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Muscleblind localizes to nuclear foci of aberrant RNA in myotonic dystrophy types 1 and 2

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The phenotypes in myotonic dystrophy types 1 and 2 (DM1 and DM2) are similar, suggesting a shared pathophysiologic mechanism. DM1 is caused by expansion of a CTG repeat in the *DMPK* gene. Pathogenic effects of this mutation are likely to be mediated, at least in part, by the expanded CUG repeat in mutant mRNA. The mutant transcripts are retained in the nucleus in multiple discrete foci. We investigated the possibility that DM2 is also caused by expansion of a CTG repeat or related sequence. Analysis of DNA by repeat expansion detection methods, and RNA by ribonuclease protection, did not show an expanded CTG or CUG repeat in DM2. However, hybridization of muscle sections with fluorescence-labeled CAG-repeat oligonucleotides showed nuclear foci in DM2 similar to those seen in DM1. Nuclear foci were present in all patients with symptomatic DM1 (*n* = 9) or DM2 (*n* = 9) but not in any disease controls or healthy subjects (*n* = 23). The foci were not seen with CUG- or GUC-repeat probes. Foci in DM2 were distinguished from DM1 by lower stability of the probe–target duplex, suggesting that a sequence related to the DM1 CUG expansion accumulates in the DM2 nucleus. Muscleblind proteins, which interact with expanded CUG repeats *in vitro*, localized to the nuclear foci in both DM1 and DM2. These results support the idea that nuclear accumulation of mutant RNA is pathogenic in DM1, suggest that a similar disease process occurs in DM2, and point to a role for muscleblind in the pathogenesis of both disorders.

INTRODUCTION

Genetic analysis has recently shown locus heterogeneity in myotonic dystrophy (DM). Myotonic dystrophy type 1 (DM1) is caused by expansion of a CTG repeat in the 3′ untranslated region (3′-UTR) of the *DMPK* gene on chromosome 19q (1). Myotonic dystrophy type 2 [DM2; proximal myotonic myopathy (PROMM)] has been linked to chromosome 3q (2,3), but linkage to this locus has been excluded in some families (4). The DM2 mutations have not been identified.

The manifestations of DM1 and DM2 are similar. Both disorders are characterized by dominantly inherited muscle weakness and myotonia (hyperexcitability of muscle fibers) (5,6). The muscle histopathology in DM2 resembles that seen in DM1 (5,7). The non-muscle manifestations of DM1 are also observed in DM2, including cataracts, cardiac arrhythmias, abnormalities of the cerebral hemispheric white matter and hypogonadism (5,7–10). Furthermore, there is evidence for anticipation in both DM1 (1) and DM2 (11). DM1 and DM2 are usually distinguished by the initial pattern of weakness in the limb muscles (proximal in DM2, distal in DM1), but this distinction is not always reliable. Some of the brain manifestations of DM1, such as hypersomnolence or mental retardation, have not been observed in DM2.

The position of the expanded CTG repeat in the 3′-UTR poses a challenge for understanding the disease mechanism in DM1. A gain of function by mutant protein is unlikely because the coding sequence of the *DMPK* gene and mRNA remain intact. Extensive genetic analysis has not identified 19q-linked DM families in which the CTG repeat is not expanded, suggesting that point mutations or deletions at the DM1 locus cannot reproduce the pathogenic effect. Disruption of the *DMPK* gene in mice causes delay in cardiac conduction (12) but fails to reproduce many aspects of the DM1 phenotype (13,14).

Recent evidence points to a pathogenic effect by the expanded CUG repeat in the mutant mRNA. Transcripts from the mutant *DMPK* allele, although fully processed and polyadenylated, remain in the cell nucleus in multiple discrete foci (15,16). In support of an RNA-mediated disease mechanism, lines of transgenic mice that express expanded CUG repeats in skeletal muscle develop a DM-like phenotype, including myotonia, histopathologic changes that resemble DM, and multifocal accumulation of expanded CUG repeats in the

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nucleus (17). However, the mechanism for nuclear retention and toxicity of transcripts that contain expanded CUG repeats is not understood. Proteins have been shown to interact with CUG repeats *in vitro* (18–21), but none have been shown to colocalize with this RNA in cells.

