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The MEF2A 3′ Untranslated Region Functions as a *cis*-Acting Translational Repressor

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Myocyte enhancer factor 2 (MEF2) proteins serve as important muscle transcription factors. In addition, MEF2 proteins have been shown to potentiate the activity of other cell-type-specific transcription factors found in muscle and brain tissue. While transcripts for MEF2 factors are widely expressed in a variety of cells and tissues, MEF2 proteins and binding activity are largely restricted to skeletal, smooth, and cardiac muscle and to brain. This disparity between MEF2 protein and mRNA expression suggests that translational control may play an important role in regulating MEF2 expression. In an effort to identify sequences within the MEF2A message which control translation, we isolated the mouse MEF2A 3′ untranslated region (UTR) and fused it to the chloramphenicol acetyltransferase (CAT) reporter gene. Here, we show by CAT assay that the MEF2A 3′ UTR dramatically inhibits CAT gene expression in vivo and that this inhibition is due to an internal region within the highly conserved 3′ UTR. RNase protection analyses demonstrated that the steady-state level of CAT mRNA produced in vivo was not affected by fusion of the MEF2A 3′ UTR, indicating that the inhibition of CAT activity resulted from translational repression. Furthermore, fusion of the MEF2A 3′ UTR to CAT inhibited translation in vitro in rabbit reticulocyte lysates. We also show that the translational repression mediated by the 3′ UTR of MEF2A is regulated during muscle cell differentiation. As muscle cells in culture differentiate, the translational inhibition caused by the MEF2A 3′ UTR is relaxed. These results demonstrate that the MEF2A 3′ UTR functions as a *cis*-acting translational repressor both in vitro and in vivo and suggest that this repression may contribute to the tissue-restricted expression and binding activity of MEF2A.

In recent years, it has become increasingly clear that posttranscriptional control is an important mechanism for regulating gene expression. The elements governing this control are usually present in the untranslated regions (UTRs) of the mRNA (9, 25, 33). Posttranscriptional control elements can occur in either the 5′ or the 3′ UTR and may help to regulate gene expression by altering mRNA stability or by influencing the translational efficiency of the message. The majority of posttranscriptional control mechanisms that have been described previously result in increased mRNA degradation or inhibition of translation (9, 25, 33). Often, the *cis*-acting elements which negatively influence the translation of eukaryotic mRNAs are present in the 3′ UTR of the transcript and function in a variety of ways to inhibit translation (9, 16, 25, 34).

The myocyte enhancer factor 2 (MEF2) family of transcription factors has been shown to play a critical role in the activation of muscle-specific gene transcription in skeletal, cardiac, and smooth muscle cells (28). In addition, MEF2 factors recently have been implicated in cell-type-specific transcription in brain and neural cells (3, 18, 21, 22). There are four vertebrate *mef2* genes, *mef2a*, *-b*, *-c*, and *-d* (5, 19, 23, 24, 26, 29, 40), whose products bind as homo- and heterodimers to an AT-rich DNA consensus sequence associated with many muscle-specific genes (12). At least one of the MEF2 factors, MEF2A, appears to be controlled at the level of translation (36, 40). MEF2A transcripts are expressed in a wide array of tissues, while MEF2A protein appears to be considerably more restricted, being abundant in skeletal muscle, heart, and brain tissues (40). Furthermore, MEF2 binding activity is restricted to the same tissues where the protein is abundant (12, 40). More recently, it was shown that MEF2A protein levels increased upon serum stimulation of vascular smooth muscle cells without changes in mRNA levels or protein stability, indicating that the regulation of MEF2A expression was at the level of translation in those cells (36).

Based on these observations, which indicated that MEF2A was, at least in part, translationally controlled, we sought to identify the sequences within the MEF2A message that were involved in this regulation. Here, we show that sequences within the 3′ UTR of MEF2A function as a *cis*-acting translational repressor in vivo and in vitro. Our results also show that the translational repression mediated by the 3′ UTR of MEF2A is regulated during muscle differentiation and suggest that sequences within the 3′ UTR of MEF2A may help to mediate the tissue-restricted expression and binding activity of MEF2A.

MATERIALS AND METHODS

Isolation of the MEF2A 3′ UTR. To isolate MEF2A cDNA clones containing the 3′ UTR, a mouse embryo cDNA library generated by random priming was screened by hybridization with a 3′ coding region cDNA probe from human MEF2A (40). Several positive clones were isolated, sequenced, and purified by standard techniques (32). One clone which contained 110 bp of coding region and approximately 600 bp of mouse MEF2A 3′ UTR was then used to screen an oligo(dT)-primed mouse embryo cDNA library to obtain the remainder of the 3′ UTR cDNA. Several positive clones were again isolated, sequenced, and purified. The PCR technique of gene splicing by overlap extension was then used to splice together two overlapping cDNA clones corresponding to the complete mouse MEF2A 3′ UTR (13). No isolated cDNA clones contained sequence extending more than 1,016 bp beyond the translational stop codon.

Plasmids, deletion constructs, and sequence analysis. 3′ UTR deletion constructs were generated by using PCR primers to amplify fragments corresponding to the indicated nucleotides. The PCR primers used contained restriction enzyme clamps to facilitate subsequent cloning steps. Each of the constructs was sequenced on both strands with an ABI 373 automated DNA sequencer to confirm that the intended deletions were correct and that no unintentional mutations were introduced by the PCR. Each of the MEF2A 3′ UTR cDNA fragments, including the full-length fragment, was cloned into plasmid pCDNA3.CAT

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(Invitrogen) as a *Bam*HI-*Not*I fragment immediately 3′ of the chloramphenicol acetyltransferase (CAT) gene. The beta-globin (β-glo) 3′ UTR used in these studies is from *Xenopus laevis* and was subcloned as a *Bgl*II-*Bam*HI fragment from plasmid pT7TS (kindly provided by Andy Johnson and Paul Krieg) into the *Bam*HI site in pCDNA3.CAT. The CAT gene is cloned into the *Hin*dIII site of plasmid pCDNA3 (Invitrogen) and is transcribed under control of the constitutively active cytomegalovirus immediate-early promoter. Plasmid pCDNA3.CAT also encodes the neomycin resistance gene (*neo*) under the control of the simian virus 40 early promoter. The *neo* gene serves as an internal control for plasmid-mediated expression. Plasmid pCDNA3.CAT also contains the promoter for bacteriophage T7 RNA polymerase immediately upstream of the CAT gene, which can be used for in vitro transcription of CAT message plus any added UTR sequences. The influence of 3′ UTR sequences on CAT expression in vivo was examined by a CAT assay and also by in vitro transcription and translation with T7 RNA polymerase.

