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Muscleblind proteins regulate alternative splicing

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Although the muscleblind (MBNL) protein family has been implicated in myotonic dystrophy (DM), a specific function for these proteins has not been reported. A key feature of the RNA-mediated pathogenesis model for DM is the disrupted splicing of specific pre-mRNA targets. Here we demonstrate that MBNL proteins regulate alternative splicing of two pre-mRNAs that are misregulated in DM, cardiac troponin T (cTNT) and insulin receptor (IR). Alternative cTNT and IR exons are also regulated by CELF proteins, which were previously implicated in DM pathogenesis. MBNL proteins promote opposite splicing patterns for cTNT and IR alternative exons, both of which are antagonized by CELF proteins. CELF- and MBNL-binding sites are distinct and regulation by MBNL does not require the CELF-binding site. The results are consistent with a mechanism for DM pathogenesis in which expanded repeats cause a loss of MBNL and/or gain of CELF activities, leading to misregulation of alternative splicing of specific pre-mRNA targets.

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Introduction

Alternative splicing is thought to occur in 41–60% of genes in the human genome (Black, 2003; Herbert, 2004). Pre-mRNAs can give rise to multiple protein isoforms with different functions and these variations contribute to protein diversity (Black, 2003). Alternative splicing also provides an additional regulatory mechanism by which vertebrates can control the expression of tissue-specific or developmental stage-specific protein isoforms. RNA-binding proteins that regulate alternative splicing bind to sequence-specific elements in the pre-mRNA to enhance or repress inclusion of alternative exons. Aberrant regulation of alternative splicing can cause the expression of inappropriate splicing patterns leading to human disease (Faustino and Cooper, 2003). An example of a disease that alters the function of RNA-binding proteins to cause misregulated alternative splicing is myotonic dystrophy (DM). Interestingly, the effects on splicing in DM1 are thought to be limited to specific pre-mRNA targets rather than a general disruption of splicing.

DM is a multisystemic disorder caused by two different microsatellite expansions. Type I myotonic dystrophy (DM1) is caused by a CTG trinucleotide expansion in the 3′ untranslated region (UTR) of the *DMPK* gene on chromosome 19 (Brook *et al*, 1992; Fu *et al*, 1992; Mahadevan *et al*, 1992), while type 2 (DM2) is caused by a CCTG expansion in intron 1 of the *ZNF9* gene on chromosome 3 (Liquori *et al*, 2001). Although the expansions are located on different chromosomes, there appears to be a common pathogenic mechanism involving the accumulation of transcripts into discrete nuclear RNA foci containing long tracts of CUG or CCUG repeats expressed from the expanded allele (Taneja *et al*, 1995; Davis *et al*, 1997; Liquori *et al*, 2001).

The RNA gain-of-function hypothesis proposes that mutant DM transcripts alter the function and localization of alternative splicing regulators, which are critical for normal RNA processing. Consistent with this proposal, misregulated alternative splicing in DM1 has been demonstrated for six pre-mRNAs: cardiac troponin T (cTNT), insulin receptor (IR), muscle-specific chloride channel (ClC-1), tau, myotubularin-related protein 1 (MTMR1) and fast skeletal troponin T (TNNT3) (Philips *et al*, 1998; Savkur *et al*, 2001; Sergeant *et al*, 2001; Buj-Bello *et al*, 2002; Charlet-B *et al*, 2002b; Kanadia *et al*, 2003). In all cases, normal mRNA splice variants are produced, but the normal developmental splicing pattern is disrupted, resulting in expression of fetal protein isoforms that are inappropriate for adult tissues. The insulin resistance and myotonia observed in DM1 correlate with the disruption of splicing of two pre-mRNA targets, IR and ClC-1, respectively (Savkur *et al*, 2001, 2004; Mankodi *et al*, 2002; Charlet-B *et al*, 2002b).

The mechanism by which expanded repeats alter the regulation of pre-mRNA alternative splicing is unclear. Two families of RNA-binding proteins have been implicated in DM1 pathogenesis: CUG-BP1 and ETR-3-like factors (CELF) and muscleblind-like (MBNL) proteins (Miller *et al*, 2000; Fardaei *et al*, 2001; Ladd *et al*, 2001). Six CELF (also called BRUNOL) genes have been identified in humans (Good *et al*, 2000; Ladd *et al*, 2001, 2004). All six CELF proteins have been shown to regulate pre-mRNA alternative splicing and two (CUG-BP1 and ETR-3/CUG-BP2) have been shown to have cytoplasmic RNA-associated functions (Timchenko *et al*, 1999; Mukhopadhyay *et al*, 2003). A functional link has been established between splicing regulation by CELF proteins and DM1 pathogenesis. CUG-BP1 regulates alternative splicing of at least three of the pre-mRNAs (cTNT, IR and ClC-1) that are misregulated in DM striated muscle (Philips *et al*, 1998; Savkur *et al*, 2001; Charlet-B *et al*, 2002b). The splicing patterns observed for all three pre-mRNAs are consistent with

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increased CUG-BP1 activity and an increase in CUG-BP1 steady-state levels in DM1 striated muscle (Philips *et al*, 1998; Savkur *et al*, 2001; Timchenko *et al*, 2001; Charlet-B *et al*, 2002b). Furthermore, cTNT minigenes expressed in DM1 muscle cultures or cTNT and IR pre-mRNAs coexpressed with CUG repeat RNA in normal cells reproduce the aberrant splicing patterns observed for endogenous genes in DM cells (Philips *et al*, 1998; Savkur *et al*, 2001). The *trans*-dominant effects of endogenous or coexpressed CUG repeat RNA on cTNT and IR splicing regulation require the intronic CUG-BP1-binding sites, indicating that binding by CUG-BP1 and/or other CELF family members to their cognate intronic regulatory elements is required for induction of aberrant splicing regulation by CUG repeat RNA (Philips *et al*, 1998; Savkur *et al*, 2001).

The other family of RNA-binding proteins implicated in DM pathogenesis is MBNL, which is homologous to *Drosophila mbl* proteins required for photoreceptor and muscle differentiation (Begemann *et al*, 1997; Artero *et al*, 1998). Three mammalian MBNL genes have been identified: *MBNL1* (formally *MBNL*), *MBNL2* (*MBLL*) and *MBNL3* (*MBXL*), located on chromosomes 3, 13 and X, respectively (Miller *et al*, 2000; Fardaei *et al*, 2002). MBNL1 was identified in HeLa cells based on its ability to bind double-stranded CUG repeats (Miller *et al*, 2000). All three MBNL gene products colocalize with the expanded repeat RNA foci *in vivo* (Miller *et al*, 2000; Mankodi *et al*, 2001; Fardaei *et al*, 2002). Loss of MBNL function due to sequestration on CUG repeat RNA is proposed to play a role in DM pathogenesis (Miller *et al*, 2000). Consistent with this proposal, Kanadia *et al* (2003) demonstrated that a mouse knockout (*Mbnl1*$^{\Delta E3/\Delta E3}$) of specific *Mbnl1*-encoded isoforms reproduces the myotonia, cataracts and misregulation of splicing observed in DM1 and DM2. Although the loss of MBNL1 function reproduces the abnormal splicing pattern observed for ClC-1 and cTNT, it is not clear if the phenotype is caused by direct or indirect effects on splicing.

