *Brain Res.* **496**, 180-186.

Prody, C.A. and Merlie, J.P. (1992) The 5' flanking region of the mouse muscle nicotinic acetylcholine receptor b-subunit gene promotes expression in cultured muscle cells and is activated by MRF4, myogenin, and MyoD. *Nucl. Acids Res.* **20**, 2367-2372.

Rafael, J.A., Tinsley, J.M., Potter, A.C., Deconinck, A.E., and Davies, K.E. (1998) Skeletal muscle-specific expression of a utrophin transgene rescues utrophin-dystrophin deficient mice. *Nature Genet.* **19,** 79-82.

Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilgenkrantz, H., Couton, D., Cartaud, J., Briand, P., Kaplan, J.C., Perricaudet, M. *et al.* (1993) Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of *mdx* mice. *Nature* **361,** 647-650.

Ranvier, L. (1888). *Traite technique d-histologie.* Paris, Savy

Renshaw, M.W., Price, L.S. and Schwartz, M.A. (1999) Focal adhesion kinase mediates the integrin signaling requirement for growth factor activation of MAP kinase. *J. Cell Biol.* **147,** 611-618.

Rieff, H.I., Raetzman, L.T. Sapp, D.W., Yeh, H.H., Siegel, R.E. and Corfas, G. (1999) Neuregulin induces GABAA receptor subunit expression and neurite outgrowth in cerebellar granule cells. *J. Neurosci.* **19,** 10757-10766.

Rimer, M. Cohen, I., Lomo, T. Burden, S.J. and McMahan, U.J. (1998) Neuregulins and ErbB receptors at neuromuscular junctions and at agrin-induced postsynaptic-like apparatus in skeletal muscle. *Mol. Cell. Neurosci.* **12**, 1-15.

Rimer, M., Cohen, I., Burden, S.J. and McMahan, U.J. (1996) Neural agrin induces aggregation of muscle-derived ARIA, and its receptors erbB2 and 3 in adult rat soleus muscle. *Soc. Neurosci. Abstr.* **22**, 1689.

Robbins (1994) MAP kinases Erk1 and Erk2: Pleiotropic enzymes in a ubiquitous signaling network. *Adv. Cancer Res.* **63**, 93-116.

Roberts, R.G., Freeman, T.C., Kendall, E., Vetrie, D.L.P., Dixon, A.K., Shaw-Smith, C., Bone, Q. and Bobrow, M. (1996) Characterization of *DRP2*, a novel human dystrophin homologue. *Nature Genet.* **13**, 223-226.

Robinson, L., Panayiotakis, A., Papas, T.S., Kola, I. and Seth, A. (1997) ETS target genes: Identification of Egr1 as a target by RNA differential display and whole genome PCR techniques. *Proc. Natl. Acad. Sci. USA* **94**, 7170-7175.

Rodius, F., Claudepierre, T., Rosas-Vargas, H., Cisneros, B., Montanez, C., Dreyfus, H., Mornet, D. and Rendon, A. (1997) Dystrophins in developing retina: Dp260 expression correlates with synaptic maturation. *NeuroReport* **8**, 2383-2387.

Rosmarin, A..G., Caprio, D.G., Kirsch, D.G., Handa, H and Simkevich, C.P. (1995) GABP and PU.1 compete for binding yet cooperate to increase CD18 (b2-integrin) transcription. *J. Biol. Chem.* **270**, 23627-23633.

Rosmarin, A..G., Luo, M., Caprio, D.G., Shang, J. and Simkevich, C.P. (1998) Sp1 cooperates with the ets-related transcription factor, GABP, to activate CD18 (b2 integrin) promoter. *J. Biol. Chem.* **273**, 13097-13103.

Roush, W. (1997). Backup gene may help muscles help themselves. *Science* **276**, 35.

Rudnicki, M.A. and Jaenisch, R. (1995) The MyoD family of transcription factors and skeletal myogenesis. *BioEssays* **17**, 203-219.

Ruegg, M.A. and Bixby, J.L. (1998). Agrin orchestrates synaptic differentiation at the vertebrate neuromuscular junction. *Trends Neurosci.* **21**, 22-27.