The phenotypic similarities raise the possibility that DM1 and DM2 share a common pathophysiologic mechanism. Here we report that fluorescence *in situ* hybridization (FISH) of muscle sections using a CAG-repeat oligonucleotide reveals nuclear foci in DM2 that are similar to those seen in DM1. Foci in DM2, however, are distinguished by the lower stability of the probe–target complex. Human muscleblind proteins, homologs of proteins required for muscle development in *Drosophila* (22), colocalize with the nuclear foci in both disorders. These results support an RNA-mediated disease mechanism in DM1 and DM2, and suggest that muscleblind proteins participate in the pathogenesis of both disorders.

RESULTS

Studies of DM1 (23), spinocerebellar ataxia type 8 (24), and HSA^{LR} transgenic mice (17) have suggested that expanded CUG repeats are pathogenic in a variety of sequence contexts, raising the possibility that DM2 could also result from expression of an expanded CUG repeat. To search for an expanded repeat in DM2, the repeat expansion detection (RED) method (25,26) was used to screen DNA from patients with DM2 (n = 9), DM1 (n = 5) or healthy controls (n = 9). Long repeat expansions (>400 bp) were detected in each patient with classical DM1 (more than 150 CTG repeats) but not in DM1 patients with fewer than 80 repeats, patients with DM2, or healthy controls. Small expansions of 180 to 360 CTG/CAG repeats were detected in DNA from four of 10 healthy controls. CTG repeats of this size are found in 29% of an unselected Swedish population (M.Schalling, unpublished data). Two of nine DM2 patients also had small (180 bp) expansions. In both cases, however, the small expansion did not segregate with DM2 in other family members. These results agree with Ranum *et al.* (2), who found that RED analysis for expanded CTG/CAG repeats was negative in a kindred with DM2.

The background frequency of CTG/CAG repeats in the human genome may limit the sensitivity of RED analysis for detecting small repeat expansions. In this regard, analysis of RNA may be advantageous for detecting transcribed CTG repeats. The frequency of CUG repeats in normal cellular RNA is very low, as estimated by database queries and slot blots with $(CAG)_{10}$ probes (C.Thornton, unpublished data). We used ribonuclease protection to assay for expanded CUG repeats in RNA isolated from myoblasts. A $(CAG)_{29}$ probe detected an expanded CUG repeat in each patient with DM1 (Fig. 1), including a patient with a minimal expansion of 77 repeats (Fig. 1, lane 2). However, this probe did not show transcripts with 15 or more uninterrupted CUG repeats in DM2 or normal myoblasts (n = 4 in each group). Similar results were obtained with RNA isolated from myotubes (data not shown).

FISH with CAG oligonucleotides reveals RNA foci in DM1 cells grown in tissue culture. In previous studies of fibroblasts, cells that expressed DMPK at a low level, the mean number of foci per nucleus was five (15). Myoblasts expressed higher levels of DMPK, and these cells had hundreds of foci per

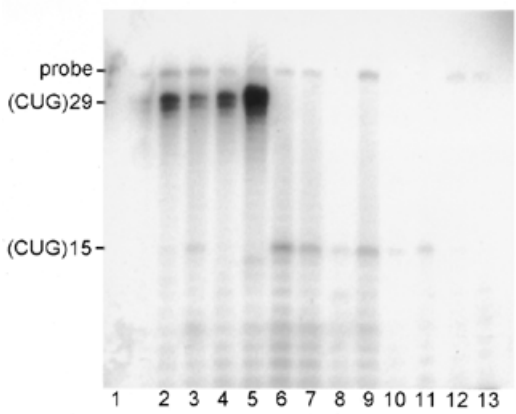

Figure 1. Ribonuclease protection assay using $(CAG)_{29}$ probe to detect CUG repeats in myoblast RNA. Hybridization of the 94 nucleotide probe (5′-GGGAGG$(AGC)_{29}$A-3′) to an expanded CUG repeat produces a protected fragment of 88 nucleotides. Lane 1 shows probe with no added RNA. Lane 2 is RNA from a DM1 patient with 77 CTG repeats, lanes 3–5 are DM1 patients with more than 100 CTG repeats, lanes 6–9 are DM2 patients from four different kindreds and lanes 10–13 are healthy subjects. A protected fragment corresponding to at least 29 uninterrupted CUG repeats was seen in all DM1 patients but not in DM2 or healthy subjects.

