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Fig. 3. Splicing of α -actinin by MBNL1. **A:** Diagram of the α -actinin minigene. The sequence of α -actinin containing NM (non-muscle) and SM (smooth muscle) alternative exons was inserted into the ORF of EGFP-C1. **B:** Upper band indicates NM- or SM-including isoform. Lower band indicates both skipped isoforms. **C:** Quantification of the ratio of both-skipped isoform. When transfected with MBNL1₄₀-myc, the both-skipped isoform was promoted. YB-1-V5 also up-regulated the isoform, although less than MBNL1. DDX1-V5 did not significantly affect the splicing. The results are expressed as the mean \pm SD of four independent experiments. * $P < 0.05$, ** $P < 0.01$.

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Production of MBNL1 Polyclonal Antibody and Endogenous Localization of MBNL1

To investigate the cellular localization of MBNL1, we raised polyclonal antibodies against MBNL1₄₀ and observed endogenous MBNL1 localization. The specificity of the antibodies was confirmed by Western blotting (Fig. 5A). Next, using these antibodies, endogenous MBNL1 localization was investigated in COS-7 cells (Fig. 5). Under normal conditions, endogenous MBNL1 localized mainly to the nucleus. After arsenite treatment, MBNL1 moved to cytoplasmic granules in the cytoplasm and strongly colocalized with TIA-1. It should be noted that MBNL1 also localized to SGs in response to heat stress in HeLa cells as well as in C2C12 myoblasts (data not shown). Therefore, the SG localization of MBNL1 would be a universal phenomenon and should have some physiological functions in SGs.

To examine whether MBNL1 localizes to SGs specifically, we subsequently examined the colocalization of MBNL1 and DCP2, a P-bodies marker. In COS-7 cells transfected with MBNL1-myc and HA-DCP2, colocalization in P-bodies was not detected in response to arsenite stress as well as in normal conditions (Fig. 5B). Thus, we concluded that the MBNL1 granules are mainly SGs,

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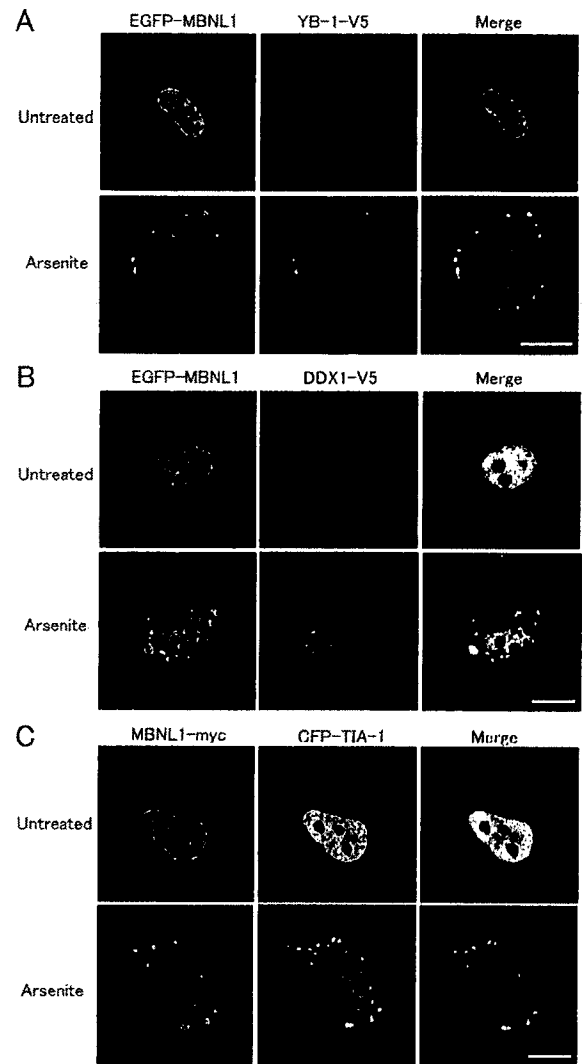


Fig. 4. Colocalization of MBNL1 and YB-1/DDX1 in HeLa. HeLa cells were cotransfected with each pair of expression plasmids. The cells were treated with sodium arsenite (0.5 mM, 45 min) 24 hr after transfection. Cell images were obtained by confocal microscopy. **A:** EGFP-MBNL1 and YB-1-V5 colocalized at cytosolic granules. **B:** DDX1-V5 colocalized with EGFP-MBNL1, as did YB-1-V5. **C:** MBNL1-myc and GFP-TIA-1 were also colocalized, as in A and B. Scale bars = 10 μ m.

but the possibility that the MBNL1 granules partially overlap with P-bodies cannot be excluded.

YB-1 but Not MBNL1 Associates With Polysomes

Several lines of evidence suggest that YB-1 associates with polysomes (Miwa et al., 2006; Nashchekin et al., 2006). Thus, to investigate further the interaction of MBNL1 and YB-1 in cytoplasmic mRNPs, we per-

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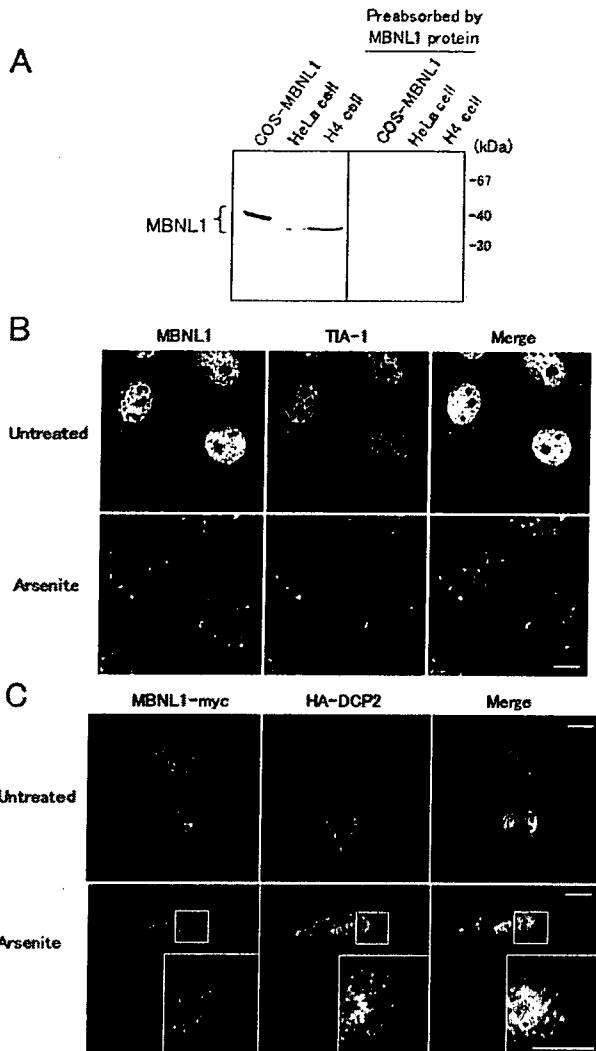


Fig. 5. Endogenous MBNL1 granules in COS-7 cells. **A:** Specificity of polyclonal antibody against MBNL1. Overexpressed MBNL1₄₀-myc has a higher molecular weight than that of endogenous MBNL1 in HeLa or H4 cells. Preabsorption by MBNL1₄₀ entirely abolished the staining. **B:** MBNL1 and TIA-1 colocalize endogenously at stress granules after arsenite stress. **C:** MBNL1 granules are distinct from P-bodies. MBNL1₄₀-myc and HA-DCP2 were cotransfected into COS-7 cells. The cells were treated with 0.5 mM arsenite for 45 min. Each of the granule types appeared in distinct locations. The MBNL1 granules are larger than those of DCP2. Scale bars = 10 μ m.

formed sucrose gradient fractionation. Although YB-1 was found to associate with polysomes, endogenous MBNL1 was not (Fig. 6). Consistent with this result, transiently transfected MBNL1-myc did not associate with polysomes. This suggests that MBNL1 associates with smaller mRNP particles. Our results for MBNL1 are similar to those for Smaug, an mRNA-binding pro-

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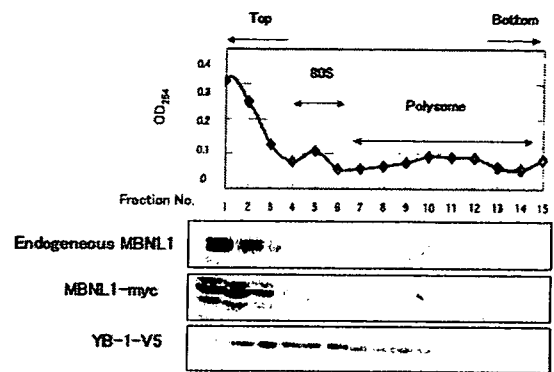


Fig. 6. Polysome analysis. Extracts of HeLa cells that had been incubated with cycloheximide were sedimented through sucrose gradients as described. The polysome and 80S single ribosome sedimentation profiles are shown by A_{254} absorbance. Western blot analysis showed that endogenous MBNL1 was present exclusively in the lighter fractions (lanes 2, 3). YB-1 was present in a broad manner in polysome fractions.

tein that forms SGs like cytoplasmic foci (Baez and Boccaccio 2005). At these foci, Smaug is said to repress the translation of mRNAs. Because YB-1 cofractionated with MBNL1, the interaction of the two proteins may occur in small mRNPs, not in polysomes.

DISCUSSION

The initial aim of this study was to identify MBNL1-binding proteins. We identified several MBNL1-interacting proteins by pull-down and mass spectrometric analyses and subsequently investigated the interaction of MBNL1 with YB-1 and DDX1. YB-1, human Y-box binding protein, belongs to a family of multifunctional nucleic acid-binding proteins. Y-box proteins contain a cold-shock domain (CSD), which is highly conserved, and function as RNA chaperones. YB-1 appears to have roles in RNA splicing (Raffetseder et al., 2003; Young et al., 2005) and translation (Pascual et al., 2006), DNA repair, and transcription (Chernukhin et al., 2000; Klenova et al., 2004; Roberts et al., 2007). Thus, it regulates gene expression at both the transcriptional and the posttranscriptional levels (Pascual et al., 2006).

During the course of our analysis of the interaction between MBNL1 and YB-1, we found that MBNL1 forms SGs in the cytoplasm, like YB-1. Our immunoprecipitation results confirmed that the interaction was mediated by RNA molecules. The two proteins cofractionated in small RNP fractions rather than as polysomes. This suggests that they may form RNP complexes before the bound mRNAs are transported to actively translating ribosomes. We also examined the functional interaction between MBNL1 and YB-1 using an α -actinin minigene splicing assay, and we found that YB-1 weakly promoted SM/NM exon skipping, like MBNL1. Recent reports have described the function of YB-1 in RNA splicing. It

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is therefore possible that MBNL1 and YB-1 function cooperatively during splicing.

The DEAD box RNA helicase family is also implicated in RNA splicing (Miller et al., 2000). Although in our system the contribution of DDX1 to alternative splicing could not be detected, it is possible that DDX1 affects the splicing activity of MBNL1 toward other targets. Micro-RNAs (miRNA) are increasingly recognized as major regulators of gene expression in eukaryotes, and DDX1 was recently identified as one of the components of the microprocessor complex (Gregory et al., 2004). It is intriguing to speculate that MBNL1 is also functionally related to miRNA biogenesis. Additionally, DDX1 was demonstrated to associate with cleavage stimulation factor and to be involved in processing the 3'-ends of pre-mRNA molecules (Bleoo et al., 2001). It is possible that MBNL1 is also part of the cleavage body in the nucleus, where it may play a role in constitutive RNA metabolism.

