Colocalization of muscleblind with RNA foci is separable from mis-regulation of alternative splicing in myotonic dystrophy

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Summary

Myotonic dystrophy type I (DM1), which is caused by a non-coding CTG-repeat expansion in the dystrophia myotonica-protein kinase (*DMPK*) gene, is an RNA-mediated disease. Expanded CUG repeats in transcripts of mutant *DMPK* form nuclear foci that recruit muscleblind-like (MBNL) proteins, a family of alternative splicing factors. Although transcripts of mutant *DMPK* and MBNL proteins accumulate in nuclear RNA foci, it is not clear whether foci formation is required for splicing mis-regulation. Here, we use a co-transfection strategy to show that both CUG and CAG repeats form RNA foci that colocalize with green fluorescent protein (GFP)-MBNL1 and endogenous MBNL1. However, only CUG repeats alter splicing of the two tested pre-mRNAs, cardiac troponin T (cTNT) and insulin receptor (IR). Using FRAP, we demonstrate that GFP-MBNL1 in CUG and CAG foci have similar half-times of recovery and fractions of immobile molecules, suggesting that GFP-MBNL1 is bound by both CUG and CAG repeats. We also find an immobile fraction of GFP-MBNL1 in DM1 fibroblasts and a similar rapid exchange in endogenous CUG RNA foci. Therefore, formation of RNA foci and disruption of MBNL1-regulated splicing are separable events.

Key words: Alternative splicing, MBNL, Muscleblind, Myotonic dystrophy

Introduction

Nuclear proteins are often segregated into distinct subnuclear compartments that can be visualized by immunofluorescence or live imaging (Zimber et al., 2004). These nuclear bodies are reorganized in cells in response to stresses and signaling. Some of these compartments, such as speckles, contain pre-mRNA splicing factors (Lamond and Sleeman, 2003). For speckles, there are approximately 25 to 50 nuclear domains located throughout a mammalian cell that contain serine/arginine (SR) proteins (Zimber et al., 2004). Although there are regions of increased SF2/ASF concentration in the nucleoplasm, protein molecules in these regions are under constant flux (Misteli et al., 1997; Phair and Misteli, 2000). Splicing factors continuously shuttle in and out, leading to variations in size and shape of the speckles (Kruhlak et al., 2000; Phair and Misteli, 2000).

Of particular interest is the relationship of splicing factors to human disease (Zimber et al., 2004). Splicing defects can be caused by cis-acting or trans-acting mutations (Faustino and Cooper, 2003). Cis-acting mutations can disrupt constitutive and alternative splice sites in the pre-mRNA. Trans-acting mutations can disrupt the basal or alternative splicing machinery.

An example of a trans mutation that affects alternative splicing factors and causes disease is myotonic dystrophy (DM), a multi-systemic disorder caused by two different micro-satellite 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), whereas 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 in DM1 and DM2, there appears to be a common disease-associated feature involving the accumulation of transcripts from the expanded allele into discrete nuclear RNA foci that contain long tracts of expanded CUG or CCUG repeats (Davis et al., 1997; Liquori et al., 2001; Taneja et al., 1995).

In DM1 myoblasts, the *DMPK* gene with expanded repeats is correctly spliced and polyadenylated (Davis et al., 1997). The half-life of the transcripts of mutant *DMPK* after exposure of cells to actinomycin D, an inhibitor of transcription, was estimated to be between 13-17 hours, suggesting stable RNA and foci (Davis et al., 1997). RNA from the normal allele in DM1 is spliced, exported to the cytoplasm, and translated. DM1 skeletal-muscle tissue, cultured fibroblasts and brain tissues have one to five foci per nucleus, whereas cultured DM1 fibroblasts converted to myoblasts with *MyoD* contain hundreds of foci per nuclei (Davis et al., 1997; Mankodi et al., 2001; Taneja, 1998).

These RNA foci do not significantly colocalize with either speckles or polyadenylated RNA (Taneja et al., 1995). Markers

for Cajal bodies (coilin), promyelocytic leukemia bodies (PML), nucleoli (nucleolin), and the perinuclear compartment (PTB) also did not show significant colocalization (Mankodi et al., 2003). Other CUG and double-stranded (ds) RNA-interacting proteins [members of the CUG-BP- and ETR-3-like factors (CELF family), protein kinase R (PKR)], have been examined for colocalization, but only muscleblind-like proteins (MBNL), a family of alternative splicing factors, have consistently shown colocalization in DM skeletal-muscle (Mankodi et al., 2003).

Loss of MBNL function owing to sequestration of CUG repeat RNA has been proposed to play a role in DM pathogenesis (Miller et al., 2000). Consistent with this proposal, small interference RNA (siRNA)-mediated depletion of MBNL1 reproduces the DM pattern of splicing for cardiac troponin T (cTNT) and insulin receptor (IR) minigenes (Ho et al., 2004). Furthermore, a mouse knockout (*Mbnl1*$^{\Delta E3/\Delta E3}$) of the specific *Mbnl1*-encoded isoforms that are recruited by CUG and CCUG expansion RNAs reproduces the myotonia, cataracts and DM1 pattern of splicing mis-regulation for cTNT, ClC-1 and TNNT3 (Kanadia et al., 2003). Disruption of MBNL function leads to mis-regulated alternative splicing of specific MBNL targets, rather than to a general disruption of splicing (Ho et al., 2004; Kanadia et al., 2003). In all cases of mis-regulated splicing in DM tissues, the normal developmental splicing pattern is disrupted, which results in the expression of fetal protein isoforms inappropriate for adult tissues. Although the pathogenesis of DM involves the loss-of-function of MBNL, it is unclear how the repeat-containing RNA causes disease and whether RNA foci formation is required for pathogenesis.

Here, we show that nuclear RNA foci form in cells that overexpress CUG repeats in the context of *DMPK* exons 11-15. The expression of CUG repeats results in the formation of RNA foci that colocalize with exogenously expressed green fluorescent protein (GFP)-MBNL1 and endogenous MBNL1. Interestingly, we also find that identical mRNAs containing CAG repeats form RNA foci, and that CAG foci also colocalize with MBNL1. Although both CUG and CAG RNA repeats form foci that contain MBNL1, only CUG repeats alter splicing of the two tested pre-mRNAs cTNT and IR. Using fluorescence recovery after photobleaching (FRAP) analysis, we demonstrate that both CUG and CAG RNA foci contain similar fractions of immobile GFP-MBNL1. The half-times of recovery of the mobile fraction of GFP-MBNL1 in CUG and CAG foci and in the nucleoplasm are not significantly different suggesting that, within the mobile fraction, there is a rapid exchange of MBNL protein in and out of either CUG or CAG RNA foci. FRAP analysis also demonstrated an immobile fraction of GFP-MBNL1 in DM1 fibroblasts and a similarly rapid exchange of MBNL protein in endogenous CUG RNA foci. In addition, the presence of this immobile fraction does not correlate with an ability to affect splicing in trans. These results demonstrate that the disruption of splicing of MBNL1 pre-mRNA targets can be uncoupled from the accumulation of MBNL1 into nuclear foci.

Materials and Methods

Plasmids

The cTNT and IR minigenes were previously described (Kosaki et al., 1998; Ladd et al., 2001; Philips et al., 1998). GFP-MBNL1 contains the 41 kDa isoform of MBNL1 inserted into pEGFP-N1 (BD Biosciences, San Jose, CA) at *Kpn*I and *Bam*HI sites (Kanadia et al., 2003; Miller et al., 2000). The plasmid was confirmed by sequencing. Plasmids expressing *DMPK* exons 11-15, containing 960 CUG and CAG repeats in exon 15, were cloned using techniques previously described (Philips et al., 1998). The interrupted repeats are composed of repeating units of the sequence $(CTG)_{20}CTCGA$ or $(CAG)_{20}TCGAG$. The integrity of the repeats was confirmed by restriction enzyme analysis.

