Distinct regions in the 3′ untranslated region are responsible for targeting and stabilizing utrophin transcripts in skeletal muscle cells

Anthony O. Gramolini, Guy Bélanger, and Bernard J. Jasmin

Department of Cellular and Molecular Medicine, Faculty of Medicine, and Center for Neuromuscular Disease, University of Ottawa; and Ottawa Health Research Institute, Ottawa, Ontario K1H 8M5, Canada

In this study, we have sought to determine whether utrophin transcripts are targeted to a distinct subcellular compartment in skeletal muscle cells, and have examined the role of the 3′ untranslated region (UTR) in regulating the stability and localization of utrophin transcripts. Our results show that utrophin transcripts associate preferentially with cytoskeleton-bound polysomes via actin microfilaments. Because this association is not evident in myoblasts, our findings also indicate that the localization of utrophin transcripts with cytoskeleton-bound polysomes is under developmental influences. Transfection of LacZ reporter constructs containing the utrophin 3′UTR showed that this region is critical for targeting chimeric mRNAs to cytoskeleton-bound polysomes and controlling transcript stability. Deletion studies resulted in the identification of distinct regions within the 3′UTR responsible for targeting and stabilizing utrophin mRNAs. Together, these results illustrate the contribution of posttranscriptional events in the regulation of utrophin in skeletal muscle. Accordingly, these findings provide novel targets, in addition to transcriptional events, for which pharmacological interventions may be envisaged to ultimately increase the endogenous levels of utrophin in skeletal muscle fibers from Duchenne muscular dystrophy (DMD) patients.

Introduction

Duchenne muscular dystrophy (DMD)* is the most prevalent inherited neuromuscular disorder affecting ~1 in every 3,500 male births (Emery, 1991). The disease results from mutations and/or deletions in the X-linked dystrophin gene which prevents production of a large cytoskeletal protein of the spectrin superfamily (Ahn and Kunkel, 1993; Worton, 1995). Although the exact function of dystrophin remains unclear, it has been shown to serve as a link between the internal actin cytoskeleton and the extracellular matrix via a complex of dystrophin-associated proteins (Matsumura and Campbell, 1994; Blake et al., 1996). Through these interactions, dystrophin is thought to maintain the integrity of muscle fibers during cycles of muscle contraction and relaxation.

Several years ago, a multiexonic gene on chromosome 6 that encodes a large cytoskeletal protein showing extensive sequence identity with dystrophin was identified (Love et al., 1989; Tinsley et al., 1992). Interestingly, this protein, now referred to as utrophin, is more ubiquitously distributed than full-length dystrophin. In skeletal muscle fibers, utrophin also displays a clear difference in its pattern of distribution as compared with dystrophin. Indeed, whereas dystrophin is known to be expressed along the entire length of the sarcolemma in normal muscle fibers, utrophin preferentially accumulates at the level of the neuromuscular junction in both normal and DMD muscles (Matsumura and Campbell, 1994; Blake et al., 1996; Gramolini et al., 2000) where it may contribute to the full differentiation of the postsynaptic apparatus (Deconinck et al., 1997; Grady et al., 1997). Because of its high degree of identity with dystrophin and its capacity to bind dystrophin-associated proteins (Matsumura et al., 1992), it has been suggested that upregulation of utrophin into extrasynaptic regions of DMD muscle fibers could serve as an
adequate therapeutic strategy for this disease (Blake et al., 1996; Gramolini et al., 2000). Indeed, a series of experiments using transgenic mouse model systems has shown that increased expression of utrophin along dystrophic muscle fibers functionally compensates for the lack of dystrophin, and hence prevents the development of the muscle pathology (see, for example, Tinsley et al., 1996). Therefore, it is of interest to understand the mechanisms presiding over utrophin expression in attempts to ultimately increase expression of the endogenous gene product throughout DMD muscle fibers.

