CELF6, a Member of the CELF Family of RNA-binding Proteins, Regulates Muscle-specific Splicing Enhancer-dependent Alternative Splicing\textsuperscript{*}

Andrea N. Ladd\textsuperscript{‡}, Nicole H. Nguyen, Kavin Malhotra, and Thomas A. Cooper\textsuperscript{§}

From the Department of Pathology, Baylor College of Medicine, Houston, Texas 77030

Received for publication, September 26, 2003, and in revised form, January 30, 2004
Published, JBC Papers in Press, February 3, 2004, DOI 10.1074/jbc.M310687200

We previously described a family of five RNA-binding proteins: CUG-binding protein, embryonic lethal abnormal vision-type RNA-binding protein 3, and the CUG-binding protein and embryonic lethal abnormal vision-type RNA-binding protein 3-like factors (CELFs) 3, 4, and 5. We demonstrated that all five of these proteins specifically activate exon inclusion of cardiac troponin T minigenes in vivo via muscle-specific splicing enhancer (MSE) sequences. We also predicted that a sixth family member, CELF6, was located on chromosome 15. Here, we describe the isolation and characterization of CELF6. Like the previously described CELF proteins, CELF6 shares a domain structure containing three RNA-binding domains and a divergent domain of unknown function. CELF6 is strongly expressed in kidney, brain, and testis and is expressed at very low levels in most other tissues. In the brain, expression is widespread and maintained from the fetus to the adult. CELF6 activates exon inclusion of a cardiac troponin T minigene in transient transfection assays in an MSE-dependent manner and can activate inclusion via multiple copies of a single element, MSE2. These results place CELF6 in a functional subfamily of CELF proteins that includes CELFs 3, 4, and 5. CELF6 also promotes skipping of exon 11 of insulin receptor, a known target of CELF activity that is expressed in kidney.

Alternative splicing allows the production of multiple mRNA species from a single gene, which often give rise to functionally distinct protein isoforms. Regulation of alternative splicing not only produces multiple mRNAs but can also modulate the levels of these different isoforms in a tissue- or developmental stage-specific manner to meet the functional needs of the cell. Numerous examples of regulated alternative splicing have been found, but few regulatory factors that control cell-specific alternative splicing have been identified. In the best-characterized vertebrate experimental systems, alternative splicing regulation is the result of dynamic antagonism between trans-acting factors binding to positive or negative elements in the pre-mRNA and promoting or repressing the use of alternative splice sites. Some of these elements bind cell-specific splicing factors. For example, neuron-specific inclusion of alternative exons in several pre-mRNAs is mediated by binding of the activator Nova-1, which is expressed only in neurons. This neuron-specific activity is antagonized by binding of the ubiquitously expressed repressor polypyrimidine tract-binding protein (PTB) in non-neuronal cells (1).

The muscle-specific alternative exon 5 of cardiac troponin T (cTNT)\textsuperscript{1} is also regulated by antagonistic activities. Muscle-specific splicing enhancers (MSEs) in cTNT pre-mRNAs regulate inclusion of exon 5 in striated muscle by binding to activators and repressors of splicing. Negative elements within MSEs upstream and downstream of exon 5 repress exon inclusion in non-muscle cells by binding PTB (2). This repression is antagonized in muscle cells by positive elements within MSEs downstream of exon 5 that bind members of the CUG-binding protein (CUG-BP) and embryonic lethal abnormal vision-type RNA-binding protein 3 (ETR-3)-like factor (CELF) family, promoting exon inclusion (2, 3). We previously described five members of the CELF family, all of which activated MSE-dependent exon inclusion of cTNT minigenes in fibroblasts (3). Expression of CELF proteins is widespread, although individual members are preferentially expressed in different cell types and developmental stages (3).

Disruption of CELF function may play a role in disease states. CUG-BP has been implicated in the pathogenesis of myotonic dystrophy (4), a neuromuscular disease caused by expansion of an unstable CTG repeat in the 3' untranslated region (UTR) of the DMPL gene (5–7) or a CCTG repeat in the first intron of the ZNF9 gene (8). The splicing of targets of CUG-BP is misregulated in myotonic dystrophy and contributes to insulin resistance (9) and myotonia (10), two clinical manifestations of the disease. Another CELF family member, ETR-3, has also recently been implicated in the misregulation of splicing in Duchenne and Becker muscular dystrophies (11) and is a candidate gene for defects associated with partial monosomy 10p (12) and familial arrhythmogenic right ventricular dysplasia (13). Thus, CELF proteins are important regulators of cell-specific alternative splicing during normal development and disease.

Here we describe a sixth member of the CELF family, CELF6. CELF6 is preferentially expressed in kidney, testis, and brain. Like the previously described CELF proteins, CELF6 activates exon inclusion in fibroblasts via MSEs in

\textsuperscript{*} This work was supported in part by grants (to T. A. C.) from the Muscular Dystrophy Association and National Institutes of Health (Grant AR45653). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{‡} Supported by a postdoctoral National Research Service Award fellowship from NIAMS, National Institutes of Health.