We tested whether MBNL family members bind and regulate splicing of pre-mRNAs that are misregulated in DM1 tissues. Our results demonstrate that all three MBNL family members are novel splicing regulators that act antagonistically to CELF proteins on the three pre-mRNAs tested: human and chicken cTNT, and human IR. Similar to CELF proteins, MBNL proteins can act as activators or repressors of splicing on different pre-mRNAs. MBNL1 binds a common motif near the human and chicken cTNT alternative exons within intronic regions, which appear to be single stranded. Mutations that prevent binding of MBNL to the human cTNT pre-mRNA dramatically reduce or eliminate responsiveness to MBNL proteins *in vivo*. CELF and MBNL proteins bind to distinct *cis*-elements and minigenes containing CELF- or MBNL-binding site mutations were used to demonstrate that regulation by one family does not require responsiveness to the other. We also show that modified cTNT and IR minigenes made nonresponsive to the *trans*-dominant effects of CUG repeat RNA still respond to MBNL depletion, suggesting that CUG repeat RNA affects splicing by a mechanism more complex than MBNL depletion alone.

Results

All three MBNL proteins regulate alternative splicing

To determine whether MBNL proteins can alter the splicing patterns of pre-mRNAs known to be abnormally regulated in DM1 striated muscle, GFP fusion proteins of all three MBNL proteins were transiently expressed with human and chicken cTNT minigenes in primary chicken skeletal muscle cultures. GFP–MBNL1, 2 and 3 strongly repressed inclusion of both human and chicken cTNT exon 5 in primary chicken skeletal muscle cultures, while expression of GFP to levels comparable to, or greater than, GFP–MBNL fusion proteins had no effect on splicing (Figure 1A and B). In addition, there were no differences in the splicing activity of GFP fusion proteins compared to Xpress epitope-tagged MBNL proteins (data not shown). Therefore, MBNL proteins are directly antagonistic to endogenous CELF activity, which activates cTNT exon inclusion in muscle (Charlet-B *et al*, 2002a).

Another pre-mRNA target that is misregulated in DM striated muscle is the IR (Savkur *et al*, 2001, 2004). To test whether the MBNL family can also regulate human IR, the three MBNL family members were coexpressed with a human IR minigene. In contrast to the inhibitory effect of MBNL on cTNT splicing, coexpression of MBNL family members with an IR minigene strongly induces exon inclusion, whereas GFP alone had no effect (Figure 1C). To determine whether the MBNL family has a general effect on alternative splicing, all three MBNL proteins were coexpressed with a clathrin light-chain minigene containing the neuron-specific exon EN. The EN alternative exon in this minigene strongly responds to overexpression of the SR family of proteins and htra2-β1, but not CELF proteins (Stamm *et al*, 1999; Singh *et al*, 2004; data not shown). Overexpression of GFP–MBNL1, 2 and 3 with the clathrin light-chain minigene had no effect on alternative splicing of exon EN (Figure 1D). MBNL expression also did not affect splicing of an artificial alternative exon flanked by splice sites from human β-globin intron 1 (data not shown). These results demonstrate that MBNL proteins do not have a general effect on alternative splicing, but rather regulate specific pre-mRNA targets.

siRNA-mediated depletion of MBNL1 alters splicing of cTNT and IR

To determine whether depletion of endogenous MBNL1 protein could also affect the splicing patterns of known DM pre-mRNA targets in human cells, siRNA constructs were designed to target MBNL1, but not MBNL2 and MBNL3. HeLa cells were chosen because they express MBNL1 (Miller *et al*, 2000) and are amenable to siRNA-mediated depletion (Elbashir *et al*, 2001). To confirm the specificity of the effects, two siRNA constructs were designed to target different regions of the MBNL1 mRNA. Independent transient transfection of each siRNA construct resulted in a knockdown of endogenous MBNL1 protein to less than 10–20%, based on comparisons to serial dilutions of the untransfected or mock-transfected lysates (Figure 2A; data not shown). Analysis of MBNL1 depletion by immunofluorescence demonstrated predominantly nuclear expression that was greatly reduced in the majority of cells by each siRNA construct (Figure 2B). In addition, the siRNA constructs silenced effectively the expression of GFP–MBNL1, but not GFP–MBNL2, GFP–MBNL3 or GFP from transiently transfected plasmids, and neither MBNL1 siRNA affected the levels of endogenous MBNL2 protein (data not shown). These results indicate that the siRNAs preferentially silence MBNL1.

To determine whether depletion of endogenous MBNL1 affected alternative splicing of cTNT, IR and clathrin light

chain, the minigenes were transfected with each siRNA construct. Depletion of MBNL1 promoted exon inclusion in cTNT, exon skipping in IR and only minimal splicing changes in the clathrin light-chain minigene (Figure 2C). These splicing effects were not caused by general activation of the mammalian RNAi machinery because siRNA targeting GFP or luciferase and nonspecific pools of siRNA had minimal effects on splicing of the three minigenes (Figure 2C; data not shown). Furthermore, the alteration in cTNT splicing caused by MBNL1 depletion in HeLa cells can be reversed by expression of GFP–MBNL2 or GFP–MBNL3, but not GFP (data not shown), demonstrating that adding back MBNL isoforms not targeted by MBNL1 siRNA rescues the splicing effects of MBNL1 deficiency.

Interestingly, siRNA-mediated depletion of MBNL1 reproduces the splicing pattern observed in DM1 for cTNT (exon inclusion) and IR (exon skipping), and is opposite to the pattern observed when MBNL1 is overexpressed. The overexpression and depletion data indicate that endogenous MBNL1 regulates the alternative splicing of cTNT and IR minigenes, and suggest that MBNL1 regulates these pre-mRNAs via specific *cis*-regulatory elements. The effects of MBNL on the cTNT and IR alternative exons are the opposite of the splicing patterns induced by CELF proteins, implying an antagonistic relationship between these protein families.

