Increased expression of utrophin in a slow vs. a fast muscle involves posttranscriptional events

ANTHONY O. GRAMOLINI, GUY BÉLANGER, JENNIFER M. THOMPSON, JOE V. CHAKKALAKAL, AND BERNARD J. JASMIN

Department of Cellular and Molecular Medicine, Faculty of Medicine, and Centre for Neuromuscular Disease, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

Received 15 March 2001; accepted in final form 5 June 2001

Gramolini, Anthony O., Guy Bélanger, Jennifer M. Thompson, Joe V. Chakkalakal, and Bernard J. Jasmin. Increased expression of utrophin in a slow vs. a fast muscle involves posttranscriptional events. Am J Physiol Cell Physiol 281: C1300–C1309, 2001.—In addition to showing differences in the levels of contractile proteins and metabolic enzymes, fast and slow muscles also differ in their expression profile of structural and synaptic proteins. Because utrophin is a structural protein expressed at the neuromuscular junction, we hypothesize that its expression may be different between fast and slow muscles. Western blots showed that, compared with fast extensor digitorum longus (EDL) muscles, slow soleus muscles contain significantly more utrophin. Quantitative RT-PCR revealed that this difference is accompanied by a parallel increase in the expression of utrophin transcripts. Interestingly, the higher levels of utrophin and its mRNA appear to occur in extrasynaptic regions of muscle fibers as shown by immunofluorescence and in situ hybridization experiments. Furthermore, nuclear run-on assays showed that the rate of transcription of the utrophin gene was nearly identical between EDL and soleus muscles, indicating that increased mRNA stability accounts for the higher levels of utrophin in slow muscles. Direct plasmid injections of reporter gene constructs showed that cis-acting elements contained within the utrophin 3′-untranslated region (3′-UTR) confer greater stability to chimeric LacZ transcripts in soleus muscles. Finally, we observed a clear difference between EDL and soleus muscles in the abundance of RNA-binding proteins interacting with the utrophin 3′-UTR. Together, these findings highlight the contribution of posttranscriptional events in regulating the expression of utrophin in muscle.

Duchenne muscular dystrophy; synaptic proteins; messenger ribonucleic acid stability; ribonucleic acid-binding proteins; neuromuscular junction

DUCHEENNE MUSCULAR DYSTROPHY (DMD) is the most prevalent inherited neuromuscular disorder, affecting 1 of every 3,500 male births (15). The disease is characterized by repetitive cycles of muscle degeneration-regeneration, with fast-contracting fibers being preferentially affected (56). Eventually, the muscle’s regenerative capacity fails, and the muscle mass is progressively replaced by adipose and connective tissues. The disease is extremely severe because children become wheelchair bound by early adolescence and death usually occurs in their second or third decade of life. DMD results from mutations/deletions in the X-linked dystrophin gene, which prevents production of a large cytoskeletal protein of the spectrin superfamily termed dystrophin (37, 58). Several approaches have been envisaged to counteract the deleterious effects of this progressive disease, including, for example, gene therapy and cell transfer. An alternative strategy consists in using a protein normally expressed in dystrophic muscle which, once expressed at appropriate levels and at the correct subcellular location, could functionally compensate for the lack of dystrophin. A good candidate for such a role is utrophin, primarily because of its high degree of sequence identity with dystrophin (53). In addition, several recent studies using transgenic mouse model systems have clearly shown the ability of utrophin to functionally compensate for the lack of dystrophin (for example, see Ref. 54).

In contrast to dystrophin, which is found along the entire length of normal muscle fibers (37, 58), utrophin accumulates preferentially at the postsynaptic membrane of the neuromuscular junction in both normal and DMD muscles (4, 24). In this context, it has been shown that local transcriptional activation of the utrophin gene in myonuclei located within the postsynaptic sarcoplasm accounts, at least partially, for the preferential expression of utrophin at the neuromuscular junction (18, 20, 21, 31, 55). Interestingly however, there are several cases in which expression of utrophin has been shown to extend well into extrasynaptic regions of muscle fibers. For example, utrophin is known to be present outside synaptic regions in small or regenerating muscle fibers of DMD patients (26, 30, 40). Moreover, increased levels of utrophin have been reported along the length of developing fibers in both embryonic and neonatal muscles (30, 32, 42, 50). Under specific conditions, therefore, utrophin presents a more homogeneous distribution along muscle fibers, indicating that additional regulatory mechanisms are likely
involved in controlling the overall levels and localization of utrophin in skeletal muscle.