\textsuperscript{§} To whom correspondence should be addressed: Dept. of Pathology, Baylor College of Medicine, One Baylor Plaza, Rm. 268B, Houston, TX 77030. Tel.: 713-798-3141; Fax: 713-798-5838; E-mail: tcooper@bcm.tmc.edu.

\textsuperscript{1} The abbreviations used are: cTNT, cardiac troponin T; CUG-BP, CUG-binding protein; ETR-3, embryonic lethal abnormal vision-type RNA-binding protein 3; CELF, CUG-BP and ETR-3-like factor; MSE, muscle-specific splicing enhancer; UTR, untranslated region; IR, insulin receptor; ORF, open reading frame; RRM, RNA recognition motif; DM, myotonic dystrophy; EST, expressed sequence tag; RNP, ribonucleoprotein; PTB, polypyrimidine tract-binding protein.
transient transfection assays. CELF6 can also activate inclusion via multiple copies of a single element, MSE2, placing it in a functional subfamily of CELF proteins that includes CELFs 3, 4, and 5. Finally, CELF6 promotes skipping of exon 11 in insulin receptor (IR), another known target of CELF activity.

MATERIALS AND METHODS
Identification of CELF6 Sequence—CELF6 was first identified in cosmids from chromosome 15 (3) and predicted from high throughput genomic sequence (Ref. 14; GenBank™ accession number AF401233). CELF6 cDNAs were amplified by polymerase chain reaction (PCR) from human adult brain cDNA (Clontech) as described previously (3). Primer sequences were ATACATTGATCCATGGCGGCGAGCAGGCGCGGGGGAGGGT and AATCGATCTCCAGTCAAGCCCGGTTGGGATATCC. PCR products were cloned into the pCR-Blunt II-TOPO plasmid using the ZeroBlunt TOPO Cloning Kit (Invitrogen), cut with EcoRI, and gel isolated using the Qiaquick Gel Extraction Kit (Qiagen). CELF6 cDNAs were sequenced full-length CELF6 cDNAs amplified by PCR were cloned into the pcDNA3.1HisC vector (Invitrogen) in-frame with the N-terminal Xpress epitope tag, and sequences were confirmed by overlapping reads of both strands.

Sequence profile and phylogenetic analyses were performed as described previously (3).

Northern and Dot Blots—Commercial Northern and RNA dot blots (Clontech) were hybridized against a CELF6 3’-UTR probe generated by PCR from human adult brain cDNA (Clontech) using the primers TGACCTGGTTTCACTGCGCAG and ATGCTCTTGGTCTCCTAAACCTC. 3’-UTR probes were likewise generated for CELF3 (GTCTGGGAGACTCCAGAGGAA and AAACCCCTAATGTGGGGAAGA), CELF4 (ACGCACATCGCCGTAAGGAC and CATCTTCTCTTCATGCTGATT), and CELF5 (TGCTCCCATCCTCGTTCTTG) and TAAATCATCACCATGCCTGCTC). The PCR products were cloned into the pCR-Blunt II-TOPO plasmid using the ZeroBlunt TOPO Cloning Kit (Invitrogen), CUG-BP was subcloned into the pcDNA3.1 vector (Invitrogen) in-frame with the N-terminal Xpress epitope tag, and CUG-BP was excised with HincII and BstBI. ETR-3 was cloned into the pcDNA3.1(+) vector (Invitrogen) from a CUG-BP/Nab50 plasmid provided by L. Timchenko (Baylor College of Medicine), and a 3’-UTR probe was excised from a phage clone provided by Dr. C. C. Liew (University of Toronto, Toronto, Ontario, Canada), and a 3’-UTR probe was excised with PvuII and NotI. Probes were end-labeled using Random Primed Labeling Kit (Amersham Biosciences). Hybridization was performed at 68 °C in Express Hyb solution (Ambion).

Cotransfection Experiments—R35C, RTBPSRAX, and M2/M2TB minigenes have been described previously (3, 15). IR minigenes have also been described previously (16) and were used previously to demonstrate regulation of IR alternative splicing by CUG-BP (9). QT35 quail fibroblasts were plated at a density of 1.8 × 10^5 cells/60-mm-diameter tissue culture dish in 3 ml of medium (F10 medium supplemented with 5% fetal bovine serum, 1% chick serum, 10% tryptose phosphate, and 2 mM L-glutamine) and cultured overnight at 37 °C in 5% CO₂, COS-M6 cells were plated in 3 ml of high-glucose DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cells were transfected with 100 ng of minigene DNA, 0–3 μg of CELF6 expression plasmid, and carrier DNA to 3.1 μg of total DNA using FuGENE 6 (Roche Applied Science). Total RNA and protein were harvested 48 h after transfection and subjected to reverse transcription-PCR and Western blotting as described previously (3), except that the amount of oligo(dT)12–18 primer used to produce cDNA was increased to 100–300 ng/reaction.

