The utrophin A 5′-UTR drives cap-independent translation exclusively in skeletal muscles of transgenic mice and interacts with eEF1A2

P. Miura1†, A. Coriati†, G. Bélanger1, Y. De Repentigny3, J. Lee2, R. Kothary1,3, M. Holcik2,4, and B.J. Jasmin1,3,*

1Department of Cellular & Molecular Medicine and Center for Neuromuscular Disease, Faculty of Medicine and 2Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, ON, Canada, K1H 8M5, 3Ottawa Hospital Research Institute, Ottawa Hospital, Ottawa, ON, Canada, K1H 8L6 and 4Apoptosis Research Centre, Children’s Hospital of Eastern Ontario, Ottawa, ON, Canada, K1H 8L1

Received November 5, 2009; Revised December 20, 2009; Accepted December 31, 2009

The molecular mechanisms regulating expression of utrophin A are of therapeutic interest since upregulating its expression at the sarcolemma can compensate for the lack of dystrophin in animal models of Duchenne Muscular Dystrophy (DMD). The 5′-UTR of utrophin A has been previously shown to drive cap-independent internal ribosome entry site (IRES)-mediated translation in response to muscle regeneration and glucocorticoid treatment. To determine whether the utrophin A IRES displays tissue specific activity, we generated transgenic mice harboring control (CMV/βGAL/CAT) or utrophin A 5′-UTR (CMV/βGAL/UtrA/CAT) bicistronic reporter transgenes. Examination of multiple tissues from two CMV/βGAL/UtrA/CAT lines revealed that the utrophin A 5′-UTR drives cap-independent translation of the reporter gene exclusively in skeletal muscles and no other examined tissues. This expression pattern suggested that skeletal muscle-specific factors are involved in IRES-mediated translation of utrophin A. We performed RNA-affinity chromatography experiments combined with mass spectrometry to identify trans-factors that bind the utrophin A 5′-UTR and identified eukaryotic elongation factor 1A2 (eEF1A2). UV-crosslinking experiments confirmed the specificity of this interaction. Regions of the utrophin A 5′-UTR that bound eEF1A2 also mediated cap-independent translation in C2C12 muscle cells. Cultured cells lacking eEF1A2 had reduced IRES activity compared with cells overexpressing eEF1A2. Together, these results suggest an important role for eEF1A2 in driving cap-independent translation of utrophin A in skeletal muscle. The trans-factors and signaling pathways driving skeletal-muscle specific IRES-mediated translation of utrophin A could provide unique targets for developing pharmacological-based DMD therapies.

INTRODUCTION

The fatal neuromuscular disease Duchenne Muscular Dystrophy (DMD) is caused by loss of dystrophin expression in the muscle fibers of affected patients (1). Utrophin is the autosomal homologue of dystrophin, and through multiple approaches it has been demonstrated that enhancing its expression can compensate for the lack of dystrophin in animal models of DMD and alleviate the muscle pathology (2–5). A major difference between dystrophin and utrophin A (the utrophin isoform expressed in skeletal muscle) is that although dystrophin is expressed along the entire sarcolemma, utrophin A expression in mature fibers is primarily restricted to post-synaptic regions of the sarcolemma (6,7). It is thus of considerable therapeutic interest to identify mechanisms by which utrophin A expression can be enhanced along the entire sarcolemma of DMD patient muscle fibers.

We have found that post-transcriptional mechanisms play an important role in enhancing expression of utrophin A at the sarcolemma. For instance, in addition to transcriptional

†These authors contributed equally to this work.

To whom correspondence should be addressed. Tel: +1 6135625800; Fax: +1 6135625636; Email: jasmin@uottawa.ca

© The Author 2010. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org
mechanisms involving calcineurin/NFAT signaling, post-transcriptional events targeting the utrophin A 3′-UTR can modulate the stability of utrophin A transcripts, thus contributing to the enhanced expression of utrophin A in extrasynaptic regions of slow-twitch, oxidative fibers when compared with fast-twitch, glycolytic fibers (8,9). Increased sarcolemmal expression of utrophin A also occurs in skeletal muscles undergoing regeneration. Interestingly, this enhancement of utrophin protein expression is not accompanied by concomitant increases in utrophin A mRNA levels, as observed both in regenerating versus control mouse skeletal muscles and in muscle biopsies of DMD patients compared with healthy subjects (10). Enhancement of utrophin A protein expression in response to muscle regeneration can be at least partially explained by increased translation initiation mediated by an internal ribosome entry site (IRES) located in the utrophin A 5′-UTR (11).

