Identification of CELF splicing activation and repression domains \textit{in vivo}

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ABSTRACT

CUG-BP and ETR-3 like factor (CELF) proteins are regulators of pre-mRNA alternative splicing. We created a series of truncation mutants to identify the regions of CELF proteins that are required to activate and to repress alternative splicing of different exons. This analysis was performed in parallel on two CELF proteins, ETR-3 (CUG-BP2, NAPOR, BRUNOL3) and CELF4 (BRUNOL4). We identified a 20-residue region of CELF4 required for repression or activation, in contrast to ETR-3, for which the required residues are more disperse. For both ETR-3 and CELF4, distinct regions were required to activate splicing of two different alternative exons, while regions required for repression of an additional third exon overlapped with regions required for activation. Our results suggest that activation of different splicing events by individual CELF proteins requires separable regions, implying the nature of the protein–protein interactions required for activation are target-dependent. The finding that residues required for activation and repression overlap suggests either that the same region interacts with different proteins to mediate different effects or that interactions with the same proteins can have different effects on splicing due to yet-to-be defined downstream events. These results provide a foundation for identifying CELF-interacting proteins involved in activated and/or repressed splicing.

INTRODUCTION

In higher eukaryotes, pre-mRNA alternative splicing is a powerful and versatile regulatory mechanism by which single genes can produce multiple mRNAs. The final draft of the human genome contains only 20 000–25 000 genes (1), which encode several fold this number of proteins. This fact, plus estimates that >60% of human genes are alternatively spliced (2,3), underscores the importance of alternative splicing for the diversity of the human proteome. Alternative splicing events are regulated by \textit{trans}-acting factors that bind to regulatory \textit{cis}-acting elements within pre-mRNAs (4,5). These \textit{trans}-acting factors can be expressed either in a tissue- or in a developmental stage-specific manner or ubiquitously, making the ratio between \textit{trans}-acting factors important in the regulation of alternative splicing (6,7).

One family of splicing regulators that controls developmentally regulated and tissue-specific splicing events is the CUG-BP and ETR-3-like factor (CELF) protein family [also known as Bruno-like (BRUNOL) proteins] (8,9). There are six CELF paralogs in human and mouse. CELF proteins are well-characterized positive regulators of human and chicken cardiac troponin T (cTNT) exon 5 alternative splicing and bind intronic elements called muscle specific elements (MSEs) downstream of the alternative exon (8,10–12). CELF proteins have also been shown to regulate splicing of human insulin receptor (IR) exon 11 (13), human muscle-specific chloride channel (ClC-1) intron 2 (14), rat N-methyl-D-aspartate (NMDA) receptor R1 exon 5 and exon 21 (15), alpha actinin NM and SM exons (16), human cystic fibrosis transmembrane conductance regulator (CFTR) exon 9, human myotubularin-related 1 (MTMR 1) exon 2.1 (17) and human polypyrimidine tract binding protein (PTB) exon 11 (18). Interestingly, CELF proteins can have either positive (e.g. cTNT, NMDA R1 exon 21 and PTB) or negative (e.g. IR, NMDA R1 exon 5 and ClC-1) effects on splicing of different target pre-mRNAs. Systematic evolution of ligands by exponential enrichment (SELEX) analysis of one CELF protein, ETR-3 (CUG-BP2, BRUNOL3, NAPOR), identified preferred U/G-rich binding sites that are found within introns near several of the exons listed above. Insertion of U/G-rich motifs, representative of the selected sequences into a cTNT minigene, made non-responsive to ETR-3 restored responsiveness not only to ETR-3 but also to two CELF paralogs, CUG-BP1 (BRUNOL2) and CELF4 (BRUNOL4) (17).

CELF proteins contain two N-terminal and one C-terminal RNA recognition motif (RRM) domains separated by...
a 160–230 residue divergent domain. The divergent domain shows limited homology between CELF protein family members and no overt homology to other proteins. To define regions required for splicing regulation, we have performed a functional analysis of two CELF protein family members, CELF4 and ETR-3, which represent the two different CELF family subgroups distinguished based on phylogenetic analysis and functional differences (8).

In this study, we identified the regions of two CELF proteins required for splicing activation and repression. Our goals are to identify residues required for positive and negative regulation, determine whether the same residues are required for positive regulation of different exons activated by CELF proteins and to determine whether residues required for activation are separate from or overlap with residues required for repression. The CELF protein family consists of two subgroups: one contains CUG-BP1 and ETR-3, which share 78% amino acids sequence identity, and the other contains CELF3 (BRUNOL1), CELF4, CELF5 (BRUNOL5) and CELF6 (BRUNOL6), which share 62–66% amino acid sequence identity to each other and ~44% identity to CUG-BP1 (8). We chose to use ETR-3 and CELF4 as representatives of each subgroup in these analyses.

