Overexpression of CUG Triplet Repeat-binding Protein, CUGBP1, in Mice Inhibits Myogenesis*

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Accumulation of RNA CUG repeats in myotonic dystrophy type 1 (DM1) patients leads to the induction of a CUG-binding protein, CUGBP1, which increases translation of several proteins that are required for myogenesis. In this paper, we examine the role of overexpression of CUGBP1 in DM1 muscle pathology using transgenic mice that overexpress CUGBP1 in skeletal muscle. Our data demonstrate that the elevation of CUGBP1 in skeletal muscle causes overexpression of MEF2a and p21 to levels that are significantly higher than those in skeletal muscle of wild type animals. A similar induction of these proteins is observed in skeletal muscle of DM1 patients with increased levels of CUGBP1. Immunohistological analysis showed that the skeletal muscle from mice overexpressing CUGBP1 is characterized by a developmental delay, muscular dystrophy, and myofiber-type switch: increase of slow/oxidative fibers and the reduction of fast fibers. Examination of molecular mechanisms by which CUGBP1 up-regulates MEF2A shows that CUGBP1 increases translation of MEF2A via direct interaction with Gcn4 repeats located within MEF2A mRNA. Our data suggest that CUGBP1-mediated overexpression of MEF2A and p21 inhibits myogenesis and contributes to the development of muscle deficiency in DM1 patients.

DM1 is a multisystem disease mainly characterized by defects in skeletal muscle with the involvement of many tissues and systems such as cardiac muscle, brain, eye, and endocrine system (1). DM1 is caused by an expansion of CTG trinucleotide repeats within the 3’-untranslated region of the myotonin protein kinase gene (2). In DM1 patients, the size of DNA CTG expansion correlates with the severity of the disease. Patients with CTG expansion containing 50–80 CTG repeats are almost asymptomatic. Individuals bearing the myotonin protein kinase gene with 100–500 CTG repeats develop a disease in adult life (classical adult form of DM1) that is characterized by a progressive muscle wasting with myotonia. The most severe form of DM1, congenital disease, affects patients before or after birth and is associated with long CTG expansions (up to 2,000 repeats). This form of disease is characterized by a delay or arrest of skeletal muscle development (3). Although there is an overlap in range of repeats between different forms of DM1, there is a clear correlation of repeat number with severity of phenotype and reduction of age of onset.

Investigation of molecular alterations in DM1 suggest that the expansion of CTG repeats causes the DM1 pathology through different mechanisms, mediated at both DNA and RNA levels. It has been shown that CTG repeats reduce expression of myotonin protein kinase in cis (4), causing abnormalities in cardiac muscle (5). CTG repeats also affect transcription of genes adjacent to myotonin protein kinase (6), leading to the development of cataracts (7, 8). A number of recent studies indicate that other symptoms in DM1 such as myotonia (9, 10), delay of skeletal muscle differentiation (11, 12), and a resistance to insulin (13) are mediated through an RNA-based mechanism. According to this mechanism, RNA CUG repeats cause the DM1 disease via specific RNA CUG-binding proteins (14).

Two families of RNA CUG-binding proteins have been identified: CUGBP (RNA-binding protein, binding to RNA CUG repeats) (15–17) and EXP (also known as MNBL, muscle blind) proteins (18). One of the members of the CUGBP family, CUGBP1, contributes to at least three symptoms of DM1: the delay of skeletal muscle differentiation (12), myotonia (19), and the insulin resistance (13). Previous studies showed that the expansion of CTG repeats in DM1 leads to a 3–5-fold elevation of CUGBP1 binding activity and protein levels (13, 19–21), perhaps by stabilizing CUGBP1 protein within RNA CUG-CUGBP1 complexes (21). CUGBP1 possesses two major biological functions: regulation of splicing and translation (12, 20–22). Translational function of CUGBP1 was investigated mainly in cultured cells. These studies showed that CUGBP1 increases translation of p21 (12) and translation of a dominant negative isoformal of transcription factor CCAAT/enhancer-binding protein β, LIP (22). It has been shown that, in tissue culture models, CUGBP1-mediated induction of p21 is required for proper differentiation of myocytes (12). However, the role of translational function of CUGBP1 in vivo has not been addressed.

To examine the role of translational activity of CUGBP1 in development of skeletal muscle deficiency in DM1 patients, we generated transgenic mice that overexpress CUGBP1 mainly in skeletal muscle and to a lesser extent in the heart. We found that elevation of CUGBP1 causes overexpression of its translational targets p21 and MEF2A (transcription factor, myocyte enhancer factor 2A) in skeletal muscle. These alterations cause...
a muscular dystrophy, an increase of slow fibers, and delay of muscle development.

**EXPERIMENTAL PROCEDURES**

**UV cross-linking—**RNA transcripts encoding 123 CUG repeats, the full-length wild type, and mutant MEF2A DNAs lacking the CUGBP1 binding site were synthesized in vitro and incubated with 10–20 μg of purified proteins as described (12). After UV cross-linking, the samples were treated with RNase A (2 units/10 μl) and separated by PAGE with 0.1% SDS. Where indicated, MEF2A riboprobe containing the (GCA)₆ repeat (CUGBP1 binding site) within MEF2A mRNA or sORF riboprobe specific for CEBP₂β was used.

**Generation of CUGBP₁ Transgenic Mice—**The experiments related to transgenic mice complied with federal guidelines and institutional policies. The full-length human CUGBP₁ cDNA was amplified from a parental plasmid (17) with CUGBP₁-specific primers. The sequence of the forward primer is as follows: 5'-TCA AAG AAA ATG AAG GCC ACC CTG-3’. The sequence of the reverse primer fused in frame with a short sequence coding for 6 histidines is as follows: 5'-ATG GTG ATG ATG GTG ATG GTA GGG CTT GCT GTC ATT CTT CGA-3'. The CUGBP₁ cDNA fused with the track of histidines on its C terminus was cloned into BS vector containing chicken β-actin promoter connected with cytomegalovirus enhancer (a gift from Dr. Schneider, Baylor College of Medicine). Transgenic construct was injected into mouse FVB inbred eggs at DNX Transgenic Sciences Co. (Princeton, NJ).