In our study, MBNL1, YB-1, and DDX1 were found to colocalize in the cytoplasm rather than in the nucleus in response to stress. A distinct feature of MBNL1 was its presence in cytosolic foci, and we demonstrated that MBNL1 colocalized with YB-1, DDX1, and TIA-1 at apparent SGs. SGs are discrete cytoplasmic aggregates that can be induced by a variety of stresses, including heat shock, hypoxia, oxidative stress, and viral infections (Anderson and Kedersha, 2002). SGs are considered to be sites of mRNA triage that regulate mRNA stability and translatability. The accumulation of RNAs into dense globules could keep them from reacting with harmful chemicals and safeguard the information that they encode. Molecules can go down one of three paths: storage, degradation, or reinitiation of translation. Recently, it was reported that YB-1, in association with the antiretroviral factor APOBEC3G, relocates from P-bodies to SGs when cells are exposed to heat shock stress. Our results show that MBNL1 granules are distinct from P-bodies but that sometimes they are located adjacent to one another. Considering these facts, when a cell is subjected to arsenite stress, YB-1 and MBNL1 colocalized in SGs. Our pull-down screen was not specific for stress conditions; thus, YB-1 and MBNL1 should also interact under normal physiological conditions. Supporting this, the interaction was observed in a nonstressed condition (Fig. 2). Among the proteins identified in our primary screen, the ribosomal protein L7a was identified as a component of SGs (Kim et al., 2006). The ribosomal protein S3 is also a component of SGs (Kedersha et al., 2002). Thus, four of the seven proteins identified in our screen are SGs-related proteins, which strongly suggests that MBNL1 is physiologically related to SGs. Because not all of the ribosomal proteins localized to SGs (e.g., L5 and L37), the components of SGs should be further studied. Investigations of the other MBNL1-interacting proteins as candidate SGs components, such as phenylalaninyl-tRNA synthetase subunits α and β and amylo-1,6-glucosidase, would also be interesting.

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The expression of most genes is regulated by multiple mechanisms, and an important control of gene expression is exerted through mRNA stability. The stability of mRNAs is regulated by a variety of signals acting on specific sequences within the RNA. Previously, muscleblind was suggested to play a role in RNA stability in the cytoplasm in *Drosophila* (Houseley et al., 2005). Our data about cytoplasmic dynamics of MBNL1 might support this novel function of muscleblind. In addition, TTP, one of the C₂H₂ zinc finger protein same as muscleblind, was shown either to promote AU-rich element (ARE)-RNA degradation or increase their stability (Houseley et al., 2005). Because TTP is one of the most related genes to MBNL1 in the point of its domain structure, it is reasonable to assume that MBNL1 also has a function in affecting the stability of mRNA in the cytoplasm. Target RNA sequence of MBNL1 is likely to be CHHG (H is A, U, or C) motif (Kino et al., 2004). Because every mRNA should have each specific sequence in 3'-UTR, it is possible that MBNL1 binds to mRNAs, which has a CHHG motif in the 3'-UTR, and controls their RNA stability.

Recently, for the RNA-binding motif protein 4 (RBM4), which plays a regulatory role in alternative splicing of precursor mRNA, it was demonstrated that its functions in translation control are modulated by cell stress (Lin et al., 2007). Although the relation and dynamics between splicing activity and translation control are unknown, it is intriguing to assume that these splicing regulators, including MBNL1, dynamically shuttle between nucleus and cytoplasm in response to cell stress. Further investigation should be done to clarify these splicing regulators dynamics.

In summary, our study demonstrates that MBNL1 formed cytoplasmic SGs and colocalized with YB-1 and DDX1. The functional significance of these events should be investigated further and may lead to a better understanding of the pathogenesis of DM.

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Overexpression of MBNL1 fetal isoforms and modified splicing of Tau in the DM1 brain : Two individual consequences of CUG trinucleotide repeats

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Abstract

Neurofibrillary degeneration is often observed in the brain of patients with type 1 myotonic dystrophy (DM1). It consists principally of the aggregation of Tau isoforms that lack exon 2/3 encoded sequences, and is the consequence of the modified splicing of Tau pre-mRNA. In experimental models of DM1, the splicing of several transcripts is modified due to the loss of Muscleblind-like 1 (MBNL1) function. In the present study, we demonstrate that the MBNL1 protein is also present in the human brain, and consists of several isoforms, as shown by RT-PCR and sequencing. In comparison with controls, we show that the adult DM1 brain exhibits modifications in the splicing of MBNL1, with the preferential expression of long MBNL1 isoforms — a splicing pattern similar to that seen in the fetal human brain. In cultured HeLa cells, the presence of long CUG repeats, such as those found in the DM1 mutation, leads to similar changes in the splicing pattern of MBNL1, and the localization of MBNL1 in nuclear RNA foci. Long CUG repeats also reproduce the repression of Tau exon 2/3 inclusion, as in the human disease, suggesting that their effect on MBNL1 expression may lead to changes in Tau splicing. However, while an overall reduction in the expression of MBNL1 mimics the effect of the DM1 mutation, none of the MBNL1 isoforms tested so far modulates the endogenous splicing of Tau. The modified splicing of Tau thus results from a possibly CUG-mediated loss of function of MBNL1, but not from changes in the MBNL1 expression pattern.

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Keywords: Neuromuscular disease; Neurodegeneration; Microtubule-associated protein Tau; Splicing; Muscleblind-like protein

Introduction

Type 1 myotonic dystrophy (DM1) is an autosomal dominant multisystemic disorder caused by the unstable expansion of CTG trinucleotide repeats located in the 3' untranslated region (UTR) of the gene encoding the myotonic dystrophy protein kinase (DMPK). The number of CTG repeats varies from 50 to

Abbreviations: DM, Myotonic dystrophy; IR, Insulin receptor; DMPK, Myotonic dystrophy protein kinase; MBNL, Muscleblind-like; PCR, Polymerase chain reaction; TNT, Troponin T; UTR, Untranslated region.

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more than 4000 in affected individuals, and is correlated with disease severity and inversely correlated with the age at onset (Harley et al., 1992; Ashizawa et al., 1992). The clinical expression of DM1 includes myotonia and progressive muscle weakness, frontal baldness, cataracts, cardiac conduction defects, hypogonadism, endocrine deficiency and cognitive impairment (Harper, 1989). A neurodegenerative process is often observed in the brain of aged DM1 patients (Vermersch et al., 1996; Kiuchi et al., 1991; Oyamada et al., 2006), characterized by the presence of neurofibrillary tangles in the neocortex and in the subcortical nuclei. These neurofibrillary tangles consist of the intraneuronal aggregation of abnormally modified microtubule-associated Tau proteins.

The etiology of DM1 implies that the disease is due to an RNA gain-of-function. CUG repeats accumulate in the nucleus in the form of ribonuclear inclusions, named foci (Taneja et al., 1995). These ribonuclear inclusions contain several proteins, among which the Muscleblind-like 1 (MBNL1) protein is predominant (Mankodi et al., 2001; Jiang et al., 2004). The sequestration of MBNL1 leads to the loss of its function. The disruption of the mouse *MBNL1* gene thus reproduces the trans-dominant effect of the CUG repeats (Kanadia et al., 2003), which in turn results in the modified splicing of a growing list of transcripts, including cardiac troponin T (cTNT), the insulin receptor (IR), the muscle-specific chloride channel (ClC-1), myotubularin-related protein 1, fast skeletal troponin T, the NMDA NR1 receptor, ryanodine receptor 1, the amyloid beta precursor protein and Tau (Jiang et al., 2004; Kanadia et al., 2003; Savkur et al., 2001; Sergeant et al., 2001; Buj-Bello et al., 2002; Charlet et al., 2002; Lin et al., 2006; Kimura et al., 2005).

In *Drosophila*, in which they were initially characterized, Muscleblind proteins have been shown to be required for the terminal differentiation of myocytes and photoreceptors (Bege-mann et al., 1997; Artero et al., 1998); for review see (Pascual et al., 2006)). In humans, the MBNL genes consist of three paralogous: MBNL1, MBNL2 and MBNL3. MBNL1 and MBNL2 are found in numerous tissues, with MBNL1 expression at its strongest in muscle and the heart (Miller et al., 2000). MBNL3 is mostly expressed in the placenta. The human *MBNL1* gene is located on chromosome 3, and alternative splicing generates several isoforms (Mankodi et al., 2001; Miller et al., 2000; Kino et al., 2004; Fardaei et al., 2002), which have been characterized in skeletal muscle (Kanadia et al., 2006).

MBNL1 proteins regulate the splicing of both cTNT and IR pre-mRNAs (Ho et al., 2004). Some MBNL1 isoforms also bind double-stranded RNA, a property that might be in balance with their role as a modulator of transcript splicing. The presence of the sequence encoded by exon 4 is essential for MBNL1 to bind to CUG repeats (Kino et al., 2004). Together, these results suggest a dual, and possibly interrelated, function for MBNL1 isoforms, as double-stranded RNA binding proteins, and as trans-regulatory splicing factors.

Although MBNL1 has been characterized in skeletal muscle to some extent (Kanadia et al., 2006), little is known about its expression in the brain, under either normal or pathological conditions. The aim of the present work was to study the expression of MBNL1 in the brain of DM1 patients and control

individuals, as well as its relationship to Tau splicing. We show here that a fetal isoform of MBNL1 is overexpressed in the brain tissue of DM1 patients. The expression of this fetal isoform can be reproduced by the ectopic expression of long CUG repeats *in vitro*. We also show that the Tau mis-splicing observed in DM1 is reproduced by the ectopic expression of CUG repeats or the RNA interference-mediated inactivation of MBNL1. In contrast, the expression of adult or fetal MBNL1 isoforms does not affect Tau exon 2/3 inclusion. Taken together, our results suggest that in the DM1 brain, MBNL1 and Tau splicing are targets of the trans-dominant effect of CUG repeats and that the silencing of MBNL1 results in alterations in Tau splicing.

Materials and methods

Human brain tissue samples

DM1 brain tissue samples were obtained at autopsy from 3 patients aged of 42, 53 and 64 years. Two patients (DM1.1 and DM1.3) had an adult form of DM1, while the third had a congenital form of DM1 (DM1.2). These cases have been previously described (Leroy et al., 2006a). Three control individuals were included in the study. The brain tissues were obtained from the Department of Neurology, University Hospital Centre of Lille, France, in accordance with the protocol of the local ethics committee. Post-mortem delays were below 48 h, and brain tissue was stored at -80°C . Four cortical brain regions, the temporal, occipital, frontal and parietal, were dissected for analysis. The genetic diagnosis of DM1 was confirmed by a long PCR assay. For the analysis of the expression of different human MBNL1 transcripts during an early stage of development, we analyzed first strand cDNA preparations from a human fetal brain (Human fetal MTC™ panel, BD Biosciences, Clontech, California, USA).

Semi-quantitative analysis

Total RNA was isolated from brain tissue using RNAgents (Promega, France) according to the manufacturer's instructions. Total RNA was isolated from cells using the Nucleospin® RNA II kit (Macherey Nagel, Düren, Germany). RNA concentration was determined by absorption at 260 nm. RT-PCR was performed in triplicate with 1 μg of total RNA using random hexamers (5 $\mu\text{M}/\text{l}$) and the M-MLV reverse transcriptase (Promega, France) according to standard protocols. No DNA amplification was observed in the RT controls. PCR was carried out in a final volume of 25 μl , with 15 pM of each primer (primer sequences are detailed in Table 1), 1.5 mM MgCl_2 and 1 U of Taq polymerase (Invitrogen), under the following conditions: 5 min at 94°C , 22 to 30 cycles of a 1 min denaturation step at 94°C , annealing for 2 min at the temperature indicated in Table 1, 2 min of extension and 7 min of final extension at 72°C . GAPDH was used as an internal control. The reaction products were resolved by electrophoresis using a 4% or 5% polyacrylamide gel, and bands were stained with SYBR Gold (Molecular Probes, Oregon, USA). The intensity of SYBR Gold luminescence was measured using a FluorImager scanner (Clarusvision, France). The mRNA signals were normalized to that of GAPDH mRNA.