IRES elements are thought to recruit the ribosome, IRES trans-acting factors (ITAFs) and other components of the translational machinery to the 5′-UTRs of some cellular mRNAs (12). IRES-mediated translation initiation occurs independently of the 7-methyl guanosine cap at the 5′ end of the mRNA, thus providing an alternative to the canonical, cap-dependent mechanism of translation initiation. This alternative mechanism allows for the cell to express a subset of proteins under stress conditions where global cap-dependent translation is suppressed, such as during viral infection, hypoxia and apoptosis (13). In the case of the utrophin A IRES its activity is enhanced during muscle regeneration and glucocorticoid treatment when global cap-dependent translation may be suppressed (11,14).

In this study, we set out to determine the tissue distribution of utrophin A IRES activity by generating and characterizing utrophin A 5′-UTR reporter transgenic mice. We also initiated experiments to identify ITAFs that interact with the utrophin A 5′-UTR and regulate its IRES activity.

RESULTS

Generation of transgenic mice harboring CMV/βGAL/CAT and CMV/βGAL/UtRA/CAT reporters

Previous work on the FGF-2 IRES has shown that tissue specificity of IRES activity in vivo is not necessarily correlated with activity in cell lines (15). We thus generated transgenic mice harboring a bicistronic utrophin A 5′-UTR reporter. For the creation of utrophin A 5′-UTR reporter transgenic mice, we used the CMV/βGAL/CAT bicistronic vector (Fig. 1A). This vector was chosen because it does not exhibit splicing or internal promoter activity under a variety of conditions when harboring the utrophin A 5′-UTR (11,16), and because previous studies have employed bicistronic reporters driven by the CMV promoter to evaluate IRES activity in transgenic mice (15). From three founder mice identified by genotyping, full analysis was performed on two utrophin A 5′-UTR reporter transgenic lines (CMV/βGAL/UtrA/CAT), namely lines 863 and 876. To serve as a control, we generated a transgenic line harboring a control bicistronic reporter (CMV/βGAL/CAT).

The utrophin A 5′-UTR drives muscle-specific translation in transgenic mice

We examined multiple tissues of the transgenic mouse lines for IRES activity. The reporter transgene provides a read-out of cap-dependent translation as β-galactosidase (βGAL) activity, whereas cap-independent, IRES-mediated translation, is reported as chloramphenicol acetyltransferase (CAT) activity. In three transgenic lines harboring the utrophin A 5′-UTR reporter, we detected appreciable levels of IRES activity (reported as a ratio of CAT to βGAL) in hindlimb skeletal muscles. Interestingly, we did not detect IRES activity in any other organs examined, including the kidney, heart, lung, liver and brain (Fig. 1B and C). In line 863, IRES activity in the tibialis anterior (TA) and gastrocnemius (gastroc) skeletal muscles was 14.5-fold and 10.7-fold above background brain levels (Fig. 1B). In line 876, a similar trend was observed, with IRES activity 7.3-fold and 6-fold greater than background brain levels in the TA and gastroc, respectively (Fig. 1C). A third line harboring the CMV/βGAL/UtrA/CAT reporter (line 881) also displayed IRES activity exclusively in skeletal muscles (data not shown).

Examination of the above data expressed as individual βGAL (Fig. 2A–C) and CAT (Fig. 2D–F) values revealed that the increase in CAT to βGAL ratio in TA and gastroc muscles of the 863 and 876 lines is due to enhanced IRES expression, and not merely a result of low βGAL levels stemming from low CMV driven transcription. These findings indicate that, in vivo, cap-independent translation driven by the utrophin A IRES occurs exclusively in skeletal muscles and no other tissues.

We performed several control experiments to ensure that the skeletal muscle-specific expression of the CAT reporter could be attributed to genuine utrophin A IRES activity. Examination of the control transgenic line (CMV/βGAL/CAT) revealed that although βGAL activity was detectable in all tissues (Fig. 2C), appreciable levels of CAT activity could not be detected (Fig. 2F). This result demonstrates that the CAT activity detected in skeletal muscles of transgenic lines 863 and 876 can be attributed solely to the utrophin A 5′-UTR.