Tissue culture cells

COSM6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% certified fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% *D*-glutamine (all from Gibco) at 37°C in 5% CO_2. DM1 fibroblasts from skin biopsies of two unrelated adults affected by DM1 (GM03987 and GM03132, with 500 and 2,000 CTG repeats, respectively) were purchased from Coriell Cell Repositiories (Camden, NJ). The length of the repeats in cell cultures was confirmed by Southern blot analysis. DM1 fibroblast cultures were maintained as previously described (Savkur et al., 2001).

Transient transfection of cells

COSM6 cells (2×10^5) or DM1 fibroblasts (3×10^5) were plated into a 6-well plate containing pre-sterilized coverslips in DMEM supplemented with 10% FBS and penicillin-streptomycin (Gibco). For minigene splicing, 24 hours after plating, cells were transfected with 100 ng of minigene, and 100 ng, 300 ng and 1 μg of repeat-expressing plasmid. The total amount of plasmid transfected per well was 2 μg, using pSP72 plasmid as a carrier and using Fugene6 (Roche, Indianapolis, IN) according to the manufacturer's directions. For western blot analysis, GFP-MBNL1 fusions were detected as previously described using JL-8 monoclonal antibody (mAb) (BD Biosciences, Palo Alto, CA) at a dilution of 1:2000 (Ho et al., 2004). The secondary antibody was a goat anti-mouse HRP conjugate (Jackson Immunoresearch, West Grove, PA) at a dilution of 1:5000.

Reverse transcriptase PCR analysis

RNA isolation and reverse transcriptase (RT)-PCR analysis for the cTNT and IR minigenes was performed as described previously (Ho et al., 2004; Kosaki et al., 1998; Ladd et al., 2001; Philips et al., 1998; Savkur et al., 2001).

Fluorescence in situ hybridization (FISH)

The detection of CUG and CAG transcripts in COSM6 cells and DM1 fibroblasts using FISH was carried out as previously published (Taneja, 1998) using Cy3-labeled 15-mer PNA probes, and $(CAG)_5$ and $(CTG)_5$ (Applied Biosystems, Foster City, CA), respectively, with the following modifications. The cells were fixed in 4% paraformaldehyde and permeabilized with 0.02% Triton X-100 in PBS, pH 7.4. For RNase treatment, cells were permeabilized then treated with 0.01 μg/ml DNase-free RNase (Roche Applied Science, Indianapolis, IN) in 50 mM Tris-HCl, 10 mM $MgCl_2$, in PBS, pH 7.4 for 1.5 hours at 37°C. For DNase treatment, the cells were treated with 1 U/μL RNase-free DNase I (Roche Applied Science, Indianapolis, IN) in cytoskeletal buffer (10 mM PIPES, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, pH 6.8) for 1.5 hours at 37°C. Additional DNA was removed by two additional washes with the ice-cold cytoskeletal buffer (Nalepa and Harper, 2004; Nickerson et al., 1997). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) using Vectashield (Vector Laboratories, Inc., Burlingame, CA). Cells were visualized using a deconvolution

microscope (Applied Precision, LLC, Issaquah, WA) and images were deconvolved using Deltavision SoftWoRx software (15 cycles).

Combined FISH/immunofluorescence

COSM6 cells (1.2×10^5) were seeded on 2-well Falcon culture slides (BD Biosciences, San Jose, CA) and transfected 24 hours after plating. Cells were washed with Hanks Balanced Salt solution and then with CSK buffer (300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1.2 mM PMSF, 10 mM PIPES, pH 6.8). The slides were incubated on ice in CSK buffer with 0.5% Triton X-100 and 10 mM vanadyl sulfate for 1 minute. They were then fixed in 4% paraformaldehyde/PBS for 10 minutes at room temperature and washed with 70% ethanol. Cells were dehydrated with 70% ethanol overnight at 4°C and then rehydrated with 40% formamide/2×SSC for 10 minutes at room temperature. Cy5-labeled oligonucleotide probes (Qiagen, Valencia, CA) $(CAG)_{10}$ and $(CTG)_{10}$ were used to detect CUG and CAG repeats, respectively. The cells were incubated in probe/hybridization buffer (40% formamide, 2×SSC, 0.2% BSA, 10% dextran sulfate, 2 mM vanadyl sulfate, 1 mg/ml yeast tRNA, 50 ng/ml probe) in a humidified chamber at 37°C. After 2 hours, the slides were washed three times with 40% formamide/2×SSC for 30 minutes at 37°C. They were then pre-blocked in 3% BSA/PBS for 15 minutes in a humidified chamber at room temperature followed by a wash with PBS. Afterwards, slides were incubated with primary anti-MBNL1 antibody 3A4 (10 mg/ml; 1:1000 dilution) in 3% BSA/PBS at room temperature for 1 hour in a humidified chamber, washed three times with PBS and incubated with the secondary antibody Alexa Fluor488-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 hour. Following the incubation, cells were washed three times with PBS and nuclei were stained with DAPI using Vectashield (Vector Laboratories, Inc., Burlingame, CA).

S1 nuclease protection analysis

The *DMPK* 3′ UTR S1 probe was generated by *Nar*I/*Bst*EII digestion of plasmid DMPKS that contains exons 11-15 of human *DMPK* in its exon 15 (Philips et al., 1998). The 567 bp fragment was 5′ end-labeled and gel purified using ProbeQuant G-50 Micro columns (Amersham Pharmacia, Piscataway, NJ). RNA (5-8 μg) was heated at 85°C for 15 minutes and then hybridized with 1 fmol of probe in 20 μl of hybridization buffer (80% deionized formamide, 40 mM PIPES, 400 mM NaCl, 1 mM EDTA, pH 6.4) at 60°C overnight (Charlet-B. et al., 2002). The hybridization was quenched with 280 μl of ice-cold S1 buffer (50 mM NaOAc, 4.5 mM $ZnCl_2$, 300 mM NaCl, pH 4.5) containing 0.2 U/μL S1 nuclease (Roche Applied Science, Indianapolis, IN) and incubated at 37°C for 50 minutes. Next, 60 μl of ice cold inactivation buffer (2 M ammonium acetate, 30 mM EDTA) and 900 μl of ice cold ethanol was added to stop the reaction. S1 resistant hybrids were recovered by ethanol precipitation and the pellets were resuspended in 10 μl of the formamide-dye mix (Ambion, Austin, TX). The samples were heated at 95°C for 6 minutes and loaded on a 5% polyacylamide; 8 M urea gel. Gels were dried and exposed to Kodak BioMax MR film and quantitated by phosphoimager analysis (Molecular Dynamics, Piscataway, NJ). The protected size fragment was 125 bp. β-Actin 3′ UTR S1 probes were generated by *Bsu*36I/*Xho*I digest of a plasmid, pCR2.1 βactin+, containing the β-actin 3′ UTR to produce a 398 bp fragment that was uniformly labeled. The size of the protected fragment was 357 bp.