For CELF4, we had previously demonstrated that RRM-1 and -2 plus 66 amino acids within the divergent domain were sufficient to function as a splicing activator for cTNT exon 5 (19). In this study, we demonstrate that this CELF4 activation domain (66 amino acids within divergent domain) retains the ability to activate cTNT alternative splicing when fused to the similar but heterologous RNA-binding domain of ETR-3. In addition, we identified a minimal 20 residues (amino acids 239–258) within the divergent domain of CELF4 that are required to activate cTNT exon 5. In contrast, a different 20 residues within the CELF4 activation domain (amino acids 279–298) were required for the activation of PTB exon 11. Meanwhile, repression of NMDA R1 exon 5 required overlapping residues within both the regions required for the activation of cTNT and PTB exons.

While the regions required for CELF4 activity are relatively well defined, deletion analysis of the two putative activation domains previously identified within ETR-3 exhibited incremental effects. These results suggest that the residues required for activation by ETR-3 are spread within two separate 40- and 90-residue regions of the divergent domain. In addition, we found significant differences in the regions of ETR-3 required for the activation of cTNT exon 5 and NMDA R1 exon 21 and repression of IR exon 11. Our results suggest a surprising degree of diversity between the regions of CELF proteins that are required to perform the same function on different pre-mRNAs and a surprising similarity in the residues required for opposite functions on different pre-mRNAs.

**MATERIALS AND METHODS**

**Plasmids**

CELF4 and ETR-3 deletion mutants truncated at the positions indicated in Figures 1–6 were generated by PCR and cloned using BamHI/XhoI into pcDNA3.1HisC (+) (Invitrogen). The chicken cTNT minigene plasmid RTB33.51 has been described previously (11). For the cloning of C2R.TGA, primers BXup (GTGGTCAGTTCGCCAGGCGGGGCGGGGCA-TGATTGAC) and BXdn (TCCAGTCAATCGATGCCCG-CGCCGCCTGGCTCCGGAAGTGCACCCAGA) were phosphorylated, annealed and cloned into BstXI/XhoI-digested CELF4 (+48) plasmid (19). C2R.232–298 contains amino acids 232–298 of the CELF4 divergent domain cloned into C2R.TGA using ClaI/XhoI. For C2R.GFP67aa,
Cell culture and transfection

Transient transfection of plasmid DNA into quail QT35 fibroblast and COS-M6 cultures and RNA extraction were performed as described previously (8,20,21). RT–PCR analysis for RTB33.51 and NMDA R1 exon 21 minigenes was performed as described previously (8). All results from RT–PCR analysis were derived from at least three independent transfections, and means (±SD) are shown. For RT–PCR analysis of the IR-F minigene, oligo(dT) (Invitrogen) was used for reverse transcription, and IR-U (5′-TAATACGACTCACTATAGGG-3′) and IR-D (5′-GGCAAAGAATTCGCCACCA-3′) were used for PCR amplification. For NMDA R1 exon 5 minigene RT–PCR analysis, oligo(dT) (Invitrogen) was used for reverse transcription, and RSV5U (5′-CATTACACACTTGGTTGG-3′) and RTRHC (5′-GGGCTTTGAGAGCGAAGG-3′) were used for PCR amplification. For RT–PCR analysis of the PTB minigene, CGRT (5′-GCTGCAATAAACAAGTTC-3′) and IR-D (5′-GGCAAAGAATTCGCCACCA-3′), and a 289 bp fusion of sTNI exons 4 and 5 and the last exon of sTNI exon 2 upstream of the SalI site and 372 bp of sTNI intron 2 were used for PCR amplification. PCRs included a kinase-labeled oligonucleotide and bands were quantified by phosphoimager analysis. Western blot analysis of expressed proteins was performed as described previously using AntiXpress antibody directed against the N-terminal epitope tag encoded by the pcDNA3.1HisC(+) vector (1:5000; Invitrogen) (22).

UV-crosslinking and immunoprecipitation of protein expressed in vivo

C2R.ETRCD87aa and C2R.ETRN71aa, the first 67 amino acids of green fluorescent protein (GFP), C-terminal 87 amino acids of ETR-3 divergent domain, or N-terminal 71 amino acids of the ETR-3 divergent domain, respectively, were cloned into the Cclal/XhoI-digested C2R.TGA vector. C2R.31aa was generated from two insertional point mutations (thymidine and adenosine) at the 1.2-kb cloning site of C2R.TGA during cloning of C2R.ETRN71aa, which shifted the open reading frame of the ETR-3 N-terminal activation domain, generating random 31 amino acids (RSILRRTKSGASSSLSRCSSTLP-PGGT). We used C2R.31aa as a negative control for the other chimeras. For the human NMDA R1 exon 21 minigene, a PCR fragment that contains 111 bp of NMDA R1 alternative exon 21 flanked by 426 bp of the upstream intron and 645 bp of the downstream intron was cloned into RHCflex using Sall and XbaI restriction sites included in the PCR primers. The final NMDA R1 exon 21 minigene contains exon 1 consisting of a 51 bp fusion of RSV 5′-untranslated region (5′-UTR) and skeletal tropinin I (sTNI) exon 2 plus 604 bp of sTNI introns 1 and 2 upstream of the Sall site and 372 bp of sTNI intron 2 and a 289 bp fusion of sTNI exons 4 and 5 and the last exon of skeletal α-actin downstream of the XbaI site. For the human NMDA R1 exon 5 minigene, a PCR fragment containing 63 bp of alternative exon 5 plus a BamHI restriction site at the 3′ end of the alternative exon flanked by 2249 bp of the upstream intron and 794 bp of the downstream intron was cloned into RHCflex using BspEII and PpuMI restriction sites. The final NMDA R1 exon 5 minigene also contains the 51 bp fusion of RSV 5′-UTR and sTNI exon 2 upstream of the BspEII site and 289 bp fusion of sTNI exons 4 and 5 and the last exon of skeletal α-actin downstream of the PpuMI site. The human PTB exon 11 minigene was obtained from Chris Smith (University of Cambridge, Cambridge, UK) and the human IR exon 11 minigene was obtained from Nicolas Webster (University of California at San Diego, La Jolla, CA). All constructs were confirmed by mapping and complete sequencing of PCR-generated fragments, except for the NMDA R1 exon 5 genomic segment which was partially sequenced.

