

Modulation of utrophin A mRNA stability in fast versus slow muscles via an AU-rich element and calcineurin signaling

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ABSTRACT

We examined the role of post-transcriptional mechanisms in controlling utrophin A mRNA expression in slow versus fast skeletal muscles. First, we determined that the half-life of utrophin A mRNA is significantly shorter in the presence of proteins isolated from fast muscles. Direct plasmid injection experiments using reporter constructs containing the full-length or truncated variants of the utrophin 3'UTR into slow soleus and fast extensor digitorum longus muscles revealed that a region of 265 nucleotides is sufficient to confer lower levels of reporter mRNA in fast muscles. Further analysis of this region uncovered a conserved AU-rich element (ARE) that suppresses expression of reporter mRNAs in cultured muscle cells. Moreover, stability of reporter mRNAs fused to the utrophin full-length 3'UTR was lower in the presence of fast muscle protein extracts. This destabilization effect seen *in vivo* was lost upon deletion of the conserved ARE. Finally, we observed that calcineurin signaling affects utrophin A mRNA stability through the conserved ARE. These results indicate that ARE-mediated mRNA decay is a key mechanism that regulates expression of utrophin A mRNA in slow muscle fibers. This is the first demonstration of ARE-mediated mRNA decay regulating the expression of a gene associated with the slow myogenic program.

INTRODUCTION

Ever since its discovery, >15 years ago, there has been a considerable amount of effort to decipher the molecular events regulating expression of the cytoskeletal protein utrophin (1,2). These efforts are fueled in part by the fact that utrophin accumulates at the neuromuscular junction where it participates in the full differentiation of the postsynaptic apparatus (3–6). In addition, directed expression of utrophin in extrasynaptic compartments of muscle fibers is considered as a prospective therapeutic strategy for the treatment of Duchenne muscular dystrophy (2,7).

Previous studies led to the notion that the state of differentiation and innervation of muscle fibers can influence the expression of utrophin A (8–10), the skeletal muscle isoform (11), mostly through transcriptional mechanisms. For example, local transcriptional activation of the utrophin A promoter accounts for the preferential accumulation of utrophin A mRNAs within synaptic regions of mature fibers (8,10,12), via activation of signaling cascades triggered by agrin and neuregulin (13–15). Although it is well established that utrophin A preferentially accumulates in synaptic regions of muscle, we noted a few years ago that slow-twitch, high oxidative, fibers express more utrophin A in their extrasynaptic compartments as compared with fast fibers (16). Subsequently, we demonstrated the involvement of signaling pathways that promote expression in muscle of the slower oxidative phenotype, in regulating expression of utrophin A (17–20). Specifically, we showed that calcineurin, a Ca^{+2} /calmodulin-regulated phosphatase

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(21,22), regulates utrophin A expression via nuclear factor of activated T-cells (NFAT) (17,19,20,23).

In recent years, it has become apparent that in addition to transcription, post-transcriptional mechanisms can influence expression of several mRNAs in all cell types including skeletal muscle (24). For instance, mRNAs encoding MyoD, myogenin, acetylcholinesterase (AChE) and α -dystrobrevin 1 are regulated at multiple post-transcriptional levels involving mRNA stability, targeting and translation (25–29). Recently, the contribution of post-transcriptional mechanisms has also been shown to play an important role in the regulation of utrophin A in muscle cells (16,30,31). Thus, although our previous studies highlighted the role of transcription in regulating the greater abundance of utrophin A in slow fibers (see above), we hypothesize that post-transcriptional events are also involved. Here, we specifically focused on the role of mRNA stability in regulating the higher levels of utrophin A mRNA seen in slow muscle. Furthermore, through a series of complementary experiments we characterized *cis*-elements within the utrophin 3'UTR, and signaling pathways involved in the post-transcriptional regulation of utrophin A mRNA in slow versus fast muscles.

MATERIALS AND METHODS

Animal care and protocols

All animal care and experimental procedures were performed in accordance with the guidelines established by the Canadian Council of Animal Care. These procedures were approved by the Institutional Animal Care and Research Advisory Committee of Laurentian University, or by the University of Ottawa Animal Care and Use Committee. For inhibiting calcineurin activity in skeletal muscle, C57 BL/6 mice were treated twice daily for 14 days with either cyclosporine A (CsA; 25 mg/kg administered subcutaneously), FK506 (5 mg/kg administered subcutaneously), or vehicle (control) as previously described (19). Soleus and extensor digitorum longus (EDL) muscles from control or treated mice were excised and frozen in liquid nitrogen. Muscles were subsequently processed for *in vitro* stability assays (see below). In separate experiments, EDL and soleus muscles of C57 BL/6 mice were directly injected with utrophin 3'UTR reporter constructs (see below) using a procedure described in detail elsewhere (8,16). These muscles were excised 7 days later, frozen in liquid nitrogen and subsequently processed for RT-PCR analysis (see below).

Cell culture

Mouse C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were plated on 6-well culture dishes coated with Matrigel (Collaborative Biomedical Products, Bedford, MA, USA) in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum, 292 ng/ml L-glutamine and 100 U/ml penicillin–streptomycin in a humidified chamber at 37°C with 5% CO₂. Confluent myoblasts were induced to differentiate into myotubes by replacing the growth

medium with differentiation medium containing 2% horse serum for 3 days (29).

In vitro stability assays

Proteins were extracted from EDL and soleus muscles from control mice, and soleus muscles of drug-treated mice (see above) using 500 μ l of a homogenization buffer [0.01 M Tris pH 8.0, 0.01 M KCl, 0.0015 M MgCl₂, 2.5% IGEPAL CA-630 (a non-ionic detergent) (Sigma-Aldrich, Oakville, ON, USA)] containing protease inhibitor complete mini-tablets as per the manufacturer's recommendations (Roche Applied Science, Laval, QC, USA). After homogenization, protein extracts were centrifuged at 3500 g for 10 min. Pellets were subsequently vortexed and incubated at 4°C in 100 μ l extraction buffer (0.02 M Tris pH 8.0, 0.45 M NaCl, 0.01 M EDTA) also containing protease inhibitor complete mini-tablets. After incubation, the pelleted fractions were centrifuged at 14 000 g for 10 min and supernatants were collected. This yielded a protein extract enriched in cytoskeletal and nuclear fractions that was used for *in vitro* stability assays. This fraction was selected because the cytoskeleton has been previously implicated in regulating utrophin expression post-transcriptionally (30). The RNA used in these assays was isolated from C2C12 myotubes using TriPure reagent (Boehringer Mannheim, Laval, QC, USA). For determination of LacZ reporter mRNA half-life, RNA was isolated from C2C12 myotubes that had been transfected with the appropriate reporter construct (see below).