Statistical Analysis—The mean values for the extent of exon inclusion accompanying each dose of CELF6 expression plasmid were compared with the means of values obtained from the minigenes alone using a two-tailed pooled F test assuming that the population variances are equal; to confirm that this assumption was valid, preliminary F tests were conducted where the α level was set at α = 0.2.

RESULTS
Identification of CELF6—We previously reported the identification of a family of five RNA-binding proteins called CELF proteins. We identified a sixth family member, CELF6, as homologous segments in cosmids sequences from chromosome 15, but no corresponding expressed sequence tags (ESTs) were available at that time (3). CELF6 (also called BRUNOL6) was also predicted from conceptual translation of a potential cDNA sequence that uses an alternative 3’ splice site in exon 9 that introduces a frameshift altering the C-terminal portion of the divergent domain and removing RRM3.
FIG. 2. CELF6 is closely related to other CELF proteins. A, evolutionary relationship of human CELF proteins. Sequence comparisons indicate that CELF6 belongs to a subgroup of the CELF family that includes CELFs 3, 4, and 5. B, an alignment of ORP1 with other CELF family members is shown. Conserved nonpolar amino acids are indicated in blue, conserved uncharged polar residues are indicated in red, conserved positively charged residues are indicated in green, conserved negatively charged residues are indicated in gold, and non-conserved residues are indicated in black.
derived from high-throughput genomic sequence (14). To obtain the actual coding region of CELF6, we amplified full-length CELF6 from human brain cDNA by PCR using primers that contained the predicted start and stop codons based on our analysis of chromosome 15 cosmids sequences. We used the sequences of the four different cDNAs obtained (Fig. 1B) to BLAST updated EST data bases and aligned our sequences, the matching ESTs, and human genomic sequence to determine the exon structure for CELF6 (Fig. 1A). CELF6 is composed of 14 exons, including a novel exon (exon 3) not previously predicted. Coding sequence ends less than 30 nucleotides upstream of the exon 13-exon 14 junction, and most of the CELF6 3′-UTR lies within exon 14.

Four CELF6 open reading frames (ORFs) generated by alternative splicing were identified (Fig. 1B). Translation of ORF1 gives rise to a full-length CELF protein that matches previously reported predicted translations (GenBank™ accession numbers AF401233 and AF425606). The domain structure of CELF6 is the same as that of other CELF family members (3): three RNP-containing RNA recognition motifs (RRMs) and a 268-amino acid divergent domain separating RRM2 and RRM3. CELF6 also possesses numerous potential phosphorylation sites, a potential nuclear localization signal at the C terminus, and an alanine-rich region within the divergent domain. By retention of an intron, inclusion of an alternative exon, or use of an alternative splice site in exon 9, ORFs 2–4 contain upstream termination codons (Fig. 1B). The retention of intron 5 in ORF2, which is otherwise identical to ORF1, introduces a frameshift that truncates RRM2 upstream of RNP1, one of two highly conserved sequences required for the RNA binding activity of RRM3 (17). ORF3 skips exon 10 but includes an upstream exon (exon 3) that contains an in-frame stop codon. ORF4 lacks exon 11 and uses an alternative 3′ splice site in exon 9 that introduces an upstream frameshift, altering the C-terminal portion of the divergent domain and removing RRM3.

Previous comparative analysis of human CELF proteins suggested that the CELF family can be divided into two subfamilies, the first containing CUG-BP and ETR-3 and the second containing CELFs 3, 4, and 5 (3). To determine how CELF6 is related to other CELF proteins, we compared full-length CELF6 (ORF1) with the other members of the CELF family (Fig. 2). CELF6 is more closely related to CELFs 3, 4, and 5 than CUG-BP and ETR-3, indicating that it falls within the second subfamily of CELF proteins.

**CELF6 and Other Members of the CELF Family Are Broadly Expressed**—To examine the expression of CELF6, commercial Northern blots containing RNAs from adult human tissues were hybridized against a 3′-UTR probe. As shown in Fig. 3, CELF6 is preferentially expressed in kidney and brain. A large mRNA of approximately 7.5 kilobases (kb) is abundant in kidney, whereas a smaller mRNA of approximately 4 kb is expressed in brain and at lower levels in kidney. Both of these mRNA isoforms are detectable at very low levels in several other tissues. The differentially spliced regions in ORFs 1–4 are not large enough to explain the difference in size between the two CELF6 mRNA isoforms seen by Northern blotting. Size differences of this magnitude between different mRNA isoforms are usually attributable to differences in polyadenylation. Indeed, there are numerous human CELF6 ESTs that contain 3′-UTR sequences that extend beyond the polyadenylated 3′ end of the published CELF6 mRNA sequence (GenBank™ accession number AF401233), suggesting that a downstream polyadenylation site is sometimes used.