FRAP analysis

COSM6 cells (2×10^5) or DM1 fibroblasts (3×10^5) were plated into Delta T culture dishes (Bioptechs Inc., Butler, PA) and transfected 24 hours after plating with 2 μg of total of plasmid DNA for COSM6 cells or 3 μg for DM fibroblasts according to the manufacturer's directions for FuGene 6. Forty-eight hours after transfection, FRAP experiments were performed on a Zeiss LSM 510 confocal microscope with an objective heater. The cells were maintained in growth media at 37°C using a peristaltic pump. For FRAP experiments, five single scans were obtained, followed by a bleach pulse. Images were taken every 0.5 seconds after bleaching. For imaging, the laser was attenuated to 0.3% of the bleach intensity for COSM6 cells and 0.8% for DM1 fibroblasts. Fluorescence intensities were calculated by the Zeiss LSM software and normalized as previously described (Phair and Misteli, 2000). Data was analyzed using Microsoft Excel and GraphPad Prism 4. Images were exported as TIFF files and processed in Adobe Photoshop 7 and Deneba Canvas 9. For $t_{1/2}$, P values were calculated using an unpaired Student's t-test comparing the half-times of recovery for CUG versus CAG foci, as well as CUG versus CAG nucleoplasm. For the immobile fraction, P values were calculated using an unpaired Student's t-test comparing the extent of recovery in foci versus nucleoplasm.

Results

Expanded CUG and CAG repeat RNAs both form foci that colocalize with GFP-MBNL1

In DM1 cells and tissues, the mutant allele expresses mRNAs containing expanded CUG repeats which accumulate into discrete nuclear RNA foci detectable by FISH (Davis et al., 1997; Fardaei et al., 2001; Fardaei et al., 2002; Liquori et al., 2001; Mankodi et al., 2001; Taneja et al., 1995). To investigate the relationship between RNA foci formation, MBNL colocalization, and the splicing mis-regulation that is observed in DM cells, truncated *DMPK* mRNAs containing 960 CUG repeats were expressed in COSM6 cells. The *DMPK* minigene plasmid contains exons 11-15 and 960 interrupted CTGs inserted at the site of the CTG expansion in exon 15 (Fig. 1A and Materials and Methods). For comparison, we used minigenes, containing either 960 or no CAG repeats, which express identical mRNAs and differ only in the presence, absence, or sequence of the repeats. Expanded CAG repeats were tested because they form similar hairpin structures as expanded CUG repeats (Sobczak et al., 2003).

As expected, the expression of mRNAs containing 960 CUG repeats resulted in the formation of punctuate nuclear foci that were detected by FISH using a Cy3-labeled antisense peptide nucleic acid (PNA) probe, $(CAG)_5$ (Fig. 1B). Unexpectedly, mRNAs containing 960 CAG repeats also formed punctuate nuclear foci that were detected with a Cy3-labeled $(CTG)_5$ antisense PNA probe (Fig. 1B). Foci that formed following the transfection of CTG- and CAG-containing plasmids were CUG and CAG RNA rather than plasmid DNA (see below).

MBNL1 has been shown to bind double-stranded CUG repeats in vitro (Kino et al., 2004; Miller et al., 2000). In DM cells, endogenous MBNL proteins and exogenously expressed GFP-MBNL1 are recruited to nuclear RNA foci that contain the expanded repeats (Fardaei et al., 2001; Mankodi et al., 2001; Miller et al., 2000). To test whether MBNL1 accumulates within the RNA foci formed from exogenously expressed RNA, we coexpressed a GFP fusion of the 41 kDa MBNL1 isoform implicated in DM pathogenesis with *DMPK* mRNAs containing 960 CUG or CAG repeats or no repeats (Fig. 1C). GFP-MBNL1 fusions have been previously demonstrated to be functional in splicing assays (Ho et al., 2004). Consistent with the results from DM cell lines, GFP-MBNL1 is localized with expanded CUG repeats in discrete

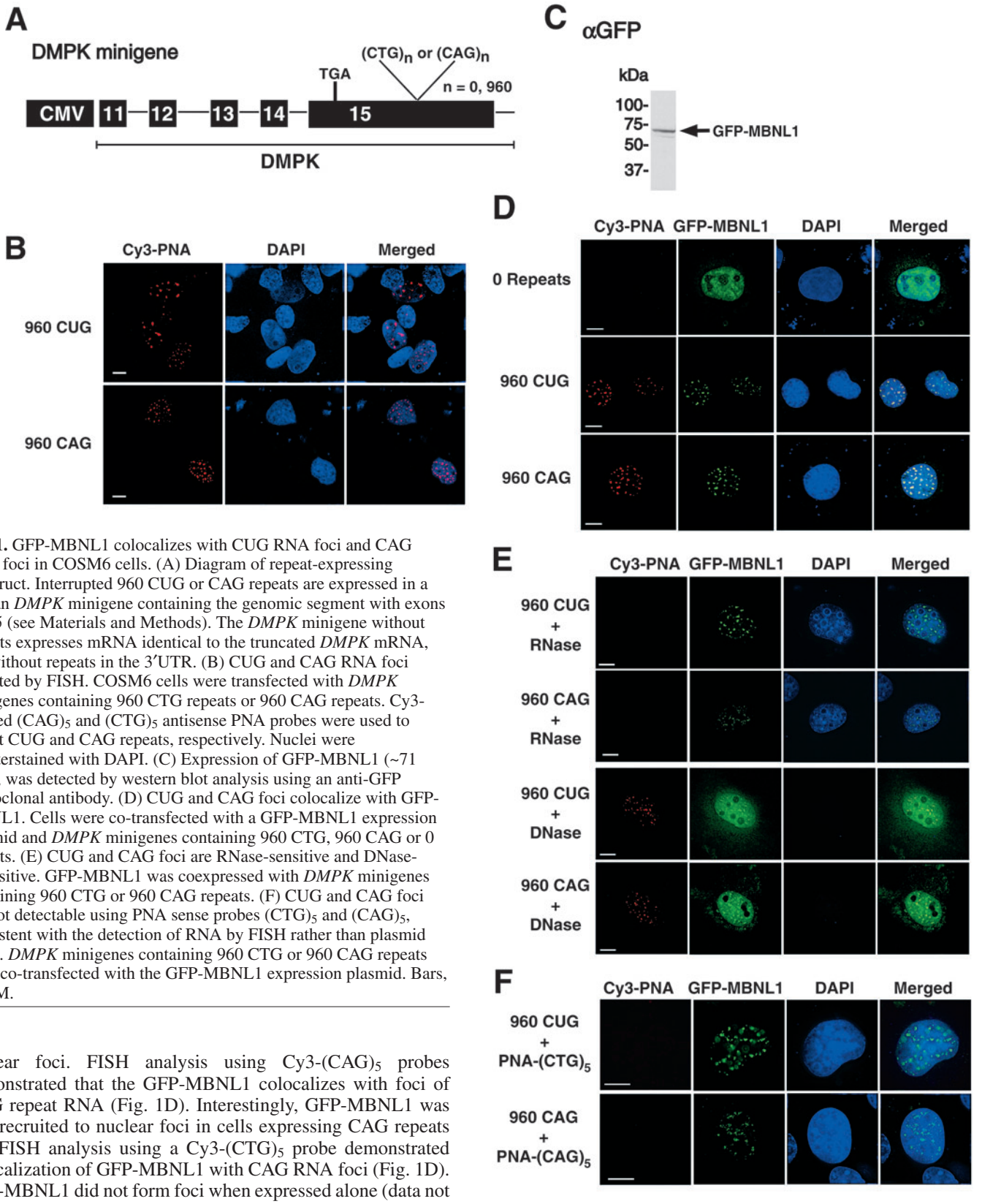


Fig. 1. GFP-MBNL1 colocalizes with CUG RNA foci and CAG RNA foci in COSM6 cells. (A) Diagram of repeat-expressing construct. Interrupted 960 CUG or CAG repeats are expressed in a human *DMPK* minigene containing the genomic segment with exons 11-15 (see Materials and Methods). The *DMPK* minigene without repeats expresses mRNA identical to the truncated *DMPK* mRNA, but without repeats in the 3′UTR. (B) CUG and CAG RNA foci detected by FISH. COSM6 cells were transfected with *DMPK* minigenes containing 960 CTG repeats or 960 CAG repeats. Cy3-labeled $(CAG)_5$ and $(CTG)_5$ antisense PNA probes were used to detect CUG and CAG repeats, respectively. Nuclei were counterstained with DAPI. (C) Expression of GFP-MBNL1 (~71 kDa), was detected by western blot analysis using an anti-GFP monoclonal antibody. (D) CUG and CAG foci colocalize with GFP-MBNL1. Cells were co-transfected with a GFP-MBNL1 expression plasmid and *DMPK* minigenes containing 960 CTG, 960 CAG or 0 repeats. (E) CUG and CAG foci are RNase-sensitive and DNase-insensitive. GFP-MBNL1 was coexpressed with *DMPK* minigenes containing 960 CTG or 960 CAG repeats. (F) CUG and CAG foci are not detectable using PNA sense probes $(CTG)_5$ and $(CAG)_5$, consistent with the detection of RNA by FISH rather than plasmid DNA. *DMPK* minigenes containing 960 CTG or 960 CAG repeats were co-transfected with the GFP-MBNL1 expression plasmid. Bars, 10 μM.