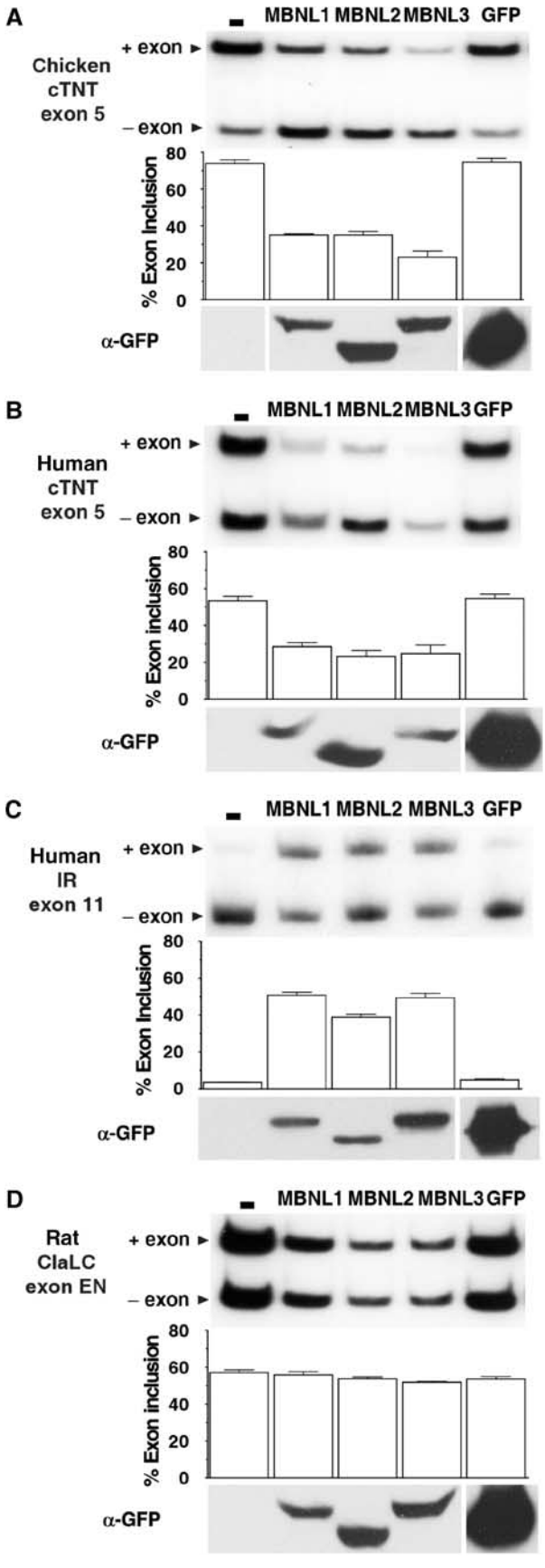

Figure 1 MBNL1, 2 and 3 regulate splicing of cTNT and IR alternative exons. Human and chicken cTNT and human IR minigenes were expressed with or without each of the three GFP–MBNL fusion proteins or with GFP alone. Duplicate transfections were used for extraction of RNA and protein. Inclusion of cTNT exon 5 or IR exon 11 was assayed by RT–PCR. Percent exon inclusion is calculated as $((\text{mRNA}+\text{exon})/(\text{mRNA}-\text{exon}+\text{mRNA}+\text{exon}))\times 100$. Results are derived from at least three independent experiments. Expression of GFP–MBNL1 (~72 kDa), GFP–MBNL2 (~58 kDa), GFP–MBNL3 (~70 kDa) and EGFP (~27 kDa) was detected by Western blot analysis using an anti-GFP monoclonal antibody. All three MBNL proteins promote exon 5 skipping of (**A**) chicken and (**B**) human cTNT exon 5 in primary skeletal muscle cultures. (**C**) All three MBNL proteins promote exon 11 inclusion in a human IR minigene in HEK293 cells. (**D**) MBNL proteins have minimal effects on splicing of exon EN in a clathrin light-chain B minigene in primary skeletal muscle cultures.

MBNL1 directly binds to introns adjacent to the human and chicken cTNT alternative exons

To determine whether the splicing effects of MBNL1 on pre-mRNA targets were direct or indirect, we performed a UV-crosslinking assay using purified recombinant GST–MBNL1 and uniformly labeled *in vitro*-transcribed segments from the human cTNT gene. The human cTNT minigene contains a 732 nucleotide (nt) cTNT genomic fragment that is necessary and sufficient to respond to MBNL1 overexpression and depletion (Figures 1 and 2C). To identify MBNL1-binding sites within this cTNT pre-mRNA region, uniformly ^{32}P-labeled, *in vitro*-transcribed RNAs covering the entire region were used for UV-crosslinking binding assays. As shown in Figure 3A, the binding of GST–MBNL1 on human cTNT was mapped to a 41 nt region within the 3′ splice site of exon 5 (compare RNAs C, D, E and F) located between a near-consensus branch point sequence and the 3′ cleavage site of the upstream intron. Scanning mutagenesis identified two MBNL1-binding sites located 18 and 36 nt upstream from exon 5 (Figure 3A). The absence of binding to long intronic segments (RNAs F and C) and RNAs containing nucleotide substitutions (RNAs H, J and M; see below) demonstrate