To examine CELF6 expression in more detail, a commercial RNA dot blot containing a greater variety of human adult as well as fetal tissues was hybridized with the 3′-UTR probe (Fig. 4A). This blot confirmed that the highest levels of CELF6 expression are in adult kidney, brain, and testis. Low levels were observed in most other tissues, although expression was not detected in several cell lines. Within the brain, CELF6 expression is widespread, being observed in all regions of the brain tested (see Fig. 4A: A, H; B, H; and 3, B and C). In fetal tissues, CELF6 expression was highest in brain but was also detectable in kidney, although at levels lower than those seen in the adult.

To develop profiles of CELF mRNA expression in these tissues, the CELF6 dot blot was compared with those of the five other CELF family members (Ref. 3 (data not shown) and Fig. 4B herein). Like CELF6, CUG-BP, ETR-3, and CELF4 are broadly expressed. CUG-BP is strongly expressed in all adult and fetal tissues tested. ETR-3 is detectable in all tissues at some level, but ETR-3 expression is highest in brain, heart, and thymus. CELF4 is highly expressed throughout the brain and in glandular tissues; moderately expressed in heart, skeletal muscle, and liver; and detectable at very low levels in all other tissues tested. CELF3 and CELF5 are restricted to brain, where they are expressed at varying levels in different regions (see Fig. 4B: A, 1–8; B, 1–6; D, 4; and G, 1). The patterns of CELF3 and CELF5 expression are nearly identical, except in pituitary gland, where CELF3 is strongly expressed, whereas CELF5 is almost undetectable.

**CELF6 Positively Regulates MSE-dependent Splicing in Vivo**—We have shown previously that members of the CELF family bind to MSEs and activate cTNT exon inclusion in an MSE-dependent manner (3). To determine whether the different ORFs of CELF6 can mediate cTNT exon inclusion, the four CELF6 ORFs were subcloned into expression vectors and co-transfected with the R35C minigene into QT35 quail fibroblast cells. R35C contains a heterologous alternative exon flanked by cTNT MSEs 1–4 and gives rise to predominantly exon-skipped

![Fig. 3. CELF6 is preferentially expressed in kidney and brain.](https://example.com/fig3.png)
A. Dot blot analysis of all six family members indicates that CELF proteins are widely expressed.  

|     | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A   | Y   | N   | Y   | N   | Y   | N   | N   | N   | N   | N   | N   | Y   |
| B   | N   | Y   | N   | N   | Y   | N   | Y   | N   | Y   | N   | N   | N   |
| C   | N   | N   | Y   | Y   | N   | Y   | N   | N   | N   | N   | N   | N   |
| D   | N   | Y   | N   | N   | Y   | N   | Y   | N   | N   | N   | N   | N   |
| E   | Y   | N   | Y   | Y   | N   | Y   | N   | N   | N   | N   | N   | N   |
| F   | Y   | N   | Y   | Y   | N   | Y   | N   | N   | N   | N   | N   | N   |
| G   | Y   | N   | Y   | Y   | N   | Y   | N   | N   | N   | N   | N   | N   |
| H   | Y   | N   | Y   | Y   | N   | Y   | N   | N   | N   | N   | N   | N   |

B. RNA dot blot of human adult and fetal tissues was hybridized with the CELF6 3'UTR probe. RNA dot blots of human adult and fetal tissues were hybridized with 3'UTR probes for the remaining CELF family members.

**Fig. 4.** Dot blot analysis of all six family members indicates that CELF proteins are widely expressed. 

A. RNA dot blot of human adult and fetal tissues was hybridized with the CELF6 3'UTR probe. B. RNA dot blots of human adult and fetal tissues were hybridized with 3'UTR probes for the remaining CELF family members.
mRNAs in fibroblasts, although exon inclusion is promoted in the presence of exogenous (3) or endogenous (2) CELF proteins. Western blots probed with antibodies against the N-terminal epitope tag confirmed that ORF1, ORF2, and ORF4 constructs all expressed proteins of the expected sizes; ORF3 was not detected at any dose on Western blots (Fig. 5). The ORF3 expression construct was confirmed by sequencing both strands; therefore, we conclude that the expressed protein is unstable. All three of the CELF6 ORFs that expressed detectable protein increased the level of exon inclusion in a dose-dependent manner (Fig. 5A). ORF3 did not affect the level of exon inclusion, consistent with the absence of detectable protein.

To determine whether enhanced exon inclusion by CELF6 is MSE-dependent, the CELF6 expression plasmids were cotransfected with the RTBPSRAX minigene containing the same alternative exon flanked by human β-globin intron 1 sequences that lack MSEs. None of the ORFs significantly enhanced exon inclusion in the absence of MSEs (Fig. 5B).