**Generation of Transgenic Mice—**Progenies were genotyped by PCR with CUGBP₁-specific primers. The sequence of forward primer is 5’-GCT GCA TTA GAA GCT CAG AAT GCT-3’. The reverse primer has a sequence 5’-AGG TTT CAT CTG TAT AGG GTG AGT-3’. Two PCR products of different size from both wild type (540 bp) and mutant alleles (78 bp) were synthesized simultaneously. The size of PCR product from mutant allele is smaller because the transgene lacks introns.

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**Tissue Culture—**Mouse C2C12 myoblasts and human primary myoblast cultures were grown in 10-cm dishes in myoblast growth medium at 37°C in 5% CO₂. Differentiation was induced by a switch from growth medium to fusion medium as described (12). Differentiating myoblasts were maintained for 5 days with medium changes occurring every day.

**Northern Analysis—**Total cellular RNA was extracted with TRI Reagent (Molecular Research Center, Inc.) from C2C12 myoblasts and myotubes at different time points of differentiation. RNA was hybridized with CUGBP₁ probe as described (15). Levels of CUGBP₁ mRNA (7 + 9 kb) were calculated as a ratio to 18 S rRNA control.

**Immunostaining of Mouse Tissues—**Paraffin and frozen sections were prepared from vastus or plantaris muscles from wild type and mutant mice with different numbers of CUGBP₁ copies of the matching age and gender. Muscle sections were stained with H&E at the Baylor College of Medicine Histology Core. Frozen sections, sections were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h and incubated for 1 h with antibodies against MEF2 (C-21; 1:1,000), myoglobin (sc-8081; 1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), fast myosin (MY-32; 1:100), and slow myosin heavy chain (1:50) (Sigma). Rhodamine-labeled anti-rabbit and antimouse immunoglobulins from Santa Cruz Biotechnology were used as secondary antibodies with dilution 1:200. Images were analyzed in Microscopic Core Facilities of Aging Center and Department of Molecular and Cellular Biology at Baylor College of Medicine.

**Isolation of Proteins and Western Blotting Analysis—**Whole protein extracts from mouse tissues were prepared with radioimmune precipitation buffer. Cytoplasmic, nuclear, and mitochondrial fractions were isolated as described (12). The primary antibodies were rabbit anti-CUGBP₁, MEF2 (C-21), myoglobin (sc-8081), and MyD (myogenic transcription factor) (C-20) and mouse p21 (H164), myogenin (CF5D), fast (MY-32) and slow myosin (NOQ7.54D), and α-tubulin (TU-02). Detection was performed using the ECL kit (Pierce). Membranes were stripped and reprobed with control antibodies (α-actin or α-tubulin), and results were quantified by densitometry analysis.

**The Effect of CUGBP₁ on MEF2A Levels in Cultured Cells—**C2C12 cells were transfected with empty pcDNA vector and with recombinant plasmid containing CUGBP₁ in pcDNA vector using FuGene 6 protocol. Protein extracts were isolated in 48 h after transfection and subjected to Western blotting with antibodies against His tag, CUGBP₁, and MEF2A. CUGBP₁ and MEF2A protein levels were calculated as a ratio to β-actin. To visualize the effects of CUGBP₁ on MEF2A levels, C2C12 cells grown in slides were transfected with GFP as control and with CUGBP₁ cloned into GFP vector. Cells were fixed and subjected to IF with antibodies against MEF2A using secondary antibodies labeled with rhodamine. CUGBP₁ (green signal) and MEF2A (red signal) were analyzed at ×40 magnification.

**Examination of MEF2A Translation in Tissue Culture—**One set of plates with C2C12 cells was co-transfected with MEF2A and CUGBP₁, and another set was co-transfected with MEF2A and empty vector. One day after transfection, the growth medium was replaced with Met-free medium. In 24 h, Met-deficient medium was supplemented with [35S]Met, and whole-cell protein extracts were prepared in 0, 1, 2, 3, and 8 h after the addition of [35S]Met. Protein extracts were diluted with phosphate-buffered saline buffer and incubated with antibodies against MEF2A (1:100) for 4 h at 4°C, following incubation with protein A-agarose for 1 h. Immunoprecipitates were collected by centrifugation at 6,000 rpm for 10 min, washed three times with 1 ml of phosphate-buffered saline, resuspended in the loading buffer, and analyzed by polyacrylamide gel electrophoresis.

**Inhibition of CUGBP₁ by siRNA—**Two RNA CUGBP₁ primers were synthesized in Qiagen to target the following CUGBP₁ sequence: 5’-AAT TTG CCT GCT GTA CTT GCT GCT-3’. The sequence of the first primer is r(UUUUGGCGUCAGACUGCUCd(TT). The sequence of the second primer is r(AGAGCGUGAGUGGCGAAAdd(TT). These primers were annealed to make double-stranded siRNA-CUGBP₁. Transfection of siRNA CUGBP₁ was performed according to the Qiagen protocol. C2C12 cells were grown at 70% density. Enhancer R was mixed with siRNA oligonucleotide and transfection reagent in a ratio of 2:1:2.5. Protein extracts were prepared from proliferating and differentiating myoblasts transfected with siRNA. In control plates, no siRNA was added.

