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Molecular mechanisms responsible for aberrant splicing of SERCA1 in myotonic dystrophy type 1

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Myotonic dystrophy type 1 (DM1) is an autosomal dominant neuromuscular disorder associated with an expansion of CTG trinucleotide repeats in the 3′-untranslated region of the *myotonic dystrophy protein kinase* (*DMPK*) gene. The RNA gain-of-function hypothesis proposes that mutant DMPK mRNA alters the function and localization of alternative splicing regulators, which are critical for normal RNA processing. Previously, we found alternative splicing variants of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1), which excluded exon 22, in skeletal muscle of DM1 patients. In the present study, we analyzed the molecular mechanisms responsible for the splicing dysregulation of SERCA1. Five 'YGCU(U/G)Y' motifs that could potentially serve as Muscleblind-like 1, (MBNL1)-binding motifs, are included downstream from the SERCA1 exon 22. Exon trapping experiments showed that MBNL1 acts on the 'YGCU(U/G)Y' motif, and positively regulates exon 22 splicing. Of the five MBNL1 motifs in intron 22, the second and third sites were important for regulation of exon 22 splicing, but the other three binding sites were not required. Overexpression of the CUG repeat expansion of DMPK mRNA resulted in exclusion of exon 22 of SERCA1. These results suggest that sequestration of MBNL1 into the CUG repeat expansion of DMPK mRNA could cause the exclusion of SERCA1 exon 22, and the expression of this aberrant splicing form of SERCA1 could affect the regulation of Ca^{2+} concentration of sarcoplasmic reticulum in DM patients.

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is the most common form of adult muscular dystrophy. It is inherited as an autosomal dominant trait and is caused by an unstable expansion of CTG trinucleotide repeats in the 3′-untranslated region (3′-UTR) of the *myotonic dystrophy protein kinase* (*DMPK*) gene (1–3). The normal *DMPK* gene contains 5–37 copies of the CTG repeat, whereas in DM1 patients the repeat is expanded in the range of 50 to several thousands. The clinical expression of DM1 includes myotonia, progressive muscle weakness, cataracts, insulin resistance and cardiac conduction defects. Repeat expansion shows a positive correlation with the severity of the disease and an inverse correlation with age at onset.

The RNA gain-of-function hypothesis proposes that mutant DMPK mRNA alters the function and localization of alternative splicing regulators, which are critical for normal RNA processing. Indeed, disrupted alternative splicing in DM1 has been demonstrated, including cardiac troponin T (cTNT), insulin receptor (IR), muscle-specific chloride channel (ClC-1), fast skeletal troponin T (TNNT3), tau and myotubularin-related protein (MTMR1) in skeletal muscle. (4–9). Disruption of ClC-1 and IR alternative splicing is believed to contribute to myotonia and insulin resistance among the major symptoms. However, the cause of progressive muscle wasting, which is the most disabling symptom, is less well defined. Here, we have focused on the two sarcoplasmic reticulum (SR) proteins, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1) and ryanodine receptor 1 (RyR1), that regulate intracellular Ca^{2+} homeostasis in skeletal muscle cells because an elevated Ca^{2+}concentration has been shown in cultured DM muscle cells and may be a

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cause of muscle degeneration. We found alternative splicing variants of SERCA1 and RyR1 in skeletal muscle of DM1 patients (10). As with cTNT, IR and ClC-1, the increase in immature isoforms of SERCA1 and RyR1 indicates dysregulation of the developmental splicing switch.

In mammals, there are three distinct genes encoding *SERCA1*, *2* and *3* (11). Transcripts from these genes are not only tissue-specific but undergo developmental regulation, including switching of isoforms that differ in their C-terminal region, which represents an important phenotypic change in the maturation of mouse and rabbit muscles (12,13). During development of fast-twitch fiber in skeletal muscle, SERCA1b is expressed in fetal/neonatal stages but is completely replaced by SERCA1a in adult muscle fiber (14,15). In humans, SERCA1 is also expressed in fast-twitch fiber in skeletal muscle, but the developmental expression of SERCA1a and 1b remains to be determined (16). Although the functional significance of controlling the switching of isoforms during development is not clear, one plausible explanation is that the Ca^{2+} transport properties of these isoforms determine the characteristics of the muscle or the developmental stage at which they are expressed. Thus, splicing regulation of SERCA1 is well known to play important roles in muscle development and maturation. In contrast, the molecular mechanisms responsible for the splicing dysregulation of SERCA1 in DM patients remain unclear.

The Muscleblind-like (MBNL) protein family belongs to the superfamily of RNA-binding proteins and is homologous to *Drosophila* mbl proteins required for photoreceptor and muscle differentiation (17,18). Three mammalian *MBNL* genes have been identified: *MBNL1* (*MBNL*); *MBNL2* (*MBLL*); *MBNL3* (*MBXL*). All three *MBNL* gene products colocalize with expanded repeat RNA foci *in vivo* (19–22). Loss of MBNL function due to sequestration of CUG repeat RNA is proposed to play a role in DM pathogenesis (23). Furthermore, it has been demonstrated that disruption of the mouse *mbnl1* gene leads to myotonia, cataracts and RNA splicing abnormalities that are characteristic of DM (24). These findings support the hypothesis that manifestations of DM can result from sequestration of MBNL by CUG repeat expansion in a mutant RNA.

In this study, we found that there are five 'YGCU(U/G)Y' motifs that could potentially serve as MBNL1-binding motifs (23) downstream from the SERCA1 exon 22. We analyzed the splicing regulation of SERCA1 exon 22 by MBNL1 and demonstrated that MBNL1 acts on the 'YGCU(U/G)Y' motif in its intron 22, and positively regulates the exon 22 splicing. These results indicate that sequestration of MBNL1 into the CUG repeat expansion of DMPK mRNA could cause the exclusion of SERCA1 exon 22, and that it is possible the neonatal form of SERCA1 expression affects the regulation of Ca^{2+} concentration of SR in the DM patients.

