Utrophin up-regulation in muscle fibers of Duchenne muscular dystrophy patients represents a potential therapeutic strategy. It is thus important to delineate the regulatory events presiding over utrophin in muscle in attempts to develop pharmacological interventions aimed at increasing utrophin expression. A number of studies have now shown that under several experimental conditions, the abundance of utrophin is increased without a corresponding elevation in its mRNA. Here, we examine whether utrophin expression is regulated at the translational level in regenerating muscle fibers. Treatment of mouse tibialis anterior muscles with cardiotoxin to induce muscle degeneration/regeneration led to a large (~14-fold) increase in the levels of utrophin A with a modest change in expression of its transcript (40%). Isolation of the mouse utrophin A 5′-untranslated region (UTR) revealed that it is relatively long with a predicted high degree of secondary structure. In control muscles, the 5′-UTR of utrophin A caused an inhibition upon translation of a reporter protein. Strikingly, this inhibition was removed during regeneration, indicating that expression of utrophin A in regenerating muscles is translationally regulated via its 5′-UTR. Using bicistronic reporter vectors, we observed that this translational effect involves an internal ribosome entry site in the utrophin A 5′-UTR. Thus, internal ribosome entry site-mediated translation of utrophin A can, at least partially, account for the dis-
transcriptional mechanisms are known to control expression of several synaptic proteins in muscle cells (20). In particular, we have previously shown that distinct elements within the utrophin 3’- UTR are important for controlling the stability of utrophin transcripts in muscle cells and for targeting them to specific subcellular locations (21, 22).

Converging lines of evidence also indicate that utrophin may be regulated at the translational and/or post-translational level. For example, we have previously demonstrated that utrophin levels are greatly up-regulated during muscle regeneration without a corresponding increase in expression of its mRNA (23). In the same study, we also noted that although utrophin levels are increased in muscle biopsy samples from DMD patients, levels of utrophin mRNA are similar to those seen in normal muscle samples (23, 24). Since these initial findings, several other laboratories have also reported discordant patterns of expression of utrophin protein and transcript. Specifically, Weir et al. (15) detected an increase in the abundance of utrophin A in muscle from mdx mice with no parallel elevation in the levels of utrophin A transcript. Similarly, utrophin protein levels in muscle change significantly during the life span of mdx mice with little alteration in the expression of utrophin mRNAs (25). Finally, results from several other studies also support the idea that utrophin is regulated via translational mechanisms (26, 27).

With this in mind, we have therefore initiated in the present study a series of experiments to examine whether the regulation of utrophin A in vivo involves translational events.

**EXPERIMENTAL PROCEDURES**

**Generation of Reporter Constructs**—To obtain the murine utrophin A 5'-UTR, reverse transcription and PCR experiments using total RNA obtained from C2C12 myotubes were carried out. The 5’ and 3’ primers amplified a 508-base pair fragment (5’ primer, 5’-TTGTTG-GAGTGCCCCT-3’; 3’ primer, 5’-CCCCATACCTTGGCCAT-3’) (15). This fragment was sequenced to confirm its identity. It was subsequently inserted into the multiple cloning site of pCMV SPORT-βGAL (Invitrogen) (called pβGAL here) to generate pUtra/βGAL (see Fig. 1 for all plasmids used). The utrophin A 5'-UTR was also inserted into the Xhol site of the bicistronic vector pβGAL/CAT (28) to generate pβGAL/Utra/CAT (see Fig. 1). The pβGAL/X-chromosome-linked inhibitor of apoptosis protein (XIAP)/CAT construct containing the ~1-kb XIAP 5’-UTR (28) was used in our experiments as a control for IRES activity. The promoterless bicistronic constructs p(-cmv)βGAL/CAT and p(-cmv)βGAL/Utra/CAT were generated by removing the CMV promoter using NruI and HindIII restriction sites. Excision of the β-galactosidase gene was accomplished by cutting with NotI and religating to create the monocistronic pUtra/CAT plasmid.