**Translation in an in Vitro Cell-free System—**MEF2A plasmid was identified by screening of a human cardiac cDNA library with a GCA triplet repeat probe. GCA deletion mutant was generated by using QuickChange site-directed mutagenesis kit (Stratagene). The sequence of forward mutagenic primer is 5’-TGCG CGG CCT GAA GCC CGA TGG GGT CAT-3’. Rabbit reticulocyte lysate was programmed with wild type and mutant MEF2A mRNAs, and increased amounts of purified CUGBP₁ were added to the incubation mixture. Translation products were subjected to SDS-PAGE and autoradiography.

**Interaction of CUGBP₁ and MEF2A mRNA with Myosin—**Myosin light chain was treated with MEF2A plasmid (20 μg) in the presence of cytoplasmic buffer (20 mM Tris·HCl, pH 7.5, 50 mM KC1, 5 mM EDTA, and 5 mM MgCl₂) containing RNase inhibitors. CUGBP₁ was immunoprecipitated with monoclonal antibodies 3B1. RNA was extracted by phenol-chloroform and reverse-transcribed with AMV using oligo(dT) primer. PCR was performed with primers specific to mouse MEF2A mRNA. The sequence of the forward primer was 5’-GAGTT-GAGCACCAGACCCCTA-3’. The sequence of the reverse primer was 5’-CTCCCGCTTTGAGCAGCTTGAGCACA-3’. The size of the PCR product with MEF2A primers was 743 bp.

**Myogenic Conversion of Fibroblasts into Myoblasts—**Mouse fibroblasts 10T1/2 were grown on four-chamber slides in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Cells grown at 60% of density were transfected with plasmids expressing MyoD, wild type and deletion mutant MEF2A, and the full-length or truncated CUGBP₁. The amount of plasmid DNA was 0.2 μg per chamber and remained constant in all experiments. When one plasmid was used instead of two or three, the amount of DNA was adjusted with pC7 DNA vector. Transfection was performed with FuGene. In 2 days after transfection, fibroblast growth medium was replaced with myoblast fusion medium. Cells were grown for 5 days with medium changed every day. CUGBP₁, fast myosin, and mitochondrial fractions were isolated as described (12). The primary antibodies were rabbit anti-CUGBP₁, MEF2 (C-21), myoglobin (sc-8081), and MyD (myogenic transcription factor) (C-20) and mouse p21 (H164), myogenin (CF5D), fast (MY-32) and slow myosin (NOQ7.54D), and α-tubulin (TU-02). Detection was performed using the ECL kit (Pierce). Membranes were stripped and reprobed with control antibodies (α-actin or α-tubulin), and results were quantified by densitometry analysis.

**RESULTS**

**Generation of CUGBP₁ Transgenic Mice—**The full-length CUGBP₁ coding region was placed under a modified β-actin promoter, which is mainly active in skeletal muscle and to a lesser extent in the heart (23), and fused on the C terminus to an oligonucleotide coding for 6 His amino acids. The resulting transgenic construct was injected into inbred eggs of FVB mice,
Fig. 1. Generation of CUGBP1 transgenic mice. A, expression of His-CUGBP1 in tissues of CUGBP1 transgenic mice. Whole cell extracts were isolated from tissues shown at the top and examined by Western blotting with antibodies to the His tag. Coomassie stain of the membrane is shown below. B, expression of His-CUGBP1 in skeletal muscle of CUGBP1-transgenic lines. Western blotting was performed with antibodies to the His tag. The membrane was reprobed with antibodies to α-actin. The numbers at the top indicate CUGBP1 transgenic lines (see Table I). C, Western blotting of His-CUGBP1 with antibodies against CUGBP1. Western blotting with protein extracts isolated from skeletal muscle of wild type, CTG transgenic (36), and CUGBP1 transgenic mice (line 36) was performed with antibodies against CUGBP1. The first lane was loaded with CUGBP1 purified from HeLa cells. The red arrows on the right show positions of hyperphosphorylated forms of CUGBP1. The bottom image shows Coomassie stain and the reprobe of the same membrane with antibodies to actin. A -fold increase of CUGBP1 (shown below) was calculated as a ratio of (endogenous + His-CUGBP1) signals to CUGBP1 signals observed in wild type animals. D, His-CUGBP1 is hyperphosphorylated in skeletal muscle. His-CUGBP1 was immunoprecipitated from skeletal muscle of CUGBP1 transgenic mice with antibodies to His tag and examined by two-dimensional gel electrophoresis followed by Western blotting with monoclonal antibodies to CUGBP1. The bottom image shows a similar analysis of His-CUGBP1 after treatment with CIP. Spots 1, 2, and 3 represent hyperphosphorylated forms.

and pups were genotyped by PCR with CUGBP1-specific primers. The expression of transgenic protein (His-CUGBP1) was verified by Western blotting with antibodies to His tag (Fig. 1, A and B). We first examined expression of His-CUGBP1 in different tissues of CUGBP1 transgenic mice. In agreement with previous publications (23), β-actin promoter fused with cytomegalovirus enhancer directs the expression of His-CUGBP1 primarily in skeletal muscle and to a lesser extent in cardiac muscle (Fig. 1A). Within the sensitivity of Western blot analysis, we did not detect His-CUGBP1 in brain, spleen, and kidney (Fig. 1A). We have generated five lines of CUGBP1 transgenic animals (Table I). The analysis of His-CUGBP1 in skeletal muscle of these animals showed that the expression of His-CUGBP1 is increased to different levels (Fig. 1B). In addition, we found that the expression of His-CUGBP1 leads to a 2-3-fold elevation of the endogenous CUGBP1 levels (Fig. 1C). This estimate was done using protein extracts from vastus muscles of 3-5 mice of each line and 17 wild type mice. The mechanisms by which the transgenic His-CUGBP1 protein induces protein levels of endogenous CUGBP1 are unknown. Our data also showed that CUGBP1 immunoreactive proteins migrate as several bands, perhaps because of different levels in phosphorylation (24). To examine whether these immunoreactive bands are isofoms of His-CUGBP1, we immunoprecipitated His-CUGBP1 with polyclonal antibodies to His and performed a two-dimensional gel electrophoresis followed by Western blotting with monoclonal antibodies to CUGBP1. Fig. 1D shows that His-CUGBP1 migrates as several spots located between pH 6.0 and 8.0. Acidic isoforms of His-CUGBP1 represent phosphorylated forms of CUGBP1, since a treatment of His-IPs with CIP shifts these isoforms to the alkali region of the strip. Given the elevation of both His-CUGBP1 and endogenous CUGBP1, as well as phosphorylated isoforms of CUGBP1, CUGBP1 levels were calculated as a ratio of all of these isoforms in transgenic animals to endo-CUGBP1 in wild type animals. These calculations showed that an increase of