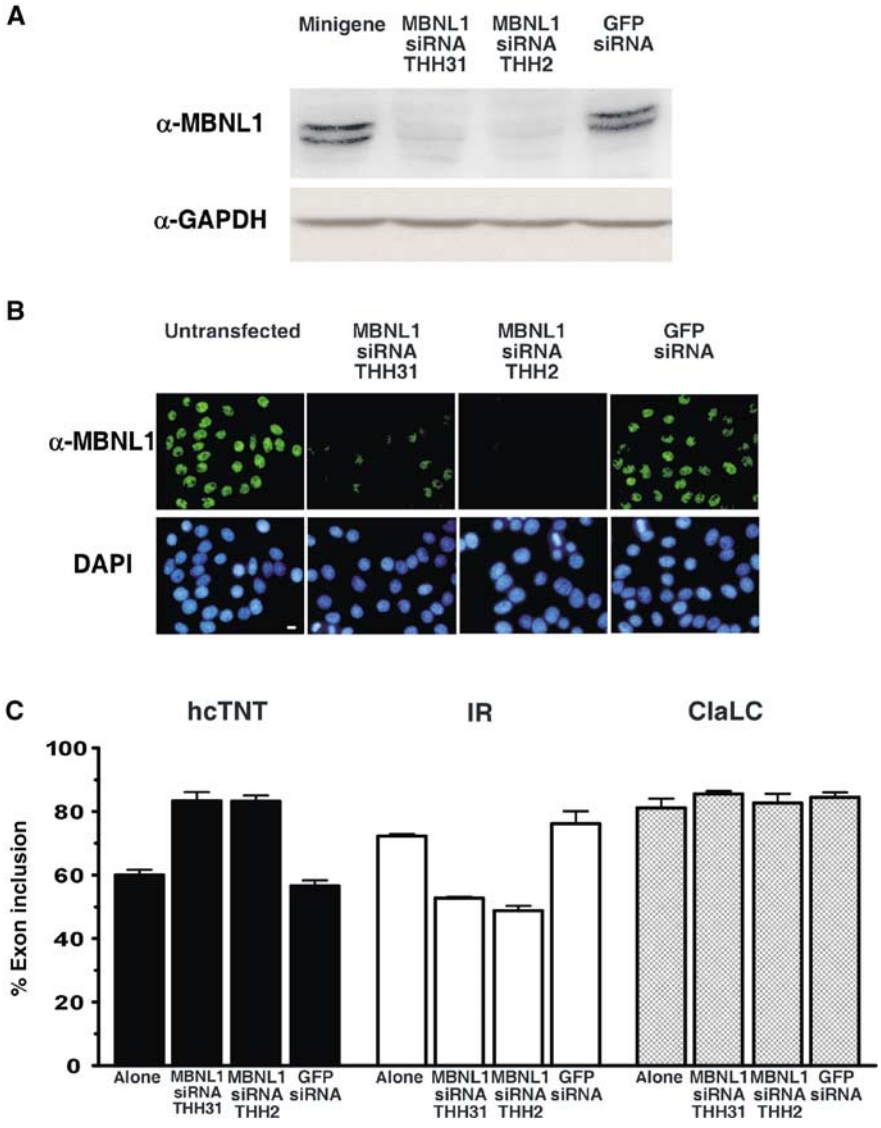

Figure 2 Endogenous MBNL1 regulates the splicing of human cTNT and IR minigenes. siRNA and minigenes were transfected into HeLa cells. (**A**) Western blot confirming depletion of endogenous MBNL1 by independent transfection of two different siRNA constructs using the MBNL1 monoclonal (mAb) 3A4, which recognizes two MBNL1 isoforms generated by alternative splicing (~41 and 42 kDa). GAPDH (~36 kDa) was used as a loading control. (**B**) Immunofluorescence using mAb 3A4 to confirm depletion of endogenous protein after independent transfection of each MBNL1 siRNA construct. Scale bar, 10 μm. (**C**) siRNA-mediated depletion of MBNL1 with two independent constructs reproduces the DM splicing patterns for cTNT and IR minigenes. RT–PCR results are from at least three transfections. GFP siRNA had no effect on splicing of any of the tested minigenes. MBNL1 siRNA had minimal effects on splicing of a rat clathrin light-chain minigene.

binding specificity. This analysis indicates that, for cTNT, the MBNL1-binding site is distinct from the CUG-BP1-binding site, which is located downstream from the alternative exon (Philips *et al*, 1998).

Nucleotide substitutions that disrupt both MBNL1-binding sites were introduced into the human cTNT minigene to test whether MBNL1 binding was required to affect responsiveness to MBNL1 expression *in vivo*. As the MBNL-binding site is located within the 3′ splice site of intron 4, only four nucleotide substitutions were introduced to reduce the effects of MBNL-binding site mutations on basal splicing efficiency (RNA M, Figure 3A). These substitutions prevented binding of recombinant MBNL1 to an RNA that is otherwise identical to RNA G containing the wild-type sequence (Figure 3B). In addition, nonlabeled RNA M was much less efficient than RNA G in competing binding of MBNL1 to labeled RNA G (Figure 3B). When introduced into the human cTNT minigene, the MBNL1-binding site mutation significantly reduced (MBNL1 and MBNL3) or eliminated (MBNL2) responsiveness to MBNL proteins (Figure 3C and D), demonstrating that loss of MBNL1 binding *in vitro* directly correlates with decreased responsiveness to MBNL1 *in vivo*. In contrast, the MBNL1-binding site mutations had little effect on responsiveness to CUG-BP1 (Figures 3C and D). GFP alone had minimal effects on splicing. We conclude that MBNL proteins regulate splicing by binding to the human cTNT pre-mRNA and that regulation by CUG-BP1 does not require the MBNL1-binding site.

UV-crosslinking analysis was performed to identify MBNL1-binding site(s) associated with the chicken cTNT