We have recently undertaken a series of experiments to gain insights into the nature of these mechanisms (Gramolini et al., 2000). In a first set of studies, we have shown that utrophin transcripts display an asymmetric distribution along skeletal muscle fibers, as they are clearly enriched within the postsynaptic sarcoplasm (Gramolini et al., 1997; Vater et al., 1998). Subsequent work further demonstrated that local transcriptional activation of the utrophin gene within myonuclei located in the postsynaptic sarcoplasm accounts, at least in part, for the preferential accumulation of utrophin mRNAs within this region of muscle fibers (Gramolini et al., 1997, 1998, 1999a; Khurana et al., 1999). However, under distinct experimental conditions we have also observed that utrophin may be regulated via posttranscriptional events (Gramolini et al., 1999b). In particular, we have recently shown that increased mRNA stability accounts for the higher levels of utrophin seen in slow versus fast skeletal muscles, thereby implicating the contribution of posttranscriptional events in the overall regulation of utrophin expression (Gramolini et al., 2001). In this context, it is interesting to note that posttranscriptional as well as translational regulatory mechanisms have recently been shown to contribute to the development and maintenance of neuromuscular junctions in Drosophila (Sigrist et al., 2000).

The targeting of distinct mRNAs to specific subcellular compartments has recently emerged as a key posttranscriptional event involved in the control of protein expression and localization. For instance, mRNAs such as bicoid, oskar, and Vg1 become localized to a specific pole in developing Xenopus and Drosophila oocytes (see, for example, Pondel and King, 1988; Kim-Ha et al., 1993; Forristall et al., 1995). Interestingly, similar mRNA targeting mechanisms have also been observed in a variety of mammalian cells (see, for example, Bruckenstein et al., 1990; Burgin et al., 1990; Cripe et al., 1993; Kislauskis et al., 1994). In some cases, the subcellular localization of mRNAs has been shown to involve their targeting to specific pools of polysomes which associate with the cytoskeleton through regulatory sequences contained within the 3′ UTR (Hesketh et al., 1994; Veyrune et al., 1996; Tsai et al., 1997; Bagni et al., 2000; Mori et al., 2000). Such subcellular localization of mRNAs is now recognized as a key step that facilitates the sorting and targeting of proteins, while also promoting their assembly into macro-molecular complexes (for review see St Johnston, 1995; Hovland et al., 1996; Bassell and Singer, 1997; Hazelrigg, 1998; Jansen, 2001). With this in mind, we have begun to examine in the present study whether posttranscriptional regulatory mechanisms control utrophin expression in skeletal muscle cells. Specifically, we have: (a) sought to determine whether utrophin transcripts are targeted to a distinct subcellular compartment; and (b) examined the role of the utrophin 3′ untranslated region (UTR) in regulating the stability and localization of utrophin transcripts in skeletal muscle cells.
Results
The utrophin 3’UTR
Cloning and sequencing of the utrophin 3’UTR revealed that it is a relatively large region spanning 1,995 nucleotides (Fig. 1 A). A detailed analysis of this RNA fragment showed that it contains several regions of interest. For example, there are six AU-rich elements located at nucleotide positions 479, 487, 613, 760, 840, and 1292, which consist of the sequence AUUUA (St Johnston, 1995; Hesketh, 1996; Veyrune et al., 1997). Alignment of the full-length 3’UTR using several data banks revealed a high degree of homology with a partial utrophin 3’UTR from rat (Fig. 1 B). Specifically, the homology between mouse and rat is ~90% through the first 281 nt, after which there is no available sequence information for the rat 3’UTR. Interestingly, significant homology was also observed between the mouse 3’UTR and a human sequence from chromosome 6 containing the utrophin gene. In this case, the overall sequence identity between the mouse and human 3’UTRs is close to 80%, with certain regions showing even higher homology (Fig. 1 B). In particular, there is ~87% homology between nucleotides 352 and 583 of the mouse and human sequences. However, our comparison also revealed that the utrophin 3’UTR is quite distinct from the dystrophin 3’UTR. Indeed, we could not find any significant homology between these two 3’UTRs (<10%) despite the high sequence identity seen in the coding regions of these two genes.

Utrophin transcripts are enriched in a cytoskeletal-bound polysomal fraction
Initially, we examined the distribution of utrophin mRNAs in different pools of polysomes by using subcellular fractionation methods (Hesketh et al., 1994; Kislauskis et al., 1994; Hovland et al., 1995; Veyrune et al., 1997). Three distinct pools, corresponding to free, cytoskeletal-, and membrane-bound polysomes, were biochemically isolated. For these experiments, we first used several approaches to confirm that we had successfully isolated different pools of polysomes. Specifically, we monitored lactate dehydrogenase (LDH) activity as previously used in this case as a positive control for the free polysomal fraction because it contains cytosolic elements (Vedeler et al., 1991; Hovland et al., 1995; Mahon et al., 1995). In addition, we examined the distribution of utrophin among the different polysomal fractions. As shown in Fig. 2 A, utrophin levels were clearly enriched within the cytoskeletal fraction as revealed by Western blot analysis. Finally, we also monitored in these fractions the presence of mRNAs encoding the acetylcholine receptor (AChR) α-subunit. Because these transcripts encode a membrane protein (Hovland et al., 1995), we observed, as expected, that AChR α-subunit mRNAs were significantly more abundant in the membrane-bound polysomal fraction (Fig. 2 B).