Figure 2. Splicing activity of human CELF4 chimeras on cTNT exon 5 inclusion. (A) Diagrams of full-length human CELF4 protein and chimeras. The light gray box indicates a (Gly)5 linker and ClaI cloning site. Regions replacing (of truncated human CELF4 proteins in QT35 quail fibroblasts. Percentages of tively. (from CELF4.48–258 (GMFNPMAIPF) or GAPDH (VGVNGFGRIG), respectively. For CELF4.48–2525 and CELF4.48-GAPDH, the last 10 residues of CELF4.48–268 (GAYGAYAQAL) were substituted with 10 heterologous amino acids of ETR-3 divergent domain, or N-terminal 71 amino acids of green fluorescent protein (GFP), C-terminal 87 amino acids were tested for RNA binding using a UV-crosslink/immunoprecipitation of proteins (UV-IP).
immunoprecipitation assay as described previously (22) with the following modifications: 20 μg of protein expression plasmid was transfected into each of the three COS-M6 150 mm plates. After 48 h, the cells were washed with phosphate-buffered saline, then 300 μl of lysis buffer (20 mM HEPES, 100 mM KCl, 0.05% Triton X-100, 20% glycerol and 1 mM DTT) was added to pooled plates. Cells were sonicated for 10 s and incubated on ice for 30 min and centrifuged at 13 000 g for 10 min. Binding reactions were performed with 500 μg of whole cell lysates and 180 000 c.p.m.
GTP and [32P]UTP-labeled RNA as described previously (22). Western blot analysis of pelleted AntiXpress-tagged proteins was performed using horseradish peroxidase (HRP)-conjugated AntiXpress (Invitrogen) at 1:1000 to prevent detection of pelleted immunoglobulin, and bands were visualized with SuperSignal Chemiluminescent femto Substrate (Pierce).

RESULTS

Identification of a minimal domain of CELF4 required for cTNT activation

Recently, we reported that either RRM1 or RRM2 of CELF4 is necessary and sufficient for binding to RNA containing the cTNT MSE regulatory elements. Additionally, we have shown

Figure 4. CELF4 contains overlapping activation and repression domains. (A) Diagram of full-length human CELF4 protein and deletion mutants. (B) Diagrams of PTB exon 11 and NMDA R1 exon 5 minigenes. The arrows above the diagrams indicate primers used for RT–PCR analysis. (C) Activation of human PTB exon 11 inclusion by transient expression of truncated forms of human CELF4. (D) Repression of human NMDA R1 exon 5 inclusion by transient expression of truncated forms of human CELF4. (E) Activation of human PTB exon 11 inclusion by transient expression of finer deletion mutants of human CELF4. (F) Repression of human NMDA R1 exon 5 inclusion by transient expression of truncated and substituted forms of human CELF4. For the information for chimeras, see the diagram in Figure 2A. Transfections were performed in COS-M6 cells. Comparable levels of protein expression were confirmed by western blotting using AntiXpress antibody (data not shown).
that RRM2 plus an additional 66 amino acids of the divergent domain are as effective as full-length CELF4 in activating MSE-dependent splicing in vivo (19). To further define the specific residues of the 66 amino acids that are required for MSE-dependent activation, we made sequential 10 amino acid deletions from the C-terminus of the smallest truncated CELF4 mutant that retained full activity (CELF4 Δ3.4, containing residues 48–298) and tested their activities on the cTNT minigene (Figure 1A). The deletion analysis demonstrated a slight but consistent loss of activity upon deletion of the 10 amino acids from positions 288 to 278 and a larger loss of activity from 268 to 258 (Figure 1B). Some activity was retained with only six residues beyond the C-terminus of RRM2 (Figure 1B, 48–238). While steady state levels of CELF4 Δ3.5 (containing residues 48–258) were consistently reduced as shown in Figure 1C, transfections showing relatively high protein expression still showed poor activation (data not shown), indicating a loss of the intrinsic splicing activity of CELF4.