nuclear foci. FISH analysis using Cy3-$(CAG)_5$ probes demonstrated that the GFP-MBNL1 colocalizes with foci of CUG repeat RNA (Fig. 1D). Interestingly, GFP-MBNL1 was also recruited to nuclear foci in cells expressing CAG repeats and FISH analysis using a Cy3-$(CTG)_5$ probe demonstrated colocalization of GFP-MBNL1 with CAG RNA foci (Fig. 1D). GFP-MBNL1 did not form foci when expressed alone (data not shown) or with an identical *DMPK* mRNA lacking repeats (Fig. 1D). Expression of GFP alone, or GFP with CUG or CAG repeats did not form GFP-labeled foci (data not shown).

To confirm that the nuclear foci that formed after the expression of the *DMPK* minigenes contain RNA and not plasmid DNA, transfected cells were treated with RNase or DNase (Fig. 1E). DNase-treated cell nuclei were counterstained with DAPI to confirm the removal of chromatin staining. For both CUG- and CAG-repeat-expressing plasmids,

the FISH signals were RNase-sensitive but DNase-insensitive, indicating that the nucleic acid component of the foci are composed of RNA. Interestingly, the GFP-MBNL1 component of the foci was unaffected by treatment with DNase and RNase suggesting that, once formed, the protein components of foci remain in the absence of intact RNA, probably as a result of paraformaldehyde fixation.

Foci generated by transfected CTG- and CAG-containing minigenes were not detectable using the Cy3-labeled sense probes $(CTG)_5$ and $(CAG)_5$, respectively (Fig. 1F), which further indicates that the PNA probes are binding to complementary RNA rather than to transfected plasmid DNA. CUG- and CAG-RNA foci formation and GFP-MBNL1 recruitment to both CUG and CAG foci was not cell line-dependent because similar results were observed in C2C12 myoblasts, NIH 3T3 fibroblasts and HeLa cells (data not shown). These results indicate that the expression of CUG and CAG repeats in normal cells can recapitulate the formation of nuclear RNA foci that contain GFP-MBNL1.

Endogenous MBNL1 colocalizes with CUG and CAG RNA foci

We next determined whether endogenous MBNL1 colocalizes with both transiently expressed CUG and CAG repeat foci. Expanded repeat RNA was expressed in COSM6 cells and the cells were analyzed for RNA foci formation by FISH using the appropriate antisense Cy5-labeled oligonucleotide probes and for endogenous MBNL1 by immunofluorescence using a monoclonal antibody that recognizes the 41 and 42 kDa isoforms of MBNL1 (Ho et al., 2004; Kanadia et al., 2003). Similar to GFP-MBNL1, endogenous MBNL1 is predominantly nuclear in COSM6 cells (Fig. 2) as described for other cultured cells (Miller et al., 2000). Consistent with our results with exogenously expressed GFP-MBNL1, both CUG and CAG repeats form nuclear RNA foci that colocalize with endogenous MBNL1 (Fig. 2). The RNA foci are not detectable with the sense oligonucleotide probes, indicating that the probes are binding to complementary RNA rather than transfected plasmid DNA (data not shown). The results indicate that the expression of either CUG or CAG RNA repeats is sufficient to sequester endogenous MBNL1.

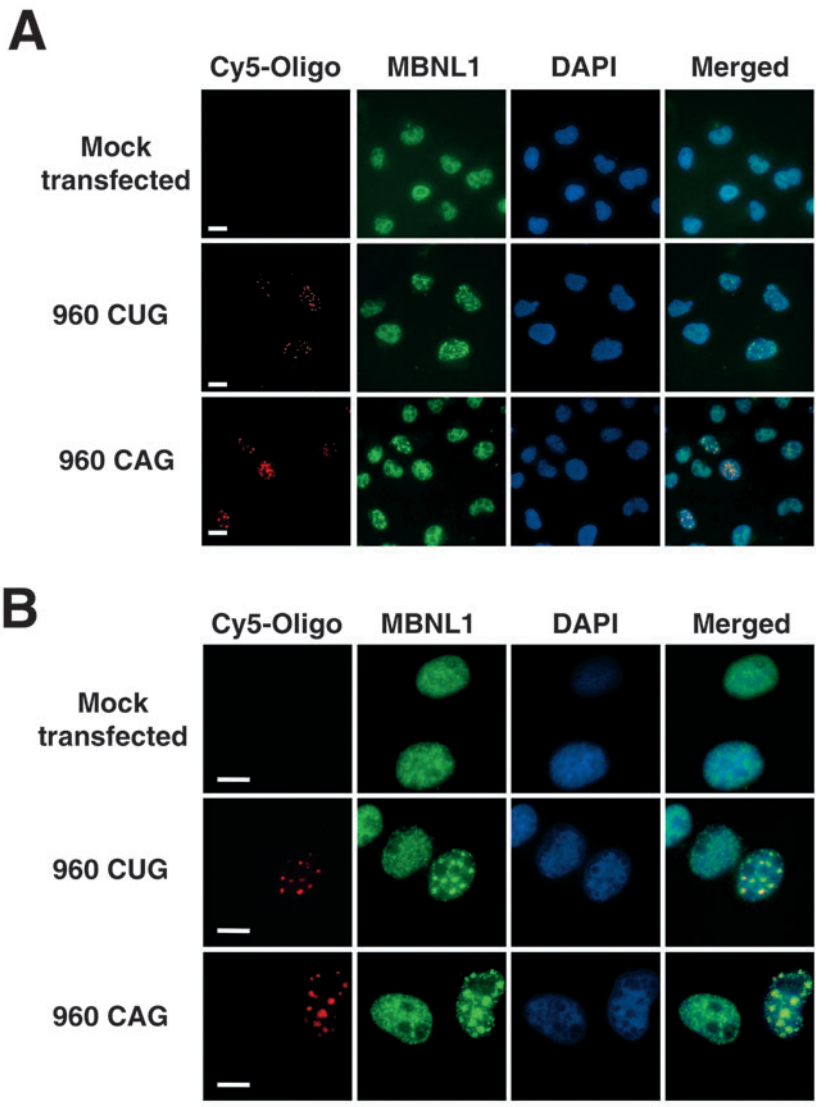


Fig. 2. Endogenous MBNL1 colocalizes with CUG and CAG foci in COSM6 cells. (A) Combined FISH/immunofluorescence using Cy5-labeled antisense oligonucleotide probes (Cy5-Oligo), MBNL1 mAb 3A4 (MBNL1) (secondary antibody is Alexa Fluor488) and DAPI staining (DAPI) demonstrating the colocalization of endogenous MBNL1 with CUG or CAG RNA. Magnification ×40. (B) Same as A with magnification ×100. Bars, 10 μM.