Ruegg, M.A, Tsim, K.W., Horton, S.E., Kroger, S. and Escher, G. (1992) The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. *Neuron* **8**, 691-699.

Rupp, F., Ozcelik, T.H., Linial, M., Peterson, K., Francke, U. and Scheller, R.H. (1992) Structure and chromosomal localization of the mammalian agrin gene. *J. Neurosci.* **12**, 3535-3544.

Sadoulet-Puccio, H.M. and Kunkel, L.M. (1996) Dystrophin and its isoforms. *Brain Pathol.* **6**, 25-35.

Salpeter, M.M., and Harris, R. (1983) Distribution and turnover rate of acetylcholine receptors throughout the junction folds at a vertebrate neuromuscular junction. *J. Cell Biol.* **96**, 1781-1785.

Sanes, J.R., Engvall, E., Butkowski, R. and Hunter, D.D. (1990) Molecular heterogeneity of basal laminae: Isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. *J. Cell Biol.* **111**, 1685-1699.

Sanes, J.R. and Lichtman, J.W. (1999) Development of the verterbrate neuromuscular junction. *Annu Rev. Neurosci.* **22**, 389-442.

Sanes, J.R., Johnson, Y.R., Kotzbauer, T., Mudd, J., Hanley, T., Martinou, J.C. and Merlie, J.P. (1991). Selective expression of an acetylcholine receptor-LacZ transgene in synaptic nuclei of adult muscle fibers. *Development* **113**, 1181-1191.

Sapru, M., Florence, S.K., Kirk, C. and Goldman, D. (1998). Identification of a neuregulin and protein-tyrosine phosphatase response element in the nicotinic acetylcholine receptor ε-subunit gene: Regulatory role of an Ets transcription factor. *Proc. Natl. Acad. Sci. USA* **95**, 1289-1294.

Sawa, C., Goto, M., Suzuki, F., Watanabe, H., Sawada, J.I. and Handa, H. (1996) Functional domains of transcription factor hgabp-beta-1/e4tf1-53 required for nuclear localization and transcription activation. *Nucl. Acids. Res.* **24**, 4954-4961.

Schaeffer, L., Duclert, N., Huchet-Dymanus, M. and Changeux, J.P. (1998) Implication of a multisubunit Ets-related transcription factor in synaptic expression of the nicotinic acetylcholine receptor. *EMBO J.* **17,** 3078-3090.

Schlessinger, J. (1994) SH2/SH3 signaling proteins. *Curr. Op. Genet. Dev.***4**, 25-30.

Schofield, J.N., Blake, D.J., Simmons, C., Morris, G.E., Tinsley, J.M., Davies, K.E. and Edwards, Y.H. (1994) Apo-dystrophin-1 and apo-dystrophin-2, products of the

Duchenne muscular dystrophy locus: Expression during mouse embryogenesis and in cultured cell lines. *Hum. Mol. Genet.* **3**, 1309-1316.

Schofield, J., Houzelstein, D., Davies, K.E., Buckingham, M. and Edwards, Y.H. (1993) Expression of the dystrophin-related protein (utrophin) gene expression during mouse embryogenesis. *Develop. Dyn.* **198**, 254-264.

Schwartz, M.A. (1994) Integrins as signal transducing receptors. In *Integrins*. Takada, Y. (ed):. CRC Press, Boca Raton, Florida. Pp. 133-145.

Scott, L.J., Bacou, F. and Sanes, J.R. (1988) A synapse-specific carbohydrate at the neuromuscular junction: Association with both acetylcholinesterase and a glycolipid. *J. Neurosci.* **8**, 932-944.

Serpinskaya, A.S., Feng, G., Sanes, J.R. and Craig, A.M. (1999) Synapse formation by hippocampal neurons from agrin-deficient mice. *Dev. Biol.* **205**, 65-78.

Seth, A. and Papas, T.S. (1990) The c-Ets-1 proto-oncogene has oncogenic activity and is positively autoregulated. *Oncogene* **5**, 1761-1767.

Sewry, C.A., Matsumura, K, Campbell, K.P. and Dubowitz, V. (1994) Expression of dystrophin-associated glycoproteins and utrophin in carriers of Duchenne muscular dystrophy. *Neuromusc. Disord.* **4**, 401-409.