Cell culture and transfections. Baby hamster kidney (BHK21) cells were maintained in Dulbecco's modification of minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) as described previously (2). C2C12 (C2), L6, and Sol8 myoblasts were maintained in the undifferentiated state in DMEM supplemented with 15% FCS and were differentiated in DMEM supplemented with 2% horse serum. Myoblasts were differentiated for 3 to 5 days in differentiation medium. Transfections of BHK21 cells were performed either by lipofection with the Lipofectin reagent (Gibco/BRL) or by calcium phosphate precipitation. Transfections of all three muscle cell lines were performed by calcium phosphate precipitation. Lipofections were performed as described elsewhere (1). Briefly, 60-mm dishes were seeded at 20% confluence in DMEM plus 10% FCS and without antibiotics 16 h prior to transfection. Cells were then rinsed once with DMEM without serum, and 3 μg of plasmid DNA and 18 μg of Lipofectin reagent were added to the monolayer in 1 ml of serum-free medium. The cells were then incubated for 5 h at 37°C. Following this incubation, 2 ml of DMEM plus 15% FCS was added to the cells, which were incubated for an additional 24 h before harvesting. Transfection by calcium phosphate precipitation was also performed as previously described (2). Cells were maintained in 9 ml of DMEM plus 10% FCS (BHK21), 15% FCS (C2 undifferentiated), or 2% horse serum (C2, L6, and Sol8 differentiated) and without antibiotics 16 h prior to transfection in 100-mm tissue culture dishes. In each transfection, 10 μg of plasmid DNA was transfected by mixing it with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2× BBS and adding this mixture to the cells (2× BBS is 50 mM BES, 250 mM NaCl, and 1.5 mM Na_2HPO_4, pH 6.95). The cells were then incubated for 16 h, washed once with phosphate-buffered saline, and incubated for 24 h in the appropriate medium before harvesting.

CAT assays. Transfected cells were harvested, and cellular extracts were prepared by three freeze-thaw cycles and heat inactivation as described previously (1). Cell lysates were then quantitated for total protein (20), and an equivalent amount of cell lysate (normalized for total protein) from each transfection was assayed for CAT activity as described previously (1). Reactions were conducted for 5 h at 37°C. Conversion to acetylated forms was analyzed by thin-layer chromatography and quantitated by PhosphorImager (Molecular Dynamics, Inc.) analysis.

In vitro transcription and translation. The influence of the MEF2A 3′ UTR on gene expression in vitro was examined with uncoupled transcription and translation reactions. Capped, in vitro-transcribed mRNAs were synthesized by linearizing pCDNA3.CAT plasmids containing no UTR, the MEF2A 3′ UTR, or the β-glo 3′ UTR with *Not*I and then transcribing in vitro with T7 RNA polymerase and the cap analog, 5′7meGppp5′G, with the mCAP kit (Stratagene) according to the manufacturer's recommendations. Transcripts were electrophoresed in a 1.4% agarose-formaldehyde gel by standard techniques (32) and stained with ethidium bromide to confirm that each transcript was full-length and was not degraded. Following purification, RNAs were quantitated by absorbance at 260 nm, and an equal number of moles of each capped transcript was added to translation reaction mixtures. By an in vitro translation system, 1.5 pmol of each full-length transcript was added to 10-μl reaction mixtures containing 50% rabbit reticulocyte lysate in the presence of [^{35}S]methionine for 90 min at 30°C as specified by the manufacturer (Promega). In some experiments, total RNA synthesized from plasmid pCDNA3 without a cDNA insert or yeast RNA was added in excess to make the total mass of RNA in each translation reaction mixture equal. No differences were seen regardless of whether filler RNA was used.

The stability of transcripts in vitro was determined by conducting in vitro transcription reactions in the presence of [α-^{32}P]rUTP and then conducting in vitro translation reactions exactly as described above except that no radiolabeled amino acids were included in the reaction mixtures. Two-microliter aliquots were removed from the cold translation reactions after 0, 30, 60, and 90 min. The aliquots were then mixed with RNA loading buffer containing 95% formamide, heated at 75°C for 5 min, and electrophoresed in 4% acrylamide–7 M urea gels. The number of radiolabeled counts of each full-length transcript for each of the constructs tested was then quantitated by PhosphorImager (Molecular Dynamics, Inc.) analysis.

RNase protection analysis (RPA). Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol method with the Trizol reagent (Gibco-BRL) according to the manufacturer's recommendations. RPA was performed with 20 μg of total RNA which was hybridized to radiolabeled CAT and *neo* probes in the same RPA reaction. Reactions and subsequent RNase digestions were conducted with the Ambion RPA II kit (Ambion, Austin, Tex.) according to the manufacturer's recommendations. Antisense CAT and *neo* probes were generated by cloning fragments of each cDNA into plasmid pBluescript SK II (+) (Stratagene). The CAT fragment used corresponds to 250 nucleotides (nt) from the 5′ end of the cDNA to the internal *Eco*RI site and was cloned as a *Hin*dIII-*Eco*RI fragment. The *neo* fragment used corresponds to 200 nt from the 5′ end of the *neo* cDNA and was cloned as a *Pst*I fragment. In both cases, the plasmids were linearized with *Xho*I and were transcribed in vitro with T3 RNA polymerase (Ambion) in the presence of [α-^{32}P]UTP (800 Ci/mmol; Amersham). In some experiments, we also used an antisense 18S rRNA probe or an antisense fragment corresponding to nt 417 to 844 of the MEF2A 3′ UTR as additional controls. Full-length riboprobes were gel purified in a 6% polyacrylamide–7 M urea gel and eluted overnight in elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.25% sodium dodecyl sulfate [SDS]). Following elution, 1.5×10^5 cpm of each probe was used in each protection reaction, and RPA reaction mixtures were hybridized at 42°C overnight and were RNase digested. Protected fragments were resolved in a 6% polyacrylamide–7 M urea gel which was subjected to autoradiography. The amount of radioactivity present in each protected band was quantitated by PhosphorImager (Molecular Dynamics, Inc.) analysis.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence reported in this paper is U94423.