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<th>Mouse line</th>
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<th>Induction of CUGBP1 protein</th>
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<td>8</td>
<td>8–10</td>
<td>55–60</td>
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<td>CUGBP1–49c</td>
<td>6</td>
<td>4–6</td>
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a The induction of CUGBP1 levels in skeletal muscle was calculated as a ratio of (His-CUGBP1 + endogenous CUGBP1) in transgenic mice to endogenous CUGBP1 in wild type littermates. b Three litters (21 mice) were weighted, p < 0.001. c Four litters (32 mice) were weighted, p < 0.001. d Four litters (28 mice) were weighted, p < 0.002. e Four litters (30 mice) were weighted, p < 0.002.
total levels of CUGBP1 correlates with the number of transgenic copies (see Table I). All analyzed animals showed a relatively narrow rate of CUGBP1 overexpression (from 2- to 10-fold), perhaps due to negative effect of CUGBP1 elevation on mouse development (Fig. 2).

**A Severity of Developmental Delay Correlates with Levels of CUGBP1 Elevation**—It has been previously shown that transgenic mice overexpressing the human DM1 region with greater than 300 CTG repeats developed myotonia and are growth-retarded (10). Given the accumulation of CUGBP1 in these mice, we performed gross analysis of CUGBP1 transgenic mice from different lines. This analysis showed that mutant mice, in which CUGBP1 is elevated to 2–3-fold (lines 29 and 32), have slight reduction of weight at birth but survive and develop normally. However, transgenic mice, in which CUGBP1 is 4–6-fold elevated, were growth-retarded and significantly underweight (Fig. 2, A and B). Analysis of three or four litters from different lines confirmed that weight of newborn CUGBP1 transgenic mice is 1/2–3/5 less than their wild type litter-mates (Table I) and that the reduction of weight depends on the levels of CUGBP1 protein. Animals with 8–10-fold elevation of CUGBP1 die in utero and are severely underdeveloped (Fig. 2B). This suggests that the overexpression of CUGBP1 in vivo might have severe impact on mouse development. Given the strongest expression of CUGBP1 transgene in skeletal muscle (Fig. 1C), we performed cross-sections through hind limb and femur of newborn wild type and mutant littermates (line 36) and found that whereas the size of femur in mutant mice is unchanged, the hind limb muscles look significantly reduced (Fig. 2C). Therefore, we further focused our studies on the histological analysis of skeletal muscle of CUGBP1 transgenic mice and on molecular pathways by which overexpression of CUGBP1 causes the disorder in skeletal muscle.

**His-CUGBP1 Precipitated from Skeletal Muscle of CUGBP1 Transgenic Mice Is Able to Regulate Translation of mRNAs**—In vitro studies have demonstrated that CUGBP1 is a key regulator of translation of p21 and C/EBPβ mRNAs (12, 21, 22). To examine whether CUGBP1 displays this activity in vivo, in tissues of transgenic mice, we first determined the RNA binding activity of His-CUGBP1 isolated from these tissues and its ability to regulate translation of mRNA targets. UV cross-link assay of skeletal muscle samples with CUG123 probe shows that His-CUGBP1, isolated from skeletal muscle cells, is able to interact with the RNA containing CUG repeats (Fig. 2D). To confirm that His-CUGBP1 binds to RNA, we immunoprecipitated CUGBP1 with antibodies to His tag and examined by gel electrophoresis. A diagram shows the structure of C/EBPβ mRNA and the position of the CUGBP1 binding site.
UV cross-link assay. As can be seen in Fig. 2D (his-IP), His-CUGBP1 interacts with the CUG123 probe. To perform a functional test for translational activity of His-CUGBP1, CUGBP1 was immunoprecipitated with antibodies to His tag from skeletal muscle and added into a cell-free translation system programmed with p21 or with C/EBPβ/H9252 mRNA. These studies demonstrated that His-CUGBP1 precipitated from skeletal muscle of transgenic animals induces translation of p21 mRNA as well as translation of the dominant negative isoform of C/EBPβ, LIP (Fig. 2E). Thus, these investigations showed that His-CUGBP1 (expressed in tissues of CUGBP1 transgenic mice) binds to RNA and potentially might regulate translation of mRNAs in vivo.

Histological Analyses of Skeletal Muscle from CUGBP1 Transgenic Mice—Histological and immunohistochemical analyses were performed on vastus and, where indicated, on plantaris muscles from the five mutant lines (five mice for line 20, six mice for line 29, five mice for line 32, seven mice for line 49) and on control littermates (21 mice). Since 2–3-fold elevation of CUGBP1 was observed in DM1 patients with mild symptoms that often are not apparent for decades, the analysis of this transgenic line was performed with aged (1.5 years) wild type and transgenic mice with 2–3-fold elevation of CUGBP1 (line 29). Myofibers from transgenic mice are variable in size and contain internal nuclei. B, H/E staining of vastus muscle from wild type and CUGBP1 transgenic mice with a 4–6-fold increase of CUGBP1 (line 49) is shown. Longitudinal sections show severe variability of myofiber size. Transverse sections indicate the presence of internal nuclei. C, H/E-stained sections through the hind limb and the femur for wild type and transgenic newborn mice (line 36) are shown. D, sections in the boxed areas show that fibers in CUGBP1 transgenic mice with 8-fold elevation of CUGBP1 are short and unfused and contain multiple nuclei. E, transverse sections of the same mutant muscle show centrally placed large nuclei in 20% of myofibers.