Sewry, C.A.,Sansome, A, Clerk, A., Sherratt, T.G., Hasson, N., Rodillo, E., Heckman, J.Z., Strong, P.N. and Dubowitz, V. (1993) Manifesting carriers of Xp21 muscular dystrophy; lack of correlation between dystrophin expression and clinical weakness. *Neuromusc. Disord.* **3**, 141-148.

Sharma, V., Xu, M., Vail, J. and Campbell, R. (1998) Comparative analysis of multiple techniques for semi-quantitation of RT-PCR amplicons. *Biotech. Tech.* **12**, 521-524.

Si, J.T., Luo, Z.J. and Mei, L. (1996) Induction of acetylcholine receptor gene expression by ARIA requires activation of mitogen-activated protein kinase. *J. Biol. Chem.* **271**, 19752-19759.

Si, J. and Mei, L. (1999) ERK MAP kinase activation is required for acetylcholine receptor inducing activity-induced increase in all five acetylcholine receptor subunit mRNAs as well as synapse-specific expression of acetylcholine receptor ε-transgene. *Mol.Brain Res.* **67**, 18-27.

Simon, A.M., Hoppe, P. and Burden, S. (1992) Spatial restriction of AChR gene expression to subsynaptic myonuclei. *Development* **114**, 545-553.

Simon, A.M. and Burden, S. (1993) An E-box mediates activation and repression of the acetycholine receptor α-subunit gene during myogenesis. *Mol. Cell. Biol.* **13**, 5133-5140.

Simoneau, J.A. and Bouchard, C. (1989) Human variation in skeletal muscle fiber-type proportion and enzyme activities. *Am. J. Physiol.* **257**, E567-E572.

St. Johnston, D. (1995) The intracellular localization of messenger RNAs. *Cell* **81**, 161-170.

Straub, V., Rafael, J.A., Chamberlain, J.S. and Campbell, K.P. (1997) Animal models for muscular dystrophy show different patterns of sarcolemmal disruption. *J. Cell Biol.* **139**, 375-385.

Straub, V. and Campbell, K.P. (1997) Muscular dystrophies and the dystrophin-glycoprotein complex. *Curr. Op. Neurol.* **10**, 168-175.

Sugiyama, J., Bowen, D.C. and Hall, Z.W. (1994) Dystroglycan binds nerve and muscle agrin. *Neuron* **13**, 103-115.

Sumarsono, S.H., Wilson, T.J., Tymms, M.J., Venter, D.J., Corrick, C.M., Kola, R., Lahoud, M.H., Papas, T.S., Seth, A. and Kola, I. (1996) Down's syndrome-like skeletal abnormalities in Ets2 transgenic mice. *Nature* **379**, 534-537.

Takemitsu, M., Ishiura, S., Koga, R., Kamakura, K., Arahata, K., Nonaka, I. and Sugita, H. (1991) Dystrophin-related protein in the fetal and denervated skeletal muscles of normal and *mdx* mice. *Biochem. Biophys. Res. Commun.* **180**, 1179-1186.

Tang, J., Ahn Jo, S. and Burden, S.J. (1994) Separate pathways for synapse-specific and electrical activity-dependent gene expression in skeletal muscle. *Development* **120**, 1799-1804.

Tanowitz, M., Si, J., Yu, D.H., Feng, G.S. and Mei, L. (1999) Regulation of neuregulin-mediated acetylcholine receptor synthesis by protein tyrosine phosphatase SHP2. *J.Neurosci.* **19**, 9426-9435.

Tansey, M.G., Chu, G.C. and Merlie, J.P. (1996) ARIA/HRG regulates AChR ∈ subunit gene expression at the neuromuscular synapse via activation of phosphatidylinositol 3-kinase and Ras/MAPK pathway. *J. Cell Biol.* **134**, 465-476.

Taverna, D., Disatnik, M.H., Rayburn, H., Bronson, R.T., Yang, J., Rando, T.A. and Hynes R.O. (1998) Dystrophic muscle in mice chimeric for expression of alpha5 integrin. *J. Cell Biol.* **143,** 849-859.

Taylor, J., Muntoni, F., Dubowitz, V. and Sewry,C.A. (1997) The abnormal expression of utrophin in Duchenne and Becker muscular dystrophy is age related. *Neuropath. Appl. Neurol.* **23**, 399-405.