RESULTS

The 3′ UTR of MEF2A is highly conserved. Initially, we suspected that elements involved in posttranscriptional control of MEF2A might be present in the 3′ UTR since expression plasmids that included portions of the 3′ UTR were less efficient in *trans*-activating a MEF2-dependent reporter than clones containing the MEF2A protein-encoding region only (4). To begin to define a possible regulatory role for the 3′ UTR of MEF2A, we isolated and sequenced MEF2A cDNA clones containing a polyadenylated 3′ UTR. The cDNA sequence of the 3′ end of the mouse MEF2A message is shown in Fig. 1. The 3′ UTR was 1,016 bp in length and, like many 3′ UTRs, was very A+U-rich (66% A+U). We believe that the UTR which we isolated was full-length since it contained a poly(A) tail and since no cDNAs containing more than 1,016 bp of 3′ UTR were isolated in our screen. However, the possibility remains that longer 3′ UTRs could exist, possibly as a result of differential splicing or differential polyadenylation. The human MEF2A 3′ UTR has been reported to be greater than 3,500 nt in length (37). No mouse 3′ UTRs of that size were isolated for the mouse MEF2A gene. Such differences in the lengths of the 3′ UTRs may account for the smaller size of mouse MEF2A message relative to the human message when analyzed by Northern blot analysis (4, 40). Shorter 3′ UTRs might also exist in vivo, as there are several putative consensus (AATAAA) or near-consensus (ATTAAA) nuclear polyadenylation sites in the 3′ UTR, but we have no evidence that these putative sites are utilized with high frequency. This may not be surprising since some mRNAs and, in some cases, those associated with tissue-specific factors utilize noncanonical polyadenylation signals (39). Additionally, the mouse MEF2A 3′ UTR contained several repeated motifs which were similar to A+U-rich elements (AREs) (16) and four long sequences which were greater than 83% A+U (Fig. 1). The mouse MEF2A 3′ UTR had very high homology to the 3′ UTR of human MEF2A (37, 40). Mouse and human MEF2As have 91% nucleotide sequence homology in the highly conserved MADS and MEF2 domains which define the MEF2 family. Outside the MADS and MEF2 domains, the homology is approximately 74% over the entirety of the coding region while the homology between human and mouse MEF2As over the entire length of the mouse 3′ UTR was greater than 91%. By comparison, the homology between mouse and human clones in the 3′ UTR of the highly conserved transcription factor MyoD was only 65%. The mouse and *Xenopus* MEF2A cDNAs also have a high degree of homology within the 3′ UTR (7).

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   1  GGCTTCCTGG TTCATGTTTG TTCTTTGTGT TACTGCAGTG AACTGCCCTA
  51  CATATCTTAA ATTGGTGAAT AAGGACATGA GTTCAATATA TTTATATGTA
 101  CATGCATACA TATATATCCC TTTACATATA TATATGTATG TTGGTGTGAG
 151  TGTGTGTGTA TGTGTGGGTG TGTGTTGCAT ACACAAAATC AGGCACTTAC
 201  TGCAAACTCC TTGTAGGTCT GCAGATGTGT GTCCACATGG CAGACAAAGC
 251  ACCCTGTAGA AACAGACCAG TCTGGCACTT CCTTGGACTA CTTGTTTCGT
 301  AAGATAAAGT TTTGCAGAGA AACGTGTACC CATATATAAT TCTTCCACAT
 351  TAGCCTGCAG AAACCTAGGG GCCCCCTATT TGATTTTATT TAACCGTGCG
 401  GTGACTGTAG TTACTTCAGA AAACAAATGC TTTGTAGGGC AGAGCAGTAG
 451  AAAAGCAGGA ACCAAGAAAG CAATACTGTA CATAAAATGT CATTTATATT
 501  AATAACCCAA CTTGGCATGG GTCTATTGCA AAGGGGTGCA TGGAAAAGGG
 551  CTGTTGATAT TAAACAAACA AACAATAAAC AAAAAGCCCC ACACATAACT
 601  GTTTTGCACG TGCAAAAAAA ATGTATTGGG TCAAAGAAGT GATTTTTAGC
 651  TATTAAAAAG AGAGAATAGA AAACACGCAT GAAATATTCA GAAATACTAG
 701  CCTAGAAATA TAGAGCATTA ACAAAATAAA ATTAATATAT TAAGTTATAA
 751  TTGGAATATG TCAGAAGTTT CTTTTACATT CATATCTTTA AAAATTAAAG
 801  AAACTGATTT TAGGTCATGT ATATTTTATA AGAAAGAAAA CACCCTTAAT
 851  GAATTGATGA CTATATATAA AATTATATCC ACTACTCGTG AACACATTCT
 901  GCTGAATTAT TTATATAAGC CAAAGCTGTA TGTTGTAACT TTTTTTTAAA
 951  GAATAGCTTT ATCTTGTTTT AACTTTTTAG TTTTATTTTA AGAGGGGAGA
1001  AAAAAAAAAA AAAAAA
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FIG. 1. Nucleotide sequence of the 3′ UTR of the mouse MEF2A cDNA. Shown is the cDNA sequence of the 1,016-bp 3′ UTR of the mouse MEF2A mRNA isolated and analyzed in this study. Nucleotide number 1 denotes the first nucleotide following the stop codon in the MEF2A cDNA. The region of the 3′ UTR responsible for maximal inhibition of gene expression (nt 417 to 844) is highlighted. Single underlines denote A+U-rich regions. Double underlines denote ARE-like elements.

While much of the *Xenopus* 3′ UTR sequence contains little or no homology with the mouse MEF2A 3′ UTR, there is a stretch of about 400 nt within the 3′ UTRs from the two species which have a level of homology comparable to the level of homology within the coding regions of the two genes (7). This region between nt 464 and 842 of the mouse 3′ UTR sequence is also highly conserved with the human sequence (37, 40). Likewise, this is the only region of homology between the human and *Xenopus* MEF2As over the entire length of the 3′ UTRs (37). The high degree of sequence conservation in the 3′ UTR of MEF2A suggested a possible conservation of function for the 3′ UTR, in particular for the region of the UTR encompassing nt 464 to 842, as this sequence was conserved over much of vertebrate evolution (37).

The 3′ UTR of MEF2A inhibits translation in vivo. To test whether the 3′ UTR of MEF2A might play a role in the regulation of gene expression, we employed a strategy in which the 3′ UTR of MEF2A was fused immediately downstream of the reporter gene encoding CAT in plasmid pCDNA3.CAT. This system offered several advantages. First, each of the CAT-UTR mRNAs is transcribed under the control of the constitutively active cytomegalovirus immediate-early promoter, and as such, transcriptional levels of each construct being compared should be equivalent. Polyadenylation of transcripts occurs from exogenous polyadenylation sequences downstream of the polylinker. This reporter gene strategy allows the influence of fused 3′ UTR sequences on the expression of the reporter to be measured by a standard CAT assay and has been employed many times previously to examine the effect of untranslated regions on the expression of the CAT reporter (16). This vector also encodes the gene for neomycin resistance (*neo*) expressed under the control of the constitutive simian virus 40 early promoter, which provides an internal control for another mRNA expressed from the same transfected plasmid but without influence from any fused 3′ UTR sequences. Finally, this vector allows for in vitro transcription of the CAT gene with the T7 RNA polymerase promoter.