Degradation assays were performed as described elsewhere, with some modifications (32,33). Briefly, total RNA from C2C12 cells (0.2 μ g/ μ l) and equal amounts of soleus and EDL protein extracts (0.25 μ g/ μ l) were incubated together in degradation buffer (10 mM Tris pH 7.4, 10 mM KOAc, 2 mM MgOAc, 2 mM DTT, 0.1 mM Spermine, 1 mM ATP, 0.4 mM GTP, 10 mM Phosphocreatine, 1 μ g creatine phosphokinase, 80 U SUPERNasin (Ambion, Austin TX), at 37°C. Time 0 was taken as RNA incubated in buffer without protein extracts for 1 min at 37°C. The reactions were stopped at different time intervals by addition of 200 μ l of phenol/chloroform. The RNA was then precipitated with isopropanol in the presence of yeast tRNA (10 μ g) (Sigma-Aldrich) as a carrier. The values of utrophin A, LacZ and S12 transcripts remaining at each time point were determined through RT-PCR analysis (see below). Values were then plotted on a logarithmic scale as a function of time, and the half-life was calculated using linear equations (34). Four separate experiments were conducted, using four different muscle extracts. Half-life values were then determined relative to appropriate controls in each individual experiment. Relative half-life values were averaged and compared between samples.

In vitro stability assays were also performed using labeled RNA probes corresponding to regions of the utrophin A 3'UTR. For these, *in vitro* transcription reactions were first performed using MAXIscript (Ambion) and a 299 to 603 nt region from the mouse utrophin 3'UTR PCR product containing a T7 RNA polymerase-binding site. This PCR product was obtained

using the following primer set: 5'-TTTTTTTTTTTTTTT TTTTTTTTTTTTAGAATGCCTGCCTACTCCACAT-3' and 5'-GATTTAATACGACTCACTATAGGGACTC AGCCATGGGAAAGTG-3'. Labeled RNA transcripts were produced by including [α -³²P] UTP (3000Ci/mmol, Amersham Biosciences) in the reaction mixtures. The radioactive probes were purified using microspin G-50 columns (Amersham Biosciences) and used in stability assays as described above with protein extracts from soleus and EDL muscles. Quantitative analyses of the intensity of the radioactive signals were performed using a Storm PhosphorImager and the accompanying ImageQuant Thv2005 software (Molecular Dynamics, Amersham; Piscataway, NJ, USA).

Plasmid construction

The 3'UTR of the mouse full-length (~2 kb) utrophin mRNA was isolated and subcloned into a LacZ reporter construct driven by the cytomegalovirus (CMV) promoter (pCMVSPORT from Invitrogen) as described previously (30). Truncated variants of the utrophin 3'UTR encompassing the first 332 and 596 nucleotides were also generated in a similar manner (30). To generate the utrophin 3'UTR fragment deleted of the conserved 16 nucleotide AU-rich element (ARE), we employed a PCR-based protocol described elsewhere (29). Briefly, the utrophin full-length 3'UTR cDNA was used as a template, and two rounds of PCR amplification were first performed with the following primers: 5'-CATAATGGTAAAACA GTCAAATAA-3' and the flanking primer 5'-TGGTCT CCTCGAGGCATCTATCCAGCCAG-3' (reaction 1); and the flanking primer 5'-TGGTCTCAAGCTTGGTA TGTAACAGATTAG-3' and primer 5'-ATCAGCCATA CCAAACGAATAGAT-3' (reaction 2). To remove the ARE region, we performed a third round of PCR, with the reaction 1 and reaction 2 PCR products, using the two flanking primers listed above to yield a product containing the utrophin 3'UTR without the conserved 16 nt ARE region (3'UTR Δ ARE). The success of this deletion approach was confirmed by sequencing.

Transfection and direct plasmid injection

Plasmid DNA was prepared using the Mega-Prep kit (Qiagen, Mississauga, ON, USA). DNA pellets were resuspended in 10 mM Tris-HCl, pH 8.5. Transfections were performed with the Lipofectamine reagent kit (Invitrogen) according to the manufacturer's instructions. Briefly, C2C12 myoblasts at ~80% confluency were transfected with 1 μ g of utrophin 3'UTR-reporter constructs together with 1 μ g of pCIneo (Promega) as a control, or with a construct containing a constitutively active variant of calcineurin (pCnA*) (17), by using lipofectamine reagent (Invitrogen). Total RNA was extracted, and the levels of reporter LacZ mRNA and neomycin mRNA (to control for transfection efficiency) were determined by RT-PCR analysis (see below). Transfections examining the effects of deleting the 16 nt ARE from the utrophin 3'UTR were done in a similar fashion. For these, 2 μ g of pCMVSPORT plasmid containing a LacZ gene fused to the SV40 late polyadenylation signal

(SV40 3'UTR), the utrophin 3'UTR full-length or the 3'UTR Δ ARE were transfected. Levels of reporter LacZ mRNA were standardized to ampicillin mRNA transcribed on the same construct by a separate promoter.

Direct gene transfer was performed using mouse EDL and soleus muscles as described in detail elsewhere (16). EDL and soleus muscles were isolated, and injected with 10 μ l of a solution containing either the utrophin 3'UTR full-length, 3'UTR Δ ARE, 3'UTR 596 and 3'UTR 332 LacZ reporter constructs, together with pCAT control plasmids used to control for transduction efficiency (concentration of 2–4 μ g/ μ l). Injected muscles were excised and immediately frozen in liquid nitrogen after seven days. Total RNA was extracted and subsequently analyzed by RT-PCR (see below). For injection of tibialis anterior (TA) muscles, 25 μ l of solution containing either 2 μ g of the utrophin 3'UTR full-length or 3'UTR Δ ARE reporter constructs, together with 2 μ g of pCnA* or pCIneo constructs were injected. Muscles were extracted and processed for RT-PCR analysis after five days (see below).

RNA extraction and RT-PCR

Total RNA was extracted by using TriPure (Boehringer Mannheim) as recommended by the manufacturer. Quantitative RT-PCR was carried out to determine the relative abundance of: (i) utrophin A and S12 in EDL and SOL muscles; (ii) utrophin A, S12 and LacZ transcripts remaining at each time point of the *in vitro* stability assays; (iii) LacZ and CAT mRNA levels after direct plasmid injections; (iv) LacZ and neomycin mRNA levels in co-transfection experiments of the utrophin 3'UTR reporter constructs with pCIneo or pCnA* in C2C12 muscle cells; and (v) Lac Z and ampicillin transcripts in C2C12 muscle cells transfected with utrophin 3'UTR full-length, parental or 3'UTR Δ ARE reporter constructs. These assays were performed using previously described protocols and primers (16,17,29). Cycle numbers varied depending on the primers used and they were all within the linear range of amplification (16,29,35). For amplification of LacZ, CAT, neomycin and ampicillin mRNAs, samples were first digested using DNase 1 to eliminate possible plasmid contamination (16,17). In all these assays, negative controls consisted of reverse transcription mixtures in which total RNA was replaced with RNase-free water. PCR products were first visualized on 1% agarose gel containing ethidium bromide.

Labeling intensity of the PCR products, which is linearly related to the abundance of cDNAs, was quantified using Kodak digital science 1D Image analysis software. For direct plasmid injections, values obtained for LacZ were standardized relative to the amount of CAT. For transfection studies in C2C12 muscle cells involving utrophin 3'UTR reporter constructs and pCnA*, values obtained for LacZ were standardized relative to the amount of neomycin. For transfection studies in C2C12 muscle cells involving the utrophin 3'UTR full-length, SV40 3'UTR and 3'UTR Δ ARE reporter-constructs, values obtained for LacZ were standardized to the amount of ampicillin (29).