Tennyson, C.N., Dally, G.Y., Ray, P.N., and Worton, R.G. (1996b) Expression of the dystrophin isoform Dp71 in differentiating human fetal myogenic cultures. *Hum. Mol. Genet.* **5**, 1559-1566.

Tennyson, C.N., Shi, Q.W. and Worton, R.G. (1996a) Stability of the human dystrophin transcript in muscle. *Nucl. Acids Res.* **24**, 3059-3064.

thi Man, N., Ellis, J.M., Love, D.R., Davies, K.E., Gatter, K.C., Dickson, G. and Morris, G.E. (1991) Localization of the DMDL gene-encoded dystrophin-related protein using a panel

of nineteen monoclonal antibodies: Presence at neuromuscular junctions, in the sarcolemma of dystrophic skeletal muscle, in vascular and other smooth muscles, and in proliferating brain cell lines. *J. Cell Biol.* **115**, 1695-1700.

Tinsley, J.M. and Davies, K.E. (1993) Utrophin: A potential replacement for dystrophin. *Neuromusc. Disord.* **3**, 537-539.

Tinsley, J., Deconinck, N., Fisher, R., Kahn, D., Phelps, S., Gillis, J.M. and Davies, K. (1998). Expression of full-length utrophin prevents muscular dystrophy in *mdx* mice. *Nature Med.* **4**, 1441-1444.

Tinsley, J.M., Blake, D.J., Roche, A., Fairbrother, U., Riss, J., Byth, B.C., Knight, A.E., Kendrick-Jones, J., Suthers, G.K., Love, D.R., Edwards, Y.H. and Davies, K.E. (1992) Primary structure of dystrophin-related protein. *Nature* **360**, 591-593.

Tinsley, J.M., Potter, A.C., Phelps, S.R., Fisher, R., Trickett, J.I. and Davies, K.E. (1996) Amelioration of the dystrophic phenotype of *mdx* mice using a truncated utrophin transgene. *Nature* **384**, 349-353.

Uchino, M., Teramoto, H., Naoe, H., Miike, T., Yoshioka, K. and Ando, M. (1994a) Dystrophin and dystrophin-related protein in the central nervous system of normal controls and Duchenne muscular dystrophy. *Acta. Neuropathol.* **87**, 129-134.

Uchino, M., Yoshioka, K., Miike, T., Tokunaga, M., Uyama, E., Teramoto, H., Naoe, H. and Ando, M. (1994b) Dystrophin and dystrophin-related protein in the brains of normal and *mdx* mice. *Muscle Nerve* **17**, 533-538.

Usdin, T.B. and Fischbach, G. (1986) Purification and characterization of a polypeptide from chick brain promotes the accumulation of acetylcholine receptors in chick myotubes. *J. Cell Biol.* **103**, 493-507.

Valenzuela, D.M., Stitt, T.N., Distefano, P.S., Rojas, E., Mattson, K., Compton, D.L., Nunez, L., Park, J.S., Stark, J.L., Gies, D.R., Thomas, S., Lebeau, M.M., Fernald, A.A., Copeland, N.G., Jenkins, N.A., Burden, S.J., Glass, D.J. and Yancopoulos, G.D. (1995). Receptor tyrosine kinase specific for the skeletal muscle lineage: Expression in embryonic muscle at the neuromuscular junction, and after injury. *Neuron* **15**, 573-584.

van der Velden, A.W. and Thomas, A.A.M. (1999) The role of the 5 ' untranslated region of an mRNA in translation regulation during development. *Int. J. Biochem. Cell Biol.* **31**,87-106.

van Kempen, G. T. H., Molenaar, P. C. and Slater, C. R. (1994) Release and synthesis of acetylcholine at ectopic neuromuscular junctions in the rat. *J. Physiol. (Lond.)* **478**, 229-238.

Vater, R., Young, C., Anderson, L.V.B., Lindsay, S., Blake, D.J., Davies, K.E., Zuellig, R.A., and Slater, C.R. (1998) Utrophin mRNA expression in muscle is not restricted to the neuromuscular junction. *Mol. Cell. Neurosci.* **10**, 229-242.