Expression of CUG RNA but not CAG RNA alters splicing of cTNT and IR minigenes

Coexpression of CUG repeat RNA with cTNT and IR minigenes in normal cells reproduces the aberrant splicing patterns observed for endogenous cTNT and IR genes in DM1 cells (Philips et al., 1998; Savkur et al., 2001). Splicing of pre-mRNAs from cTNT and IR minigenes is regulated by GFP-MBNL1 expression and siRNA-mediated depletion of endogenous MBNL1 (Ho et al., 2004). To test the hypothesis that the accumulation of MBNL1 in foci is the primary event compromising MBNL function and thus altering splicing, we tested whether foci formation is sufficient to alter splicing of a cTNT minigene in cells expressing truncated *DMPK* mRNAs that contain 960 CUG repeats, 960 CAG repeats or no repeats. A constant amount of the cTNT minigene was co-transfected with increasing amounts of plasmids expressing *DMPK* mRNAs containing CUG, CAG or no repeats. Coexpression of *DMPK* mRNAs containing CUG repeats promoted cTNT exon 5 inclusion (Fig. 3A). The IR gene expresses another alternatively spliced pre-mRNA that is mis-regulated in DM striated muscle (Savkur et al., 2001; Savkur et al., 2004). The *DMPK* mRNA containing CUG repeats coexpressed with a IR minigene reproduced the DM pattern of splicing as was

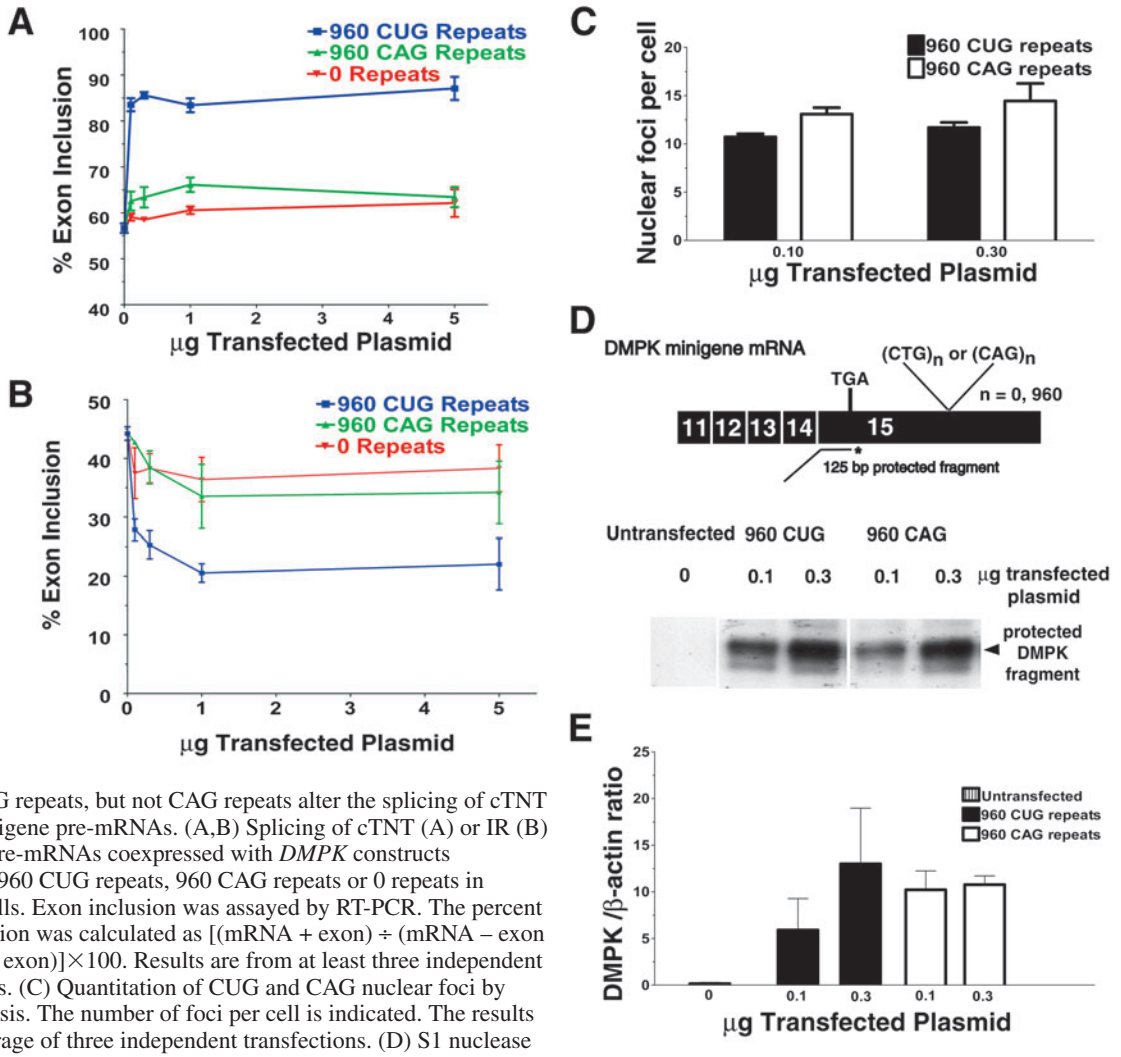


Fig. 3. CUG repeats, but not CAG repeats alter the splicing of cTNT and IR minigene pre-mRNAs. (A,B) Splicing of cTNT (A) or IR (B) minigene pre-mRNAs coexpressed with *DMPK* constructs containing 960 CUG repeats, 960 CAG repeats or 0 repeats in COSM6 cells. Exon inclusion was assayed by RT-PCR. The percent exon inclusion was calculated as [(mRNA + exon) ÷ (mRNA – exon + mRNA + exon)]×100. Results are from at least three independent experiments. (C) Quantitation of CUG and CAG nuclear foci by FISH analysis. The number of foci per cell is indicated. The results are the average of three independent transfections. (D) S1 nuclease protection analysis of repeat RNA expression in cells transfected with 0, 100 or 300 ng of repeat-expressing plasmid. Top: diagram of the *DMPK* S1 probe. The 567-bp probe end-labeled with ^{32}P hybridizes to the 3′ UTR of *DMPK* to yield a 125 bp protected fragment. The probe detects transcripts expressed from the *DMPK* minigenes that contain 960 CUG repeats, 960 CAG repeats or 0 repeats. The labeled end is denoted with a *. Bottom: representative results show that cells express equivalent steady-state levels of CUG and CAG repeat RNA. (E) Quantitation of S1 nuclease protection analysis using endogenous β-actin as an internal standard. S1 probes specific to the 3′ UTR of *DMPK* or β-actin were quantitated by phosphoimager analysis and the ratio of *DMPK* to β-actin signal was calculated. The results of three independent experiments were averaged. *P* values comparing CUG and CAG repeat expression at 100 ng and 300 ng were 0.3770 and 0.7539, respectively.

observed with the cTNT minigene (increased exon 11 skipping). However, its coexpression with mRNAs containing CAG repeats or no repeats had only minimal effects on splicing (Fig. 3B).