To determine the effect of the MEF2A 3′ UTR on CAT gene expression, we compared cells transfected with the plasmid encoding the CAT mRNA fused to the MEF2A 3′ UTR to cells transfected with the plasmid encoding the CAT mRNA fused to the 3′ UTR of the β-glo gene. We also analyzed cells transfected with a plasmid encoding CAT without a fused exogenous 3′ UTR. For this analysis, we transfected the kidney cell line BHK21. We chose to examine the effect of the MEF2A 3′ UTR on gene expression in a kidney cell line since it has been reported that MEF2A transcripts are easily detectable in kidney tissue while MEF2A protein expression was barely detectable, suggesting that strong translational control of MEF2A expression might be occurring in that tissue (40). Therefore, we predicted that this cell line might demonstrate a translation control response mediated by the MEF2A 3′ UTR. Following transfection, cells were harvested and assayed for CAT activity. The results showed that the MEF2A 3′ UTR caused a dramatic inhibition of activity when fused to CAT compared to the activity that occurred when CAT was fused either to no UTR or to the β-glo 3′ UTR (Fig. 2A). No differences in CAT activities were observed between the CAT construct containing no added UTR and that with the β-glo 3′ UTR. Similar results were obtained in transfection analyses in every nonmuscle cell type that we examined, including Cos-1 and 10T1/2. These results indicate that the MEF2A 3′ UTR negatively affects gene expression in a *cis*-acting manner in vivo.

We hypothesized that the inhibition of gene expression shown in Fig. 2A was due to translational repression since earlier observations had demonstrated that the expression of endogenous full-length MEF2A message is regulated at the level of translation (36). However, it was possible that the MEF2A 3′ UTR might destabilize the CAT mRNA or that the fusion to CAT might inhibit transcription in vivo. To rule out these possibilities, we measured the steady-state level of CAT mRNA produced by BHK21 cells transfected with each of the CAT-MEF2A 3′ UTR fusions analyzed in Fig. 2A. In addition, we measured the steady-state level of *neo* message produced from the pCDNA3.CAT plasmids. Using RPA, we examined the steady-state levels of both CAT and *neo* mRNAs and observed no significant reduction in the level of CAT mRNA resulting from any of the MEF2A 3′ UTR fusions (Fig. 2B). We quantitated the CAT/*neo* mRNA ratios and found no significant differences in the level of CAT mRNA relative to the level of the *neo* mRNA internal control for any of the three constructs tested. The mean CAT/*neo* ratios for the no-UTR construct, the β-glo 3′ UTR construct, and the MEF2A 3′ UTR construct from three independent experiments were 5.0×10^{-2}, 4.2×10^{-2}, and 4.7×10^{-2}, respectively. The total amount of protected *neo* message was much greater than the total level of protected CAT message in these experiments, reflecting the greater stability of the *neo* transcript relative to the CAT transcript.

The 3′ UTR of MEF2A negatively influences translation in vitro. We next examined whether the MEF2A 3′ UTR could inhibit CAT expression in vitro. To do this, plasmids containing the MEF2A, β-glo, or no 3′ UTR fused to CAT were linearized immediately downstream of the 3′ UTR in plasmid

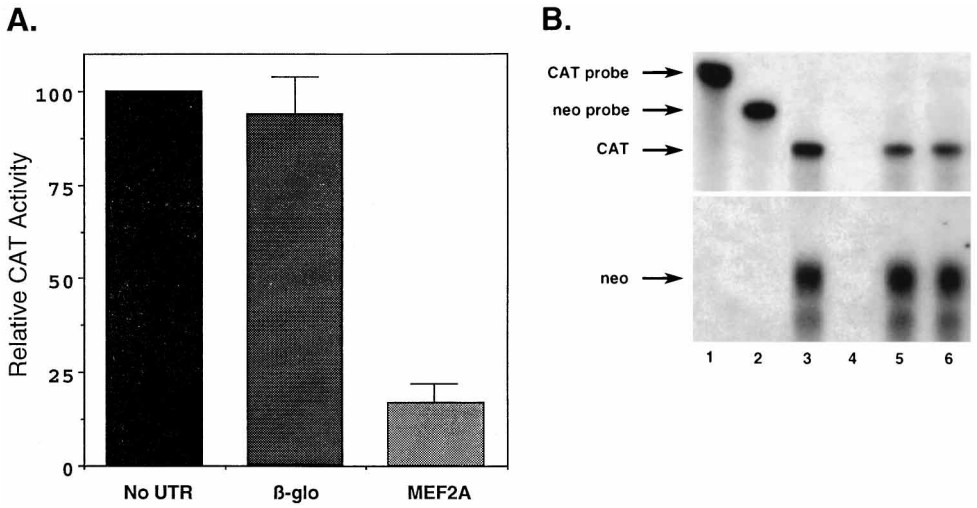

FIG. 2. (A) Analysis of the effect of the MEF2A 3′ UTR on CAT gene expression in vivo. BHK21 cells were transfected with plasmid pCDNA3.CAT with no UTR fused to CAT (No UTR), with the β-glo 3′ UTR fused to CAT (β-glo), or with the MEF2A 3′ UTR fused to CAT (MEF2A). Cells were harvested 24 h posttransfection, and the CAT activity of cell extracts was determined by the conversion of [^{14}C]chloramphenicol to acetylated forms as analyzed by thin-layer chromatography and quantitated by phosphorimager analysis. The results shown represent the mean values obtained in five independent transfections and analyses. Error bars represent the standard errors of the means for the five experiments. The mean levels of CAT enzyme produced by each of the three constructs were 0.35, 0.33, and 0.06 U for the no-UTR, β-glo UTR, and MEF2A UTR fusions, respectively. One unit of CAT enzyme generates approximately 50% conversion of chloramphenicol to acetylated forms under the conditions used in these experiments. (B) RPA of the steady-state level of CAT mRNA in transfected BHK21 cells. Twenty micrograms of total cellular RNA was subjected to RPA with CAT and *neo* antisense riboprobes as described in Materials and Methods. In some experiments, antisense riboprobes to 18S rRNA were also used to confirm the integrity and quantity of total RNA present in each sample (data not shown). Following protection, RNAs were digested with RNases A and T, resolved on a 6% denaturing urea–polyacrylamide gel, dried, and subjected to autoradiography. Results from a representative experiment are shown. Nearly identical results were obtained from experiments performed with RNAs isolated from three independent sets of transfections. The portion of the gel that shows the CAT-protected fragment (upper panel) was exposed to film for 24 h, and the portion that shows the *neo*-protected fragment (lower panel) was exposed to film for 6 h. Lanes 1 and 2, undigested CAT and *neo* probes, respectively; lane 3, RNA isolated from cells transfected with pCDNA3.CAT without a fused 3′ UTR protected by both CAT and *neo* probes; lane 4, untransfected-cell RNA protected with both probes; lanes 5 and 6, RNA isolated from cells transfected with pCDNA3.CAT containing a fused β-glo 3′ UTR or a fused MEF2A 3′ UTR, respectively, and protected with both probes. Protected fragments were quantitated by PhosphorImager (Molecular Dynamics, Inc.) analysis. No significant reduction in the CAT/*neo* mRNA ratio was observed for any of the constructs, nor were any differences observed for the level of 18S rRNA from any of the transfections (data not shown).

pCDNA3.CAT. CAT-UTR mRNA fusions were then transcribed in vitro with T7 RNA polymerase. Transcripts were examined by formaldehyde-agarose gel electrophoresis, which confirmed that they were full-length (data not shown). Equal moles of full-length CAT, CAT–β-glo 3′ UTR, and CAT-MEF2A 3′ UTR mRNAs were then translated in vitro in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3A). The results of the in vitro translation analysis showed that the 3′ UTR of MEF2A also exerted its negative effect on gene expression in vitro (Fig. 3A). The expression of CAT in vitro when fused to the MEF2A 3′ UTR (Fig. 3A, lane 5) ranged from 25 to 40% of the level of CAT with no UTR fused to it (lane 3) and from 20 to 30% compared to the β-glo UTR fusion (lane 4). The slightly higher expression of CAT when fused to the β-glo UTR compared to when it was fused to no UTR was expected, as the β-glo 3′ UTR is known to increase translational efficiency in vitro (15).