nucleus (17). Mature skeletal muscle also expresses DMPK at relatively high levels. However, only one to three foci per nucleus were observed in the single patient whose muscle tissue was examined (15). To confirm and extend this observation, we examined sections of DM1 muscle tissue using FISH. 2-*O*-methyl substituted RNA oligonucleotides were used in these experiments to enhance probe stability and competition against secondary structure in target RNAs (27,28). An unexpected finding in the initial experiment was that CAG repeat probes also revealed nuclear foci in the DM2 controls, prompting us to undertake a more extensive survey of nuclear foci in myotonic dystrophies and other muscle diseases.

Nuclear foci were present in muscle tissue from each of seven individuals with classical DM1 (more than 100 CTG repeats; Fig. 2). The number of foci was typically one to three per nucleus. The foci in DM1 had round, curvilinear or bean-shaped profiles. Foci were present in skeletal muscle from a 34-year-old individual with 180 CTG repeats whose only manifestation was myotonia. His strength was normal and his muscle biopsy showed no other histopathologic abnormality, indicating that foci were present early in the disease process. In individuals having small expansions of the CTG repeat, the detection of foci depended on the length of the expansion. No foci were observed in two DM1 patients with 60 and 61 CTG repeats (aged 57 and 46 years, respectively). Neither patient had clinical or histologic signs of muscle disease. In both cases, PCR analysis indicated that the numbers of CTG repeats in muscle and peripheral blood leucocytes were equivalent. In contrast, infrequent foci were observed in two individuals with 74 and 77 repeats (aged 71 and 73 years, respectively) who had mild, late-onset weakness. The repeat expansions in leucocytes and muscle were similar by PCR and Southern analysis in both cases. These results suggest that nuclear foci are closely associated with the development of muscle disease in DM1.