It is well established that adult fast and slow skeletal muscles differ markedly in their physiological characteristics as a result of pronounced differences in the expression of numerous contractile proteins and metabolic enzymes (for review, see Refs. 44, 48, and 51). In addition to these well-known differences, recent studies have also demonstrated that fast and slow muscles contain varying amounts of structural proteins. In particular, slow muscles express significantly more spectrin (38, 57) and dystrophin (11, 28). Interestingly, there is also evidence showing that differences between fast and slow muscles extend even to the pattern of expression of transcripts encoding synaptic proteins. Indeed, mRNAs encoding acetylcholinesterase (AChE) (39), acetylcholine receptor (AChR) subunits (34), and ColQ (33) are found expressed at significant levels in extrasynaptic regions of slow muscle fibers. Because utrophin is a structural protein preferentially expressed at the neuromuscular junction, we hypothesize in the present study that the expression and localization of utrophin is different between fast and slow muscles. Because our results indicated that slow muscles do, in fact, contain more utrophin than fast muscles, we also became interested in identifying the molecular mechanisms that account for this difference.

METHODS

Animal care. Control C57BL/6 mice were obtained from Charles River Laboratories (St. Constant, Quebec, Canada) and housed in the University of Ottawa Animal Care Facility. All surgical procedures were performed in accordance with the strict guidelines established by the Canadian Council on Animal Care. For most of these studies, the slow soleus and fast extensor digitorum longus (EDL) muscles were used. In some cases, the lateral and medial gastrocnemius muscles were also excised. Because we were also interested in determining utrophin levels in the fast and slow regions of a given muscle, these muscles were further dissected into their red and white compartments. After excision, muscles were either flash frozen in liquid nitrogen or embedded in optimum cutting temperature compound and frozen in melting isopentane precooled with liquid nitrogen.

Characterization of reporter gene constructs and direct gene transfer. The full-length utrophin 3′-untranslated region (3′-UTR) was generated by RT-PCR using RNA isolated from C2C12 cells and primers designed against the mouse utrophin sequence (25). The PCR product was sequenced and compared with sequences available in data banks (Gramolini, Bélanger, and Jasmin, unpublished observations). The full-length utrophin 3′-UTR (1,995 nt) was subsequently inserted downstream of the reporter gene LacZ in the pCMV SPORT β-galactosidase expression vector (GIBCO BRL, Burlington, Ontario, Canada) in the forward or reverse (used as a control) orientation.

Direct gene transfer was performed as described in detail elsewhere (10, 20, 21) with the exception that soleus and EDL muscles were injected bilaterally with plasmid DNA instead of tibialis anterior muscles. Briefly, a small incision was made on the lateral side of both hindlimbs while the mice were anesthetized. The soleus and EDL muscles were carefully isolated and injected with 25 μl of the appropriate plasmid cDNAs (2–4 μg/μl) under a dissecting microscope. The wounds were then closed using autoclips, and the animals were returned to their cage. Seven to ten days later, injected muscles were excised and immediately frozen in liquid nitrogen. The injection solution contained, in addition to the appropriate reporter constructs (see above), a constitutively expressed chloramphenicol acetyltransferase (CAT) plasmid (Promega), which was coinjected to monitor the efficiency of transduction. In all these experiments, plasmid DNA was prepared using the Qiagen Mega-prep and/or Mini-prep procedures (Chatsworth, CA).

RNA extraction and RT-PCR. Total RNA was extracted from muscles using TriPure as recommended by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Briefly, soleus and EDL muscles were homogenized in 1 ml of TriPure reagent using a Polytron. After chloroform addition, the homogenates were centrifuged at 12,000 g for 10 min at 4°C. The resulting aqueous phase was transferred to a fresh microfuge tube, and 0.5 ml of ice-cold isopropanol was added. The RNA pellets were isolated by centrifugation at 12,000 g for 15 min and thoroughly washed with 70% isopropanol. The pellets were then dried in 1 ml of RNA-free water and stored at −20°C. Before RT-PCR, RNA samples were treated with RQ DNase I (Promega, Madison, WI) at 37°C for 90 min and heated at 65°C for 20 min to terminate the reaction.

