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Expression of the utrophin gene during myogenic differentiation

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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 homolog 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 h 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 post-mitotic 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 acetylcholine receptor (AChR), the neural cell-adhesion molecule (NCAM) and the enzyme acetylcholinesterase (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 (reviewed in 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 (19–22).

In 1989 Love and colleagues (23) identified an autosomal homolog 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 post-synaptic 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 homolog to dystrophin, and (ii) the utrophin promoter contains an E-box (42).

METHODS AND MATERIALS

Tissue culture

C2C12 muscle cells were cultured and maintained as described previously (37). Experiments were performed on either

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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, CA) 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, IN). 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 min at 42°C and the mixture was heated to 99°C for 5 min to terminate the reaction. Negative controls were prepared by substituting the 2 μl of total RNA for RNase-free water. Utrophin cDNAs of 548 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, 30–34 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'-GACTATGGAGGAGTGAAA-AA-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 <10% variation between them (data not shown) indicating that equivalent amounts of total RNA were indeed analyzed.

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 Phosphor-Imager (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 chemi-fluorescence 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) 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 & 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 (1× 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 (NEN) for 4 h (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 h), 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

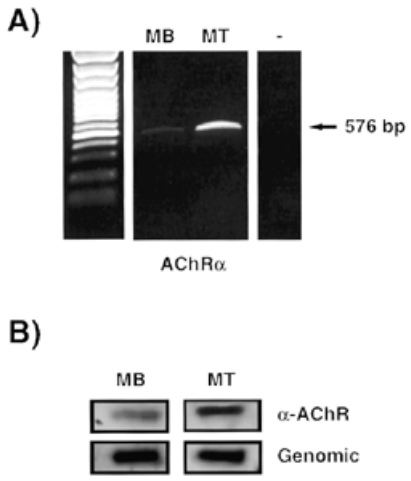

Figure 1. Differentiation of C2C12 muscle cells leads to a significant increase in AChR α-subunit expression. (**A**) Shown is a representative example of an ethidium bromide-stained agarose gel of RT–PCR products corresponding to AChR α-subunit cDNAs obtained from myoblasts (MB) and myotubes (MT). Left 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.

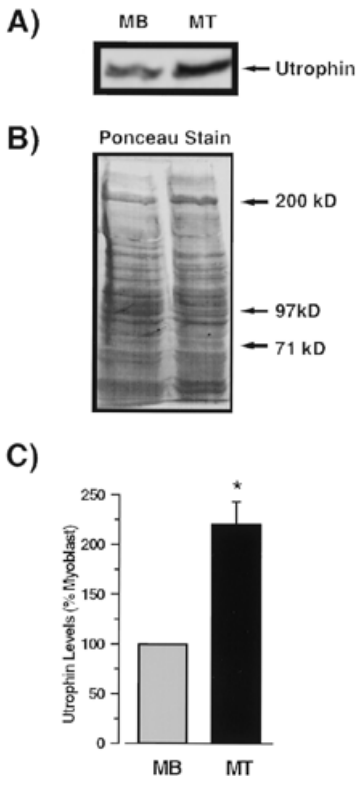

Figure 2. Utrophin protein levels increase during myogenesis. Muscle cells were solubilized and protein extracts were subjected to immunoblotting. (**A**) A representative example of a western blot showing utrophin levels in myoblasts (MB) versus myotubes (MT). (**B**) The same membrane was reprocessed for Ponceau staining to stain total protein. Relative molecular masses are indicated to the right. (**C**) Utrophin levels were quantitated and expressed as a percent of the levels seen in confluent myoblasts. Shown are the results obtained with four independent experiments. All data are expressed as mean ±SEM. The asterisk denotes a significant difference (Student's *t*-test, $P < 0.05$).

(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 ($P < 0.05$) (Fig. 1A), and that the rate of transcription for this gene increased similarly under these conditions (up to 8-fold; $P < 0.05$, $n = 9$) (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 3B). Differentiation of the myoblasts into myotubes resulted in a further increase in the levels of utrophin transcripts (Fig. 3A). In agreement with the immunoblot data, the abundance of utrophin transcripts increased by ~2-fold in myotubes as compared to myoblasts (Fig. 3B). Consistent with these results obtained with mouse myotubes, we noted that myogenic differentiation of human skeletal muscle cells also led to a 1.5–2-fold increase in utrophin (Fig. 4A) and its mRNA (Fig. 4B) (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 (Fig. 3A and B), we determined that expression of the utrophin gene increased during myogenic differentiation (Fig. 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 (Fig. 5B). By contrast, the rate of transcription of the GAPDH gene remained largely unchanged during myogenesis (9).