RESULTS

Aberrant splicing of SERCA1 by DMPK expanded CUG repeat

We previously reported that the SERCA1 splicing variant excluding exon 22 (SERCA1b) was exclusively observed in the muscles of all the DM1 patients we investigated, but not in those of normal control or the other disease patients (10) (Fig. 1A). The structures of these two splicing variants are shown in Figure 1B. The alternative splicing of exon 22 affects the translation of seven amino acids located at the C-terminal of the SERCA1 protein (Fig. 1B). To test whether aberrant splicing of the SERCA1 exon 22 is caused by expression of the DMPK transcript with expanded CUG repeats, we examined the effects of the CUG repeats on the alternative splicing of SERCA1 exon 22. When the expression vectors for DMPK expanded CUG repeat (130 repeats) were introduced into C2C12 myoblast cells, RT–PCR using a specific primer set for amplification of endogenous SERCA1 showed that the levels of an endogenous SERCA1 transcript that included exon 22 were decreased while those of an isoform lacking exon 22 were increased (Fig. 1C). However, introduction of DMPK cDNA with normal repeat of CUG (five repeats) into cells never affected the alternative splicing of exon 22, indicating that the expression of DMPK expanded-repeat affects the alternative splicing of SERCA1 exon 22.

To determine the *cis*-acting elements responsible for the aberrant splicing of SERCA1 by expanded repeat of CUG in *DMPK* gene, we searched the specific sequences for splicing in the exon 22 and its franking intron sequences. We found five repeats of the specific sequences for MBNL1-binding, YGCU(U/G)Y, in intron 22 (23) (Fig. 1D). However, the other *cis*-elements of interest such as binding motifs of CUG binding protein (CUG-BP) (4,25) and FOX-1 (26), that regulate splicing, in exon 22 and its franking intron sequences were not observed. The alignment of the nucleotide sequence in intron 22 and the location of MBNL1-binding motifs are shown in Figure 1D. Lin *et al*. (27) previously reported the splicing misregulation of the SERCA1 exon 22 in *MBNL1* knockout mice. These informations raise the possibility that MBNL1 could regulate the alternative splicing of the SERCA1 exon 22 by binding to the MBNL1-binding motifs in intron 22 and that the DMPK transcript with expansion of CUG repeats could affect the splicing regulation by MBNL in DM muscle.

MBNl promotes the splicing of SERCA1 exon 22 and expanded poly CUG repeat inhibits its effects

To examine the splicing regulation of SERCA1 exon 22, we constructed a mini-gene including the mouse genome sequences of *SERCA1* exon 21–intron 21–exon 22–intron 22–exon 23 cloned into a pcDNA3.1 vector. When the mini-gene was transfected into C2C12 cells, RT–PCR using plasmid-specific primer sets (pcDNA forward and SERCA1 reverse primers, see Materials and Methods) showed that the mini-gene expressed mRNAs both with inclusion and exclusion of exon 22 (Fig. 2B, lane 2). The endogenous SERCA1 mRNA was predominantly expressed with exon 22 when RT–PCR analysis was carried out using a specific primer set for amplification of endogenous SERCA1 (Fig. 2B, lane 1). The ratios of exon inclusion when using a mini-gene cloned into pcDNA were lower than those of endogenous SERCA1 mRNA in C2C12 cells. The differences indicate that there may be some *cis*-acting elements that regulate the splicing of SERCA1 exon 22 in regions that are different from the mini-gene sequences. However, because the mini-gene showed a significant