The fact that the CELF4 deletion mutant retaining only six residues of the divergent domain (48–238) had residual activity suggested the possibility that the N-terminal segment containing RRM1 and 2 might contribute to splicing activation in addition to RNA binding. We created the construct C2R.TGA that truncates CELF4 at position 231, the penultimate residue of RRM2. This construct contains a linker of five glycines ([Gly]₅) and convenient restriction sites immediately following RRM2, which allows cloning and testing of regions for the activation of cTNT exon 5. The C2R. TGA-encoded protein could not promote inclusion of cTNT exon 5 (Figure 2B). Replacing residues 232–298 with the N-terminus of GFP abolished the activity similar to C2R. TGA (Figure 2B, C2RGFP67aa), indicating that the RRMs alone are not sufficient to activate cTNT exon 5. Because previous results indicate that GFP fusions with CELF proteins do not inhibit splicing activity (23) (data not shown), we conclude that the divergent domain of CELF4 contains the residues that are necessary and sufficient for the full extent of activation of cTNT exon 5 inclusion. To confirm that the linker does not disrupt activation, we tested C2R.232–298, which contains the identical region of the divergent domain as CELF4 Δ3.4. C2R.232–298 is nearly as active as CELF4 Δ3.4 in activating cTNT exon 5 inclusion (compare Figures 1 and 2), indicating that the (Gly)₅ linker does not interfere with splicing activation.

To determine whether the reduced splicing activity resulted from the loss of residues required for activation or from non-specific effects of creating truncated proteins, such as protein misfolding, reduced protein stability or translational inefficiency, we created several constructs in which residues defined as required by the sequential deletion analysis were replaced by substitutions. Deletion of residues 268–258 significantly decreased splicing activation (Figure 1); however, when these 10 residues were replaced with 10 heterologous amino acids (residues 249–258) from CELF4 (CELF4.48-2525) or GAPDH (CELF4.48-GAPDH), splicing activity was restored (Figure 2B). In contrast, substitution of residues 248–268 and 238–268 with the N-terminal residues of GFP caused a loss of activation. Taken together, these results demonstrate that residues 232–258 are required for the activation of cTNT exon 5. The majority of this activity resides within the 20 residues from 239 to 258. These results also demonstrate the importance of substitution analysis relative to deletions alone, because in Figure 1, 10 residues between 258 and 268 were considered as critical residues for splicing activity, but when we replaced them with other 10 heterologous residues, the splicing activity was restored as shown in Figure 2.

The CELF4 activation domain is active in a heterologous context

Previously, we reported that non-overlapping N- and C-terminal regions of the ETR-3 divergent domain independently serve as activation domains of cTNT exon 5 [ETR-3 Δ3.4 and ETR-3 Δ5.7, see Figure 5, below and (19)]. Having functionally defined a putative activation domain of CELF4 in the N-terminal segment of the divergent domain and two putative activation domains within the N- and C-terminal regions of the ETR-3 divergent domain, we next tested the ability of each of these domains to function in a heterologous context. First, we replaced the N-terminal ETR-3 activation domain (residues 188–258) with the CELF4 activation domain (residues 232–298) (Figure 3A). These residues of CELF4 retained the ability to activate splicing when placed with ETR-3 RRM1 and 2.
The decreased activity of the ETR-CELF4 chimera relative to CELF4 Δ3.4 seems to be at least partly due to the reduced protein expression level (Figure 3C). We conclude that the CELF4 activation domain can function in the context of the ETR-3 RNA binding domains.

We next replaced the CELF4 divergent domain (232–298) in C2R.232–298 with either the N-terminal (188–258, C2R.ETRND71aa) or the C-terminal (286–372, C2R.ETRCD87aa) activation domain of ETR-3 (Figure 3D). As a negative control, we replaced the CELF4 activation domain with a random 31 amino acids (C2R.31aa) (Figure 3D). Constructs containing either segments of the ETR-3 divergent domain showed only very weak splicing activity (Figure 3E). We tested binding of the chimeric proteins expressed in vivo to uniformly labeled in vitro transcribed RNA containing cTNT MSEs 2–4 (the last 16 nt of exon 5 and the first 149 nt of intron 5 of chicken cTNT) which contains the binding site for ETR-3 and CELF4 (22) (data not shown). Expression plasmids were transiently expressed in COS-M6 cells. Whole cell lysates were prepared after 48 h and were used in UV-crosslinking/immunoprecipitation assays with uniformly labeled MSE RNA as described previously (19). Binding was detected