Veyrune, J.L. Hesketh, J., Blanchard, J.M. (1997) 3' untranslated regions of c-myc and c-fos mRNAs: multifunctional elements regulating mRNA translation, degradation and subcellular localization in *Progress in Molecular and Subcellular Biology, Volume 18.* Ph. Jeanteur (editor). Berlin: Springer-Verlag.

Walke, W., Staple, J., Adams, L., Gnegy, M., Chahine, K.G. and Goldman, D. (1994) Calcium dependent regulation of rat and chick muscle nicotinic acetyltcholine receptor (nAChR) gene expression. *J. Biol. Chem.* **269**, 19447-19456.

Wallace, B.G. (1989) Agrin-induced specializations contain cytoplasmic, membrane and extracellular matric-associated components at the postsynaptic apparatus. *J. Neurosci.* **9**, 1294-1302.

Wallace, B.G. (1995) Regulation of the interaction of nicotinic acetylcholine receptors with the cytoskeleton by agrin-activated protein tyrosine kinase. *J. Cell Biol.* **128**, 1121-1129.

Wang, X.M., Tsay, H.J. and Schmidt, J. (1990) Expression of the acetylcholine receptor delta-subunit gene in differentiating chick muscle cells is activated by an element that contains two 16 bp copies of a segment of the alpha-subunit enhancer. *EMBO J.* **9**, 783-790.

Wasylyk, B., Hahn, S.L. and Giovane, A. (1993) The Ets family of transcription factors. *Eur. J. Biochem.* **211**, 7-18.

Watkins, S.C., Hoffman, E.P., Slayter, H.S. and Kunkel, L.M. (1988) Immunoelectron microscopic localization of dystrophin in myofibers. *Nature* **333**, 863-866.

Webster, C., Silberstein, L., Hays, A.P. and Blau, H.M. (1988) Fast muscle fibers are preferentially affected in Duchenne muscular dystrophy. *Cell* **52**, 503-513.

Weintraub, H. (1993) The MyoD family and myogenesis: Redundancy, networks, and thresholds. *Cell* **75**, 1241-1244.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T.K., Turner, D., Rupp, R. and Hollenberg, S. (1991) The MyoD gene family: Nodal point during specification of the muscle cell lineage. *Science* **251**, 761-766.

Williamson, R.A., Henry, M.D., Daniels, K.J., Hrstka, R.F., Lee, J.C., Sunada, Y., Ibraghimov-Beskrovnaya, O. and Campbell, K.P. (1997) Dystroglycan is essential for early embryonic development: Disruption of Reicharts membrane in Dag1-null mice. *Hum. Mol. Genet.* **6**, 831-841.

Willis, A.E. (1999) Translational control of growth factor and proto-oncogene expression. *Int. J. Biochem. Cell Biol.* **31**, 73-86.

Wilson, L.A., Cooper, B.J., Dux, L., Dubowitz, V. and Sewry, C.A. (1994) Expression of utrophin (dystrophin-related protein) during regeneration and maturation of skeletal muscle in canine X-linked muscular dystrophy. *Neuropath. Appl. Neurol.* **20**, 359-367.

Wilson, J., Putt, W., Jiminez, C. and Edwards, Y.H. (1999) Up71 and Up140, two novel transcripts of utrophin that are homologues of short forms of dystrophin. *Hum.Mol.Genet.* **8**, 1271-1278.

Winder, S.J. (1997) The membrane-cytoskeleton interface: The role of dystrophin and utrophin. *J. Muscle Res. Cell Motil.* **18,** 617-629.

Winder, S.J. and Kendrick-Jones, J. (1995) Calcium/calmodulin -dependent regulation of the NH_2-terminal F-actin binding domain of utrophin. *FEBS* **357,** 125-128.

Winder, S.J. and James, M. (1999) Tyrosine phosphorylation of β-dystroglycan regulates binding to utrophin. *Soc. Neurosci.* **25,** 161A.

Witzemann, V., Brenner, H.R. and Sakmann, B. (1991) Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. *J. Cell Biol.* **114,** 125-141.

Won, S., Si, J.T., Colledge, M., Ravichandran, K.S., Froehner, S.C. and Mei, L. (1999) Neuregulin-increased expression of acetylcholine receptor epsilon-subunit gene requires ErbB interaction with Shc. *J. Neurochem.* **73,** 2358-2368.