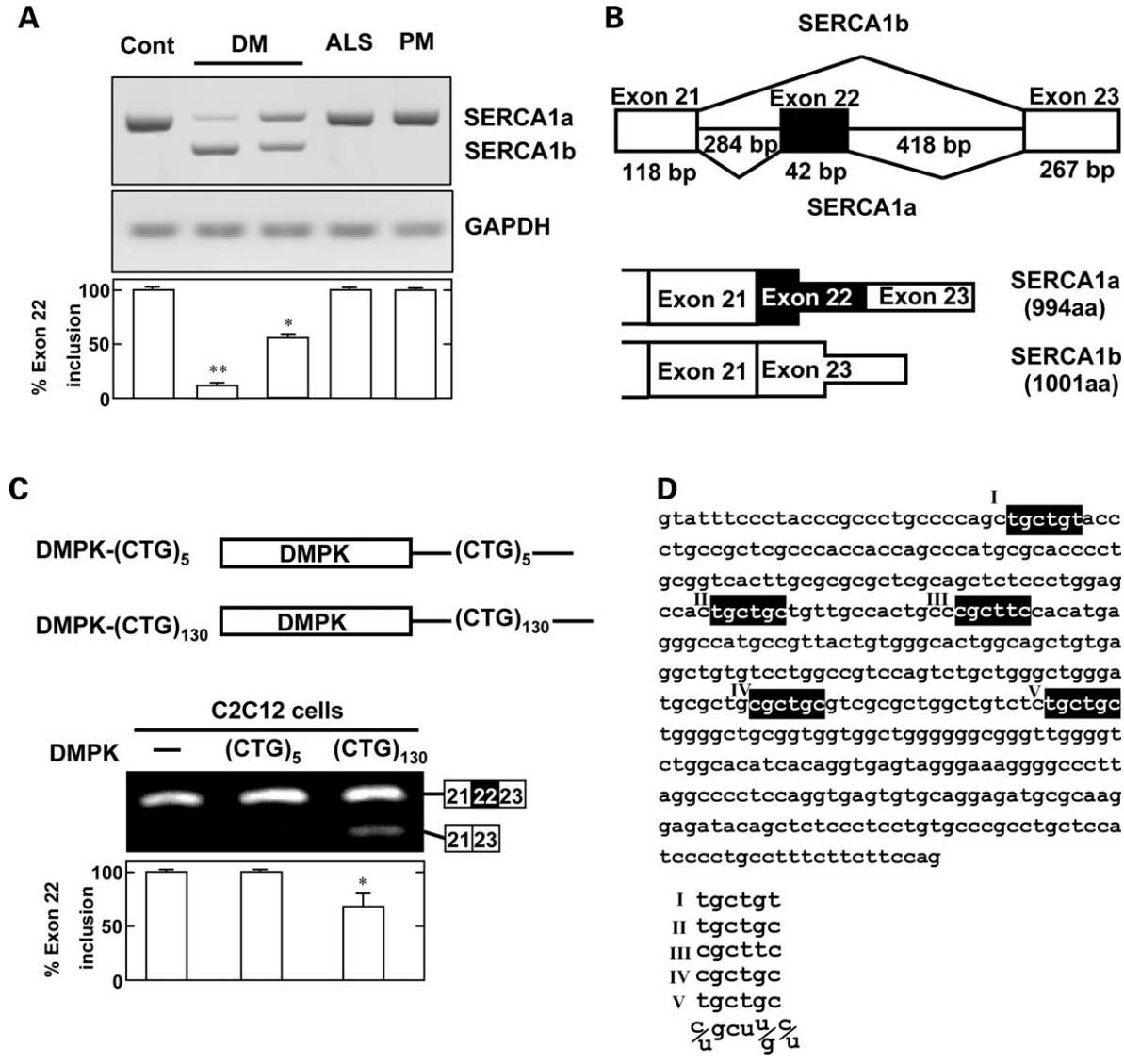

Figure 1. Aberrant splicing of SERCA1 in DM1. (**A**) An increase in SERCA1b (neonatal form) in DM1. RT–PCR analysis of endogenous SERCA1 mRNA from skeletal muscle tissues of DM1, normal control (Cont), amyotrophic lateral sclerosis (ALS) and polymyositis (PM) patients. SERCA1 exon 22 exclusion was analyzed by RT–PCR using specific primer set for amplification of endogenous SERCA1. Lower panel: the percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from control levels ($^{**}P < 0.01$; $^{*}P < 0.05$) (Student's *t* test). (**B**) Schematic representation of the *SERCA1* gene (exon 21–intron 21–exon 22–intron 22–exon 23) (upper panel). Schematic representation of the SERCA1 mRNAs (exon 21–exon 22–exon 23, exon 21–exon 23) (lower panel). Exons represented as thick boxes are translated segments and thin boxes indicate untranslated segments. (**C**) CUG repeat expansion of DMPK mRNA induces exclusion of SERCA1 exon 22 in C2C12 cells. Schematic structures of DMPK-$(CTG)_5$ and DMPK-$(CTG)_{130}$ constructs used in this study. The DMPK cDNAs contain DMPK full coding sequences with CTG trinucleotide repeats of 5 or 130 (upper panel). C2C12 cells were transfected with pcDNA/DMPK-$(CTG)_5$ or pcDNA/DMPK-$(CTG)_{130}$. SERCA1 exon 22 exclusion was analyzed by RT–PCR using a specific primer set for amplification of endogenous SERCA1 (lower panel). The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from control levels ($^{*}P < 0.05$) (Student's *t* test). (**D**) The mouse genome sequences in intron 22 of *SERCA1*. Five MBNL1-binding motifs are highlighted by black boxes (upper panel). Alignment of five MBNL1-binding motifs in intron 22 of SERCA1 (lower panel).

expression of transcript including exon 22, the construct should be useful for determining the MBNL *cis*-acting elements in this mini-gene.

The construct was transiently co-transfected with the expression vector for MBNL1, MBNL2 or MBNL3 that were fused with GFP into HEK-293 cells that express endogenous SERCA1 excluding exon 22 (Fig. 2B). After collection of total RNA from the cells, RT–PCR was performed using a plasmid-specific primer set to detect RNA processing of SERCA1 in transfected HEK-293 cells. The expression of each MBNL protein promoted inclusion of SERCA1 exon 22 compared with control GFP expression vector transfected cells (Fig. 2B). In contrast, the transfection of the expression vectors of CUG-BP or FOX-1, or both, which are RNA-binding splicing factors, did not affect the splicing of SERCA1 exon 22 (Fig. 2D). Moreover, we introduced the