**Cell Culture**—C2C12 myoblasts were cultured in growth media in 35-mm plates as previously described in detail elsewhere (29). Cells were transfected when they reached 60–80% confluence. Two µg of pβGAL and pUtra/βGAL constructs were co-transfected with 2 µg of pCAT control vector (Promega, Mississauga, Canada) using 7.5 µl of Lipofectamine (2 mg/ml) according to the manufacturer’s instructions. Transfections using the bicistronic vectors were performed using 4 µg of plasmid with 8 µl of Lipofectamine (2 mg/ml). Cells were lysed 24 h after transfection using 400 µl of 1× reporter lysis buffer according to the manufacturer’s instructions (Promega), and the reporter activity was determined as described below.

**Direct Plasmid Injection and Cardiotoxin Treatment**—For in vivo studies, 25 µl of cardiotoxin (Latoxan, Rosans, France) at 10−5 M were injected into the right tibialis anterior (TA) muscle of 4-week-old C57BL/10 mice (Charles River Laboratories, St. Constant, Canada) to induce muscle degeneration and regeneration as previously described (23). Seven days later, cardiotoxin-treated and control, untreated TA muscles were excised and frozen in liquid nitrogen. For immunofluorescence experiments (see below), some TA muscles were frozen in melting isopentane precooled with liquid nitrogen.

To examine the expression of the reporter constructs during muscle regeneration, 25 µl of plasmid at 2 µg/µl in PBS was directly injected in both regenerating and control TA muscles 3 days after cardiotoxin treatment. Four days later, TA muscles were excised and frozen in liquid nitrogen. TA muscles injected with plasmids were homogenized using a Polytron homogenizer in 1 ml of 1× reporter lysis buffer (Promega), freeze-thawed twice, and centrifuged for 20 min at 12,000 × g. Supernatants were collected and frozen until further analysis.

**Reporter Assays**—Assays for βGAL enzymatic activity were performed on homogenized TA muscles or C2C12 myoblast lysates using the β-galactosidase enzyme assay system according to the manufacturer’s instructions (Promega). CAT activity was measured by analyzing the conversion of chloramphenicol to butyryl chloramphenicol by incorporation of [14C]butyryl coenzyme A using the CAT enzyme kit (Promega). Counting was performed in a liquid scintillation counter (Wallac 1414 liquid scintillation counter with 1414 W Environspect Windows software). Background levels for both reporter assays were determined by analyzing reporter activity in nontransduced TA muscle and nontransfected C2C12 cells.

**RNA Extraction and RT-PCR**—Total RNA was isolated from C2C12 cells or TA muscle using TRIzol reagent (Invitrogen) as recommended by the manufacturer. To quantitate the levels of endogenous transcripts in cardiotoxin-treated and untreated TA muscles, RT-PCR was performed to amplify utrophin A and S12 ribosomal RNA as previously described in detail elsewhere (18). Negative controls consisted of RT mixtures in which total RNA was replaced by sterile, diethylpyrocarbonate-treated water. For quantitation of endogenous utrophin A mRNA and S12 ribosomal RNA levels, PCR products were separated on Vista Green (Amersham Biosciences)-containing agarose gels, and the intensity of the signal was quantitated using ImageQuant version 5.1 (Amersham Biosciences). The relative levels of utrophin A transcripts were standardized according to the amount of S12 ribosomal RNA levels present in the same samples.

For RT-PCR analysis of reporter expression in transfected C2C12 cells and transfected TA muscles, Trizol-extracted RNA was first DNase I-treated (Invitrogen) for 1 h to eliminate plasmid contamination according to the manufacturer’s instructions. For TA muscles injected with pβGAL and pUtra/βGAL and co-transduced with pCAT, RT-PCR was carried out with primers to amplify a portion of CAT (5’-TG-GCAATGAAAGACGGTGAG-3’; 5’-GAACAGGGGGGCAGAAGT-3’), and a portion of βGAL (5’-GTGACGGCGCATTCGCTGAGTA-3’). A negative control consisted of an RT mixture that had the reverse transcriptase replaced with diethylpyrocarbonate-treated water. Denaturation was performed at 94 °C for 45 s, followed by an annealing step of 55 °C of 1 min and an extension step at 72 °C for 1 min for both βGAL and CAT PCR. For βGAL amplification, 20–27 cycles were used for quantitative analysis, whereas 23–28 cycles were used for CAT. Cycling was followed by a final extension step at 72 °C for 10 min. It is important to note that for all RT-PCR experiments used to quantify the relative abundance of transcripts, cycle numbers were optimized to be within the linear range of amplification as previously described in detail (22, 30, 31).