Again, we hypothesized that the inhibition of gene expression caused by the MEF2A 3′ UTR in the in vitro translation experiments in Fig. 3A was due to translational control. Because the transcripts were generated in vitro and an equal number of moles of each full-length transcript was added to the translation reaction mixtures, we knew there were no differences in transcription in these experiments. To confirm that there were no differences in mRNA degradation in vitro, we performed a time course analysis of full-length mRNA levels present in each of the translation reactions after 0, 30, 60, and 90 min of translation (Fig. 3B). The stability of transcripts in vitro was determined by conducting in vitro transcription reactions in the presence of [α-^{32}P]rUTP and then conducting cold in vitro translation reactions with the radiolabeled mRNAs for the indicated times. The data in Fig. 3B show the mean levels of mRNA remaining at each time point from three independent mRNA preparations in three independent translation reactions. The results of these experiments show that there was a slight degradation of each of the transcripts during in vitro translation. However, there were no significant differences in the rates of mRNA turnover for any of the three constructs analyzed. These results, taken together with the results shown in Fig. 3A, confirm that the inhibition of gene expression seen in the in vitro translation reactions in Fig. 3A was at the level of translation.

Translational control of the MEF2A 3′ UTR is modulated during muscle differentiation. The results of Fig. 2 and 3 demonstrated that the MEF2A 3′ UTR inhibited translation in a *cis*-acting fashion. To determine if translational control of the MEF2A 3′ UTR might be involved in regulated expression of MEF2A expression during muscle cell differentiation, we transfected the CAT-UTR fusions examined in Fig. 2 and 3 in C2C12 muscle cells grown under proliferation and differentiation conditions (Fig. 4A and B). We also examined the CAT-UTR fusions in two other muscle cell lines, L6 and Sol8, grown under differentiation conditions (Fig. 4C and D, respectively). The data in Fig. 4 show the mean CAT activity generated by each of the CAT-UTR fusions in three independent transfections and analyses. The results of Fig. 4A show that the MEF2A 3′ UTR when fused to CAT mediated a strong level of translation repression in proliferating myoblasts compared to CAT genes with no fused UTR or with a fused β-glo 3′ UTR.

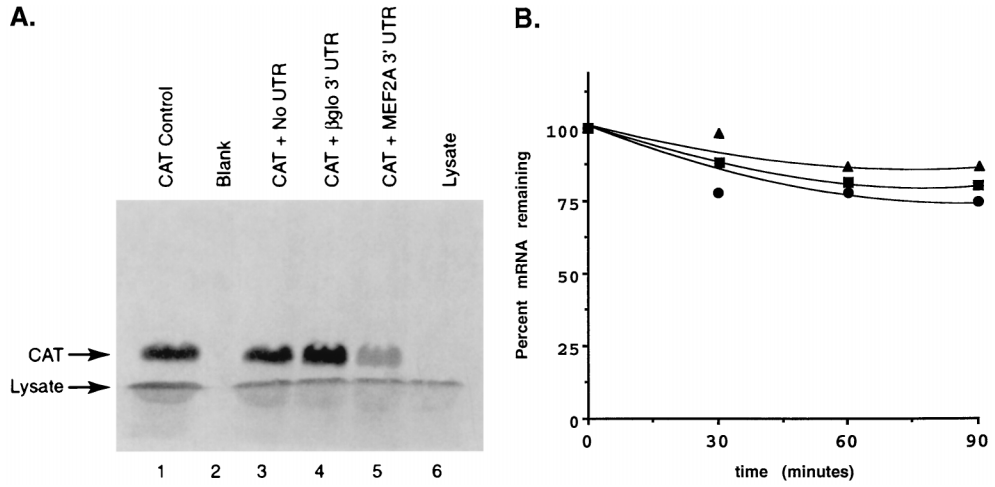

FIG. 3. (A) In vitro translation analysis of the effect of the MEF2A 3′ UTR on CAT gene expression. CAT-UTR transcripts were transcribed in vitro from the T7 RNA polymerase promoter in plasmid pCDNA3.CAT, which had been linearized and purified. Full-length transcripts were then purified and quantitated. Equal numbers of moles of each transcript were translated in vitro in rabbit reticulocyte lysate in the presence of [^{35}S]methionine and analyzed by SDS-polyacrylamide gel electrophoresis, an autoradiogram of which is shown. Lane 1, CAT protein control synthesized with a coupled transcription-translation system; lane 2, blank; lane 3, in vitro translation of CAT transcripts with no fused UTR; lane 4, in vitro translation of CAT transcripts fused to the β-glo 3′ UTR; lane 5, in vitro translation of CAT transcripts fused to the MEF2A 3′ UTR. Lane 6 shows an in vitro translation reaction with transcripts synthesized from plasmid pCDNA3 lacking a CAT gene, which shows the lack of a CAT band and the presence of a nonspecific lysate band, both of which are denoted at the left of the figure. The size of the CAT protein was confirmed by comparison to the migration of Bio-Rad kaleidoscope high-molecular-weight markers (not shown). In some experiments, an excess of yeast RNA was included in each translation reaction mixture as filler RNA. No differences in the results were observed regardless of whether excess filler RNA was present. Results of a representative experiment are shown. Comparable results were obtained in six independent sets of translations performed with two separate preparations of in vitro-transcribed mRNA. (B) Time course of mRNA degradation during in vitro translation. To confirm that there were no differences in mRNA degradation in vitro, we performed a time course analysis of full-length mRNA levels present in each of the translation reactions after 0, 30, 60, and 90 min of translation. The stability of transcripts in vitro was determined by conducting in vitro transcription reactions in the presence of [^{32}P]rUTP and then conducting cold in vitro translation reactions with radiolabeled in vitro-transcribed CAT plus no UTR (●), CAT plus β-glo 3′ UTR (■), and CAT plus MEF2A 3′ UTR (▲) mRNAs for the indicated times. The data represent the relative levels of each mRNA remaining at each time point. The values are from three independent mRNA preparations subjected to three independent translation reactions. The results show that there were no significant differences in the rates of mRNA turnover for any of the three constructs analyzed.