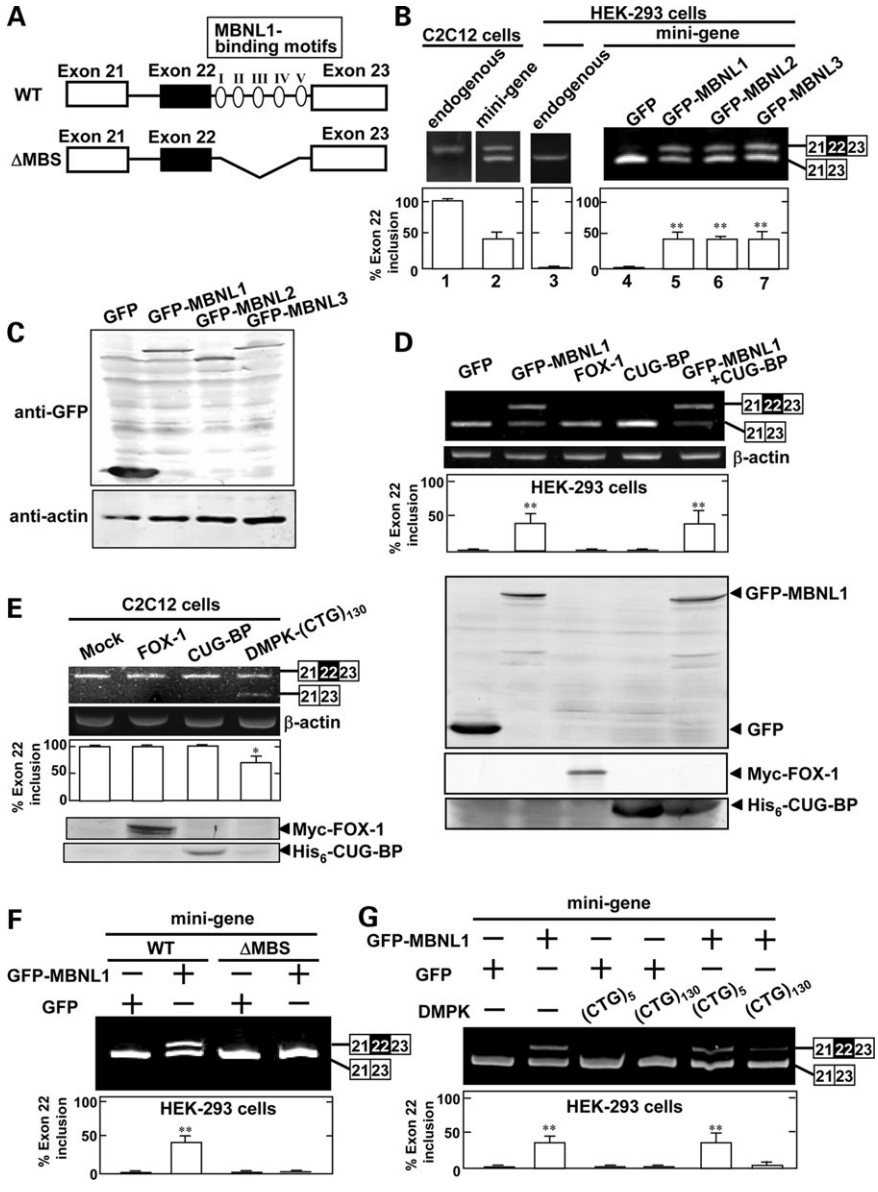

Figure 2. Effects of MBNL on the splicing of SERCA1 exon 22 in cultured cells. (**A**) Schematic structures of each mini-gene. WT, wild-type; ΔMBS, deletion of all MBNL1-binding motifs. The positions of MBNL1-binding motifs are shown by the five circles. (**B**) RT–PCR analysis of endogenous SERCA1 mRNA in C2C12 cells (lane 1). C2C12 cells transfected with the WT SERCA1 mini-gene were analyzed for the ratios of SERCA1 exon 22 inclusion by RT–PCR using a plasmid-specific primer set (lane 2). RT–PCR analysis of endogenous SERCA1 mRNA in HEK-293 cells (lane 3). The WT SERCA1 mini-gene was transfected into HEK-293 cells with pEGFP-C1, pEGFP-C1/MBNL1, pEGFP-C1/ MBNL2 or pEGFP-C1/ MBNL3. The ratios of SERCA1 exon 22 inclusion were analyzed by RT–PCR using a plasmid-specific primer set (lanes 4–7). The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from the control transfected with GFP ($^{**}P < 0.01$) (Student's *t* test). (**C**) Lysates (20 μg of protein) of C2C12 cells expressing the indicated proteins were probed with the anti-GFP and anti-actin antibodies, respectively. (**D**) WT SERCA1 mini-gene was transfected into HEK-293 cells with pEGFP-C1/MBNL1, pcDNA3/Myc-FOX-1, pcDNA3.1HisC/CUG-BP or pEGFP-C1/MBNL1 and pcDNA3.1HisC/CUG-BP. The SERCA1 exon 22 inclusion was analyzed by RT–PCR, using a plasmid-specific primer set. The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from the control transfected with GFP ($^{**}P < 0.01$) (Student's *t* test) (upper panel). The expression level of each protein was determined by western blotting with anti-GFP, anti-Myc and anti-His_6 antibodies, respectively (lower panel). (**E**) C2C12 cells were transfected with mock vector, pcDNA3/Myc-FOX-1, pcDNA3.1HisC/CUG-BP or pcDNA/DMPK $(CTG)_{130}$. SERCA1 exon 22 exclusion was analyzed by RT–PCR, using a specific primer set for amplification of endogenous SERCA1. The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from the mock transfected control ($^{*}P < 0.05$) (Student's *t* test) (upper panel). Lower panel shows western blotting with anti-Myc and anti-His_6 antibodies, respectively. (**F**) WT SERCA1 mini-gene or ΔMBS mini-gene was transfected into HEK-293 cells with or without pEGFP-C1/MBNL1. The ratios of SERCA1 exon 22 inclusion were analyzed by RT–PCR, using a plasmid-specific primer set. The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from the control transfected with GFP ($^{**}P < 0.01$) (Student's *t* test). (**G**) WT SERCA1 mini-gene and pEGFP-C1/MBNL1 were transfected into HEK-293 cells with pcDNA/DMPK-$(CTG)_5$ repeat or pcDNA/DMPK-$(CTG)_{130}$ repeat. The ratios of SERCA1 exon 22 inclusion were analyzed by RT–PCR using a plasmid-specific primer set. The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from the control transfected with GFP ($^{**}P < 0.01$) (Student's *t* test).

expression vectors for CUG-BP or FOX-1 into C2C12 cells to examine whether these RNA-binding splicing factors induce skipping of endogenous SERCA1 exon 22. RT–PCR using a specific primer set for amplification of endogenous SERCA1 showed that overexpression of CUG-BP or FOX-1 did not induce exclusion of the exon 22 (Fig. 2E). Taken together, these findings suggest that MBNLs could specifically promote the splicing of the exon 22 acting on specific *cis*-elements in a mini-gene.

Next, to examine whether increased inclusion of exon 22 is due to the effects of MBNL on the MBNL1-binding sequences in the intron 22, we constructed a mini-gene having deleted the five MBNL1-binding sequences (ΔMBS, Fig. 2A). When HEK-293 cells were transfected with the ΔMBS mini-gene and MBNL1 expression vector, RT–PCR using a plasmid-specific primer set showed that MBNL1 did not promote inclusion of SERCA1 exon 22 (Fig. 2F), indicating that MBNL1 could act on these MBNL1-binding sequences in intron 22 and promote the inclusion of exon 22.

As mentioned earlier, the introduction of the DMPK expanded CUG repeat into myoblast cells resulted in inhibition of exon 22 splicing of SERCA1 (Fig. 1C). To experimentally verify this phenomenon, the expression vector for DMPK expanded CUG repeat was co-transfected with the constructs of a mini-gene containing SERCA1 exons 21–23 and the MBNL1 expression vector. RT–PCR using a plasmid-specific primer set showed that transfection of the DMPK expanded CUG repeat caused a reduction of MBNL1-dependent inclusion of SERCA1 exon 22 (Fig. 2G). However, DMPK with the non-expanded CUG repeat [DMPK-$(CUG)_5$] did not affect the MBNL1-dependent splicing of SERCA1. These results were consistent with the effects of the DMPK-CUG repeats on the alternative splicing of endogenous SERCA1 exon 22 (Fig. 1C) and, taken together, suggest that sequestration of MBNL into the CUG repeat of DMPK mRNA could lead to the exclusion of SERCA1 exon 22.

Identification of MBNL1-binding sites in intron 22 of SERCA1

To identify the MBNL1-binding sites in SERCA1 intron 22, we constructed various mini-genes having deleted the MBNL1-binding sequences (Fig. 3A). When HEK-293 cells were co-transfected with the ΔMBS (I–III) mini-gene, in which the first three MBNL1-binding motifs were deleted, and the MBNL1 expression vector, MBNL1 did not promote inclusion of SERCA1 exon 22. In contrast, in the case of the ΔMBS (IV–V) mini-gene, in which the fourth and fifth MBNL1-binding motifs were deleted, MBNL1 promoted the inclusion of exon 22 similar to that of the wild-type (WT) mini-gene. These results indicate that the 5′ region containing the first three MBNL1-binding sequences is important for MBNL1-dependent inclusion of the exon 22 (Fig. 3B). The possibility that deletion of the long region in intron 22 may have affected the splicing regulation of SERCA1 exon 22 cannot be excluded. Therefore, we mutated the MBNL1-binding motifs and replaced them with the sequence AUAAUA (Fig. 3A). When the mutant (I), in which the first MBNL1-binding motif was mutated, was introduced into HEK-293 cells, MBNL1 promoted the inclusion of exon 22 similar to the case of the WT mini-gene. In contrast, in the cases of the mutant (II), in which the second MBNL1-binding motif was mutated, and (III), in which the third MBNL1-binding motif was mutated, the ratios of exon 22 inclusion were both ∼50% compared with those of the WT mini-gene (Fig. 3C). As well, in the cases of the mutant (I/II), in which the first and second MBNL1-binding motifs were mutated, and (I/III) mini-genes, in which the first and third MBNL1-binding motifs were mutated, MBNL1 promoted the inclusion of exon 22, but the ratios of exon 22 inclusion were ∼50% compared with those of the WT mini-gene. In the case of the mutant (II/III) mini-gene, in which the second and third MBNL1-binding motifs were mutated, MBNL1 could not promote inclusion of exon 22, as was the case with the SERCA1 mutant (I/II/III) mini-gene (Fig. 3D). We also introduced the mutated mini-genes of mutants (I/II, I/III, II/III, I/II/III) into C2C12 cells and examined the splicing of exon 22 (Fig. 3E). Basically, the same results were obtained as in the experiments in which mutated mini-genes and the expression vectors for MBNL1 were transfected into HEK-293 cells (Fig. 3D). Therefore, we concluded that the second and third MBNL1-binding motifs are necessary for MBNL1-dependent inclusion of exon 22 (Fig. 3C).