To control for the presence of an intact bicistronic reporter transcript following C2C12 transfections and direct plasmid injection into TA muscles with pβGAL/CAT and pβGAL/Utra/CAT plasmids, RT-PCR using total RNA was performed using a 5’ primer spanning 142 nucleotides of the LacZ gene and a 3’ primer spanning 459 nucleotides of the CAT gene that flanked the utrophin A 5’-UTR (5’-TGGATCCGGATT-

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**Utophin A 5’-UTR Confers IRES-mediated Translation**

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TGCTCAAC-3' 5'-GAGAAACTCCAGGATG-3'). This RT-PCR strategy is summarized in Fig. 6. The RT-PCR products were subcloned using the TOPO TA cloning kit (Invitrogen) and sequenced to verify their identity.

For quantitative RT-PCR, reverse transcription was carried out using the First-Strand cDNA Synthesis kit (Amersham Biosciences) with Not-d(T)18 primers. The quantitative real time RT-PCR was performed using the QuantiTect SYBR green RT-PCR kit (Qiagen) and analyzed on an ABI Prism 7000 sequence detection system using the ABI Prism 7000 SDS Software. Quantitative PCRs were carried out to detect βGAL (5'-ACTATCCCACCGCCTTACT-3', 5'-CTCTAGCGGCTGATTTGAAA-3') and CAT (5'-GGCTGTTACGTTGAAA-CCT-3', 5'-GGCGCAAGAAGTGTGTCATA-3') for muscle samples transduced with the bicistronic vectors. In order to control for DNA contamination, quantitative PCRs were performed directly on RNA samples.

Northerm Blotting—For Northern blot analysis, electrophoresis of total RNA was performed using a denaturing agarose/formaldehyde gel followed by transfer onto a Hybond, positively charged nylon membrane (Amersham Biosciences) for 2.5 h using downward alkaline transfer. The membranes were preincubated for 4 h at 42 °C in a shaking water bath with 10 ml of DIG easy hyb buffer (Roche Applied Science). The membrane was then incubated overnight with a linearized cDNA probe for CAT (301 bp) or βGAL (333 bp) labeled with Redivue [α-32P]dCTP (Amersham Biosciences) using the Rediprime II random prime labeling system (Amersham Biosciences). Washes were carried out for 1 × 5 min and 1 × 10 min at room temperature with 2× SSC, 0.1% SDS, and then for 1 × 2 min at 65 °C with 0.1× SSC, 0.1% SDS. The blot was rinsed in 2× SSC and exposed to Kodak BioMax MR film (Eastman Kodak Co.).

Western Blotting—A polyclonal antibody directed against utrophin A was raised as previously described (18). This utrophin A antibody was purified from production bleed of antisera using the SulfoLink Kit (Pierce), according to the manufacturer’s instructions. For Western blots, cardiotoxin-treated and untreated TA muscles from 4-week-old mice were homogenized in a previously described extraction buffer (32) using a Dounce homogenizer and then boiled and centrifuged for 10 min at 10,000 × g. One hundred μg of the protein extracts were separated using a 5% SDS-PAGE gel with a 4% stacking gel at 200 V for 5 h. Gels were transferred to Immobilon-P polyvinylidine fluoride membranes (Millipore Corp., Bedford, MA) overnight at 4 °C. Membranes were incubated with Tris-buffered saline with 0.1% Tween 20 and 5% dried skim milk for 1 h and then incubated for 1 h with the primary antibody (1:10,000) diluted in 5% milk Tris-buffered saline buffer. The membranes were washed thoroughly in Tris-buffered saline and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) secondary antibody (1:25,000) for 1 h. Protein detection was carried out using SuperSignal West Dura extended duration substrate (Pierce) and exposed to Kodak BioMAX MR film. The average intensities of the bands were quantitated using Kodak digital science 1D version 3.0.2 image analysis software. Equal loading of protein was confirmed with the SYPRO Ruby protein blot stain (Molecular Probes, Inc., Eugene, OR), as recommended by the manufacturer, and was further quantified using a monoclonal anti-actin antibody (Sigma).