Additional control experiments were performed to ensure that IRES activity observed in skeletal muscle was not due to an aberrant splicing event or cryptic promoter activity. The presence of internal promoter activity or aberrant splicing could result in the expression of transcripts other than the intact, full-length bicistronic mRNA, potentially leading to a false-positive indication of IRES activity. We performed qRT–PCR analysis on RNA extracted from the 876 and 863 lines (CMV/βGAL/Utra/CAT) to ensure that βGAL and CAT cDNA could be amplified in an equal ratio in both tissues that displayed IRES activity (gastroc and TA) and tissues that had no IRES activity (heart and brain). Indeed, we found that the ratio of amplified CAT to βGAL cDNA in all tissues examined was not statistically different (P > 0.05; Fig. 1D and E). The enhanced CAT activity in skeletal muscles of the CMV/βGAL/UtrA/CAT reporter mice can thus be attributed to increased cap-independent translation driven by events that target the utrophin A 5′-UTR.
Identification of RNA-binding proteins that interact with the utrophin A 5'-UTR

Since IRES-mediated translation is regulated by the binding of trans-factors, we set out to identify RNA-binding proteins that interact with the utrophin A 5'-UTR. We first examined whether proteins isolated from regenerating muscle versus control muscle could bind selectively to the utrophin A 5'-UTR since utrophin A IRES activity is enhanced in regenerating muscle (11). To induce skeletal muscle degeneration and regeneration, we injected TA muscles of mice with the snake venom cardiotoxin and isolated the muscles after 7 days. At this time-point, utrophin A IRES activity is induced and its protein levels are 14-fold above control levels (11). Using northwestern analysis, we found that a RNA probe corresponding to a portion of the utrophin A 5'-UTR (nucleotides 147–363) associated with several proteins preferentially in regenerating muscle extracts (CTX; Fig. 3A). Thus, conditions under which the utrophin A IRES is highly activated are associated with the binding of multiple proteins to the utrophin A 5'-UTR.

eEF1A2 interacts with the utrophin A 5'-UTR

Various RNA-binding proteins are involved in cellular IRES-mediated translation; however, each particular IRES appears to be regulated by a distinct set of trans-factors, and no single universal factor has been shown to be necessary for cap-independent translation of all IRES. An RNA affinity chromatography approach (17,18) was employed to isolate and identify proteins that interact with the utrophin A 5'-UTR. For these experiments, we used a biotinylated RNA probe containing the same region of the utrophin A 5'-UTR found to associate with several proteins in regenerating skeletal muscles (nucleotides 147–363, Fig. 3A). The probe was linked to avidin-agarose beads and incubated with regenerating skeletal muscle protein lysates. After extensive washing, SDS–PAGE was performed. Analysis of the SYPRO ruby-stained gel revealed the presence of a band, migrating at 50 kDa, which was only present in the sample containing the 5'-UTR probe and not in the sample lacking the biotinylated RNA (Fig. 3B). The band was excised and analyzed by MALDI-TOF, which identified the protein to be eukaryotic elongation factor 1A2 (eEF1A2).

To confirm that the 50 kDa protein was indeed eEF1A2, we performed additional RNA affinity chromatography experiments, transferred the denatured proteins to a PVDF membrane and performed western analysis using an anti-eEF1A antibody that recognizes both eEF1A1 and eEF1A2. Western analysis revealed the presence of a band at the expected molecular mass in samples that were incubated with the utrophin A 5'-UTR probe (Fig. 3C). We verified that this interaction between the utrophin A 5'-UTR and eEF1A protein(s) was not caused by non-specific binding to RNA by performing additional experiments in which a non-related RNA probe was used (Fig. 3C). We also performed the chromatography
experiment using lysates from HEK293T cells. Association of the probe to eEF1A was not detected using these lysates (Fig. 3D). Since HEK293T cells express eEF1A1 and not, or very limited amounts of, eEF1A2 (12-fold difference as determined by qRT–PCR), these experiments provide evidence that eEF1A2 is a factor that interacts with the utrophin A5'0-UTR.