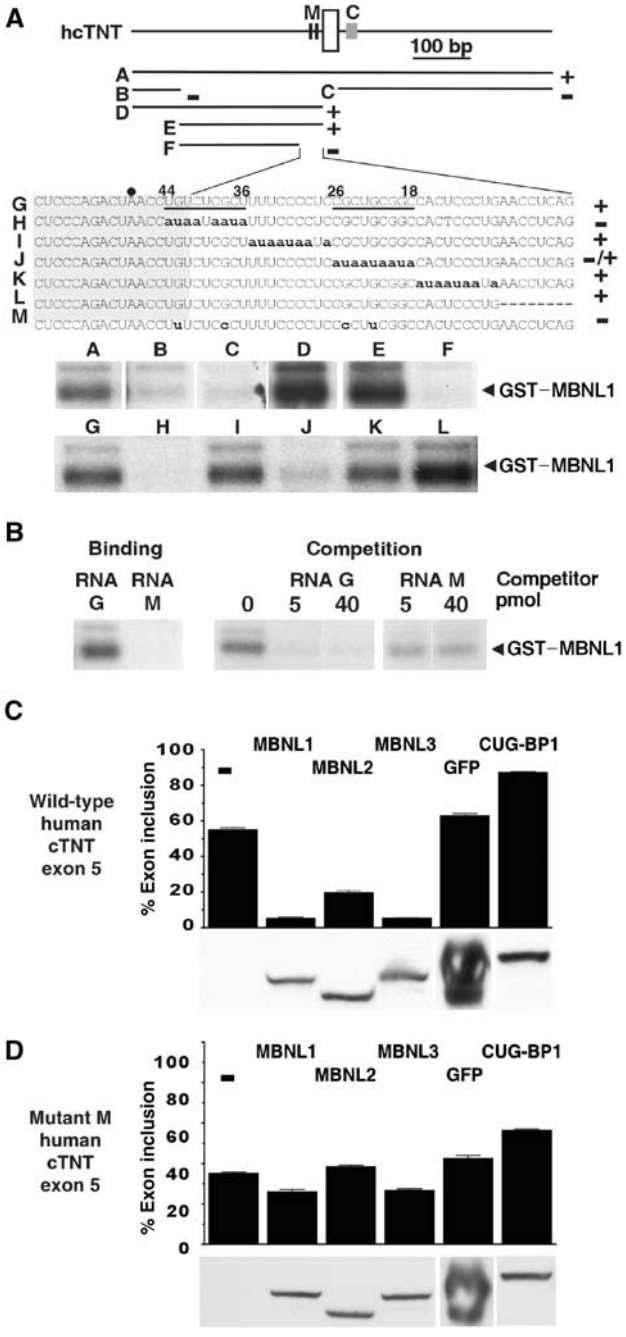

Figure 3 MBNL1 binds upstream of exon 5 in human cTNT at a site distinct from the CUG-BP1-binding site. (**A**) Binding of recombinant GST–MBNL1 to uniformly ^{32}P-labeled RNA was assayed by UV crosslinking. Scanning mutagenesis was performed by replacing 6 nt blocks with AUAAUA and identified two binding sites 18 and 36 nt upstream of the alternative exon. The MBNL1-binding sites (M) and the CUG-BP1-binding site (C) are located on opposite sides of exon 5. (+) and (−) indicate binding; (●) indicates a putative branch point adenosine. (**B**) Four nucleotide substitutions significantly reduce binding of recombinant MBNL1 detected by UV crosslinking. Competition of GST–MBNL1 binding to ^{32}P-labeled RNA G by the indicated picomoles of nonlabeled RNAs G or M shown in A). (**C, D**) MBNL1-binding site mutations reduce responsiveness to MBNL1, MBNL2 and MBNL3 coexpression but not CUG-BP1 in COSM6 cells. Human cTNT minigenes containing the natural sequence (C) or the four nucleotide substitutions (mutation M in A) in the MBNL1-binding site (D) were coexpressed with GFP or the indicated GFP fusion proteins. Exon inclusion was assayed by RT–PCR.

alternative exon 5. The genomic segment of chicken cTNT that responds to MBNL expression contains 99 and 142 nt of upstream and downstream introns flanking the alternative exon, respectively. Within the intronic segments are four muscle-specific splicing enhancers (MSEs, Figure 4A) previously shown to be required for enhanced exon inclusion in embryonic striated muscle (Ryan and Cooper, 1996; Cooper, 1998) and required for regulation by all the six CELF family members (Ladd *et al*, 2001, 2004). RNAs containing MSEs 1–4 or individual MSEs were transcribed *in vitro* as uniformly ^{32}P-labeled RNAs and used for UV crosslinking. GST–MBNL1 bound strongly to MSE4 and slightly to MSE1 (Figure 4A). In competition studies, nonlabeled MSE1 RNA poorly competed in the binding of GST–MBNL1 to RNA containing MSE1–4, while MSE4 effectively competed in binding (Figure 4B), consistent with the UV-crosslinking results. The absence of competition by MSE2 or MSE3 demonstrates the sequence specificity of MBNL1 binding (Figure 4B). To define the MBNL1-binding site(s) within MSE4, scanning mutagenesis was performed. Two regions required for MBNL1 binding were identified at 94 and 120 nt downstream from the exon (Figure 4C). Alignment of the four MBNL1-binding sites in chicken and human cTNT revealed a common motif of YGCU(U/G)Y (Figure 4D).

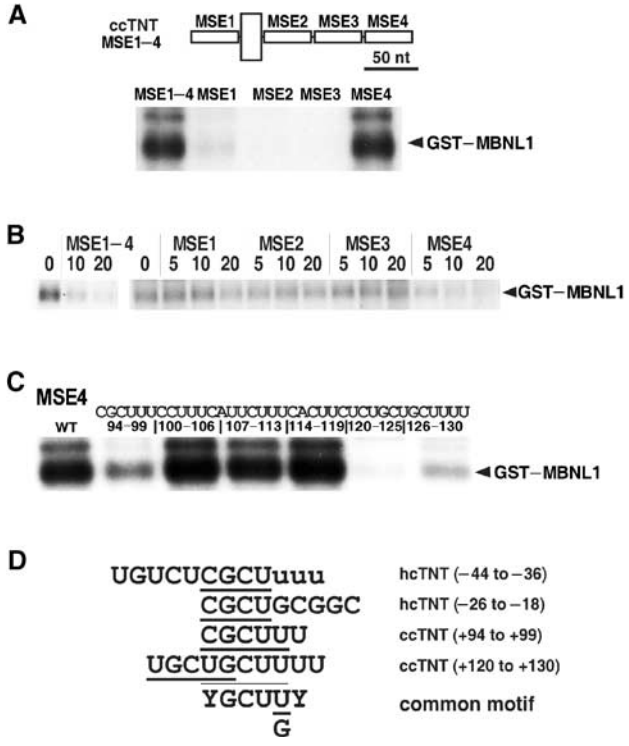

Figure 4 MBNL1 binds to *cis*-elements in chicken cTNT intron 5 required for muscle-specific splicing. (**A**) The chicken cTNT MSE1–4 RNA contains an alternative exon flanked by four MSEs. GST–MBNL1 bound weakly to MSE1 and strongly to MSE4 in UV-crosslinking assays. (**B**) Competition of GST–MBNL1 binding to labeled chicken cTNT MSE1–4 RNA by nonlabeled MSE RNAs. Picomoles of competitor RNA are indicated. (**C**) Scanning mutagenesis identified two MBNL1-binding sites within MSE4. (**D**) Alignment of the four MBNL1-binding motifs in human and chicken cTNT reveals a common motif.

CELF protein cis-regulatory elements in cTNT and IR are not required for regulation by MBNL1

The CUG-BP1-binding site located downstream from exon 5 in the human cTNT minigene is required for regulation by all six CELF proteins (Philips *et al*, 1998; T Ho, unpublished data), and is distinct from the MBNL-binding site mapped in Figure 3. The results shown above demonstrate that CUG-BP1 regulates minigenes in which MBNL1-binding site mutations have greatly reduced or eliminated MBNL responsiveness (Figure 3D). To determine whether MBNL1 can regulate minigenes lacking the CUG-BP1-binding site, GFP–MBNL1 or MBNL1 siRNA was cotransfected with a human cTNT minigene containing mutated CUG-BP1-binding sites. The overexpression and depletion results demonstrate that cTNT minigenes containing the mutant and wild-type CUG-BP1-binding sites are equally responsive to MBNL1 (Figure 5A and B). GFP–MBNL2 and 3 also showed similar regulation of wild-type and mutant human cTNT minigenes (data not shown).