Quantitative RT-PCR was used to strictly determine the relative abundance of utrophin transcripts in fast vs. slow muscles. This assay was performed as described in detail previously (18, 20, 29, 39). Reverse transcription was carried out at 42°C using 100 ng of total RNA and random hexamers. After 45 min, the RT mixtures were heated to 99°C for 5 min to terminate the reaction. Primers that selectively amplified utrophin (20, 29) and S12 rRNA (6, 16) were designed on the basis of available sequences. These primers amplified a 548-bp and a 368-bp fragment from the mouse utrophin and S12 cDNAs, respectively. To examine expression of utrophin A and B mRNAs, primers against the unique 5′-UTR of these transcripts were designed on the basis of available sequences (8). These primers amplify a 530-bp and a 101-bp fragment in the utrophin A and B transcripts, respectively. cDNAs encoding β-galactosidase were amplified using primers that generate a 506-bp fragment (5). PCR amplification was performed in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT). Each cycle of amplification consisted of denaturation at 94°C for 1 min, primer annealing at 65°C for 1 min, and extension at 72°C for 1 min. In these assays, negative controls consisted of reverse transcription mixtures in which total RNA was replaced with RNase-free water.

The PCR products were visualized on 1% agarose gel containing ethidium bromide. The 100-bp molecular weight markers (MBI Fermentas, Flamborough, Ontario, Canada, and GIBCO BRL) were used to estimate the size of the PCR products. For quantitative experiments, PCR products were separated and visualized on agarose gels containing the fluorescent dye Vistra Green (Amersham, Arlington Heights, IL) (20). The labeling intensity of the PCR product, which is linearly related to the amount of DNA, was subsequently quantitated using a Storm PhosphorImager and the accompanying Imagequant software (Molecular Dynamics, Sunnyvale, CA). Values obtained for utrophin were standardized relative to the corresponding level of rRNA in the same sample. All RT-PCR measurements aimed at determining the relative abundance of selected transcripts were performed during the linear range of amplification (see, for example, Refs. 9 and 39). Typically, the cycle numbers were 26 to 30. RT-PCR conditions (primer concentrations, input RNA, choice of RT primer, cycling conditions) were initially...
optimized, and these were identical for all samples. Appropriate precautions were taken to avoid contamination and RNA degradation. All samples as well as negative controls were prepared using common master mixes containing the same RT and PCR reagents, and they were always run in parallel. In all experiments, PCR products were never detected for the negative controls.

**In situ hybridization.** Longitudinal serial cryostat sections (12 μm) from EDL and soleus muscles were cut in a cryostat and placed on Superfrost microscope slides (VWR Canlab). Slides were first processed for AChE histochemistry, and the regions containing neuromuscular junctions were photographed. The sections were then subjected to in situ hybridization using synthetic oligonucleotides for detection of utrophin transcripts as described (21). The in situ hybridization experiments were performed using two antisense oligonucleotides complementary to mouse utrophin transcripts (21).

Quantitative analysis of in situ hybridization labeling was performed using an image analysis system equipped with Northern Eclipse software (Empix Imaging, Mississauga, Ontario, Canada). Briefly, labeled cDNA density in synaptic vs. extrasynaptic regions was determined by measuring the pixel intensity within circular fields of similar sizes in muscle fibers and by subtracting background values determined as the signal seen in regions external to the fibers (21, 23, 41).

For these analyses, both EDL and soleus muscle sections were placed on the same slide and processed for in situ hybridization simultaneously. Three separate experiments were performed using a minimum of ten muscle sections per condition. A minimum of four measurements were performed on each cryostat section.