Table 1
Primers and PCR reactions

Sequence amplified	Sense primers	(Exon location)	Antisense primers	(Exon location)	PCR conditions	Fragment size (bp)
MBNL1 _{41/43}	5'-ATTACAACCCGTGCCAATGT-3'	(3/4)	5'-CACCATGGGAACAACACTTGTA-3'	(8/9)	1.0 mM MgCl ₂ 60 °C	676/730
MBNL1 _{40/42}	5'-CCCCATTACAACCCGTGCCAAT-3'	(3/4)	5'-ATGGGAACTGGTGGGAGAA-3'	(7/9)	1.5 mM MgCl ₂ 60 °C	640/694
MBNL1 exon 5	5'-AATTGCAACCGAGGAGAAAA-3'	(5)	5'-CAGCCTGGTTGACCTGGTAT-3'	(5)	1.5 mM MgCl ₂ 60 °C	199
MBNL1 exon 6	5'-AATTGCAACCGAGGAGAAAA-3'	(5)	5'-AGGGTTCGCTTCAGTGATTT-3'	(6)	1.5 mM MgCl ₂ 60 °C	269
MBNL1 exon 8	5'-CAAAGAGGCCTGCTCTTGAA-3'	(7)	5'-CAACACTTGGTAGCGGGTGC-3'	(8)	1.5 mM MgCl ₂ 60 °C	159
MBNL1 exon 6–8	5'-GCAACCTTTGACCTGGGAAT-3'	(6/7)	5'-ATTGAGCCTGGTGGGAGAA-3'	(7/8)	1.5 mM MgCl ₂ 60 °C	167
MBNL1 exon 3–11	5'-ACTAGCCAATGCCATGATGC-3'	(3)	5'-TTGTGGCTAGTCAGATGTTTCG-3'	(11)	1.5 mM MgCl ₂ 60 °C	588–882
hcTNT exon 5	5'-CATTACCACATTGGTGTGC-3'		5'-AGGTGCTGCCGCCGGCGGTGGCTG-3'		1.5 mM MgCl ₂ 64 °C	109, 139
Tau exon 2/3	5'-TACGGTTGGGGGACAGGAAAGAT-3'	(1)	5'-GGGGTGTCTCCAATGCCTGCTTCT-3'	(4)	1.5 mM MgCl ₂ 65 °C	112, 199, 286
DMPK 3'UTR	5'-TGTCTGTCCCCACCTAGGAC-3'		5'-GCTAGCTCTCCAGACCTT-3'		1.5 mM MgCl ₂ 60 °C	171
GAPDH	5'-CCATGGCACCGTCAAGGCTGA-3'		5'-GCCAGTAGAGGCAGGGATGAT-3'		1.5 mM MgCl ₂ 62 °C	440

List of primers used to analyze the expression of MBNL1 isoforms and the alternative splicing of Tau exon 2 and 3 and hcTNT exon 5. The first column indicates the sequences that were amplified. The second and third columns are the sequence of the forward and reverse primers used, and their exonic location. The concentration of MgCl₂, annealing temperature and size of the fragments are also indicated.

Real time RT-PCR

The expression levels of MBNL1 exon 6, exon 8 and exon 5 were studied by real time RT-PCR. The primers used are indicated in Table 1. We analyzed the expression of MBNL1 transcripts containing both exon 6 and exon 8 (exons 6–8) using a forward primer overlapping exons 6 and 7, and a reverse primer overlapping exons 7 and 8 (Table 1). The levels of MBNL1 were assessed relative to the level of β -actin. The PCR was performed with a Light Cycler (Roche, Mannheim, Germany). The master mix contained Taq DNA polymerase and SYBR Green I dNTPs (Light Cycler® FastStart DNA Master SYBR Green, Roche, Mannheim, Germany). The primers were used at a final concentration of 0.5 μ M. The conditions required for amplification included 2.5 μ M of MgCl₂ and annealing at 62 °C for exon 6, and 4 μ M of MgCl₂ and annealing at 64 °C for exon 8. The amplification of exon 5 and exons 6–8 required 2.5 μ M of MgCl₂ and annealing at 60 °C, over 40 cycles.

Antiserum production and purification

Antibodies were raised by immunizing rabbits (Eurogentec, Liege, Belgium) with a specific MBNL1 N-terminal peptide, 9-RDTKWLTLEV-18 (numbering according to MBNL1₄₂; Swiss-Prot ref. MBNL1_Human Q9NR56), which was coupled at its C-terminal end to keyhole limpet hemocyanin. The specificity of the antiserum was tested using HeLa cells transfected with MBNL1 or non-transfected controls (Kino et al., 2004).

Cloning of MBNL1 isoforms and molecular constructs

To screen for the different MBNL1 isoforms, we selected primers delimiting the coding sequence between exon 2 and exon 11 of MBNL1. These exons are common to the isoforms previously described (Kino et al., 2004). Total cDNA sequences were obtained by amplification of DM1 and control cDNA, with DYNAszyme EXT™ Taq polymerase (Finnzymes, Espoo, Finland). PCR products were cloned into the PCR-TOPO TA Cloning vector (Invitrogen, California, USA) for sequencing. Plasmid DNA was purified using the Nucleobond® AX kit (Macherey Nagel, Düren, Germany). Other plasmids were constructed for each MBNL1 isoform, with the addition of N-terminal GFP and V5-tags to the coding sequence. Briefly, MBNL1 cDNAs in TOPO vectors were subcloned into the Gateway® system (Invitrogen) and inserted into pDEST 53-GFP and pcDNA 3.1-nV5. All constructs were double-strand sequenced. Plasmids containing the 3'UTR of DMPK with 5 or 200 CTG repeats were derived from previously described constructs under a rodent ROSA promoter (Amack et al., 1999). The constructs were subcloned into pcDNA 3.1 under the control of the CMV promoter; the empty pcDNA plasmid was used as a control during transfection. The plasmid containing the 3'UTR of DMPK with 960 interrupted CTGs was a minigene construct also under the control of the CMV promoter. It contained the last 5 exons of DMPK followed by the full length DMPK 3'UTR, with 960 CTG repeats interrupted at every 20th repeat by CTCGA motif (Ho et al., 2005). The conservation of the number of CUG repeats was verified by a long PCR assay. The RTB300 minigene containing

exon 5 of human cTNT (hcTNT) has been previously described (Cooper, 1998).

Cell culture and transfection

HeLa or COS cells were grown in monolayer cultures in 6 well plates in Dulbecco's Modified Essential Medium (DMEM) (Invitrogen, California, USA) supplemented with 10% FBS and 0.5% Penicillin/Streptomycin, at 37 °C in a humidified CO₂ (5%)

incubator. Cells grown to ~50% confluence were transiently transfected with 2 µg of plasmid DNA, using the Superfect ExGene 500 transfection reagent (Fermentas), or growing doses of plasmids (from 0.5 to 4 µg). Total RNA was analyzed 48 h post-transfection, with GAPDH as an internal loading standard except where otherwise mentioned. For silencing experiments, siRNAs were synthesized by Eurogentec. The sense strand sequences of the siRNAs designed to target MBNL1 mRNA transcripts were as follows: siMBNL1a 5'-CGCAGUUGGAGAUAAAUGG-3',

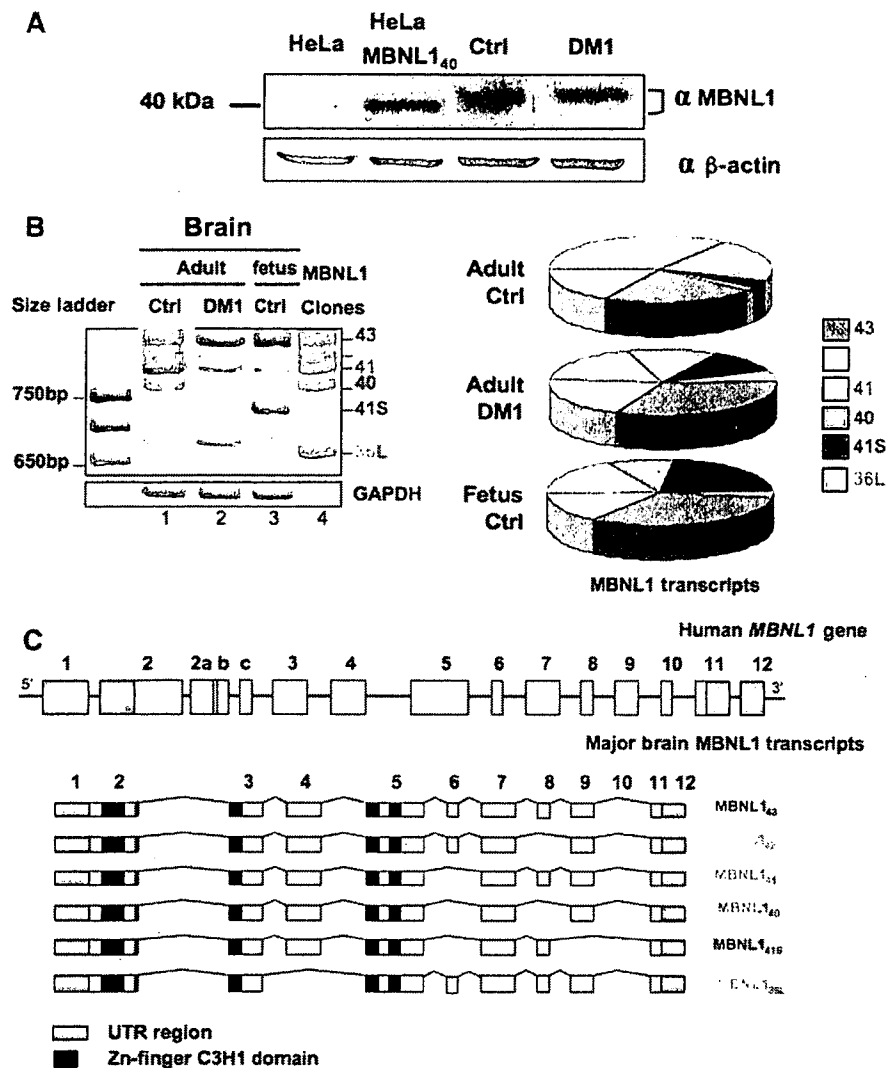


Fig. 1. MBNL1 expression in control and DM1 brain tissues. A. MBNL1 protein expression. Western blotting of protein extracts from untransfected HeLa cells (lane 1) or cells transfected with the MBNL1₄₀ expression vector (lane 2). MBNL1 protein expression was also analyzed in control (lane 3) and DM1.3 (lane 4) frontal cortical homogenates. Proteins were transferred onto a nitrocellulose membrane and probed with our MBNL1 antiserum (α MBNL1), then stripped and re-probed with a β-actin antibody (α β-actin). B. MBNL1 transcript expression. Left panel: RT-PCR analysis of total RNA from adult control (lane 1), DM1.3 (lane 2) and fetal control (lane 3) brain tissue using oligonucleotides complementary to MBNL1 exons 3 and 11. Five brain transcripts (36L, 40, 41, 42 and 43) were identified by RT-PCR followed by cDNA cloning and sequencing (lane 4), as described in the Materials and methods. MBNL1_{41S} was identified by isolating the band from the acrylamide gel and sequencing. GAPDH was used as an internal loading standard. Pie chart showing the distribution of the six major MBNL1 transcripts (36L, 40, 41, 41S, 42 and 43) in the control (Ctrl), DM1 and fetal brain. C. Representation of the MBNL1 gene and major brain transcripts. The human MBNL1 gene is located on chromosome 3q25. The MBNL1 primary transcript contains 15 exons. Grey boxes represent untranslated regions (UTRs). Exons are represented as boxes and introns as lines. Exon 1 and part of exon 2 correspond to the 5'UTR. Two thirds of exon 11 and the entire exon 12 encode the 3'UTR. Black boxes represent the four C3H1 type Zinc-finger motifs which bind to RNA. The Zinc-finger motifs are encoded by exons 2, 3 and 5. Only the five major brain transcripts with a molecular mass ranging from 36 to 43 kDa are represented. The nomenclature used corresponds to that of Kino et al. (2004) and Kanadia et al (2006).

and siMBNL1b 5'-CAGACAGACUUGAGGUAUG-3' (Ho et al., 2004). A scrambled siRNA and an siRNA against β -actin were also designed as controls. The siRNAs were transfected using JetSI[®]-Endo (Eurogentec) according to the manufacturer's instructions. Growing concentrations were used (1–200 nM) for the dose response effect.