Fig. 3. Muscle deficiency in CUGBP1 transgenic mice. A, H/E staining of transverse and longitudinal sections of vastus muscle from aged (1.5 years) wild type and transgenic mice with 2–3-fold elevation of CUGBP1 (line 29). Myofibers from transgenic mice are variable in size and contain internal nuclei. B, H/E staining of vastus muscle from wild type and CUGBP1 transgenic mice with a 4–6-fold increase of CUGBP1 (line 49) is shown. Longitudinal sections show severe variability of myofiber size. Transverse sections indicate the presence of internal nuclei. C, H/E-stained sections through the hind limb and the femur for wild type and transgenic newborn mice (line 36) are shown. D, sections in the boxed areas show that fibers in CUGBP1 transgenic mice with 8-fold elevation of CUGBP1 are short and unfused and contain multiple nuclei. E, transverse sections of the same mutant muscle show centrally placed large nuclei in 20% of myofibers.

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genic mice are variable in length and are shorter than those from wild type mice (Fig. 3D). Muscle fibers in this line are characterized by prominent nuclei. Analysis of transverse muscle sections showed that 21–33% cells in mutant mice have large nuclei occupying the central position (Fig. 3E). Several muscle spindles in mutant mice contain clusters of poorly organized fibers with prominent large nuclei (data not shown).

Overexpression of CUGBP1 in Skeletal Muscle Leads to Increased Expression of MEF2A and p21—We next examined the molecular basis for the alterations observed in skeletal muscles of CUGBP1 transgenic mice. The presence of short fibers in mutant mice with 8-fold elevation of CUGBP1 (Fig. 3D) suggests that CUGBP1 may disrupt major regulators of late myogenesis, such as myogenin (25) and/or MEF2 factors, which cooperate with basic helix-loop-helix myogenic factors to activate the differentiation program (26, 27). In a parallel search for the possible regulators of myogenesis that might be potential binding sites for CUGBP1, we identified a member of MEF2 family transcription factors, MEF2A, containing a GC-rich region in the position of nucleotide 1565. The other members of this family also contain extensive GC-rich sequences that might be potential binding sites for CUGBP1. Since CUGBP1 up-regulates p21 and MEF2A in cultured cells (12) (see Fig. 6), we examined whether these translational targets of CUGBP1 are affected in transgenic mice. As can be seen in Fig. 4A, both p21 and MEF2A are increased in skeletal muscle of CUGBP1 transgenic mice. This result was confirmed by immunostaining of transverse muscle sections for plantaris muscle from wild type (WT) and mutant mice (line 46) with antibodies against myoglobin and slow and fast myosin heavy chains. Western blotting with antibodies against CUGBP1, MEF2, and actin shows that protein extracts from DM1 patients with 857 (1) or 300 (2) CTG repeats and from patients with late-onset myopathy (M) were analyzed with antibodies against CUGBP1, MEF2A, and myogenin as the loading control. Western analysis with CUGBP1 Abs detects CUGBP1 and a weak upper band corresponding to IgG. C upper image, immunostaining of transverse muscle sections for plantaris muscle from wild type (WT) and mutant mice (line 46) with antibodies against myoglobin and slow and fast myosin heavy chains. Bottom image, the switch of fiber type in skeletal muscle from CUGBP1 transgenic (TR) mice. Total skeletal muscle proteins isolated from vastus muscle of wild type and mutant mice (lines 20, 29, and 46) were examined by Western blotting with antibodies shown on the left. Each membrane was stripped and reprobed with antibodies to actin (not shown) or stained with Coomassie to verify protein loading.

FIG. 4. Elevation of CUGBP1 in skeletal muscle leads to the induction of MEF2A, myogenin, and p21 protein levels and to alterations of myofiber type. A, elevation of CUGBP1 in skeletal muscle increases levels of MEF2A, p21, and myogenin. Total proteins from vastus muscles were analyzed by Western blotting with various antibodies as indicated. To verify protein loading, each membrane was reprobed with antibodies against actin and stained with Coomassie Blue. Actin control is shown for myogenin and MEF2A filter. Right image, immunostaining of transverse sections of vastus muscle with antibodies against MEF2A. Sections from wild type and transgenic mice with 4–6-fold elevation of CUGBP1 (line 49) at 8 months of age were analyzed under identical conditions. B, MEF2A, myogenin, and p21 levels are elevated in skeletal muscle of DM1 patients. Protein extracts were isolated from skeletal muscle of normal control and DM1 patient with large CTG expansion (1,500 repeats) and analyzed by Western blotting. Right, CUGBP1, MEF2A, and p21 levels are changed in skeletal muscle of DM1 patients but not in muscle from patients with late onset myopathy. Total protein extracts from DM1 patients with 857 (1) or 300 (2) CTG repeats and from patients with late-onset myopathy (M) were analyzed with antibodies against CUGBP1, MEF2A, and actin as the loading control. Western analysis with CUGBP1 Abs detects CUGBP1 and a weak upper band corresponding to IgG. C upper image, immunostaining of transverse muscle sections for plantaris muscle from wild type (WT) and mutant mice (line 46) with antibodies against myoglobin and slow and fast myosin heavy chains. Bottom image, the switch of fiber type in skeletal muscle from CUGBP1 transgenic (TR) mice. Total skeletal muscle proteins isolated from vastus muscle of wild type and mutant mice (lines 20, 29, and 46) were examined by Western blotting with antibodies shown on the left. Each membrane was stripped and reprobed with antibodies to actin (not shown) or stained with Coomassie to verify protein loading.
MEF2A, and p21 levels in DM1 muscle are not due to general dystrophy, because the levels of these proteins were unchanged in muscle from patients with late onset myopathy (Fig. 4B). Thus, the overexpression of CUGBP1 in DM1 patients and in CUGBP1 transgenic mice affects MEF2A and p21 proteins, which play a significant role in myogenesis.