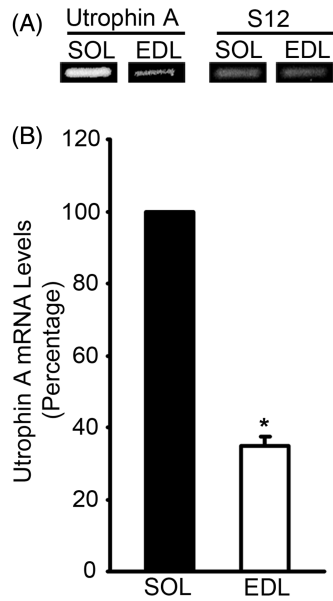


Figure 1. Levels of utrophin A mRNA are higher in slow versus fast muscles. (A) Examples of ethidium bromide-stained agarose gels showing utrophin A and S12 PCR products from EDL and soleus (SOL) muscles. (B) Quantitation of utrophin A mRNA levels in EDL muscles expressed as a percentage of those found in SOL muscles and standardized to S12 transcripts. Note the significantly greater amount of utrophin A mRNA in SOL muscles. *Indicates a significant difference from EDL ($P < 0.05$; $n = 4$ independent experiments).

Statistical Analysis

Analysis of variance (ANOVA) and *F*-tests, or two-tailed Student's *t*-tests were used to analyze the data. Means \pm SEM are presented throughout. All statistics were done using Microsoft Excel Analysis ToolPak.

RESULTS

Levels of utrophin A mRNA are greater in slow versus fast muscles due also to enhanced stability

In an initial series of experiments, we examined the levels of utrophin A mRNA in fast and slow muscles. As shown in Figure 1A, levels of utrophin A mRNA in slow soleus muscles were much higher in comparison with fast EDL muscles. Quantitative analysis revealed that utrophin A transcripts were \sim 3-fold higher ($P < 0.05$) in soleus muscles (Figure 1B). These higher levels of utrophin A mRNA in slow muscles are in excellent agreement with previous observations (10,12,16,17).

To determine whether the stability of utrophin A transcripts is different in fast versus slow muscles, we initially chose to employ a previously described *in vitro* stability assay (32,33). The use of an *in vitro* protocol under highly controlled and reproducible conditions is warranted since our preliminary work revealed that measuring the stability of transcripts in skeletal muscle of living animals using transcription inhibitors such as actinomycin D, is unreliable and fraught with difficulties (data not shown). In fact, limitations of this approach have been discussed previously (34,36) and they

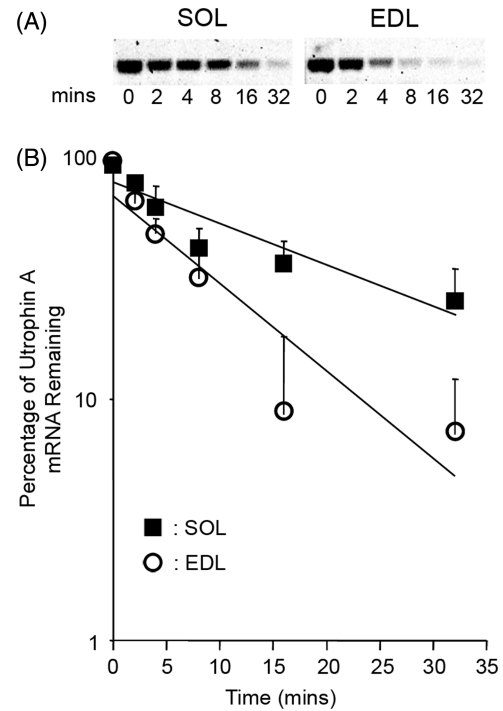


Figure 2. Utrophin A mRNA decays at a faster rate in the presence of fast muscle protein extracts. (A) *In vitro* stability assays were performed with protein extracts from EDL and soleus (SOL) muscles and RNA isolated from 3 day-old C2C12 myotubes. Examples of ethidium bromide-stained-agarose gels display utrophin A PCR products following 0, 2, 4, 8, 16 and 32 min incubation with protein extracts. Note the greater amount of utrophin A PCR products remaining upon incubation with the slow SOL muscle protein extracts versus with fast EDL muscle extracts. (B) Quantitation of the amounts of utrophin A mRNA at each time point, plotted in a semi-log scale as percentage of RNA seen at time 0. This analysis revealed that the half-lives seen in the presence of EDL versus soleus protein extracts were 3.55 min and 11.41 min, respectively ($n = 4$ independent experiments).

include: (i) the toxicity of the drug which kills the animals in a few hours making extrapolations of half-life of relatively stable mRNAs questionable and (ii) the direct effect of these drugs on the mRNA decay process which may artificially alter the rate of degradation of specific cellular transcripts.

With the use of this *in vitro* stability assay, we examined the degradation of utrophin A mRNA in the presence of fast (EDL) or slow (soleus) skeletal muscle protein extracts. Total RNA from 3 day-old C2C12 myotubes was incubated with either fast or slow muscle protein extracts for different time periods in a degradation buffer. The amount of utrophin A mRNA remaining at each time point was subsequently determined and used to calculate mRNA decay kinetics (see Materials and Methods section). In the presence of slow muscle protein extracts, utrophin A mRNA appeared to degrade at a slower rate in comparison to mRNA incubated with fast muscle extracts (Figure 2A). Evaluation of mRNA half-life values revealed that utrophin A degraded at approximately three times the rate ($P < 0.05$) in the presence of fast muscle protein extracts relative to incubation with slow muscle extracts (Figure 2B). Using qPCR, we also observed a 2 to 3-fold