**CELF6 Promotes Exon Inclusion via MSE2 Alone**—CELFs 3, 4, and 5 are all able to promote exon inclusion via multiple copies of a single element, MSE2, but CUG-BP and ETR-3 cannot (3). To determine whether CELF6 can promote exon inclusion via MSE2 alone, ORFs 1–4 were cotransfected with the M2/M2TB minigene, which contains a 52-nucleotide alternative exon flanked on either side by three copies of MSE2. As before, Western blots confirmed expression of ORF1, ORF2, and ORF4, but not ORF3, in these experiments (Fig. 6). ORF1, ORF2, and ORF4 all promoted exon inclusion (Fig. 6), placing CELF6 in the functional subfamily suggested by comparison of CELF protein sequences (Fig. 2A).

**CELF6 Promotes Skipping of IR Exon 11**—In addition to regulating cTNT exon 5 inclusion, CUG-BP has been shown to promote skipping of the alternative exon 11 of IR (9). To determine whether CELF6 also promotes IR exon 11 skipping, ORFs 1, 2, and 4 were cotransfected with the IR-N minigene into COS-M6 cells (Fig. 7A). COS-M6 cells were used for these experiments because the basal level of IR exon 11 inclusion in QT35 cells is already ~10% (data not shown). ORF3 was not included in this experiment because ORF3 protein is not expressed at detectable levels at any dose. Western blots confirmed ORF1, ORF2, and ORF4 protein expression (Fig. 7). Like CUG-BP, the full-length CELF6 ORF1 promoted IR exon 11 skipping in a dose-dependent manner (Fig. 7A). ORF4 also promoted exon skipping, al-
though to a much lesser extent than ORF1, despite expressing protein levels at least comparable with those seen at 1 µg of ORF1 expression plasmid. ORF2, in contrast, had no effect on the level of exon inclusion at any dose.

A 1.8-kb region within IR that is required for responsiveness to CUG-BP and contains a CUG-BP binding site has been identified (9). To determine whether reduced exon 11 inclusion by CELF6 is also dependent on this region, CELF6 ORF1, ORF2, and ORF4 expression plasmids were cotransfected into COS-M6 cells with the IR-E minigene lacking this region (Fig. 7B). Interestingly, CELF6 ORF1 and ORF4 both promote exon skipping, despite the lack of the CUG-BP regulatory region, suggesting that CELF6 acts through different sequences than CUG-BP on some substrates. The response of the IR-E minigene to ORF4 was greater than the response to ORF1, although this may be attributable to the higher level of protein expressed for ORF4 relative to ORF1 in this set of experiments.

**DISCUSSION**

A New Member of the CELF Family—In this study, we isolated cDNAs and performed functional analysis of protein isoforms of CELF6 (also called BRUNOL6), a sixth member of the CELF family whose existence was previously only predicted (3, 14). Four CELF6 open reading frames generated by alternative splicing were found, one of which includes a novel exon, exon 3. Although ORFs 2–4 encode truncated CELF6 proteins, and the proteins from ORF2 and ORF4 regulate splicing, it is not clear whether any of these proteins are made in vivo. The premature termination codons introduced in all three ORFs lie more than 50 nucleotides upstream of an exon-intron junction and thus would likely lead to destruction of these mRNAs via the nonsense-mediated decay pathway (18). Thus, alternative splicing of CELF6 could lead to down-regulation of its expression. The relative abundance of the four CELF6 mRNA isoforms is unknown because they were isolated by non-quantitative PCR. It is possible that CELF6 undergoes differential alternative splicing in a tissue-specific or developmental stage-specific manner as a means of regulating levels of CELF6 protein.

Interestingly, two of the truncated proteins, ORFs 2 and 4, promoted MSE-dependent exon inclusion to levels similar to those promoted by the full-length CELF6 when expressed at high levels in transient transfection assays. Furthermore, ORF4 (but not the shorter ORF2) promoted exon 11 skipping in IR minigenes. Regardless of whether the truncated proteins are naturally expressed, this suggests that the C terminus is not required to influence alternative splicing. Consistent with this result, it has been reported that another CELF protein, ETR-3 (also called BRUNOL3), binds to RNA via the first two RRRMs (14). Furthermore, deleitional analysis of human ETR-3 and CELF4 indicate that the two N-terminal RRRMs plus a small portion of the adjacent divergent domain are sufficient for full activity (19). Although the C terminus of CELF6 is not required for activity, this does not mean that it does not normally contribute to its function. Deletion mutants of ETR-3 consisting of only the C-terminal RRM plus a portion of the adjacent divergent domain are also active (19), suggesting that the C termini of CELF proteins may also participate in splicing regulation.