Immunofluorescence—Immunofluorescence experiments were performed as previously described (18). Briefly, the polyclonal anti-utrophin A antibody was used to identify utrophin A expression on longitudinal sections of untreated or cardiotoxin-treated TA muscles. Alexa 488-conjugated α-bungarotoxin 594 (Jackson ImmunoResearch, West Grove, PA) was used to label acetylcholine receptors and, hence, to identify the presence of neuromuscular junctions.

Statistical Analysis—The presence of significant differences between group means was determined using Student’s t test. The level of significance was set at \( p < 0.05 \). Means ± S.E. are shown throughout.

RESULTS

Discordant Expression of Utrphin A and Its mRNA in Regenerating Muscle—Treatment of skeletal muscle with the snake venom cardiotoxin induces severe myonecrosis and subsequent muscle regeneration while leaving the innervating nerve intact. This is a well established model to study muscle regeneration (33, 34). Under these conditions, we have previously shown that the content of total utrophin (using an antibody that recognized both utrophin A and B isoforms) is significantly up-regulated several days after cardiotoxin treatment (23). Here, we examined the specific effect of cardiotoxin treatment on expression of the utrophin A isoform, which is selectively expressed in skeletal muscle (15, 18). Western blot analysis of muscles treated with cardiotoxin for 3–10 days revealed that the greatest levels of utrophin A expression were obtained at 7 days after cardiotoxin treatment. At this time point, we found that utrophin A expression was increased ~14-fold \( (p < 0.05) \) compared with untreated control muscles (Fig. 2, A and B). Immunofluorescence experiments revealed that whereas utrophin A expression in control muscles is confined to neuromuscular junctions (Fig. 3, A and B) (10–12), the increased expression of utrophin A in regenerating muscle fibers was observed along the entire length of the sarcolemma (Fig. 3, E and F). However, in sharp contrast to this dramatic induction of protein, utrophin A mRNA levels were modestly increased in regenerating muscle (40% increase; Fig. 2C). This relatively small increase in the abundance of utrophin A mRNA is in stark contrast to the dramatic increase in protein levels, thereby suggesting that changes in protein stability and/or in the translational efficiency of utrophin A mRNA could have an important regulatory function.

The Utrphin A 5'-UTR Confers Translational Regulation—On the basis of these findings, we next examined the possibility that utrophin is translationally regulated and decided to specifically assess whether mechanisms involving the utrophin A 5'-UTR could account for the discrepancy between the levels of protein and transcript observed during muscle regeneration. To this end, we isolated the murine utrophin A 5'-UTR from total RNA obtained from C2C12 myogenic cells and found that it was relatively long (15). Using the mFOLD algorithm, this sequence was predicted to have a high degree of secondary structure (35). Long 5'-UTRs often confer an inhibitory effect on cap-dependent translation. Therefore, we inserted this fragment into a reporter vector upstream of the β-galactosidase reporter gene to examine whether the utrophin A 5'-UTR causes translational inhibition (see schematics of constructs in Fig. 1).

The utrophin A 5'-UTR reporter construct (pUtrA/βGAL) or a parental control (pβGAL) was directly injected into control mouse TA muscles. An indication of the role that a 5'-UTR of interest plays in translational regulation can be obtained by measuring protein reporter activity and standardizing to the relative levels of reporter mRNA expressed from this plasmid (36, 37). Results of these experiments are thus expressed as a ratio of reporter protein activity to reporter transcript levels. As shown in Fig. 4A, in control TA muscles utrophin A stimulation in the ratio of protein to transcript for muscles transduced with the utrophin A 5'-UTR reporter construct (pUtrA/βGAL) compared with muscles transduced with the parental vector (pβGAL). This demonstrates that the 5'-UTR of utrophin A confers an overall inhibitory effect on translation in skeletal muscle fibers under control, steady-state conditions.
In order to study the effects of muscle regeneration on the ratio of reporter protein activity to transcript levels, we preinjected TA muscles with cardiotoxin 3 days prior to direct plasmid injection of the reporter constructs. Muscles were excised 7 days after the initial cardiotoxin injection. In these experiments, we reasoned that if the 5′-UTR of utrophin A plays a role in the discordant expression of endogenous utrophin A protein and transcript during muscle regeneration (see Figs. 2 and 3), then there should be a significant increase in the ratio of protein activity to transcript levels in regenerating muscles injected with pUtrA/BGAL. In accordance with our hypothesis, the relative ratio of protein to mRNA levels was dramatically increased in regenerating muscles injected with the construct containing the utrophin A 5′-UTR (Fig. 4B). Together, these results demonstrate that the inhibitory effect exerted by the utrophin A 5′-UTR on translation in control muscles was mitigated in cardiotoxin-treated TA muscles. These experiments further indicate that the increase in utrophin A protein expression observed in regenerating muscles (Figs. 2 and 3) involves a derepression of translation inhibition conferred by the long and highly structured utrophin A 5′-UTR.