Table 1. LDH activity in various polysomal fractions

| Fraction | Control (n = 12) | COL (n = 4) | Cy-D (n = 8) | 3’UTR (n = 12) | Myoblast (n = 7) |
|----------|----------------|-----------|-------------|--------------|----------------|
| Free     | 95.0 ± 0.6     | 90.0 ± 3.7| 95.1 ± 0.8  | 93.1 ± 1.0   | 93.2 ± 1.5     |
| Cytoskeletal | 3.3 ± 0.6    | 9.0 ± 3.8 | 3.5 ± 0.5   | 4.1 ± 0.8    | 3.9 ± 1.1      |
| Membrane | 0.7 ± 0.2      | 1.1 ± 0.6 | 1.4 ± 0.4   | 3.0 ± 0.5    | 3.0 ± 0.5      |

Aliquots from each polysomal fraction were assayed for LDH. As expected, the preferential activity of LDH within the free (cytosolic) polysomal fraction.

Figure 2. Characterization of the polysomal fractions. Markers of the three distinct pools of polysomes were used to ensure the validity of the subcellular fractionation procedure. Activity of the cytosolic enzyme LDH was determined for the free polysomal fraction (text and Table I). (A) shows the levels of utrophin in the free (F), cytoskeletal (C)-, and membrane (M)-bound polysomal fractions as determined by Western blot analysis. (B) shows a representative ethidium bromide–stained agarose gel of RT-PCR products corresponding to α-AChR mRNAs obtained from the three fractions. The left lane is a 100-bp molecular mass marker. (−), negative control lane. Note the accumulation of utrophin in the cytoskeletal-bound fraction (B) and the enrichment of transcripts encoding the α-subunit of AChR in the membrane-bound polysomal fraction (C).
free, cytoskeletal-, and membrane-bound polysomal fractions. Interestingly, in these experiments we observed that utrophin mRNAs were enriched within the cytoskeletal-bound polysomal fraction in 4-d-old myotubes (Fig. 3 A). In fact, our analysis revealed that 65% of the total content of utrophin transcripts was present within this fraction as compared with the free and membrane-bound fractions (Fig. 3 B).

Utrophin transcripts associate with actin filaments
To further characterize the association of utrophin transcripts with cytoskeletal-bound polysomes, we next disrupted the integrity of actin filaments or microtubules. Short-term treatments of myotubes with cytochalasin D (Cy-D) resulted in the depolymerization of actin filaments as shown by fluorescence labeling using rhodamine-conjugated phalloidin (Fig. 4, compare A and B). Examination of the relative content of utrophin transcripts in polysomal fractions isolated from control versus treated myotubes revealed a dramatic redistribution of utrophin mRNAs. In comparison to the findings seen in control myotubes, utrophin transcripts were clearly enriched in the cytoplasmic fraction after Cy-D treatment. These findings not only confirm the association of utrophin mRNAs with the cytoskeleton (Fig. 3), but further indicate that actin filaments play an essential role in maintaining a link between these transcripts and cytoskeletal-bound polysomes (Fig. 4 C). In these experiments, we verified that the 3-h Cy-D treatment did not affect the overall steady-state levels of utrophin mRNA, thereby demonstrating that the disappearance of utrophin transcripts from the cytoskeletal-bound polysomal fraction could not be ascribed to a change in mRNA stability, and that it was caused by a redistribution of the existing transcripts.