Protein extraction and western blot analysis

DM1 and control brains were homogenized with a Dounce homogenizer in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% deoxycholate) with the addition of 1 mM Na_3VO_4 , 125 nM okadaic acid, protease and DNase inhibitors. The resulting homogenates were sonicated, and spun at 13,000 g for 20 min at 4 °C. The supernatants were transferred and the concentration of proteins measured. The pellets were sonicated again, warmed and centrifuged. The supernatants were diluted with Laemmli Buffer with or without RNAse (40 $\mu\text{g}/\text{ml}$ ribonuclease A, Fermentas, Canada). The protein extracts (50 μg each) were used for SDS-PAGE (4–12%; Nupage[®] Bis-Tris gels, Invitrogen, California, USA) and transferred onto Hybond nitrocellulose membranes (G&E Amersham) using the XCell[™] II Blot Module (Invitrogen). The proteins were reversibly stained with Ponceau red in order to control for the amount of protein loaded. After blocking with 5% of skimmed milk in TBS-T (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.1% Tween-20), the membranes were incubated overnight at 4 °C with MBNL1 antiserum (1:1000) in the same buffer. Membranes were subsequently incubated with a peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) (1:4000 in the same buffer) and antibody complexes were revealed using ECL[™] and Hyperfilms (G&E Amersham) according to the manufacturer's instructions.

Immunofluorescence microscopy

HeLa or COS cells were grown in cell culture chamber slides (Labtek) in DMEM supplemented with 10% FBS. HeLa cells were transfected with either (CTG)₅-pcDNA or (CTG)₉₆₀-pcDNA alone, or co-transfected with the MBNL1 isoforms pDEST 53GFP-36L, pDEST 53GFP-40, pDEST 53GFP-42 or pDEST 53GFP-43. COS cells were transfected with MBNL1 isoforms alone. After 48 h, cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS) for 30 min at room temperature, and permeabilized with 0.25% Triton X-100 in PBS. After blocking with 1% bovine serum albumin (BSA) in PBS, the fixed cells were incubated for 2 h at room temperature with the primary antibody in PBS with 1% BSA and 0.25% Triton X-100. The anti-GFP antibody was used to probe for the MBNL1 isoforms and to study their localization. After washing, cells were incubated with Alexa Fluor[®] 568-conjugated secondary antibodies (Invitrogen). Nucleic acids were stained using TOPRO[®]-3. The coverslips were mounted onto slides with Vectashield (Vector laboratories). Images were acquired with a Leica TCS NT laser scanning confocal microscope. All data were analyzed using Leica image analysis software.

Statistical analyses

Statistical analyses were performed using the non-parametric Mann-Whitney test, with the help of Prism Software (GraphPad Software Inc., San Diego, USA).

Results

MBNL1 protein expression is not decreased in the DM1 brain

To investigate any change in MBNL1 expression in DM1, we first compared the expression of MBNL1 protein in the frontal cortex of control and DM1 brains by western blot analysis using a

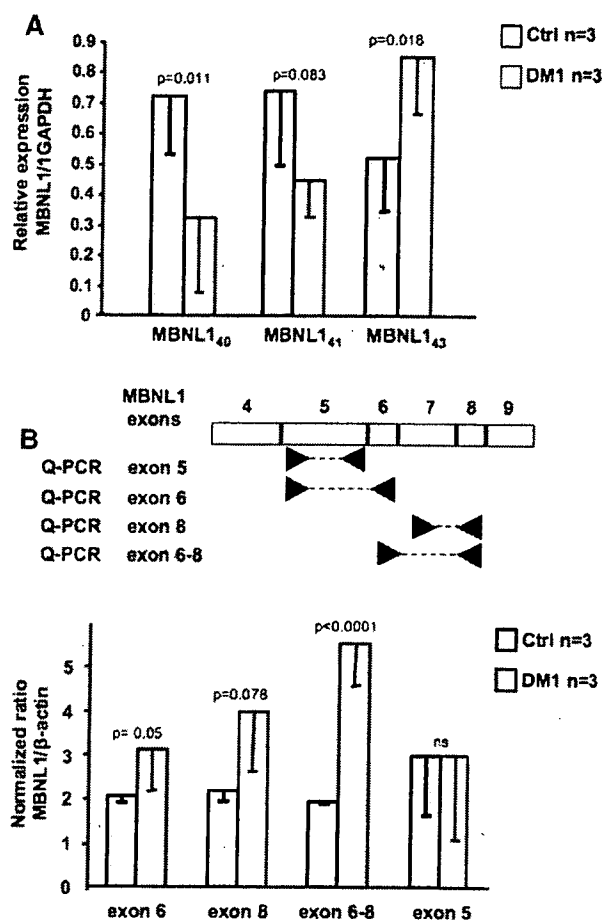


Fig. 2. Semi-quantitative analysis of exon 6 and 8 in control and DM1 tissues. A. Quantification of MBNL1 splice variants by RT-PCR of total RNA from normal and DM1 brain tissues. Primer sets MBNL1_{41/43} and _{40/42} (Table 1) were used to amplify selected sets of MBNL1 isoforms. Each primer set simultaneously amplified two transcripts that could be distinguished by their size, according to the insertion or deletion of exon 6. After polyacrylamide gel electrophoresis, the bands were quantified and the results expressed relative to GAPDH, used as an internal loading standard. The histogram represents the mean \pm standard deviation of measurements obtained from three different brain samples for each patient. B. Quantitative RT-PCR of MBNL1 transcripts. The relative positions of the primers used are indicated above the histogram in the form of arrows. The first primer set, located in the constitutive exon 5, was used to quantify the total level of MBNL1 transcripts. The other primer sets allowed the specific quantification of MBNL1 transcripts containing exon 6, exon 8 or both exons 6 and 8. Actin was used as the internal standard. All results were obtained from at least three independent experiments.

rabbit polyclonal antibody against the N-terminal region of MBNL1 (see Materials and methods; Fig. 1A). As a control for antibody specificity, we used protein extracts from HeLa cells transfected with the MBNL1₄₀ expression vector. A single band at 40 kDa was detected by immunoblotting using our MBNL1 antiserum in these extracts, whereas no immunoreactivity was observed in extracts from non-transfected cells. In extracts from human brain tissue, the antibody revealed a broad band with an apparent molecular mass of 41–43 kDa. No difference in expression levels was seen between control and DM1 brains (Fig. 1A). There did appear to be a slight variation in electrophoretic mobility. However, SDS-PAGE followed by western blotting was insufficient to discriminate between the different MBNL1 isoforms.

MBNL1 splicing is modified and the expression of a fetal isoform increased in the DM1 brain

We analyzed MBNL1 transcript expression in control, DM1 and fetal brain tissues (Fig. 1B), by polyacrylamide gel electrophoresis of the RT-PCR products. As shown in Fig. 1B (lanes 1 and 2) several RT-PCR products were obtained using MBNL1

primers located in exon 3 and 11 (Table 1). After cloning and sequencing, we identified several human MBNL1 isoforms (Fig. 1B, lane 4). The relative distribution of these different MBNL1 brain isoforms is represented in the form of pie charts (Fig. 1C). The major transcripts expressed in the adult control brain are MBNL1₄₀, MBNL1₄₁ and MBNL1₄₃ (Fig. 1B lane 1 and Fig. 1C). In the DM1 brain, MBNL1₄₀ and MBNL1₄₁ isoforms are significantly less abundant whereas MBNL1₄₃ is overexpressed (Fig. 1B, lanes 1–2, and Fig. 1C). Interestingly, we observed that MBNL1₄₃ is also one of the major fetal brain transcripts (Fig. 1B, lane 3, and Fig. 1C). The MBNL1₄₃ transcript corresponded to the MBNL1₄₂ of Kino's classification (Kino et al., 2004), with the addition of exon 8 (Fig. 2D). Such a transcript has also been identified recently by Kanadia et al. in mouse skeletal muscle (Kanadia et al., 2006). MBNL1_{36L} corresponds to MBNL1₃₆, with the inclusion of the exon 6 sequence (Fig. 2C).

Increased inclusion of MBNL1 exon 6 and 8 in DM1 brain

After qualitative analysis of the pattern of MBNL1 transcript expression in control and DM1 brains, we conducted a semi-

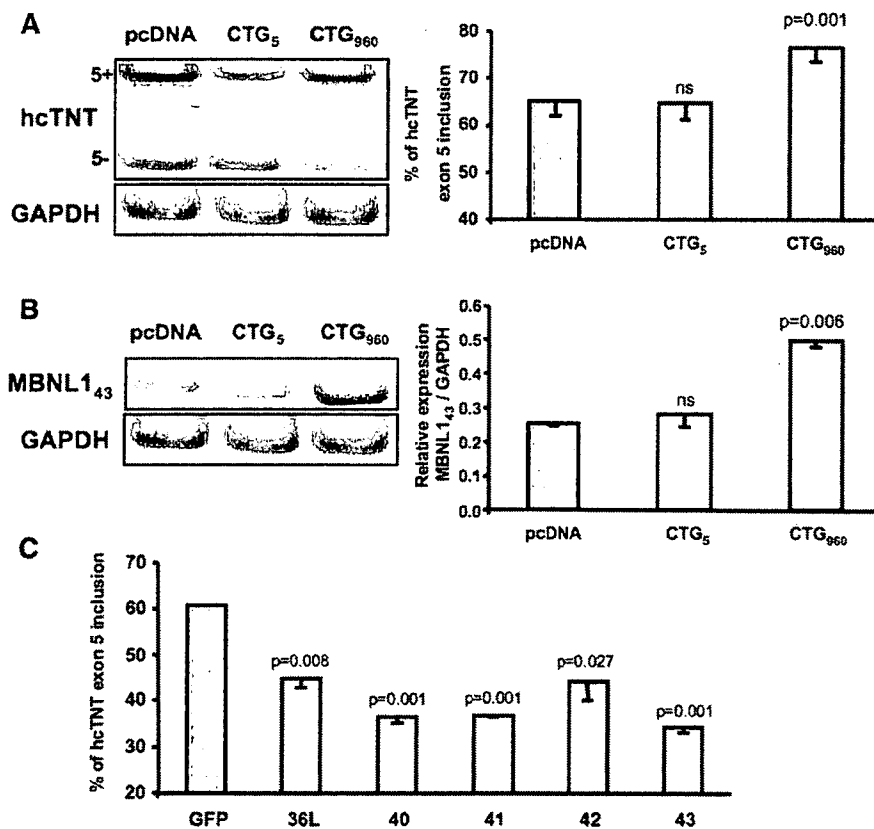


Fig. 3. Effect of long CUG repeats on MBNL1 and hcTNT splicing in HeLa cells. A. Regulation of human hcTNT exon 5 splicing by DMPK 3'UTR CUG repeats. Total RNA was analyzed from HeLa cells co-transfected with the hcTNT minigene and the 3'UTR of human DMPK containing the number of CTG indicated above each lane. The histogram is representative of three individual experiments, and represents the percentage of transcripts that include exon 5. B. Regulation of human MBNL1 by DMPK 3'UTR CUG repeats. Total RNA was analyzed from HeLa cells co-transfected with MBNL1 and the 3'UTR of the human DMPK containing the number of CTG indicated above each line. Results are expressed as the level of MBNL1₄₃ expression relative to GAPDH. Histograms represent the mean and SD of three individual experiments. C. hcTNT exon 5 inclusion is similarly repressed by several MBNL1 isoforms. HeLa cells were co-transfected with hcTNT minigene and MBNL1 isoforms 36L, 40, 41, 42 or 43, and exon 5 splicing analyzed by RT-PCR. The histogram is representative of three individual experiments and the percentage of transcripts that include exon 5 is expressed as the mean±standard deviation of the three experiments.