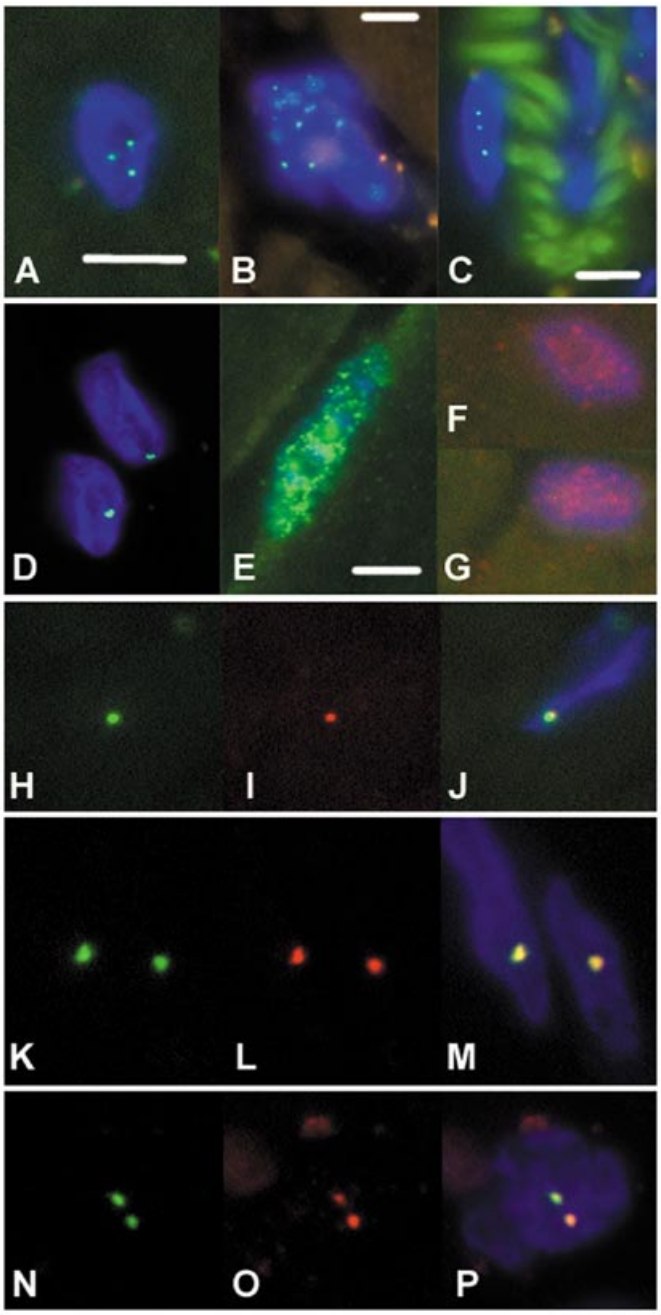

Figure 2. FISH of muscle tissue. Fluorescence-labeled CAG probe shows RNA foci (green) in the nucleus (blue, counterstained with DAPI). (**A**) A nucleus in the center of a muscle fiber shows foci in DM1. (**B**) Multiple nuclei in a pyknotic nuclear clump show RNA foci in DM1 (brown material is autofluorescent lipofuscin). (**C**) Foci are present in the nucleus of a medial smooth muscle cell that lies directly beneath internal elastic lamina (bands of green autofluorescence) of a small intramuscular artery in DM1. Foci are present in muscle nuclei in DM2 (**D**), and in HSA^{LR}-20b mice that express expanded CUG repeats (**E**). FISH combined with immunofluorescence for muscleblind (red) in normal muscle shows a speckled distribution of muscleblind in the nucleus and no nuclear foci (**F** and **G**). Nuclear foci of RNA (**H** and **K**) and muscleblind (**I** and **L**, polyclonal antibody to muscleblind) are colocalized in the merged images in DM1 (**J**) and DM2 (**M**). The results are similar using mAb 3B10 in DM2 (**N**–**P**). Bars: 5 μm. Bar in (A) applies to other panels, except where indicated.

Given that DMPK is also expressed in smooth muscle and that blood pressure is relatively low in DM1 (29), it was interesting to note that nuclear foci were also present in the smooth muscle cells of intramuscular arteries (Fig. 2C).

CAG-repeat probes also revealed nuclear foci in muscle tissue from each of nine patients with DM2 (Fig. 2D), including one patient whose only clinical manifestation was mild myotonia. The shape and number of foci in DM2 were similar to those seen in DM1. However, the foci tended to be larger and more intensely fluorescent in DM2. In contrast, nuclear foci were not observed in muscle tissue from 15 subjects with other muscle diseases or eight healthy controls. Detection of foci was tolerant to various fixation conditions, including conditions expected to eliminate the RNA binding activities of proteins. For example, foci were detected with precipitating fixative (Carnoy's, which eliminated the immunodetection of nuclear proteins) or cross-linking fixative [paraformaldehyde (PFA)]. Increasing the length of fixation time (3% PFA for times ranging from 10 to 60 min, Carnoy's for 10–30 min) did not eliminate FISH signals. Furthermore, hybridization with sense (CUG repeat) or GUC repeat probes did not show foci in any subjects. These results make it unlikely that foci result from probe binding to nuclear proteins or DNA, and suggest that the FISH signals in DM2 nuclei result from hybridization of probe to a disease-specific RNA.

Nuclear foci were detected in three DM2 patients whose myoblast RNAs had not shown an expanded CUG repeat by RNase protection. We postulated that DM2 muscle cells express an aberrant RNA with sufficient complementarity to allow hybridization with a CAG probe. However, mismatches or bulge-loops in the probe–target duplex could render it sensitive to cleavage in the RNAse protection assay. Consistent with this interpretation, we found that a higher stringency wash (65°C versus 45°C, 30% formamide) eliminated all nuclear foci in patients with DM2, without affecting the FISH signals in DM1.