**Immunoblotting.** Muscles were homogenized using a Polytron in Tris-HCl, pH 8.0 (1% sodium deoxycholate, 5% SDS, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM iodoacetamide, 2 mg/ml aprotinin, 100 mM Tris-HCl, 140 mM NaCl, and 0.025% Na3VO4) and subjected to immunoblotting as described (20). For some of these experiments, rat soleus and EDL muscles were also used with similar results. Briefly, equivalent amounts of proteins (up to 200 μg) were separated on a 6% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Sigma, St. Louis, MO). After transfer, the membranes were incubated with a monoclonal antibody directed against utrophin (Novocastra Laboratories, Newcastle upon Tyne, UK) and revealed using a commercially available chemiluminescence kit (REN Life Sciences, Boston, MA). To ensure that equivalent amounts of proteins were loaded for each sample, membranes were also stained with Ponceau S (Sigma).

**Immuno-fluorescence.** Immunofluorescence experiments were performed on longitudinal serial cryostat sections (12 μm) of EDL and soleus muscles placed on the same slide. Urophin immunoreactivity was detected using a monoclonal anti-utrophin antibody (Novocastra) and a goat anti-mouse Cy3-conjugated secondary antibody (Jackson Laboratories; Bio/Can Scientific, Mississauga, Ontario, Canada). Fluorescein isothiocyanate-conjugated α-bungarotoxin (Molecular Probes, Eugene OR) was used to label AChR at the neuromuscular junctions.

**Isolation of nuclei and in vitro transcription assays.** Nuclei were isolated from soleus and EDL muscles, and in vitro transcription assays were performed as described (5, 9, 22). Briefly, pools of 12–14 muscles were first washed with PBS and 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 dithiothreitol (DTT), and 1 μM PMSF. The nuclei were then isolated by centrifugation. They were resuspended in a solution containing 5% 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-32P]UTP (Amer sham) to label nascent transcripts for 30 min at 27°C. After 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 (41). After hybridization, the membranes were washed thoroughly (1× standard saline citrate, 0.1% SDS) at 42°C and subjected to autoradiography. The signal intensities were quantitated using a Storm PhosphorImager, and the intensity of the utrophin signals was standardized according to the corresponding signal seen with genomic DNA.

**In vitro transcription and ultraviolet crosslinking.** The utrophin 3'-UTR was subcloned into pCR2.1-TOPO vector (Invitrogen). In vitro RNA-binding studies were performed as previously described (2, 5). Briefly, 32P-labeled sense RNA was generated using the T7 polymerase. Labeled RNA (~5 × 109 counts/min) was then incubated with 100 μg of total protein isolated from the muscles. To obtain protein extracts, muscles were homogenized using a Polytron in 10× (mass/volume) of a buffer containing 0.3 M sucrose, 60 mM NaCl, 15 mM Tris (pH 8.0), 10 mM EDTA, 0.1 mM β-mercaptoethanol, 0.01 mM PMSF, 0.01 mM benzamidine, 1 μg leupeptin, 10 μg pepstatin A, and 1 μg apro tin (2). The extracts were centrifuged at 14,000 g for 15 min at 4°C. The supernatants were then removed, and the protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce Laboratories, Rockford, IL). Samples were stored at −80°C until further analysis.

**RESULTS**

**Slow muscles contain more utrophin.** To determine whether utrophin expression varies between different muscle types, we first examined the levels of utrophin in the slow soleus as well as fast EDL muscles. As shown in Fig. 1, immunoblotting experiments revealed that the levels of utrophin are approximately three- to fourfold higher (P < 0.05) in soleus muscles compared with the levels seen in EDL muscles. To ascertain that this difference between these muscles could be extended to other muscles and that it was indeed related to muscle type and not to muscle function, i.e., ankle extensors vs. ankle flexors, we also examined the relative levels of utrophin in the red and white portions of the lateral and medial gastrocnemius muscles. As illustrated in Fig. 1C, the red portions of the medial and
lateral gastrocnemius muscle contain considerably more utrophin compared with the white, faster-contracting adjacent regions (Fig. 1C).