Regions of the utrophin A 5'-UTR that contain IRES activity also bind eEF1A2

In order to delineate the regions of the utrophin A 5'-UTR that interact with eEF1A2, purified eEF1A2 was incubated with radiolabeled RNA probes containing various truncations of the utrophin A 5'-UTR and then cross-linked by UV radiation. A specific interaction was identified for probes 1–228 and 147–363, but not 1–70, as indicated by the presence of a shifted complex (Fig. 4). This interaction was specific since the shifted complexes detected using the 1–228 and 147–363 probes could be competed away by the addition of cold probe.

To determine whether the regions of the utrophin A 5'-UTR that bind to eEF1A2 also harbor IRES activity, we performed transient transfections of utrophin A 5'-UTR bicistronic reporters or an empty vector control in C2C12 myoblasts. The full-length utrophin A 5'-UTR displayed ~14-fold IRES activity (normalized to empty vector control; Fig. 5A). The 1–228 region showed 59% of the activity of the full-length utrophin A 5'-UTR and the 147–363 region showed 25% of full-length activity (8.3-fold for 1–228, 3.4-fold for 147–363 above empty vector control levels; P < 0.05). In contrast, the 1–70 regions showed no significant IRES activity over control levels (P > 0.05). These experiments demonstrate that regions of the utrophin A 5'-UTR that bind to eEF1A2 are the same regions able to drive cap-independent translation in C2C12 myoblasts.

eEF1A2 enhances utrophin A IRES activity and its expression pattern correlates with IRES activity in transgenic mice

To ascertain whether eEF1A2 can directly regulate utrophin A IRES activity, we attempted to knockdown eEF1A2 in C2C12 cells by using siRNA and shRNA based protocols. Due to the abundant expression of eEF1A2 in C2C12 cells, we were unable to achieve significant knockdown of this protein (data not shown). As an alternative approach, we obtained a rat fibroblast cell line that lacks eEF1A2, and one that is stably transfected to overexpress eEF1A2 (19). Transient transfection experiments revealed that the utrophin A IRES had ~1.4-fold greater activity in the cells expressing eEF1A2 (1A2) compared with controls lacking eEF1A2 (CTL; P < 0.05; Fig. 5B).

Figure 2. Individual levels of bGAL and CAT reporter activity in utrophin A 5'-UTR and control bicistronic reporter transgenic mice. (A–C) Relative bGAL activity in transgenic mice from transgenic lines 863, 876 and control normalized to reporter levels in brain. (D–F) Relative CAT activity in transgenic mice from transgenic lines 863, 876 and control normalized to reporter levels in brain. Note that CAT activity is found at high levels only in skeletal muscles (TA and Gastroc) in both 863 and 876 lines and that no tissue of the control line displays CAT activity, although bGAL activity can be detected. *P < 0.05, relative to brain. Control line, n = 4; 876 line, n = 5; 863 line, n = 6.
To assess whether eEF1A2 expression correlates with utrophin A IRES activity in tissues of the utrophin A 5'-UTR reporter transgenic mice, we examined mRNA levels of eEF1A2 and eEF1A1 in various tissues by real-time quantitative RT-PCR. This technique was used because antibodies that distinguish between the two isoforms are not available. In accordance with previous studies (20,21), we found that eEF1A2 was expressed in skeletal muscle, heart and brain, but not kidney, lung and liver (Fig. 5C). Examination of the relative abundance of eEF1A2 to eEF1A1 revealed that although eEF1A2 is expressed in heart and brain (tissues that do not contain utrophin A IRES activity), there are higher levels of eEF1A1 in these organs compared with skeletal muscle, which contains nearly undetectable levels of eEF1A1 (Fig. 5C). The relative abundance of the two eEF1A isoforms shown as a ratio in Table 1 reveals that skeletal muscles contain ~2- and 6-fold the ratio of eEF1A2 to eEF1A1 when compared with heart and brain tissues, respectively.