On the other hand, treatment of myotubes with colchicine (COL) failed to induce a redistribution of utrophin transcripts among the distinct pools of polysomes. In these experiments, we did verify by immunofluorescence that the COL treatment had successfully induced the depolymerization of microtubules (unpublished data). Because the results are similar to those observed in control myotubes (Fig. 3), these data indicate that the preferential localization of utrophin mRNAs within the cytoskeletal-bound pool of polysomes does not require intact microtubules.
the distribution of utrophin transcripts between the three pools of polysomes in myoblasts versus myotubes. As shown in Fig. 5, utrophin mRNAs are clearly not enriched in the cytoskeletal-bound polysomal fraction in myoblasts. By comparison to myotubes (Fig. 3), in which ~65% of all utrophin mRNAs are found within cytoskeletal-bound polysomes, <20% are present in this polysomal fraction in myoblasts. Therefore, these data suggest that the association of utrophin transcripts with cytoskeletal-bound polysomes and actin microfilaments is regulated during myogenic differentiation. In contrast, agrin treatment of myotubes, which is known to induce the formation of AChR clusters that contain other synaptic components (Burden, 1998; Sanes and Lichtmann, 1999) including utrophin (Campanelli et al., 1994; Guo et al., 1996), failed to alter the distribution of utrophin mRNAs amongst the three pools of polysomes (compare Figs. 5 C and 3).

**Role of the 3’UTR in targeting utrophin transcripts to cytoskeletal-bound polysomes**

Given that previous studies have indicated that mRNAs associate with the cytoskeleton via their 3’UTR (for review see Veyrune et al., 1997), we next investigated the role of the 3’UTR in the targeting of utrophin transcripts in skeletal muscle. To this end, we isolated the utrophin 3’UTR by PCR and subsequently generated β-galactosidase reporter constructs by inserting the 3’UTR in forward or reverse (used as a control) orientation, downstream of the LacZ gene (see Fig. 7 C). These expression vectors were then transfected into skeletal muscle cells grown in culture. The three distinct polysomal fractions were subsequently isolated and the presence of β-galactosidase transcripts was analyzed in each fraction.

In these experiments, analysis of the various subcellular fractions showed that the utrophin 3’UTR, cloned in the forward orientation, was capable of directing β-galactosidase transcripts to the cytoskeletal pool of polysomes (Fig. 6 A). Quantitative analyses revealed that ~65% of the total content of β-galactosidase transcripts was present within the cytoskeletal-bound polysomal fraction (Fig. 6 B), a value nearly identical to that observed for endogenous utrophin transcripts (Fig. 3 B). In addition, we noted in these experiments that disruption of the actin filament network with Cy-D, led to a redistribution of β-galactosidase transcripts as observed with the endogenous utrophin mRNAs (compare Figs. 4 and 6 A). In contrast to the localization within the cytoskeletal-bound polysomal fraction of β-galactosidase transcripts containing the utrophin 3’UTR in the forward orientation, cultures transfected with β-galactosidase expression constructs containing the 3’UTR in the reverse orientation failed to be targeted to this pool of polysomes (Fig. 6 A). With the reverse orientation construct, we observed that ~75% of all β-galactosidase transcripts were enriched within the free polysomal fraction (Fig. 6 B). This pattern of distribution was also seen with the parental β-galactosidase expression vector that only contains its own 3’UTR (unpublished data). As a control for these experiments, we verified in these myotubes (except for those treated with Cy-D) that endogenous utrophin mRNAs were indeed present within cytoskeletal-bound polysomes. Together, these results indi-

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**Redistribution of utrophin transcripts during myogenic differentiation**

Because the association of specific mRNAs with specific subcellular domains may be developmentally regulated (Cripe et al., 1993), and because utrophin expression is moderately increased during myogenic differentiation (Gramolini and Jasmin, 1999), we also determined in separate experiments...
cated that the 3’UTR is critical in targeting utrophin transcripts to their appropriate subcellular location.

**Domains necessary for targeting utrophin transcripts**

Having established that the full-length 3’UTR leads to an association of utrophin mRNAs with cytoskeletal-bound polysomes and actin filaments, we became interested in further characterizing the region(s) within the 3’UTR necessary for this targeting. Therefore, we generated several additional reporter constructs that contained, downstream of the LacZ gene, truncated fragments of the utrophin 3’UTR (Fig. 7C). These constructs were subsequently transfected into muscle cells, and polysomal fractions were isolated and analyzed for the presence of β-galactosidase transcripts. As illustrated in Fig. 7, A and B, we determined in these experiments that elements contained within nucleotides 969 to 596 account for ~1/2 of the localization of chimeric mRNAs to cytoskeletal-bound polysomes. Similarly, the 3’UTR fragment containing nucleotides 969–332 appeared responsible for the remaining targeting effect, as reporter constructs containing the first 332 nucleotides, or less (with constructs 161 and 86) of the 3’UTR, failed to direct β-galactosidase mRNAs to this pool of polysomes. Therefore, a region containing >600 nucleotides appears critical for targeting β-galactosidase transcripts to cytoskeleton-bound polysomes.