**CELF-mediated Regulation of Alternative Splicing in Kidney and Brain**—Although CELF6 can activate splicing of cTNT minigenes, CELF6 is not expressed at high levels in striated muscle and thus is unlikely to be a key regulator of endogenous cTNT alternative splicing. CELF6 also promoted exon skipping in minigenes of IR, however, which is endogenously expressed at high levels in kidney and brain (20). The pattern of CELF6 expression suggests that it plays a role in regulating alternative splicing in kidney and brain. Members of the CELF family have distinct but overlapping patterns of expression, and every tissue examined to date has been shown to express at least one member of the CELF family (Fig. 4; Ref. 3), suggesting that regulation of alternative splicing by CELF proteins is widespread. In the kidney, CELF6 may be the primary regulator of CELF-mediated alternative splicing events. By RNA dot blot,
CELF3 and CELF5 expression is restricted to brain, and although other CELF mRNAs are detectable in kidney, by Western blot ETR-3 and CELF4 proteins are expressed at very low levels in kidney, whereas CUG-BP is not detectable at all (3).

In contrast, all six known members of the CELF family are expressed in brain (Fig. 4; Ref. 3). Regulation of alternative splicing in brain has been functionally linked to at least one member of the CELF family. ETR-3 has been implicated in the regulation of brain region-specific alternative splicing of exons 5 and 21 of the N-methyl-D-aspartate receptor (NMDA R1) in the rat (21). ETR-3 promotes NMDA R1 exon 21 inclusion and represses exon 5 inclusion in cotransfection assays, and high levels of endogenous exon 21 inclusion and exon 5 skipping correlate with high ETR-3/CUG-BP expression in rat forebrain. Whereas CELF6 expression in the brain is not restricted to a particular region (Fig. 4), it is quite likely that different CELF family members are responsible for the splicing regulation of different subsets of neural pre-mRNAs. The CELF family can be divided into two subfamilies, based on both sequence similarity and function: CUG-BP and ETR-3 make up one subfamily, and CELF3–6 make up the other subfamily. The ability of CELFs 3–6 to activate exon inclusion via MSE2 alone and the failure of CUG-BP and ETR-3 to do so suggest that these subfamilies have different requirements for activation of alternative splicing. Although all six CELF proteins promote MSE-dependent splicing, the binding sites have been mapped for only CUG-BP and ETR-3 (2, 22). It is not known to what extent the preferred binding sequences recognized by the CELF proteins differ. The ability of CELF6 to influence IR splicing in the absence of the known CUG-BP binding site suggests that the different CELF proteins may act through different sites on at least some substrates. It is also not known whether the CELF proteins interact with different partner proteins to form distinct activation or repression complexes on target pre-mRNAs. Furthermore, CELF proteins may interact with each other in varying combinations to influence alternative splicing in different contexts. It will be interesting to examine the potential redundancy and distinctiveness of CELF proteins in neural and other cell types in future studies.

Potential Role of CELF6 in the Pathogenesis of Myotonic Dystrophy—Myotonic dystrophy (DM) is the most common form of adult-onset muscular dystrophy. It is an autosomal dominant disease caused by expansion of CTG (type 1, or DM1) or CCTG (type 2, or DM2) repeats in the 3′-UTR of the DMPK gene or intron 1 of the ZNF9 gene, respectively (5–8). DM is characterized by myotonia (the inability to relax contracted muscle as a result of membrane hyperexcitability) and muscle wasting. In addition to these muscular problems, DM patients experience cardiac conduction defects, insulin resistance, cataracts, testicular atrophy, and cognitive dysfunction (23). Congenital forms of DM display even more severe symptoms, including deficiencies in muscle formation and mental retardation (23).

The mechanism of DM pathogenesis is not well understood, but several lines of evidence suggest that RNA expressed from the expanded allele has a gain-of-function activity that disrupts normal processing of other pre-mRNAs (24). This activity appears to be mediated at least in part by CELF proteins. Levels of CUG-BP protein are elevated in the striated muscle of DM1 patients (9, 25). The alternative splicing of three targets of CELF protein regulation is altered in DM1 muscle (9, 10, 22), and patterns of all three are consistent with elevated CELF activity in DM muscle. It is unknown whether the normally low levels of CELF6 expression in muscle are elevated in DM patients, but it is possible that it and other members of the CELF family in addition to CUG-BP contribute to the misregulation of muscle-specific splicing in DM.