**DISCUSSION**

In recent work, we have shown that the utrophin A 5'-UTR contains an IRES that is activated in response to a variety of conditions (11,14). Here, we analyzed transgenic mice harboring utrophin A 5'-UTR reporter constructs to determine whether the activity of the utrophin A IRES is tissue specific. Interestingly, we found that the utrophin A 5'-UTR can direct cap-independent translation exclusively in skeletal muscles, and no other tissues. This expression pattern suggested that factors specifically expressed or activated in skeletal muscle could be important in mediating utrophin A IRES activity. In accordance with this prediction, RNA-affinity chromatography experiments identified eEF1A2, the isoform of eEF1A1 expressed in mature skeletal muscle, as a factor that interacts with the utrophin A 5'-UTR. Additional binding experiments and reporter activity assays demonstrated the importance of this interaction in mediating utrophin A IRES activity.

Utrophin A is expressed in a variety of different organs and tissues, including lungs, kidney, brain, heart and skeletal muscle (6,7,22). At the transcriptional level, cis-elements in the promoter region direct expression of utrophin A to multiple tissues, and an intrinsic enhancer appears to contribute to utrophin A mRNA expression in heart and skeletal muscle (23–25). In contrast to the wide expression pattern of utrophin A mRNA and protein, here we found utrophin A IRES activity only in skeletal muscles. Although this observation does not rule out the involvement of translational control mechanisms in regulating utrophin A in other tissues, it does strongly suggest that translational regulation of utrophin A by its 5'-UTR is particularly important in skeletal muscle. Indeed, we have already uncovered that, like several other IRES regulated transcripts, utrophin A cap-independent translation can be driven under conditions of stress where the overall levels of cap-dependent translation are reduced (11,13,14). Given these results, we predict that in skeletal muscle, additional physiologically important ‘stress’ stimuli might target the utrophin A 5'-UTR to allow for the rapid synthesis of protein from a pre-existing pool of utrophin A transcripts.

It is well established that utrophin A is more highly expressed in slow-oxidative muscles, such as the soleus, compared with fast-glycolytic muscles, such as the extensor digitorum longus (EDL) (8,9). Interestingly, we observed that IRES-activity in line 876 was 6.8-fold greater in the more fast-glycolytic EDL muscles when compared with soleus muscles ($P < 0.05$). A similar trend was observed in line 863; however, the difference was not statistically significant. These observations suggest that utrophin A IRES activity is greater in muscle fibers expressing fast, type II myosin heavy chain isoforms. Future experiments using bicistronic vectors harboring fluorescent protein reporters will be necessary to confirm whether there are clear fiber type differences in utrophin A IRES activity. This question could not be addressed using our transgenic mice since antibodies do not reliably detect the CAT reporter by indirect immunofluorescence. Fluorescent bicistronic reporters will also be useful to investigate the subcellular localization of IRES activity within skeletal muscles; in particular to determine whether IRES-activity is enhanced at the neuromuscular junction.

A limited number of studies have examined the relevance of cellular IRES elements in vivo. Studies performed on *Drosophila* harboring bicistronic reporters for ultrabithorax and antennapedia 5'-UTRs display spatial and temporal regulation of IRES activity (26). Mice harboring a c-myc IRES reporter
display high activity in embryonic tissues, but low or undetectable activity in adult tissues (27). Several studies have examined activity of the FGF-2 IRES in transgenic mice. IRES-activity in these mice is regulated in response to hyperglycemia and aging (28,29), and activity is particularly high in the brain, with certain structures displaying more activity than others (15,30). Interestingly, a factor highly expressed in brain, hnRNP A1, is important for FGF-2 IRES activity (31). From these limited number of studies, it appears that cellular IRES elements are highly regulated in a tissue-specific manner, suggesting an important role for trans-factors that have tightly controlled expression patterns.

Since the utrophin A 5’-UTR drives IRES-mediated translation only in skeletal muscles, it is likely that the complex of proteins that permits this type of translation is present or active exclusively in skeletal muscles. We provide evidence suggesting that one of the factors is eEF1A2. The two eEF1A isoforms are eEF1A1 and eEF1A2. These share 92% sequence identity and appear to be functionally equivalent with regard to their effects on protein synthesis activity in vitro (32). Although the canonical role for eEF1A isoforms is in shuttling aminoacyl-tRNA during translation elongation, distinct ‘moonlighting’ roles for these proteins have also been identified (33). eEF1A1 is important in regulating cytoskeletal
organization (34), protein nuclear export (35) and mRNA stability (36,37). In contrast, eEF1A2 protects against apoptosis (38,39), and plays a role in actin remodeling and oncogenesis (19,40). Distinct roles for these two proteins are also suggested by their tissue specific expression pattern. Although eEF1A1 is expressed almost ubiquitously, eEF1A2 is expressed in skeletal muscle, heart and brain (20,41). Interestingly, mice lacking functional eEF1A2 (wasted mice, wst/wst) exhibit muscle wasting and motor neuron degeneration resulting in premature death (21,42).