We next performed immunofluorescence experiments to confirm these observations using light microscopy and to gain insights into the distribution of utrophin in slow vs. fast muscles. In these experiments, we observed, as expected, an accumulation of utrophin at the neuromuscular junctions from both soleus and EDL muscles (Fig. 2). However, we also determined that utrophin is present at low levels throughout the sarcolemma of soleus muscle fibers. These findings indicate, therefore, that the greater amount of utrophin detected in soleus muscles results from an increase in its expression in extrasynaptic regions of muscle fibers.

Increased expression of utrophin transcripts in soleus vs. EDL muscles. To determine whether these differences in the abundance of utrophin were paralleled by changes in the expression of its mRNA, we next performed a series of RT-PCR analyses and in situ hybridization experiments. For these experiments, we chose to focus on the soleus and EDL as representative of slow and fast muscles, respectively, since these two muscles are of similar size. In addition, they are easily accessible, thereby increasing the reliability of the dissection procedure and injection experiments (see below). Consistent with the immunoblotting results, we observed that the levels of utrophin mRNAs were higher in soleus vs. EDL muscles (Fig. 3A). Quantitative measurements of the relative abundance of utrophin transcripts revealed that, compared with their fast-twitch counterparts, soleus muscles contain significantly more ($P < 0.05$) utrophin mRNAs (Fig. 3B). Because skeletal muscles express two distinct utrophin transcripts termed A and B, which arise from different promoters (8), we also determined whether the expression of both transcripts was similarly increased in slow

Fig. 1. Expression of utrophin in fast vs. slow muscles. A: an example of a Western blot showing the presence of utrophin in extract from extensor digitorum longus (EDL; fast) and soleus (SOL; slow) muscles. Coomassie blue staining of the gel confirmed equal loading (not shown). B: quantitation indicating that, compared with fast muscles, slow muscles contain approximately 3- to 4-fold more utrophin. Shown are results obtained with 4 independent experiments. *Significant difference between the 2 groups ($P < 0.05$). C: examples of Western blots showing the levels of utrophin in the red (R) and white (W) portions of the medial and lateral gastrocnemius muscles. Similar to the findings with soleus muscles, the red portions of the gastrocnemius muscle contain more utrophin. Shown are examples obtained with a minimum of 5 muscles.

Fig. 2. Localization of utrophin in EDL vs. soleus muscles. Shown are representative examples of photomicrographs of EDL (A and B) and soleus (C and D) muscles processed for double-fluorescence experiments using fluorescein-conjugated $\alpha$-bungarotoxin, which recognizes acetylcholine receptors (AChR) located at the neuromuscular junctions (A and C), and an antibody against utrophin (B and D). In addition to the expected junctional accumulation, note the expression of utrophin at the sarcolemma in extrasynaptic compartments of soleus muscle fibers. Bar, 50 $\mu$m.
muscles. To this end, we selectively amplified the utrophin A or B mRNA by PCR using primers designed against the unique 5′-UTR of these transcripts (8). These experiments showed that both utrophin A and B transcripts were more abundant in the slow soleus muscle (data not shown).

To further characterize the increase in utrophin mRNA levels in soleus muscles and to confirm the RT-PCR data, we also performed in situ hybridization experiments aimed at localizing utrophin transcripts along single soleus and EDL muscle fibers. Because our RT-PCR data showed that the utrophin A and B transcripts were similarly increased in soleus muscles, we used synthetic oligonucleotides that recognize both mRNA species (21). Although utrophin transcripts are enriched at the neuromuscular junction in both muscle types (Fig. 4; see also Refs. 21 and 55), we observed a clear increase in the abundance of utrophin transcripts in extrajunctional regions of soleus fibers. Quantitative analyses revealed, in agreement with our immunofluorescence data, that the synaptic enrichment of utrophin mRNAs was similar in fast and slow muscles,
whereas in extrajunctional compartments utrophin transcripts were approximately threefold ($P < 0.05$) more abundant in soleus muscle fibers (Fig. 4C).