The interaction of MBNL1 with MBNL1-binding motifs in SERCA1 intron 22

To confirm that MBNL1 directly interacts with the MBNL1-binding motifs of intron 22 in SERCA1, ^{32}P-labeled *in vitro*-transcribed SERCA1 intron 22 and MBNL1 fused to maltose-binding protein (MBP) expressed in *Escherichia coli* were mixed and UV cross-linked. In this experiment, as shown in Figure 4A, we used synthetic RNAs that were transcribed from templates of 372-bp sequences containing partial sequences of intron 22 with MBNL1-binding motifs (I–V) or with mutations in the first three MBNL1-binding motifs, partial sequences intron 21 and full-sequence of exon 22. As shown in Figure 4D, SERCA1 intron 22 RNA directly interacted with MBP-MBNL1 but not with MBP. In contrast, RNA with mutations in the first three MBNL1-binding sites did not bind to MBP-MBNL1. The direct binding of intron 22 and MBNL1 was reduced by adding non-labeled expanded CUG repeat RNA (Fig. 4D, *lane 4*). These results suggest that the CUG repeat expansion competes with the SERCA1 intron 22 for binding to MBNL1.

DISCUSSION

Previously, we found the alternative splicing of SERCA1 exon 22 in the skeletal muscle of DM1 patients (10). In this study, we attempted to determine the molecular mechanisms for aberrant splicing of SERCA1 exon 22. We searched specific sequences for splicing around exon 22 of the *SERCA1* gene and found that there were five 'YGCU(U/G)Y' motifs in intron 22. These motifs have been shown to be an MBNL1-binding sequence, and there are two motifs upstream from human cTNT exon 5 (−44 to −36 and −26 to −18) within intronic regions (23). MBNL1 directly interacts with