Figure 6. Mapping of ETR-3 residues required for the activation of NMDA exon 21 inclusion and repression of IR exon 11 inclusion. (A) Diagram of full-length human ETR-3 protein and the N- and C-terminal deletion series. (B) Diagrams of NMDA R1 exon 21 minigene and IR exon 11 minigene. Primers used for RT–PCR analysis are indicated by arrows. (C) Activation of human NMDA R1 exon 21 inclusion by transient expression of full-length and truncated form of human ETR-3 assayed by RT–PCR. (D) Repression of human IR-F exon 11 inclusion by transient expression of full-length and truncated forms of human ETR-3. Transfections were performed in COS-M6 cells. Comparable levels of protein expression were confirmed by western blotting using AntiXpress antibody (data not shown).
by immunoprecipitation of protein–RNA adducts using the AntiXpress antibody followed by autoradiography. The same blot was then used for western blot analysis probed with HRPCoujugated AntiXpress antibody to determine the amount of the immunoprecipitated protein. C2R.232–298, which activates splicing (Figure 3E), binds to the MSE RNA (Figure 3F, lane +Ab, P). C2R.TGA and C2R.31aa, which do not activate splicing (Figures 2B and 3E, respectively), also bind to the RNA consistent with the retention of RNA binding activity but loss of a splicing activation domain in these proteins. Surprisingly, even though C2R.ETRCD87aa and C2R.ETRND71aa contain the same RRMs as those proteins that bind to the RNA, these two proteins showed minimal, if any, binding despite strong protein expression. These results explain the loss of splicing activity of C2R.ETRCD87aa and C2R.ETRND71aa. It is important to note that neither the (Gly)3 linker alone nor the presence of heterologous downstream sequence generally disrupts binding since three of the five constructs retain binding activity. This result indicates that RNA binding by CELF4 RRMs 1 and 2 can be strongly influenced by the downstream sequence.

Separate residues within the CELF4 divergent domain function to activate splicing of different alternative exons

CELF proteins have been shown to regulate a number of different alternative splicing events. Interestingly, CELF proteins activate splicing of some pre-mRNA targets and repress splicing of other targets [(13–15,18,24) and see Introduction]. We next used the deletion series of CELF4 and ETR-3 to address two questions. First, are the residues required for the activation of cTNT exon 5 the same as those required for the activation of other splicing events activated by CELF4 proteins? Second, are the residues required for repression the same or different from those required for activation?

The residues in CELF4 required for the activation of cTNT exon 5 were well defined to a 20-residue region within the divergent domain (239–258) (Figure 2). To determine whether CELF4 uses the same residues to activate splicing of a different pre-mRNA, we tested a PTB minigene, in which exon 11 inclusion is activated by CELF4 (18). In the analysis of the CELF4 N-terminal deletion mutants (Figure 4A), we found that in contrast to chicken cTNT exon 5 (19), PTB exon 11 was not activated by the N-terminal CELF4 Δ5.1 deletion (Figure 4C). It should be noted that CELF4 Δ5.1 was active in repressing splicing of NMDA R1 exon 5 (see below) and in activating splicing of cTNT exon 5 (19), indicating that this protein is functional on two other pre-mRNAs. CELF4 Δ5.2 activated splicing of PTB exon 11 as observed previously with cTNT exon 5 (19). CELF4 N-terminal deletion mutants lacking the two complete N-terminal RRMs were inactive (Figure 4A and C, Δ5.3–Δ5.7) consistent with the loss of RNA binding reported previously (19). Analysis of C-terminal deletions demonstrated an increase in activity relative to CELF4 (+48) as the C-terminus was removed. Constructs CELF4 Δ3.1–Δ3.3 demonstrated activation that was significantly higher than CELF4 (+48). These deletions had a similar effect on the activation of cTNT exon 5 (19). Upon further deletion, activation of PTB exon 11 inclusion was lost between residues 258 and 298 as was observed for cTNT. To further define the activation domain between 258 and 298, we tested the same constructs that we used for Figures 1 and 2. Interestingly, in contrast to our results for cTNT exon 5, deletion of 289–298 significantly decreased the ability of CELF4 to activate PTB exon 11, and deletion of 279–288 completely abolished the splicing activity (Figure 4E). We conclude that distinct residues are required for the activation of two CELF protein pre-mRNA targets.

Residues within CELF4 required for splicing repression overlap with residues required for splicing activation

To determine whether the CELF4 residues required for splicing repression are the same or different from those required for splicing activation, we constructed a minigene containing the human NMDA R1 exon 5. Exon 5 of the rat NMDA R1 gene has been shown to be repressed by ETR-3 (15). A fragment of the human NMDA R1 gene consisting of the last 51 nt of exon 4 to the first 55 nt of exon 6 was PCR amplified and cloned into our standard minigene (Figure 4B). In this construct, splicing of the human NMDA R1 exon 5 inclusion is repressed by ETR-3 (data not shown), although very weakly compared with the rat exon (15). Coexpression of CELF4 (+48) with the minigene also repressed inclusion of this exon (Figure 4D, +48). Coexpression of the NMDA R1 exon 5 minigene with the CELF4 deletion series revealed that the regions of CELF4 required for NMDA R1 exon 5 repression corresponded to the regions required for the activation of cTNT exon 5 and PTB exon 11. An N-terminal deletion that removes RRMs 1 and 2 (CELF4 Δ5.3) lost the ability to repress NMDA R1 exon 5 (Figure 4D), presumably due to the loss of RNA binding (19).

Deletions within the divergent domain between CELF4 Δ3.4 (position 298) and CELF4 Δ3.5 (position 258) defined this region as required for full repression activity. This same region is required for the activation of cTNT exon 5 [Figure 1 and (19)] and PTB exon 11 (Figure 4C). To further define the residues required for repression, we tested the deletion constructs used to define the residues required for the activation of cTNT and PTB, as described above. Interestingly, deletion of 279–288 significantly decreased the splicing activity and deletion of 239–248 completely abolished the splicing activity (Figure 4F). Therefore, the set of residues required for the activation of cTNT and the set required for the activation of PTB contributed to full repression activity on NMDA R1 exon 5. We conclude that residues 278–288 and 239–248 of CELF4 are required for the repression of NMDA R1 exon 5 inclusion, residues 279–289 of CELF4 are required for the activation of PTB exon 11, while residues 239–258 are required for the activation of chicken cTNT exon 5.