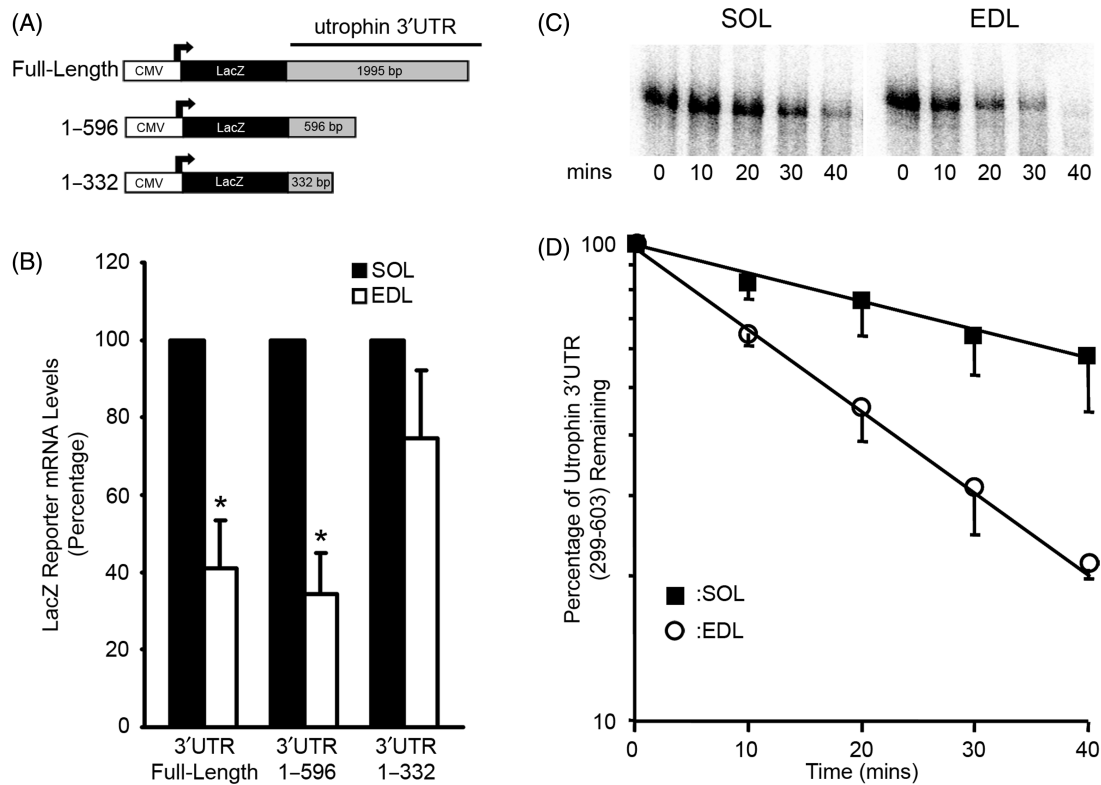


Figure 3. A region between nucleotides 332–596 in the utrophin 3'UTR decreases reporter mRNA expression in fast muscles. (A) Schematic representation of reporter constructs containing the full-length utrophin 3'UTR, and the first 596 and 332 inserted downstream of the LacZ reporter gene. (B) Mouse EDL and soleus (SOL) muscles were transfected with expression plasmids containing the various lengths of the 3'UTR. Note that LacZ reporter mRNA levels are ~60% lower in EDL muscles when fused to either the utrophin full-length 3'UTR or the first 596 nucleotides. However, this difference is lost when LacZ mRNAs are fused to the first 332 nucleotides indicating the presence between nucleotides 332 and 596 of an instability element active in fast EDL muscles. *Indicates a significant difference from EDL ($P < 0.05$; $n = 4-5$ animals for each construct and in independent experiments). (C) Example of an *in vitro* stability assay using a radiolabeled RNA probe corresponding to nucleotide 299–603 of the utrophin 3'UTR incubated for various time periods with protein extracts from EDL and SOL muscles. (D) The intensity of the radioactive signals seen in these assays was quantified and plotted on a semi-log scale as a percentage of RNA seen at time 0 ($n = 4$ independent experiments). Note that the half-lives seen under these conditions were 17.36 min and 50.56 min when the radiolabeled RNA probe was incubated with fast EDL versus slow SOL protein extracts, respectively.

longer half-life of utrophin mRNA upon incubation with slow versus fast protein extracts. As a control, we observed in separate experiments no difference in the rate of degradation of S12 transcripts upon incubation with either fast or slow muscle extracts ($P > 0.05$; <10% difference in half-life measurements between fast and slow protein extracts). These data indicate that in addition to transcriptional events, increased mRNA stability also contributes to the differential abundance of utrophin A transcripts in slow versus fast muscles.

A *cis*-element in the utrophin 3'UTR differentially regulates reporter expression in slow versus fast muscles

The 3'UTRs of host mRNAs can function to regulate the levels of transcripts present in a cell (37,38). This ability to control mRNA levels stems from the presence of *cis*-acting elements often contained within the 3'UTR. Next, we therefore sought to determine, *in vivo*, the contribution of specific *cis*-acting elements contained within the mouse utrophin 3'UTR in conferring differences in mRNA expression between fast and slow muscles using direct plasmid injection of reporter constructs in EDL and soleus

muscles of mice (Figure 3A). Direct plasmid injection of a LacZ reporter construct containing the utrophin full-length 3'UTR led to a ~2.5-fold ($P < 0.05$) higher level of reporter mRNA in soleus versus EDL muscles (Figure 3B).

Similar to the results obtained with the construct containing the utrophin full-length 3'UTR, direct injection of a reporter construct with the first 596 nucleotides of the utrophin 3'UTR also led to a 2.5-fold ($P < 0.05$) increase in reporter mRNA expression in soleus versus EDL muscles (Figure 3B). In contrast, levels of LacZ reporter mRNAs fused to the first 332 nucleotides of the utrophin 3'UTR were not significantly different ($P > 0.05$) between soleus and EDL muscles (Figure 3B). Interestingly, relative levels of reporter mRNAs fused to the first 332 nucleotides of the utrophin 3'UTR in EDL muscles increased to values comparable to those seen in soleus muscles. LacZ reporter mRNA levels from soleus muscles injected with the full length, 1–332 and 1–596 constructs did not differ significantly ($P > 0.05$). Collectively, these data suggest that nucleotides 332–596 of the utrophin 3'UTR contain elements capable of suppressing reporter mRNA levels in EDL muscles thereby indicating the