It could be expected that since eEF1A2 is expressed in skeletal muscle, heart and brain, utrophin A 5' UTR reporter transgenic mice would show IRES activity in all these tissues. One explanation for the lack of IRES activity in brain and heart tissue can be inferred from the greater ratio of eEF1A2 to eEF1A1 found in skeletal muscle compared with heart and brain (Fig. 5C and Table 1). In tissues that express both isoforms, competition for binding to the utrophin A 5' UTR may occur, with eEF1A1 opposing the positive effect of eEF1A2 on IRES-mediated translation. On the other hand, skeletal muscle-specific accessory factors that bind eEF1A2 (for example (43)), might be needed for IRES-mediated translation to occur. Alternatively, eEF1A2 might be only one of several proteins needed for skeletal muscle-specific utrophin A IRES activity. On the basis of expression pattern, it seems that eEF1A2 is not responsible for the enhancement of utrophin A IRES activity during muscle regeneration, since it is not upregulated after myotoxin injection (Miura et al., unpublished observations; (44)). Thus, additional factors or protein modifications are likely required for enhancement of utrophin A IRES activity under various stress conditions.

It is unknown how eEF1A2 is involved in the mechanism of utrophin A IRES-mediated translation and what additional factors are required. Given the ability of eEF1A2 to bind aminoacyl-tRNA, and the presence of tRNA like structures in several viral IRES elements (45,46), we hypothesized that the protein might interact with structural elements in the utrophin A 5' UTR that resemble tRNA; however, using the bioinformatic analysis of Baird et al. (12), we could detect no such structural elements. Cricket paralysis virus and hepatitis C virus both contain tRNA-like structures and can interestingly be translated via their IRES elements in an in vitro system that contains ribosomes, tRNA and elongation factors, but lacks translation initiation factors (47,48). In light of our findings presented here, one could speculate that eEF1A2 plays a role in translation initiation for these viruses. Further investigation into regulation of IRES-mediated translation by eEF1A2 may provide insights into the mechanistic commonalities of translation initiation of viral and cellular IRESes.

Several groups, including our own, have been performing pre-clinical screening of small molecules for their ability to enhance activity of the utrophin A promoter in hopes of finding an effective drug treatment for DMD. Our results presented here, combined with previous work demonstrating that glucocorticoid treatment can stimulate utrophin A translation via its 5'-UTR (14), provide a rationale to screen for compounds that can activate expression of utrophin A at the translational level. In this regard, utrophin A 5'-UTR reporter transgenic mice could serve as a useful tool to validate promising compounds in vivo. Given the skeletal-muscle specific pattern of utrophin A IRES activity, therapeutics that target the utrophin A 5'-UTR would have the advantage of stimulating protein expression specifically in skeletal muscles and no other tissues.

### MATERIALS AND METHODS

#### Cell culture and transfections

C2C12 cells were cultured under standard conditions (11). Rat2 cells and Rat2 cells overexpressing eEF1A2 were cultured as previously described (19). The bicistronic reporter constructs containing various truncations of the utrophin A 5'-UTR were transiently transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as previously described (14).