Nuclear retention and pathogenicity of expanded CUG repeats may involve interactions with RNA binding proteins. Miller *et al.* (21) have identified a family of muscleblind proteins that recognize expanded CUG repeats in preference to non-expanded CUG repeats, expanded CAG repeats or other structured RNAs. Although the nuclear distribution of muscleblind is altered in cultured DM1 cells, no proteins have been shown to colocalize with expanded CUG repeats in cells or tissue. We used a polyclonal antibody raised against recombinant human muscleblind to examine the distribution of muscleblind proteins in sections of skeletal muscle. In normal muscle, muscleblind in the myonucleus had a speckled distribution (Fig. 2F and G). However, the nuclear distribution of muscleblind was markedly altered in DM. Myonuclei in DM1 and DM2, but not in disease controls, showed focal accumulations of muscleblind. When immunofluorescence was combined with FISH, the accumulations of muscleblind were localized precisely to the nuclear foci of RNA (Fig. 2H–M). In some nuclei, it appeared that muscleblind in the nucleus was entirely redistributed to the nuclear foci. Studies using monoclonal antibody (mAb) 3B10, also raised against recombinant human muscleblind, gave similar results (Fig. 2N–P). By comparison, immunofluorescence for eight other nuclear proteins did not show colocalization with nuclear foci (A.Mankodi, M.Krym and C.Thornton, manuscript in preparation).

DISCUSSION

The detection of nuclear foci in DM2 by FISH was a robust finding in15 separate experiments and in every case that was examined. Foci were present in a family linked to the DM2 locus on chromosome 3q, and also in a family that probably is not linked to this locus (LOD score <–2; R.Krahe, A.Mitchell and K.Johnson, unpublished data). These observations suggest that nuclear accumulation of aberrant RNA is a general phenomenon in the myotonic dystrophies and that FISH may be useful in the diagnosis of DM2.

What target RNA sequence is recognized by CAG- but not by GUC- or CUG-repeat probes in DM2? The answer awaits the identification of mutations that are responsible for DM2. The report of anticipation in DM2 (11) suggests that the mutation involves an unstable microsatellite repeat. Possibilities would include UUG triplet repeats, non-triplet microsatellite repeats which contain a CUG motif (and form bulge loops when bound to CAG probes), or unrelated sequences that bind CAG probes through non-canonical interactions. Our demonstration of a disease-specific probe–target interaction may facilitate the identification of the DM2 gene(s). It is possible that hybridization-based methods using CAG or related probes could be used to enrich for mutant mRNA/cDNA.

In situ hybridization of DM1 myoblasts shows hundreds of foci per nucleus (16). We confirm, however, that nuclei in DM1 muscle tissue have only one to five foci. It seems unlikely that restricted access of probe to the nucleoplasm or secondary structure in the target has limited the detection of nuclear foci; the 2-*O*-methyl RNA probes used here should compete favorably against secondary structure (27,28), the length of the probe (20 nucleotides) is shorter than in previous studies, and our results were not affected by modification of the FISH procedure (e.g. longer periods of hybridization, acetone versus Triton-X permeabilization, mild fixation). Morever, similar methods revealed more than 50 foci per nucleus in muscle tissue from transgenic mice that express expanded CUG repeats (17) (Fig. 2E). We conclude that expanded CUG repeats are distributed to a very limited volume of the nucleus in DM1 skeletal muscle. Further studies are necessary to determine whether these foci associate with a specific nuclear structure or domain, and whether pathogenicity is generated by the transcripts in the foci or a fraction of mutant mRNA that remains free. The present study defines a threshold length for formation or detection of nuclear foci *in vivo*. Repeat lengths below 70 did not show nuclear foci or signs of muscle disease. Repeat lengths between 70 and 100 showed sparse foci and mild, late-onset myopathy. Repeat lengths that exceed these thresholds, however, failed to show an obvious connection between the number or size of nuclear foci and the repeat length or severity of muscle disease.