In contrast, the MEF2A 3′ UTR caused only a weak inhibition of translation in differentiated muscle cells compared to no UTR or β-glo UTR fusions (Fig. 4B to D). These data demonstrate that the 3′ UTR of MEF2A inhibits translation in all cell types but that this level of inhibition varies in different cell types. The strongest inhibition of translation is observed in nonmuscle kidney cells, at only ~17% of the no-UTR control (Fig. 2A). In proliferating muscle cells, the translational control exerted by the MEF2A 3′ UTR is weaker than that in kidney cells but is still strong at ~33% of the no-UTR control (Fig. 4A). However, in differentiated muscle cells (Fig. 4B to D), translational repression mediated by the MEF2A 3′ UTR is quite weak and significant amounts of CAT protein are produced (67 to 75% of control). The regulated control of translation by the MEF2A 3′ UTR is most evident in C2 myoblasts compared to C2 myotubes (Fig. 4A and B). Strong inhibition of translation is observed in undifferentiated C2 muscle cells (Fig. 4A). This inhibition is similar in strength to the inhibition seen in nonmuscle cells (Fig. 2A). However, when those muscle cells are differentiated to form multinucleated myotubes, translational inhibition is dramatically reduced (Fig. 4B). The failure of the MEF2A 3′ UTR to inhibit translation in differentiated muscle cells is further supported by the observations in two additional myogenic cell lines maintained under differentiation conditions (Fig. 4C and D). Therefore, taken together with the results of Fig. 2, the data presented in Fig. 4 strongly suggest that translational regulation by the 3′ UTR is likely to play a role in the increased expression of MEF2A during muscle differentiation.

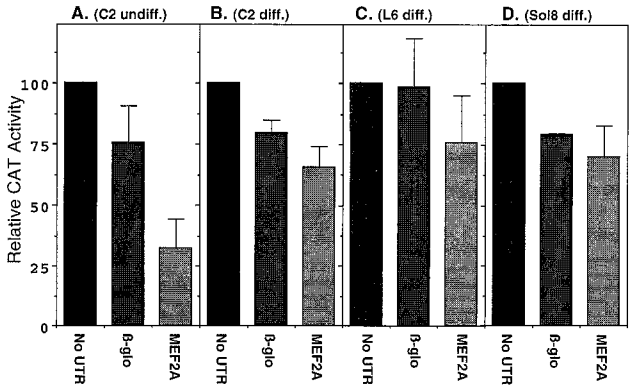

FIG. 4. Modulation of translational control by the 3′ UTR of MEF2A during muscle differentiation. C2, L6, and Sol8 muscle cell lines were grown under proliferation conditions (undiff.) or differentiation conditions (diff.) and were transfected with plasmid pCDNA3.CAT with no UTR fused to CAT (No UTR), with the β-glo 3′ UTR fused to CAT (β-glo), or with the MEF2A 3′ UTR fused to CAT (MEF2A). Cells were harvested 24 h posttransfection, and the CAT activities of cell extracts were determined by the conversion of [^{14}C]chloramphenicol to acetylated forms as analyzed by thin-layer chromatography and quantitated by PhosphorImager (Molecular Dynamics, Inc.) analysis. The results shown represent the mean values obtained in three independent transfections and analyses. Error bars represent the standard errors of the means for each of the sets of three experiments.

An internal fragment of the MEF2A 3′ UTR is sufficient to maximally inhibit translation. The results of Fig. 2 showed that the full-length MEF2A 3′ UTR inhibited translation of the CAT reporter gene in vivo in a *cis*-acting manner relative to a CAT gene with no fused 3′ UTR or to a CAT gene fused to the β-glo 3′ UTR. To further characterize the inhibition of translation mediated by the 3′ UTR of MEF2A and to more precisely map the regions within the UTR which were respon-