The utrophin gene appears transcribed at a similar rate in EDL and soleus muscles. To determine whether the increased utrophin mRNA and protein expression seen in soleus muscles results from a greater transcriptional activity of the utrophin gene, we performed run-on transcription assays using nuclei isolated from EDL and soleus muscles. In these experiments, we observed within the sensitivity of this approach that the rate of transcription for the utrophin gene appeared nearly identical in both muscle types (Fig. 5A). Densitometric analysis of these results confirmed that the transcriptional activity of the utrophin gene in soleus muscles was not significantly different ($P > 0.05$) from the activity observed in EDL muscle (Fig. 5B). As a positive control for these assays, we observed that transcription of the gene encoding the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was, as expected, higher in EDL samples (not shown). Furthermore, in parallel experiments using the same approach, we recently described significant changes in gene transcription (see, for example, Ref. 1). Therefore, our findings indicate that enhanced stability of existing transcripts likely accounts for the higher levels of utrophin mRNAs in soleus muscles (see Fig. 3).

The utrophin 3'-UTR regulates expression of a chimeric reporter construct in soleus muscles. On the basis of these observations, we next performed a series of experiments aimed at examining the importance of posttranscriptional mechanisms in the regulation of utrophin in fast vs. slow muscles. We thus performed direct plasmid injection experiments using constitutively expressed LacZ reporter constructs engineered to contain the utrophin full-length 3'-UTR, which is common to both A and B transcripts. Soleus and EDL muscles were injected with this construct and with a CAT plasmid used to monitor the efficiency of transduction. Seven to ten days later, expression of LacZ transcripts was examined by RT-PCR. As shown in Fig. 6A, levels of the reporter transcript were clearly higher in soleus muscles compared with the levels seen in the EDL. In fact, quantitative analysis ($n = 8$) revealed that LacZ expression, normalized to CAT mRNA levels, was approximately two- to threefold higher in slow muscles. By contrast, we failed to detect any significant difference in the expression of LacZ transcripts in soleus and EDL muscles injected with a reporter construct containing the utrophin 3'-UTR in the reverse orientation and used, in this case, as a control (Fig. 6B).

On the basis of these results, we expected that the pattern of RNA-protein interactions would be different between fast and slow muscles. To examine this, we performed UV-cross-linking experiments by incubating protein extracts from soleus or EDL muscles, with in vitro-transcribed $^{32}$P-labeled RNA corresponding to the utrophin 3'-UTR. Using this approach, we determined the number of possible cytoplasmic factors that could bind to the 3'-UTR as well as their molecular mass. In protein extracts from soleus muscles, we readily detected two protein complexes (arrows in Fig. 7). The presence of these complexes was completely abolished by preincubation of the protein extracts with an excess of cold unlabeled probe. In agreement with our hypothesis and with the results presented above, we also observed an increase in the expression of these RNA-binding proteins in fast muscles because the intensity of the most abundant complex was clearly greater in EDL muscles.

DISCUSSION

Although our recent studies (18, 20, 21), as well as those of others (31, 55), have shown that transcriptional regulatory mechanisms account, at least partially, for the preferential synaptic accumulation of utrophin, it is nonetheless possible to envisage that posttranscriptional events are also involved in the reg-
ulation of utrophin in muscle fibers. In this context, we have shown that distinct regions in the 3′-UTR control the targeting and stability of utrophin mRNAs in cultured muscle cells (Gramolini, Bélanger, and Jasmin, unpublished observations). Additionally, a recent study has nicely highlighted the contribution of posttranscriptional mechanisms in the development and maintenance of neuromuscular junctions in Drosophila (49). In agreement with these findings, we now show that utrophin is more abundant in soleus vs. EDL muscles as a result of an increase in the stability of its mRNAs, which appears mediated by cis-acting elements in the 3′-UTR and by the availability of RNA-binding proteins. Together, these data show, therefore, that in addition to transcriptional regulation, posttranscriptional events also play a key role in controlling the expression and localization of utrophin in skeletal muscle.

In our experiments, we also observed that the red portion of both the medial and lateral gastrocnemius muscle contains more utrophin compared with their respective adjacent white compartment. Therefore, it appears likely that the importance of posttranscriptional mechanisms generally operates in slow muscle fibers, thereby regulating the abundance and localization of utrophin. Even though there appears, on the basis of these data, to be a good correlation between muscle type, i.e., fast vs. slow, and utrophin expression, additional work remains to be done to refine this relationship by correlating, for example, utrophin levels with the expression of proteins known to be excellent markers of the contractile speed or fatigue characteristics of individual muscle fibers.