**A distinct region in the 3’UTR regulates the stability of utrophin transcripts**

In a final series of experiments, we used the same reporter constructs to determine the impact of the utrophin 3’UTR on the stability of β-galactosidase transcripts. For these studies, we transfected muscle cells in culture with the various expression vectors and subsequently exposed the cells to actinomycin D to inhibit transcription. At different time points thereafter, muscle cell cultures were analysed to determine the levels of β-galactosidase mRNAs. In comparison to parental β-galactosidase mRNAs, which displayed a half-life of ~4 h under these conditions, these experiments revealed that the utrophin 3’UTR was capable of conferring a more stable half-life to β-galactosidase transcripts (Fig. 8). Indeed, we determined that the half-life of β-galactosidase transcripts that contained the full-length utrophin 3’UTR was ~21 h (Fig. 8), a value similar to that seen recently under similar experimental conditions for endogenous utrophin mRNAs (Gramolini and Jasmin, 1999). Furthermore, we also identified in these experiments a region that appears critical for conferring this relatively stable half-life to β-galactosidase transcripts. Using the different reporter constructs containing different lengths of the utrophin 3’UTR (Fig. 7C), we determined that constructs 86 and 161 failed to extend the longevity of β-galactosidase mRNAs, whereas the reporter constructs containing the first 332, 596, and 969 nucleotides showed similar half-lives as those seen using the full-length 3’UTR. Therefore, it appears that the element(s) contained within nucleotides from position 161 to 332 in the 3’UTR is necessary to regulate the relatively stable half-life of utrophin transcripts in skeletal muscle cells.

**Discussion**

Similar to several other transcripts encoding synaptic proteins in muscle (for review see Ducleart and Changeux, 1995; Burden, 1998), utrophin mRNAs display an asymmetric distribution along skeletal muscle fibers as they accumulate preferentially within the postsynaptic sarcoplasm (Gramolini et al., 1997; Vater et al., 1998). Such a pattern of distri-
bution has been shown to result, at least partially, from the
local activation of the utrophin gene in myonuclei located in
the vicinity of the postsynaptic apparatus (Gramolini et al.,
1997, 1998). However, previous studies using several other
experimental systems, have led to the notion that the com-
partmentalized expression of mRNAs in specific cellular
compartments requires their interaction with cytoskeletal el-
ements (Bassell and Singer, 1997; Bassell et al., 1999; Lip-
shitz and Smibert, 2000). Additionally, it has become appar-
ent that in the cytoplasm, specific mRNAs are localized to a
distinct class of polysomes that are attached to cytoskeletal
elements rather than localizing to free polysomes (Hesketh,
1996; Bassell and Singer, 1997). Because the postsynaptic
sarcoplasm of muscle fibers contains a differentiated set of
microtubules (Jasmin et al., 1990) as well as a specialized ac-
tin meshwork (Hall et al., 1981), we became interested in
determining whether utrophin transcripts were enriched in a
particular subcellular compartment of polysomes through
interactions with the cytoskeleton. Using a sequential deter-
genent/salt extraction procedure, we show that in myogenic
cells, utrophin mRNAs are concentrated in a pool of cyto-
skeletal-bound polysomes and that this specific enrichment
depends on the integrity of the actin microfilaments. There-
fore, it is tempting to speculate that these mechanisms also
operate in vivo and act to complement the local transcrip-
tional regulation of the utrophin gene in order to ensure the
efficient local expression of utrophin mRNAs at the neuro-
muscular junction.

In our experiments, we also observed that the association
of utrophin transcripts with cytoskeletal elements is under
developmental influence because, as opposed to our findings
with myotubes, utrophin mRNAs were clearly not enriched
within the cytoskeletal-bound polysomal fraction in undif-
ferentiated myoblasts. Interestingly, recent studies have indi-