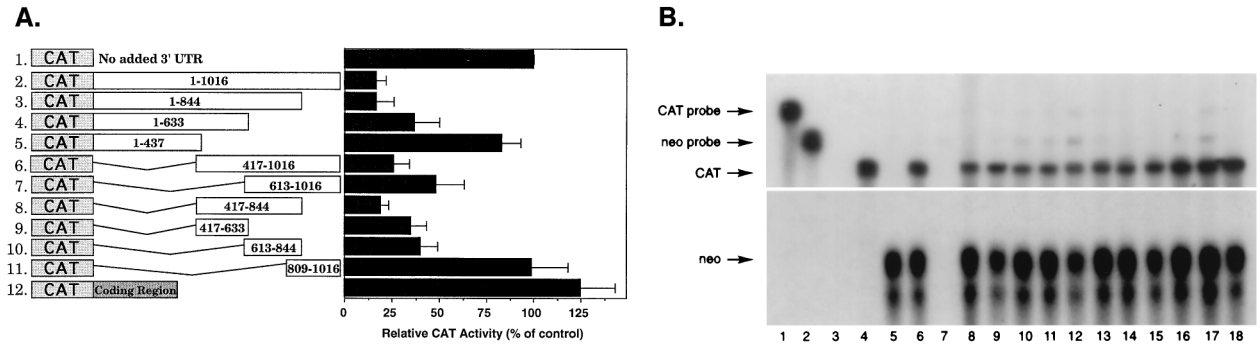

FIG. 5. (A) Reporter gene analysis of effects of MEF2A 3′ UTR deletions on CAT gene expression in vivo. BHK21 cells were transfected with plasmid pCDNA3.CAT with no UTR fused to CAT (lane 1) or with various fragments of the MEF2A 3′ UTR fused to CAT (lanes 2 to 11). Lane 12 shows a control in which the final 110 nt of MEF2A coding region cDNA were fused to CAT. The data are expressed as the percentages of activity obtained with each CAT-UTR construct relative to that of the pCDNA3.CAT construct containing no added 3′ UTR (lane 1). The nucleotide positions (from Fig. 1) of each MEF2A fragment analyzed are indicated on the schematic portion of the figure. Cells were harvested 24 h posttransfection, and the CAT activities of cell extracts were analyzed by thin-layer chromatography and quantitated by PhosphorImager (Molecular Dynamics, Inc.) analysis. The data are the averages of five independent transfections and analyses. Error bars represent the standard errors of the means for the five experiments. (B) RPA of the steady-state level of CAT mRNA in transfected cells. BHK21 cells were transfected with plasmid pCDNA3.CAT with no UTR fused to CAT or with the same UTR fusions analyzed in panel A. At 24 h posttransfection, cells were harvested and total cellular RNA was isolated. Twenty micrograms of total RNA was then subjected to RPA with CAT and *neo* antisense riboprobes as described in Materials and Methods. In some experiments, antisense riboprobes specific to 18S rRNA were also used to confirm the integrity and quantity of total RNA present in each sample (data not shown). Following protection, RNAs were digested with RNases A and T, resolved on a 6% denaturing urea–polyacrylamide gel, dried, and subjected to autoradiography. The portion of the gel that shows the CAT-protected fragment (upper panel) was exposed to film for 12 h, and the portion that shows the *neo*-protected fragment (lower panel) was exposed to film for 1 h. Lanes 1 and 2, undigested CAT and *neo* probes, respectively; lane 3, blank; lanes 4 to 6, pCDNA3.CAT-transfected cell RNA protected by CAT probe only (lane 4), *neo* probe only (lane 5), or both probes (lane 6); lane 7, untransfected-cell mRNA protected with both probes. Lanes 8 to 18 show mRNA isolated from pCDNA3.CAT plasmid-UTR fusion-transfected cells protected with both CAT and *neo* probes. The same fragments were analyzed here as in the CAT assays in panel A. The UTR fragments fused to CAT in each lane are as follows: lane 8, nt 1 to 1016; lane 9, nt 1 to 844; lane 10, nt 1 to 633; lane 11, nt 1 to 437; lane 12, nt 417 to 1016; lane 13, nt 613 to 1016; lane 14, nt 417 to 844; lane 15, nt 417 to 633; lane 16, nt 613 to 844; lane 17, nt 809 to 1016; lane 18, MEF2A coding region control. Protected fragments were quantitated by PhosphorImager (Molecular Dynamics, Inc.) analysis. No significant reduction in CAT mRNA relative to the protected fragments from CAT without a fused UTR (lanes 4 and 6) was observed, nor were any differences observed for the levels of *neo* or 18S rRNA from any of the transfections (data not shown). Nearly identical results were obtained in four experiments from RNAs isolated from two independent transfections.

sible for this inhibition of gene expression, we created a series of MEF2A 3′ UTR deletion constructs (Fig. 5A). These deletions were fused to the CAT gene in plasmid pCDNA3.CAT, as before, and their effect on gene expression was examined by transfection analysis in BHK21 cells. Following transfection, cells were harvested and assayed for CAT activity (Fig. 5A). The level of CAT activity generated by each of the 3′ UTR fusions was compared to the level of CAT activity generated by a CAT construct containing no fused UTR (lane 1). As an additional control, we used a fragment of the MEF2A gene corresponding to a 3′ portion of the MEF2A coding region (lane 12). Thus, in this experiment, we compared various fragments of the MEF2A cDNA fused to CAT to each other and to a construct containing no fused UTR. The region of the MEF2A 3′ UTR between nt 417 and 844 (lane 8) caused maximal inhibition of CAT expression (about sixfold). Relative to a CAT gene with no UTR fused to it (lane 1), in every case in which a CAT-MEF2A 3′ UTR construct contained the inhibitory region from nt 417 to 844, an average five- to sixfold inhibition of CAT activity occurred (lanes 2, 3, 6, and 8). Constructs which contained either the 5′ (nt 417 to 633) or 3′ (nt 613 to 844) portion of this inhibitory region caused approximately threefold inhibition of CAT activity (lanes 4, 7, 9, and 10). In contrast, CAT genes which contained only the 5′ portion of the MEF2A 3′ UTR (nt 1 to 437) caused no significant change in the level of CAT expression (lane 5). Likewise, CAT genes that had the most 3′ portion of the MEF2A UTR (nt 808 to 1016) or a small portion of the MEF2A coding sequence caused no decrease in the level of CAT gene expression (lanes 11 and 12). These experiments demonstrate that an internal portion of the MEF2A 3′ UTR inhibits expression to the same extent as the full-length 3′ UTR. These experiments also show that, while some regions of the MEF2A 3′ UTR cause maximal inhibition (nt 417 to 844), others (nt 1 to 437 and 809 to 1016) cause no inhibition. In addition, we examined the ability of the inhibitory fragment of the MEF2A 3′ UTR (nt 417 to 844) to inhibit translation in vitro and found that this fragment was equally as effective as the full-length MEF2A 3′ UTR in inhibiting the translation of CAT in vitro (data not shown). Interestingly, the region of the 3′ UTR conferring maximal inhibition of translation (nt 417 to 844) encompasses the region of the 3′ UTR with high sequence homology to the *Xenopus* 3′ UTR (nt 464 to 842) (7). Also, there is no appreciable conservation between the mouse and *Xenopus* 3′ UTRs from nt 1 to 437, and as predicted, this region of the mouse 3′ UTR was unable to inhibit gene expression when fused to CAT (lane 5).

The results of the CAT assays in Fig. 5A demonstrated that a portion of the 3′ UTR of MEF2A from nt 417 to 844 potently down-regulated CAT gene expression in vivo. Since the results of Fig. 2 and 3 showed that the full-length MEF2A 3′ UTR inhibited gene expression at the level of translation in vivo and in vitro, respectively, we hypothesized that the inhibition of gene expression mediated by the MEF2A 3′ UTR fragment from nt 417 to 844 was also due to translational repression. However, the possibility existed that some of the fragments of the MEF2A 3′ UTR might destabilize the CAT mRNA or that the fusion to CAT might inhibit transcription in vivo. To determine whether the inhibition of gene expression observed for the UTR-CAT fusions analyzed in Fig. 5A was at the level of translation, we measured the steady-state levels of CAT and *neo* messages produced from the pCDNA3.CAT plasmids by RPA. We observed no significant reductions in the levels of either CAT or *neo* transcripts resulting from any of the MEF2A 3′ UTR fragment fusions (Fig. 5B). In addition, we measured the amount of 18S rRNA by RPA and saw no differences in any sample, confirming that each reaction mixture contained an equivalent amount of total RNA and that the

RNA samples present in each protection assay were not degraded (data not shown). Quantitation of the level of protected message present in each of the transfections confirmed that no reduction in CAT mRNA resulted from fusion of MEF2A 3′ UTR fragments. While there were no decreases in the steady-state levels of CAT mRNA resulting from any of the MEF2A 3′ UTR fusions (lanes 8 to 18) relative to the no-UTR control (lanes 4 and 6), the 3′-most MEF2A UTR fragment (nt 808 to 1016) and the coding region control fusions (lanes 17 and 18) actually caused a slight increase in the level of CAT message. These slight increases are most likely due to a minimal stabilizing effect on the message and probably account for the slightly higher CAT activity observed for these constructs in the CAT assays shown in Fig. 5A. To confirm that the region of the MEF2A 3′ UTR from nt 417 to 844 is produced by the transfected plasmids containing that fragment, we also performed RPA on transfected-cell RNA from BHK21 cells transfected with the full-length MEF2A 3′ UTR-CAT fusion and the MEF2A 3′ UTR (nt 417 to 844)-CAT fusion. Both of these constructs produced similar levels of mRNA which contained the 428-nt region protected by an antisense MEF2A RNA probe containing nt 417 to 844, confirming that this inhibitory region was transcribed and was stable in vivo. This result was further confirmed by RNA blot analysis which showed that the region from nt 417 to 844 was present in mRNA produced by transfected cells (data not shown). Likewise, the same fragment was protected in RPAs and was present on RNA blots from blotting performed on endogenous RNA isolated from C2C12 myotubes (data not shown). Taken together, the data in Fig. 5 show that the highly conserved fragment of the MEF2A 3′ UTR from nt 417 to 844 is sufficient to maximally inhibit gene expression in a *cis*-acting manner and that this inhibition is at the level of translation.