ETR-3 residues required for cTNT exon 5 activation are widely distributed

We have previously shown that non-overlapping N- and C-terminal segments of ETR-3 activated inclusion of cTNT exon 5 in a sequence-specific manner (19) (Figure 5A, ETR-3 Δ5.7 and ETR-3 Δ3.4). To further define residues within both regions that are important for the activation of cTNT exon 5, we sequentially deleted 10 amino acids from the termini of the active N- and C-terminal fragments (Figure 5A). While the ETR-3 Δ3.4 deletion retained most of the activity of
full-length, sequential 10 amino acid deletions from this N-terminal segment of ETR-3 gradually decreased the splicing activity. Similarly, sequential deletions from ETR-3 Δ5.7 resulted in a gradual loss of residual activity (Figure 5B). From this analysis, we conclude that unlike CELF4, the residues within ETR-3 that are required for the activation are dispersed throughout the previously defined activation domains.

Distinct domains of ETR-3 are required to activate and repress splicing of different pre-mRNA targets

To determine whether the activation of a second pre-mRNA target exhibited similar or different requirements compared with cTNT (e.g. activation by non-overlapping N-terminal and C-terminal segments), we coexpressed the ETR-3 deletion series with a minigene containing human NMDA R1 exon 21. At first, we tried to use the same minigenes that we used for the analysis of CELF4, but neither human PTB exon 11 nor human NMDA R1 exon 5 minigenes responded strongly to ETR-3. As mentioned above, ETR-3 did repress NMDA R1 exon 5 inclusion, but its repression activity was not as strong as that of CELF4. These results demonstrate different specificities of different CELF proteins to different alternative exons. Recently, ETR-3 has been shown to activate inclusion of rat NMDA R1 exon 21 (15) and we found that as for rat, the human NMDA R1 exon 21 responds strongly to coexpressed full-length ETR-3 (Figure 6C).

Sequential deletions from the N- and C-termini of ETR-3 demonstrated that the regions required for the activation of human NMDA R1 exon 21 differed from those required for the activation of chicken cTNT exon 5. Whereas the RNA binding domains plus segments of the adjacent divergent domain at either end of ETR-3 were sufficient to activate cTNT exon 5 (ETR-3 Δ3.4 and Δ5.7, Figure 6A), activation of human NMDA R1 exon 21 was nearly lost by deletion of only one N-terminal RRM (Δ5.1) and was abolished by the first deletion that inactivates the C-terminal RRM by removing the highly conserved RNP1 hexamer motif (Δ3.1) (Figure 6C). We conclude that unlike cTNT exon 5, activation of NMDA R1 exon 21 requires domains in both the N- and C-termini of the protein. To determine whether the complete RRM3 was required to maintain its RNA binding activity, we tested a natural splice variant of ETR-3 that lacks the N-terminal two-thirds of RRM3, including the RNP2 octamer, which is required for RNA binding activity (ETR-3 ΔJ). This protein activated NMDA R1 exon 21 inclusion comparably with Δ5.1 and Δ5.2, indicating that RNA binding activity of RRM3 is not absolutely required and that the requirement for this domain is indicative of another function.

To identify regions within ETR-3 that are required for the repression of exon inclusion, we coexpressed the ETR-3 deletion series with a human IR minigene. ETR-3 repressed IR exon 11 splicing (Figure 6D) just as it has been previously shown for the closely related CELF protein, CUG-BP1 (13). Interestingly, the deletion that removed part of RRM1 (ETR-3 Δ5.1) lost most of the ability to repress exon inclusion; however, removal of an additional 40 residues (ETR-3 Δ5.2) restored splicing activity. Further N-terminal deletions expected to inactivate RNA binding of the N-terminal RRMs (starting with Δ5.3) completely abolished splicing activity. Except for the weak residual activity of the first C-terminal deletion, which is predicted to inactivate RRM3 (Δ3.1), C-terminal deletions lost the ability to repress inclusion of IR exon 11 (Figure 6D), despite the fact that ETR-3 Δ3.1–Δ3.4 were still able to activate cTNT exon 5 (19). These results indicate that the regions of ETR-3 that are sufficient for the activation of cTNT exon 5 are not individually sufficient for either the activation of NMDA R1 exon 21 or the repression of IR exon 11.