Figure 7. **Targeting domain within the utrophin 3' UTR.** (A) Representative examples of ethidium bromide–stained agarose gels corre-
sponding to β-galactosidase RT-PCR products obtained from free (F), cytoskeletal (C), and membrane (M)-bound polysomal fractions after
transfections with reporter constructs containing truncated fragments of the 3' UTR. Note that β-galactosidase constructs containing nucle-
otide sequence 332–596 are still targeted to cytoskeletal-bound polysomes and that this localization is lost with a smaller 3' UTR fragment
(332 nt). (B) The levels of β-galactosidase transcripts for each polysomal fraction were determined and expressed as percentage of the total
β-galactosidase mRNA levels detected. Shown are representative examples of a minimum of five experiments. (C) shows the reporter
constructs used in these studies. The utrophin 3' UTR sequences were inserted downstream of the LacZ gene. Nucleotide positions are
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notated on the right.
The utrophin 3' UTR regulates the stability of β-galactosidase transcripts. β-Galactosidase reporter constructs containing the full-length utrophin 3' UTR were transfected into muscle cells in culture. The stability of β-galactosidase transcripts was determined by exposing cultures to actinomycin D at time zero. Samples were collected at various time points and total RNA was subsequently extracted and subjected to RT-PCR. A shows a representative ethidium bromide-stained agarose gel corresponding to β-galactosidase RT-PCR products. (−), negative lane. (B) Quantitation of these results revealed that the half-life of β-galactosidase transcripts containing the full-length utrophin 3' UTR is ~22 h (C; line 1). Similar results were obtained with constructs containing the first 332 nt of the utrophin 3' UTR (lines 2, 4, and 5). Note also that the stability of the chimeric mRNAs was reduced with smaller fragments (lines 6 and 7) or when the region from nt 1 to 969 was absent from the 3' UTR (line 3). Shown are the pooled results from four independent experiments.

cated that other transcripts that are found in association with distinct polysomal fractions are also regulated during myogenic development. For example, transcripts encoding vimentin or the poly (a+)-binding protein do not show any association with the cytoskeleton in myoblasts; however, following myogenic differentiation, these transcripts become preferentially localized to the cytoskeleton (Adamou and Bag, 1992; Cripe et al., 1993). Thus, it appears that the subcellular localization of some mRNAs, including utrophin, undergoes profound changes during remodelling of the cytoskeleton which accompanies myogenic differentiation. Consistent with such a mechanism, a recent study has further revealed that actin polymerization during the early stages of synaptogenesis acts as an intracellular scaffold that orchestrates the assembly of the postsynaptic apparatus during the formation and growth of the neuromuscular junction (Dai et al., 2000). As a result, it is possible that the sorting of utrophin mRNAs to specific subcellular compartments could in fact play an important role in establishing and maintaining the local synthesis of utrophin at the neuromuscular junction during synapse formation.

Having established that utrophin mRNAs are specifically targeted to a particular subcellular compartment in skeletal muscle cells, we next investigated the mechanism by which these transcripts are separated from those translated on the rough endoplasmic reticulum or free polysomes. We focussed on the 3' UTR as this region is known to contain cis-acting elements that are responsible for directing the intracellular trafficking of various mRNAs (Kim-Ha et al., 1993; Hesketh et al., 1994; Kislauskis et al., 1994; Partridge et al., 1999; Keibler and DesGroseillers, 2000). In our experiments, the ability of the utrophin 3' UTR to redirect the localization of β-galactosidase mRNAs to cytoskeletal-bound polysomes indicates that utrophin transcripts are subject to an active intracellular sorting and targeting process. To characterize more precisely the cis-acting elements involved in this targeting mechanism, we generated several reporter constructs that contain deleted fragments of the utrophin 3' UTR and examined the polysomal association of the chimeric LacZ transcripts. These studies highlighted the crucial contribution of the regions between nucleotides 332 and 969 of the utrophin 3' UTR for the targeting of LacZ mRNAs to cytoskeletal-bound polysomes. A detailed examination of elements contained within this region revealed the presence of several AU-rich regions, including two adjacent ones, that have been shown previously to be necessary for the appropriate targeting of c-myc transcripts to cytoskeletal-bound polysomes in fibroblasts (Veyrune et al., 1996). Therefore, it appears likely that these consensus sequences are also critical in regulating the targeting of utrophin transcripts to the cytoskeleton in skeletal muscle cells.