Structural properties and protein interactions of expanded CUG repeats may underlie their pathogenic effects. Expanded CUG repeats form stable hairpins *in vitro* (20,30,31). Various proteins interact with CUG repeats *in vitro*, including CUGBP1 (18), ETR3 (19), PKR (20) and muscleblind proteins (21). There has been uncertainty, however, about which of these proteins, if any, interact with expanded CUG repeats *in vivo*. Muscleblind was selected for these studies because of its strong preferential binding to expanded CUG repeats compared with other structured RNAs, and because of its relative abundance in skeletal and cardiac muscle (21). Furthermore, muscleblind is the human homolog of a protein required for terminal differentiation of muscle and photoreceptor cells in *Drosophila* (22), suggesting a model in which sequestration of muscleblind in nuclear foci plays a role in DM1 pathogenesis. In showing that muscleblind is localized to nuclear foci in both DM1 and DM2, the present studies are consistent with this model. More information about the function of muscleblind and its cytoplasmic and nuclear distribution in DM is needed to evaluate its potential role in pathogenesis.

The pleiomorphic manifestations of DM1 may represent a composite phenotype resulting from several distinct pathogenic mechanisms, such as loss of function for DMPK, silencing of the flanking gene *SIX5*, and gain of function by mutant mRNA. This view is supported by the appearance of partial DM-like phenotypes in various mouse models. For example, there is delayed cardiac conduction (12) and reduced muscle force generation (13) in *Dmpk* knockouts, cataracts in *Six5* knockouts (32,33), and myotonia and myopathy in mice that express expanded CUG repeats in skeletal muscle (17). The view of DM1 as a mechanistically complex, multi-genic disease, however, is difficult to reconcile with the observation that many of its manifestations are reproduced in DM2, which maps to a locus that shows no obvious homology or functional relationship with the DM1 locus [reviewed by Tapscott (34)]. The present results raise the possibility that RNA gain of function is a unifying mechanism which underlies all forms of DM, including the non-muscle manifestations. Alterations in *DMPK* or *SIX5* expression, however, may have important disease-modifying roles in DM1.

MATERIALS AND METHODS

Subjects

The diagnosis of DM2 (n = 9, age 44–68 years, mean age 55 years, four men and five women) was based on the lack of an expanded CTG repeat at the DM1 locus, cataracts before the age of 50 years, weakness that included proximal muscles, myotonia demonstrable by electromyography, a family history of multi-generation disease, and a muscle biopsy confirming the histologic findings of DM2 (chronic myopathy with a marked increase in central nuclei and numerous pyknotic nuclear clumps) in the subject or a first degree relative. The exception was that one DM2 patient had myotonia but no weakness. The diagnosis of DM1 (n = 11, age 25–78 years, mean age 51 years, eight men and three women) was based on weakness or myotonia with genetic confirmation by PCR or Southern blot as described previously (35). The exceptions were two subjects with small expansions (60 and 61 repeats) who had no signs of muscle disease. Control muscle biopsies included eight healthy subjects (age 21–72 years, mean age 46 years, four men) and 15 disease controls (age 14–68 years, mean age 42 years, 12 men) with the following diagnoses: chloride channel myotonia (n = 2), mitochondrial myopathy (n = 3), Becker muscular dystrophy (n = 1), limb girdle dystrophy (n = 3), inflammatory myopathy (n = 3), fascioscapulohumeral muscular dystrophy (n = 1), amyotrophic lateral sclerosis (n = 1) and oculopharyngeal dystrophy (n = 1).

Muscle tissue and myoblast cultures

Needle biopsies of quadriceps muscle were obtained and prepared for frozen sections as described by Dubowitz (36). Primary myoblast cultures were established as described previously (37). Myogenic differentiation to myotubes was induced by withdrawal of growth factors.

RED

RED analysis was performed as described previously (25,26). The analysis was performed in a blinded fashion on coded samples. The nine DM2 patients were from six different kindreds, of which one has been linked to the DM2 locus on chromosome 3q (A.Mitchell, R.Krahe and K.Johnson, unpublished data).