quantitative analysis, using three pairs of primers designed to selectively amplify MBNL1₄₀, MBNL1₄₁, and MBNL1₄₃ isoforms (Table 1). The significant increase in MBNL1₄₃ ($p=0.018$), and decrease in MBNL1₄₀ ($p=0.011$) expression levels (Fig. 2A) confirmed our previous observations (Fig. 1B, pie charts). A decrease in the expression of MBNL1₄₁ was also observed, but was not found to be statistically significant (Fig. 2A, $p=0.083$).

MBNL1₄₃ contains both exon 6 and exon 8 whereas MBNL1₄₀ lacks these exons, suggesting a possible change in the splicing of exon 6 and 8. These exons were quantified as described above, and compared to exon 5, which is constitutive (Fig. 2B). We observed a significant increase in the inclusion of exon 6 ($p=0.05$) between control and DM1 brains. A 2-fold increase in the inclusion of exon 8 was also observed in the DM brain when compared to healthy controls. While this difference was not found to be statistically significant in the current study, subsequent comparisons with a larger number of brains might prove that exon 8 as well as exon 6 are affected in DM1. At the present time, however, the results suggest that the disrupted splicing of MBNL1 is the result of the increased inclusion of exon 6, or of a combination of exon 6 and 8 ($p<0.0001$). It is worth noting that no difference was observed between control and DM1 brains in the level of MBNL1 exon 5 (Fig. 2B), thus confirming previous data showing that the overall expression of MBNL1 was not altered.

Expanded CUG repeats modify MBNL1 splicing and promote the expression of a fetal transcript

The trans-dominant effect of expanded CUG repeats in DM1 has been directly related to the disrupted splicing of several transcripts, such as hcTNT or IR. Based upon the evidence we found for altered MBNL1 isoform expression in the DM1 brain, we asked whether or not the CUG repeats were also responsible for the modified splicing of MBNL1. HeLa cells were used throughout this study. Unlike the neuroblastoma cell lines tested so far (e.g. SY-5Y, N2a, hNT), we observed an alternative splicing of Tau exon 2/3 in HeLa cells. As a positive control for the trans-dominant effect of expanded CUG repeats, the hcTNT minigene was co-transfected with the 3'UTR of human DMPK, containing 5 or 960 CTG repeats expressed under the control of the CMV immediate early promoter (Fig. 3A). The expression of CUG repeats was assessed by RT-PCR of the 3'UTR of DMPK. We also verified that the number of CTG repeats remained unchanged 48 h after transfection (data not shown). A 15% increase in the inclusion of hcTNT exon 5 was observed with CTG₉₆₀, but not with the CTG₅ minigene. HeLa cells were then transfected with CTG₉₆₀ alone (Fig. 3B) and MBNL1₄₃ transcript expression was examined by RT-PCR with the primers MBNL1_{41/43} (Table 1). In the latter cells, we observed a 1.5 fold increase in the expression of MBNL1₄₃ (Fig. 3B), demonstrating that endogenous MBNL1₄₃

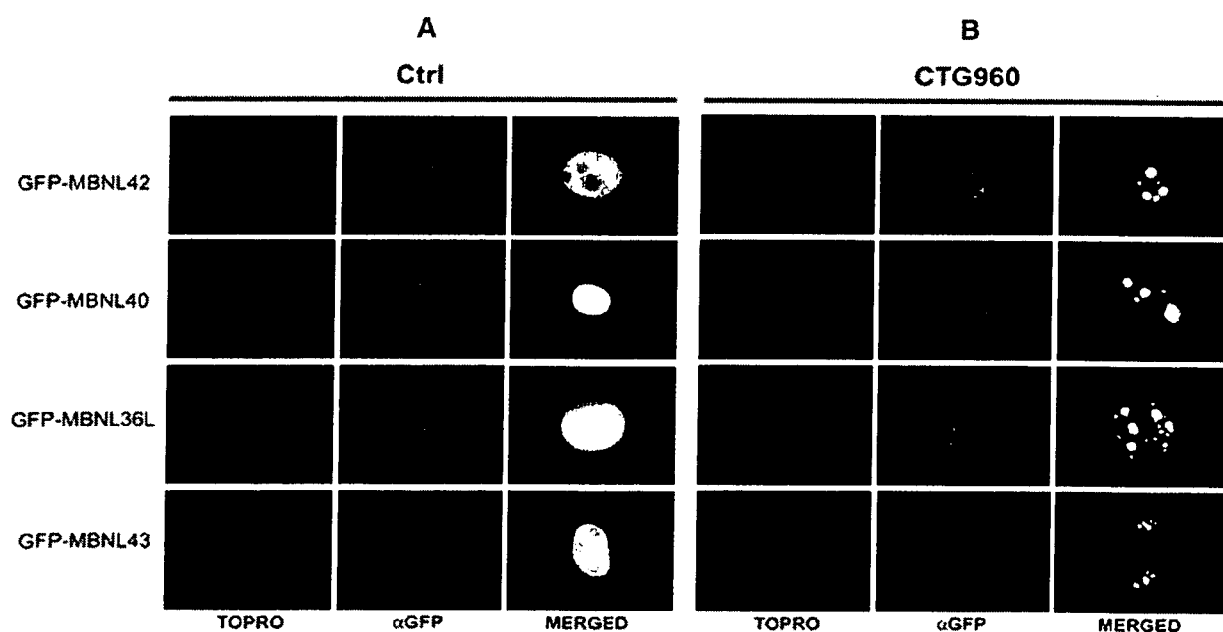


Fig. 4. Intracellular localization of MBNL1 isoforms in the presence or absence of long CUG repeats. HeLa cells were transfected with GFP-MBNL₄₂, GFP-MBNL₄₀, GFP-MBNL_{36L} and GFP-MBNL₄₃ alone (panel A) or together with pcDNA-CTG₉₆₀ (panel B). In each panel, the first column shows the cell nucleus stained with TOPRO. The second column shows the distribution of the GFP-tagged protein, revealed by an anti-GFP antibody. The last column corresponds to the merged images obtained from TOPRO and anti-GFP staining, showing the localization of MBNL1 isoforms in nuclear RNA foci when co-expressed with long CUG repeats. GFP fluorescence completely overlapped with that observed with an anti-GFP antibody. Cells with a moderate level of expression were selected to illustrate the localization of MBNL1 isoforms, as high cellular expression levels resulted in partial cytosolic localization (data not shown), possibly due to the titration of nuclear import factors.

is overexpressed as a result of the trans-dominant effect of long CUG repeats.

Trans-repression of hcTNT exon 5 inclusion by MBNL1 isoforms, and cellular localization

The modified pattern of MBNL1 isoform expression in DM1 led us to consider whether the MBNL1 isoforms had similar or opposing splicing functions. We examined the trans-regulatory function of these isoforms on hcTNT exon 5 splicing. HeLa cells were co-transfected with the hcTNT minigene and GFP-MBNL1 isoforms (Fig. 3C) or V5-MBNL1 isoforms (data not shown). As a positive control for these constructs, we confirmed the decreased inclusion of hcTNT exon 5 induced by MBNL1₄₀ (Fig. 3C). We observed that MBNL1₄₃ and MBNL1_{36L} decreased exon 5 inclusion, in a manner similar to MBNL1₄₀, MBNL1₄₁ and MBNL1₄₂.

We next investigated the possibility that another function, such as cell localization, or the propensity of CUG repeats to recruit MBNL1 isoforms in nuclear foci, was also modified. All GFP-MBNL1 isoforms showed diffuse nuclear staining and were excluded from the nucleoli (Fig. 4A, control panel). We also tested our constructs in COS cells since Lin et al. recently

showed that MBNL1 isoform lacking the sequence encoded by exon 7 had a nucleocytoplasmic localization (Lin et al., 2006). MBNL1₄₀ isoform which lacks the sequence encoded by exon 7 is localized in the cytoplasm and nucleus of COS whereas the other MBNL1_{43, 42} and _{36L} isoforms, having the exon 7 encoded sequence, were preferentially observed in the nucleus (supplementary Fig. 1). Control HeLa cells transfected with GFP alone or co-transfected with pcDNA-CTG₅ also revealed the diffuse cellular distribution of GFP (data not shown). In contrast, in HeLa cells co-transfected with GFP-MBNL1 isoforms in addition to pcDNA-CTG₉₆₀, GFP-MBNL1 staining was concentrated at large foci in the nucleus (Fig. 4B), even though the level of protein expression of the GFP-MBNL1 isoforms was found to be invariant between the different experimental groups (western blotting; data not shown).