In general, it has become evident that, for a number of genes, transcriptional regulation cannot solely account for the observed changes in mRNA levels known to occur under a variety of conditions. Thus the importance of mRNA stability is becoming increasingly recognized as a key regulatory step in the control of gene expression (12, 45, 46). In this context, it is known that the half-life of distinct mRNAs within the same cell can vary by more than one order of magnitude, ranging from minutes for relatively labile transcripts to hours or even days in the case of unusually stable mRNAs. More interesting is the fact that these half-lives can be specifically altered according to environmental and/or intracellular influences (35, 45). Indeed, there are numerous examples using cultured cells that have shown that hormones, growth factors, and ionic concentrations can affect the stability of presynthesized mRNAs.
thereby leading to rapid and dramatic changes in gene expression (46).

In skeletal muscle cells, it has also become clear that posttranscriptional mechanisms are indeed important in dictating the abundance of specific mRNAs (for example, see Refs. 52 and 59). Interestingly, there are even a few cases where the importance of posttranscriptional events has been implied in the expression of genes encoding synaptic proteins in muscle. In particular, a series of studies have shown that the increased expression of AChE mRNA that occurs during differentiation of myogenic cells in culture results in part from the enhanced stability of existing transcripts (3, 17). Consistent with these observations, we have recently determined that the ~10-fold reduction in AChE mRNA levels that occurs in denervated rat muscles results from a reduction in the half-life of existing transcripts with little change in the rate of transcription of the AChE gene (5). Similarly, the contribution of mRNA stability has to be taken into account in the response of AChR subunit genes to denervation since the ~5- to 10-fold increase in transcription, known to occur in denervated muscles, is transient and cannot fully account for the much greater increases seen in the abundance of AChR transcripts (7, 14). Finally, in a collaborative effort, we have shown that the synaptic accumulation of a-dystrobrevin 1 transcripts in muscle fibers likely involves posttranscriptional mechanisms as opposed to a local transcriptional activation of the dystrobrevin gene in myonuclei located in the postsynaptic sarcoplasm (41). Taken together with our present findings, results of these studies indicate therefore that, in addition to transcriptional regulation (7, 14, 47), posttranscriptional mechanisms represent key events for controlling the expression and localization of synaptic proteins in muscle fibers.

Although there is now more information illustrating the importance of mRNA stability in the control of gene expression, there are relatively few examples where the precise molecular mechanisms have been clearly defined. For instance, several unstable mRNAs, including c-fos and granulocyte-macrophage colony-stimulating factor, contain adenosine-uridine-rich elements (AURE) in their 3′-UTR that regulate their turnover through interactions with members of the AURE-binding protein family. Additionally, mRNAs encoding the transferrin receptor contain iron-responsive elements that bind a cytosolic protein referred to as IRP, which stabilizes these transcripts by preventing their degradation during iron deficiency (for review, see Refs. 45 and 46).

In the present study, we used direct plasmid injection to examine whether the utrophin 3′-UTR contains elements that may confer differential stability to reporter transcripts. Our data show that the utrophin 3′-UTR can indeed increase the levels of chimeric LacZ mRNAs in soleus muscles by an extent similar to that seen for endogenous utrophin transcripts. This suggests, therefore, that although the 5′-UTR and the coding regions can also contribute to the stability of specific mRNAs (45, 46), it appears that in the case of utrophin transcripts the 3′-UTR is primarily responsible for controlling their turnover rate. This view is, in fact, further supported by the observation that expression of the A and B transcripts, which differ mostly in their 5′-UTR (8), are both increased in soleus muscles.