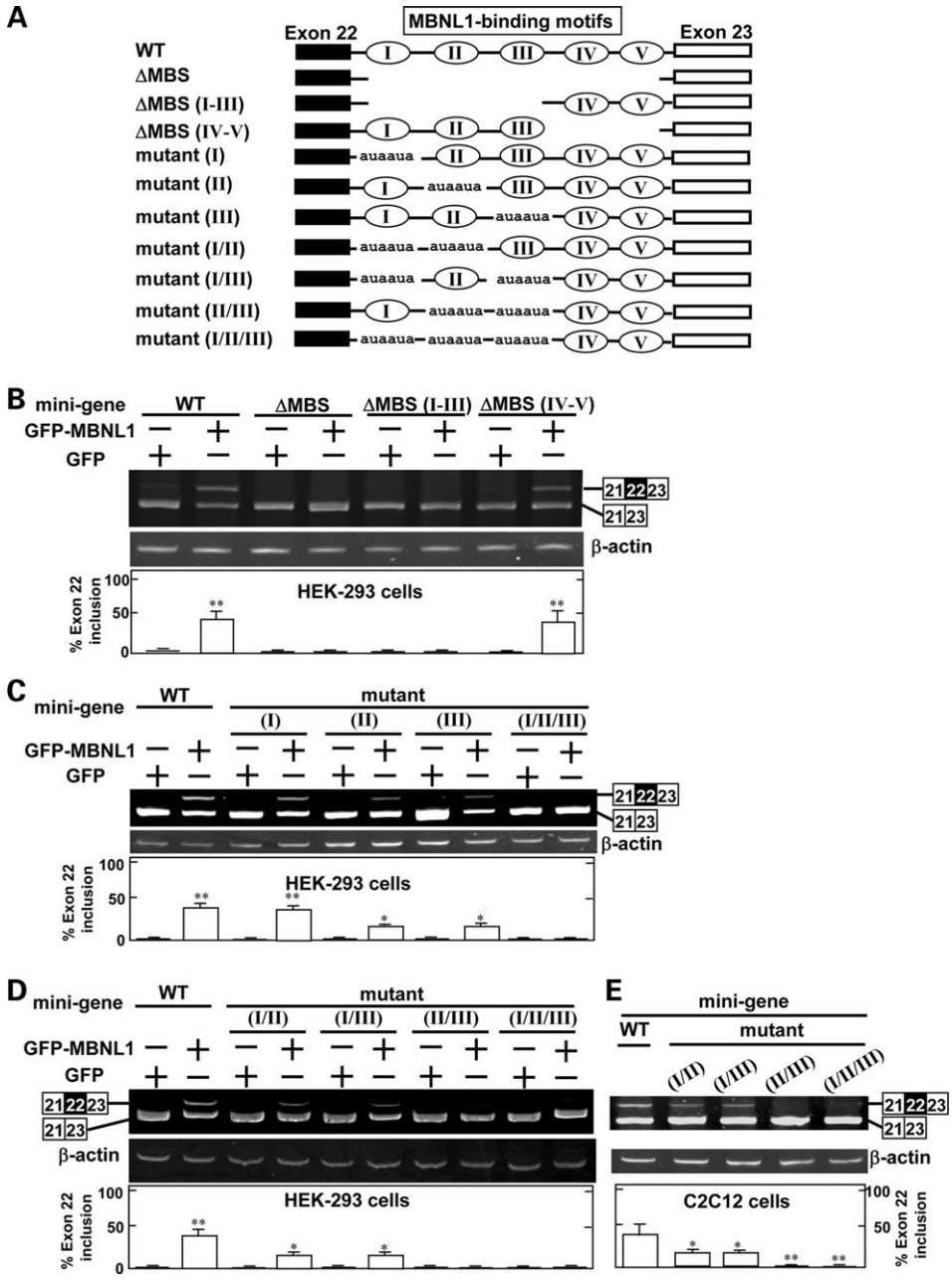

Figure 3. Identification of the binding-sites of SERCA1 mRNA that binds to MBNL1. (**A**) Schematic structures of each mini-gene. WT, wild-type; ΔMBS, deletion of all MBNL1-binding motifs; ΔMBS (I–III), deletion of the first three MBNL1-binding motifs; ΔMBS (IV–V), deletion of the fourth and fifth MBNL1-binding motifs; mutant (I), mutation of the first MBNL1-binding motif; mutant (II), mutation of the second MBNL1-binding motif; mutant (III), mutation of the third MBNL1-binding motif; mutant (I/II), mutation of the first and second MBNL1-binding motifs; mutant (I/III), mutation of the first and third MBNL1-binding motifs; mutant (II/III), mutation of the second and third MBNL1-binding motifs; mutant (I/II/III), mutation of the first three MBNL1-binding motifs. (**B**) WT SERCA1 mini-gene, ΔMBS, ΔMBS (I–III) or ΔMBS (IV–V) were transfected into HEK-293 cells with or without pEGFP-C1/MBNL1. The ratios of SERCA1 exon 22 inclusion were analyzed by RT–PCR using a plasmid-specific primer set. The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from each control transfected with GFP ($^{**}P < 0.01$) (Student's *t* test). (**C**) WT SERCA1 mini-gene, mutant (I), mutant (II), mutant (III) or mutant (I/II/III) were transfected into HEK-293 cells with or without pEGFP-C1/MBNL1. The ratios of SERCA1 exon 22 inclusion were analyzed by RT–PCR using a plasmid-specific primer set. The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from each control transfected with GFP ($^{**}P < 0.01$; $^{*}P < 0.05$) (Student's *t* test). (**D**) WT SERCA1 mini-gene, mutant (I/II), mutant (I/III), mutant (II/III) or mutant (I/II/III) were transfected into HEK-293 cells with or without pEGFP-C1/MBNL1. The ratios of SERCA1 exon 22 inclusion were analyzed by RT–PCR using a plasmid-specific primer set. The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from each control transfected with GFP ($^{**}P < 0.01$; $^{*}P < 0.05$) (Student's *t* test). (**E**) WT SERCA1 mini-gene, mutant (I/II), mutant (I/III), mutant (II/III) or mutant (I/II/III) were transfected into C2C12 cells. The ratios of SERCA1 exon 22 inclusion were analyzed by RT–PCR using a plasmid-specific primer set. The percentages of exon 22 inclusion relative to the total transcripts are represented (means ± SD of three independent experiments). Asterisks indicate a significant difference from the WT control ($^{**}P < 0.01$; $^{*}P < 0.05$) (Student's *t* test).

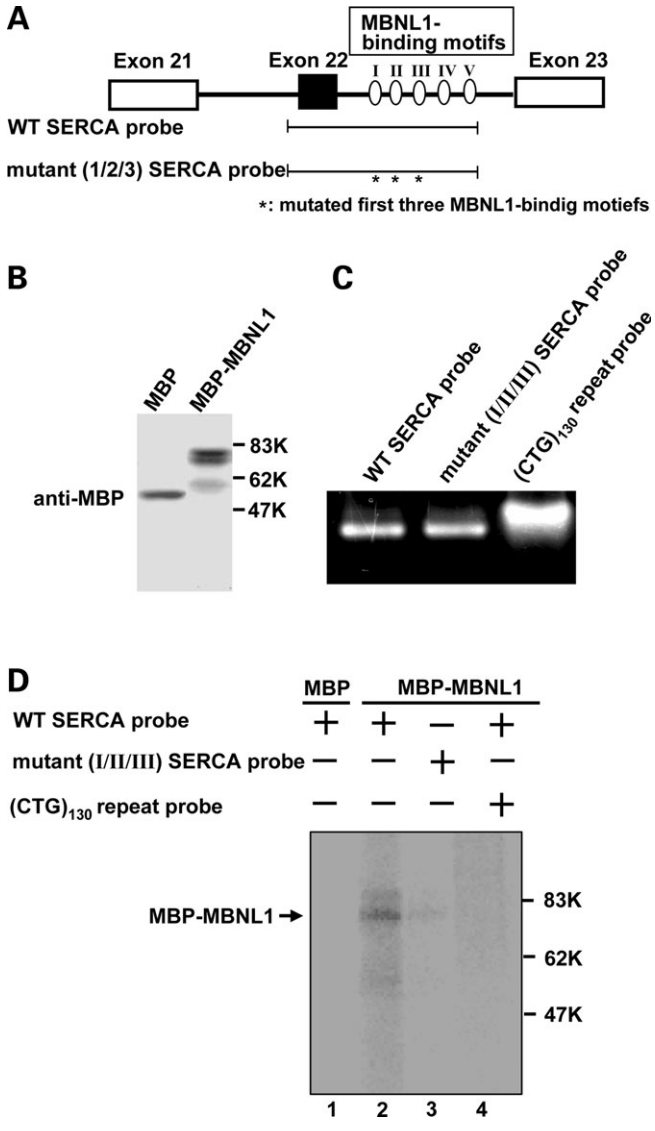

Figure 4. Direct binding of MBNL1 protein to SERCA1 intron 22. (**A**) The construction of each probe. A WT SERCA1 probe was transcribed from the template of 372-bp sequences containing partial sequences of intron 22 with five MBNL1-binding motifs and intron 21 and exon 22, and a mutant (I/II/III) SERCA1 probe was also synthesized from the template with mutations of the first three MBNL1-binding motifs. (**B**) Purified MBP and MBP-MBNL1 proteins were probed with the anti-MBP antibody. The expected molecular weight of MBP-MBNL1 is about 80 kDa. (**C**) Synthesized WT SERCA1 probe, mutant (I/II/III) probe and $(CUG)_{130}$ repeat probe were subjected to gel electrophoresis. (**D**) Radiolabeled WT SERCA1 probe was incubated with MBP (lane1) or MBP-MBNL1 (lane 2). Radiolabeled mutant (I/II/III) probe was incubated with MBP-MBNL1 (lane 3). Non-labeled CUG_{130} repeat probe was pre-incubated with MBP-MBNL1 before the addition of the radiolabeled WT SERCA1 probe (lane 4). Sizes are indicated in kiloDaltons.