Loss of MBNL1 expression is responsible for the Tau splicing pattern observed in DM1

We have previously described the preferential expression and aggregation of fetal Tau protein in degenerating neurons in the brain of DM1 patients (Sergeant et al., 2001). The increased expression of fetal Tau isoforms results from the reduced

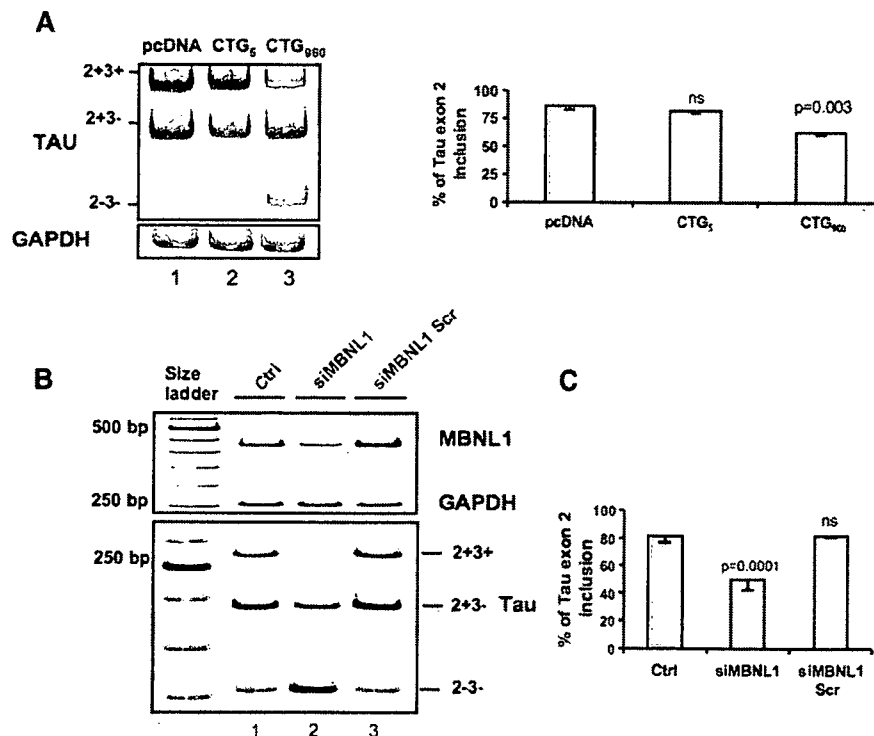


Fig. 5. Effect of long CUG repeats and loss of MBNL1 expression levels on the inclusion of Tau exon 2. A. Reduced Tau exon 2 inclusion in the presence of long CUG repeats. The proportion of Tau transcripts including exons 2 and 3 was analyzed in HeLa cells transfected with the 3'UTR of the human DMPK gene containing 5 or 960 CUG repeats, by RT-PCR. The bands were quantified and the results expressed as the percentage of transcripts including exon 2. GAPDH was used as the internal loading standard. B. Loss of MBNL1 expression reduces Tau exon 2 inclusion. An RNA duplex (siMBNL1a) against human MBNL1 (siMBNL1, lane 2) or a scrambled duplex (siMBNL1 Scr, lane 3) were used for transfection into HeLa cells. In controls (Ctrl, lane 1), the transfection agent alone was used. Note the reduced expression of MBNL1 in presence of the siMBNL1 and not of the siMBNL1 Scr (upper panel B, lane 3). RT-PCR using primers surrounding exon 2/3 were used to analyze the pattern of Tau splicing (lower panel B). Tau isoforms are indicated on the right. C. Histogram of the percentage of transcripts including exon 2. The mean and standard deviation were calculated from three individual experiments. Histograms are representative of the mean±standard deviation of three independent experiments in A and C.

inclusion of Tau exon 2/3. As shown here, MBNL1 isoform expression is also modified in the DM1 brain, raising the question of whether it is the CUG repeats or the MBNL1 fetal isoforms that are responsible for the modified splicing of Tau. To answer this question, we first analyzed the consequences of the ectopic expression of CUG repeats on the splicing of Tau in HeLa cells (Fig. 5A). Under control conditions, endogenous Tau exon 2/3 were alternately spliced in HeLa cells (Fig. 5A, lane 1). As Tau exon 3 never appears independently of exon 2,

three PCR products are observed, corresponding to 2+3+, 2+3– and 2–3– transcripts. However, the expression of CUG₉₆₀ (Fig. 5A, lane 3), but not CUG₅ (Fig. 5A, lane 2) by HeLa cells induced a significant 25% decrease in exon 2 inclusion, demonstrating that the reduced inclusion of Tau exon 2/3 resulted from the trans-dominant effect of CUG repeats (Fig. 5A).

We next examined whether or not the loss of MBNL1 expression induced the preferential exclusion of Tau exon 2/3. Two siRNAs against human MBNL1 and two scrambled RNA

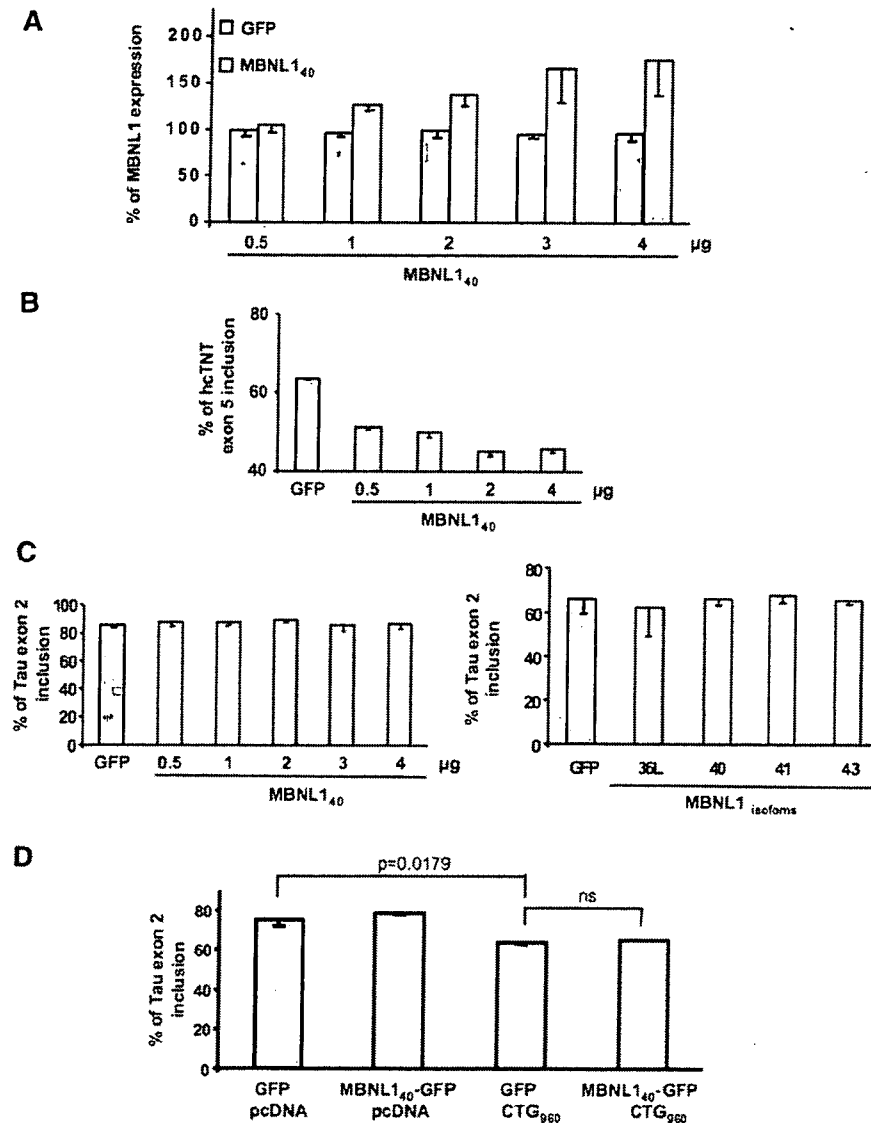


Fig. 6. Effect of MBNL1 isoforms ectopic expression on hcTNT exon 5 and Tau exon 2 splicing. A. Dose expression of MBNL1₄₀. A growing quantity of MBNL1₄₀ plasmid (in microgram) was transfected. The amount of MBNL1₄₀ mRNA expressed is represented as the percentage to the control expression. A plasmid containing the GFP alone was used as a control of ectopic expression (grey bars). Note that GFP does not affect endogenous MBNL1 expression. B. Dose response exclusion of hcTNT exon 5 in presence of growing amount of MBNL1. A growing quantity of MBNL1₄₀ plasmid was co-transfected with hcTNT minigene. Exon 5 splicing was analyzed by RT-PCR and results are represented as the percentage of exon 5 inclusion. C. Tau exon 2 inclusion is not modulated by MBNL1₄₀ expression nor by MBNL1 isoforms. Endogenous splicing of Tau exon 2 was analyzed after transfection of HeLa cells with an increased amount of MBNL1₄₀ plasmid. Two microgram of MBNL1 isoforms (36L, 40, 41 and 42) were transfected in HeLa and endogenous splicing of Tau exon 2 was analyzed by RT-PCR. The plasmid expressing GFP was used as a control. D. Tau exon 2 inclusion is unmodified by MBNL1₄₀ ectopic expression in presence or absence of long CUG repeats. The level of Tau exons 2 and 3 was analyzed by RT-PCR in HeLa cells transfected with the human DMPK 3'UTR containing 960 CTG repeats, in the presence or absence of cotransfection with MBNL1₄₀. The bands were quantified and results were expressed as percentage of transcripts that included exon 2. Histograms are representative of the mean \pm standard deviation of three independent experiments in A to D.

duplexes were used (only one is shown). MBNL1 expression level (Fig. 5B, upper panel) and the splicing pattern of endogenous Tau (Fig. 5B, lower panel) were determined by RT-PCR. The MBNL1-specific RNA duplex reduced the expression level of MBNL1 mRNA (Fig. 5B, lane 2). We also observed a reduction in the expression of Tau isoforms including exon 2/3, and the increased expression of Tau isoforms lacking these exons, with this RNA duplex (Fig. 5B, lane 2), whereas the scrambled siRNA had no obvious effect on Tau splicing (Fig. 5B, lane 3). Quantitative analysis showed that the MBNL1 duplex induced a significant decrease ($p=0.0001$) in the level of exon 2 inclusion, when compared to either non-RNA controls or a scrambled RNA duplex (Fig. 5C). This decrease was not induced by an RNA duplex against β -actin (not shown). To further ascertain that the reduced inclusion of Tau exon 2 is due to the loss of MBNL1 expression, we performed a dose response effect of the loss of MBNL1 expression on Tau exon 2 splicing (supplementary Fig. 2). HeLa cells were transfected with a growing concentration of siMBNL1 duplex and endogenous MBNL1 expression and Tau exon 2 inclusion were assessed by RT-PCR. From 1 to 100 nM of duplex there was a linear decrease of MBNL1 mRNA expression (supplementary Fig. 2). In parallel, we observed a decreased percentage of Tau exon 2 inclusion from 5 to 40%, demonstrating that the reduced Tau exon 2 inclusion is in close relationship with the loss of MBNL1 expression and reproduces the splicing pattern of Tau observed in the DM1 brain.

Loss of MBNL1 function mimics DM1 phenotype and supposes that MBNL1 regulates Tau exon 2 splicing. To further address this question, a growing quantity of MBNL1₄₀ was expressed in HeLa cells. Fig. 6A shows that we could reach a 50% increase of MBNL1 expression by increasing the quantity of plasmid transfected. This incremental expression of MBNL1 induced a progressive decreased of hcTNT exon inclusion (Fig. 6B). The splicing of Tau exon 2 was insensitive to MBNL1₄₀ expression, whatever the level of expression (Fig. 6B). Moreover, none of the isoforms tested modified the splicing of Tau exon 2 (Fig. 6C) showing that splicing of Tau exon 2 is not affected by a specific isoform, as the fetal MBNL1₄₃ isoform. In order to investigate a recent claim that the overexpression of MBNL1 reverses the trans-dominant effect of CUG repeats by correcting for MBNL1 functional deficits (Kania et al., 2006), we co-transfected HeLa cells with CUG₉₆₀ and MBNL1₄₀, and analyzed the endogenous splicing pattern of Tau exon 2/3. We observed that the overexpression of MBNL1₄₀ did not restore normal endogenous splicing of Tau to control levels (Fig. 6D).