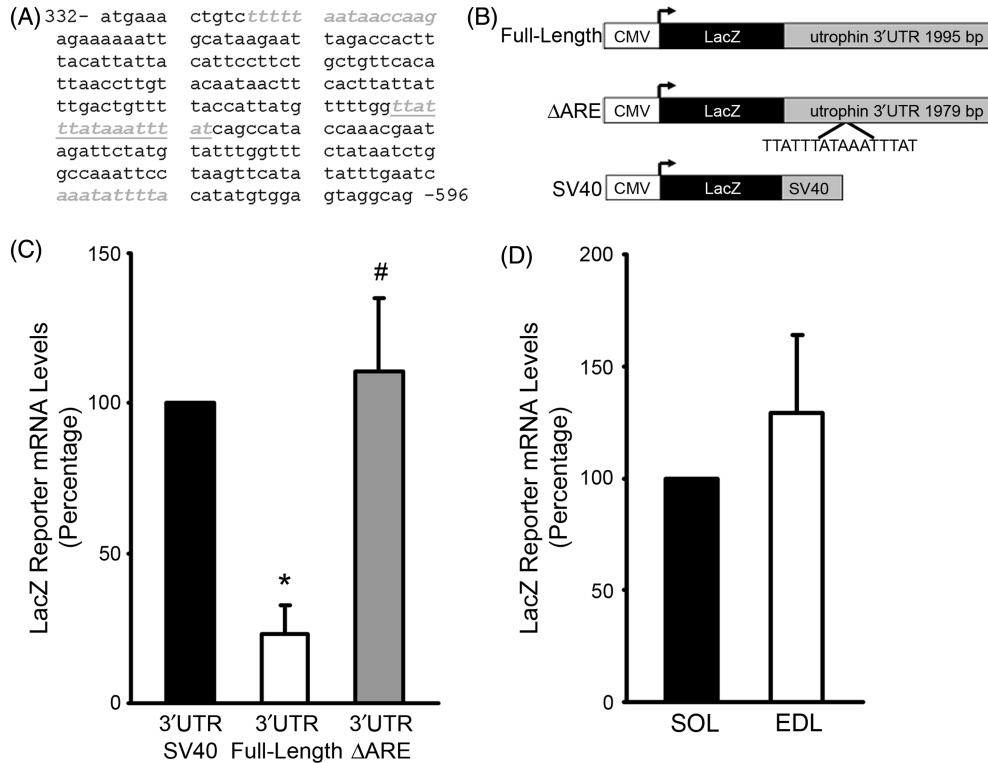


Figure 4. The utrophin 3'UTR contains a conserved ARE that acts as a destabilizing element. (A) Sequence between nucleotides 332 and 596 of the utrophin 3'UTR. Grey characters denote three potential ARE. The underlined one is an ARE conserved between mouse and human which we subsequently deleted for further analysis. (B) Schematic representation of the utrophin full-length 3'UTR, the full-length version deleted of the conserved ARE (3'UTR ΔARE) and the SV40 3'UTR inserted downstream of the LacZ reporter construct. (C) Analysis of LacZ mRNA levels from 3 day-old C2C12 myotubes transfected with one of the three reporter constructs. Note the decrease in LacZ reporter mRNA expression when fused to the utrophin full-length 3'UTR relative to the SV40 3'UTR. Importantly, note also the loss of this effect upon deletion of the conserved ARE. *Indicates a significant difference relative to SV40 3'UTR, #indicates a significant difference relative to full-length 3'UTR [$P < 0.05$; $\#P < 0.01$] $n = 3$ independent experiments done in triplicate]. (D) Mouse EDL and soleus (SOL) muscles were injected with plasmids containing the utrophin 3'UTR ΔARE reporter constructs. Note that in contrast to injection with reporter constructs containing the utrophin full-length 3'UTR (Figure 3), expression levels of LacZ mRNA are not different between EDL and SOL muscles upon deletion of the conserved ARE ($P > 0.05$); $n = 5$ animals used in independent experiments.

presence of a destabilizing element active *in vivo*, preferentially in fast muscle fibers.

Given these findings, we sought to illustrate further the importance of this region in determining the stability of utrophin A transcripts in fast versus slow muscles. To this end, we generated a radiolabeled RNA probe corresponding to nucleotides 299–603 of the utrophin 3'UTR and performed *in vitro* stability assays using protein extracts from soleus and EDL muscles. As shown in Figure 3C, the radiolabeled RNA appeared to decay at a faster rate in the presence of proteins from EDL muscles. Quantitative analyses of these results showed that indeed, the half-life of this RNA incubated with fast muscle protein extracts was approximately three times lower than that seen upon incubation with extracts from soleus muscles (Figure 3D). These findings are important since they further show the key function of this region within the utrophin 3'UTR in controlling the stability of these transcripts in fast versus slow muscles.

The 332–596 region contains multiple ARE

Sequence analysis of the 332–596 region in the utrophin 3'UTR revealed that 72% of the nucleotides are either

adenosine or uridine. Within these 265 nucleotides, we found three potential ARE known to play key roles in regulating the turnover of mRNAs (Figure 4A, grey characters) (39–41). Species comparison revealed that one of the three AREs is conserved between mouse and human utrophin 3'UTR sequences (Figure 4A, underlined grey characters).

To determine whether the conserved ARE contributes to the regulation of utrophin A mRNAs, we generated full-length utrophin 3'UTR LacZ reporter constructs deleted for this minimal region (ΔARE; Figure 4B). To initially examine the effects of deleting the ARE, we first transfected C2C12 muscle cells with reporter constructs containing either the utrophin full-length 3'UTR, the 3'UTR ΔARE or the LacZ gene fused to the SV40 3'UTR (see Materials and Methods section). After 3 days of differentiation, transfected cells were harvested and reporter mRNA levels were examined. RT-PCR analysis of total mRNA isolated from transfected muscle cells revealed that the presence of the utrophin full-length 3'UTR inhibited reporter mRNA expression as evidenced by an ~80% decrease in LacZ transcript levels ($P < 0.05$) relative to the levels of reporter mRNA fused to the SV40

3'UTR (Figure 4C). In contrast, levels of reporter mRNA fused to the utrophin 3'UTR Δ ARE were \sim 4-fold ($P < 0.05$) higher relative to the levels seen with the utrophin full-length 3'UTR (Figure 4C). Furthermore, levels of reporter mRNA were not different ($P > 0.05$) between cells transfected with constructs containing the utrophin 3'UTR Δ ARE or the SV40 3'UTR (Figure 4C). Together, these findings show the presence of an instability element with the utrophin 3'UTR.

Next, we determined if the conserved ARE could also function *in vivo*, particularly in a physiological context. For this, we performed direct plasmid injection of reporter constructs containing either the full-length 3'UTR or the deleted ARE version into EDL and soleus muscles. Direct plasmid injection of a reporter construct containing the utrophin full-length 3'UTR resulted in an \sim 2.5-fold ($P < 0.05$) increase in reporter mRNA expression in soleus muscles as compared with EDL muscles (Figure 3B). In contrast, injection of the 3'UTR Δ ARE reporter construct did not lead to a significant difference ($P > 0.05$) in LacZ mRNA expression between EDL and soleus muscles (Figure 4D). These observations are consistent with the idea that the conserved ARE in the utrophin 3'UTR acts as a destabilizing element preferentially active in fast muscles, as also suggested by experiments presented in Figure 3.

The utrophin conserved ARE functions as a key regulatory element in the presence of fast and slow muscle protein extracts

Since utrophin A displays differential mRNA stability in the presence of either fast or slow muscle protein extracts, we next established whether the utrophin conserved ARE could confer differences in mRNA stability under similar conditions. Therefore, we performed *in vitro* stability assays whereby mRNA collected from C2C12 muscle cells transfected with LacZ reporter constructs containing either the utrophin full-length 3'UTR or the 3'UTR Δ ARE, were incubated with fast or slow muscle protein extracts (as described earlier). Consistent with the ability of the utrophin 3'UTR to promote higher levels of reporter mRNA expression in slow muscles relative to fast ones (Figure 3), these assays demonstrated greater reporter mRNA expression over time for the construct containing the full-length 3'UTR in the presence of soleus protein extracts relative to EDL extracts (Figure 5A). Quantitative comparisons of the half-life of utrophin full-length 3'UTR reporter mRNAs demonstrated a 50% ($P < 0.05$) decrease upon incubation with fast versus slow muscle extracts (Figure 5B).

Deletion of the ARE from the utrophin 3'UTR, which did not significantly affect the stability of reporter mRNAs incubated with slow muscle extracts, had a marked stabilizing influence on reporter mRNA expression incubated with fast muscle protein extracts (Figure 5A). Indeed, quantitative analyses revealed that deletion of the ARE from the utrophin 3'UTR led to an \sim 2-fold ($P < 0.05$) increase in reporter mRNA half-life relative to the half-life seen with mRNAs fused to the utrophin full-length 3'UTR upon incubation with fast EDL muscle extracts (Figure 5B). Of note, the half-life of reporter