Elevation of CUGBP1, p21, and MEF2A in Skeletal Muscle Is Associated with Alterations of Myofiber Type—At earlier stages of the disease, DM1 patients have an increased number of slow myofibers, which become preferential fibers at later stages of disease progression. Given that p21 and MEF2A are similarly induced in DM1 and CUGBP1 transgenic mice, we examined whether myofiber type is changed in skeletal muscle from CUGBP1 transgenic mice. IF analysis of transverse muscle sections with antibodies against fast myosin shows that in plantaris muscle of 8-month-old wild type mice, fast and intermediate fibers represent 70–85%. IF analysis of similar sections from mutant littermates (line 49) showed that the number of these fibers is significantly reduced (50%) (Fig. 4C, Fast). The number of slow fibers determined by IF with antibodies to fast myosin was 15–30% greater, respectively, in CUGBP1 transgenic mice. The increase of slow fibers in CUGBP1 transgenic mice was confirmed by Western analysis of myoglobin and fast and slow myosin. Fig. 4C shows that, in plantaris muscle from CUGBP1 transgenic mice, the levels of slow myosin are increased, whereas the levels of fast myosin are reduced. It is known that slow fibers contain increased levels of myoglobin, and this is in contrast to fast fibers containing lower levels of myoglobin. We determined myoglobin levels by Western blot analysis and IF assay and found that plantaris muscle from CUGBP1 transgenic mice (line 49) has elevated levels of myoglobin, suggesting that the number of slow fibers is greater in CUGBP1 mutant mice. These data suggest that the elevation of CUGBP1 causes alterations in fiber-type composition in skeletal muscle with an increase of slow fibers and reduction of fast fibers similar to DM1 disease.

CUGBP1 Interacts with GCA Repeats Located within MEF2A mRNA—The elevation of MEF2A in skeletal muscle from CUGBP1 transgenic mice prompted us to examine molecular mechanisms by which CUGBP1 increases MEF2A levels. UV cross-link assay with full-length MEF2A mRNA showed that CUGBP1 binds directly to this mRNA and that the affinity of this interaction is similar to that for RNA probe containing CUG123 (Fig. 5A). CUGBP1 binds to CAG repeats within MEF2A mRNA, since the deletion of these repeats abolishes the interaction. To confirm the specificity of the interaction, cold MEF2A mRNA and (CAG)10 and AU-rich RNA oligomers were incorporated into the binding reactions. As can be seen in Fig. 5A, MEF2A and CAG10 compete for the binding, whereas AU-rich oligomer does not.

We next determined which RNA-binding domains (RBDs) of CUGBP1 bind to MEF2A mRNA. UV cross-link analysis indicates that full-length CUGBP1 and RBD3 bind to MEF2A mRNA, whereas truncated forms of CUGBP1 containing RBD1–2 and RBD2 do not interact with MEF2A mRNA (Fig. 5B). Given the in vitro interaction of CUGBP1 with MEF2A mRNA, we asked whether this interaction takes place in vivo. For this goal, we immunoprecipitated CUGBP1 from differentiated C2C12 myotubes and examined the presence of MEF2A mRNA in CUGBP1 IPs using the reverse transcriptase-PCR procedure. In these experiments, we applied primers that cover the GCA repeat within MEF2A mRNA and produce a 743-kb DNA fragment. As can be seen, MEF2A mRNA is detectable in CUGBP1 IPs, whereas MEF2A mRNA is not observed in control precipitates with agarose only (Fig. 5C). Thus, these studies revealed that CUGBP1 interacts with MEF2A mRNA in vitro and in vivo.

CUGBP1 Increases Translation of MEF2A in a Cell-free Translation System and in Cultured Cells—We next examined whether the interaction of CUGBP1 with MEF2A mRNA affects expression of MEF2A in a cell-free translation system. As can be seen in Fig. 6A, CUGBP1 increases protein levels of MEF2A in the cell-free translation system programmed with the wild type full-length MEF2A mRNA. Deletion of GCA repeats (CUGBP1 binding site) diminishes binding of CUGBP1 to MEF2A mRNA (Fig. 5A) and leads to a failure of CUGBP1 to induce the translation of mutant MEF2A mRNA (Fig. 6A). Thus, we conclude that CUGBP1 increases the translation of
MEF2A through the interaction with GCA repeats located within MEF2A mRNA.

We next examined whether CUGBP1 increases MEF2A expression in cultured cells using two approaches: transient transfection assay and a stable clone line containing antisense CUGBP1 mRNA under Lac repressor control (21). The stable clone line contains the inducible antisense CUGBP1 mRNA under control of the Lac repressor. Examination of MEF2A protein levels after the addition of isopropyl-1-thio-/beta-D-galactopyranoside revealed that the inhibition of CUGBP1 causes the reduction in the protein levels of MEF2A (Fig. 6B). To confirm these observations, His-CUGBP1 was transfected into C2C12 cells, and levels of MEF2A were determined by Western blotting. Fig. 6C shows that the levels of His-CUGBP1 expressed from the transfected plasmid are significantly higher than the levels of endogenous CUGBP1 protein. In cells overexpressing CUGBP1, protein levels of MEF2A are dramatically induced. Densitometric analysis of multiple experiments demonstrated an 8–10-fold induction of MEF2A by CUGBP1 as a ratio to /beta-actin. Immunostaining of transfected cells also revealed that protein levels of MEF2A are dramatically increased in cells overexpressing GFP-CUGBP1 (Fig. 6C, right). This effect is specific to GFP-CUGBP1, since GFP alone does not increase MEF2A levels. Thus, studies in the cell-free translation system and in cultured cells show that CUGBP1 increases protein levels of MEF2A.