Altered splicing regulation is also seen in the central nervous system of DM patients, where all six of the CELF proteins are normally expressed. The microtubule-associated protein, tau, is normally alternatively spliced in the adult brain to give rise to six protein isoforms. Alteration of the stoichiometry of tau isoforms contributes to the pathology of a number of neurodegenerative disorders (26). A pathological tau protein expression profile is seen in the brains of DM1 patients (27), mirrored by changes in tau pre-mRNA splicing (28). In transgenic mice carrying expanded CTG repeats in the context of the human DMPK region, tau expression profiles were similarly altered (29). Although it has not been demonstrated that tau is a direct target of CELF activity, these results suggest that a similar mechanism gives rise to both muscular and neural pathogenesis in DM patients. Consistent with this model, the alternative splicing of a known CELF target, NMDA R1 exon 5, is also misregulated in DM1 patients, whereas the alternative splicing of other pre-mRNAs is unaffected. All six CELF family members including CELF6 are highly expressed in the fetal and adult brain, perhaps revealing a role for CELF proteins in the mental retardation of congenital DM patients in addition to later cognitive impairments seen in adult-onset cases.

Finally, it is important to note that in addition to altered splicing regulation, the effects of the RNA gain of function may also include disruption of other potential RNA processing functions of the CELF family (see “Other Potential Functions of CELF6”), which may in turn contribute to the pathogenesis of DM.

Other Potential Functions of CELF6—It is likely that CELF6 plays a role in the regulation of other RNA processing events in addition to alternative splicing. The other CELF proteins CUG-BP, ETR-3, and CELF4 have been demonstrated to localize to the cytoplasm as well as the nucleus (30, 31). The subcellular distribution of CELF6 protein has not been determined, but interestingly, both Reinhardt’s NCNN method and the k-NN nearest-neighbor algorithms predict that CELF6 will be predominantly cytoplasmic, despite the presence of a putative nuclear localization signal near the C terminus. CELF6 may shuttle between the nucleus and cytoplasm, with steady-state levels in each compartment determined by the needs of the cell. Indeed, it has been proposed that members of the CELF family act as shuttling proteins to regulate both nuclear and cytoplasmic mRNA processing (3, 14).

Phylogenetic analysis indicates that the CELF family is most closely related to the Hu proteins (3), which are known to regulate mRNA stability and translation (32). Human ETR-3 and CUG-BP proteins have been shown to bind to a Bruno response element, a cis-element involved in translational control of oskar mRNA in Drosophila (14), and share sequence similarity to Bruno, the Drosophila protein that mediates this process. It has also been shown that ETR-3 binds to the 3′-UTR of cyclooxygenase-2 messages, affecting both translation and mRNA stability (31), and to a poRNA, regulating its C to U editing (33). The Xenopus homolog of CUG-BP, EDEN-binding protein, mediates sequence-specific deadenylation of Eγ5 mRNA (34). Other splicing regulators are known to be involved in multiple aspects of mRNA processing or transport, including PTB, a known antagonist of CELF activity (2), which has been implicated in translational regulation (35). It remains to be determined whether the model of dynamic antagonism between PTB and CELF proteins that was developed to explain...
the regulation of alternative splicing (2) will also hold true in other RNA processing contexts.

Acknowledgments—We thank Rajesh Savkur and Paige Jennings for technical assistance; C. C. Liew, Lubov Timchenko, and Nicholas Webster for providing reagents; and André Faustino and Thai Ho for helpful discussions on the manuscript.