Worton, R. (1995) Muscular dystrophies: Diseases of the dystrophin-glycoprotein complex. *Science* **270,** 755-760.

Yang, J.F., Ng, Y.P., Pun, S., Ip, N.Y. and Tsim, K.W.K. (1997) The EGF-like domain of chick acetylcholine receptor aggregating activity (ARIA) contains its full biological activity. *FEBS* **403**, 163-167.

Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G. and Campbell, K.P. (1995) SH3 domain-mediated interaction of dystroglycan and Grb2. *J. Biol. Chem.* **270**, 11711-11714.

Yoshida, M. and Ozawa, E. (1990) Glycoprotein complex anchoring dystrophin to sarcolemma. *J. Biochem.* **108**, 748-752.

Young, C., Lindsay, S., Vater, R. and Slater, C.R. (1998) An improved method for the simultaneous demonstration of mRNA and esterase activity at the human neuromuscular junction. *Histochem. J.* **30**, 7-11.

Zhao, J., Yoshioka, K., Miyatake, M. and Miike, T. (1992) Dystrophin and a dystrophin-related protein in intrafusal muscle fibers, and neuromuscular and myotendinous junctions. *Acta. Neuropathol.* **84**, 141-146.

Zhao, J.E., Lochumuller, H., Nalbantoglu, J., Allen, C., Prescott, S., Massie, B. and Karpati, G. (1997) Study of adenovirus-mediated dystrophin minigene transfer to skeletal

muscle by combined microscopic display of adenoviral DNA and dystrophin. *Hum. Gene Ther.* **8**, 1565-1573.

Zhu, X., Lai, C., Thomas, S., and Burden, S.J. (1995) Neuregulin receptors Erb3 and ErbB4 are localized at neuromuscular synapses. *EMBO J.* **14**, 5842-5848.

Zubrzycka-Gaarn, E.E., Bulman, D.E., Karpati, G., Burghes, A.H., Belfall, B., Klamut, H.J., Talbot, J., Hodges, R.S., Ray, P.N. and Worton, R.G. (1988) The Duchenne muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle. *Nature* **333**, 466-469.

CHAPTER 9

CURRICULUM VITAE: A.O. GRAMOLINI

DEPARTMENT OF CELLULAR AND MOLECULAR MEDICINE
FACULTY OF MEDICINE, UNIVERSITY OF OTTAWA,
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Education

DOCTORATE OF PHILOSOPHY (PHD) 1996 - PRESENT
CELLULAR AND MOLECULAR MEDICINE
University of Ottawa, Ottawa, ON

MASTERS OF SCIENCE (MSC) 1993 - 1995
PHYSIOLOGY
University of Ottawa, Ontario, ON

BACHELORS OF SCIENCE (BSC) 1989 -1993
PHYSIOLOGY
University of Saskatchewan, Saskatoon, SK

Scholarships

2000-2003	Medical Research Council of Canada Postdoctoral Fellowship	$33,000/yr
1999-2000	Strategic Area Development Scholarship, University of Ottawa	$10,000
1998-99	Ontario Graduate Scholarship, Ministry of Education and Training	$11,995/yr
1996-98	Arthur Minden Pre-Doctoral Fellowship, Muscular Dystrophy Association of Canada	$20,150/yr
1996-99	University of Ottawa, Graduate Studies and Research Excellence Scholarships	$4,750/yr
1996	University of Ottawa, Graduate Studies and Research Special Research Assistantship	$3,000
1996	University of Ottawa, Graduate Studies and Research Admission Scholarship	$4,050
1995/96	Department of Physiology, University of Ottawa Entrance Scholarship (PhD)	$500
1993/94	Department of Physiology, University of Ottawa Entrance Scholarship (MSc)	$1,100
1990/91	Saskfirst Athletic Scholarship	$1,000
1989/90	University of Ottawa Science Scholarship (applicant declined as attended U. of Sask.)	$1,500
1989/90	Carleton University Science Scholarship (applicant declined as attended U. of Sask.)	$2,000

Awards and Citation

External Awards

Travel Fellowship. Travel award from the International Society for Neurochemistry to cover expenses at the Xth International Symposium on Cholinergic Mechanisms, Arcachon, France, September 1-5, 1998.