Identification of IRES Activity in the Utrophin A 5′-UTR in Regenerating Muscle—The above data are coherent with the idea that utrophin A is translationally regulated in regenerating muscles by mechanisms that target the 5′-UTR. Most commonly, translational regulation occurs at the point of translation initiation. One possible mechanism through which specific eukaryotic mRNAs are translated during periods of “cellular stress” involves translation initiation mediated by the presence of an IRES (39). We therefore speculated that the utrophin A 5′-UTR contains an IRES that functions during the “stress” of cardiotoxin-induced muscle regeneration. A well-established approach to assess IRES activity of 5′-UTRs consists in using bicistronic reporter constructs (40, 41). Bicistronic vectors contain two reporter proteins, which enable the identification of IRES activity in a sequence of interest inserted between the two cistrons. The downstream cistron is only expressed if the sequence of interest contains an IRES. To test this hypothesis, we thus inserted the utrophin A 5′-UTR into a bicistronic vector (pβGAL/CAT; see Fig. 1) previously used to identify IRESs (28). The first cistron (βGAL) of this vector measures cap-dependent translation, whereas the second cistron (CAT) represents cap-independent translation.

Cardiotoxin-treated and control TA muscles were injected with the bicistronic reporter constructs using the same time frame as for injection with monocistronic reporter constructs (see above). As shown in Fig. 5, IRES activity is reported as a ratio of CAT/βGAL activity. A ratio significantly higher than that seen with the parental vector is reflective of IRES activity. In control muscles transduced with the bicistronic construct, no IRES activity above background was detected with the utrophin A 5′-UTR (Fig. 5B). However, there was a striking increase of more than 9-fold (p < 0.05) in the ratio of CAT/βGAL in regenerating muscles injected with the bicistronic vector containing the utrophin A 5′-UTR (Fig. 5A). The ratio of CAT/βGAL in pβGAL/UtrA/CAT-injected regenerating muscles was ~6-fold greater than that of pβGAL/CAT-injected regenerating muscles.
Utrophin A 5’-UTR Confers IRES-mediated Translation

Further analysis of the data expressed as individual levels of CAT and βGAL revealed that the increased CAT/βGAL ratio seen for the parental vector in regenerating muscles was caused by a decrease in the level of the βGAL reporter and not by an increase in CAT (Fig. 5B). Thus, the increased ratio seen for the parental vector in Fig. 5A is not reflective of spurious IRES activity but rather indicates a decrease in cap-dependent translation (determined by βGAL). In contrast, the increased ratio of CAT/βGAL mediated by the presence of the utrophin A 5’-UTR between the cistrons and seen in cardiotoxin-treated muscles was clearly due to an increase in CAT activity (Fig. 5B). Similar to what was observed with the pβGAL/CAT constructs, we also detected a decrease in βGAL activity with the pβGAL/Utra/CAT construct to an extent similar to that seen with the parental vector (Fig. 5B). It should be noted that the levels of βGAL for pβGAL/CAT and pβGAL/Utra/CAT in the cardiotoxin-treated muscle were not statistically different, whereas the
levels of CAT were 8.3-fold greater in pβGAL/UtrA/CAT-injected regenerating muscles compared with pβGAL/CAT-injected regenerating muscles. Therefore, the utrophin A 5′-UTR mediates cap-independent translation of the CAT reporter but only in cardiotoxin-treated muscle. This demonstrates that the utrophin A 5′-UTR contains an inducible IRES that becomes activated during muscle regeneration.

In parallel experiments, we also performed direct plasmid injection into TA muscles using a bicistronic vector containing the 5′-UTR of the XIAP, which is well characterized to contain an IRES element (pβGAL/XIAP/CAT) (28, 42). Interestingly, XIAP IRES activity was dramatically lower in cardiotoxin-treated muscles compared with control muscles (data not shown), suggesting that the IRES-activating effect of muscle regeneration is gene-specific.