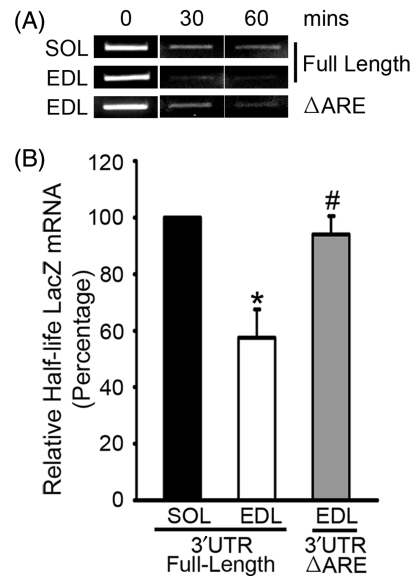


Figure 5. The conserved ARE in the utrophin 3'UTR increases the rate of decay of reporter mRNAs in fast muscle. (A) Example of ethidium bromide-stained agarose gels showing LacZ PCR products obtained following an *in vitro* stability assay at 0, 30 and 60 min of incubation with EDL or soleus (SOL) protein extracts. Note the greater amount of LacZ mRNA fused to the utrophin full-length 3'UTR that remains after 30 and 60 min incubations with the slow SOL muscle protein extracts. Also, note the higher levels of LacZ mRNA remaining after 30 and 60 min incubations with EDL protein extracts upon deletion of the conserved ARE in the utrophin 3'UTR (compare full-length 3'UTR to 3'UTR Δ ARE from EDL samples). (B) Quantitation of the relative half-life expressed as a percentage of the values obtained for LacZ mRNA fused to the utrophin full-length 3'UTR and incubated with SOL protein extracts. Note the significant decrease in the half-life for LacZ mRNA fused to the utrophin full-length 3'UTR upon incubation with EDL protein extracts relative to SOL. Also, note the significant increase in the half-life of LacZ mRNA upon deletion of the conserved ARE in the utrophin 3'UTR, relative to the utrophin full-length 3'UTR in samples incubated with EDL protein extracts. *Indicates a significant difference from SOL; #indicates a significant difference from other EDL samples ($P < 0.05$; $n = 4$ independent experiments).

mRNAs containing the utrophin 3'UTR Δ ARE incubated with fast muscle extracts were not significantly different ($P > 0.05$) from the half-life seen with constructs containing the full-length 3'UTR incubated with slow muscle extracts (Figure 5B). It should also be noted that control experiments showed no difference in the degradation of reporter mRNAs fused to the SV40 3'UTR upon incubation with either fast or slow muscle protein extracts (data not shown). Therefore, the conserved ARE in the utrophin A 3'UTR serves as an instability element in the presence of fast muscle protein extracts.

Calcineurin regulates the stability of utrophin A mRNA

The phosphatase calcineurin has been shown to have important roles in maintaining the expression of genes reflective of the slower, high oxidative, myofiber program (21,22). Recently, we showed that stimulation of calcineurin signaling promotes expression of utrophin A mRNA through modest increases in transcription acting through an NFAT site located within the utrophin A promoter region (10,17,19). Here, we sought to determine

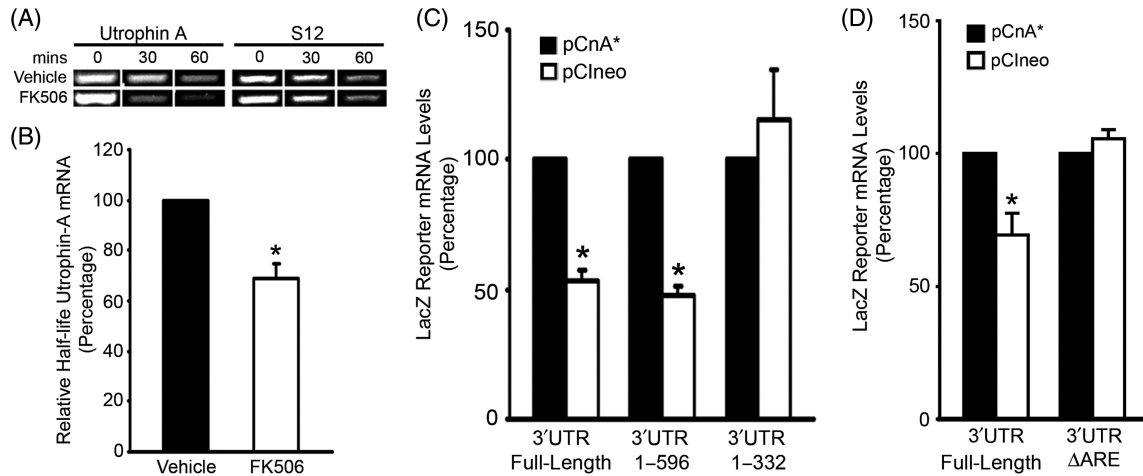


Figure 6. Calcineurin signaling regulates the stability of utrophin A mRNA through its 3'UTR. *In vitro* stability assays were performed with soleus (SOL) protein extracts from mice treated with vehicle or the calcineurin inhibitor FK506, and RNA isolated from 3-day old C2C12 myotubes. (A) Example of ethidium bromide-stained agarose gels displaying utrophin A and S12 PCR products following 0, 30 and 60 min incubation with protein extracts. Note the greater amount of utrophin A mRNA remaining after 30 and 60 min incubations with SOL extracts from vehicle-treated mice as compared with SOL extracts from mice treated with FK506. (B) Quantitation of the relative half-life expressed as a percentage of values obtained for utrophin A incubated with SOL extracts from vehicle treated mice. Note the significant decrease in the half-life of utrophin A upon incubation with SOL extracts from FK506-treated mice. *Indicates a significant difference from vehicle ($P < 0.05$; $n = 3$ independent experiments). (C) C2C12 myoblasts were transfected with reporter constructs containing either the utrophin full-length 3'UTR, or the first 596 or 332 nucleotides (Figure 2A), together with an empty vector (pCIneo) or a construct containing a constitutively active variant of calcineurin (pCnA*). Note that LacZ reporter mRNA levels expressed from the utrophin full-length 3'UTR and the first 596 nucleotide constructs, are 50% higher in myotubes expressing CnA*. This difference is lost when LacZ mRNAs are fused to only the first 332 nucleotides indicating the presence of a calcineurin-responsive element between nucleotides 332 and 596. *Indicates a significant difference between pCIneo and pCnA*transduced muscles ($P < 0.05$; $n = 4$ experiments done in triplicate). (D) Tibialis anterior muscles were injected with plasmids containing the utrophin full-length 3'UTR or the utrophin 3'UTR Δ ARE reporter constructs along with the pCIneo or pCnA*constructs. LacZ reporter mRNA levels from the full-length 3'UTR construct are significantly increased when muscle fibers have overexpressed pCnA*, whereas no increase is observed in muscle fibers injected with the 3'UTR Δ ARE reporter construct. *Indicates a significant difference between pCIneo and pCnA*transduced muscles ($P < 0.05$; $n = 3-4$ mice).

whether calcineurin signaling also affects utrophin A mRNA turnover.

To this end, we first performed *in vitro* stability assays. For these, mRNA from C2C12 myotubes was incubated with fast or slow muscle protein extracts taken from mice treated with the specific calcineurin inhibitor FK506 or vehicle (as control). FK506 (tacrolimus) acts as a specific inhibitor of calcineurin by forming a complex with the endogenous immunophilin protein FKBP12, thus sterically hindering substrates from accessing the catalytic site (42-44). As shown in Figure 6A, utrophin A transcripts decayed at an accelerated rate in the presence of soleus muscle extracts obtained from FK506-treated mice relative to vehicle controls. Quantitative assessment of half-life values revealed an $\sim 30\%$ ($P < 0.05$) decrease in the amount of time required for utrophin A mRNA to decay to half-maximal values when incubated with soleus muscle extracts from FK506-treated mice as compared to vehicle controls (Figure 6B). Similar results were obtained when *in vitro* stability assays were conducted with another muscle protein extracts from mice treated with another calcineurin inhibitor, cyclosporine A (CsA) ($\sim 25\%$ reduction; $P < 0.05$). Analysis of S12 transcript decay did not reveal any differences upon incubation with soleus muscle protein extracts taken from vehicle- or FK506-treated mice (Figure 6A).