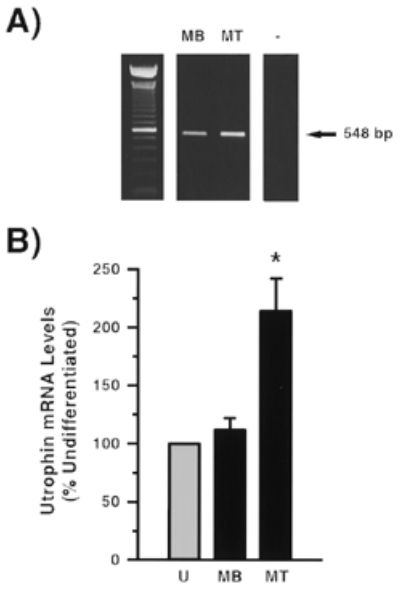

Figure 3. Myogenic differentiation increases utrophin transcript levels. (**A**) A representative ethidium bromide-stained agarose gel of RT–PCR products corresponding to utrophin cDNAs obtained from myoblasts (MB) and myotubes (MT). The negative control lane is marked with a minus sign. The left lane 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. The asterisk denotes a significant difference from undifferentiated myoblasts (Student's *t*-test, $P < 0.05$).

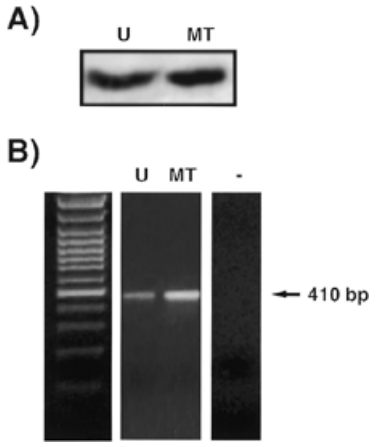

Figure 4. Utrophin protein and mRNA levels in human skeletal muscle cells are increased during myogenic differentiation. (**A**) A representative immunoblot revealing that utrophin levels increase during muscle cell development from undifferentiated myoblasts (U) to myotubes (MT). (**B**) A representative 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.

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 h and RNA samples were collected and analyzed 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 h. In addition, we observed that the half-life of utrophin transcripts was ~20 h in myoblasts and that it remained largely unaffected in myotubes (Fig. 6A and B). In a second experimental approach, we performed pulse–chase experiments. In these assays, we determined that the halflife of utrophin mRNAs was also ~20 h in both myoblasts and myotubes (Fig. 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 are regulated, at least partially, by MyoD family members that interact with E-box motifs located within the 5' flanking region of these genes (reviewed in 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 (reviewed in 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 the 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

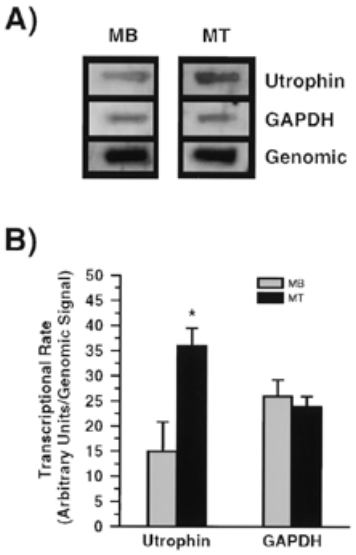

Figure 5. Myogenic differentiation results in an increase in transcription of the utrophin gene. (**A**) Shown are representative autoradiograms of run-on assays using nuclei obtained from myoblasts (MB) and myotubes (MT). (**B**) Quantitation of the nuclear run-on assays. Hybridization signals were 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, $P < 0.05$).

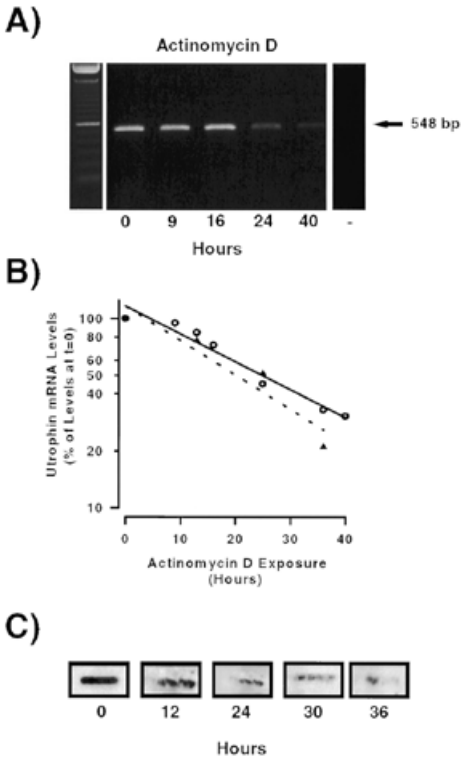

Figure 6. Half-life determination of utrophin transcripts in myogenic cultures. (**A**) Inhibition of RNA synthesis was achieved by exposing cultures to actinomycin D at time zero. Shown is a representative ethidium bromide-stained agarose gel of utrophin PCR products following actinomycin D exposure 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 h in undifferentiated myoblasts and is largely unchanged in myotubes (~24 h). Closed circle, utrophin transcript levels at time zero for both myoblasts and myotubes; triangles, myoblasts; dashed line, linear regression for myoblast data; open circles, 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 h following exposure to ^{3}H-uridine.

(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 (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 life span of a muscle fiber, 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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