Although data in a cell-free translation system suggest that CUGBP1 increases translation of MEF2A, the CUGBP1-mediated increase of MEF2A levels in transfected cells could be also due to a stabilization of MEF2A protein. To examine this possibility, we determined the half-life of MEF2A protein in C2C12 cells transfected with CUGBP1 or with empty vector as the control. Experiments with cyclohexamide block of protein synthesis showed that MEF2A is a very stable protein and that...
CUGBP1 does not affect MEF2A stability (Fig. 6D). We then analyzed whether CUGBP1 increases MEF2A protein translation by measuring newly synthesized radioactive MEF2A protein. C2C12 cells were co-transfected with MEF2A and CUGBP1 or with empty vector, [35S]Met was added, and [35S]MEF2A was precipitated and analyzed by gel electrophoresis. In cells transfected with CUGBP1, the incorporation of [35S]Met into MEF2A (translation) was significantly greater than the incorporation of [35S]Met in cells transfected with the empty vector at each time point analyzed in our studies (Fig. 6E). Taken together, these investigations show that CUGBP1 increases translation of MEF2A mRNA in a cell-free translation system and in cultured cells.

CUGBP1 Enhances the MEF2A-dependent Myogenic Conversion—The analysis of MEF2A under conditions of overexpression of CUGBP1 showed that CUGBP1 increases translation of MEF2A. We next tested the hypothesis that CUGBP1 might regulate MEF2A during a differentiation course of C2C12 myoblasts. Northern analysis showed that the levels of two major isoforms of CUGBP1 mRNA (7 and 9 kb) are 3–6-fold elevated at days 3 and 5 of differentiation (Fig. 7A). Western analysis revealed that protein levels of CUGBP1 are also elevated in cytoplasm (Fig. 7B, left) and in nuclei of differentiating C2C12 cells (data not shown). Analysis of p21 and MEF2A expression in the same cell extracts shows an increase of the protein levels of MEF2A and p21, which correlates with the induction of CUGBP1 (Fig. 7B, left). We next examined whether CUGBP1 interacts with MEF2A mRNA in differentiated myotubes. CUGBP1 was immunoprecipitated from cytoplasm with monoclonal antibodies, and CUGBP1 IPs were examined by reverse transcriptase-PCR with primers specific to MEF2A mRNA. The position of a PCR product (743 bp) is shown. Right, inhibition of CUGBP1 by siRNA reduces levels of MEF2A and p21 in differentiating C2C12 myoblasts. C, DM1 cells fail to induce MEF2A during differentiation. Left, MEF2A Western blotting with protein extracts isolated from control cells and from DM1 cells growing in differentiation medium for 0, 3, and 5 days is shown. The membrane was reprobed with β-actin antibodies. Right, 4′,6-Diamidino-2-phenylindole (DAPI) staining of normal and DM1 myoblasts and myotubes is shown. D, CUGBP1 enhances MEF2A-dependent myogenic conversion. Left, fibroblasts were co-transfected with MyoD or wild type or mutant MEF2A and CUGBP1. Cells were subjected to double immunostaining with antibodies against MyoD (red) and myosin (green). Right, bar graph shows a summary of three independent experiments.

**Fig. 7.** CUGBP1 increases levels MEF2A during myoblast differentiation. **A**, CUGBP1 mRNA is increased in C2C12 cells at later stages of differentiation. The upper panel shows CUGBP1 mRNA levels. Ethidium bromide staining of the gel is shown in the middle. The bottom panel shows the reprobe of the same filter with 18 S rRNA probe. **B**, protein levels of CUGBP1 and MEF2A are increased during differentiation of C2C12 cells. Left, Western blotting was performed with proteins isolated from C2C12 cells at days 0, 3, and 5 after the initiation of differentiation. The membrane was reprobed with Abs to β-actin, and MEF2A levels were calculated as ratios to β-actin. Antibodies to MEF2 (C-21; Santa Cruz Biotechnology) in addition to MEF2A recognize MEF2C. CUGBP1-IP. MEF2A mRNA is associated with CUGBP1 after initiation of differentiation. CUGBP1 was precipitated from cytoplasm with monoclonal antibodies, and CUGBP1 IPs were examined by reverse transcriptase-PCR with primers specific to MEF2A mRNA. The position of a PCR product (743 bp) is shown. Right, inhibition of CUGBP1 by siRNA reduces levels of MEF2A and p21 in differentiating C2C12 myoblasts. C, DM1 cells fail to induce MEF2A during differentiation. Left, MEF2A Western blotting with protein extracts isolated from control cells and from DM1 cells growing in differentiation medium for 0, 3, and 5 days is shown. The membrane was reprobed with β-actin antibodies. Right, 4′,6-Diamidino-2-phenylindole (DAPI) staining of normal and DM1 myoblasts and myotubes is shown.
CUGBP1 as a Translational Regulator of Myogenesis