Due to the negative effects of calcineurin inhibition on the stability of utrophin A mRNA, we further examined how altered activity of this enzyme could impact the

ability of the utrophin 3'UTR to regulate mRNA expression. First, we studied the effects of increased calcineurin activity on expression of LacZ reporter mRNAs containing the utrophin full-length 3'UTR in cultured C2C12 muscle cells. For these experiments, we transfected a construct containing a transgene that encodes a constitutively active variant of calcineurin (pCnA*) together with the LacZ construct harboring the utrophin full-length 3'UTR (17). In agreement with the ability of calcineurin to regulate the stability of utrophin A transcripts, we observed that co-transfection of pCnA* with the reporter construct containing the utrophin full-length 3'UTR, led to an ~ 2 -fold ($P < 0.05$) increase in LacZ mRNA expression in comparison to empty vector controls (pCIneo) (Figure 6C). Similarly, co-transfection of pCnA* with a deletion reporter construct containing only the first 596 nucleotides of the utrophin 3'UTR also led to an ~ 2 -fold ($P < 0.05$) induction in reporter mRNA levels in comparison to controls (Figure 6C). Notably, this difference was eliminated upon further truncation of the 3'UTR to the first 332 nucleotides (Figure 6C).

To demonstrate whether this regulation by calcineurin also occurs *in vivo*, we injected primarily fast TA muscles of mice with either the full-length 3'UTR or the deleted ARE version, along with pCnA* or an empty vector control (pCIneo). Muscles injected with the full-length reporter along with pCnA* showed an ~ 1.4 -fold enhancement of LacZ mRNA expression compared with pCIneo co-injected controls ($P < 0.05$). In contrast, co-injection of

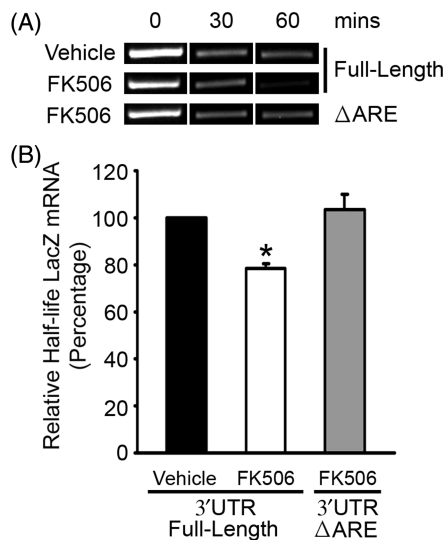


Figure 7. Calcineurin signaling regulates utrophin 3'UTR reporter mRNA stability through the conserved ARE. *In vitro* stability assays were performed with soleus (SOL) extracts obtained from mice treated with vehicle or FK506, and RNA isolated from 3 day-old C2C12 myotubes transfected with reporter constructs containing the full-length 3'UTR or the ARE-deleted version (3'UTR Δ ARE). (A) Example of ethidium bromide-stained agarose gels displaying LacZ PCR products following 0, 30 and 60 min incubation with protein extracts. Note the greater amount of LacZ mRNA fused to the utrophin full-length 3'UTR that remain after 30 and 60 min incubations with SOL extracts from vehicle-treated mice versus incubation with extracts from FK506-treated mice. Also, note the loss of this effect upon deletion of the conserved ARE. (B) Quantitation of the relative half-life expressed as a percentage of values obtained for LacZ mRNA fused to the utrophin full-length 3'UTR incubated with SOL protein extracts from vehicle-treated mice. Note the significant decrease in the half-life of LacZ mRNA with the full-length 3'UTR upon incubation with SOL extracts from FK506-treated mice relative to SOL extracts from vehicle-treated mice. Also, note the loss of this response to FK506 upon deletion of the ARE. *Indicates a significant difference from utrophin full-length 3'UTR ($P < 0.05$; $n = 3$ independent experiments).

the pCnA construct did not affect LacZ mRNA reporter levels from the 3'UTR Δ ARE reporter construct. Therefore, nucleotides 332–596 in the utrophin 3'UTR, in addition to functioning as a specific region required to maintain differences in mRNA expression between fast and slow muscles, is also a calcineurin-responsive element.

Lastly, we wanted to determine the effects of altered calcineurin signaling on mRNA stability mediated by the utrophin 3'UTR. Therefore, we performed *in vitro* stability assays with soleus muscle protein extracts obtained from mice treated with either FK506 or vehicle, in the presence of RNA isolated from C2C12 muscle cells transfected with reporter constructs containing either the utrophin full-length 3'UTR or the 3'UTR Δ ARE reporter constructs. The stability of utrophin full-length 3'UTR reporter mRNAs incubated with soleus muscle extracts of FK506-treated mice decreased relative to vehicle controls (Figure 7A). This observation is entirely consistent with the effect of calcineurin inhibitors on the stability of utrophin A mRNA and with the ability of activated calcineurin to promote reporter expression via the utrophin 3'UTR. Quantitative assessment over time of

reporter mRNAs fused to the utrophin full-length 3'UTR revealed that upon incubation with soleus protein extracts from FK506-treated mice, the half-life of these transcripts decreased by $\sim 25\%$ ($P < 0.05$) relative to vehicle-treated controls (Figure 7B). In contrast, the half-life of reporter mRNAs containing the utrophin 3'UTR Δ ARE incubated in the presence of soleus protein extracts obtained from FK506-treated mice was similar to those obtained for reporter mRNAs fused to the utrophin full-length 3'UTR incubated with soleus extracts from vehicle-treated mice (Figure 7B). These results show that inhibition of calcineurin signaling promotes degradation of utrophin mRNAs through the conserved ARE found within the 3'UTR.

DISCUSSION

We used a combination of approaches to gain insights into the molecular events controlling expression of utrophin A in fast and slow muscles. Here, we focused on the contribution of post-transcriptional mechanisms. Using an *in vitro* stability assay, we determined that utrophin A mRNA degrades at a faster rate in the presence of fast muscle proteins. In agreement with this, we also observed that reporter mRNAs fused to the utrophin 3'UTR decayed at a faster rate upon incubation with fast muscle extracts. Within the utrophin 3'UTR, we uncovered a conserved ARE with a prominent role in conferring higher levels of utrophin A mRNA in slow muscles relative to fast muscles. Finally, we demonstrated that calcineurin affects the stability of utrophin A mRNAs via the conserved ARE. These results, together with our previous observations (10,16,17), establish that both transcriptional and post-transcriptional mechanisms act in concert to promote increased expression of utrophin A in slower, oxidative muscle fibers. In fact, it appears that the 3–4 fold enrichment in utrophin A mRNA and protein levels seen in slow versus fast muscle can be accounted for by equal contributions of transcriptional and post-transcriptional events.