Pre- and Post-Doctoral Trainee Award. First place standing for Excellence in the Poster Presentation at meeting of "Neural and Neuromuscular Aspects of Muscle Fatigue: A Satellite Symposium to Precede the 24th Annual Neuroscience Meeting" Miami, Florida. November 12-14, 1994.

Internal Awards

Gerry Taichman Award. Best Research Achievement (PhD Program), Department of Cellular and Molecular Medicine, University of Ottawa. May 14, 1999.

Travel Award. Travel award from the School of Graduate Studies and Research, University of Ottawa, to cover expenses at the Annual Meeting for the Society for Neuroscience, New Orleans, LA, October 25-29, 1997.

Outstanding Student Seminar. First place standing for PhD research presentation, Department of Physiology, University of Ottawa. May 2, 1997.

Certificate of Achievement. First place standing for non-research MSc seminar, Department of Physiology, University of Ottawa. May 6, 1994.

Taichman Poster Award. Second place standing in University of Ottawa, Department of Physiology for MSc research poster presentation. May 6, 1994.

Publications (Papers)

1. S. Newey, **A.O. Gramolini,** J. Wu, B.J. Jasmin, Davies, K.E. and Jasmin, B.J. (2000) A novel mechanism for modulating synaptic gene expression: Differential localization of α-dystrobrevin transcripts in skeletal muscle. (In preparation)

2. **A.O. Gramolini,** G. Belanger and B.J. Jasmin (2000) Stabilization and targeting of utrophin transcripts in skeletal muscle cells is dependent upon the 3' untranslated region (UTR). (In preparation).

3. **A.O. Gramolini,** J. Wu. and B.J. Jasmin (2000) Regulation and functional significance of utrophin expression at the mammalian neuromuscular synapse. *Microscopy Research Techniques*. (In press)

4. **A.O. Gramolini** and B.J. Jasmin. (1999) Expression of the utrophin gene during myogenic differentiation. *Nucleic Acids Research* **27**, 3603-3609.

5. **A.O. Gramolini**, L.A. Angus, L. Schaeffer, E.A. Burton, J.M. Tinsley, K.E. Davies, J.P. Changeux and B.J. Jasmin (1999) Induction of utrophin gene expression by heregulin: Role of the N-box motif and GABP. *Proceedings of the National Academy of Science U.S.A.* **96**, 3223-3227.

6. **A.O. Gramolini**, G. Karpati and B.J. Jasmin. (1999) Discordant expression of utrophin and its transcript in human and mouse skeletal muscle. *Journal of Neuropathology and Experimental Neurology* **58**, 235-244.

7. **A.O. Gramolini** and B.J. Jasmin. (1998) Molecular mechanisms and putative signaling events controlling utrophin expression in mammalian skeletal muscle fibers. *Neuromuscular Disorders* **8**, 351-361.

8. B.J.Jasmin, **A.O. Gramolini**, F. Adatia, C. Boudreau-Lariviere, R.Y.Y. Chan, A.M. Krupa, J. A. Lunde and F.A. Mankal. (1998) Nerve-derived trophic factors and transcriptional regulatory mechanisms at the neuromuscular junction of skeletal muscle. *Canadian Journal of Applied Physiology* **23**, 366-376.

9. **A.O. Gramolini,** E.A. Burton, J.T. Tinsley, M.F. Ferns, A. Cartaud, J. Cartaud, K.E. Davies, J.L. Lunde and B.J. Jasmin. (1998) Muscle and neural isoforms of agrin increase utrophin expression in cultured myotubes via a transcriptional regulatory mechanism. *Journal of Biological Chemistry* **273**, 736-743.

10. **A.O. Gramolini** and B.J. Jasmin (1997) Duchenne muscular dystrophy and the neuromuscular junction: The utrophin link. *BioEssays* **19**, 747-750.

11. **A.O. Gramolini**, C.L. Dennis, J.M. Tinsley, G.S. Robertson, J.Cartaud, K.E. Davies and B.J. Jasmin (1997) Local transcriptional control of utrophin expression at the neuromuscular synapse. *Journal of Biological Chemistry* **272**, 8117-8120.