DISCUSSION

Definition of the minimal region required for CELF protein splicing activity

CELF proteins regulate alternative splicing of several pre-mRNAs by binding specific sequences within the introns adjacent to the alternative exons (8,13–16,22). Several alternative splicing events are regulated either positively or negatively by CELF proteins, depending on the pre-mRNA targets. Using a series of sequential N- and C-terminal deletions of ~40 amino acids, we previously mapped CELF protein domains required for the activation of chicken cTNT exon 5. These results demonstrated that the first 66 residues of the CELF4 divergent domain (residues 233–298) serve as a putative ‘activation domain’ when present with RRM1 and 2 or with RRM2 alone (19). Deletion analysis of ETR-3 identified two non-overlapping regions of the divergent domain (189–258 with RRM1 and 2; 286–404 with RRM3) that were sufficient for splicing activation of chicken cTNT exon 5 (19). In this study, we delineated the minimal regions required for the activity of all three regions. In addition, we tested whether the same or different domains are required for splicing activation and repression of different pre-mRNA targets. We note that deletions caused a loss of CELF4 function, yet substitution of the same residues demonstrated them not to be required for function (Figure 2). Results from deletion analyses should be interpreted with caution and substitutions are preferable to deletions for the delineation of functional domains.

The results for CELF4 are summarized in Figure 7. CELF4 activates chicken and human cTNT exon 5, human PTB exon 11 and represses human NMDA R1 exon 5 [(8,18) and this study]. The same 40-residue region of the CELF4 divergent domain between constructs CELF4 Δ3.4 and CELF4 Δ3.5 (residues 259–298) was required to activate both cTNT exon 5 and PTB exon 11. However, finer deletion/ substitution analysis demonstrated that the specific residues required to activate the two exons were different: residues 239–258 were required for cTNT exon 5 activation (Figures 1 and 2) and residues 279–298 were required for the activation of PTB exon 11 (Figure 4E). We also show that a protein containing only RRMs 1 and 2 binds to at least one target pre-mRNA (chicken cTNT) (Figure 3F, C2R,TGA). Since the regions required for splicing regulation are not required for RNA binding, they are most likely to function by binding other factors required for splicing activation or repression. The finding that distinct residues of CELF proteins separated by 20 amino acids are required for the activation of different alternative exons suggests several possibilities. One is that the activation of different alternative exons requires interactions with separate factors. Another is that the same CELF-interacting factor activates cTNT and PTB exons by binding
to separate regions of the CELF protein. In this case, the two domains required for cTNT and PTB activation would represent redundant protein–protein interaction sites. Consistent with this possibility, the two regions contain methionine and glutamine-rich motifs. It is possible that the activation of PTB exon 11 requires more copies of a redundant interaction domain than cTNT exon 5. Further analysis is ongoing to identify the interacting factors required for activation.

Interestingly, the regions of the CELF4 protein required for the repression of human NMDA R1 exon 5 completely overlap with those required for the activation of chicken cTNT or human PTB (Figure 4D). The large deletion series revealed that residues 259–298 were required for splicing repression. Finer deletion and substitution analysis revealed that 10 residues between 279 and 288 are required for repression of chicken cTNT exon 5 by CELF4, with an additional requirement for residues 239–248 for full activity (Figure 4F). These results suggest that the protein–protein interactions with CELF4 required for splicing repression are the same as those that mediate splicing activation. Therefore, whether the CELF complex that assembles on the pre-mRNA activates or represses splicing could be determined by features other than the proteins that directly interact with CELF4. For example, where the complex assembles relative to the regulated alternative exon could be determinative for repression or activation of splicing. The CELF4 binding sites required to regulate human PTB exon 11 and NMDA R1 exon 5 have not yet been identified. It is also possible that different proteins required for activation or repression interact with the same region of CELF4, altering the effect of the regulatory complex on recruitment or stabilization of the basal splicing machinery. Zebrafish Fox-1 (ataxin-2 binding protein 1, A2BP1) has been demonstrated to positively regulate splicing of one pre-mRNA and negatively regulate another. While only an N-terminal portion of Fox-1 is required for positive regulation, a C-terminal segment is required for positive and negative regulation (25). These results suggest the need for different interacting proteins to mediate different effects.

In the case of ETR-3, we were not able to further define minimal activation domains for cTNT exon 5 splicing for either the N- or C-terminal regions of the divergent domain, since sequential deletions within the previously defined domain only gradually reduced splicing activity. Therefore, the activation domains of ETR-3 appear to be spread out over larger regions of the divergent domain and differ significantly from the relatively small regions within CELF4.

We showed that ETR-3 increases inclusion of the human NMDA R1 exon 21 (Figure 6C) consistent with results reported for the rat exon (15). We also found significant differences in the domains required for the activation of human NMDA R1 exon 21 and chicken cTNT exon 5. While the non-overlapping N- and C-terminal segments of ETR-3 containing RRM3s plus different regions of the divergent domain were capable of sequence-specific activation of cTNT exon 5 (Figure 5) (19), both ‘ends’ of ETR-3 were required to activate NMDA R1 exon 21. Deletions of either the N- or C-terminal portions of the protein inactivated its effects on NMDA R1 exon 21 (Figure 6C). That these proteins retained the ability to regulate splicing was demonstrated previously on chicken cTNT (19). To test another mutant lacking RRM3 binding activity, we used a natural splice variant (ETR-3 ΔJ) lacking two-thirds of RRM3, including the RNP2 motif which is required for RNA binding. This variant activated splicing, albeit at a reduced level, indicating that the RNA binding activity of RRM3 is not required for full splicing activation of NMDA R1 exon 21. These results suggest that ETR-3 requires multiple protein–protein interactions within different regions of the divergent domains and perhaps RRM3 to promote exon inclusion.