#### Bicistronic transgenic mice

Six- to 8-week-old C6B3F1 mice were purchased from Charles River Laboratories (Boston, MA, USA). They were cared for in approval with the University of Ottawa Animal Care and Use Committee who is compliant with the Guidelines of the Canadian Council on Animal Care and the Animals for Research Act. The bicistronic vectors, βGal/UtrA/CAT and pβGal/CAT were digested with SalI and ApaI restriction enzymes to generate 6528 and 6020 nucleotide products, respectively. These fragments included the CMV promoter and both reporter genes. Products were electrophoresed on 1% agarose gels containing ethidium bromide. Bands were excised and purified using QiaEXI gel extraction kit (Qiagen, Chatsworth, CA, USA). To generate transgenic mice, hybrid C6B3F1 mice were used as donors for fertilized one-cell embryos. CMV/βGal/UtrA/CAT and CMV/βGal/CAT DNA fragments were separately microinjected into the pronucleus of donor embryos. Pseudopregnant females were used as recipients for the modified zygotes. Potential founders were weaned at 3 weeks after birth. Tail biopsies were collected, minced and incubated overnight in proteinase K (20 mg/ml) at 55 °C. Genomic DNA was extracted by a standard phenol/chloroform/isoamyl alcohol extraction and subjected to PCR genotyping. PCR was performed using a 5' primer spanning 142 nt of the Lacz gene and a 3' primer spanning 459 nt of the CAT gene (5'-TTTTT CCCGATTTGGCTACA-3'; 5'-TGAAAATCTCACCCAGGG).
ATTG-3'). PCR products were visualized on 2% agarose gel containing ethidium bromide. Founders were bred with C6B3F1 wild-type mice and their progeny were examined for transgene expression using βGAL assay. The three CMV/βGAL/Utra/CAT (876, 881 and 863) and one CMV/βGAL/CAT transgenic lines were backcrossed with C6B3F1 wild-type mice for several generations.

**Reporter activity assays**

Various tissues were excised from transgenic mice and immediately frozen in liquid nitrogen after euthanizing the mice with CO2. For reporter assays, protein from tissues was extracted with reporter lysis buffer (Promega) as previously described (11) and protein concentration was quantified by Bradford Standard Assay. Protein samples were diluted to a final concentration of 4 μg/μl prior to reporter assays. βGAL enzymatic assays were performed using the βGAL enzyme assay system as recommended by the manufacturer (Promega). To measure CAT activity, we analyzed the conversion of chloramphenicol to butyryl chloramphenicol by incorporation of [14C] butyryl coenzyme A (11). Back-conversion of chloramphenicol to butyryl chloramphenicol (Promega). To measure CAT activity, we analyzed the conversion of chloramphenicol to butyryl chloramphenicol by incorporation of [14C] butyryl coenzyme A (11). Background levels for both reporter assays were determined by analyzing reporter activity in tissues from mice not harboring a transgene.

**RNA extraction and qRT–PCR analysis**

Total RNA was isolated from tissues of CMV/βGAL/Utra/CAT and CMV/βGAL/CAT transgenic mice using TRIzol reagent (Invitrogen) as recommended by the manufacturer. TRIzol extraction was followed by a one hour DNAse I (Invitrogen) treatment to eliminate possible plasmid and genomic DNA contamination. To control for the presence of an intact bicistronic reporter transcript in CMV/βGAL/Utra/CAT transgenic mice, qRT–PCR was performed with previously described βGAL and CAT primers (11). Negative controls consisted of an RT mixture that had the reverse transcriptase replaced by sterile water. Quantitative real-time RT–PCR was performed on reverse transcribed RNA using QuantiTect SYBR green PCR kit (Qiagen) on a Stratagene MX3005p, and samples were separated on Tris-glycine-polyacrylamide gels. Desalted eEF1A2 protein (100 ng) and 50 000 cpm of RNA probe were transcribed from PCR templates containing T7 RNA polymerase binding site using the MAXIScript in vitro transcription kit (Ambion, Austin, TX, USA), and [α-32P]UTP (800 Ci/mmol; Perkin Elmer, Boston, MA, USA). For northwestern analysis, 60 μg of cardiotoxin-treated TA muscle extract was ran on a 10% SDS–PAGE gel and transferred to PVDF membrane. Equal loading was confirmed by staining with Ponceau S. Membranes were presoaked, incubated with the 1 × 106 cpm [α-32P]UTP labeled utrophin A 5′-UTR RNA probes and washed as previously described (51). Binding of the RNA probe to proteins was visualized by autoradiography. For UV-crosslinking experiments, recombinant eEF1A2 was produced as previously described (52), desalted with a Sepharose G25 column (Sigma, USA) and quantified by BCA kit (Pierce, Rockford, IL, USA). MEGA-Short Script kit (Ambion, Austin, TX, USA) was used to produce non-radiolabeled RNA probes. Unincorporated nucleotides were removed by Sepharose G25 column and purified by electrophoresis on 6% Tris-glycine-polyacrylamide gels. Desalted eEF1A2 protein (100 ng) and 50 000 cpm of RNA probe were pre-incubated for 30 min at room temperature, in UV-binding buffer (10 mM Tris–HCl pH 7.5, 50 mM KCl, 10% glycerol, 5 mM DTT and 10 ng/μl of yeast tRNA). For competition experiments, non-radiolabeled RNA was added 15 min prior to the end of the pre-incubation step. Samples were then exposed to 254 nm UV light for 30 min at 4°C and resuspended in RNA loading buffer. Samples were separated on Tris-glycine-polyacrylamide gels using 4% w/v acrylamide/bis-acrylamide (19:1). Following electrophoresis, the gels were dried and subjected to autoradiography.