12. **A.O. Gramolini** and J-M. Renaud (1997) Blocking $K^+_{(ATP)}$ channels during metabolic inhibition impairs skeletal muscle contractility. *American Journal of Physiology (Cell Physiology)* **272**, C1936-1946.

13. J-M. Renaud, **A.O. Gramolini**, P. Light and A. Comtois (1996) Modulation of muscle contractility during fatigue and recovery by ATP-sensitive potassium channels. *Acta Physiologica Scandinavica* **156**, 203-212.

Publications (Abstracts)

(1) **A.O. Gramolini**, G. Bélanger, J. Wu, and B.J. Jasmin. The 3'UTR regulates the stability and targeting of utrophin RNAs in skeletal muscle cells. *Society for Neuroscience* **26** (In press)

(2) **A.O. Gramolini**, J.A Lunde, J. Wu, and B.J. Jasmin. Regulation of utrophin expression during development of skeletal muscle cells. Xth International Symposium on Cholinergic Mechanisms. Arcachon, France, September 1-5, 1998. *Journal of Physiology (Paris)* **92**, 434.

(3) **A.O. Gramolini**, J.M. Tinsley, K.E. Davies,and B.J. Jasmin. Transcriptional regulation of utrophin in skeletal muscle by a DNA element and agrin. Society for Neuroscience Annual Meeting. New Orleans, Louisiana, October 25-29, 1997. *Society for Neuroscience* **23**, 35.

(4) **A.O. Gramolini**, J.M. Tinsley, G.S. Robertson, K.E. Davies, J.Cartaud, and B.J. Jasmin. Localization of utrophin mRNA in skeletel muscle fibers and its regulation by agrin. Society for Neuroscience Annual Meeting. Washington, D.C., November 16-21, 1996. *Society for Neuroscience* **22**, 535.

(5) **A.O. Gramolini**, D.J. Parry, and B.J.Jasmin. Utrophin expression in skeletal muscle fibers and its role in synaptogenesis. *Ontario Exercise Physiology Winter Meeting*, Orillia, Ontario, February 2-4, 1996.

(6) **A.O. Gramolini** and J-M. Renaud. $K^+_{(ATP)}$ channel activators fail to induce a faster rate of fatigue and/or recovery in skeletal muscle of the frog, *Rana Pipiens*. Canadian Society of Exercise Physiology Annual Meeting, Quebec, Quebec, October 26-28, 1995. *Canadian Journal of Applied Physiology*, **20 Supplement**, 18P.

(7) **A.O. Gramolini** and J-M. Renaud. Changes in skeletal muscle excitability and contractility with

$K^+_{(ATP)}$ channel modulators during metabolic inhibition: Implications for fatigue. Neural and Neuromuscular Aspects of Muscle Fatigue: A Satellite Symposium to Precede the 24th Annual Neuroscience Meeting, Miami, Florida, November 11-14, 1994. *Muscle & Nerve* **Supplement 4**, S41.

(8) **A.O. Gramolini** and J-M. Renaud. Changes in skeletal muscle excitability and contractility with $K^+_{(ATP)}$ channel modulators during metabolic inhibition. Canadian Society of Exercise Physiology Annual Meeting, Hamilton, Ontario, October 15-19, 1994. *Canadian Journal of Applied Physiology.* **19 Supplement**, 19P

(9) **A.O. Gramolini** and N. McDuffie. Unscheduled DNA synthesis and glycoconjugate modification caused by organochloride pesticide addition to endothelial cell cultures. *Society for Complex Carbohydrates*, San Juan, Puerto Rico, November 16-20, 1993.

Invited Seminars

A. Gramolini "*Cellular and molecular mechanisms controlling utrophin expression at the mammalian neuromuscular junction.*" Department of Cell Biology, Duke University, North Carolina. Feb 18, 1999.

A. Gramolini "*Developing therapies for Duchenne muscular dystrophy (DMD)*" Rehabilitation Medicine Grand Rounds, Ottawa General Hospital. Feb 25, 2000.

CHAPTER 10

JOURNAL OF
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June 22, 1999

Anthony Gramolini
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Dear Dr. Gramolini:

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June 18, 1999

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