To determine whether the IRES activity that we observed in regenerating muscles could be linked to aberrant splicing events, several control experiments were performed to ensure that CAT expression was due to genuine IRES-mediated translation of an intact bicistronic transcript. Due to the relatively low efficiency of transduction in mouse skeletal muscle, it is necessary to use a combination of PCR experiments to control for the possibility of splicing. First, RT-PCR was used to amplify the central region of the bicistronic RNA using primers flanking the utrophin A 5′-UTR sequence and portions of the βGAL and CAT reporter mRNAs (see Fig. 6A). As shown in Fig. 6B, we amplified RT-PCR products of the expected size using RNA extracted from cardiotoxin-treated muscles injected with pβGAL/UtrA/CAT. Sequencing of these PCR products confirmed their identity. Using this sensitive approach, we failed to detect smaller, aberrantly spliced mRNA, further demonstrating that IRES-mediated translation of utrophin A occurs during muscle regeneration.

To further control for the possibility of aberrant splicing, additional experiments were performed. Specifically, we used quantitative RT-PCR to amplify βGAL and CAT cistrons of the bicistronic mRNA (Fig. 6C). We reasoned that if the CAT activity from muscles transduced with the pβGAL/UtrA/CAT plasmid was due to IRES-mediated translation from intact, full-length bicistronic transcript, then βGAL and CAT cDNAs would be amplified by the quantitative PCR in a ratio of 1:1. Conversely, spurious splicing of the bicistronic mRNA would result in an increase of the CAT cistron RNA relative to βGAL cistron. This approach has in fact been used recently by others (43). We therefore processed RNA from control and cardiotoxin-treated muscles transduced with the parental or 5′-UTR containing plasmids. For all samples, we found a ratio of 1:1 (Fig. 6C). This indicates that only full-length mRNAs were transcribed from both parental and 5′-UTR containing plasmids in both control and cardiotoxin-treated TA muscles.

The Utrophin A IRES Is Also Active in Myoblasts—In separate experiments, we also assessed the activity of the utrophin A IRES in muscle cell culture. To this end, we transfected C2C12 myoblasts with the parental or utrophin A 5′-UTR-containing bicistronic vectors, harvested the cells 24 h later, and assessed IRES activity by determining reporter activity. We found that the presence of the utrophin A 5′-UTR conferred a ~9-fold (p < 0.05) induction in the ratio of CAT/βGAL compared with the ratio seen using the parental vector (Fig. 7A). This demonstrates that the utrophin A IRES element also mediates cap-independent translation in myoblasts. Interestingly, transfected cells that were induced to undergo myogenic differentiation for 4 days showed no IRES activity mediated by the utrophin A 5′-UTR (data not shown).

To ensure that expression of the downstream cistron was caused by genuine internal ribosome entry, we also examined the βGAL and CAT data individually. As shown in Fig. 7B, the increase ratio noted in cells transfected with the pβGAL/UtrA/CAT construct was caused by a large increase (p < 0.05) in CAT activity. To further verify that this increased CAT activity was not due to the presence of a cryptic promoter in the utrophin A 5′-UTR, transfections of bicistronic reporter constructs in which the CMV promoter had been deleted were performed. If the utrophin A 5′-UTR contains a cryptic promoter, CAT expression would occur in transfected cells despite the deletion of the viral promoter.
FIGURE 7. The utrophin A IRES is also active in C2C12 myoblasts. A, bicistronic reporter constructs were transfected in C2C12 myoblasts, and levels of reporter proteins were assayed 24 h later. Note that in comparison with cells transfected with the parental construct, cells transfected with the construct containing the utrophin A 5'-UTR display a large increase (+874%) in IRES activity. These data are reported as a ratio of CAT activity, measuring cap-independent translation, to βGal activity, measuring cap-dependent translation. A higher ratio of CAT/βGal is indicative of IRES activity. B shows the individual levels of CAT and βGal activity used to calculate the ratios presented in A. Note that the increased ratio is due to an increase in CAT activity. *, a significant difference between cells transfected with βGal/CAT versus βGal/UtrA/CAT-transfected cells. Mean values ± S.E. are shown; p < 0.05.