Similarly, for the IR minigene, regulation by CUG-BP1 requires a CUG-BP1-binding site in a 110 nt region located upstream of IR exon 11 (Savkur *et al*, 2001). A mutant IR minigene lacking the CUG-BP1-binding site was coexpressed with GFP–MBNL1, 2 and 3 in HEK293 cells (Figure 6A) or MBNL1 siRNA constructs in HeLa cells (Figure 6B) to determine whether regulation by MBNL proteins requires the CUG-BP1-binding site. The mutant IR minigenes displayed regulation by MBNL proteins, which was comparable to the wild-type IR minigenes (compare Figures 6A and 1C and 6B and 2C). We conclude that regulation of human cTNT and IR by MBNL proteins does not require the CUG-BP1-binding site.

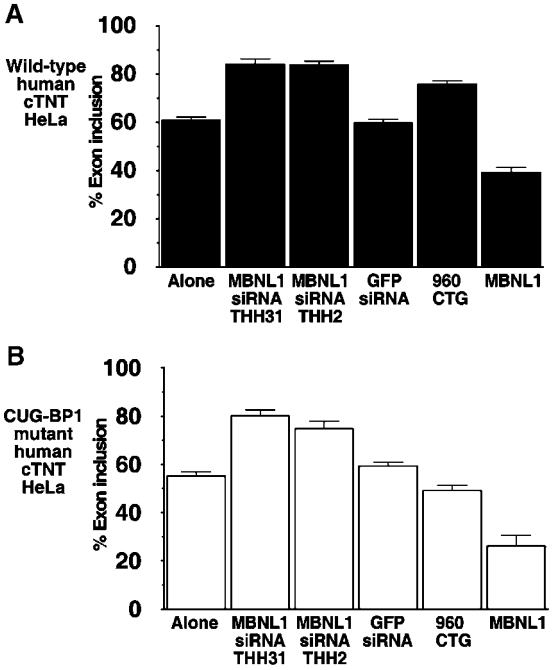

Figure 5 Regulation of human cTNT by MBNL1 is independent of CELF regulation. The (**A**) wild-type cTNT minigene or a (**B**) mutant cTNT minigene with point mutations that prevent CUG-BP1 binding and regulation were cotransfected with the indicated siRNA constructs, a plasmid expressing a DMPK minigene with 960 CUG repeats (Philips *et al*, 1998) or a GFP–MBNL1 expression plasmid in HeLa cells.

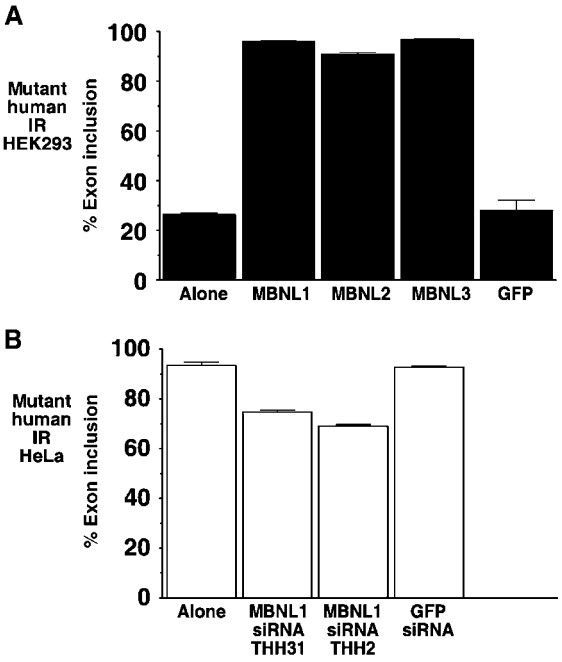

Figure 6 Deletion of the human IR CUG-BP1-binding site does not affect regulation by MBNL1. (**A**) All the three MBNL proteins promote exon 11 inclusion of a mutant human IR minigene lacking the CUG-BP1-binding site in HEK293 cells. (**B**) RNAi depletion of MBNL1 in HeLa cells using the indicated siRNA constructs promotes exon 11 skipping in a human IR minigene lacking the CUG-BP1-binding site.

Mutant cTNT and IR minigenes lacking the CUG-BP1-binding site respond as strongly as nonmutated minigenes to MBNL1 depletion by RNAi (Figures 5B and 6B). However, neither of these minigenes respond to the *trans*-dominant effects of coexpressed CUG repeat RNA as do the nonmutated minigenes (Philips *et al*, 1998; Savkur *et al*, 2001; 960CTG, Figure 5). The RNAi results demonstrate that the mutated cTNT and IR minigenes are 'competent' to respond to MBNL1 depletion and yet they do not respond to coexpression of CUG repeat RNA. Therefore, while our results demonstrate that MBNL proteins are alternative splicing regulators of cTNT and IR alternative exons, this result suggests that MBNL depletion by CUG repeat RNA is not sufficient to account for the *trans*-dominant effect of CUG repeat RNA on splicing (see Discussion).

Discussion

The MBNL family of proteins regulate alternative splicing

In this paper, we show that MBNL proteins function as potent target-specific regulators of pre-mRNA alternative splicing. This finding has particular relevance to the molecular mechanism of DM pathogenesis in which loss of MBNL function due to sequestration on expanded CUG repeat RNA has been proposed to play a major role (Miller *et al*, 2000; Kanadia *et al*, 2003). We employed overexpression and siRNA-mediated depletion to demonstrate that all three *MBNL* genes encode factors that regulate splicing of the three pre-mRNAs that were tested, human cTNT, chicken cTNT and human IR. MBNL1 binds to the introns flanking exon 5 in

both chicken and human cTNT pre-mRNAs, and point mutations that eliminate binding *in vitro* also eliminated or decreased responsiveness of the human cTNT minigene to MBNL1, MBNL2 and MBNL3 coexpression. These results demonstrate that regulation by MBNL protein is mediated via binding the pre-mRNA, and suggest that all three MBNL proteins regulate human cTNT splicing by binding to the same site.

Proteins from all three MBNL genes contain two pairs of Cys3His zinc-finger-related motifs with identical spacing between cysteine and histidine residues in fingers 1 and 3 (CX7CX6CX3H) and fingers 2 and 4 (CX7CX4CX3H) (Miller *et al*, 2000; Fardaei *et al*, 2002; Squillace *et al*, 2002). The Cys3His-type zinc-finger is an evolutionarily conserved motif found in a number of proteins that perform diverse RNA-processing functions, and mutation of this motif results in a loss of RNA binding and disrupts protein function (Bai and Tolias, 1996, 1998; Lai *et al*, 1999; Stoecklin *et al*, 2002). MBNL1 was described originally as a double-stranded RNA-binding protein and has been shown to bind expanded CUG repeats that form an extended hairpin *in vitro* (Michalowski *et al*, 1999; Miller *et al*, 2000; Sobczak *et al*, 2003). However, the binding sites within human and chicken cTNT introns are not predicted to form an identifiable secondary structure. We conclude that MBNL1 also binds to specific sequences within single-stranded RNA, consistent with the results from other Cys3His zinc-finger proteins (Cheng *et al*, 2003; Michel *et al*, 2003). Among the four binding sites mapped, we delineated a variable motif YGCU(U/G)Y, where Y = U or C. We note that this motif is found in expanded CUG repeats, to which MBNL1 has high affinity. However, MBNL genes have multiple splice variants, and different protein isoforms may have different binding affinities, binding specificities and/or splicing activities (Fardaei *et al*, 2002; Kanadia *et al*, 2003; Kino *et al*, 2004).