The different trans-acting effects of CUG and CAG repeat RNA on alternative splicing could reflect differences in RNA expression, foci formation or the relative affinities of MBNL proteins for CUG versus CAG repeats. To compare the amount of foci formed by expression of the CTG or CAG expression plasmids, nuclear foci were identified by FISH and the number of nuclear foci per cell were counted. Different trans-acting splicing effects of CUG and CAG repeat RNA are evident at 0.10 and 0.30 μg of transfected *DMPK* plasmids so these conditions were used for quantitative analysis of RNA expression and foci formation. The results shown in Fig. 3C demonstrate that similar numbers of nuclear foci per cell were counted for CUG and CAG repeat expression plasmids. The amount of *DMPK* mRNA containing CUG or CAG repeats was quantified by S1 nuclease protection analysis using a 5′-end ^{32}P-labeled probe specific to the human *DMPK* 3′ UTR (Fig. 3D). S1 nuclease analysis was performed on the same RNAs for which RT-PCR analysis demonstrated altered splicing of cTNT when CUG but not CAG repeat RNA was expressed; the amount of repeat RNA was normalized to endogenous β-actin RNA (Fig. 3E). Steady-state levels of CUG and CAG repeat RNA were comparable at 0.1 μg and 0.3 μg of transfected plasmid (Fig. 3D). We conclude that transfection of CUG and CAG expression plasmids results in comparable amounts of expressed RNA (P>0.05) and comparable levels of foci formation. Both CUG and CAG repeats colocalize with MBNL1 in foci, but only CUG repeats reproduced the DM splicing pattern in cTNT and IR minigenes.

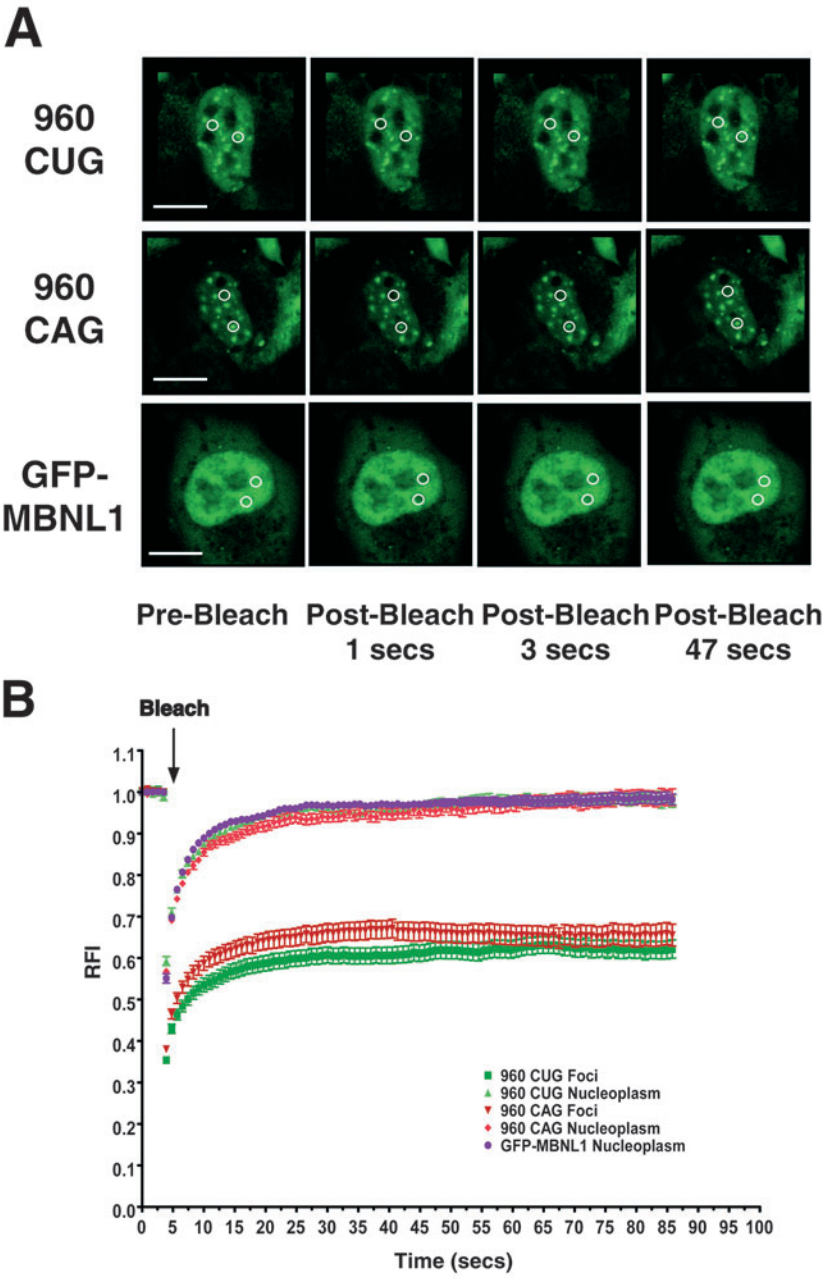


Fig. 4. FRAP analysis reveals an immobile fraction of GFP-MBNL1 molecules in both CUG and CAG foci. COSM6 cells were transfected with GFP-MBNL1 alone or with *DMPK* minigenes containing 960 CTG or CAG repeats. (A) Photobleaching of foci and nucleoplasm in 960 CUG repeat and CAG repeat or 0 repeat plasmids. White circles denote the photobleached regions in the foci and nucleoplasm. Bars, 10 μM. (B) Recovery curves of photobleached foci and nucleoplasm in cells expressing CUG or CAG repeats. Five images were taken pre-bleach, followed by bleaching pulses as indicated on the graph. The half-time of recovery was calculated from at least three independent experiments. The duration of the experiment was 100 seconds. The graph represents the data collected from 42, 34 and 45 cells for the expression 960 CUG repeats, 960 CAG repeats and GFP-MBNL1, respectively. RFI, recovery of fluorescence intensity.

Quantitative analysis of fluorescence recovery of GFP-MBNL1 in foci and nucleoplasm

Gel-retardation analysis indicates that, in vitro, GST-MBNL1 has a lower affinity for CAG RNA compared with CUG RNA (Miller et al., 2000). A difference in the mobile fraction or the mobility of GFP-MBNL1 in CUG and CAG foci could account for the different trans-acting effects on splicing, secondary to the extent of MBNL1 sequestration. To assess relative binding of MBNL1 to CUG and CAG RNA foci in vivo, we tested the mobility of GFP-MBNL1 in CUG and CAG foci using FRAP. COSM6 cells were transfected with GFP-MBNL1 and CTG- or CAG-repeat-containing *DMPK* minigenes. Circular regions containing either foci or non-foci nucleoplasm within the same nucleus were simultaneously photobleached to compare the mobility of GFP-MBNL1 (Fig. 4A). This analysis was performed on cells expressing CUG or CAG RNA to compare GFP-MBNL1 mobility on both RNA repeats. FRAP analysis was also performed in cells expressing GFP-MBNL1 alone. Because foci are mobile, an optimal area that would encompass foci for the duration of the experiment was empirically determined.