REFERENCES
1. Grabowski, P. J., and Black, D. L. (2001) Prog. Neurobiol. 65, 289–308
2. Charlet-B, N., Logan, P., Singh, G., and Cooper, T. (2002) Mol. Cell 9, 649–658
3. Ladd, A., Charlet-B, N., and Cooper, T. (2001) Mol. Cell. Biol. 21, 1285–1296
4. Timchenko, L. T., Miller, J. W., Timchenko, N. A., Devore, K. V., Lin, L. J., Roberts, R., Caskey, C. T., and Swanson, M. S. (1996) Nucleic Acids Res. 24, 4407–4414
5. Brook, J., McCurrach, M., Harley, H., Buckler, A., Church, D., Aburatani, H., Hunter, K., Stanton, V., Thirion, J., Hudson, T., Sohn, R., Zemelman, B., Snell, R., Rundle, S., Davies, J., Shelbourne, P., Buxton, J., Jones, C., Juvonen, V., Johnson, K., Harper, P., Shaw, D., and Housman, D. (1992) Cell 68, 799–808
6. Fu, Y., Pizzuti, A., Fenwick, R., King, J., Rajnarayan, S., Dunne, P., Dubel, J., Nasser, G., Ashizawa, T., Dejong, P., Wieringa, B., Korneluk, R., Perryman, M., Epstein, H., and Caskey, C. (1992) Science 255, 2717–2726
7. Mahadevan, M., Tsiflidis, C., Sabourin, L., Shutler, G., Amemiya, C., Jansen, G., Neville, C., Narang, M., Barcelo, J., Ohoy, K., Leblond, S., Earlamosdonald, J., Dejong, P., Wieringa, B., and Korneluk, R. (1992) Science 255, 1255–1258
8. Liquori, C., Ricker, K., Moseley, M., Jacobsen, J., Kress, W., Naylor, S., Day, J., and Ranum, L. (2001) Science 293, 864–867
9. Savkur, R., Phillips, A., and Cooper, T. (2001) Nat. Genet. 29, 40–47
10. Charlet-B, N., Savkur, R., Singh, G., Philips, A., Grice, E., and Cooper, T. (2002) Mol. Cell 10, 45–53
11. Sironi, M., Cagliani, R., Comi, G., Pozzoli, U., Bardoni, A., Giorda, R., and Bresolin, N. (2003) FEBS Lett. 537, 30–34
12. Lichtner, P., Attié-Bitach, T., Schuffenhauer, S., Henwood, J., Bouvagnet, P., Scambler, P., Meitinger, T., and Vekemans, M. (2002) J. Mol. Med. 80, 431–442
13. Li, D., Bachnaski, L., and Roberts, R. (2001) Genomics 74, 396–401
14. Good, P., Chen, Q., Warner, S., and Herring, D. (2000) J. Biol. Chem. 275, 28583–28592
15. Cooper, T. A. (1998) Mol. Cell. Biol. 18, 4519–4525
16. Kisksi, A., Nelson, J., and Webster, N. J. G. (1998) J. Biol. Chem. 273, 10031–10037
17. Burd, C., and Dreyfuss, G. (1994) Science 265, 615–621
18. Sun, X., Moriarty, P. M., and Maquat, L. E. (2000) EMBO J. 19, 4734–4744
19. Singh, G., Charlet-B, N., Han, J., and Cooper, T. A. (2004) Nucleic Acids Res. 32, 1232–1241
20. Goldstein, B. J., Muller-Wieland, D., and Kahn, C. R. (1987) Mol. Endocrinol. 1, 759–766
21. Zhang, W., Hauing, L., Kyouhga, H., and Grabowski, P. (2002) RNA (N. Y.) 8, 671–685
22. Philips, A., Timchenko, L., and Cooper, T. (1998) Science 280, 737–741
23. Harper, P. (1998) in Genetic Instabilities and Hereditary Neurological Diseases (Wells, R., and Warren, S., eds), pp. 115–130, Academic Press, Boston
24. Faustino, N. A., and Cooper, T. A. (2003) Genes Dev. 17, 419–437
25. Timchenko, N. A., Cai, Z.-J., Welm, A. L., Reddy, S., Ashizawa, T., and Timchenko, L. T. (2001) J. Biol. Chem. 276, 7820–7826
26. Delacourte, A., and Baeze, L. (2000) Curr. Opin. Neurol. 13, 371–376
27. Verniersch, P., Sergeant, N., Ruchoux, M. M., Hofmann-Radvanyi, H., Wattez, A., Petit, H., Dwailey, P., and Delacourte, A. (1996) Neurology 47, 711–717
28. Sergeant, N., Sahlonniere, B., Schraen-Maschke, S., Ghastem, A., Maurage, C.-A., Wattex, A., Verniersch, P., and Delacourte, A. (2003) Hum. Mol. Genet. 10, 2143–2155
29. Seznec, H., Agblou, T., Sergeant, N., Savouret, C., Ghastem, A., Tabti, N., Willer, J.-C., Outhl, L., Durco, C., Briese, E., Fouquet, C., Butler-Browne, G., Delacourte, A., Junien, C., and Guoerdn, G. (2001) Hum. Mol. Genet. 10, 2717–2726
30. Timchenko, N. lako, P., Cai, Z.-J., Smith, M., and Timchenko, L. (2001) Mol. Cell. Biol. 21, 6927–6938
31. Mukhopadhyay, D., Houchen, C., Kennedy, S., Dieckglaes, B., and Anant, S. (2003) Mol. Cell 11, 113–126
32. Keene, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5–7
33. Anant, S., Henderson, J., Mukhopadhyay, D., Navaratnam, N., Kennedy, S., Min, J., and Davidson, N. (2001) J. Biol. Chem. 276, 47338–47351
34. Paillard, L., Omili, P., Legagneur, V., Bassez, T., Manley, D., and Osborne, H. B. (1998) EMBO J. 17, 275–287
35. Kaminski, A., Hunt, S. L., Patton, J. G., and Jackson, R. J. (1995) RNA (N. Y.) 1, 924–938
CELF6, a Member of the CELF Family of RNA-binding Proteins, Regulates Muscle-specific Splicing Enhancer-dependent Alternative Splicing

Andrea N. Ladd, Nicole H. Nguyen, Kavin Malhotra and Thomas A. Cooper

J. Biol. Chem. 2004, 279:17756-17764.
doi: 10.1074/jbc.M310687200 originally published online February 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M310687200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 17 of which can be accessed free at http://www.jbc.org/content/279/17/17756.full.html#ref-list-1