**RNA binding experiments**

Cardiotoxin-treatment of C57BL/10 mice was performed as previously described (11) and TA muscles were excised 7 days after toxin injection. Isolation of proteins binding the utrophin A 5′-UTR was performed using an RNA-affinity chromatography protocol (18). For these experiments, 60 μg of biotinylated RNA was conjugated to avidin-agarose beads and incubated with 4 mg of protein extract from regenerating TA muscle extracts. In-gel trypsin digestion and mass peptide fingerprinting was performed at the Protein Function Discovery Centre (Queen’s University, Kingston, ON, Canada). To confirm eEF1A2 as an interacting protein, we performed RNA-affinity chromatography using 15 μg of biotinylated RNA and 1.6 mg of TA muscle or HEK293T cell extracts, transferred proteins from the gel to a PVDF membrane, and performed western blot using an eEF1A antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA probes were transcribed from PCR templates containing T7 RNA polymerase binding site using the MAXIScript in vitro transcription kit (Ambion, Austin, TX, USA), and [α-32P]UTP (800 Ci/mmol; Perkin Elmer, Boston, MA, USA). For northwestern analysis, 60 μg of cardiotoxin-treated TA muscle extract was ran on a 10% SDS–PAGE gel and transferred to PVDF membrane. Equal loading was confirmed by staining with Ponceau S. Membranes were presoaked, incubated with the 1 × 106 cpm [α-32P]UTP labeled utrophin A 5′-UTR RNA probes and washed as previously described (51). Binding of the RNA probe to proteins was visualized by autoradiography. For UV-crosslinking experiments, recombinant eEF1A2 was produced as previously described (52), desalted with a Sepharose G25 column (Sigma, USA) and quantified by BCA kit (Pierce, Rockford, IL, USA). MEGA-Short Script kit (Ambion, Austin, TX, USA) was used to produce non-radiolabeled RNA probes. Unincorporated nucleotides were removed by Sepharose G25 column and purified by electrophoresis on 6% Tris-glycine-polyacrylamide gels. Desalted eEF1A2 protein (100 ng) and 50 000 cpm of RNA probe were pre-incubated for 30 min at room temperature, in UV-binding buffer (10 mM Tris–HCl pH 7.5, 50 mM KCl, 10% glycerol, 5 mM DTT and 10 ng/μl of yeast tRNA). For competition experiments, non-radiolabeled RNA was added 15 min prior to the end of the pre-incubation step. Samples were then exposed to 254 nm UV light for 30 min at 4°C and resuspended in RNA loading buffer. Samples were separated on Tris-glycine-polyacrylamide gels using 4% w/v acrylamide/bis-acrylamide (19:1). Following electrophoresis, the gels were dried and subjected to autoradiography.

**Statistical analysis**

For transgenic mice reporter activity analysis, one-tailed t-tests were used to determine statistical significance. For all other experiments, two tailed t-tests were used. Level of significance was set at P < 0.05.

**ACKNOWLEDGEMENTS**

We would like to thank Amanda Shaver for assistance in breeding mice and genotyping. We also thank John A. Lunde, Steven Lewis, Tyson Graber and Dixie Pinke for technical assistance and Dr S. Lee (University of Ottawa) for providing the anti-EF1A antibody.

Conflict of Interest statement. None declared.