MBNL and CELF proteins are antagonistic regulators of alternative splicing

Of particular interest is the observation that CELF and MBNL proteins are antagonistic regulators of cTNT and IR splicing. CELF proteins induce inclusion of both human and chicken cTNT exon 5 and exon skipping of human IR exon 11 (Philips *et al*, 1998; Ladd *et al*, 2001; Savkur *et al*, 2001; Charlet-B *et al*, 2002a). In contrast, MBNL proteins induce skipping of human and chicken cTNT exon 5 and inclusion of human IR exon 11. MBNL and CELF proteins mediate their effects via distinct *cis*-acting elements in human and chicken cTNT, indicating that antagonism is not due to direct competition for the same binding site. CUG-BP1 regulates splicing of minigenes with MBNL1-binding site mutations that block MBNL1 responsiveness. Similarly, MBNL regulates splicing by a mechanism that does not require responsiveness to CELF proteins, since minigenes that are nonresponsive to CELF proteins due to binding site mutations remain responsive to MBNL. Therefore, antagonism does not appear to involve an effect of MBNL on CELF function or *vice versa*. Rather, MBNL and CELF proteins most likely have separate 'inputs' into the decision of whether or not the exon is to be recognized by the basal splicing machinery.

Polypyrimidine tract-binding protein (PTB) also acts antagonistically to CELF proteins to regulate chicken cTNT alternative splicing (Charlet-B *et al*, 2002a). PTB usually functions as a splicing repressor to prevent expression of cell-specific splicing patterns in inappropriate cell types (Ashiya and Grabowski, 1997; Chan and Black, 1997; Zhang *et al*, 1999; Jin *et al*, 2000; Polydorides *et al*, 2000; Charlet-B *et al*, 2002a; Wagner and Garcia-Blanco, 2002). In contrast, MBNL proteins activate as well as repress splicing, depending on the pre-mRNA substrate. PTB binds chicken cTNT MSE1, MSE2 and MSE4 to repress exon 5 inclusion, while MBNL1 binds MSE4 (Charlet-B *et al*, 2002a). Mutations that prevent PTB binding to MSE4 are distinct from those that prevent MBNL1 binding, strongly suggesting that these two proteins have similar effects on cTNT splicing by distinct but adjacent binding sites.

The identification of two protein families with opposing activities on different splicing patterns provides a unique opportunity to understand regulatory mechanisms controlling splice site selection. One aspect will be to understand the mechanism by which MBNL and CELF proteins activate splicing of one substrate and repress splicing of another. Another will be to determine whether antagonistic regulation by MBNL and CELF family members is mechanistically linked such that all pre-mRNAs regulated by one family are also regulated by the other. Alternatively, it could be that some pre-mRNAs such as cTNT and IR are regulated by both, while other pre-mRNAs will be targets of one or the other family. This distinction has implications with regard to the consequences of disruption of MBNL and CELF function in DM.

The role of MBNL in DM pathogenesis

Expression of expanded CUG and CCUG repeats is proposed to have *trans*-dominant effects, which disrupt the function of proteins that regulate alternative splicing and alter splicing of their target pre-mRNAs. A role for loss of MBNL function in DM pathogenesis is indicated by physical evidence linking the MBNL proteins to the CUG and CCUG repeat foci, and genetic evidence that the loss of specific MBNL isoforms results in several aspects of DM, including aberrant splicing regulation (Miller *et al*, 2000; Fardaei *et al*, 2002; Mankodi *et al*, 2002; Kanadia *et al*, 2003). Here we demonstrate that MBNL proteins directly regulate splicing of two of the misregulated genes in DM and that loss of MBNL splicing activity reproduces the splicing patterns observed in DM.

The splicing patterns observed for cTNT and IR in DM are consistent with increased CELF activity and/or loss of MBNL activity. The striking similarities in phenotype and splicing abnormalities observed in the *Mbnl1*$^{\Delta E3/\Delta E3}$ mice and HSA^{LR} mice expressing long CUG repeat RNA strongly support a role for the loss of MBNL due to sequestration on expanded repeats and unopposed CELF activity (Mankodi *et al*, 2000; Kanadia *et al*, 2003). Another possibility is that expression of expanded CUG and CCUG repeats results in increased CELF activity, such as by causing increased CUG-BP1 steady-state levels by an unknown mechanism (Savkur *et al*, 2001; Timchenko *et al*, 2001). These possibilities are not mutually exclusive. Increased CELF plus decreased MBNL activities would have combined effects on pre-mRNAs regulated antagonistically by both families as well as effects on pre-mRNAs that are regulated by only one family.

Our results demonstrate that MBNL proteins regulate alternative splicing of specific pre-mRNAs that are misregulated in DM1, and that the splicing patterns observed in DM1

are consistent with MBNL1 depletion. However, we also present evidence that MBNL depletion is not sufficient to completely explain the *trans*-dominant splicing effects of CUG repeat RNA on cTNT and IR splicing. We have shown previously, and in Figure 5B here, that cTNT and IR minigenes made insensitive to CELF regulation by mutations in the CUG-BP1-binding site no longer respond to expanded CUG repeat RNA, suggesting that the *trans*-dominant effect is mediated at least in part via an intact CUG-BP1-binding site (Figure 5B; Philips *et al*, 1998; Savkur *et al*, 2001). Here we show that the mutated cTNT and IR minigenes are competent to respond to MBNL depletion by RNAi as strongly as the nonmutated minigenes, yet they do not respond to CUG repeat RNA. If expanded CUG repeats affected cTNT and IR splicing simply by sequestering and depleting MBNL, then the coexpression of CUG repeats should have affected splicing of the mutated as well as nonmutated minigenes. These results suggest that the repeats have a *trans*-dominant effect on splicing by a mechanism more complex than MBNL depletion alone.