Discussion

MBNL1 expression is central to DM1 pathophysiology, and the loss of MBNL1 function leads to the altered splicing of several transcripts in the muscle tissue of DM1 patients. Several transcripts, including those of Tau, are modified in the brain tissue of DM1 patients. However, our knowledge of MBNL1 in the human brain is limited to the fact that it is expressed at low levels (Miller et al., 2000) and is localized in the nuclear RNA

foci of neocortical neurons in DM1 patients (Jiang et al., 2004). We show here that in adult human brain tissue, long MBNL1 protein isoforms, especially MBNL1₄₁ and/or MBNL1₄₂, are preferentially expressed. We characterize several transcripts and show a modification in the splicing pattern of MBNL1 in the brain tissue of DM1 patients. In addition, we show that this splicing pattern is induced by long CUG repeats, and therefore is a result of the trans-dominant effect of RNA bearing the DM1 mutation. However, this observation further adds to the complexity of the pathophysiology of DM1. For instance, the trans-dominant effect of the CUG repeats could result either from the loss of MBNL1, or from the re-expression of MBNL1 isoforms that are normally expressed early in development.

The altered splicing of MBNL1 has also been reported recently in DM1 and DM2 muscle tissue by Lin et al. (Lin et al., 2006). In the DM1 brain, in addition to the altered splicing of exon 6 seen in muscle (called exon 7 in Lin et al. (2006)), we demonstrate the preferential inclusion of MBNL1 exons 6 and 8. Moreover, we show that an increased number of MBNL1 isoforms are expressed in the DM1 brain, and that these isoforms include both exons 6 and 8, with or without exon 4. A consequence of the presence of CUG repeats is the expression of a combination of adult and fetal protein isoforms due to the trans-dominant effect. The altered splicing of MBNL1 in the human brain is consistent with the increased expression of a fetal isoform of MBNL1. MBNL1 isoforms including exons 6 and 8 are preferentially found in the fetal human brain, whereas MBNL1 isoforms lacking exon 6 are expressed in adult tissue. For instance, the predominant adult isoform is MBNL1₄₁.

The identification of additional MBNL1 isoforms raises the question of their cellular localization and trans-regulatory splicing properties. Are these isoforms neutralized by expanded CUG repeats? In HeLa cells, all MBNL1 isoforms in our study showed diffuse nuclear localization. However, it has previously been shown that the cellular localization of MBNL1 in COS cells is modulated by the presence or absence of exon 6 but not that of exon 8 (Lin et al., 2006). In both studies, MBNL1 isoforms were fused to GFP. However, in the present work, GFP has been placed at the N-terminus of the MBNL1 isoforms, whereas Lin et al. (Lin et al., 2006) used MBNL1 isoforms with GFP fused to the C-terminus. By transfecting GFP–MBNL1 fused constructs in COS cells, we confirm that exon 6 contributes to the nuclear localization of MBNL1. GFP position does not interfere with MBNL1 cell localization. In contrast, we show here that MBNL1 cell localization is cell-type specific as the nuclear localization of MBNL1 isoforms was not affected in HeLa. Therefore, both MBNL1 primary sequence and the cell system used should be considered to investigate MBNL1 nucleocytoplasmic shuttling. Although a difference in trans-regulatory function could not be proven using the hcTNT exon 5 splicing assay, we cannot exclude the hypothesis that MBNL1 isoforms have different trans-regulatory properties toward other targets. Therefore, the altered pattern of MBNL1 expression may contribute further to the multisystemic expression of DM1 than the mere loss of its function due to titration by CUG repeats.

In vitro experiments have suggested that the spacer between the two Zinc-finger domains is essential for the binding of MBNL1 to

CUG repeats (Kino et al., 2004). Interestingly, in our study, all the MBNL1 isoforms examined, including MBNL1_{36L}, which lacks the exon 4-encoded spacer between the two Zinc-finger domains, are localized to nuclear foci in the presence of long CUG repeats. Our results suggest therefore that *in vivo*, all MBNL1 isoforms, even those lacking the exon 4-encoded sequence, associate with double-stranded RNA.

In the present study, we show that the modified splicing of Tau pre-mRNAs is induced by CUG repeats, as previously observed in the DM1 brain (Sergeant et al., 2001). However, this effect only occurs with very long expansions (CUG₉₆₀) and not with moderate ones (CUG₂₀₀) (not shown). Our results are consistent with those previously shown for hCNT exon 5, where the level of inclusion of exon 5 appears to follow CUG repeat length (Savkur et al., 2001; Philips et al., 1998). This suggests that, depending on the number of repeats, different sets of transcripts might be affected. In particular, in tissues where somatic mosaicism is important and expansion increases with age, we can hypothesize that some transcripts will only be altered at more advanced ages, leading to the late expression of specific clinical signs. This is the case in the DM1 brain, where we have previously demonstrated the importance of somatic instability; the number of CUG repeats is heterogeneous between brain regions, and very large expansions of over 4000 CUG repeats are observed (Sergeant et al., 2001).

The mechanism by which CUG repeat RNAs exert their effect is thought to involve the interaction of the CUG repeats with proteins, especially the MBNL1 protein family. One possibility is that MBNL1 sequestration in foci leads to a loss of function of MBNL1 and the disrupted splicing of several target pre-mRNAs. In the present study, we show that the modified splicing of Tau pre-mRNA is induced by long CUG repeats (see also (Sergeant et al., 2001)) or by the loss of MBNL1 expression, as observed in the DM1 brain. Moreover, the reduced Tau exon 2 inclusion is dose-dependant since we showed that a growing loss of MBNL1 expression results in a growing decrease of Tau exon 2 inclusion. Conversely, incremental expression of MBNL1 or the expression of several MBNL1 isoforms does not modify the splicing pattern of Tau, whereas these isoforms and the increment expression of MBNL1 have similar trans-regulatory properties towards hCNT exon 5 splicing. Moreover, the reduced inclusion of Tau exon 2/3 induced by the presence of long CUG repeats is not reversed by the overexpression of MBNL1₄₀, supporting the hypothesis that the trans-dominant effect of CUG repeats toward Tau splicing is mediated by a complete loss of MBNL1 function. A recent study demonstrated coordinated physical and functional interactions between hnRNPH, CUG-BP1 and MBNL1 in the regulation of IR splicing (Paul et al., 2006). Although the mechanism of Tau exon 2/3 splicing is currently unclear, MBNL1 could be one element of a protein complex that regulates spliceosome activity. We have recently shown that ETR-3, a member of the CELF family of splicing factors, acts as a strong repressor of Tau exon 2/3 inclusion (Leroy et al., 2006b). Other proteins potentially involved in MBNL1-mediated Tau splicing could be CUG-BP1 or hnRNPH.

A growing body of evidence, including our results, supports the hypothesis that the DM1 phenotype is in part related to a

change in the splicing program due to the trans-dominant effect of CUG repeats, resulting in the mixed expression of adult and fetal isoforms of various proteins, including Tau and MBNL1. Moreover, the reactivation of fetal-like splicing machinery in adult neurons is also a concept that should be explored in the case of Tauopathies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.expneurol.2007.08.047.

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Quantitative Analysis of CUG-BP1 Binding to RNA Repeats

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CUG-binding protein 1 (CUG-BP1) is a member of the CUG-BP1 and ETR-3-like factors (CELF) family of RNA-binding proteins, and is involved in myotonic dystrophy type 1 (DM1). Several mRNA targets of CUG-BP1 have been identified, including the insulin receptor, muscle chloride channel, and cardiac troponin T. On the other hand, CUG-BP1 has only a weak affinity for CUG repeats. We conducted quantitative-binding assays to assess CUG-BP1 affinities for several repeat RNAs by surface plasmon resonance (SPR). Although we detected interactions between CUG-BP1 and CUG repeats, other UG-rich sequences actually showed stronger interactions. Binding constants of CUG-BP1 for RNAs indicated that the affinity for UG repeats was far stronger than for CUG repeats. We also found that N-terminal deletion mutant of CUG-BP1 has UG repeat-binding activity in a yeast three-hybrid system, although C-terminal deletion mutant does not. Our data indicates that CUG-BP1 specifically recognized UG repeats, probably through cooperative binding of RNA recognition motifs at both ends of the protein. This is the first report of a binding constant for CUG-BP1 calculated *in vitro*.

Key words: binding constant, CUG-BP1, myotonic dystrophy, surface plasmon resonance, triplet-repeat.

Abbreviations: 3-AT, 3-amino triazole; CELF, CUG-BP and ETR3-like factor; DM, myotonic dystrophy; DMPK, DM protein kinase; RRM, RNA recognition motif.

CUG-BP and ETR3-like factor (CELF) proteins, also known as Bruno-like (BRUNOL) proteins, are a family of highly conserved RNA-binding proteins (1, 2). All mammalian CELF proteins contain three RNA recognition motifs (RRMs; also referred to as RNP domains or consensus RNA-binding domains) and have a similar organization: two closely spaced N-terminal RRM, a divergent hinge region of 60–90 residues, and a C-terminal RRM (1). RRM-containing proteins represent the largest family of RNA-binding proteins and perform various functions in post-transcriptional gene regulation. CUG-binding protein 1 (CUG-BP1) was the first discovered member of the CELF proteins and acts as a regulator of alternative splicing (1, 3–5), translation (6, 7) and deadenylation (8, 9).

Several reports have shown the involvement of this protein in myotonic dystrophy (dystrophia myotonica or DM) type 1 (DM1) (3–6, 10 and 11 for review). As indicated by its name, the binding of CUG-BP1 to expanded CUG repeats was first demonstrated in the onset of DM (12, 13). Indeed, CUG-BP1 was first identified as a protein binding to a (CUG)₈ probe in a gel retardation assay (12, 13). However, whether this protein does actually bind expanded repeats is controversial. For example, it does not specifically co-localize with nuclear RNA foci, formed by expanded repeats

in DM1 cells (14). Moreover, contrary to its name, CUG-BP1 actually appears to specifically bind to UG motifs, rather than to CUG repeats, in a yeast three-hybrid system (15, 16).

Muscleblind-like proteins, another group of RNA-binding proteins, have recently emerged as more plausible proteins for the sequestration model (14, 16). Nevertheless, CUG-BP1 is still an important factor in DM1, because reports have shown up-regulation of CUG-BP1 protein levels in DM1 cells by still unknown mechanisms, and its elevated activity is thought to cause abnormalities in DM1 (4).

CUG-BP1 may contribute to the aberrant splicing of multiple genes, a hallmark of DM1. Alternative splicing of the insulin receptor (IR), muscle chloride channel (CLCN1), cardiac troponin T (cTNT) and other genes is regulated by CUG-BP1, and the splicing patterns of these genes are altered in DM1 patients (3–5, 17).

In addition to splicing defects, it has also been suggested that CUG-BP1 is involved in the altered translation of p21 and MEF2A in DM1 cells, and leads to defects in myogenic progression (6). Furthermore, over-expression of CUG-BP1 in mice recapitulated some abnormalities similar to those observed in DM1 patients, such as aberrant splicing and histological impairment of the muscle (10). It is also described that mice over-expressing normal DMPK 3'-untranslated region had increased levels of CUG-BP1 in skeletal muscle, as seen in individuals with DM1 (18). Thus, understanding CUG-BP function and its target genes is important for an understanding of DM pathogenesis.