DISCUSSION

The results of this study demonstrate that the 3′ UTR of MEF2A mediates translational repression in a *cis*-acting fashion when linked to a CAT reporter gene. This conclusion is supported by the results of the CAT assay and RPA in Fig. 2, which showed that the MEF2A 3′ UTR mediated a strong inhibition of gene expression in vivo without a corresponding decrease in the steady-state level of CAT mRNA. Likewise, the results of Fig. 3 demonstrated that translational repression by the 3′ UTR of MEF2A also occurred in vitro. This translational repression is modulated during muscle cell differentiation (Fig. 4). As muscle cells differentiate, the translational repression exerted by the MEF2A 3′ UTR diminishes, and this inhibition of translation maps to a 428-nt fragment of the 3′ UTR (Fig. 5). Taken together, all of these results demonstrate a translational control mechanism mediated by the MEF2A 3′ UTR which is regulated during muscle cell differentiation. Interestingly, the level of repression exerted by the 3′ UTR is very close to the level of translational control seen for endogenous MEF2A in vascular smooth muscle cells (36) and suggests that this region of the 3′ UTR may be solely responsible for the translational control of MEF2A.

The sequences within the 3′ UTR that mediated translational repression lay between nt 417 and 844, as this fragment alone was able to mediate maximal inhibition of CAT activity (Fig. 5A). We analyzed this region for motifs known to result in translational repression and noted several A+U-rich sequences that were similar to consensus AREs (Fig. 1). AREs are A+U-rich sequences which may be degenerate and have been shown to mediate translational repression (16, 25). Overall, like many inhibitory 3′ UTRs, the entire MEF2A 3′ UTR is very A+U-rich, and this enrichment is even higher within the inhibitory region between nt 417 and 844 (Fig. 1). We consider it likely that this entire region of the 3′ UTR is required to confer the maximal inhibition of translation since the conservation among the mouse, *Xenopus*, and human 3′ UTRs extends over that entire sequence and since dissection of the inhibitory region of the 3′ UTR into two parts reduced the inhibitory effect (Fig. 5A). This conclusion is further supported by the observation that the inhibitory region identified here corresponds to the only region of the *Xenopus* 3′ UTR containing extensive homology to the mouse and human 3′ UTRs (7, 37). It remains a possibility that longer mouse MEF2A 3′ UTRs may exist, particularly since longer human 3′ UTRs have been identified (37). However, we were unable to isolate any longer UTRs from mouse libraries. Our failure to isolate longer 3′ UTRs suggests that the 3′ UTR that we isolated is indeed full length. This difference in the length of the 3′ UTRs may account for the observation that the mouse MEF2A mRNA is significantly smaller in size than the human MEF2A mRNA when analyzed by Northern blotting (4, 40). Even the possible existence of longer 3′ UTR sequences would not alter the conclusions of this study, since the region of the 3′ UTR which has been conserved throughout most of vertebrate evolution is present in the mouse UTR sequences we have analyzed, and this sequence maximally inhibits translation.

There are several potential mechanisms by which the 3′ UTR might regulate translational control. One possible mechanism involves the potential regulation of *trans*-acting RNA-binding factors during muscle differentiation. Models such as this, which might allow the MEF2A message to be more efficiently translated either by actively promoting translation during muscle differentiation or by releasing the message from control of a ubiquitous translational repression factor, have been proposed (33, 34). Another potential mechanism involves the regulation of polyadenylation. While we have no evidence for differential polyadenylation of the MEF2A message, it remains a possibility that this may occur during muscle differentiation. This hypothesis is supported by the observation that several putative polyadenylation signals exist within the MEF2A 3′ UTR (Fig. 1) but apparently are not used with high frequency, possibly because they lack canonical downstream signal sequences (38, 39). However, if these signals are used, even at low frequency, during differentiation, significant release of translational repression might result. Differential polyadenylation mechanisms have been proposed previously to be involved in alternative, tissue-specific mRNA processing (38, 39). 3′ UTRs have previously been shown to play a role in muscle differentiation. The 3′ UTR of the alpha-tropomyosin gene has been shown to be involved in muscle differentiation by partially rescuing a mutant myoblast cell line incapable of undergoing differentiation (30) and by conversion of chicken embryonic fibroblasts to a muscle phenotype (17). The 3′ UTR of the alpha-tropomyosin gene is capable of tumor suppression in vivo (31), which likely occurs as a result of its ability to activate the RNA-dependent protein kinase PKR (8). We believe that this is unlikely to be the mechanism of action observed in this study since the MEF2A 3′ UTR inhibits translation in a *cis*-acting manner while the alpha-tropomyosin 3′ UTR functions in *trans* (8, 30, 31).

The role of translational control in the regulation of MEF2A gene expression has been controversial. While some studies have shown that MEF2A mRNA is ubiquitous and that the protein is tissue restricted (40), other studies have suggested that the protein is also widespread (10). We believe that MEF2A expression is regulated at multiple levels. We have observed that MEF2A mRNA is expressed in a wide range of

tissues, and while mRNA levels are slightly increased in muscle, brain, and cardiac cells, the increases that we have observed cannot account for the much larger changes in protein level (4). During mouse embryonic development, strong expression of MEF2A mRNA is restricted to cells of the developing muscle and neural lineages (11, 21), and this mRNA expression pattern correlates with the distribution of MEF2A protein in the developing embryo (35). However, in neonatal and adult tissues, mRNA expression becomes widespread while MEF2A protein expression remains restricted (4, 40). Thus, it is clear that the regulation of MEF2A is complex. The results of this study and of others (36, 40) suggest that MEF2A expression is translationally controlled. Other evidence suggests that MEF2A is also regulated transcriptionally (10, 29, 40), and posttranslationally (6). Tight control of MEF2 gene expression is important since even small changes in expression levels may have dramatic consequences within the cell. By employing multiple levels of control, a cell ensures that MEF2A is expressed only at the proper level, time, and place. This type of tight control is important in light of the role of MEF2 factors in potentiating cell-type-specific transcription in brain and muscle where the decision to differentiate is irreversible (3, 14, 22, 27).

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