Stability of utrophin A mRNA in skeletal muscle

Post-transcriptional regulation of transcripts is becoming increasingly recognized as an important regulatory event in skeletal muscle maintenance and development [see (24) for review]. Fast and slow skeletal muscles, in addition to differing in the profiles of mRNAs and proteins expressed, also differ in the patterns of activity they receive from innervating motoneurons (45). Our previous work established that utrophin is a relatively stable mRNA, demonstrating a half-life of ~ 20 h that is unchanged during myogenic differentiation of C2C12 muscle cells in culture (46). Here, we show that stability of utrophin A mRNAs *in vivo* is clearly different between fast and slow muscles, suggesting that the different patterns of activity can modulate the half-life of specific mRNAs in muscle. Similar to utrophin A, expression of other transcripts in muscle is also known to be regulated post-transcriptionally via activity. For example, denervation of muscle fibers leads to an almost complete disappearance

of transcripts encoding AChE due primarily to a reduced half-life of pre-synthesized mRNAs (25,47,48). Moreover, it is becoming well established that post-transcriptional mechanisms also have important roles in promoting expression of the mitochondrial protein cytochrome c in response to increased skeletal muscle activity particularly in the early induction phase following electrical stimulation (49,50). These observations, together with our current findings, illustrate the importance of mRNA turnover in regulating expression of specific transcripts in skeletal muscle in response to altered levels of activity and among muscle fiber types.

Implication of a conserved ARE in the utrophin 3'UTR

It is now well recognized that ARE-mediated mRNA turnover is an important regulatory process that controls expression of distinct transcripts in all cell types [see for review (38,51)]. A large percentage of mRNAs have now been identified to contain AREs within their 3'UTRs [up to 5%, see (52)], emphasizing the importance of this regulatory site in controlling the abundance of multiple transcripts in cells placed under varying conditions. Close inspection of the ARE contained within the utrophin 3'UTR reveals the presence of two AUUUA motifs within a conserved region of 16 nucleotides in length, overlapping with a UUAUUUA (U/A) (U/A) nonamer. The presence of the two AUUUA motifs and the nonamer suggest that this particular ARE shares characteristics consistent with motifs involved in mRNA degradation. Deletion of this element resulted in an increase in the stability of the reporter mRNA, thereby indicating that indeed, this confined region of the 3'UTR acts as a destabilizing element. Our additional studies further showed that this element mediates its destabilizing effects preferentially in fast muscle.

Mechanisms regulating the stability of utrophin A mRNA

It has been shown that ARE-containing transcripts are rapidly deadenylated and degraded by one of two processes in other cell systems. One involves the association of the exosome, a large multiprotein enzymatic complex, with ARE-containing transcripts (41,53). This association of the exosome subsequently targets ARE-containing mRNAs for degradation. The second pathway involves a dynamic equilibrium, whereby ARE-containing mRNAs are targeted to cellular entities [i.e. processing (P) bodies or stress granules; (54,55)]. The 265 nucleotides within the 332–596 region of the utrophin 3'UTR which contains the conserved ARE, has previously been shown to have an important role in targeting utrophin mRNAs to cytoskeletal-bound polysomes during myogenesis (30). Since here we have shown that the same region in the utrophin 3'UTR can regulate the stability of mRNAs *in vivo*, it appears likely that in the context of fast and slow muscles, stability and targeting of utrophin A transcripts are linked. Therefore, deciphering the intracellular sites where utrophin A transcripts are enriched in fast and slow muscle fibers may provide important insights into the mechanisms that regulate their turnover.

The ability of AREs to affect the turnover rates of host mRNAs involves recruitment of ARE-binding proteins (ARE-bp) [see for review (38,56)]. The binding of these proteins to AREs can lead to either stabilization or accelerated degradation of mRNAs. ARE-bp such as AUF1 family members and KRSP are examples of trans-acting factors that promote degradation of ARE-containing mRNAs (57,58). Conversely, the Hu family of proteins, namely HuR, B, C and D, are the most well characterized ARE-bps known to promote stabilization of mRNAs (59). In skeletal muscle cells in culture, HuR has been shown to associate with the AREs found in the 3'UTR of transcripts encoding MyoD, myogenin, p21 and AChE (27,29,60). In this context, the levels of HuR as well as its subcellular localization have been shown to play important roles in stabilizing these various mRNAs during myogenesis. Conversely, activation of p38 MAPK signaling promotes stabilization of MyoD, myogenin and p21 mRNAs by preventing interactions between the destabilizing protein KRSP with ARE (28).

Previous work has shown that chronic stimulation of skeletal muscle leads to increased expression of cytochrome c accompanied by the loss of binding of a potentially destabilizing ~37kDa protein to the cytochrome c 3'UTR (49). Considering that chronic stimulation promotes conversion of skeletal muscles to a slower oxidative phenotype (45), these latter observations are consistent with the notion that fast muscle protein extracts contain one or more factors that promote destabilization of normally low abundance target mRNAs. Previously, using UV crosslinking assays, we showed greater binding to the utrophin 3'UTR of a 37kDa protein from fast muscle relative to slow muscle protein extracts (16). Collectively, these observations thus suggest that conversion and/or promotion of the slow myofiber program leads to a loss in the association of destabilizing factor(s) to ARE sites found in the 3'UTR of mRNAs that reflect this particular phenotype including cytochrome c and utrophin A transcripts.

Role of calcineurin in mRNA stabilization

It is now well established that calcineurin takes part in maintaining and promoting expression of the slower, high oxidative, myofiber phenotype by regulating at the transcriptional level, expression of slower myofiber genes (22,61). In this widely accepted model, different patterns of electrical activity controlled by innervating motor neurons lead to distinct intracellular waves of Ca^{+2} which activate calcineurin. In turn, activated calcineurin dephosphorylates NFAT and MEF2 that become free to stimulate transcription of a subset of genes via direct interactions with DNA regulatory motifs found within their promoter regions. However, recent detailed examination of calcineurin-null mice has led to the suggestion that this phosphatase regulates expression of the slower myofiber phenotype through additional factors other than NFAT and MEF2 (62).

In skeletal muscle, changes in intracellular calcium have been linked previously to alterations in the turnover of mRNAs encoding AChE and the inwardly rectifying

potassium channel 1 (63,64). In this context, alterations of calcineurin activity in skeletal muscle cells grown in culture have been shown to influence the stabilization/degradation of AChE transcripts during myogenic differentiation (65). Interestingly, calcineurin has also been shown previously to regulate specifically ARE-mediated mRNA turnover. In particular, calcineurin inhibition leads to the destabilization of IL-3 mRNAs and, notably, this effect is lost upon deletion of the ARE from the IL-3 3'UTR (66). In addition, inhibition of calcineurin activity has been shown to alter parathyroid mRNA levels while also promoting interactions between AUF1 and the parathyroid 3'UTR (67). In the present study, we demonstrate that calcineurin activity can influence the turnover of utrophin A mRNA through an ARE-dependent mechanism. Therefore, these results suggest that post-transcriptional events operating at the level of ARE-mediated mRNA decay provide additional mechanisms whereby calcineurin can promote expression of genes that characterize the slower oxidative myofiber program. Together with its known transcriptional effects on expression of slower, high-oxidative genes in muscle, it appears highly efficient and optimal for calcineurin signaling to concomitantly participate in the post-transcriptional mechanisms regulating the turnover of these target mRNAs in muscle fibers *in vivo*.

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