Comparison of the utrophin 3' UTR across species revealed a very high degree of sequence homology, particularly between nucleotides 352 and 583 of the mouse and human sequences (Fig. 1 B). This high level of conservation suggests that this region may indeed be critical for regulating the targeting of utrophin transcript in both mouse and human skeletal muscle. The exact mechanism by which these sequences act to target transcripts to cytoskeletal-bound polysomes remains unknown, but it is likely that specific RNA-binding proteins interact with these cis-acting elements, resulting in the recruitment and targeting of transcripts to the intracellular actin network. As utrophin mRNAs are specifically targeted to cytoskeletal-bound polysomes in myotubes but not in myoblasts, it appears plausible that the abundance of these RNA-binding proteins may vary during myogenic differentiation as to correctly target utrophin transcripts to their appropriate subcellular compartment. Therefore, it will be important in future studies to identify the putative RNA-binding proteins that interact with the utrophin 3' UTR in attempts to further characterize the role of the 3' UTR in the control of utrophin expression. Similarly, it may be interesting to examine whether the 5' UTR and coding sequence also plays a role in targeting utrophin transcripts in muscle cells, although the large size of the coding region may make it difficult to study in its entirety.
It is well established that in addition to directing the subcellular localization of mRNAs, the 3’UTR controls the turnover rate of pre-synthesized mRNAs through interactions with trans-acting factors and cytoskeletal elements (Bassell and Singer, 1997; Tsai et al., 1997; Veyrune et al., 1997). In recent work, we have shown that the half-life of utrophin transcripts in skeletal muscle cells is ~20 h (Gramolini and Jasmin, 1999). In the present study, we determined that specific regions of the utrophin 3’UTR were sufficient to confer a similar and relatively stable half-life to the LacZ transcript. In fact, deletion analyses revealed that nucleotides between position 161 and 332 of the utrophin 3’UTR are important for mediating this effect. Interestingly, this region, responsible for mediating transcript stability, is different than the elements responsible for targeting utrophin transcripts to the cytoskeletal-bound polysomes (nucleotides 332–969; see above). Nonetheless, this observation is entirely consistent with previous studies that have examined the localization and stability of c-myc transcripts in fibroblasts (Veyrune et al., 1996). In particular, mutations of specific AU-rich regions in the c-myc 3’UTR were shown to disrupt the subcellular targeting of the transcript without affecting mRNA stability (Veyrune et al., 1996). It should also be pointed out that since we were able to confer a relatively stable half-life to the reporter transcripts that was nearly identical (~20 h) to that measured for endogenous utrophin mRNAs under similar conditions (Gramolini and Jasmin, 1999), it appears that the 3’UTR contains most, if not all, the regulatory elements necessary to control the stability of utrophin transcripts in skeletal muscle cells.

Based on utrophin’s ability to functionally compensate for the lack of dystrophin in animal models of DMD, a number of studies have examined the regulatory events controlling utrophin expression in skeletal muscle. Until now, these studies have focussed primarily on transcriptional mechanisms (see, for example, Gramolini et al., 1997, 1998, 1999a; Burton et al., 1999; Khurana et al., 1999; Galvagni and Oliviero, 2000). Taken together with our recent findings obtained in vivo and demonstrating the importance of mRNA stability in the control of the levels of utrophin and its transcript in slow versus fast muscles (Gramolini et al., 2001), the results of the present study illustrate the key contribution of post-transcriptional events in the overall regulation of utrophin in skeletal muscle cells. Accordingly, these findings provide novel targets, in addition to transcriptional events, for which pharmacological interventions may be envisaged to ultimately increase the endogenous levels of utrophin in DMD muscle fibers.

Materials and methods

Generation of utrophin 3’UTR reporter constructs

The full-length utrophin 3’UTR was generated by reverse transcription (RT)-PCR using RNA isolated from C2C12 cells and primers designed against the mouse utrophin sequence (Fig. 1 A; Blake et al., 1995; Guo et al., 1996). The 3’UTR fragment was subsequently inserted downstream of the reporter gene LacZ in the pCMV Sport β-galactosidase expression vector (GIBCO BRL) using available NcoI and EcoRI cloning sites. Additional constructs containing varying lengths of the utrophin 3’UTR (Fig. 7 C) were also generated by PCR amplification. All plasmids were sequenced to verify the identity of the constructs. For both transfection and direct gene transfer experiments, plasmid DNA was prepared using QIAGEN kits.

Tissue culture and cellular fractionation

Mouse C2C12 muscle cells were cultured, maintained, differentiated, and transfected using calcium phosphate, as described in detail previously (Gramolini et al., 1998). In our studies, myoblasts were examined when they were 50% confluent, whereas myotubes were analyzed after 4 d in differentiation media. Agin treatment of myotubes was performed as described in Gramolini et al. (1998). For experiments involving the inhibition of RNA synthesis, cultures were incubated with 4 μg/ml of actinomycin D in culture media (Tennyson et al., 1996; Gramolini and Jasmin, 1999). Samples were collected for up to 40 h after drug exposure. Unless otherwise specified, all culture reagents were obtained from GIBCO BRL.