In an attempt to determine whether the difference in utrophin mRNA stability in fast vs. slow muscles involves differential expression of RNA-binding proteins, we performed UV-crosslinking experiments using cytoplasmic extracts from soleus and EDL muscles. Our results indicate that both muscle types express a similar pattern of proteins that can bind to the utrophin 3′-UTR. However, we noted in these experiments a clear difference in the relative content of these RNA-binding proteins. Indeed, the extract from fast EDL muscles appears to contain significantly more protein factors that interact with the utrophin 3′-UTR. This would indicate that these complexes represent destabilizing factors because EDL muscle expresses fewer utrophin transcripts. In this context, it is interesting to note that the utrophin 3′-UTR contains six AUREs (Gramolini, Belanger, and Jasmin, unpublished observations). It is therefore possible that these elements may preferentially destabilize utrophin transcripts in fast muscle via interactions with members of the AURE-binding protein family. Experiments are currently under way to see whether expression of these RNA-binding proteins differ between fast and slow muscles.

A key issue that also deserves further attention deals with the nature of the signaling events that ultimately control expression of these RNA-binding proteins. In this context, we have recently shown that elimination of electrical activity via surgical denervation induces an increase in the amount of cytoplasmic factors that interact with the 3′-UTR of AChE (5). In addition, Booth and colleagues (59) have previously demonstrated that increased contractile activity induces a significant decrease in RNA-protein interactions in the 3′-UTR of cytochrome c mRNA. On the basis of these observations, it appears that electrical activity represents an initiating signal that ultimately influences, via yet to be determined signaling cascades, the abundance of RNA-binding proteins, which, in turn, affect the stability of specific transcripts. Accordingly, it is tempting to speculate that the differences in the amount and pattern of electrical activity, known to exist between soleus and EDL muscles (27), are responsible for controlling the levels of cytoplasmic factors that interact with the utrophin 3′-UTR. In this scenario, calcium may constitute a key signal, linking membrane events to changes in mRNA expression, because the continuous activation pattern of soleus muscles results in sustained elevations of the intracellular concentration of calcium.

Recent studies have led to the idea that calcineurin, a calcium-regulated protein phosphatase, may serve as a key player in the control of the slow muscle phenotype by acting via nuclear factor of activated T cells (NFAT) transcription factors (for review, see Ref. 43). Interestingly, calcineurin has also been implicated in
the regulation of mRNA stability in different cell types. In skeletal muscle for example, Taylor and colleagues (36) have shown that calcineurin can modulate the stability of AchE transcripts in differentiating C2C12 cells maintained in culture. Along those lines, we have recently observed that overexpression of a constitutively active form of calcineurin in transgenic mice results in an increased expression of utrophin in soleus muscles (13). Together with the findings of the present study, these data suggest that the greater stability of utrophin transcripts in soleus muscles may result from the sustained intracellular levels of calcium seen in soleus muscles, which, in turn, activate calcineurin, thereby regulating the stability of utrophin mRNAs.

The results of the present study showing that slow muscles contain more utrophin may explain, in fact, why fast fibers are preferentially affected in DMD patients (56). Indeed, in the absence of dystrophin, extrasynaptic utrophin may help protect slow fibers against the damage elicited by repetitive mechanical stress. In addition, our findings that utrophin mRNAs appear more stable in slow muscles provide an in vivo model system with which to begin characterizing precisely cis-acting elements and trans-activating factors that control the half-life of utrophin transcripts in skeletal muscle fibers. The results of the present studies should therefore lead to the identification of novel posttranscriptional targets for which pharmacological manipulations may be envisaged to ultimately increase the endogenous levels of utrophin in DMD muscle fibers. Accordingly, experiments remain to be done that will nicely complement ongoing studies aimed at increasing utrophin levels via interventions on transcriptional events.

We thank J. A. Lunde for expert technical assistance and D. J. Parry for helpful discussion.

This work was supported by grants from the Association Française Contre les Myopathies, the Muscular Dystrophy Association of America, and the Canadian Institutes of Health Research (CIHR). During the course of this work, A. O. Gramolini was supported by a Strategic Area of Development Fellowship from the University of Ottawa and is now supported by a Postdoctoral Fellowship from CIHR. B. J. Jasmin is a CIHR Investigator.

Present address of A. O. Gramolini: Howard Hughes Medical Institute and Dept. of Cell Biology, Duke Univ. Medical Center, Durham, NC 27710.

REFERENCES


26. Helllwell TR, Man NT, Morris GE, and Davies KE. The dystrophin-related protein, utrophin, is expressed on the sarcolemma of regenerating human skeletal muscle fibres in dystro-