The N- and C-terminal ETR-3 deletions that prevented activation of NMDA exon 21 also prevented repression of IR exon 11 (Figure 6D). Deletion analysis of chicken ETR-3 demonstrated that in contrast to results obtained for human ETR-3, deletion of the N-terminal quadrant of the divergent domain or deletion of the last third of RRM3 impaired its splicing activation of chicken cTNT (21). Although human and chicken ETR-3 protein sequences are 97% identical, inclusion of six additional amino acids (VAQMLS) in the divergent domain of human ETR-3 at the exon 10/exon 11 boundary and other small differences could be responsible for these functional discrepancies. Detailed functional comparisons of chicken and human ETR-3 are in progress.

We compared the defined CELF4 activation or repression domain with the comparable region of other CELF family members (Figure 7B). The comparable region within ETR-3 was also shown to activate cTNT exon inclusion (19). Glutamine residues are most conserved among all six CELF
proteins, and methionine, arginine, proline, leucine and alanine residues are also conserved. It remains to be determined whether these residues are specifically required for splicing activation or repression. Interestingly, the C-terminal region with the ETR-3 divergent domain that is sufficient for the activation of cTNT exon 5 does not contain obvious residue commonalities with this N-terminal region, suggesting that at least for cTNT, the different regions use different protein–protein interactions to mediate splicing activation (19).

Another noticeable finding in this study is that closely related proteins, such as CELF4 and ETR-3, have different pre-mRNA targets. We have shown that CELF4 activated PTB exon 11 inclusion and repressed NMDA R1 exon 5 inclusion, while neither of these minigenes were strongly regulated by ETR-3. ETR-3 has been reported to promote rat NMDA R1 exon 21 inclusion and exon 5 skipping (15), but for human NMDA R1 exon 5 skipping, ETR-3 regulation was weak. Similarly, while ETR-3 strongly repressed IR-F exon 11 inclusion, CELF4 had only a weak effect. It is unclear whether these differences reflect differences in their RNA sequence recognition and binding affinity or accessibility to target RNA and/or their interacting protein counterparts.

**Context-dependent activities of CELF activation domains**

To determine whether the three defined activation domains of CELF4 and ETR-3 function when linked with heterologous RNA binding domains, we placed the activation domain of CELF4 with the N-terminal segment of ETR-3 containing RRMs 1 and 2 (Figure 3A, ETR-CELFD87aa and C2R.ETRND71aa). The two activation domains of ETR-3 were separately fused to the N-terminal segment of CELF4 containing RRMs 1 and 2 of CELF4 (Figure 3D, C2R.ETRCD87aa and C2R.ETRND71aa). Both CELF4 and ETR-3 have been shown to bind the same mSEs flanking chicken cTNT exon 5 (22) (data not shown). These chimera were tested for their ability to activate chicken cTNT in vivo. The ETR-CELFD4 chimera activated cTNT exon 5 inclusion in a dose-dependent manner, although its splicing activity was decreased compared with the same activation domain in the context of CELF4 RRMs 1 and 2. This could reflect, at least in part, the lower expression of the ETR-CELFD4 chimera protein compared with CELF4 Δ3,4 (Figure 3C). We conclude that the activation domain of CELF4 functions in heterologous RNA binding domains.

In contrast, neither the N-terminal (188–258, 71 amino acids) nor the C-terminal (286–372, 87 amino acids) activation domains of ETR-3 functioned when they replaced the CELF4 activation domain (232–298) (Figure 3D–F). This result does not necessarily mean that the divergent domain of CELF4 is required for RNA binding, since proteins lacking this region (C2R.TGA and C2R.31aa) did bind to target RNA (Figure 3F). It is unclear why only ETR-3 divergent domains prevent CELF4 RRMs from binding to target RNA. It is possible that these fragments alter the structure of the protein in a manner that is incompatible with RNA binding. Studies by other investigators have demonstrated that non-RRM domains of CELF proteins affect the RNA binding affinity. The *Xenopus* ortholog of CUG-BP1, embryo deadenylation element-binding protein (EDEN-BP), which has 87% homology to human CUG-BP1, binds specifically to the EDEN motif in the 3′-UTRs of specific maternal mRNAs. Sequence-specific binding of EDEN-BP requires the two N-terminal RRMs and at least the first 167 amino acids of the divergent domain (26). Recently, Delaunay et al. (27) showed that the Bruno (the *Drosophila* ortholog of CUG-BP1) paralog Bru-3 specifically binds the EDEN element. They also showed that the divergent domain of Bru-3 is required for specific RNA binding of the C-terminal RRM of Bru-3 to the EDEN element.

Taken together, our results demonstrate that CELF proteins influence splicing of diverse pre-mRNAs using distinct regions of the protein. The activation and repression domains defined in this study will be used to identify proteins that interact with the CELF proteins and will be a foundation to understand the splicing mechanism of CELF protein activation and repression of splicing activity.

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