DISCUSSION

Proper myogenesis requires an orchestrated cascade of expression of muscle specific genes; certain proteins should be expressed at certain stages of muscle development and at certain levels. Such programmed expression of muscle genes is controlled at different levels, including the regulation of transcription and post-transcriptional control. Protein levels of CUGBP1 and RNA binding activity of CUGBP1 are elevated in DM1 patients by expansion of CUG RNA repeats (12, 13, 19–21). In this paper, we examine the role of CUGBP1 elevation in DM1 muscle pathology using transgenic mice. The analysis of CUGBP1 transgenic mice shows that the overexpression of CUGBP1 above biological levels is sufficient to disrupt myogenesis in vivo. We found a strong correlation between levels of CUGBP1 elevation and severity of muscle defects. Muscle in mutant mice with 2–3 fold elevation of CUGBP1 shows mild variability in fiber size and increase of internal nuclei only at 1.5–2.0 years of age. Histological muscle phenotype in these mice is similar to that observed in DM1 patients (adult form of DM1) during earlier changes of disease progression. In skeletal muscle of mice with greater increase of CUGBP1 (4–6-fold, line 49), a larger number of cells was variable in size with accumulation of internal nuclei. Comparison of two transgenic lines shows that mutant mice in line 49 develop muscle histopathology earlier in life (6–8 months). Elevation of CUGBP1 up to 8-fold causes immaturity of myofibers: central nuclei, increase of nuclei number, shortening of fibers, and reduction in myofiber density. These mice do not survive. Such a phenotype is partially reminiscent of congenital DM1, since immaturity of fibers and fibers with centrally positioning nuclei were found in congenital DM1. The phenotype in this line is stronger than one in congenital DM1, perhaps because the elevation of CUGBP1 in this line is higher than in congenital patients that we analyzed.

Another important similarity between muscle abnormalities in DM1 and in CUGBP1 mice is a myofiber-type switch: increase of slow fibers and reduction of fast fibers. A similar change in fiber type composition can be seen in experimental mice affected with myotonia (29), which is a hallmark of DM1. Elevation of MEF2A in CUGBP1 transgenic mice is consistent with recent data from Dr. Olson’s laboratory indicating that MEF2 activity is elevated in slow fibers in mouse muscles with myotonia (29). In these myotonic mice, ~80% of fibers are oxidative or slow. The elevation of MEF2 activity in muscle of myotonic mice clearly indicates that hyperexcitability of myotonic muscle is associated with up-regulation of MEF2, resulting in increase of slow fibers.

A number of proteins have been shown to play a key role in differentiation and development of skeletal muscle. One of these proteins, p21, in collaboration with p57 is involved in the irreversible growth arrest of skeletal muscle cells (30). Our data from CUGBP1 transgenic mice demonstrate that, in vivo, elevation of CUGBP1 causes an increase of p21 accompanied by delay of muscle development and dystrophic histopathology in muscle. Since CUGBP1 increases translation of p21 in cultured cells (12), it is likely that translation of p21 is also induced in skeletal muscle of DM1 patients. On the other hand, alterations in p21 mRNA may also contribute to increased levels of p21 protein. What is a possible mechanism by which increase of p21 might delay myogenesis in DM1 and in CUGBP1 transgenic mouse muscles? It has been recently shown that p21 may play opposite roles in the regulating cell proliferation: 1) arrest of proliferation by inhibiting Cdk2-cyclin E and 2) promotion of proliferation by stimulating the assembly of active Cdk4-cyclin D1 complexes (31). Therefore, elevated levels of p21 in DM1 tissues might lead to both inhibition and promotion of proliferation depending on the protein environment at the cell cycle stage when p21 is elevated. Since differentiation requires a tightly regulated cascade of gene expression and precise amounts of the proteins, we suggest that CUGBP1-mediated overexpression of p21 in DM1 tissues inhibits differentiation of skeletal muscle.

Myogenesis is also regulated by myogenic basic helix-loop-helix transcription factors (MyoD, Myf-5, myogenin, and

![CUGBP1 as a Translational Regulator of Myogenesis](http://www.jbc.org/)
MFR4) and their co-activators, MEF2 factors. An increase of myogenic transcription factors and their co-activators is required for the activation of myogenesis. In CUGBP1 transgenic mice, however, an increase of MEF2A and myogenin is associated with a delay of myogenesis. We hypothesize that such a paradox occurs because of misregulation of myogenic transcription factors. For proper myogenesis, these factors should be regulated in a very precise manner. However, forced increase of CUGBP1 in mice might result in unscheduled activation of myogenic factors at the wrong time of development, delaying myogenesis. It is interesting to note that both deletion and overexpression of myogenin in mice leads to neo- or postnatal death (32, 33), showing that normal development requires a tight regulation of myogenin.

A similar phenomenon has also been described for MEF2A. Since MEF2A activates transcription of many muscle proteins, precise timing and levels of MEF2A expression are required to provide distinct levels of specific proteins at the correct stage of muscle development. Such regulation has been earlier described for MEF2A. It has been found that the 3'-untranslated region of MEF2A mRNA is responsible for regulating its own mRNA translation during differentiation (34). This regulation leads to low levels of MEF2A in proliferating myoblasts via inhibition of MEF2A translation and high levels of MEF2A in differentiating myoblasts due to lack of repression of MEF2A translation. Another publication also found that proper regulation of MEF2A levels is required for differentiation (35). In this paper, the authors show that too little or too much MEF2A activity leads to the same result: disruption of differentiation. Taking into account these observations, we suggest that too much CUGBP1 activity in CUGBP1 transgenic mice delays myogenesis.

CUGBP1 is located in nuclei and cytoplasm and is able to regulate both RNA splicing and mRNA translation. In this paper, we focused our studies on the translational function of CUGBP1. Based on our observations, we suggest a hypothetical pathway by which the cytoplasmic portion of CUGBP1 contributes to DM1 pathology in skeletal muscle (Fig. 8). We hypothesize that in normal differentiating cells, CUGBP1 causes an increase of MEF2A and p21 at the correct time. In DM1 patients, basal overexpression of CUGBP1 caused by CUG repeats leads to overall overexpression of MEF2A and p21 at the correct time. In this paper, we focused our studies on the translational function of CUGBP1. Based on our observations, we suggest a hypothetical pathway by which the cytoplasmic portion of CUGBP1 contributes to DM1 pathology in skeletal muscle (Fig. 8). We hypothesize that in normal differentiating cells, CUGBP1 contributes to DM1 pathology in skeletal muscle (Fig. 8). 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Overexpression of CUG Triplet Repeat-binding Protein, CUGBP1, in Mice Inhibits Myogenesis

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