RNase protection assay for expanded CUG repeats

Total cellular RNA was isolated from myoblasts or myotubes of subjects with DM2, DM1 or healthy subjects ($n = 4$ in each group) using the acid phenol-guanidinium method (Trireagent, Molecular Research, Cincinnati, OH). Ribonuclease protection assays were performed using kits (RPAII, Ambion, Austin, TX). A CAG repeat probe synthesized using T7 RNA polymerase and ^{32}P-dCTP was purified on denaturing polyacrylamide gels as described previously (20). The template for probe synthesis, plasmid pASU29 (20), contained 29 uninterrupted CAG repeats fused directly to the promoter for T7 polymerase. Assays were carried out with 10 µg total RNA and 3×10^5 c.p.m. probe, and analyzed on 20 cm 8% denaturing polyacrylamide gels.

FISH

Frozen sections (6 µm) of quadriceps muscle were dried for 30 min, fixed in modified Carnoy's (73% ethanol, 25% acetic acid, 2% formalin) at 4°C for 10–30 min, and washed in 3× phosphate-buffered saline (PBS) followed by 1× PBS for 10 min at 20°C. Alternatively, sections were fixed in PFA PBS at 20°C. For most experiments, sections were fixed in 3% PFA for 30 min, but fixation times ranging from 2% PFA for 10 min up to 3% PFA for 60 min were also effective. PFA fixation was followed by washing five times in PBS for 2 min and permeabilization in 2% acetone PBS (pre-chilled at –20°C) for 5 min. Sections were placed in 30% formamide and 2× SSC for 10 min, hybridized with probe (1 ng/µl) for 2 h at 37°C in buffer (30% formamide, 2× SSC, 0.02% BSA, 66 µg/ml yeast tRNA, 2 mM vanadyl complex), and then washed for 30 min in 30% formamide/2×SSC at 45°C or 65°C followed by 1× SSC and 33 nM diamidino-2-phenylindole (DAPI) for 30 min at 20°C. Sections were then mounted in Prolong (Molecular Probes, Eugene, OR). Probes were HPLC-purified 2-*O*-methyl RNA 20-mers (IDT, Coralville, IA) having CAG-, CUG- or GUC-repeats and 5′ end-labeled fluorescein.

FISH-immunofluorescence (FISH-IF)

For the preparation of anti-muscleblind polyclonal antibodies, a DNA fragment encoding a novel muscleblind isoform, hEXP41 (accession no. AF401998), was PCR synthesized using primers MSS789 and MSS790 and subcloned into pET15b (Novagen, Madison, WI) as described previously (21). The resulting His-tagged hEXP41 protein (His-hEXP41) was expressed in BL21-Gold (Stratagene, La Jolla, CA), and purified by His-Bind (Novagen) chromatography using 6 M guanidine-HCl according to the manufacturer's recommendation. Denatured His-hEXP41 (250 mg/injection) was used for rabbit immunization (Cocalico Biologicals, Reamstown, PA). Following three injections, this antiserum recognized a 41 kDa protein in both HeLa and lymphoblastoid cell line (LCL) whole cell extracts. These anti-hEXP41 antibodies also immunopurified hEXP41 photocrosslinked to (CUG)97 RNA *in vitro* (21). The His-hEXP41 fusion protein was also used to prepare the mAb 3B10 using previously described methods (18). By immunoblot analysis, mAb 3B10 recognized a major 41 kDa MBNL isoform in human LCLs and mouse C2C12 myoblasts as well as a 40/41 kDa doublet in Hela whole cell extracts (C.R.Urbinati and M.S.Swanson, unpublished data). For FISH-IF, muscle sections were dried for 30 min then fixed in 3% PFA PBS for 30 min at 20°C, washed five times in PBS for 2 min, and then permeabilized in 2% acetone PBS (pre-chilled at –20°C) for 5 min. FISH procedures were carried out as described above except for omission of DAPI from 1× SSC wash. Following the 1× SSC post-hybridization wash, sections were placed in primary antibody (1:1000 dilution of polyclonal antibody) overnight at 4°C, washed five times in PBS for 2 min, placed in secondary antibody (Alexa 568-labeled goat anti-rabbit polyclonal, Molecular Probes) and 33 nM DAPI for 30 min at room temperature, washed five times in PBS and then mounted in Prolong.

NOTE ADDED IN PROOF

The DM2 mutation was recently shown to be an expanded CCTG repeat (38).

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