Removal of the CMV promoter from the parental and utrophin A 5'-UTR vectors (p(-cmv)βGal/CAT and p(-cmv)βGal/UtrA/CAT, respectively) resulted in the loss of both βGal and CAT reporter activity (data not shown). In agreement with these findings, no expression of an mRNA containing the CAT sequence could be detected by Northern blots performed with total RNA extracted from cells transfected with the p(-cmv)βGal/UtrA/CAT construct (data not shown). This further shows that the IRES activity of the utrophin A 5'-UTR is not due to cryptic promoter activity.

To control for the possibility of splicing events causing the expression of the CAT cistron from the pβGal/UtrA/CAT vector in the transfected cells in culture, we performed RT-PCR and Northern blot analyses to verify the presence of an intact bicistronic transcript. RT-PCR analysis of RNA from the pβGal/CAT- and the pβGal/UtrA/CAT-transfected C2C12 cells amplified single transcripts of the expected sizes (Fig. 8A). The larger PCR product seen in cells transfected with pβGal/UtrA/CAT is due to the presence of the utrophin A 5'-UTR between the two cistrons. Northern blotting using a 301-bp probe for CAT was performed on RNA extracted from cells transfected with the pβGal/CAT construct (see Fig. 8A). The arrowhead denotes where RNA from cells transfected with a monocistronic CAT vector would migrate (not containing the utrophin A 5'-UTR). The 28 S marker corresponds to 4.7 kb, whereas the 18.5 marker corresponds to 1.9 kb.

DISCUSSION

In recent years, several reports have implicated that utrophin is regulated via translational and/or post-translational mechanisms (see Introduction). Specifically, the discordant patterns of expression in the levels of utrophin and its mRNA in muscle cells placed under a number of experimental conditions indicate that utrophin expression is regulated by translational mechanisms and/or protein stability. The current study was designed to examine this issue by focusing on the possibility that utrophin expression, in addition to being controlled by transcriptional mechanisms, is also subject to important translational events that ultimately modulate the overall abundance of utrophin in muscle fibers. Here, we show that the large increase in the expression of utrophin A during muscle regeneration is clearly not accompanied by a parallel increase in utrophin A transcripts. Additionally, we show that the utrophin A 5'-UTR has an inhibitory effect on translation of a reporter transcript in intact skeletal muscle fibers. Strikingly, a marked derepression of this inhibition is observed in muscle fibers undergoing regeneration. Finally, using several complementary approaches, we show that the utrophin A 5'-UTR contains an IRES whose activity becomes highly stimulated in regenerating muscle.

IRES-mediated translation is an alternative to cap-dependent trans-
Utrophin A 5′-UTR Confers IRES-mediated Translation

lication, the main mechanism of translation initiation in eukaryotes (44). In cap-dependent translation, the eukaryotic initiation factor eukaryotic initiation factor 4E binds to the 5′-methyl guanosine-capped structure at the 5′-end of an mRNA. Upon association of other initiation factors, Met-tRNA\textsubscript{Met} and the 40 S ribosomal subunit, scanning is believed to occur in a 5′ to 3′ direction until a start codon in a favorable sequence context is encountered, and protein synthesis begins (45). By contrast, in IRES-mediated translation, the 5′-UTR harbors specific sequences that are thought to recruit ribosomes directly to the vicinity of initiation codon independently of the 5′-capped structure (39). Whereas cellular mRNAs are normally translated in a cap-dependent manner, there is a growing list of cellular mRNAs that are now known to be translated via cap-independent, IRES-mediated translation. Often, these mRNAs contain long and highly structured 5′-UTRs.

Using bicistronic reporter vectors, we showed that the utrophin A 5′-UTR displayed, as expected, no IRES activity in intact muscles. However, in muscle induced to degenerate and regenerate by cardiotoxin treatment, the utrophin A 5′-UTR mediated a 9-fold increase in the expression of the downstream reporter. Several control experiments revealed that the bicistronic mRNA did not undergo aberrant splicing, while also showing that the utrophin A 5′-UTR does not contain cryptic promoter activity. Based on these findings, it appears therefore that the induction of the muscle regenerative program serves as an appropriate signal to create an environment causing activation of the utrophin A IRES. Thus, cap-independent translation via the utrophin A 5′-UTR IRES can at least partially account for the large discrepancy in protein and transcript levels observed in regenerating skeletal muscle.