these motifs and regulates the alternative splicing of cTNT. MBNL1 was originally identified as a double-stranded RNA binding protein and has been shown to bind to expanded CUG repeats that form an extended hairpin structure (19,28,29). The prediction of the RNA secondary structure was reported to be that the 'YGCU(U/G)Y' motif within the cTNT intron does not form a hairpin structure (23). We analyzed the higher-order structure of MBNL1-binding sequences and their 5′- and 3′-franking regions using the MFOLD program (30). We confirmed that the sequences do not form a hairpin structure as in the case of the cTNT intron (data not shown). Therefore, it is possible that MBNL1 could bind to the specific motif within a single-stranded RNA sequence of 'YGCU(U/G)Y', although the precise mechanisms of its regulation are unclear. Of these five motifs, MBNL1 specifically acts on the second and third motifs from the 5′ region of SERCA1 intron 22, but does not act on the first, fourth or fifth motifs. The sequence of the second 'YGCU(U/G)Y' is completely in accord with the fifth sequence (Fig. 1D). Therefore, MBNL1 does not simply recognize a primary sequence of 'YGCU(U/G)Y' motif, but also recognizes the position of the motif and the high-order structure, which is constituted by the motif and its around sequence. Further studies will be necessary to better understand details of the interaction between MBNL1 and the 'YGCU(U/G)Y' motif.

CUG-BP is a member of the CELF family that regulates the alternative splicing of cTNT, IR and ClC-1 by recognition of intronic elements containing U/G-rich motifs (4,5,8,25,31). In the intronic regions of human cTNT around exon 5, there is one U/G-rich region and two MBNL1-binding motifs. CUG-BP and MBNL1 bind to their respective specific *cis*-elements and antagonistically regulate alternative splicing of the cTNT exon 5 (23). In this study, U/G-rich motifs are not included around exon 22 of the *SERCA1* gene. Furthermore, co-expression of MBNL1 and CUG-BP did not affect the MBNL1-dependent SERCA1 exon 22 inclusion. From these results, it is possible that CUG-BP is not associated with the regulation of alternative splicing of SERCA1 exon 22 and that MBNL solely modulates its splicing.

The RNA gain-of-function hypothesis proposes that DM1 is caused by the expansion of the CUG triplet repeat in the 3′-UTR of DMPK. This expanded repeat is thought to alter the function and localization of alternative splicing regulators, such as CUG-BP, elav-like RNA binding protein 3 (ETR-3), MBNL and double-stranded RNA-dependent protein kinase (PKR) (19,32–34). Endogenous and exogenously expressed MBNL proteins co-localize with the expanded repeat RNA in nuclear foci in DM1 cultured cells and skeletal muscle tissue (19–22). Disruption of the mouse *MBNL1* gene leads to muscle, eye and RNA splicing abnormalities that are characteristic of DM disease (24). These findings support the hypothesis that DM1 results from the sequestration of MBNL1 to CUG repeats expansion. We demonstrated that overexpression of the DMPK-expanded CUG repeat into C2C12 myoblast cells affects the alternative splicing of SERCA1 exon 22. Furthermore, our results showed that the SERCA1 intron 22 directly interacted with MBNL1 and that this interaction was inhibited by the expanded CUG repeat. Taken together, these findings suggest that recruitment of MBNL1 proteins to the CUG repeat expansion leads to inhibition of MBNL1 binding to the intron 22 of SERCA1, resulting in the skipping of SERCA1 exon 22.

It has been reported that the mammalian SERCA1 has two major splice variants, SERCA1a (adult form) and SERCA1b (neonatal form). These isoforms are mainly expressed in fast-twitch fiber in skeletal muscle (12–15). The difference

between these two isoforms results from the alternative splicing of exon 22 and consequently affects seven amino acids located at the C-terminal. SERCA1a contains exon 22 and encodes 994 amino acids, while SERCA1b encodes 1001 amino acids because of exon 22 skipping. Although the functional difference between SERCA1a and 1b is not clear, it is possible that the C-terminal difference affects the binding of muscle-specific factors that influence ATPase activity. Sarcolipin is an integral membrane protein that copurifies with SERCA1a in fast-twitch fiber of skeletal muscle (35). It has been suggested that sarcolipin regulates the ATPase activity of SERCA1 and may interact with transmembrane helices in SERCA1 molecules (36). Thus, the difference in C-terminal splicing could alter the interaction between SERCA1 and sarcolipin, and may change the ATPase activity of SERCA1. Indeed, an increase in resting Ca^{2+} concentration has been reported in cultured DM myotubes (37). It will be important for future studies to clarify functional differences between SERCA1a and 1b in muscle fibers and/or cultured skeletal muscle cells of DM patients.

In summary, we found five 'YGCU(U/G)Y' motifs that could potentially serve as MBNL1-binding motifs, in intron 22 of SERCA1. Among these five motifs, the second and third sites were important for regulation of exon 22 splicing. MBNL1 directly bound to SERCA1 pre-mRNA and regulated its correct splicing; however, the expanded CUG repeat RNA inhibited MBNL1-dependent splicing and resulted in the exclusion of exon 22 of SERCA1. Thus, it is possible that a change in the high-order structure at the C-terminal caused by protein translated from mRNA without SERCA1 exon 22 could affect the original functions of SERCA1 in the SR of skeletal muscle tissue in DM patients.

MATERIALS AND METHODS

Cell cultures

HEK-293 and C2C12 cells were used for *in vivo* splicing assays. HEK-293 cells were grown in 10% fetal bovine serum/Dulbecco's modified Eagle's medium and C2C12 cells were cultured in α-minimum essential medium with 10% fetal bovine serum. Prior to transfection, the cells were plated at a density of 60–80% confluency on 60-mm-diameter dishes.