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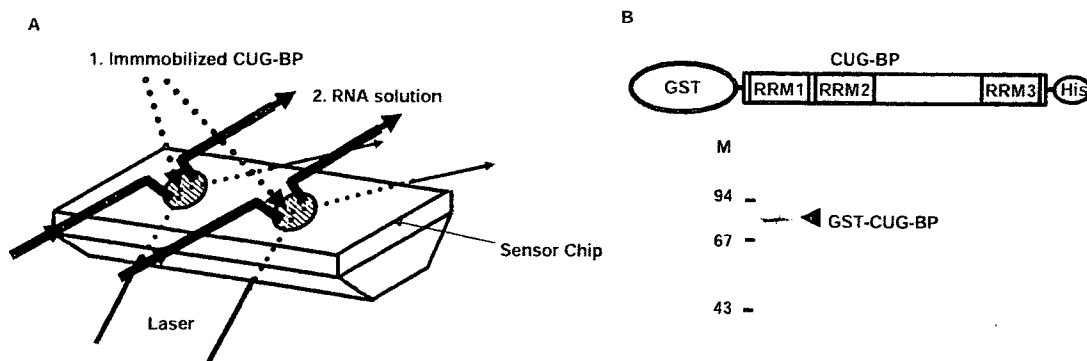


Fig. 1. Design of SPR experiment and purified protein used in this study. A: Schematic illustration of the SPR analysis. First, purified CUG-BP1 was immobilized on the gold layer of the sensor chip. RNA solution flowed through the sensor chip surface. CUG-BP1-RNA interactions were detected as the difference in resonance angle of the surface plasmon, elicited by a laser. B: Recombinant CUG-BP1. (Top) Structure of

recombinant CUG-BP1. CUG-BP1 was fused with glutathione S-transferase at the N-terminus and a 6 \times His-tag at the C-terminus. (Bottom) Purified GST-CUG-BP1 used in this study. The purified fraction was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Molecular weight is indicated by M.

Despite its importance, the RNA-binding specificity of CUG-BP1 remains to be determined, primarily because of the lack of biochemical analysis directly addressing the binding constants. In this article, we conducted an *in vitro* binding analysis between CUG-BP1 and CUG and other repetitive nucleotides to examine whether CUG-BP1 could interact with CUG repeats and to find specific target sequences of CUG-BP1.

MATERIALS AND METHODS

Synthesis of DNAs Containing Repetitive Sequences—DNA fragments of repetitive sequences such as (CUG)₁₄₀, (UG)₂₄, (UG)₄₁ and (UAUG)₁₄, were synthesized by a non-template polymerase chain reaction (PCR) method with Pfu Turbo polymerase (Stratagene) through 5–25 cycles of amplification (96°C for 1 min, 60°C for 30 s and 72°C for 90 s) after 10 rounds of 96°C for 1 min, 60°C for 30 s and 72°C for 1 min (19). DNAs for other short repeat fragments were purchased from Proligo, Japan.

In vitro Transcription—DNA fragments with repetitive sequences were ligated into the *Hinc*II site of a pBluescriptII SK(+)-derived vector containing an *Ap*I–*Eco*52I site deletion and a *Hinc*II site insertion. Using DNA fragments from these constructs as templates, RNAs were transcribed with the MEGAscript T7 or T3 kits (Ambion) in a 20 mL reaction. The quantity of the synthesized mRNAs was checked by the absorbance at 260 and 280 nm. Their quality was checked by denaturing agarose gel electrophoresis.

SPR Biosensor Analysis—In the SPR biosensor analysis, using SPR-MACS (Moritex), the sensor chip was coated with 4,4'-dithiodibutyric acid (DDA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), according to the manufacturer's protocol. CUG-BP1 was immobilized on the sensor chip by amino-coupling, and RNAs were passed over the protein surface (Fig. 1A). The binding reaction was performed in a running buffer of 10 mM HEPES

(pH 8.0), 150 mM NaCl, 1 mM dithiothreitol (DTT), at a flow rate of 10 ml/min. Association and dissociation were measured in arbitrary units and displayed on a graph (sensorgram). The RNAs binding to the protein surface were released with 9 mM NaOH as a regeneration solution (Fig. 2). After this treatment, the next binding experiment was performed. Binding constants were calculated based on the sensorgram using Method 2 software (Moritex).

Bacterial Expression and Purification of Recombinant Double-Tagged CUG-BP1 and Mutants—An expression vector, human CUG-BP1 in pET-GX, has been previously described (16). BL21(DE3) competent cells (Stratagene) were transformed with this construct and used for protein expression. A single colony was grown overnight at 37°C in LB medium with 50 mg/ml ampicillin, diluted 1:20 in fresh medium, and then grown at 37°C until A_{600} reached 0.25–0.30. IPTG was then added to the culture to a final concentration of 0.1 mM and incubated for a further hour at 37°C. The cells were harvested as a pellet and were resuspended in 30 ml PBS containing 1/500 volume of proteinase inhibitor mixture (Wako), 1 mM PMSF and 1 mM DTT, and were lysed in a French press (Ohtake Works, Co.). The total cell lysate was centrifuged (15 min, 15,000 g). The supernatant was subjected to affinity purification using GSTrap FF affinity columns (Amersham Pharmacia Biotech) with an FPLC system and 300 ml (bed volume) of BD Talon affinity resin (Clontech), according to the manufacturer's protocol. The protein sample was dialysed twice against HBS buffer (10 mM HEPES-NaOH pH 8.0, 150 mM NaCl, 1 mM DTT). The quantity and purity were checked on SDS-polyacrylamide electrophoresis gels, stained with Coomassie brilliant blue (CBB; Fig. 1B).

Yeast Three-Hybrid System—To make CUG-BP1 RRM mutants, conserved residues were changed by site-directed mutagenesis, K59Q and F63A in Mut1; R148Q and F152A in Mut2 and K438Q and F442A in Mut3.

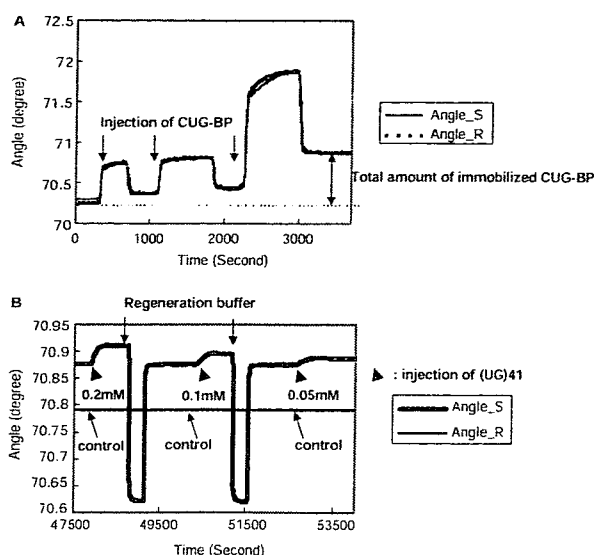


Fig. 2. SPR measurement. A: Immobilization of CUG-BP1. The signals of the resonance angle were monitored during the injection and immobilization of purified CUG-BP1 protein on the gold layer. Angle S and Angle R indicate the resonance angles at the measurement spots for the sample and the reference control, respectively. Arrows indicate the time when CUG-BP1 solution was injected. Multiple injections enhanced the amount of immobilized CUG-BP1. B: An example of SPR measurement of CUG-BP1 and (UG)₄₁ repeat. Arrows show the time point when (UG)₄₁ RNA solution at the concentration indicated was injected. Each time after measurement, regeneration buffer was injected to wash off RNAs bound to CUG-BP1. Injection of a higher concentration of (UG)₄₁ resulted in more rapid and larger changes in the signals.

Mut(1+2), Mut(2+3) and Mut(1+3) contained two combined RRM mutations. To make deletion mutants, fragments of CUG-BP1 (Fig. 4), were amplified by PCR. • RRM1, • RRM2, • RRM3 and • linker constructs have been described previously (15). In the HIS3 assay, yeast strain L40-coat was transformed with pGAD and pIII/MS2-2 vectors encoding CUG-BP1 mutants and RNAs, respectively, and selected on plates lacking leucine and uracil. Yeast transformants were picked and spotted onto selection plates lacking leucine, uracil and histidine, with or without 0.5, 1, 2.5, 5 or 10 mM 3-amino triazole (3-AT) (16). The plates were incubated at 30°C for about 1 week and the viability of the yeast transformants was analysed. We classified the binding activity as (++++), (++++), (+++), (++) and (+) when yeast growth was observed on the plates containing 10, 5, 2.5, 1 and 0.5 mM 3-AT, respectively; no growth of yeast transformants with empty vectors was observed on 0.5 mM 3-AT plates.

RESULTS

SPR Analysis—We used a recombinant CUG-BP1 protein, fused with glutathione S-transferase (GST) at the N-terminus and a histidine tag at the C-terminus of the protein (Fig. 1B). For measurements, CUG-BP1 was

immobilized on the sensor chip and then RNA solution was passed over the chip (Fig. 1A). By detecting changes in the sensorgram, we could confirm the immobilization of CUG-BP1 (Fig. 2A) and the binding of RNAs to CUG-BP1 (an example is shown in Fig. 2B).

Comparative Analysis of CUG-BP1's RNA-Binding in an SPR Assay—First, we determined whether CUG-BP1 could bind to the (CUG)₈ or (CUG)₁₄₀ repeats. We detected weak binding of CUG-BP1 to (CUG)₈ and (CUG)₁₄₀ repeats, with K_d values of 27.2 mM and 6 mM, respectively, but detected no binding of CUG-BP1 to a (CAG)₁₀ repeat (Fig. 3, Table 1).

Next, we compared UG repeat sequences to CUG repeats, because the former interacted with CUG-BP1 in previous yeast three-hybrid experiments (15,16). We detected stronger binding of CUG-BP1 to UG repeats than to CUG repeats (Fig. 3, Table 1). Thus, CUG-BP1 actually bound tighter to a UG motif than to a CUG motif.

UG Dinucleotide is a Preferred Binding Motif of CUG-BP1—We next asked which was more important for the binding of CUG-BP1, the content of 'U' and 'G' nucleotides in the RNA sequence or that of 'UG' dinucleotides. Although (UG)₁₅ and (UUGG)₇ contain 15 Us and 15 Gs (Table 2), the binding of CUG-BP1 to (UUGG)₇ was weaker than to (UG)₁₅. Remarkably, another RNA ((UUUGG)₅), which has the same number of Us and Gs as (UG)₁₅ and (UUGG)₇, showed no detectable binding to CUG-BP1. These results suggest that the content of the UG dinucleotide motif, but not the number of U and G nucleotides, is important for recognition by CUG-BP1.

CUG-BP1 can bind to an RNA sequence, named embryonic deadenylation element (EDEN), which is composed of repetitive UAUG motifs (20). Thus, UA may be another recognition motif for CUG-BP1. To test this, we examined (UAUG)₇ as a probe. Compared to (UG)₁₅, (UAUG)₇ showed decreased binding to CUG-BP1, implying a weaker affinity for the UA motif than for a UG motif. Consistently, one substitution of U to A in one of the middle UG motifs in (UG)₁₅ (UG15mut, Table 2) doubled the value of K_d . Thus, UA is not as preferred a binding motif for CUG-BP1 as UG. Furthermore, we could detect no binding to a pure UA repeat, (UA)₁₅. This may reflect intramolecular or intermolecular base-pairing between UA repeats, which may affect the binding of CUG-BP1.

Finally, we examined the binding of CUG-BP1 to UGG (GUG/GGU) and UUG (UGU/GUU) motifs, because both GUG and UGU motifs can be found in a UG dinucleotide repeat. Also, repeats of both UGG/GUG/GGU and UUG/UGU/GUU motifs contain UG motifs. In both (UGG)₁₀ and (UUG)₁₀, the number of non-overlapping UG motifs was smaller than that of (UG)₁₅, while the number of GUG and UGU motifs, respectively, was larger than in (UG)₁₅. This comparison enabled the determination of whether the GUG or UGU motifs were more preferred than UG. Both (UGG)₁₀ and (UUG)₁₀ showed lower affinities than (UG)₁₅ (Table 2). From these results, we conclude that CUG-BP1 recognizes a UG (or GU) dinucleotide repeat as a binding motif, rather than other UG-rich repeats.