Skeletal muscle regeneration that follows after injury or chemical insult involves the activation of quiescent satellite cells and promotion of their entry into the mitotic phase. These cells proliferate, terminally differentiate, and fuse to form newly formed multinucleated muscle fibers (33). Interestingly, and in agreement with our in vivo findings, we observed in the present studies that the utrophin A 5′-UTR also displayed IRES activity in myoblasts growing in culture at 9.1-fold over parental levels (Fig. 7). In contrast, after transfected myoblasts were induced to differentiate to form multinucleated myotubes, we observed no significant IRES activity (data not shown). This is unlikely to be coincidental, since before they fuse to form myotubes, C2C12 myoblasts are similar to satellite cells that are activated during muscle regeneration. Our results obtained with cultured cells as well as with muscle in vivo are strongly supported by the fact that cap-dependent translation is often down-regulated during cell cycle activation and differentiation, whereas IRES-mediated translation of specific genes persists under these conditions (46, 47). Indeed, βGAL reporter levels, which in our experimental system are indicative of cap-dependent translation, were reduced in cardiotoxin-treated samples.

Many of the mammalian IRES elements found to date mediate cap-independent translation of proteins that are expressed during periods of “cellular stress” when cap-dependent translation may become less efficient (39, 44, 48). An interesting possibility therefore is that the “stress” of muscle regeneration causes the induction of IRES activity for a variety of mRNAs. In this context, several other proteins that are known to be involved in muscle regeneration have also been shown to be regulated via IRES-mediated translation. Specifically, it is known that injection of fibroblast growth factor II in muscles of mdx mice improves regeneration (49). Additionally, expression of insulin growth factor I receptor is up-regulated during muscle regeneration (50). It is therefore highly relevant to note that the fibroblast growth factor II 5′-UTR contains an IRES (51) and that the receptor for insulin growth factor I is also subject to IRES-mediated translational control (52). In addition, both the leader 2 5′-UTR of insulin growth factor II (53) and the A and C leaders of fibroblast growth factor I (54) can cause translation initiation via internal ribosome entry. Thus, muscle regeneration may indeed represent a type of “cellular stress” that promotes IRES-mediated translation of a subclass of mRNAs whose expression may be crucial to ensure the success of the regenerative response.

In recent years, there has been an emergence of studies identifying translational regulation at specific postsynaptic sites. The majority of such work has focused on translational events that occur in dendrites and that impact on synaptic plasticity (55–57). Such local regulation provides a mechanism for protein synthesis to occur rapidly in a distinct subcellular compartment under the influence of incoming inputs from presynaptic nerve terminals. Map 2 and Arc are two mRNAs that are localized to dendrites and whose translation can be regulated by the presence of IRES elements in their 5′-UTRs (58). These findings are important and supportive of our current results, since (i) utrophin A is an important component of the postsynaptic apparatus in muscle fibers, and (ii) both Map 2 and Arc are also cytoskeletal proteins.

There is solid evidence now indicating that translational regulation does occur at the Drosophila neuromuscular junction (59, 60). Furthermore, there are indirect observations implicating translational regulation as an important control step in the synthesis of synaptic proteins in mammalian muscle. For instance, it has been shown that expression of the acetylcholine receptor α-subunit is regulated at the translational level in rat primary muscle cells (61). Moreover, a decrease in translation has been shown to account for the reduction in acetylcholinesterase expression in skeletal muscle following glucocorticoid treatment (62). Finally, we have recently demonstrated that staufen, a double-stranded RNA-binding protein involved in mRNA targeting and translation, accumulates within the postsynaptic membrane domain of the neuromuscular junction (63).

In summary, we have demonstrated that the utrophin A 5′-UTR can drive IRES-mediated translation during muscle regeneration. This finding provides an explanation for the discrepancy between utrophin A protein and transcript levels observed in various studies. Although these findings do not exclude the possibility that post-translational mechanisms, such as alterations in protein stability, may also partially account for these discrepancies between protein and mRNA levels (25, 27, 64), our data add considerably to the growing literature highlighting the importance of translational control for the regulation of synaptic proteins in muscle and nerve. Additionally, our results provide an additional target from which pharmacological interventions may be rationally designed to stimulate utrophin A expression in muscle fibers from DMD patients in attempts to alter the progression of this neuromuscular disorder and provide functional benefits.

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