Materials and methods

Plasmids

The cTNT, IR and clathrin light chain B minigenes were previously described (Kosaki *et al*, 1998; Philips *et al*, 1998; Stamm *et al*, 1999; Ladd *et al*, 2001). GFP fusions with MBNL1, 2 and 3 were provided by Dr JD Brook (Fardaei *et al*, 2002). GFP–MBNL1 was found to have a novel MBNL1 isoform lacking exons 7, 9 and 10 and containing a frameshift in exon 12. Plasmids expressing DMPK exons 11–15 containing 960 interrupted CUG repeats in exon 15 were cloned using techniques as previously described (Philips *et al*, 1998). The MBNL mutant human cTNT minigene was generated by inverse PCR.

Transfection of siRNA

Two custom siRNA duplexes were designed for RNAi against human MBNL1 using the Dharmacon siDESIGN program (www.dharmacon.com), and were synthesized by Dharmacon. The sequences are as follows: THH31 mRNA target (AA-N19 format 5′→3′) AACAGACAGACUUGAGGUAUG, THH2 mRNA target (AA-N19 format 5′→3′) AACACGGAAUGUAAAUUUGCA, GFP siRNA duplex (Dharmacon, Lafayette, CO cat. no. D-001300-01-20). 300 000 HeLa cells were plated in 2 ml of antibiotic-free growth media (DMEM supplemented with 10% FBS) per well in a six-well plate. At 12 h after plating, the media was exchanged with 800 µl serum-free media (DMEM) per well. siRNA duplex (2.66 µg) was transfected using Oligofectamine (Invitrogen, Carlsbad, CA). 1 ml of 3 × serum-containing media (DMEM supplemented with 30% FBS) was added after 4 h. After 12 h, the 3 × serum-containing media was replaced with antibiotic-free growth media and the cells were transfected with 1 µg of minigene and 2.66 µg of siRNA duplex using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The media was exchanged with antibiotic-free growth media 6 h later. RNA and protein were harvested 48 h after transfection of the minigene.

Transient transfection and RT–PCR analysis

HEK293 cells were plated at 500 000 cells per well in a six-well plate in DMEM supplemented with 10% FBS and Gibco penicillin–streptomycin. At 24 h after plating, the cells were transfected with 1 µg of minigene and 2 µg of protein expression plasmid using Fugene6 (Roche, Indianapolis, IN), according to the manufacturer's directions. Protein and RNA were harvested 36–48 h after transfection. Chicken primary muscle cultures were prepared, maintained and transfected as previously described, using 0.5 µg minigene reporter and 1 µg expression plasmid (Xu *et al*, 1993). COSM6 cells were plated at 150 000 cells per well in a six-well plate in DMEM supplemented with 10% FBS, Gibco penicillin–streptomycin and L-glutamine. At 24 h after plating, the cells were transfected with 500 ng of minigene and 1 µg of protein expression plasmid using Fugene6 (Roche, Indianapolis, IN) according to the manufacturer's directions. Protein and RNA were harvested 36–48 h after transfection. RNA isolation and RT–PCR analysis for the cTNT, IR, and clathrin light-chain B minigenes were performed as described previously (Philips *et al*, 1998; Stamm *et al*, 1999; Savkur *et al*, 2001).

Western blot analysis

Cells were harvested in protein loading buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol and 5% 2-β-mercaptoethanol) and the protein concentration was quantitated with the Non-Interfering Protein Assay (Genotech, St Louis, MO). Total protein lysates from HEK293 (20 µg) and primary chicken skeletal (30 µg) cultures were loaded on a 12.5% acrylamide gel and transferred to Immobilon-P membranes (Millipore, Bedford, MA). GFP was detected using JL-8 monoclonal antibody (BD Biosciences, Palo Alto, CA) at a dilution of 1:2000. The secondary antibody was a goat anti-mouse HRP conjugate (Jackson Immunoresearch, West Grove, PA) at a dilution of 1:5000. To detect endogenous MBNL1, HeLa (50 µg) protein lysates were loaded on a 12.5% acrylamide gel. Blots were probed with the monoclonal 3A4 (16 mg/ml) at a dilution of 1:500. The secondary antibody was a sheep anti-mouse HRP conjugate (Amersham Biosciences, Piscataway, NJ) at a dilution of 1:5000. For GAPDH in HeLa cells, 15 µg of total protein lysates was run on a 12.5% acrylamide gel, transferred to membranes and detected using the 6G5 monoclonal (Biogenesis, Kingston, NH) at a dilution of 1:100 000. The secondary antibody was a goat anti-mouse HRP conjugate (Jackson Immunoresearch, West Grove, PA) at a dilution of 1:5000.

Immunofluorescence

HeLa cells were grown on coverslips in six-well plates and transfected with 2.66 µg siRNA using Oligofectamine. The coverslips were washed with cold PBS (pH 7.4) and fixed in 4% paraformaldehyde/PBS for 15 min. After three washes with PBS, the cells were dehydrated with 70% ethanol overnight at 4°C. The coverslips were then rehydrated with PBS for 10 min and incubated with 3% BSA/PBS for 15 min at room temperature. The cells were washed once with PBS and incubated with the primary antibody 3A4 (10 mg/ml) at a dilution of 1:1000 in 3% BSA/PBS at room temperature for 1 h. The cells were then washed three times with PBS and incubated with the secondary antibody, Alexa Fluor-labeled goat anti-mouse IgG (2 mg/ml, Molecular Probes, Eugene, OR), at a dilution of 1:100 in 3% BSA/PBS at room temperature for 1 h. The cells were then washed with PBS three times, counterstained with DAPI (Molecular Probes, Eugene, OR) and mounted for visualization by fluorescence microscopy.

In vitro transcription and UV crosslinking

Uniformly ^{32}P-labeled RNAs were transcribed *in vitro* using [α-^{32}P]GTP and [α-^{32}P]UTP (Perkin-Elmer, Wellesley, MA) from PCR products or cloned regions of the human or chicken introns 4 and 5, as represented in Figures 3 and 4. UV-crosslinking assays were performed using radiolabeled transcripts standardized for picomoles of G and U. UV-crosslinking assays included 1 µg of purified GST–MBNL1 in the presence of 1 µg BSA, 1 µg tRNA, 0.3 µg heparin, 0.3 mM magnesium acetate, in 2 mM magnesium acetate, 2 mM ATP, 16 mM HEPES (pH 7.9), 65 mM potassium glutamate, 0.16 mM EDTA, 0.4 mM DTT and 16% glycerol. Binding was for 10 min at 30°C. Recombinant GST–MBNL1 protein was produced as described (Miller *et al*, 2000). Competitions were performed as described previously (Singh *et al*, 2004). The indicated amounts of nonlabeled competitor RNAs were added to the binding reaction 10 min prior to addition of labeled substrate RNA.

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