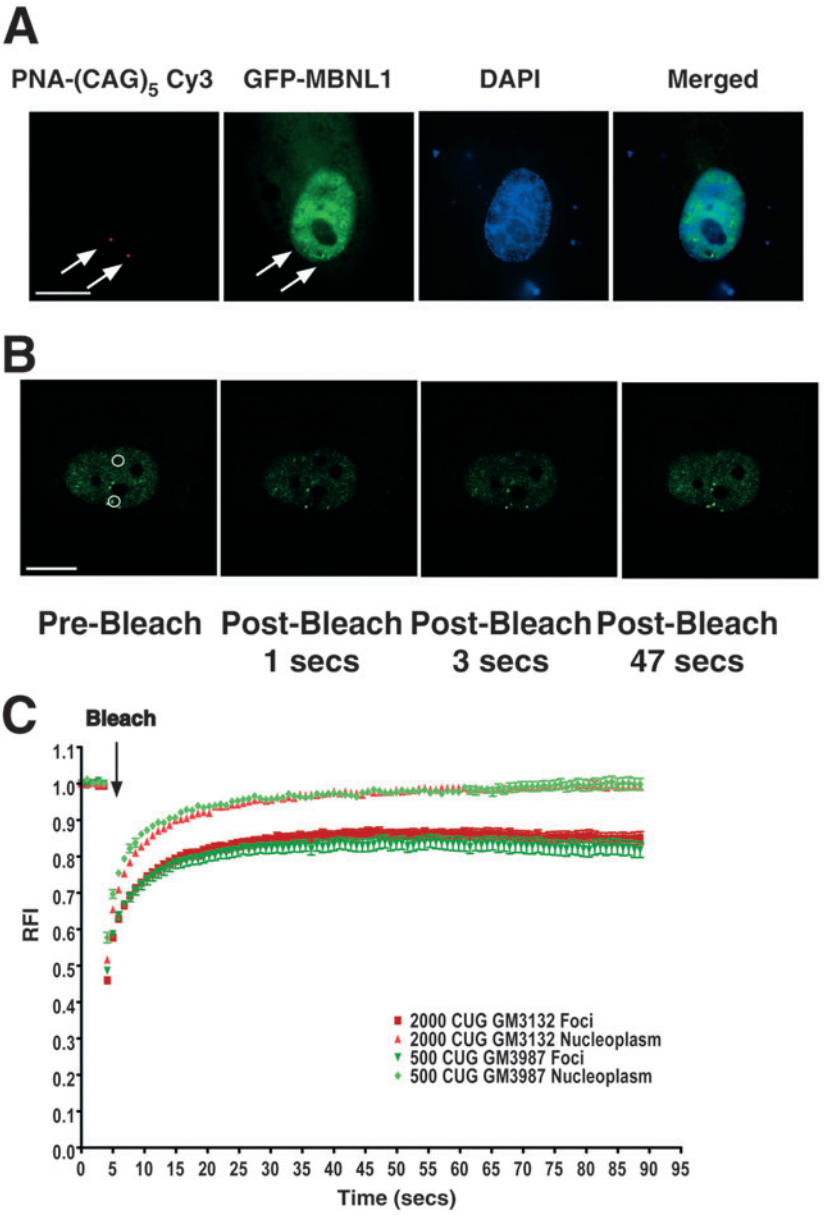
The extent of GFP-MBNL1 fluorescence recovery (relative to the pre-bleach intensity) was different in foci compared with nucleoplasm. The nucleoplasm recovered to ~96% of the initial fluorescence whereas the foci recovered to ~65% (Fig. 4B and Table 1), suggesting that there was an immobile fraction in foci. It is important to notice, however, that recovery in CUG and CAG foci was not significantly different (P=0.1718, Table 1). Therefore, both CUG and CAG foci contained comparable immobile fractions of GFP-MBNL1. For both CUG and CAG foci, GFP-MBNL1 recovered fluorescence in a pattern that reflected the original size and shape of the pre-bleach foci rather than the diffuse pattern observed in the nucleoplasm (Fig. 4A).

For the mobile fraction, there was no statistically significant difference between the half-time of recovery in CUG versus CAG foci and CUG versus CAG nucleoplasm (Fig. 4B and Table 1). In fact, the half-time of recovery was similar in cells coexpressing GFP-MBNL1 and CUG or CAG repeat RNA when compared with cells expressing GFP-MBNL1 alone (Fig. 4B and Table 1). Coexpression of GFP-MBNL1 with either CUG or CAG repeats did not affect total expression of GFP-MBNL1 as shown by western blot analyses (data not shown). These results indicate two features of GFP-MBNL1 in these cells. First, a similar immobile fraction of GFP-MBNL1 in both CUG and CAG foci, which does not recover fluorescence over the course of the experiment. Second, a similar half-time of recovery of the mobile fraction in foci and nucleoplasm for both CUG and CAG repeats.

Table 1. Half-time of recovery values for GFP-MBNL1

Cell type	Photobleached region	$t_{½}$ (seconds) of recovery ± 2× s.e.m.	*P* value (Student's *t*-test)	Immobile fraction ± 2× s.e.m.	*P* value (Student's *t*-test)
COSM6 960 CTG repeats	CUG foci (*n*=42)	3.7±0.3	0.6427	37.2±3.7	<0.0001
COSM6 960 CTG repeats	CUG nucleoplasm (*n*=42)	3.1±0.3	0.0867	2.5±1.6	0.2428
COSM6 960 CAG repeats	CAG foci (*n*=34)	3.6±0.4		33.2±4.4	<0.0001
COSM6 960 CAG repeats	CAG nucleoplasm (*n*=34)	3.5±0.3		3.9±2.7	0.7358
COSM6	GFP-MBNL1 alone (*n*=45)	3.3±0.1	0.1137	3.5±0.9	
GM03132 DM patient fibroblast (2000 CTG)	CUG foci (*n*=37)	3.5±0.4	0.1890	13.9±2.8	0.3221
GM03132 DM patient fibroblast (2000 CTG)	CUG nucleoplasm (*n*=37)	3.5±0.2	0.0066	2.1±1.6	0.7644
GM03987 DM patient fibroblast (500 CTG)	CUG foci (*n*=31)	3.1±0.3		16.2±3.6	
GM03987 DM patient fibroblast (500 CTG)	CUG nucleoplasm (*n*=31)	3.0±0.3		1.8±2.0	

P values for the half-times of GFP-MBNL1 recovery for foci and nucleoplasm were calculated using an unpaired Student's *t*-test comparing CUG and CAG foci or nucleoplasm of CUG, CAG and GFP-MBNL1 alone. For $t_{½}$, *P* values were calculated using an unpaired Student's *t*-test comparing the half-times of recovery for CUG vs CAG foci, and CUG vs CAG nucleoplasm. For the immobile fraction, *P* values were calculated using an unpaired Student's *t*-test comparing the extent of recovery (relative to pre-bleach intensity) in foci vs nucleoplasm.



Quantitative analysis of fluorescence recovery of GFP-MBNL1 in DM1 fibroblasts

We also used FRAP to analyze the mobility of GFP-MBNL1 in the CUG RNA foci and the nucleoplasm of fibroblast cell lines from DM1 patients. These cell lines have fewer foci (~1-5) and less *DMPK* RNA relative to the transiently transfected COSM6 cells (Davis et al., 1997) (data not shown). Two DM1 cell lines were used whose mutated *DMPK* allele contains 500 or 2000 CTG repeats, which were confirmed by Southern blot analysis (Savkur et al., 2001). In DM1 fibroblasts that express 2000 CTG repeats, FISH experiments confirmed colocalization of GFP-MBNL1 with the endogenous *DMPK* RNA foci (Fig. 5A). For FRAP analysis, the photobleached areas were identical in size to those used in COSM6 cells (Fig. 5B). Similar to transiently transfected COSM6 cells, CUG RNA foci contained an immobile fraction of GFP-

Fig. 5. FRAP analysis of GFP-MBNL1 mobility in DM1 fibroblasts. DM cells (GM3987, GM3132) with CTG repeats of different lengths (500 and 2000 repeats, respectively) were transfected with GFP-MBNL1. (A) GFP-MBNL1 colocalizes with nuclear foci of endogenously expressed CUG repeat RNA. Arrows indicate the location of the foci. CUG-containing RNA was detected using Cy3-labeled $(CAG)_5$ antisense PNA probe, nuclei were counterstained with DAPI. Bar, 10 μm. (B) Photobleaching of foci and nucleoplasm in DM1 fibroblasts (~2000 CTG repeats) transfected with GFP-MBNL1. Circles denote the photobleached regions in the foci and nucleoplasm. Bar, 10 μM. (C) Recovery curves of photobleached foci and nucleoplasm in DM1 fibroblasts. GM3987 and GM3132 fibroblasts were transfected with GFP-MBNL1. The graph represents data collected for 37 GM3132 fibroblasts and 31 GM3987 fibroblasts over three independent experiments.