The procedure to isolate subcellular fractions was similar to that described by Hesketh et al. (1994) and Howland et al. (1995). In brief, muscle cells were washed three times with PBS and harvested in 1.0 ml of lysis buffer containing 10 mM Tris, pH 7.6, 0.25 M sucrose, 25 mM KCl, 5 mM MgCl2, 0.5 mM CaCl2, and 0.5% Nonidet P-40. Lysates were then centrifuged at 1,000 × g for 5 min and the supernatant, containing the free polynucleosomal fraction, was removed and stored on ice. To isolate the cytoskeletal-bound polynucleosomal fraction, the pellet was washed in lysis buffer, resuspended in a second lysis buffer that contained 130 mM KCl and 0.5% NP-40, and then incubated for 10 min at 4°C. The extract was centrifuged at 2,000 × g for 5 min and the supernatant removed and stored on ice. The membrane-bound polynucleosomal fraction was harvested by incubating at room temperature for 15 min the cell pellet in a third lysis buffer containing 130 mM KCl, 0.5% Nonidet P-40, and 0.5% deoxycholate. This cell suspension was centrifuged at 3,000 × g for 5 min and the resulting supernatant yielding the membrane-bound fraction, was removed and placed on ice. An aliquot of each of the three fractions was taken to measure the activity of LDH using a commercially available kit (Sigma-Aldrich). All fractions were stored at −80°C for further analysis.

To examine the association of utrophin transcripts with the cytoskeleton, myotube cultures were treated for 3 h, before the collection of subcellular fractions, with compounds known to disrupt the integrity of distinct components of the cytoskeleton. Specifically, myotubes were treated with 2 μM Cy–D (Tsakiridis et al., 1994) or 50 μM COL (Basell et al., 1994) to depolymerize actin filaments or the microtubule network, respectively. To verify that these drug treatments had indeed resulted in the depolymerization of microfilaments and microtubules, myotubes were processed for fluorescence experiments using phalloidine-conjugated phallolidin or a monoclonal antibody directed against α-tubulin (Molecular Probes), respectively.

RNA extraction and RT-PCR

Total RNA was extracted from muscle cells in culture and from the polysonomal fractions using TriPure as recommended by the manufacturer (Boehringer Mannheim). Final RNA pellets were washed twice with 75% ethanol, resuspended in 20 μl RNase-free water, and stored at −20°C. From each RNA sample, the RNA was further diluted to a final concentration of 50 ng/μl. 2 μl of this final dilution was used for RT-PCR. For cultures transfected with β-galactosidase expression constructs, the RNA samples were treated with RNase free D (Promega) at 37°C for 1.5 h and then heated at 65°C for 20 min (Boudreaud-Lariviére et al., 2000).

RT-PCR was used to determine the relative abundance of transcripts among the different polysonomal fractions and was performed as described in detail elsewhere (Jasmin et al., 1995; Gramolini et al., 1998). In brief, RT was performed for 45 min at 42°C using random hexamers, and heated to 99°C for 5 min to terminate the reaction. Utophin cDNAs were specifically amplified by PCR using primers designed on the basis of the mouse utrophin sequence as described previously (Jasmin et al., 1995; Gramolini et al., 1998). These primers generate a 548-bp fragment. cDNAs encoding the α-subunit of the ACHR were amplified using primers designed on the basis of the mouse sequence (5′-GACTATCAAGGGAGTTACAA-3′; and 3′-GGACGGTTGAAGGCTAGC-3′) that generate a 576-bp PCR product. cDNAs encoding β-galactosidase were amplified as described (Boudreaud-Lariviére et al., 2000). The primers used to amplify β-galactosidase were 5′-CTGACGTCTTACGGTCC-3′; and 3′-CATTGATCTACGCAC-3′. 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. Typically, 26–30 cycles of amplification were performed. In separate experiments, we ascertained that these cycle numbers were within the linear range of amplification. Negative controls consisted of RT reactions in which total RNA was replaced with RNase-free water. PCR products were visualized on a 1% agarose gel containing ethidium bromide. The 100-bp molecular mass markers (MBI Fermentas and GIBCO
BRL) were used to estimate the molecular mass of the PCR products. For quantitative PCR experiments, PCR products were separated and visualized on agarose gels containing the fluorescent dye Vistra green (American Pharmacia Biotech) as described in Gramolini et al., (1998). 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 software (Molecular Dynamics).

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