Construction of plasmids

To construct the mini-gene including SERCA1 exons 21–23, the mouse genome sequence of *SERCA1* containing exon 21–intron 21–exon 22–intron 22–exon 23 was generated by PCR using the following primer sets: forward (5′-GGA TCC GAT CTT CAA GCT CCG GGC CCT G-3′) and reverse (5′-GAA TTC AGC AAT CAG CTA GTC AGT TG-3′). The PCR product was digested with *Bam*HI and *Eco*RI, and inserted into the pcDNA3.1(+) mammalian expression vector (Invitrogen). Various deletion mutants of mini-gene including SERCA1 exons 21–23 were generated by PCR using the template as a pcDNA3.1(+)/SERCA1 exons 21–23 vector. The primers for the amplification of the fragments were as follows: forward (5′-TGG GGC TGC GGT GGT GGC-3′) and reverse (5′-GGG CAG GGC GGG TAG-3′) for ΔMBNL1-binding sequences (ΔMBS), forward (5′-TGG GCA CTG GCA GCT GTG AGG-3′) and reverse (5′-GGG CAG GGC GGG TAG-3′) for ΔMBS (I–III), forward (5′-TGG GGC TGC GGT GGT GGC-3′) and reverse (5′-GTA ACG GCA TGG CCC TCA TG-3′) for ΔMBS (IV–V). The MBNL1-binding site mutations were generated by PCR using the template as a pcDNA3.1(+)/SERCA1 exons 21–23 vector with mutagenized oligonucleotide primers: forward (5′-ATA ACC CTG CCG CTC GCC CAC CAC CAG-3′) and reverse (5′-TAT GCT GGG GCA GGG CGG GTA G-3′) for mutant (I), forward (5′-ATA TGT TGC CAC TGC CCG CTT CCA CAT G-3′) and reverse (5′-TAT GTG GCT CCA GGG AGA GCT GC-3′) for mutant (II), forward (5′-ATA CAC ATG AGG GCC ATG CCG TTA C-3′) and reverse (5′-TAT GGC AGT GGC AAC AGC AGC AGT G-3′) for mutant (III). These PCR products were ligated by a BKL kit (Takara, Japan). The pGEM/SERCA1 (WT probe) was generated by PCR using the template as a pcDNA3.1(+)/SERCA1 exons 21–23 vector with the following primer sets: forward (5′-CTC TGT CCT CTC TGG CCA TAG-3′) and reverse (5′-CTC ACC TGT GAT GTG CCA GAC-3′). Standard recombinant DNA techniques were used to construct the following plasmids: pcDNA3.1(+)/DMPK-$(CTG)_5$; pcDNA3.1(+)/DMPK-$(CTG)_{130}$; pGEM/$(CTG)_{130}$; pMAL-CRI/MBNL1. All of the constructs were sequenced before use in the experiments.

In vivo splicing

Constructs of mouse SERCA1 mini-genes (3.0 μg) were transfected into cells using LipofectAMINE2000 reagent (Invitrogen) according to the manufacturer's protocol. Transfected cells were lysed in buffer RLT (Qiagen), and the total cellular RNA was purified using the RNeasy mini kit (Qiagen). First strand cDNA was synthesized in a 20-μl reaction volume using a random primer (TaKaRa) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR amplification analysis of the plasmid-derived cDNAs was performed using the primer set, pcDNA forward (5′- CGA AAT TAA TAC GAC TCA CTA TAG GGA G-3′) and SERCA1 reverse (5′-CAG CTT TGG CTG AAG ATG CA-3′). Total RNA preparations and cDNA syntheses from human skeletal muscle tissues were performed as described previously (10). To analyze endogenous splicing of SERCA1, RT–PCR was performed using the following primer sets: forward (5′- ATC TTC AAG CTC CGG GCC CT-3′) and reverse (5′-CAG CTT TGG CTG AAG ATG CA-3′) for mouse SERCA1; forward (5′- ATC TTC AAG CTC CGG GCC CT-3′) and reverse (5′- CAG CTC TGC CTG AAG ATG TG -3′) for human SERCA1. PCR was performed with *Taq* DNA polymerase (Promega) as follows: an initial denaturation step (94°C for 5 min), 32 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 1 min), and a final extension step (72°C for 5 min). The reaction products were resolved by electrophoresis through an 8% acrylamide gel. PCR products were cloned into the pGEM-T vector (Promega) and sequenced. Quantification of the density of each band was carried out using a densitography program (ATTO). The ratios of inclusion of exon 22 were quantified and expressed as percentages of inclusion relative to

the total intensities. Data were shown as the mean ± SD of three independent experiments.

Western blotting

Cells were washed with PBS, harvested and lysed in Triton X-100 lysis buffer [0.5% Triton X-100, 10 mM HEPES pH 7.9, 50 mM NaCl, 100 mM EDTA, 0.5 M sucrose and 0.1% protease inhibitor cocktail (Sigma)]. The lysates were then incubated on ice for 30 min and centrifuged at 8000*g* for 10 min. Equal amounts of protein were subjected to 10–15% SDS polyacrylamide gel electrophoresis, transferred to PVDF membranes and immunoblotted with each primary antibody. The membranes were washed with PBS/Tween-20, and then incubated with a peroxidase-conjugated secondary antibody. The corresponding bands were detected using an ECL plus kit (Amersham Biosciences Corp., Piscataway, NJ).

Synthesis of RNA probes and competitors

Radiolabeled RNA probes and unlabeled competitor RNAs were synthesized according to the manufacturer's instructions, using T7 RNA polymerase (Promega) on linearized DNA templates in the presence of 2.5 mM each of ATP, GTP and CTP, and 50 μCi [α-^{32}P]UTP and 0.1 mM UTP (radiolabeled) or 2.5 mM UTP (unlabeled). Templates used for synthesis of the RNAs: pGEM/SERCA1 (WT probe) (372 bp) and pGEM/SERCA1 (mutant (I/II/III) probe) (372 bp) linearized with *Nde*I; pGEM/$(CTG)_{130}$ linearized with *Eco*RI. One hour after the synthesis, the templates were removed by DNase I (Promega) treatment (1 U in 21 μl reaction mixture for 15 min at 37°C) and the RNAs were separated from enzymes by Micropure™-EZ (MILLIPORE) and from unincorporated nucleotides by Sephadex G25 spin dialysis. The integrity of the RNAs was confirmed by gel electrophoresis.

Analysis of RNA–protein interactions

MBP and MBP-fused MBNL1 were purified from *E. coli* according to the manufacturer's instructions. Internally labeled WT SERCA1 probe and its mutant probe were used for UV cross-linking assays. Approximately 2000 cpm of the radiolabeled probe were mixed with 100 ng of MBP or MBP-MBNL1 protein in 30 μl of binding buffer (10 mM Tris–HCl, pH 7.6, 150 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) for 30 min at 25°C. In competition experiments, the competitor non-labeled CTG_{130} repeat RNA was pre-incubated with 100 ng of MBP-MBNL1 protein for 30 min before the addition of the radiolabeled SERCA1 probe. RNA complexes were UV-cross-linked by 300 000 μJ of UV radiation at room temperature, using a CL-1000 Ultraviolet cross-linker (UVP). Samples were then treated with 40 μg of RNase A for 45 min at 37°C. Samples were then subjected to SDS–PAGE followed by autoradiography.

RNA structure analyses

The RNA secondary structures and free energies were predicted using the GCG version of the MFOLD program (30).

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