MBNL1 when compared with the nucleoplasm (Table 1). The immobile fraction in DM1 fibroblasts was smaller than in transfected COSM6 cells, probably because of the smaller amount of RNA expressed. In addition, we did not detect a significant difference in the half-time of recovery of the mobile GFP-MBNL1 fraction in foci or nucleoplasm (Fig. 5C, Table 1). We also did not detect significant differences in the half-time of recovery of immobile fractions in cell lines that express RNAs containing 500 or 2000 repeats (Table 1). However, FRAP analysis might not be sensitive enough to detect differences in GFP-MBNL1 sequestration in these two cell lines. We therefore conclude that, the features determined for transiently expressed CUG and CAG repeat RNA accurately reflects endogenous CUG containing RNA.

Discussion

Here, we show that expression of truncated *DMPK* mRNAs containing either CUG or CAG repeats in normal cells results in the formation of nuclear RNA foci that colocalize with endogenous MBNL1. We also show that exogenously expressed GFP-MBNL1 accumulates in both CUG and CAG nuclear RNA foci. Expression of an identical *DMPK* mRNA without repeats does not alter the subcellular distribution of GFP-MBNL1 or MBNL1. Although endogenous MBNL1 colocalizes with both CUG and CAG foci, only the expression of CUG repeats induces the DM1 pattern of splicing for the two tested pre-mRNAs, cTNT and IR.

To determine whether differences in association of endogenous MBNL with CUG and CAG foci can explain differences in the trans-acting effects on alternative splicing, we compared the mobility of GFP-MBNL1 associated with these foci in live cells. Using FRAP, we examined the extent of GFP-MBNL1 recovery and the half-time of recovery of the mobile fraction. We detected a difference in the mobility of GFP-MBNL1 in both CUG and CAG foci compared with nucleoplasm, which indicates that both CUG and CAG RNA foci are associated with an immobile fraction of GFP-MBNL1. However, no significant difference between the immobile fraction associated with CUG and CAG foci was detected. Surprisingly, for the mobile fraction, the half-times of recovery of GFP-MBNL1 were the same in the absence of repeat RNA, in CUG or CAG RNA foci, or the nucleoplasm of CUG and CAG expressing cells. FRAP analysis of DM1 cell lines expressing either 500 or 2000 CUG repeats also detected an immobile fraction and had similar half-times of recovery for GFP-MBNL1, as observed in foci of exogenous expressed RNA. These results strongly suggest that the re-localization of MBNL1 into RNA foci and the disruption of splicing of MBNL1 pre-mRNA targets by CUG repeat RNA are separable events.

The role of altered subcellular localization of MBNL in DM pathogenesis

In DM, the transcribed mutant allele forms RNA foci that colocalize with MBNL1, MBNL2 and MBNL3 (Fardaei et al., 2002; Mankodi et al., 2003; Mankodi et al., 2001). The mean MBNL1 immunofluorescence intensity of non-foci nucleoplasm was estimated to be approximately 2.3-fold lower in DM1 neurons compared with non-DM1 controls, suggesting that either sequestration or increased degradation of MBNL proteins results in reduced nucleoplasmic MBNL (Jiang et al., 2004). DM splicing patterns are reproduced in *Mbnl1*$^{\Delta E3/\Delta E3}$ mice that lack most MBNL1 protein isoforms and in cell cultures in which endogenous MBNL1 is reduced by 80-90% using siRNAs (Ho et al., 2004; Kanadia et al., 2003). Consistent with a loss of MBNL1 function, the similarities of clinical phenotype and splicing patterns between the *Mbnl1*$^{\Delta E3/\Delta E3}$ mice and HSA^{LR} transgenic mice (that express 250 CUG repeats), clearly implicates the MBNL family in the pathogenesis of DM. The formation of RNA foci might affect the activity of other RNA binding proteins as well, however, several lines of evidence suggest that the mis-regulation of splicing in DM involves signaling and/or regulatory pathways other than MBNL sequestration alone. First, the effect of the repeats on splicing mis-regulation requires an intact CUG-BP1-binding site (CUG-BP1 response element) within affected pre-mRNAs. cTNT and IR mutant minigenes containing CUG-BP1-binding site mutations no longer responded to expanded CUG repeat RNA. This suggests that the trans-dominant effect is mediated, at least in part, through an intact CUG-BP1-binding site (Ho et al., 2004; Philips et al., 1998; Savkur et al., 2001). The mutant minigenes still responded to siRNA-mediated depletion of endogenous MBNL1, demonstrating that the trans effects of CUG repeat RNA is distinct from the loss of MBNL function alone. Elevation of CUG-BP1 levels in DM cultures might be an independent event from MBNL1 sequestration (Dansithong et al., 2004). Second, in a C2C12 cell culture model, foci formation was not correlated with the defect in myoblast differentiation observed in DM cultures, suggesting that foci formation was not sufficient to disrupt myogenic differentiation (Amack and Mahadevan, 2001; Furling et al., 2001). Similarly, in a transgenic *Drosophila melanogaster* model expressing 162 CTG repeats, the presence of CUG foci colocalizing with *Drosophila* muscleblind protein did not cause muscle defects or alterations in locomotive activity (Houseley et al., 2005). Third, although the foci are associated with DM in muscle, DM tissues contain small numbers of foci (1-3) relative to cultured myoblasts (>20) and it is unknown whether these are sufficient to quantitatively inactivate a large fraction of cellular MBNL protein (Davis et al., 1997; Mankodi et al., 2003; Mankodi et al., 2001). There is a decrease in MBNL1-immunostaining of nucleoplasm in DM tissue compared with non-DM tissue, but it is unclear whether the decrease in signal is proportional to the length of the expanded repeats, number of foci or size of foci (Jiang et al., 2004; Mankodi et al., 2001). Fourth, the results reported here: Although MBNL1 accumulates in both CUG and CAG repeat nuclear RNA foci, only the expression of CUG repeat RNA affects splicing (Fig. 1B, Fig. 3A and Fig. 4). FRAP analysis showed no significant differences in MBNL1 mobility in cells expressing CUG or CAG RNA. Similar mobile and immobile fractions were found on CUG and CAG RNA, suggesting that GFP-MBNL1 was equivalently recruited into both foci (Fig. 5B and Table 1). In a recent study, siRNA-mediated depletion of MBNL1 in DM1 myoblasts reduced the formation of RNA foci, suggesting a role for MBNL1 in foci maintenance (Dansithong et al., 2004). These observations suggest that the formation of RNA foci might not be the primary pathogenic event in DM. Indeed, recent studies on Huntington's disease suggest that, the formation of intranuclear

inclusion bodies is beneficial coping response to the expression of toxic polyglutamine (Arrasate et al., 2004).

We have shown previously that MBNL proteins directly regulate splicing of cTNT and IR gene pre-mRNAs (whose splicing is mis-regulated in DM1), and that this regulation is antagonistic to the effect of a second family of splicing regulators called CUG-BP1 and ETR-3 like factors (CELF) proteins (Ho et al., 2004). Ten alternative splicing events have been shown to be mis-regulated in DM striated muscle and brain tissues, and all are splicing events that are normally regulated developmentally – during tissue remodeling – from embryonic to adult isoforms (Buj-Bello et al., 2002; Charlet-B. et al., 2002; Jiang et al., 2004; Kanadia et al., 2003; Mankodi et al., 2002; Philips et al., 1998; Savkur et al., 2001; Sergeant et al., 2001). In all ten of these splicing events, embryonic isoforms are not replaced with adult isoforms in DM tissues. One working model is that, MBNL and CELF protein families are mediators of one or more programs of developmentally regulated splicing, and that the expression of CUG or CCUG repeats (in DM1 and DM2, respectively) either initiates an aberrant signaling event or interferes with a natural signaling cascade for which MBNL and CELF proteins are downstream mediators.

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